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Lectins Associated With the Feeding Organs of the Oyster *Crassostrea virginica* Can Mediate Particle Selection

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Abstract. Despite advances in the study of particle selection in suspension-feeding bivalves, the mechanisms upon which bivalves rely to discriminate among particles have not been elucidated. We hypothesized that particle sorting in suspension-feeding bivalves could be based, in part, on a biochemical recognition mechanism mediated by lectins within the mucus that covers the feeding organs. Using *Crassostrea virginica*, the Eastern oyster, our investigations demonstrated that lectins from oyster mucus can specifically bind several microalgal species as well as different types of red blood cells (RBC), triggering their agglutination. Agglutination of microalgal species and RBC varied with the source of mucus (gills *vs.* labial palps). Hemagglutination and hemagglutination inhibition assays emphasized that mucus contains several lectins. In feeding experiments, *Nitzschia closterium* and *Tetraselmis maculata* were separately incubated with mucus before being fed to oysters. Results showed that pre-treating these microalgae with mucus significantly alters the ability of oysters to sort particles. In another experiment, oysters were fed a mixture of microspheres coated with either bovine serum albumin (BSA) or glucosamide-BSA. Results show that oysters preferentially ingest microspheres with bound carbohydrates, highlighting probable interactions between lectins and carbohydrates in the mechanisms of microalgae recognition. This study confirms the presence of lectins in mucus that covers the feeding organs of oysters and suggests a new concept with regard to particle processing by suspension-feeding bivalves: specific interactions between carbohydrates on the surface of particles and lectins within the mucus mediate the selection and rejection processes.

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Introduction

The capability of suspension-feeding bivalves to sort and select among captured particles is well known (Newell and Jordan, 1983; Shumway *et al.*, 1985; Ward *et al.*, 1997; Cognie *et al.*, 2003; Ward and Shumway, 2004; Beninger *et al.*, 2004). Although some aspects of the selection process have been elucidated, the actual mechanism by which particles of poor quality are rejected and those of higher quality are diverted toward the mouth and ingested remains unknown. Among several theories advanced in the literature, some studies support the idea that bivalves use chemical cues to discriminate among particles (Kiørboe and Møhlenberg, 1981; Newell and Jordan, 1983; Shumway *et al.*, 1985; Ward and Targett, 1989; Pales Espinosa *et al.*, 2007, 2008). Chemical communication among plants and animals is widespread in marine environments, and thousands of metabolites have been identified in seawater. In numerous physiological processes, such as defense (Selander *et al.*, 2006), reproduction (Ianora *et al.*, 1995; Alimenti *et al.*, 2002), or feeding (Power *et al.*, 2002), chemical signals are used to mediate interactions among organisms (Hay, 1996, and references therein). In the case of the interactions between suspension-feeding bivalves and microalgae, Ward and Targett (1989) demonstrated that a mussel, *Mytilus edulis*, is able to select and preferentially ingest synthetic microspheres coated with metabolites produced by microalgal species. Moreover, the great scallop, *Pecten maximus*, is capable of selecting *Coscinodiscus perforatus* by detecting the presence of external organic components on its siliceous

shells (frustules) (Beninger and Decottignies, 2005) and rejecting the cleaned frustules of the same diatom species (Beninger *et al.*, 2004). More recently, we demonstrated that the oyster *Crassostrea gigas* can selectively ingest alginate microcapsules containing live microalgal cells or reject them in pseudofeces, depending upon the microalgal species used to produce the microcapsules (Pales Espinosa *et al.*, 2007). These findings suggest that microalgal cells produce extracellular metabolites that are specifically recognized by the bivalve. The functional mechanisms involved in identifying and responding to these metabolites remain, however, obscure. The existence of possible chemosensory cells on the labial palps of *Crassostrea virginica* has been reported (Dwivedy, 1973), but the methodology and results of this study have been criticized (Beninger *et al.*, 1990; Beninger, 1991). We suggest that, instead of chemosensory cells directly identifying particles of interest, bivalves secrete metabolites that mediate the process of recognition and selection.

The soft body of bivalves is covered by a layer of mucus produced by the pallial organs such as foot and mantle (Calabro *et al.*, 2005), gills (Foster-Smith, 1975), and labial palps (Beninger *et al.*, 1995; Beninger and St-Jean, 1997a). In suspension-feeding bivalves, mucus is abundantly secreted throughout the feeding process, particularly during particle transport on feeding organs (Kiørboe and Møhlenberg, 1981; Beninger *et al.*, 1993; Ward *et al.*, 1993, 1997) and rejection in biodeposits (Urrutia *et al.*, 2001). Mucus is composed largely of water, inorganic salts, mucopolysaccharides, and bioactive compounds (Davies and Hawkins, 1998, and references therein), including lysozymes (McDade and Tripp, 1967), proteases (Brun *et al.*, 2000), and agglutinins (Fisher, 1992). Among molecules known for non-self recognition, lectins are a group of sugar-binding proteins that specifically and reversibly bind to sugar molecules covering living cells (Sharon and Lis, 2004). They are ubiquitously distributed in nature—found in viruses, bacteria, fungi, plants, invertebrates, and vertebrates (Iguchi *et al.*, 1985; Nyholm and McFall-Ngai, 2003). In bivalves, they have been identified in body extracts (Bulgakov *et al.*, 2004; Gourdine and Smith-Ravin, 2007), in hemolymph (Fisher and DiNuzzo, 1991; Tripp, 1992; Minamikawa *et al.*, 2004), and in mucus of the pallial cavity (Fisher, 1992). Several studies have demonstrated that by binding to specific carbohydrates, these proteins play significant roles in recognizing and neutralizing foreign substances (Vasta and Marchalonis, 1984; Sminia and Van der Knaap, 1986). Carbohydrates represent a universal component in the cell surface of most plants and animals, allowing organisms to interact with each other through the involvement of lectins (Olafsen, 1986). If lectins play a role in recognition of non-self cells, they could also be participating in particle selection through specific binding to sugars coating the cell wall of microalgae. In fact, lectins have been widely used in research for the identification of microalgal species (Hori *et al.*, 1996; Takahashi *et al.*, 2002; Tien *et al.*, 2005). Moreover, in discussing the role of mucus adhesion in particle selection in deposit-feeding invertebrates including polychaetes, an amphipod, a holothurian, and the bivalve *Macoma baltica*, Taghon (1982) strongly suggested that the adhesive interactions between particles and mucus could be very specific.

In this study, we hypothesized that the mucus covering the feeding organs of oysters contains lectins that are able to recognize carbohydrate compounds on the cell surface of microalgae and mediate the process of particle selection. To test this hypothesis, we designed a series of *in vitro* and *in vivo* experiments to address the following questions: (1) Does oyster mucus contain lectins that can interact with carbohydrate molecules on the surface of microalgae? (2) What is the carbohydrate specificity of such lectins? (3) Can lectins be used by oysters to discriminate among particles? We also examined some of the physical-chemical surface characteristics of the cells and particles used in the feeding assays. In particular, surface charge and wettability, two parameters that are widely used to characterize surfaces (Hunter, 1980; Loder and Liss, 1985; Hiemenz, 1986; Razatos *et al.*, 1998), were measured. These parameters have been shown to play a role in particle capture in animals that function at low Reynolds numbers (*e.g.,* Gerritsen and Porter, 1982; LaBarbera, 1984; Monger and Landry, 1990; Solow and Gallager, 1990; Hernroth *et al.*, 2000). Very little, however, is known about how surface properties affect particle selection. Therefore, we considered these parameters when drawing conclusions from the results of our study.

Materials and Methods

Organisms

Microalgal strains investigated in the study (Table 1) were obtained from the Milford Marine Laboratory collection (Connecticut), or were provided by Dr. Nicholas Fisher (Stony Brook University, New York). Microalgae were separately grown in F/2 medium (Guillard, 1982) at 15 °C under a light/dark cycle of 14 h:10 h. Juvenile (43.8 ± 4.7 mm in length) and adult (88.4 ± 8.3 mm in length) oysters

Table 1

List of the microalgal species used in the agglutination and feeding experiments

Species	Class	Ecological type	Size (μm)
Isochrysis sp.	Prymnesiophyceae	planktonic	5–6
Nitzschia closterium	Diatom (pennate)	benthic/planktonic	17–20
Tetraselmis maculata	Prasinophyceae	planktonic	10–18

were obtained from a commercial source (Frank M. Flower and Sons Oyster Company, Oyster Bay, New York). Their shells were cleaned and epibionts removed. All animals were acclimated in the laboratory for a minimum of 1 week (salinity of 28, 21 °C) where they were fed daily using DT's Live Marine Phytoplankton (15% dry weight; DT's Plankton Farm, Sycamore, IL), as described by Pales Espinosa and Allam (2006). They were purged in filtered (0.45 μm) seawater for a day prior to being used in the feeding experiments.

Agglutination of microalgae by pallial mucus

Six large oysters were V-notched at the posterior-ventral edge of the shell without damaging the mantle. The mantle cavity fluid was drained and the pallial cavity refilled, using a pipette, with artificial seawater (ASW: salinity of 30, filtered at 0.22 μm). The notch was then sealed with Parafilm, and the oysters were inverted several times to bathe the tissue surfaces. They were left at room temperature for 1 h before the pallial wash was recovered, centrifuged (300 $\times$ *g*, 5 min, 4 °C), and filtered (1-μm syringe filters) to remove debris. Additionally, six oysters were carefully opened and their pallial organs rinsed with ASW. Mucus covering the gills and labial palps was carefully and separately collected using sterile cotton-tipped swabs. Swabs were then immersed in 60 ml of ASW and stirred at ambient temperature for 1 h on a rotating shaker. The resulting fluid (mucus extract) was centrifuged at 400 $\times$ *g* for 15 min, filtered (0.22-μm syringe filters) to remove debris, and maintained at 4 °C until use, typically within the following hour. A 25-μl aliquot of plasma, pallial wash, and mucus extract were used to determine protein concentrations with a Pierce BCA protein assay reagent kit (Pierce, Rockford, IL) as per manufacturer's recommendations.

Microalgal cultures were centrifuged at 400 $\times$ *g* for 15 min, washed once, and resuspended in ASW. Agglutination assays were conducted in 96-well microtiter plates by mixing 50 μl of tested fluids (pallial wash or mucus extracts) with 50 μl of a suspension containing microalgal cells at a concentration of $5 \times 10^6 \cdot \text{ml}^{-1}$. Control wells contained 50 μl of ASW and 50 μl of algal cell suspensions. A second set of control cells contained 50 μl of ASW and 50 μl of tested fluids. In addition to these negative controls, two sets of positive controls were included. The first set of positive controls used horse and rabbit red blood cells (RBC) instead of microalgal cells, since these cells are well known to be agglutinated by bivalve lectins (Olafsen, 1986; Fisher and DiNuzzo, 1991). Red blood cells (10% suspensions) were obtained from Lampire Biological Laboratories (Pipersville, PA). They were centrifuged at 300 $\times$ *g* for 5 min, washed once in Alsever's solution and twice in ASW (salinity of 8.5), and re-suspended in ASW (salinity of 8.5; 2% RBC final concentration). Fifty microliters of this suspension was added to each well containing 50 μl of test fluid (pallial wash or mucus extracts). The second set of positive controls used oyster plasma instead of pallial wash or mucus extract because plasma has a known ability to agglutinate foreign particles (Olafsen, 1986; Fisher and DiNuzzo, 1991). In brief, hemolymph was withdrawn from the adductor muscle of each oyster with a syringe equipped with a 22-gauge needle and centrifuged at 300 $\times$ *g* for 5 min to remove hemocytes. The resulting supernatant (= plasma) was used in the agglutination assays as described above. To determine the carbohydrate specificity of the lectins, hemagglutination-inhibition assays were performed using selected carbohydrates (Table 2; Sigma Aldrich, USA, and V-labs, Inc., USA). Carbohydrates were added to mucus extracts from gills and labial palps and incubated for 1 h at room temperature before RBC were added.

Each agglutination assay was performed in triplicate. After incubation (2 h at room temperature), plates were examined under an inverted microscope and agglutination levels in each well determined using a semiquantitative scale ranging from 0 (no agglutination) to +++ (strong agglutination) (adapted from Allen *et al.*, 1977).

Feeding experiments

Effect of pallial mucus on particle selection. Two microalgal species, *Nitzschia closterium* and *Tetraselmis maculata*, were used in feeding experiments with oysters. Despite the fact that they present major differences (taxo-

Table 2

Effect of 12 different carbohydrates on hemagglutination activity of the pallial mucus extract from gills and labial palps of Crassostrea virginica

		Inhibition of hemagglutination	
Carbohydrates	Concentration	Mucus from gills	Mucus from labial palps
Arabinose-BSA	20 $\mu g \cdot ml^{-1}$	+	+
Fetuin	2 $mg \cdot ml^{-1}$	++	++
Galactose	36 $mg \cdot ml^{-1}$	NI	NI
Galactose-BSA	20 $\mu g \cdot ml^{-1}$	+	+
GalNAc	44.2 $mg \cdot ml^{-1}$	+	++
Glucosamide-BSA	20 $\mu g \cdot ml^{-1}$	++	++
Glucose	36 $mg \cdot ml^{-1}$	NI	NI
LPS	2 $mg \cdot ml^{-1}$	+	++
Mannan	2 $mg \cdot ml^{-1}$	+++	++
Mannose	36 $mg \cdot ml^{-1}$	NI	NI
Mannose-BSA	20 $\mu g \cdot ml^{-1}$	+	+
Mannopyranosylphenyl-BSA	20 $\mu g \cdot ml^{-1}$	++	++

Agglutination inhibition was tested with horse RBC. + = poor inhibition, ++ = moderate inhibition, +++ = strong inhibition; NI = no inhibition.

nomic group, shape, size), these two species were chosen because they are differentially selected by several bivalves: *T. maculata* is preferentially ingested, whereas *N. closterium* is preferentially rejected by the oyster *Crassostrea gigas* and other bivalves (Bougrier *et al.*, 1997; Barillé *et al.*, 2003; Pales Espinosa *et al.*, 2007). Both microalgae were separately incubated either in ASW (control) or in mucus extract (from both gills and labial palps), collected from oysters as described in the agglutination assays (above); protein concentrations were normalized to 800 $\mu g \cdot ml^{-1}$ with ASW. After incubation, microalgae were centrifuged ($400 \times g$, 15 min) and washed three times with ASW before being resuspended in ASW. Washing removed excess mucus that could interfere with the feeding experiment. Three diets were tested: Treatment 1—*N. closterium* and *T. maculata* incubated with ASW (control); Treatment 2—*N. closterium* incubated with mucus and control *T. maculata* cells; and Treatment 3—control *N. closterium* cells and *T. maculata* incubated with mucus. Equal concentrations of both microalgae were suspended in filtered seawater (total cell concentration $1 \times 10^5 \cdot ml^{-1}$). Adult oysters were placed in individual 3-l trays and delivered 1 l of a given treatment. Microalgae were kept in suspension using a magnetic stirrer. Pseudofeces were collected from each individual oyster 2 h after onset of production. Pseudofeces were homogenized to disperse aggregations and fixed with 10% buffered formalin. The samples were analyzed by counting a minimum of 300 microalgal cells under a microscope.

Uptake of carbohydrate-bound microspheres. Glucosamide-BSA and BSA were covalently bound to two types of carboxylated, polystyrene microspheres (6-μm diameter; Bangs Laboratories, Inc., Fishers, Indiana): fluorescent dragon green (excitation/emission = 480/520 nm) and fluorescent flash red (excitation/emission = 660/690 nm). The PolyLink Protein Coupling kit was used according to the manufacturer's instructions. The glucosamide-BSA was selected for use in the feeding experiment because it clearly inhibits the hemagglutination activity of lectins present in oyster mucus (see Results section for more details). About 500 μg of BSA and glucosamide-BSA were bound to 10^8 microspheres.

Equal concentrations of both types of microspheres were suspended in filtered seawater (total cell concentration $2 \times 10^5 \cdot ml^{-1}$). Juvenile oysters were placed in individual 1-l trays and delivered 800 ml of the microsphere suspension. Microspheres were kept in suspension using a magnetic stirrer. For each oyster, pseudofeces and feces were separately collected by micropipette 2 h after onset of production, homogenized to disperse aggregations, and analyzed using a flow cytometer (FACSCalibur, BD Biosciences, CA). The 488-nm argon and the 635-nm red diode lasers were used for excitation, and microspheres were identified using fluorescent parameters. A minimum of 10^4 events were analyzed.

Surface properties of phytoplankton and microspheres

To examine the influence of surface properties of cells and particles on selection, charge and wettability of phytoplankton and microspheres were determined. Surface charge estimates were based on electrophoretic mobility of the control and treated particles in filtered seawater (0.2 μm, similar to that used in feeding assays) at a salinity of 15 (pH 7.8). A Zetasizer-nano-ZS instrument (Malvern Instruments, Inc.) was employed that can determine mobility and calculate zeta potential of particles in solutions with a maximum ionic strength equivalent to a salinity of 15. Standard operating procedures were used with an attenuation ≤ 11, a monomodal setting, a run temperature of 20 °C, and 19 sampling runs per determination. A new disposable capillary cell was used for each replicate determination because the high ionic conditions caused corrosion of the cell's electrodes. Zeta potential was calculated by the instrument using the Smoluchowski-equation setting. Surface wettability was estimated from contact angle measurements with Milli-Q water (Hiemenz, 1986). Suspensions of each particle type (phytoplankton, microspheres) were passed through individual 3-μm polycarbonate filters (2.5-cm diameter) to form a particle pad that completely covered the filter. Pads were rinsed with 5 ml of isotonic ammonium formate to remove salts and dried at 70 °C overnight. A 4-μl drop (Della Volpe *et al.*, 2002) of Milli-Q water was carefully placed on each pad and immediately photographed with a digital camera mounted on a horizontal dissecting scope (*ca.* 35× magnification). Surface roughness was assumed to be similar for all particle pads.

The two phytoplankton species used in feeding assays, *Nitzschia closterium* and *Tetraselmis maculata*, were grown at a salinity of 30. Zeta potential, however, could only be determined at a maximum ionic strength equivalent to a salinity of 15. Therefore, to determine if surface charge of phytoplankton was altered when transferred from a salinity of 30 to 15, we also determined zeta potential of the two phytoplankton species grown at a salinity of 15. Culture concentrations of *N. closterium* and *T. maculata* ranged from 33 to 38 $\times 10^6 \cdot ml^{-1}$ and 4 to 8 $\times 10^6$ ml^{-1}, respectively. Samples of each culture were treated with mucus or seawater as described above. For charge estimates, subsamples were diluted to appropriate cell concentrations (*ca.* $2 \times 10^6 \cdot ml^{-1}$) in individual 2-ml microcentrifuge tubes just prior to analysis. For cultures grown at a salinity of 30, ionic strength was also adjusted with dropwise additions of Milli-Q to obtain a salinity of 15. A 1-ml syringe was used to transfer each suspension to a disposable capillary cell and analyzed immediately. Two replicate determinations were made for each suspension. For wettability

estimates, 2- to 4-ml subsamples of each experimental condition (two species, mucus and control treated, salinity of 15 and 30) were used to make each pad. At least two pads were made for each species and treatment combination. Controls were also prepared by passing several milliliters of filtered seawater (salinity of 15 or 30) through polycarbonate filters and rinsing with ammonium formate.

Microspheres with covalently attached BSA or glucosamide-BSA were prepared as described above. For charge estimates, 5 μl of each microsphere stock (sphere concentration *ca.* $1.2 \times 10^8 \cdot ml^{-1}$) were placed in individual 2-ml microcentrifuge tubes and diluted with 1 ml of filtered seawater (salinity of 15). Each suspension was transferred, using a 1-ml syringe, to a disposable capillary cell and analyzed immediately. Three replicate determinations were made for each suspension. For wettability estimates, about 0.8 ml of each microsphere stock was placed in individual 15-ml Falcon tubes and diluted with 5 ml of filtered seawater (salinity of 15). Tubes were then spun and supernatant removed; 10 ml of ammonium formate was added, and tubes were sonicated for 15 to 30 s in a bath sonicator to resuspend beads. The solution was then used immediately to make two pads (5 ml per pad).

Data analysis

The concentrations of protein in pallial wash samples, mucus extracts from gills, and extracts from labial palps were compared using a one-way analysis of variance (ANOVA). Data were examined for normal distribution and equality of variance prior to comparison.

For both feeding experiments, two series of goodness-of-fit tests (*G* test) were performed using raw counts to compare the proportion of each type of particle in diets and pseudofeces or feces samples. The first series of tests ensured that replicates within each treatment were homogeneous. The second series tested the null hypothesis that, within each treatment, the proportion of each type of particle (*i.e.,* the two species of microalgae or the two types of microspheres) was the same in diets and pseudofeces or feces samples.

In addition to the comparison of raw data, a sorting efficiency (SE) index was calculated in order to examine particle selection (Iglesias *et al.*, 1992). This index was defined as

$$SE = 1 - (P/D)$$

where P and D represent the proportion of the particle of interest in the pseudofeces and diet, respectively. A positive SE for a given particle type indicates that it is preferentially ingested (particle type is depleted in the pseudofeces, compared to diet), a negative SE indicates rejection (particle type is enriched in the pseudofeces compared to diet), and zero indicates the absence of active selection. After confirming their normal distributions, calculated SEs were individually compared to zero using a one-sample Student's *t*-test (two-tailed). The null hypothesis was that the selection efficiencies were equal to zero (*i.e.,* no selection). A second set of tests (one-way ANOVAs) compared SEs obtained for each type of experimental particles among different treatments. In all statistical tests, results were considered significant if $P < 0.05$. Significant ANOVA results were followed with Fisher's least significant difference (LSD) *post hoc* test for pairwise comparisons.

Zeta-potential data for the two phytoplankton species were first analyzed for differences between the two salinity conditions (15 and, 30) within each species by treatment condition using a two-sample *t*-test. No significant differences were found ($P > 0.05$), so data were pooled and subsequently analyzed with a two-way ANOVA (Zar, 1984; Systat ver. 1.1; Systat Software, Inc.). Phytoplankton species (*N. closterium, T. maculata*) and treatment (seawater, mucus) were used as the independent variables. Zeta-potential data for the two microspheres were analyzed using a two-sample *t*-test. Contact angles were analyzed with a similar design, but procedures for circular-scale data were used (Zar, 1984; Oriana software ver. 2.02; RockWare, Inc.). Differences between independent variables were examined using a Watson-Williams *F*-test (multisample and pairwise comparisons). Unless otherwise indicated, all data are given as mean $\pm$ standard deviation (SD).

Results

Agglutination of microalgae by pallial mucus

Protein concentration in plasma averaged 4955 ± 410 $\mu g \cdot ml^{-1}$. Lower protein levels were measured in the pallial wash (358 ± 26 $\mu g \cdot ml^{-1}$) and mucus extracts from gills (406 ± 27 $\mu g \cdot ml^{-1}$) and labial palps (374 ± 23 $\mu g \cdot ml^{-1}$). Oyster plasma, pallial wash, and extract of gill mucus agglutinated both horse and rabbit erythrocytes with comparable intensities (Figs. 1 and 2). Agglutination levels ranged from 2.9 ± 0.5 to 3.4 ± 0.7 in plasma, 3.1 ± 0.8 to 3.4 ± 1.0 in pallial wash, and 2.8 ± 0.8 to 2.8 ± 1.0 in mucus extract from gills. Agglutination levels were noticeably lower when mucus extracts from labial palps were incubated with rabbit RBC (0.7 ± 0.3), as compared to incubation with horse RBC (2.4 ± 0.3). No agglutination was found in the ASW controls. Both plasma and pallial wash of *Crassostrea virginica* agglutinated three tested microalgal species belonging to three different classes (Fig. 1). The agglutination level ranged from 0.8 ± 0.3 to 2.7 ± 0.2 in plasma, and 2.6 ± 0.5 to 4.1 ± 0.1 in pallial wash. Extracts of mucus from gills and labial palps also agglutinated several microalgal species. Agglutination levels ranged from 0.1 ± 0.1 to 4.4 ± 0.3 for *Isochrysis* sp. and *Tetraselmis maculata*, respectively. It is noteworthy that, for the same algal species, agglutination level varied ac-

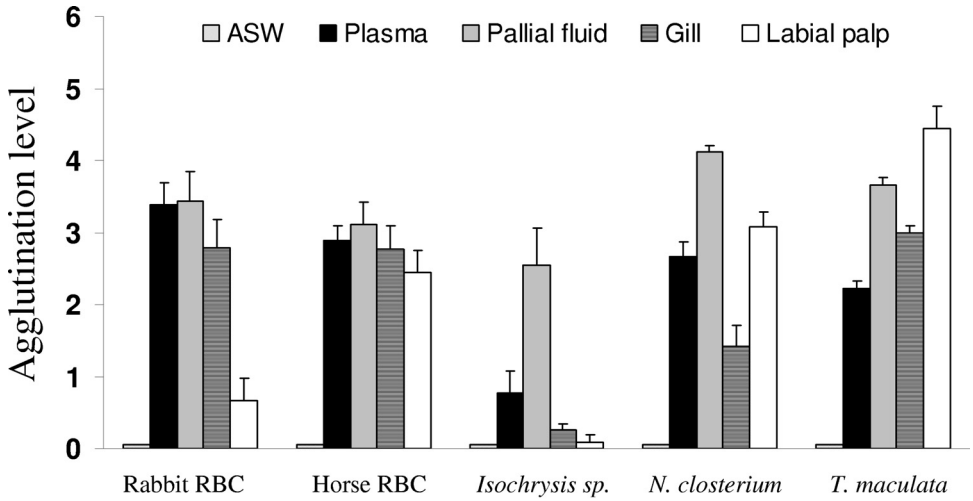

Figure 1. Agglutination activity of artificial seawater (ASW), plasma, pallial wash, and mucus extracts from gills and labial palps on rabbit and horse red blood cells (RBC), and several microalgae species. Data are mean ± SD for 3 replicate measurements.

cording to the origin of mucus. Thus, the agglutination level obtained with *Isochrysis sp.* was higher when tested with mucus collected from gills. In contrast, *Nitzschia closterium* and *T. maculata* were more agglutinated by mucus originating from labial palps.

Carbohydrate-binding specificity of lectins contained in mucus

Results of hemagglutination (HAG)-inhibition experiments showed that lectin activities were not inhibited by monosaccharides such as galactose, glucose, or mannose (Table 2). Extracts of mucus from gills and labial palps HAG were poorly inhibited by the presence of arabinose-BSA, mannose-BSA, galactose-BSA, GalNAc (*N*-acetyl-galactosamine), and lipopolysaccharide. The highest HAG inhibition was obtained in the presence of mannan, glucosamide-BSA, and fetuin.

Feeding experiments

Effect of pallial mucus on particle selection. Total microalgal cell concentrations in Treatments 1, 2, and 3 were $1.1 \pm 0.1 \times 10^5 \cdot ml^{-1}$, $1.0 \pm 0.1 \times 10^5 \cdot ml^{-1}$, and $1.1 \pm 0.1 \times 10^5 \cdot ml^{-1}$, respectively. Although the targeted proportion of each microalga was 50%, an *a posteriori* count revealed the following proportions of *Nitzschia closterium* and *Tetraselmis maculata*: 49.1 (±2.4%):50.9 (±2.3%) for Treatment 1; 48.9 (±1.4%):51.1 (±2.7%) for Treatment 2; and 48.0 (±3.4%):52.0 (±2.8%) for Treatment 3 (Fig. 3). Goodness-of-fit tests revealed, however, that these slight variations were not significantly different. The proportions remained stable over the course of the experiment, indicating that differential settling of microalgal species had not occurred.

When *Crassostrea virginica* received Treatment 1 (control, both microalgal species incubated in ASW), the proportion of *Nitzschia closterium* and *Tetraselmis maculata* changed significantly in the pseudofeces compared to the diet ($n = 8$, G test, $P < 0.001$). The proportion of *N. closterium* increased to 90.9% ± 1.5%, and the proportion of *T. maculata* decreased to 9.1% ± 1.5% (Fig. 3A). When oysters were delivered Treatments 2 and 3 (one of the microalgal species incubated with mucus), the proportion of *N. closterium* also increased significantly in the pseudofeces compared to the diets (Fig. 3B, C), but these increases were diminished. The proportion of *N. closterium* in the pseudofeces was 85.7% ± 1.7% and 69.8% ± 5.9% for Treatments 2 and 3, respectively (*cf.* Treatment 1 above). Sorting efficiencies (SE) confirmed the above results. For all three treatments, oysters preferentially ingested *T. maculata* while they rejected *N. closterium* (Table 3, $n = 7–8$, *t*-test, $P < 0.001$). SE for *T. maculata* in Treatment 1 was, however, significantly higher than those in Treatments 2 and 3 (Table 3, one-way ANOVA followed by a Fisher LSD *post hoc* test, $P < 0.001$).

Uptake of carbohydrate-bound microspheres. Results obtained in this experiment support the assertion that lectins associated with pallial organ mucus are involved in the particle selection mechanisms of *Crassostrea virginica*. Total concentration of microspheres in diet was $1.8 \pm 0.2 \times 10^5 \cdot ml^{-1}$, and flow cytometric analysis revealed a proportion of 53.1% ± 3.1% BSA-coated microspheres to 46.9% ± 3.1% glucosamide-BSA-coated microspheres in the suspension (Fig. 4). These proportions remained stable over the

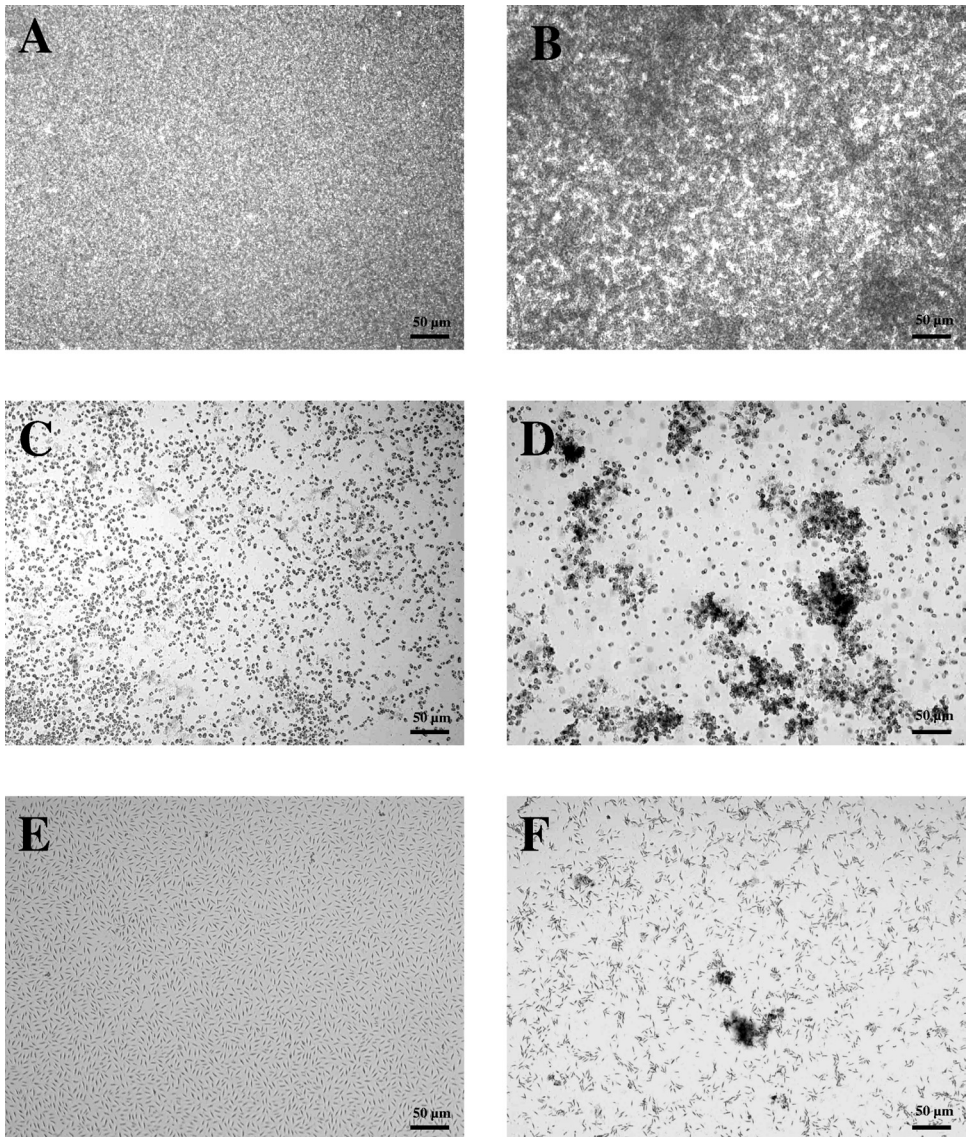

Figure 2. Agglutination of red blood cells and microalgae by mucus covering pallial organs of the oyster *Crassostrea virginica.* Rabbit erythrocytes incubated with ASW (A, control) or pallial wash (B); *Tetraselmis maculata* incubated with ASW (C, control) or mucus extracts from labial palps (D); and *Nitzschia closterium* incubated with ASW (E, control) or mucus extracts from gills (F). Scale bar = 50 μm.

course of the experiment. When oysters were delivered the two types of microspheres, the proportion of BSA-coated spheres to glucosamide-BSA-coated microspheres changed significantly compared to the diet ($n = 8$, G test, $P < 0.001$). In the pseudofeces, the proportion of BSA-coated spheres was higher (60.2% ± 4.3%), whereas the proportion of the glucosamide-BSA-coated microspheres was lower (39.8% ± 4.3%). In the feces, the inverse was found. These biodeposits contained a higher proportion of glucosamide-BSA-coated (58.8% ± 5.6%) than BSA-coated microspheres (41.2% ± 5.6%). The SE values confirmed that oysters preferentially ingested glucosamide-BSA-coated microspheres (SE = 0.13 ± 0.10), and rejected BSA-coated microspheres (SE = −0.11 ± 0.09; $n = 8$, t-test, $P < 0.001$).

Surface properties of phytoplankton and microspheres

There was no significant effect of species (*N. closterium, T. maculata*) or treatment (seawater, mucus) on zeta potential ($n = 8$–9, two-way ANOVA, $P > 0.05$). Similarly, these two independent variables had no effect on contact angle (Table 4, $n = 8$–9, Watson-Williams F, $P < 0.05$). Contact angles of all phytoplankton pads were significantly higher than those of seawater-treated, blank filters (no phytoplankton; mean vector = 59.4° ± 3.0° circular SD, $n =$

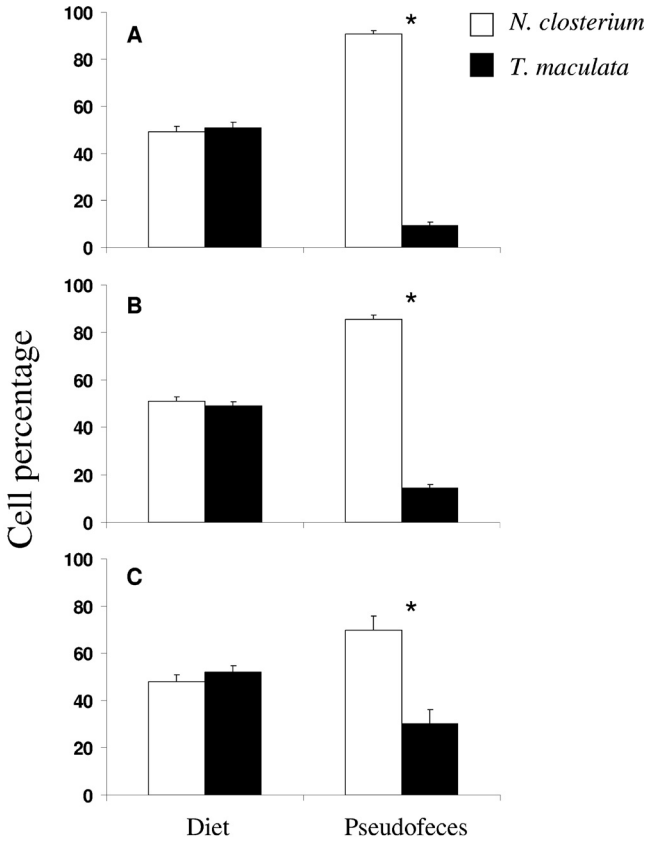

Figure 3. Percentage (mean ± SD) of *Nitzschia closterium* and *Tetraselmis maculata* in food supply (Diet) and pseudofeces (PF) of *Crassostrea virginica* fed with a mixture of (A) untreated algae cells (control, Treatment 1, $n = 8$); (B) *N. closterium* cells incubated in mucus and untreated *T. maculata* (Treatment 2, $n = 7$); or (C) untreated *N. closterium* and *T. maculata* incubated in mucus (Treatment 3, $n = 8$). * indicates significant difference for each microalgal species between diet and pseudofeces (goodness-of-fit G test, $P < 0.001$).

9). Zeta potentials of the two microspheres were significantly different (i = 3, t-test, $P < 0.001$), with BSA-coated microspheres having a higher mean value (-8.8 ± 1.0) than glucosamide-BSA-coated microspheres (-4.6 ± 0.8). Similar results were obtained for contact angle, with BSA-coated microspheres having a significantly higher ($n = 4$, t-test, $P < 0.001$) mean vector value (88.2° ± 5.0° circular SD) than glucosamide-BSA-coated microspheres (42.6° ± 5.5° circular SD).

Table 3

Sorting efficiency (mean ± SD) of Nitzschia closterium *and* Tetraselmis maculata *in* Crassostrea virginica *fed each of the three treatments*

	Sorting efficency	
Treatment	*N. closterium*	*T. maculata*
1	−0.85 ± 0.03	0.82 ± 0.03
2	−0.75 ± 0.03	0.72 ± 0.03
3	−0.45 ± 0.12	0.42 ± 0.11

SE was determined from 8, 7, and 8 oysters in Treatment 1, 2, and 3 respectively. SE values for each algal species were significantly different among the three treatments (ANOVA; $P < 0.001$).

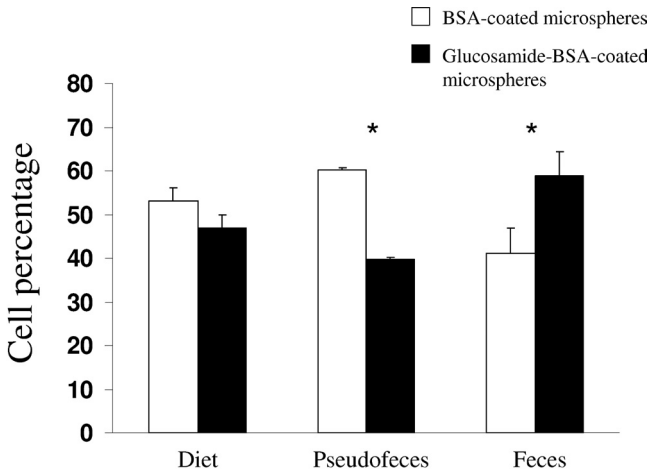

Figure 4. Percentage (mean ± SD, $n = 8$ oysters) of BSA-coated microspheres and glucosamide-BSA-coated microspheres in food supply (Diet), pseudofeces, and feces of *Crassostrea virginica*. * indicates significant difference for each type of microsphere between diet and pseudofeces or feces (goodness-of-fit G test, $P < 0.001$).

Discussion

This study provides evidence that substances within the mucus from the pallial organs of the Eastern oyster, *Crassostrea virginica*, are involved in the mechanism of recognition and qualitative selection of food particles. The results suggest a new concept with regard to particle processing by the feeding organs: specific interactions between carbohydrates on the surface of particles and lectins within the mucus can mediate the selection and rejection processes.

For instance, our results showed that substances con-

Table 4

Surface properties of the microalgae Nitzschia closterium *and* Tetraselmis maculata *treated with either filtered seawater (0.2 μm; control) or mucus from gill and labial palps of oysters*

	Zeta potential (mV)		Contact angle (°)	
Species	Control	Mucus	Control	Mucus
N. closterium	−10.2 (1.1)[a]	−10.4 (0.7)[a]	92.9 (4.3)[a]	95.5 (2.5)[a]
T. maculata	−10.8 (0.4)[a]	−9.2 (2.2)[a]	93.1 (5.4)[a]	91.3 (3.0)[a]

Zeta potentials are presented as means of 4 to 5 replicate measurements ± standard deviation in parentheses. Contact angle data are given as mean vector (μ) of 8 to 9 replicate measurements ± circular standard deviation in parentheses. Within each data set, means with the same letters are not significantly different at the $\alpha = 0.05$ level.

tained in the mucus were able to agglutinate vertebrate erythrocytes (rabbit and horse) and several marine microalgae from different families, confirming the results of Fisher (1992), who demonstrated that agglutinins present in the pallial mucus of the same oyster species clumped together vertebrate erythrocytes as well as several marine bacteria. Additionally, our results clearly demonstrated that the agglutination of vertebrate erythrocytes and microalgae was inhibited by carbohydrates. These two observations fulfill the definition for lectins proposed by Goldstein *et al.* (1980) as "sugar-binding proteins of non-immune origin that agglutinate cells."

Further results of the feeding experiments strongly emphasize the role of mucus lectins in the particle selection process. In the first set of feeding experiments, microalgal cells were incubated with mucus from the pallial cavity. The expectation was that lectins in the mucus would bind to and saturate carbohydrate residues on the surface of the microalgal cells. Results of these experiments showed that selection indices significantly decreased when oysters were fed a diet composed of microalgae incubated in mucus compared to indices obtained from oysters fed the control diet (same microalgal species, not incubated in mucus). Therefore, oysters, which are known to ingest *Tetraselmis maculata* and reject *Nitzschia closterium* preferentially (Pales Espinosa *et al.*, 2007), were less able to discriminate between these two microalgae after they were treated with mucus. Importantly, the surface charge and wettability of the two phytoplankton species were not significantly different and did not change after being treated with mucus, indicating that these parameters did not influence selection. Instead, we suggest that the change in selective capability resulted from the saturation of binding sites on algal cells during pre-incubation in mucus. Thus, saturated algal cells were less reactive to the lectins present in mucus produced by the pallial organs.

Additional evidence for the role of lectins in particle selection was obtained when oysters were fed microspheres coated with a carbohydrate known to inhibit hemagglutination activity. When presented in equal proportions in the diet, microspheres coated with glucosamide-BSA were significantly ingested, whereas microspheres coated with BSA alone were rejected in pseudofeces. The use of microspheres of the same size and material greatly reduced the variability associated with the use of living cells. Although the surface properties (charge, wettability) of the BSA and glucosamide-BSA-coated microspheres were different, comparison with the surface properties of the microalgae adds additional information. For example, the surface properties of the particles that were preferentially ingested (*T. maculata* and glucosamide-BSA-coated microspheres) were significantly different (zeta potential: ANOVA followed by Fisher's LSD, $P < 0.01$; contact angle: Watson-Williams F-Test, $P < 0.01$); nevertheless, both were preferentially ingested. The surface properties of *T. maculata* and BSA-coated microspheres were not significantly different (zeta potential: ANOVA, $P > 0.05$; contact angle: Watson-Williams F-Test, $P > 0.05$), yet *T. maculata* was preferentially ingested whereas BSA-coated microspheres were rejected. These results further support the idea that, in this study, the surface properties of particles had little influence on the selection of particles. Overall, these results confirmed our *a priori* expectation that spheres with a coating of glucosamide would be preferentially ingested. Moreover, covalent binding (efficient chemical attachment) on polystyrene-carboxyl microspheres allowed us to study the role of a single carbohydrate in particle selection.

Our hypothesis is also supported by several studies reporting the presence of lectins in bivalve molluscs and their role in mechanisms involving recognition. Thus, several lectins have been identified in bivalves, particularly in plasma (Suzuki and Mori, 1989; Tripp, 1992; Bulgakov *et al.*, 2004; Takahashi *et al.*, 2008), hemocytes (Tasumi and Vasta, 2007), and mucus covering the pallial organs (Fisher, 1992). Lectins have also been found in the gills of the symbiotic clam *Codakia orbicularis* by Gourdine and Smith-Ravin (2007). Lectins are well known to play an important role in the recognition of foreign particles and more specifically in the defense system of vertebrates and invertebrates (Sharon and Lis, 2004). Additionally, the role of lectins has also been examined in other mechanisms in marine organisms, involving interactions between microalgae and invertebrates, such as symbiosis (Jimbo *et al.*, 2000; Wood-Charlson *et al.*, 2006) or feeding process. Recently, Wootton *et al.* (2007) clearly identified a mannose-binding lectin that is involved in the feeding process of the dinoflagellate *Oxyrrhis marina*.

Results of the hemagglutination inhibition assays reported here suggest that several types of lectins may be present in pallial mucus, and their activities are more specific to fetuin, mannan, and glucose-related residues. The probable diversity of lectins in pallial mucus would not be surprising given the diversity of food particles selected by oysters. In addition, we showed that mucus from gills and labial palps does not have the same agglutination profile. Thus, agglutination activity displayed by mucus from gills was more intense against horse erythrocytes, whereas agglutination activity displayed by mucus from labial palps was higher when rabbit erythrocytes were used. Similar results were obtained for microalgal cells. Agglutination activity against *Isochrysis* sp. was higher when tested with mucus from gills, whereas *Nitzschia closterium* and *Tetraselmis maculata* were more agglutinated by mucus originating from labial palps. Such results suggested that the mucus covering gills and labial palps have different lectin profiles, which might reflect the different functions of these two organs in the particle selection process.

The difference observed between the agglutination spec-

ificity raises questions concerning the origin of lectins and their role in particle selection in the oyster. Fisher (1992) proposed that agglutinating substances could be produced by mucus cells, or mucocytes, on the mantle and gill. Moreover, mucocytes present in pallial organs produce different types of mucus according to the function of each organ (Beninger and St-Jean, 1997b, and references therein; Beninger *et al.*, 2004). In five bivalves belonging to different families, viscous mucus is associated with indiscriminate transport on the ventral tracts of the gill and rejection in pseudofeces. Mucus with intermediate viscosity is involved in the transport of particles destined for ingestion, and mucus with low viscosity is found in areas such as the labial palps (Beninger and St-Jean, 1997b), where reduction of mucus viscosity is necessary. Thus, these authors demonstrated that mucuses derived from gills and labial palps differ in terms of their chemical composition. Beninger *et al.*, (2005) have also shown that distinct types of mucocytes are present at different locations on the gill of the Pacific oyster (*Crassostrea gigas*). For instance, at the apex of the ordinary filaments of the gill are two ciliated tracts associated with different types of mucus-producing cells: the frontal lateral tract, in which cilia beat dorsally, is associated with mucus with intermediate viscosity; the frontal median tract, in which cilia beat ventrally, is associated with mucocytes producing high-viscosity mucus. These differences in mucus viscosity could also be associated with variations in the composition of agglutinating substances.

The implication of multiple lectins in the selection process requires further investigations, but we can speculate that these bioactive molecules associated with gill mucus bind target cells and participate in their transport toward the labial palps. In *Crassostrea virginica*, small microalgal species (*Tetraselmis* sp. and *Rhodomonas lens*) are directed to the dorsal tract, whereas detrital particles with similar size are transferred to the ventral tract (Ward *et al.*, 1997). Additionally, in the oyster *Crassostrea gigas*, the intact diatom *Actinoptychus senarius*, which is small enough to enter the gill principal filament, is directed to the dorsal tract, whereas clean empty frustules (no organic coating) of the same diatom are transferred to the ventral tract (Beninger *et al.*, 2008). These results suggest that lectins associated with gills could bind with particles and mediate the direction of transport (dorsal, ventral). When transferred from the gill to the labial palps of *C. virginica*, mucus is reduced in viscosity and particles are dispersed (Ward *et al.*, 1994; Ward, 1996), probably due to mechanical or chemical action between the palp lamellae (Newell and Jordan, 1983; Ward, 1996). Once liberated from the embedded mucus, particles could then interact with lectins associated with palp mucus, resulting in their ingestion or their rejection in pseudofeces. This scenario may represent a mechanistic explanation for the sorting of large microalgae (>70 μm), such as the diatom *Coscinodiscus perforatus*, that are frequently and naturally ingested by oysters and, after transit through the ventral pathway, may be subjected to a second selection at the labial palps of the oysters (see Cognie *et al.*, 2003).

This is the first study to demonstrate the involvement of lectins in the mucus covering the feeding organs in the particle sorting process of oysters. We propose that the mechanism of selection in suspension-feeding bivalves in general is at least partially mediated by interactions between lectins and food particles. These molecules need to be isolated from mucus, characterized, and individually tested for their involvement in particle sorting. The exact role of mucus lectins in the selection process also requires further investigation, in particular an evaluation of lectin diversity, mapping of lectin-producing cells on feeding organs, and determination of the pathways of these molecules. Future studies should also investigate the potential influence of the surface properties of particles (charge, wettability) on the selection process, since these factors have been shown to affect particle capture in bivalve larvae (Gallager *et al.*, 1988; Solow and Gallager, 1990), zooplankton (Gerritsen and Porter, 1982; LaBarbera, 1984; Monger and Landry, 1990), and mussels (Hernroth *et al.*, 2000). Although the effects of surface properties on particle selection by adult bivalves are not known, these parameters may play a role in the discrimination of certain types of particles.

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