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Microalgal Cell Surface Carbohydrates as Recognition Sites for Particle Sorting in Suspension-Feeding Bivalves

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Abstract. Cell surface carbohydrates play important roles in cell recognition mechanisms. Recently, we provided evidence that particle selection by suspension-feeding bivalves can be mediated by interactions between carbohydrates associated with the particle surface and lectins present in mucus covering bivalve feeding organs. In this study, we used lectins tagged with fluorescein isothiocyanate (FITC) to characterize carbohydrate moieties on the surface of microalgal species and evaluate the effect of oyster mucus on lectin binding. These analyses revealed that concanavalin A (Con A), one of six lectins tested, bound to *Isochrysis* sp., while *Nitzschia closterium* reacted with *Pisum sativum* agglutinin (PNA) and peanut agglutinin (PEA). The cell surface of *Rhodomonas salina* bound with PNA and Con A, and *Tetraselmis maculata* cell surface was characterized by binding with PNA, PEA, and Con A. Pre-incubation of microalgae with oyster pallial mucus significantly decreased the binding of FITC-labeled lectins, revealing that lectins present in mucus competitively blocked binding sites. This decrease was reversed by washing mucus-coated microalgae with specific carbohydrates. These results were used to design a feeding experiment to evaluate the effect of lectins on sorting of microalgae by oysters. *Crassostrea virginica* fed with an equal ratio of Con A-labeled *Isochrysis* sp. and unlabeled *Isochrysis* sp. produced pseudofeces that were significantly enriched in Con A-labeled *Isochrysis* sp. and depleted in unlabeled microalgae. Selection occurred even though two physical-chemical surface characteristics of the cells in each treatment did not differ significantly. This work confirms the involvement of carbohydrate-lectin interaction in the particle sorting mechanism in oysters, and provides insights into the carbohydrate specificity of lectins implicated in the selection of microalgal species.

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Abbreviations: Con A, concanavalin A; FITC, fluorescein isothiocyanate; GalNAc, *N*-acetylgalactosamine; PEA, *Pisum sativum* agglutinin; PNA, peanut agglutinin: SBA, soy bean agglutinin; UEA 1, *Ulex europaeus* agglutinin; WGA, wheat germ agglutinin.

Introduction

Microalgae represent one of the most important sources of food for suspension-feeding bivalves, even if some species are known to ingest and digest detritus, bacteria, or protozoans (Kreeger and Newell, 2001). In *Crassostrea virginica*, the eastern oyster, some microalgal species (*e.g., Tetraselmis* sp. and *Rhodomonas lens* Pascher et Ruttner) are preferentially ingested, whereas detrital particles with similar size are transferred to the ventral tract and rejected in pseudofeces (particles expelled without having passed through the digestive tract) (Ward *et al.,* 1997). Furthermore, suspension-feeding bivalves are well known to select among microalgal species within the same size range. For example, *Tetraselmis maculata* is preferentially ingested whereas *Nitzschia closterium* is preferentially rejected by the oyster *C. gigas* and other bivalves (Shumway *et al.*, 1985; Bougrier *et al.*, 1997; Barillé *et al.*, 2003; Pales Espinosa *et al.*, 2007). Despite abundant descriptive evidence for a particle sorting process (Ward and Shumway, 2004), the mechanisms upon which bivalves rely to discriminate among particles have not been elucidated. Some studies have, however, demonstrated that microalgal chemicals represent important factors mediating particle selection in

bivalves (Shumway *et al.*, 1985; Ward and Targett, 1989; Pales Espinosa *et al.,* 2007, 2009). For example, ectocrines on the surface of microalgae or in a boundary layer surrounding cells are thought to be essential in particle selection (Ward and Targett, 1989; Pales Espinosa *et al.*, 2007). Investigations by Beninger *et al.* (2008) in the Pacific oyster *C. gigas* showed that the intact diatom *Actinoptychus senarius* Ehrenberg and clean empty frustules (no organic coating) of the same diatom are processed differently by the gills. Our recent investigations focused on the study of the interactions between the organic coating of food particles and mucus covering feeding organs, where we demonstrated the presence of lectins in the mucus that covers gills and labial palps of the oyster *Crassostrea virginica*, and proposed the involvement of these molecules in the mechanisms of microalgal recognition and sorting in this bivalve (Pales Espinosa *et al.*, 2008, 2009).

Lectins are sugar-binding proteins of nonimmune origin that agglutinate cells. They are known to play a significant role in several physiological mechanisms involving cell-cell or cell-matrix interactions. Several lectins have been identified in marine invertebrates and more specifically in bivalve plasma (Suzuki and Mori, 1989; Bulgakov *et al.*, 2004; Takahashi *et al.*, 2008) and hemocytes (Vasta *et al.*, 1982; Tasumi and Vasta, 2007) where they are involved in the defense system against pathogens. Lectins are also involved in other interactions involving biorecognition, such as symbiotic associations (Jimbo *et al.,* 2000; Koike *et al.*, 2004). Thus, Wood-Charlson *et al.* (2006) have provided evidence that lectin-glycan are influential during the onset of symbiosis between coral larvae and their endosymbiotic dinoflagellate. In bivalves, lectins have been found associated with the gills of the symbiotic clam *Codakia orbicularis* by Gourdine and Smith-Ravin (2007), who hypothesized that these molecules have a role in symbiont (sulfo-oxidant bacteria) acquisition. The involvement of lectins in the selection of food particles by aquatic organisms has been hypothesized (Fisher, 1992) but demonstrated in only a few studies (Allen and Dawidowicz, 1990; Wootton *et al.*, 2007). As an example, Wootton *et al*. (2007) have clearly identified a mannose-binding lectin on the dinoflagellate *Oxyrrhis marina* that is involved in recognition of prey microalga such as *Isochrysis galbana* Parke.

In this study, our working hypothesis was that carbohydrate signatures present on the cell surface of microalgae are involved in particle selection by the oyster *C. virginica*. During suspension feeding, the mucillaginous matrix of microalgal cells comes in contact with mucus on the feeding organs of bivalves (*i.e.,* gills and labial palps; Ward *et al.*, 1993); therefore, the carbohydrate moieties could bind with lectins associated with this mucus and mediate the selection process. To test this hypothesis, we addressed the following questions: (1) What carbohydrate moieties are present on the surface of microalgae known to be ingested or rejected by oysters? (2) Do lectins contained in pallial mucus of oysters bind carbohydrates present on the surface of microalgae? (3) Does blocking of carbohydrates moieties on the surface of a microalga affect particle recognition by oysters, or affect the physical-chemical surface characteristics (*i.e.,* surface charge and wettability) of the cells?

Materials and Methods

Organisms

Microalgal species investigated in this study were obtained from the collection of the Milford Laboratory of the NOAA Northeast Fisheries Science Center (Milford, Connecticut). Algal species were grown separately in F/2 medium (Guillard, 1982) at 15 °C under a cycle of 14-h light/10-h dark. Oysters (*Crassostrea virginica* Gmelin) belonging to two size classes (43.8 ± 4.7 mm and 88.4 ± 8.3 mm in length) were obtained from a commercial source (Frank M. Flower and Sons Oyster Company, Oyster Bay, New York), and their shells were cleaned of epibionts. Oysters 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% of dry weight), (Sycamore, IL; Pales Espinosa and Allam, 2006). One day prior to being used in the feeding experiments, oysters were purged in filtered seawater (0.45 μm).

Binding of FITC-labeled lectins to microalgae

Procedures used to bind lectins to microalgae were adapted from Gauthier *et al.* (2004). Microalgal cultures were centrifuged at 400 × *g* for 10 min, washed once, and resuspended in FSW. FITC-conjugated lectins (Sigma Aldrich, USA; Table 1) were diluted in FSW (filtered artificial seawater, 0.22 μm) to 1 mg · ml^{-1}. One 50-μl aliquot of lectin (or FSW control) was added to each of three replicate microcentrifuge tubes containing 1 ml of washed microalgae (10^6 cells). Microalgae were then incubated in the dark at room temperature for 1 h. Carbohydrate specificity was evaluated for lectin that bound to each microalgae species with the highest affinity. Lectins (50 μl) were separately mixed with 950 μl of competitive carbohydrates (150 mmol l^{-1} final concentration in 150 mmol l^{-1} NaCl) and incubated for 30 min before the addition of washed microalgae (10^6 cells, final concentration). Tested carbohydrates included D-glucose, D-mannose, D-galactose, *N*-acetylgalactosamine, and α-lactose (Sigma). After 1 h, microalgae were washed three times by centrifugation (400 × *g* for 10 min) with FSW and resuspended in FSW for flow cytometric analysis. Each assay was performed in triplicate.

Effect of pallial mucus on the binding of FITC-labeled lectins

In this experiment we determined if lectins present in mucus from the pallial organs of oysters competitively

Table 1

Lectins used in the study and their carbohydrate specificity

Lectin	Abbreviation	Source	Specificity
Concanavalin agglutinin	ConA	*Canavalia ensiformis*	Methyl α-mannopyranoside; D-Mannose; D-Glucose
Pisum sativum agglutinin	PEA	*Pisum sativum*	α-Methyl mannoside; α-Methyl glucoside
Peanut agglutinin	PNA	*Arachis hypogaea*	α-Lactose; D-Galactose; *N*-Acetylgalactosamine
Soybean agglutinin	SBA	*Glycine maxima*	N-Acetylgalactosamine; D-Galactose; Methyl α-D-galactopyranoside
Ulex europaeus agglutinin	UEA I	*Ulex europaeus*	L-Fucose
Wheat germ agglutinin	WGA	*Triticum vulgaris*	N-Acetylglucosamine

affected the binding of commercial lectins labeled with fluorescein isothiocyanate (FITC) to microalgae, and determined carbohydrate specificity of the mucus-associated lectins. Four groups of large oysters were collected in June 2007, December 2007, January 2008, and February 2008. Bivalves were carefully opened and the pallial organs rinsed with FSW. Mucus covering the gills and labial palps was separately collected using sterile, cotton-tipped swabs. Special care was taken to avoid damaging the epithelial layer covering the organs. Swabs were then immersed in 60 ml of FSW and stirred at 4 °C for 1 h on a rotating shaker. The resulting fluid (gill and labial palp mucus extract) was centrifuged at 400 × *g* for 15 min, filtered (0.22-μm syringe filters) to remove debris, and maintained at 4 °C until used, which was typically within 1 h. A 25-μl aliquot of mucus extract was used to determine protein concentrations with a Pierce BCA protein assay reagent kit (Pierce, Rockford, IL) as per the manufacturer's recommendations. Protein concentration in mucus samples was normalized to 750 $\mu g \cdot ml^{-1}$ by adding FSW before being used to treat microalgae. Microalgae were prepared as indicated above, and cells (10^6) were mixed with 1 ml of either gill or labial-palp mucus extract or with FSW as a control and incubated at room temperature for 1 h. Microalgae were then washed three times with FSW (400 × *g*, 10 min) and labeled with FITC-conjugated lectins as described above. To test carbohydrate specificity of mucus-associated lectins, microalgae were incubated with mucus extracts before being treated with selected competitive carbohydrates. Following incubation (30 min at 21°C), microalgae were washed three times with FSW, labeled with FITC-conjugated lectins as described above, and analyzed by means of a flow cytometer. Each assay was performed in triplicate.

Feeding experiments

To evaluate the involvement of carbohydrates on the cell surface of microalgae in particle selection by oysters, cells of *Isochrysis* sp. were treated with either seawater or a commercially available FITC-lectin. Our working hypothesis was that by saturating the cell-surface carbohydrates, these moieties would be unavailable to bind with mucus-associated lectins produced by oysters during the feeding process, thus enhancing their rejection (or alternatively, ingestion) by bivalves. An exponential culture of *Isochrysis* sp. (4×10^6 cell $\cdot$ ml^{-1}) was washed as indicated above, resuspended in FSW, and divided into two aliquots. One aliquot was then incubated with FITC-labeled Con A in FSW (50 $\mu g \cdot ml^{-1}$ per 10^6 cells) for 1 h in the dark at room temperature. After incubation, all microalgal aliquots were washed three times with FSW (400 × *g*, 10 min) and resuspended in FSW. This labeling step did not affect cell viability, size, or intracellular complexity, as confirmed using microscopy and flow cytometric analyses. Similarly, labeling with Con A did not affect the surface properties of the *Isochrysis* sp. cells (see below). Labeled and unlabeled cells of *Isochrysis* sp. and polystyrene carboxyl microspheres (6-μm diameter, Bangs Laboratories, Inc., IN) were used to formulate three experimental diets as follows: Treatment 1, unlabeled *Isochrysis* sp. and polystyrene microspheres; Treatment 2, labeled *Isochrysis* sp. and microspheres; Treatment 3, labeled and unlabeled *Isochrysis* sp. Equal concentrations of each particle type were suspended in filtered seawater (1×10^5 cells $\cdot$ ml^{-1} final concentration) and delivered to small oysters maintained in individual 1-l tanks. Microspheres were kept in suspension by means of gentle disruption with a micropipette every 15 min, and water samples were taken periodically to determine sedimentation. Pseudofeces were collected from each tank 2 h after onset of production. Pseudofeces were homogenized to disperse aggregations and fixed with 10% buffered formalin, and their composition was determined using flow cytometry. Prior to the analysis, pseudofeces samples were vortexed to disrupt particle aggregates and passed through a 35-μm, nylon-mesh sieve.

Surface properties of Isochrysis sp.

To determine the effects of Con A treatment on the surface properties of *Isochrysis* sp. cells, surface charge and wettability were measured. Surface charge estimates were based on electrophoretic mobility of the control and treated cells and determined by means of a Zetasizer-nano-ZS instrument (Malvern Instruments, Inc.), which 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 of ≤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 corroded 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). Samples of cultures 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 of Milli-Q water was carefully placed on each pad (Della Volpe *et al.*, 2002) and immediately photographed with a digital camera mounted on a horizontal dissecting scope (35× magnification). Surface roughness was assumed to be similar for all particle pads.

For the feeding experiments, *Isochrysis* sp. was 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 the surface charge of microalgal cells was altered when transferred from a salinity of 30 to 15, we also determined Zeta potential of *Isochrysis* sp. cells grown at a salinity of 15 and run at the same salinity. Samples of cultures (3–7 $\times$ 10^6 cells ml^{-1}) were treated with FSW or Con A and washed as described above. For charge estimates, subsamples were diluted to appropriate concentrations (*ca.* 2 $\times$ 10^6 cells ml^{-1}) with filtered seawater at a salinity of 15 (0.2 μm, similar to that used in feeding assays) in individual 2-ml microcentrifuge tubes just prior to analysis. For cultures grown at a salinity of 30, ionic strength also was adjusted with dropwise additions of Milli-Q to obtain a salinity of 15 (pH 7.8). Each suspension was transferred to a disposable capillary cell with a 1-ml syringe and analyzed immediately. Two replicate determinations were made for each suspension. For wettability estimates, 3-to 6-ml subsamples of each experimental condition (FSW- and Con A-treated, salinity of 15 and 30) were used to make each pad. At least two pads were made for each treatment combination. Blanks were also prepared by passing several milliliters of filtered seawater (salinity of 15 or 30) through polycarbonate filters and rinsing with ammonium formate.

Flow cytometry analysis

Microalgae and microspheres were analyzed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, CA). A minimum of 10^4 events were analyzed. The 488-nm argon laser was used for excitation, and test particles (microspheres and labeled and unlabeled microalgae) were identified and characterized on the basis of one or more of the following parameters: forward (FSC) and side (SSC) light scatters, FITC fluorescence (FL1, 535 nm), and chlorophyll auto-fluorescence (FL3, 675 nm). Microalgal size (FSC) and intracellular complexity (SSC) were evaluated after each treatment (mucus, carbohydrates, and lectins) to check for cell alteration.

Data treatment and statistical analysis

All data are presented as mean ± standard deviation. Results obtained for microalgae treated with FITC-labeled lectins are presented as ratios between fluorescent intensities (geometric mean of channel number) of treated cells and the baseline auto-fluorescence in FL1. Additionally, the geometric mean fluorescence values were divided by the FITC/protein ratio determined for each lectin (relative fluorescence intensity) in order to compare across lectin types. Statistical analyses of these data, however, were performed on raw flow cytometric fluorescence histograms using a Kolmogorov-Smirnov (K-S) analysis that is packaged in the CellQuest Pro software in BD FACStation software for Mac OS X, ver. 5.2.1(Becton Dickinson Immunocytometry Systems). The K-S test compares the distributions of fluorescence histograms, calculates a D value that represents the greatest difference between the two curves, and establishes the probability (P) that the two histograms are different.

For the feeding experiments, data were analyzed using goodness-of-fit tests (G test). Two series of tests were performed comparing the proportion of each type of particle in samples of the diet and pseudofeces collected from the oysters. The first series of tests ensured that within each treatment, replicate samples of the diet and pseudofeces were homogeneous. The second series tested the null hypothesis that, within each treatment, the proportion of each particle type (*i.e., Isochrysis* sp. cells treated or untreated, or microspheres) in the diet and the proportion of each in the pseudofeces were not different. In addition to the comparison of raw counts, a sorting efficiency (SE) index was calculated to examine particle selection (Iglesias *et al.*, 1992). This index was defined as:

$$SE = 1 - (PP/DP),$$

where PP and DP 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 SE values obtained for each of the two particles in each treatment were compared to zero using a one-sample Student's t-test (two-tailed). The null hypothesis was that the selection

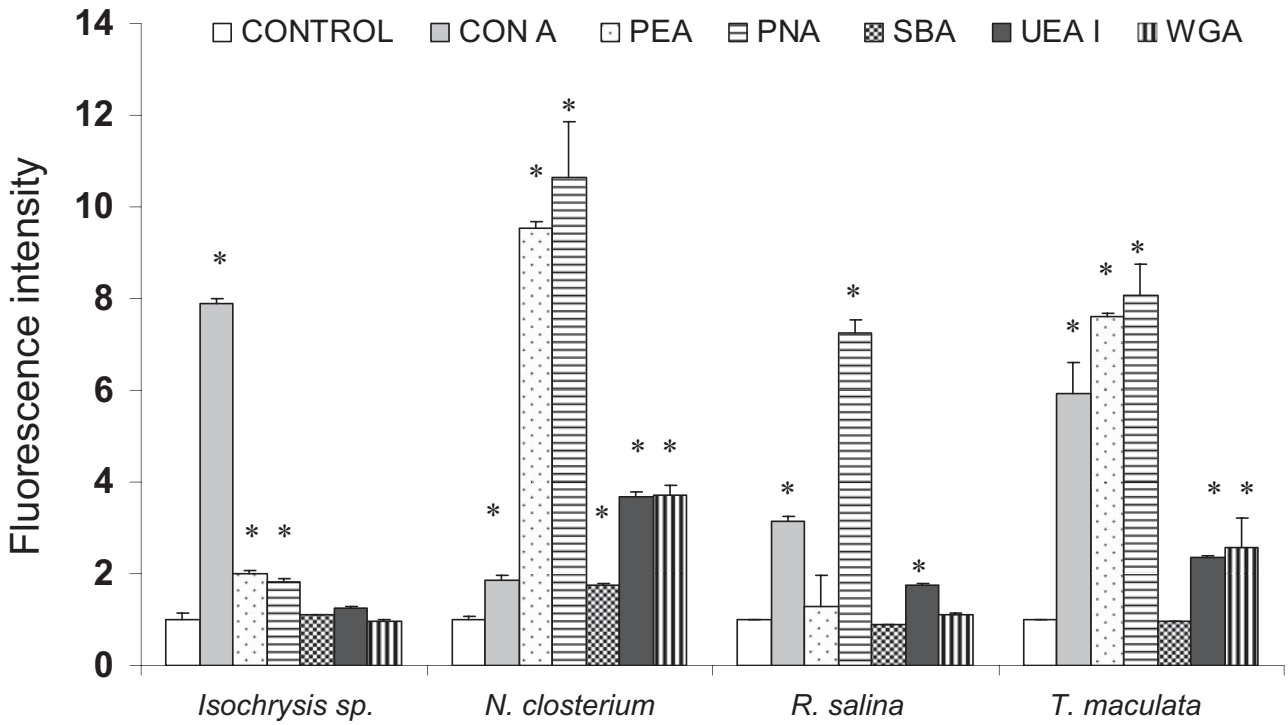

Figure 1. Binding of FITC-labeled lectins (fluorescence units, mean ± standard deviation, $n = 6$) to *Isochrysis* sp., *Nitzschia closterium*, *Rhodomonas salina,* and *Tetraselmis maculata*. Abbreviations of lectins are defined in Table 1. Control: negative controls made with filtered seawater; * indicates significant difference between the negative control and the tested FITC lectins within each species (K-S test, $P < 0.001$).

efficiencies were equal to zero (*i.e.,* no selection). Two-sample *t*-tests were then used to examine differences in SE between the two particle types in each treatment.

Zeta-potential data for cells of *Isochrysis* sp. were first analyzed for differences between cultures grown at a salinity of 15 and 30 within each experimental condition using a two-sample *t*-test. No significant differences were found ($P > 0.05$), and data were pooled. Differences in the zeta potential of cells in the two treatments (FSW-, Con A-treated) were then examined (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 (salinity or treatment) were examined using a Watson-Williams *F*-test (multisample and pairwise comparisons). For all statistical tests, a significance level of alpha = 0.05 was used.

Results

Binding of FITC-labeled lectins to microalgae

Microscopy observation and flow cytometry measurements of light-scatter parameters confirmed that microalgae labeling procedures did not alter cell viability, size, or intracellular complexity. Fluorescence intensity ratios showed strong binding of the lectin Concanavalin A (Con A) to the surface of *Isochrysis* sp. (Fig. 1, $D = 0.97$, K-S test, $P < 0.001$), revealing the presence of methyl α-mannopyranoside, D-mannose, and D-glucose. Weaker binding was observed for *Pisum sativum* agglutinin (PEA) and/or peanut agglutinin (PNA), while no significant binding was detected for either soy bean agglutinin (SBA), *Ulex europaeus* agglutinin (UEA 1), or wheat germ agglutinin (WGA). Fluorescence ratios of *Nitzschia closterium* showed significant binding of all the tested lectins to the cell surface, even though the binding of PNA ($D = 0.86$, $P < 0.001$) and PEA ($D = 0.81$, $P < 0.001$) was more intense, revealing the presence of α-lactose, D-galactose, *N*-acetylgalactosamine (GalNAc), α-methyl mannoside, and α-methyl glucoside. Fluorescence ratios showed significant binding of the lectin PNA and Con A to the surface of *Rhodomonas salina* ($D = 0.95$ and $D = 0.87$ respectively, $P < 0.001$), revealing the presence of α-lactose; D-galactose, GalNAc, and methyl α-mannopyranoside, D-mannose and D-glucose. The lectin UEA 1 also bound significantly to *R. salina* cells ($D = 0.54$, $P < 0.001$) indicating the presence of L-fucose. In *Tetraselmis maculata,* fluorescence ratios showed significant binding of PNA (α–lactose, D-galactose, and GalNAc), PEA (α-methyl mannoside and α-methyl glucoside), and Con A (methyl α-mannopyranoside, D-mannose, and D-glucose)

Table 2

Effect of selected carbohydrates on the binding of FITC-labeled lectins (relative fluorescence units, mean ± standard deviation in parentheses, n = 6) to Isochrysis *sp.,* Nitzschia closterium, Rhodomonas salina, *and* Tetraselmis maculata

	CON A			
Species	Negative control	Positive control	Glucose	Mannose
Isochrysis sp.	1.0 (0.0)	5.0 (0.1)	4.6 (0.1)*	2.9 (0.1)*

	PNA				
	Negative control	Positive control	GalNAc	Galactose	Lactose
Nitzschia closterium	1.0 (0.0)	4.5 (0.1)	1.7 (0.1)*	1.9 (0.1)*	1.5 (0.0)*
Rhodomonas salina	1.0 (0.0)	15.6 (0.4)	6.0 (0.1)*	5.7 (0.1)*	1.6 (0.0)*
Tetraselmis maculata	1.0 (0.0)	5.7 (0.4)	1.5 (0.1)*	1.2 (0.1)*	1.2 (0.0)*

The FITC-labeled lectins used are Con A for *Isochrysis sp*., and PNA for *N. closterium*, *R. salina,* and *T. maculata*. Abbreviations of lectins are defined in Table 1. The negative and positive controls represent microalgae incubated either with filtered seawater (autofluorescence in FL1) or with the corresponding FITC lectins. For each microalgal species, * indicates significantly lower signals compared to the positive control (K-S test, $P < 0.001$). GalNAc: *N*-acetyl D-galactosamine.

($D = 0.91$. $D = 0.88$ and $D = 0.85$ respectively, $P < 0.001$). Relative fluorescence of lectins (geometric value divided by the FITC/protein ratio) calculated for *Isochrysis* sp., *N. closterium, R. salina,* and *T. maculata* showed the same tendency as described above.

The specificity of the binding was further analyzed for lectins that bound to the four tested microalgae with the highest intensity (Table 2). The binding of Con A to the surface of *Isochrysis* sp. was inhibited by a 150 mmol l^{-1} solution of D-mannose ($D = 0.67$, K-S test, $P < 0.001$), and to a lesser extent by 150 mmol l^{-1} of D-glucose ($D = 0.06$, $P < 0.001$). Binding of PNA to the surface of *N. closterium* was inhibited by 150 mmol l^{-1} of α–lactose ($D = 0.72$, $P < 0.001$), and by 150 mmol l^{-1} of GalNAc and D-galactose ($D = 0.54$ and $D = 0.52$ respectively, $P < 0.001$). Binding of PNA to the surface of *R. salina* was inhibited by 150 mmol l^{-1} α–lactose ($D = 0.88$; $P < 0.001$), and to a lesser extent by 150 mmol l^{-1} D-galactose and GalNAc ($D = 0.54$, $D = 0.52$ respectively, $P < 0.001$). Binding of PNA to the surface of *T. maculata* was inhibited by 150 mmol l^{-1} of D-galactose and α–lactose ($D = 0.54$ and $D = 0.54$ respectively, $P < 0.001$), and to a lesser extent by 150 mmol l^{-1}. GalNAc ($D = 0.30$; $P < 0.001$).

Effect of pallial mucus on the binding of FITC-labeled lectins

Pre-incubation of microalgae with mucus from the pallial organs of oysters significantly reduced the binding of FITC-labeled lectins to their surface ligands (Fig. 2). All *D* values comparing green fluorescence histograms in untreated (positive control) to mucus-treated microalgae were significant (K-S test, $P < 0.001$). In addition, the binding of fluorescent lectins varied according to the origin of mucus. For instance, mucus collected from labial palps significantly decreased the binding of FITC-labeled lectins to *Isochrysis* sp., *T. maculata,* and *R. salina* compared to the mucus from gills. In contrast, mucus collected from gills significantly decreased the binding of PNA to *N. closterium* compared to mucus from labial palps (Fig. 2).

The effect of pallial cavity mucus (collected in January 2008) was moderated when microalgae subsequently were washed with specific carbohydrates (Table 3), providing evidence for the carbohydrate specificity of mucus binding. Binding of Con A to mucus-treated *Isochrysis* sp. increased after a washing step with a 150 mmol l^{-1} solution of D-glucose ($D = 0.25$, K-S test, $P < 0.001$), and to a lesser extent following treatment with a 150 mmol l^{-1} of D-mannose ($D = 0.12$, $P < 0.001$). Binding of PNA to the surface of mucus-treated *N. closterium* increased following a washing step with a 150 mmol l^{-1} of α–lactose ($D = 0.48$, $P < 0.001$), and to a lesser extent following treatment with a 150 mmol l^{-1} solution of GalNAc or D-galactose ($D = 0.44$, $D = 0.38$ respectively, $P < 0.001$). Binding of PNA to the surface of *R. salina* increased when washed with a 150 mmol l^{-1} of GalNAc, D-galactose, or α–lactose ($D = 0.24$, $D = 0.12$, $D = 0.11$ respectively, $P < 0.001$). Binding of PNA to the surface of *T. maculata* increased when washed with 150 mmol l^{-1} of D-galactose or α–lactose ($D = 0.06$, $D = 0.07$ respectively, $P < 0.001$).

Feeding experiments

Total microalgal concentration in Treatments 1, 2, and 3 were $1.15 \pm 0.09 \times 10^5$ cell · ml^{-1}, $1.05 \pm 0.12 \times 10^5$ cell · ml^{-1}, and $1.17 \pm 0.14 \times 10^5$ cell · ml^{-1}, respectively. Although the targeted proportion of each microalgae was 50%, an *a posteriori* count revealed the following proportions:

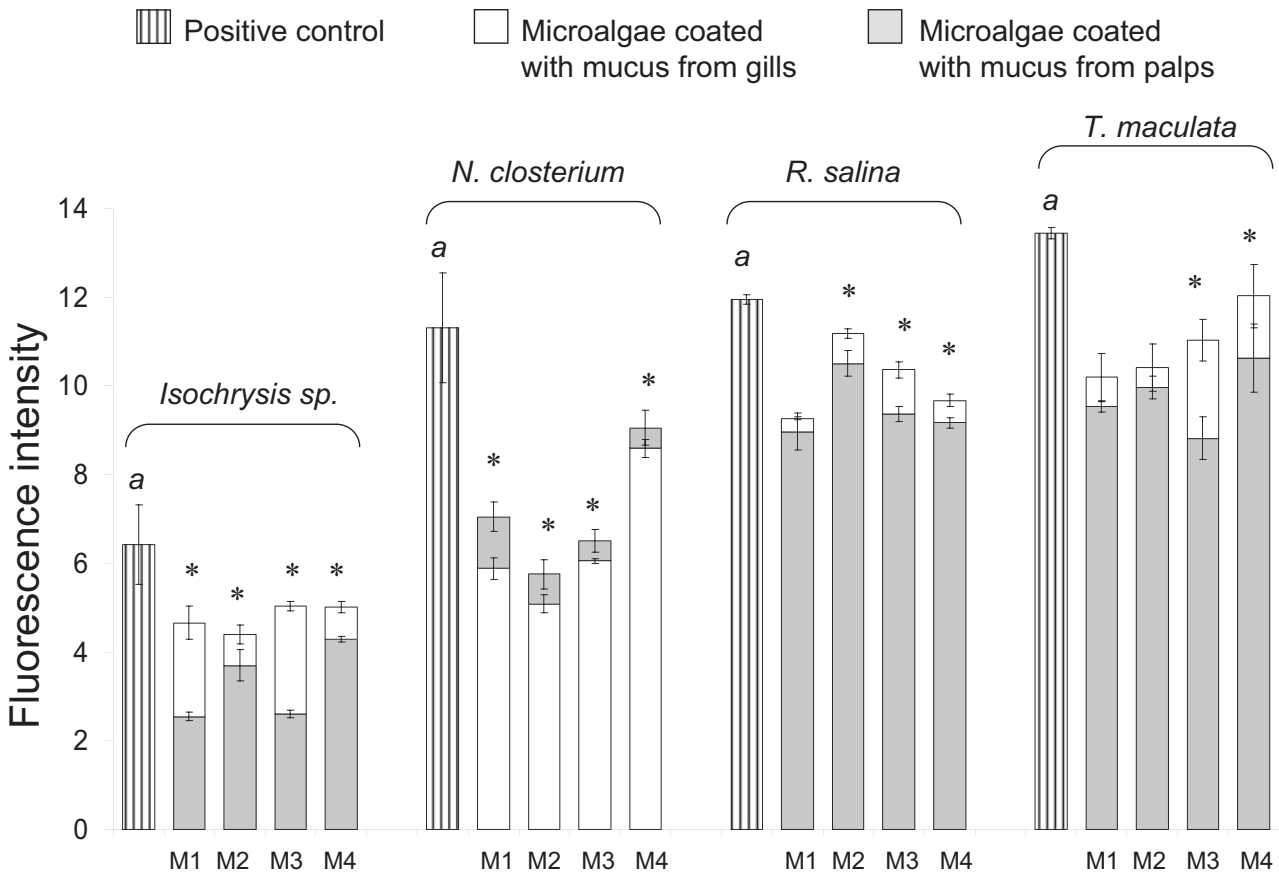

Figure 2. Effect of mucus from gills and labial palps on the binding of FITC-labeled lectins (relative fluorescence units, mean ± standard deviation, $n = 3$) to *Isochrysis* sp., *Nitzschia closterium*, *Rhodomonas salina,* and *Tetraselmis maculata*. Con A was used to label *Isochrysis* sp.; PNA was used to label *N. closterium*, *T. maculata,* and *R. salina.* Abbreviations of lectins are defined in Table 1. Mucus samples were collected on 23 June 2007 (M1), 7 December 2007 (M2), 23 January 2008 (M3), and 29 February 2008 (M4). For each micoalgal species, * designates significant difference between gill and palp mucus (K-S test, $P < 0.001$), whereas *a* designates significantly higher signals in the positive control as compared to all mucus treatments (K-S test, $P < 0.001$). Mucus from labial palps significantly decreased the binding of FITC-labeled lectins to *Isochrysis* sp., *R. salina,* and *T. maculata* compared to the mucus from gills. In contrast, mucus collected from gills significantly decreased the binding of PNA to *N. closterium* compared to mucus from labial palps.

52.9% ± 4.3%:47.1% ± 3.3% for unlabeled *Isochrysis* sp. and microsphere, respectively, in Treatment 1; 44.8% ± 7.7%: 55.2% ± 7.7% for labeled *Isochrysis* sp. and microspheres, respectively, in Treatment 2; and 50.9% ± 0.6%:49.1% ± 0.6% for unlabeled *Isochrysis* sp. and labeled *Isochrysis* sp., respectively, in 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 microalgae and microspheres had not occurred.

When *Crassostrea virginica* was fed Treatment 1 (unlabeled *Isochrysis* sp. and microspheres) and Treatment 2 (Con A-labeled *Isochrysis* sp. and microspheres), the proportion of microspheres increased significantly in the pseudofeces compared to the diet (Fig. 3A and B, $n = 18$ and 13, G test, $P < 0.001$). When oysters were fed Treatment 3 (unlabeled *Isochrysis* sp. and Con A-labeled *Isochrysis* sp.), the proportion of labeled *Isochrysis* sp. increased significantly in the pseudofeces as compared to diets (57.6% ± 0.8%, $n = 10$, $P < 0.001$, Fig. 3C). Sorting efficiencies (SE) confirmed the above results. For Treatments 1 and 2, oysters preferentially ingested unlabeled or labeled *Isochrysis* sp. (SE = 0.40 ± 0.05 and 0.31 ± 0.07, respectively; Table 4), while they rejected microspheres (SE = −0.44 ± 0.05; SE = −0.25 ± 0.09, $n = 18$ and 13, t-test $P < 0.001$). SE values were, however, lower in Treatment 2 than in Treatment 1. SE indices in Treatments 3 showed that oysters preferentially ingested unlabeled *Isochrysis* sp. (SE = 0.17 ± 0.05, Table 4), while they rejected Con A-labeled *Isochrysis* sp. (SE = −0.17 ± 0.06, $n = 10$, $P < 0.001$).

Table 3

Combined effects of lectins contained in gill mucus (collected on 01/23/07) and selected carbohydrates on the binding of FITC-labeled lectins to Isochrysis *sp.,* Nitzschia closterium, Rhodomonas salina, *and* Tetraselmis maculata

	CON A				
				Mucus + Carbohydrates	
Species	Negative control	Positive control	Mucus	Glucose	Mannose
Isochrysis sp.	1.0 (0.0)	5.4 (0.3)	3.3 (0.1)*	5.3 (0.0)†	4.7 (0.2)†

	PNA					
				Mucus+ Carbohydrates		
	Negative control	Positive control	Mucus	GalNAc	Galactose	Lactose
Nitzschia closterium	1.0 (0.0)	13.1 (0.5)	6.1 (0.1)*	10.3 (0.1)†	9.7 (0.2)†	12.8 (0.2)†
Rhodomonas salina	1.0 (0.0)	7.3 (0.1)	4.6 (0.2)*	5.8 (0.1)†	5.2 (0.1)†	5.2 (0.1)†
Tetraselmis maculata	1.0 (0.0)	7.0 (0.1)	2.4 (0.1)*	2.3 (0.1)	3.1 (0.0)†	2.9 (0.0)†

Con A was used to label *Isochrysis* sp.; PNA was used to label *N. closterium*, *R. salina,* and *T. maculata.* Abbreviations of lectins are defined in Table 1. The negative and positive controls represent microalgae incubated either with filtered seawater (autofluorescence in FL1) or with the corresponding FITC lectins. For each microalgal species, * indicates significantly different signals in samples treated with mucus compared to the positive control, whereas † indicates significantly different signals in samples treated with mucus and carbohydrates as compared to mucus alone (K-S test, $P < 0.001$). GalNAc: *N*-acetyl D-galactosamine.

Surface properties of Isochrysis *sp.*

Treating *Isochrysis* sp. with the lectin Con A had no significant effect on either zeta potential or contact angle (Table 5, *t*-test and Watson-Williams *F*, respectively, $P > 0.05$). Contact angles for both unlabeled and Con A-labeled *Isochrysis* sp. were, however, significantly higher than those of seawater-treated, blank filters (mean vector = 59.4° ± 3.0° circular SD, $n = 9$; Watson-Williams *F*, $P < 0.01$).

Discussion

Although the ability of suspension-feeding bivalves to select among captured particles is well known, the mechanism, or mechanisms, by which particles of poor quality are rejected and those of higher quality are diverted toward the mouth and ingested remains unknown (Ward and Shumway, 2004). Recently, we proposed that the mechanism of selection in these bivalves is, at least partially, mediated by interactions between carbohydrates present at the surface of food particles and lectins present in the mucus covering feeding organs (Pales Espinosa *et al.*, 2009). The present study provides further evidence for the involvement of carbohydrate-lectin interactions in particle selection by suspension-feeding bivalves. First, we characterized carbohydrates present at the cell surface of four microalgal species known to be ingested or rejected by suspension-feeding bivalves. We then used this information to demonstrate that lectins contained in pallial mucus competitively and reversibly bind to sites recognized by commercially available lectins. Finally, we revealed that binding lectins to the surface of *Isochrysis* sp. cells alters the ability of oysters to sort this microalga.

Results from lectin-binding experiments indicated the presence of a significant amount of mannose on the surface of *Isochrysis* sp. cells, confirming the finding of Wootton *et al.* (2007) for *Isochrysis galbana*. The presence of α-lactose, D-galactose, and *N*-acetylgalactosamine (GalNAc) as well as α-methyl mannoside and α-methyl glucoside residues on the surface of *Nitzschia closterium* cells can be deduced from peanut agglutin (PNA) and *Pisum sativum* agglutinin (PEA) binding, respectively. Although comparison between microalgal species is not always possible due to the highly specific nature of carbohydrate patterns within a genus (Rhodes *et al.*, 1995; Cho *et al.*, 2001; Cho, 2003; Hou *et al.*, 2008) or the variation of cell surface carbohydrates within a given species in different physiological states (Waite *et al.*, 1995), it is, however, interesting to discuss our results in light of other studies. Results reported by Cho (2003) for *Nitzschia* sp. corroborate our findings that there is strong binding of the fluorescein isothiocyanate (FITC)-conjugated lectins PNA and PEA. Additionally, extracellular polymeric substances (EPS) present at the surface of *Cylindrotheca closterium* (Ehrenberg) Lewin & Reimann and *Nitzschia* sp. cells—two species related to *N. closterium*—were characterized by glucose, galactose, and xylose/mannose (de Brouwer and Stal, 2002). Binding of concanavalin A (Con A) and PNA to the cell surfaces of *Rhodomonas salina* indicated the presence of GalNAc,

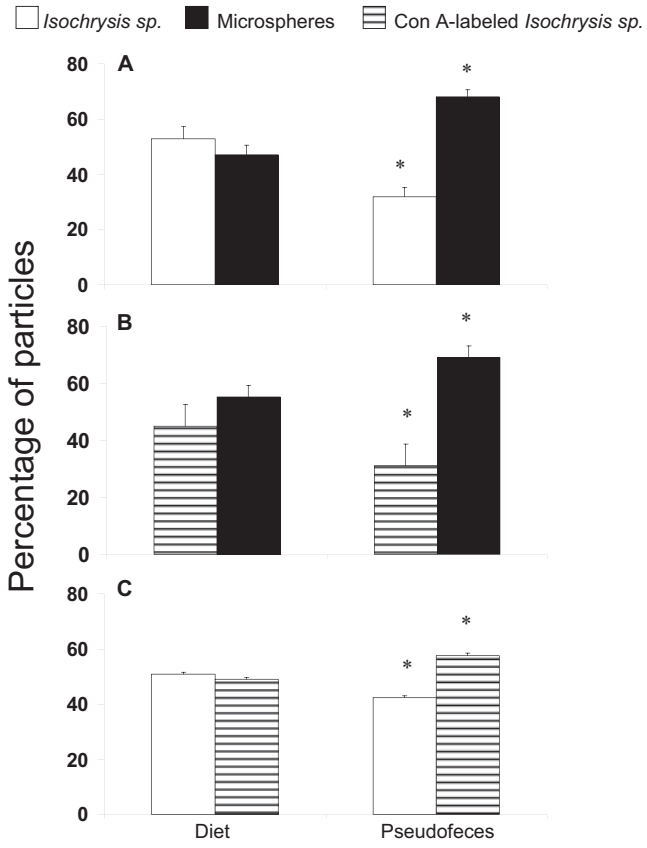

Figure 3. Percentage (mean ± standard deviation) of the two particle types in the food supply (diet) and pseudofeces of *Crassostrea virginica* fed with Treatment 1 (unlabeled *Isochrysis* sp./Microspheres), Treatment 2 (Con A-labeled *Isochrysis sp.*/Microspheres), and Treatment 3 (unlabeled *Isochrysis* sp./Con A-labeled *Isochrysis* sp.). For each treatment, * indicates a significant difference between the particle type in the diet and pseudofeces (G test, $P < 0.001$).

α-lactose, and D-galactose as well as methyl α-mannopyranoside, D-mannose, and D-glucose. Comparatively, the Cryptophyceae *Plagioselmis* sp. binds with wheat germ agglutin (WGA) and DBA (*Dolchos biflorus* agglutinin which binds GalNAc residues) as well as with several macroalgal lectins that recognize mannose residues (Hori *et al.*, 1996). Finally, the cells of *Tetraselmis maculata* bind PNA, PEA, and Con A, which indicates the presence of GalNAc, α-lactose, D-galactose, α-methyl mannoside, α-methyl glucoside, methyl α-mannopyranoside, D-mannose, and D-glucose. Interestingly, another study has shown that several strains of the genus *Tetraselmis* bind GNA (*Galanthus nivalis* agglutinin lectin, which binds terminal mannose) and PNA (Becker *et al.*, 1995). This binding, however, was only observed on the flagella: no fluorescence of the theca or intracellular structures was observed (Becker *et al.*, 1995; Costas *et al.*, 1996). In our study, observations under an epifluorescent microscope revealed that the cell wall of *T. maculata* was weakly labeled by both PEA and PNA. These observations were confirmed by flow cytometry, which is more powerful than microscopy as it allows the detection of lower fluorescence signals. Finally, Tasumi and Vasta (2007) demonstrated that the galectin (CvGal) from *C. virginica* hemocytes, in the family of β-galactoside-binding lectins, strongly binds to cells of *Tetraselmis* spp.

Our results also demonstrate that lectins in mucus from the gills and labial palps of *Crassostrea virginica* competitively bind carbohydrate moieties on the surface of several microalgal species. The effect of mucus-associated lectins was reversible, as demonstrated by the higher binding of commercially available lectins after treatment of microalgae with specific carbohydrates. Interestingly, the binding pattern of commercially available lectins on the various microalgae species was affected by the source of the mucus (gills *vs.* labial palps; Fig. 2). For instance, the binding of FITC-labeled Con A to *Isochrysis* sp. and PNA to *T. maculata* and *R. salina* was higher when microalgae were incubated with mucus from the gills than with mucus from labial

Table 4

Sorting efficiency (mean ± standard deviation) of unlabeled Isochrysis *sp., microspheres, and Con A-labeled* Isochrysis *sp. in* Crassostrea virginica *fed with different treatments*

	Sorting efficiency		
Treatment	*Isochrysis* sp.	Microspheres	Con A-labeled *Isochrysis* sp.
1	0.40 ± 0.05	−0.44 ± 0.05	NA
2	NA	−0.25 ± 0.09	0.31 ± 0.07
3	0.18 ± 0.01	NA	−0.17 ± 0.02

Treatment 1 (Unlabeled *Isochrysis* sp./Microspheres), Treatment 2 (Microspheres/Con A-labeled *Isochrysis* sp.), and Treatment 3 (Unlabeled *Isochrysis* sp./Con A-labeled *Isochrysis* sp.). All SE values were significantly different from zero (one-sample t-test, $P < 0.001$). Within each treatment, SE of the preferentially-ingested (positive SE) particle was significantly different from the SE of rejected (negative SE) particle (two-sample t-test, $P < 0.001$).

Table 5

Surface properties of the phytoplankton Isochrysis *sp. unlabeled and labeled with the lectin Con A*

	Surface property	
Treatment	Zeta potential (mV)	Contact angle (°)
Unlabeled	−7.2 (0.8)	85.3 (3.2)
Con A-labeled	−7.4 (1.0)	83.5 (3.5)

Zeta potentials are presented as means of 4 (labeled) or 6 (unlabeled) replicate measurements ± standard deviation in parentheses. Contact angle data are given as mean vector (μ) of 8 (unlabeled) or 11 (labeled) replicate measurements ± circular standard deviation in parentheses. Treatment had no significant effect on either zeta potential or contact angle measurements (t-test, Watson-Williams F, respectively, $P > 0.05$).

palps. This result may indicate that lectins in gill mucus did not block as many binding sites as lectins from mucus of the palps. The opposite trend was observed in *N. closterium*. In this case, binding of PNA was higher when the microalgal cells were incubated with mucus from labial palps than with mucus from the gills. It is intriguing that among the microalgal species tested, *N. closterium* is the only one significantly rejected in the pseudofeces by several bivalves, including *C. virginica* (see discussion in Pales Espinosa *et al.*, 2007). In contrast, *R. salina* and several strains of the genera *Isochrysis* and *Tetraselmis* are considered to be the industry standard for supporting bivalve growth or for use as positive controls in feeding studies (Wikfors *et al.*, 1996; Hegaret *et al.*, 2007). The differential effects of mucus from the gills compared to from the palps on binding efficiency of FITC-labeled lectins to microalgal cells could be due to qualitative or quantitative differences in the composition of lectins in the mucus. Such results suggest that mucus covering gills and labial palps could either contain the same lectin at different concentration or could contain different lectins (Pales Espinosa *et al.*, 2008 and 2009).

Furthermore, results of the feeding experiments strongly emphasize the role that the cell surface carbohydrates of microalgae and their specific interaction with lectins contained in the mucus of oysters play in the process of particle selection. The methods we used to test for the effects of carbohydrate-lectin interactions on feeding were inspired by previous researchers who blocked surface carbohydrates of prey (Ucko *et al.*, 1999). In that study, feeding by a predatory dinoflagellate, *Crypthecodinium cohnii*-like, was inhibited when its prey, the microalga *Porphyridium* sp., was treated with Con A or a specific antiserum. The biorecognition mechanism involving a surface polymer of the dinoflagellate, which may be a ligand for the glycoprotein, is yet to be studied (Shrestha *et al.*, 2004). Using the same approach, we demonstrated that oysters preferentially rejected Con A-labeled cells of *Isochrysis* sp. compared to unlabeled cells. The sorting efficiencies calculated in this experiment are low but statistically different. This result could be explained by the fact that some Con A binding sites on the cell surfaces of the algae were not bound and remained available for mucus lectin. In addition, mucus contains several lectins with different carbohydrate affinities (Pales Espinosa *et al*., 2009) that would also be able to bind *Isochrysis* sp. and participate in the selection. It is important to mention that the binding of lectins to microalgae did not affect cell viability (microscopy observations), size, or complexity (flow cytometry data). Similarly, the surface charge and wettability of *Isochrysis* sp. did not change after treatment with Con A, indicating that these parameters did not influence selection. The fact that cells of *Isochrysis* sp. labeled with Con A were significantly rejected in pseudofeces whereas unlabeled cells were ingested constitutes evidence for the involvement of carbohydrate-lectin interactions in particle selection by oysters.

Supporting this conclusion are the results from the lectin-binding experiments. First, the binding of Con A to cells of *Isochrysis* sp. was significantly reduced by gill and, particularly, palp mucus, suggesting that the lectins in mucus have specificity similar to that of Con A (*i.e.,* bind to methyl α-mannopyranoside, D-mannose, and D-glucose). Second, among our tested microalgal species, those known to be ingested preferentially by bivalves (*Isochrysis* sp., *R. salina,* and *T. maculata*) were noticeably labeled with Con A, whereas *N. closterium*, a species known to be rejected by several bivalves, was only weakly labeled with this lectin. Specific molecular recognition of prey has been suggested or demonstrated previously using protozoa as models. For example, the planktonic protozoan *Actinophrys sol* produces a 40-kDa glycoprotein that mediates the capture and ingestion of the flagellate prey *Chlorogonium elongatum* (P.A. Dangeard) Francé (Sakaguchi *et al.*, 2001). Similarly, Wilks and Sleigh (2004) found lectin-binding sites on the food vacuolar membranes and cytosome of the ciliate *Euplotes mutabilis*, and suggested that these sites are involved in selective capture of food. More interestingly, Wootton *et al.* (2007) demonstrated the involvement of a mannose-binding lectin as a feeding receptor for recognizing prey in the marine dinoflagellate *Oxyrrhis marina*. Blocking this receptor using mannose-BSA clearly inhibited ingestion of *Isochrysis galbana* as well as mannose-BSA coated beads.

In summary, this study demonstrates that lectins in pallial mucus bind carbohydrate moieties associated with microalgal cell surface. Blocking of Con-A binding sites on the microalgal cell surface significantly reduced sorting efficiency of *Isochrysis* sp. by oysters, suggesting that a mucus lectin with affinity to mannose, glucose, or both, is involved in particle selection in *C. virginica*. Additionally, the surface charge and wettability of *Isochrysis* sp. treated with Con A did not change, indicating that selection was not influenced by these parameters. With information from our previous studies (Pales Espinosa *et al.*, 2008, 2009), these new results provide evidence that particle selection in *C. virginica* (and likely other suspension-feeding bivalves) is, at least partially, mediated by interactions between microalgal cell surface carbohydrates and lectins present in the mucus covering bivalve pallial organs. The role of mucus lectins in the sorting process requires further investigations, particularly to evaluate lectin diversity and map the lectin-producing cells on feeding organs.

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