Physicochemical surface properties of microalgae and their combined effects on particle selection by suspension-feeding bivalve molluscs

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Abstract

The capabilities of bivalve molluscs to feed selectively have been well documented, and physicochemical properties of particles have been implicated as possible factors in the selection process. In this study, the surface-property profiles of nine different microalgal species were determined by characterizing the surface charge, wettability (= contact angle), and surface carbohydrate moieties. Three fluorescein isothiocyanate (FITC) conjugated lectins were used to characterize carbohydrate moieties, including concanavalin A (ConA), *Pisum sativum* agglutinin (PEA), and wheat germ agglutinin (WGA). Distinct surface-property profiles were identified using linear discriminant analysis (DA) and used to design mixed-algal feeding experiments to assess particle selection by the blue mussel *Mytilus edulis* and the eastern oyster *Crassostrea virginica*. Results demonstrated preferential ingestion of some algal species over others, with strong rejection of some species (e.g. *Pavlova lutheri* and *Prasinocladus marinus*). These data were then used to develop DA and multiple linear regression models that considered the quantified surface properties and microalgal fates (rejected, ingested, or no selection) to examine determinants of selection. The DA model correctly classified 58% of the selection outcomes in mussels and 57% in oysters. Wettability was the most important factor in predicting selection in mussels, and surface charge was most important for oysters. In the multiple linear regression

analyses, lectin affinity and wettability were the strongest predictors of particle selection, explaining ca. 90% of the variability in electivity index for mussels and 94% of the variability for oysters. This study characterizes both physical and chemical surface properties of several microalgae used as food for suspension-feeding bivalves. Results demonstrate that multiple surface-property characteristics need to be considered in order to develop meaningful models of particle selection in bivalves, and future research should take into account species-specific differences in selection.

Keywords: microalgae; bivalve molluscs; surface characteristics; feeding selectivity; *C. virginica*; *M. edulis*

1. Introduction

The capabilities of suspension-feeding bivalve molluscs to select some particles over others for ingestion have been well documented (see Shumway et al. 1985; Cognie et al. 2003; Ward and Shumway 2004). Prior studies have demonstrated that chemical substances on particle surfaces can mediate selection in these organisms (e.g., Newell and Jordan 1983; Ward and Targett 1989; Ward et al. 1997; Pales Espinosa et al. 2007), and physicochemical properties, such as electrostatic charge and wettability, also have been suggested to play roles in selection (Newell RC et al. 1989; Beninger 1991; Hernroth et al. 2000; Rosa et al. 2013). Charged particles, for example, have been shown to be more readily captured than particles with a neutral charge by both the brittle star *Ophiopholis aculeate* (LaBarbera 1978) and by larvae of the northern quahog (= hard clam) *Mercenaria mercenaria* (Solow and Gallagher 1990). Hernroth et al. (2000) examined the influence of the surface charge of radioactive-labeled *Salmonella typhimurium* cells upon particle selection by the blue mussel *M. edulis* and found that cells with

a lower net-negative charge were more likely to be captured than cells with a higher negative charge. Wettability, a weak force dependent upon hydrophobic-hydrophilic interactions between a surface and a liquid, can also act to mediate feeding in invertebrates (Mihm and Loeb 1992, Conova 1999). In the crustacean *Daphnia magna*, particle capture was related to wettability, with wettable (= hydrophilic) particles being retained at a higher proportion than non-wettable (=hydrophobic) particles (Gerritsen and Porter 1982). In a more recent study, Rosa et al. (2013) demonstrated that the eastern oyster, *Crassostrea virginica*, and the blue mussel, *Mytilus edulis*, can discriminate between synthetic particles of the same size based upon the surface charge and wettability of particles. These findings suggested that non-specific physicochemical interactions play a role in mediating a passive selection mechanism. Most of the studies examining effects of physicochemical properties upon selection, however, have used synthetic particles (e.g., polystyrene and silica). To understand fully how physicochemical characteristics of particles mediate the feeding process, surface properties of natural food particles (e.g., microalgae) need to be characterized and assessed in selection experiments.

More specific chemical interactions (e.g., lectin-sugar) also contribute to particle discrimination. Scientists have long recognized the role of lectins as signaling molecules in marine bivalves (e.g. Renwranz and Stahmer 1983; Gauthier et al. 2004), and research by Pales Espinosa et al. (2009) provided some of the first evidence that lectins are involved in selective feeding. Working with mucus isolated from the pallial organs of oysters, *Crassostrea virginica*, and mussels, *Mytilus edulis*, commercially available lectins, and neoglycoproteins covalently attached to synthetic microspheres, Pales Espinosa et al. (2009, 2010a, 2010b) demonstrated that 1) the cell surfaces of four different microalgal species contain several different sugars that vary in abundance, 2) lectins present in pallial-organ mucus interact with sugars on the surface of

microalgal cells, and 3) sugar-lectin interactions are involved in mediating particle sorting in both *C. virginica* and *M. edulis*. These findings suggest that sugars act as recognition molecules for mucosal lectins produced by the feeding organs to mediate particle discrimination.

Different chemical substances (e.g., proteins, carbohydrates, humics) likely change the surface characteristics of organic and inorganic particles, affecting the aforementioned physicochemical characteristics (charge and wettability), as well as the adhesive interactions with mucus on the feeding structures. Waite et al. (1995) measured sugar-containing compounds that accumulated on the cell surface of two microalgal species. By binding FITC-labeled Concanavalin A (Con A) lectins to the cell surfaces of the microalga *Thalassiosira pseudonana* and *Chaetocerus neogracile*, the authors showed that the accumulation of sugars increased with growth phase and varied between the two microalgae. The red microalga *Porphyridium* sp. was found to have an overall negative surface charge, attributed to the presence of sulfate groups and glucuronic acid associated with the cell wall (Shrestha et al. 2004). Dam and Drapeau (1995) used a mesocosm study to examine the dynamics of a phytoplankton bloom and reported a strong correlation between the presence of cell surface carbohydrates and aggregation rates (= cell stickiness). Results of these studies suggest a correlation between cell-surface carbohydrates and physicochemical properties that can vary between species and among living and non-living particles (Waite et al. 1995). Understanding how different carbohydrate moieties (= groups) affect the surface properties of microalgal cells can help determine which properties serve to mediate selection in different bivalve species.

To understand more clearly the mechanisms mediating selection, an examination of the effects of all measurable particle characteristics, and how they form a profile for different microalgal species, is necessary. Even when suspension-feeders exhibit a strong preference for

one particular particle type over another, calculated selection indices are not 100% (e.g. Shumway et al. 1985; MacDonald and Ward 1994; Ward et al. 1997), indicating discrimination is not an absolute process. Therefore, it is necessary to determine which characteristics are more important in determining what makes one particle more likely to be ingested compared to another. This project was designed to quantify the differences in surface properties of microalgae that have often been used in aquaculture settings and in prior studies with suspension-feeding bivalves. Understanding differences in surface properties will help to determine if certain surface characteristics typically lead to rejection or ingestion, and if general rules for particle discrimination can be developed. Finally, results of this study will be used to explore whether statistical models can be developed to predict selection by bivalve molluscs fed different algal species. This study is the first to examine the influence a range of physicochemical surface properties of microalgae (e.g. charge, wettability, carbohydrate moieties) has on particle selection by suspension-feeding bivalve molluscs.

2. Methods

2.1 Microalgal characterization – surface charge and wettability

To develop surface-property profiles for the types of algae that typically are rejected versus preferentially ingested by bivalves, the surface charge and wettability of nine different microalgae species were determined (Table 1). Algal species were chosen based on their previous use in selection studies (e.g., Møhlenberg and Riisgård 1978; Newell and Jordan 1983; Shumway et al., 1985) and as feed for commercially-important bivalve species (Wikfors, 2000). Each species was grown in biological triplicate, and were obtained from the NOAA NMFS, Northeast Fisheries Science Center Milford Marine Microalgal Culture Collection. All triplicate

cultures were grown aseptically using enriched, sterilized seawater from Milford Harbor (7.8 pH, salinity of 15), under a 24-hr light regime, and were harvested in early stationary-phase.

Characterization of microalgal surface properties was carried out using previously described methods (Rosa et al. 2013). Briefly, zeta potential was determined for each microalgal species using a Zetasizer Nano ZS[®] (Malvern Instruments Inc., UK). The instrument measures electrophoretic mobility using laser-Doppler electrophoresis (Pashley et al. 1985), and zeta potential was calculated by means of the Smoluchowski equation (Sze 2003) using values for viscosity and dielectric constant of the solution, as well as the measured electrophoretic mobility. In a standard solution such as that used in this analysis (i.e., pH 8.0, salinity 15) zeta potential is an indication of surface charge and henceforth is designated as such.

Wettability of the algae was determined by measuring contact angle of dried cells (Hiemenz 1986). Microalgal cultures were vacuum-filtered through 3- μ m polycarbonate filters to form a pad of cells. Pads were rinsed with isotonic ammonium formate to remove salts and dried overnight at 70 °C. Control pads also were produced by passing filtered seawater (0.2 μ m) through the polycarbonate filters, rinsing with ammonium formate, and drying overnight to test whether or not the contact angles of the microalgae changed significantly during the washing treatment. A drop of MQ water (4 μ l) was then placed on the dried pad and photographed using a digital camera attached to a side-mounted dissecting microscope (i.e., goniometer; Mohammadi et al. 2003). Image J was used to measure the contact angle formed between the water droplet and the microalgal surface. By convention, surfaces with a contact angle greater than 90° are classified as non-wettable (= hydrophobic), and conversely, surfaces with a contact angle less than 90° are classified as wettable (= hydrophilic) (Volpe et al. 2006). At least three technical replicates were used for characterization of each biological replicate of each microalgal species.

2.2. Microalgal characterization – carbohydrate profiles

Chemical properties of microalgae used in the feeding experiments were characterized further by determining some of the carbohydrate moieties associated with the cell walls. Three distinct FITC-conjugated lectins were used; concanavalin A (Con A) derived from *Canavalia ensiformis*, wheat germ agglutinin (WGA) derived from *Triticum vulgaris*, and *Pisum sativum* derived agglutinin (PEA). The carbohydrate specificities of these lectins are outlined in Table 2. Microalgae were treated and tagged with FITC-conjugated lectins following previously-described methods (Gauthier et al. 2004; Pales Espinosa et al. 2010b). Briefly, biological triplicates of algal cultures, grown in the same conditions described above, were centrifuged at 400 x g for 10 min, washed once with filtered seawater (0.22 µm, FSW), and resuspended in FSW to a cell density of 10⁶ cells/ml. FITC-conjugated lectins were diluted in artificial, filtered seawater (0.22 µm, ASW) to a concentration of 1 mg ml⁻¹. A 50-µl aliquot of each lectin or FSW control then was added to 1mL of the washed microalgae. Microalgae were incubated with each individual lectin or FSW control at room temperature and in the dark for 1hr. Incubated microalgal samples were analyzed using an Accuri C6 flow cytometer (BD Instruments). The fluorescence intensity of each of the 3 lectins was determined based upon FITC fluorescence in the FL1 detector of the FCM. Fluorescence intensity for microalgae incubated with the lectins was calculated by dividing mean fluorescence of the conjugated algae by the control algae incubated in filtered seawater (FSW). All lectin-binding assays were repeated in triplicate for each biological replicate of each microalgal culture.

2.3 Experimental animals

Eastern oysters *Crassostrea virginica* (Gmelin 1791) (80.8 ± 2.9 mm shell length) and blue mussels *Mytilus edulis* L. (1758) (50.4 ± 3.6 mm shell length) were used in the study. Oysters were obtained from the Cornell Cooperative Extension, located in Suffolk County, NY, and mussels were harvested locally from wild populations at the Avery Point Campus in Groton, CT. All animals were cleaned of fouling material and maintained in flow-through raceway tanks at the Northeast Fisheries Science Center Milford Laboratory seawater facility (Milford Harbor, CT) for 2 weeks prior to the start of the experiments.

2.4 Feeding experiments with microalgal pairings

Characterized surface properties of microalgae (surface charge, contact angle, lectin-binding affinity) were used to design feeding experiments and to test the effects, if any, of differences and similarities upon selection (Table 3). The characterized algae were used in 11 different pairings with mussels *Mytilus edulis* (n= 8-10) and oysters *Crassostrea virginica* (n= 6-13). Feeding experiments were conducted at the Milford Laboratory (Milford Harbor, CT). A flow-through, multi-chamber system was used for the feeding experiments (MacDonald and Ward 1994; Bacon et al. 1998). The flow-through system used a common head tank/mixing chamber to deliver by gravity equal cell concentrations (10^4 cells ml^{-1}) of microalgal suspension to replicate rectangular feeding chambers holding one bivalve each. The system provided a constant head pressure so that animals were exposed to a steady flow (~ 100 ml min^{-1}) maintained using flow restrictors. This flow-through system also ensures a steady concentration of algae throughout the experiment. The accuracy of flow-through methods in bivalve feeding studies has been critically evaluated (Filgueira et al. 2006), and has been found to provide better results than those of static systems. Seawater was pumped directly from Milford Harbor and filtered to a

nominal pore size of 0.45µm (FSW), and animals were acclimated in the chambers for 1 hr prior to the start of an experiment.

Each combination of algae was introduced to the head tank by peristaltic pump to maintain the desired concentration, and animals were given up to 1 hr to open and show signs of feeding. Once feeding began (T_0), animals were allowed to feed for up to an additional hour while pseudofeces (pf) were collected. As calculation of electivity indices is not time dependent, if 2 ml of pf were collected prior to one hour, the trial was cut short and the total time noted. The collected same samples were homogenized by vigorous shaking, passed through a 100-µm mesh to eliminate large solid particles, and then analyzed at the end of each feeding trial using an Accuri C6 flow cytometer. Use of the C6 instrument, and flow cytometric techniques to distinguish living microalgal cells and detrital particles is well documented (Cucci et al. 1985, Newell et al. 1989, Li 1997). For algal pairings that included *P. lutheri* (MONO), water samples were collected at the chamber entrance ("in") and the chamber overflow ("out") to calculate capture efficiency (CE). This algal species was less than half the size (3-4 µm) of the other species used in selection assays, and below the threshold size (ca. 6 µm; Vahl 1972; Møhlenberg and Riisgård 1978; Riisgård 1988) for ca. 100% capture efficiency. Calculating CE ensured that any differences in selection were not attributable to differential capture of *P. lutheri* by the mussels and oysters.

2.5 Data processing

Selection (or lack thereof) of the different algae was quantified by using a modified electivity index (EI) (Jacobs 1974):

$$EI = \frac{S - W}{(S + W) - (2SW)}$$

Where S is the proportion of algal cells in the pseudofeces sample and W is the proportion of algal cells in the water (=diet) sample. A positive EI indicates the pseudofeces sample is replete with the algal cells, and rejection is taking place. Conversely, a negative EI indicates the sample is depleted of the algal cells, and preferential ingestion is taking place. An EI of zero indicates no selection (rejection *or* preferential ingestion) is taking place. The CE for microalgal pairings involving *P. lutheri* was calculated as:

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

where PC_f is the algae concentration exiting each individual chamber (“out”), and PC_c is the algae concentration entering each individual chamber (“in”). A CE of zero indicates the algae were not efficiently captured, and a CE of one indicates maximum capture efficiency for the algae.

2.6 Statistical analysis

Fluorescence data from the FITC-conjugated lectins were tested against controls using a non-parametric ranks test (Kolmogorov-Smirnov, K-S; Zar 1984). Significant differences in mean fluorescence between microalgae incubated with the FITC-conjugated lectins and the FSW controls indicated the presence of specific carbohydrates. A mixed-model analysis of variance (ANOVA) was performed to determine if there were differences in fluorescence intensity within and among different microalgal species. A linear discriminant analysis (DA) was performed on the characterized surface properties (surface charge, contact angle, lectin affinity) of the microalgae to determine if microalgal species had distinct characteristics, and if these could be used to distinguish among species. Autocorrelation among the independent variables was checked prior to analysis, and only those variables not correlated were included. A classic linear

model (Systat 13©) was applied and Wilks' Lambda statistic was used to determine significance of the model. Canonical correlations and discriminant functions were calculated to determine the proportion of total variability explained by the models and determine which of the surface properties (=factors) was the most important in classifying the algal species.

Calculated electivity indices (EI) were arcsine transformed (Zar 1984) prior to statistical analyses and compared to zero (no selection) using a one-sample t-test (Bonferroni-adjusted P-value used). A second DA model was performed on the selection results with the absolute delta of characterized surface properties (surface charge, contact angle, lectin affinity) between the microalgae to determine if differences in these properties could be used to classify selection (e.g., ingestion, rejection, or no selection). As described in our previous work (see Rosa et al. 2013), the absolute differences in the surface properties between algae used in each experimental pairing were used. Use of delta values in the model accounts for the same algae being used in several different feeding experiments, with potentially different EI values depending upon other species with which it was paired. A classic linear model (Systat 13©) was applied, and Wilks' Lambda statistic was used to determine significance of the model. Canonical correlations and discriminant functions were calculated to determine which of the surface properties was more important in classifying selection.

A multiple linear regression procedure was used to analyze the effects of physicochemical surface properties on selection. Because only two algal species were used for each experiment, results of the model for rejection were the inverse of results for preferential ingestion. Accordingly, only results for rejection (positive EI values) are presented. Mean electivity-index data were used as the dependent variable, with surface charge, contact angle, and intensity of lectin binding (Con A, WGA, PEA) as the independent variables. The independent

variables were standardized (converted to zero mean, unit variance) prior to use in the model. To avoid overfitting bias, the dependent variable model residuals were weighted by the inverse standard deviation of the mean electivity-index data. A collinearity analysis was performed on the independent variables prior to generating the model (Belsley et al. 2013), and only those not collinear included. Model residuals were examined and a linear model was determined to be an appropriate fit for the data.

To define better the characteristics of particles that were always rejected or preferentially ingested regardless of pairings, single-factor linear regression analyses were performed on a subset of data (endpoints). For these analyses the actual surface-property values were used as predictors to determine which surface characteristics (surface charge, contact angle, or lectin affinity) best explained microalgal fate. PEA was selected for the analysis because it was the most important lectin in predicting selection (see results below). Prior to generating the model, predictor data were standardized (converted to zero mean, unit variance). For oysters, the endpoints included data for particles produced from ground, aged *Spartina alterniflora*, with animals fed the same concentrations of microalgae and detrital particles as in this study (Ward et al. 1998). All statistical procedures were performed using Systat 13 with a significance level of $\alpha = 0.05$.

3. Results

3.1 Microalgal characterization

A range of physicochemical surface characteristics was found for the individual microalgal species tested (Tables 1, 4). Surface charges ranged from -24.1 (± 2.4 SD) for *P. marinus* to -7.5 (± 1.2 SD) for *T. chui*. Contact angles ranged from 66.3 (± 3.6 SD) for *P. lutheri*

to 106.0 ($\pm$ 2.1 SD) for *C. carterae*. The mean fluorescence intensity for microalgae incubated with lectins was significantly higher than background fluorescence of the microalgae alone in all cases but ConA coupled with *R. salina* (Figure 1; mixed model ANOVA, $0.01 < P < 0.05$). The highest binding reaction was found for PEA with *C. carterae*, indicating a high presence of α -methyl mannoside and α -methyl glucoside on the scaled cell coating of this haptophyte. The chlorophyte *Chlamydomonas sp.* and the micromonadophyte *T. chui* also had high affinities for this lectin. Additionally, *C. carterae* had the highest affinity for WGA, which indicates the presence of N-acetyl-glucosamine on the cell surface. The halophilic chlorophyte *D. salina* and the scaled micromonadophyte *P. marinus* had high affinities for WGA. The cryptophyte *R. lens* had the lowest binding affinity to ConA and WGA, and the dinoflagellate *P. minimum* had the lowest affinity to PEA. Binding affinity to ConA was low for all of the species tested, indicating that these microalgae had low levels of methyl α -mannopyranoside, D-mannose, and D-glucose on their cell surfaces.

Three independent variables (surface charge, contact angle & PEA lectin-binding affinity) were used in the discriminant analysis (DA) to determine distinct characteristics of the nine different microalgal species. These predictors were chosen because no significant correlations were found between surface charge, contact angle, and binding affinity of ConA, WGA or PEA for the characterized microalgae ($P > 0.05$). Significant correlations, however, were found in the level of binding affinity of the three FITC-conjugated lectins (Pearson Correlation, $P < 0.001$). Therefore, data for only PEA were chosen for the model because this lectin showed the largest differences in binding affinity among the microalgal species (Figure 1; Tukey's HSD $0.01 < P < 0.05$; Table 4). The classification model (DA) was found to be statistically significant (Wilks' lambda $p < 0.001$), explaining 96% of the data variability

(canonical correlation of 0.966). In total, 78% of the algal species (7 of 9) were distinctly classified using the model. Discriminant functions (with 1 being the highest) were 0.126 (PEA), 0.162 (angle) and 0.720 (charge), indicating that charge was the most important factor in classifying microalgae. A DA using Class instead of species to classify groups also was performed (data not shown). This model was significant, but separation between groups and canonical correlations were not improved by classification by taxonomic Class, i.e., there were no surface characteristics that could reliably define a microalgal Class.

3.2 Feeding experiments with microalgal pairings

Seven of the 11 feeding assays with mussels, and six of the 11 feeding assays with oysters resulted in significant preferential ingestion of one of the two microalgal species (Bonferroni adjustment $0.01 < P < 0.05$; Figure 2). Degree of selection varied between the two bivalve species, and, except for the *R. lens* vs. *D. salina* algal pairing, the same algal species were rejected or preferentially ingested by both animals. Capture efficiency (CE) of *P. lutheri* by mussels and oysters did not differ significantly from CE of the other paired algae (Table 5). Thus, differences in the calculated electivity indices for these feeding experiments were attributable to post-capture selection between the algal species.

3.3 Statistical modelling of selection

As indicated above (section 3.1), the lectin binding affinities of ConA, WGA and PEA to the microalgal species were significantly correlated. Therefore, only data for PEA lectin-binding intensity were used as an independent variable for the second DA model (along with surface charge & contact angle). The binding affinities of WGA and PEA were also highly collinear.

Therefore, WGA was not used in the analyses and only ConA and PEA were used in subsequent regression models. Substituting WGA for PEA yielded identical results to those reported below. For mussels, the calculated EI resulted in a significant classification model (DA, Wilks's Lambda, $P < 0.001$). Microalgal cell properties that resulted in no selection were correctly classified by the model 82% of the time; whereas, characteristics resulting in ingestion were correctly classified 59% of the time, and rejection 0% of the time. Overall, the model correctly classified 58% of the cases, and explained 64% of the variability in the data. Based upon the discriminant functions, contact angle (measure of wettability) was the most important particle surface characteristic in identifying selection ($F(40, 63) = 39.3$, $P < 0.001$), with surface charge and PEA having almost identical weight. A DA of the algae that were only ingested or not selected (rejection data not included) resulted in an improved and significant model (Wilks's lambda $P < 0.001$), correctly classifying 87% of the selection cases and explaining 75% of the observed variability. This model classified surface charge as the most important factor in determining selection, followed by PEA affinity and finally wettability. Regression analysis of positive EI data (rejection) with the physicochemical surface properties (Δ -charge, Δ -angle, Δ -ConA, Δ -PEA) produced a model that was able to account for 90% of the variability in EI ($F(4, 6) = 24.73$, $P < 0.001$, $R^2 = 0.90$). Affinity of PEA was determined to be the factor best predicting rejection ($P = 0.001$), followed by ConA ($P = 0.006$), and finally contact angle ($P = 0.038$). Surface charge had no significant effect on EI ($P > 0.05$). The abundance of a given microalgae species in the pseudofeces in relation to its availability in the water (EI value) was negatively correlated to differences in contact angle and ConA between the pairs of microalgae, and positively correlated to differences in PEA affinity between the algae. The regression model took the form:

$$EI = 0.18 - 0.06Dangle - 0.10DConA + 0.13DPEA$$

For oysters, the calculated EI resulted in a significant classification model (DA, Wilks's Lambda, $P < 0.001$). Microalgal cell properties that resulted in no selection were correctly classified by the model 100% of the time, but characteristics resulting in ingestion were correctly classified 54% of the time, and rejection 13% of the time. Overall, the model correctly classified 57% of the cases, and explained 42% of the variability in the data. Based upon the discriminant functions, surface charge was the most important particle surface characteristic in identifying selection ($F(10, 314) = 15.5$, $P < 0.001$), followed by PEA and finally wettability. As with the mussels, a DA of the algae that were only ingested or not selected (rejection data not included) was performed, resulting in a slightly-improved, significant model (Wilks's lambda $P < 0.001$), correctly classifying 69% of the cases, but only explaining 40% of the variability in the data. Regression analysis of positive EI data (rejection) with the physicochemical surface properties (Δ -charge, Δ -angle, Δ -ConA, Δ -PEA) produced a model that was able to account for 94% of the variability in EI ($F(4, 5) = 39.8$, $P < 0.001$, $R^2 = 0.94$). PEA affinity was determined to be the factor best predicting ingestion ($P = 0.018$), followed by ConA ($P = 0.020$), and finally contact angle ($P = 0.032$). Surface charge had no significant effect on EI ($P > 0.05$). The abundance of a given microalgae species in the pseudofeces in relation to its availability in the water (EI value) was negatively correlated to differences in ConA between the pairs of microalgae, and positively correlated to differences in contact angle and PEA affinity between the algae. The regression model took the form:

$$EI = 0.46 + 0.20Dangle - 0.33DConA + 0.33DPEA$$

3.4 Analyses of endpoint data

Evaluation of EI endpoint data included microalgae that were always rejected or preferentially ingested regardless of its pairing with other algal species. For mussels, four microalgal species (*C. carterae*, *P. lutheri*, *P. marinus*, *T. chui*) were used. For oysters, the same microalgal species, and one detrital particle produced from *Spartina alterniflora*, were used. For mussels, EI was significantly dependent on contact angle ($R^2 = 0.17$, $0.01 < P < 0.05$) and PEA binding affinity ($R^2 = 0.64$, $P < 0.01$), but not dependent on charge. Similar results were obtained for oysters, with EI being significantly dependent on angle ($R^2 = 0.38$, $0.001 < P < 0.01$) and PEA binding affinity ($R^2 = 0.80$, $0.001 < P < 0.01$), but not dependent on charge. Results of these single-factor regressions demonstrated that particles with higher contact angles (= hydrophobic surfaces) and higher PEA affinity were selected for ingestion by both mussels (Figure 3) and oysters (Figure 4).

4. Discussion

This study demonstrates that both physical and chemical surface properties of microalgae affect selection processes of two species of suspension-feeding bivalve molluscs. Mussels (*Mytilus edulis*) and oysters (*Crassostrea virginica*) use these surface properties to discriminate between microalgal species, with differences in a particular surface property among different algal species resulting in strong selection, i.e., preferential ingestion or rejection. Results from this study also reveal that different species of microalgae have distinctive physicochemical surface properties, varying in the degree of wettability and negative zeta potential. Binding affinity abundance and distribution of the three tested lectins to carbohydrates on the cell surface of microalgae were significantly correlated, with *Pisum sativum* agglutinin (PEA) having the

greatest differences in binding affinity among the nine algal species tested. Data for microalgal characteristics were used to generate significant statistical models for predicting selection in both bivalve species. Ambiguity in the models was a result of different selection outcomes for some microalgae, i.e., some species were selected or not selected based upon the specific algal-pairings used. Difference between the particle-selection models generated for mussels and oysters could be a result of the loci of selection, which are different in these two species of bivalve (Beninger et al. 1993, Ward et al. 1997, 1998).

Surface properties of the nine microalgal species examined displayed a wide range of characteristics. All had negative surface charges, a finding consistent with what has been reported for other marine organic particles and microalgae (Neihof and Loeb 1972; Ozkan and Berberoglu 2013). Generally, inorganic particles (e.g. glass, resin, clay) have been reported to have a lower range of measured surface charge than organic particles (Neihof and Loeb 1972), and the few reports of positively-charged particles in seawater were for inorganic sediments (Pravdic 1970). Differences in charges between organic and inorganic particles have been attributed to adsorbed organic constituents (Neihof and Loeb 1974; Hunter 1980; Hunter and Liss 1982; Karickhoff 1984). Interest in cation interactions with algal cell coverings decades ago, specifically divalent heavy metals, led to a large number of articles describing adsorptive and absorptive processes of metals interacting with algal cells (see Kuyucak and Volesky, 1989). Adsorptive processes essentially are ion-exchange reactions in which metal ions and other divalent cations, e.g., calcium, compete for binding sites with negatively-charged carboxyl groups on the cell surface. The net-negative charges found for microalgae in the current study are consistent with the affinity for metals to adsorb on algal-cell coverings and confirm that the negative, pericellular charge is a general feature of microalgae in seawater.

Most microalgal species characterized in this study had slightly hydrophobic surfaces, a finding consistent with results for colony-forming and benthic microalgal species (Ozkan & Berberoglu 2013). Previously characterized inorganic particles (i.e., alumina, Rosa et al. 2013; silica, Ozkan and Berberoglu 2013), in contrast, have been wettable, a characteristic that is indicative of hydrophilic surfaces. These findings suggest a general trend in the marine environment; inorganic particles tend to have surfaces that are weakly charged and more hydrophilic; whereas, organic particles tend to have surfaces that are strongly charged and more hydrophobic. Generally, bivalves preferentially ingest living and organic particles compared to non-living, inorganic particles (see Kiørboe et al. 1980; Ward et al. 1997; Ward and Shumway 2004; Beninger et al. 2008). Accordingly, if wettability is correlated with the presence of an organic coating on particles, and if this trend holds true for most particles in marine systems, a possible correlation between particle discrimination and passive selection is suggested. Significantly, the most hydrophilic microalga used in this study (*Pavlova lutheri*) was strongly rejected in all pairings tested.

Similarities in binding profiles of the three lectins, previously identified in mucus of bivalves (Bayne 1990; Gauthier et al. 2004; Pales Espinosa et al. 2009, 2010b), were found among the nine microalgal species studied. All of the tested microalgae, for example, had low binding affinity to concanavalin agglutinin (ConA). Seven of the tested microalgae had similar binding affinities to wheat germ agglutinin (WGA). In contrast, only three of the microalgae had similar binding affinity to *Pisum sativum* agglutinin (PEA). One species of microalga, *Cricosphaera cartarae*, had the highest binding affinity to all three of the lectins. Generally, the patterns of binding affinities of the lectins used in this study indicate that the distribution and abundance of the surface sugars targeted in this study were very similar among microalgae

species. These findings are consistent with lectin-binding profiles recently reported for several microalgae (Pales Espinosa et al. 2016), with some lectins (e.g. PEA) binding strongly to the sugars on these cell surfaces and others having high specificity for one or two algal species only. As a result of the high degree of collinearity in binding affinity and abundance among the three lectins to each of the microalgae species tested in this study, data for only one lectin (PEA) could be used in the multiple regression models. Pales Espinosa et al. (2016) also found that binding-affinity profiles of the lectins ConA, WGA and PEA for 16 species of microalgae were correlated. Further, these authors noted differences in FITC-binding intensity within biological replicates of several microalgae species, something found with only one species (*Rhodomonas lens*) in the current study. These findings highlight some of the drawbacks of using only lectin profiles in studying selection in bivalves. Lectin molecules are ubiquitous, and with the number of different types of sugars with which they can potentially bind, finding a particular one that produces distinct binding-profiles across a range of microalgae species can be difficult. Further, growth conditions, growth phase, and nutrient availability have been shown to affect lectin profiles (Pales Espinosa et al. 2009; Feng et al. 2015). Results of the current study, for example, demonstrated that the nine microalgal species tested had low binding affinities for the lectin ConA; whereas, the same species have been reported to have higher binding affinity for the same lectin (Pales Espinosa et al. 2016). Such differences in lectin-binding affinity, using similar methods (adapted from Gauthier et al. 2004) and the same microalgal species, indicate that more work is needed to determine intra- and interspecific variation in sugar moieties on the cell surfaces of microalgae. Further characterization might also help to target a lectin that acts as a more predictable effector of particle selection in bivalves.

No pattern in the distribution of surface characteristics (charge, wettability, or lectin affinity) was apparent between microalgae with differing cell surfaces (scales, vs. membrane, vs. glycoprotein wall). Members of the same class are not always similar in charge and wettability, which may be attributable to their cell characteristics e.g., *Chlamydomonas sp* has a glycoprotein wall, but *D. salina* lacks a wall, so the membrane is the outermost surface. Descriptions of cell structures and coverings are independent of phylogeny (Okuda 2002). Five of the microalgae used in this study had membranes (= “naked”), with no discernable pattern in selection. The two scaled microalgae tested (*P. marinus* and *T. chui*) were rejected by both bivalve species when paired against other microalgae, and when fed together no selection occurred. This suggests that scales may promote rejection or interfere with detection of cell-membrane compounds by feeding organs. In a survey of biochemical composition of microalgal strains used in aquaculture, Brown and co-workers (1997) concluded that variation in sugar content within a taxonomic class generally is larger than differences between classes. Nevertheless, some classes did exhibit unusual sugar profiles, e.g., prymnesiophytes surveyed had exceptionally large arabinose contents relative to other classes. It remains to be determined, however, how total cellular sugar composition may relate to extra-cellular sugar composition that can interact with the feeding organs of bivalves.

The regression and classification models generated in the current study demonstrate that the microalgal surface characteristics that are most important in particle selection by *M. edulis* and *C. virginica* are different. For mussels, the regression model found significant effects of PEA, ConA and wettability on EI, with PEA being the most important factor. The DA model found that wettability was most important factor. Addition of the other two lectins (ConA and WGA) to the DA model did not increase the classification capabilities. In oysters, the regression

model found that PEA, ConA, and wettability significantly affected EI, with PEA also being the most important factor. The DA model, on the other hand, found that charge was most important factor. The fact that the two species do not rely on the same particle characteristics to the same extent is not surprising. Mussels and oysters possess gills with very different architectures and have distinct loci of selection (Ward et al. 1997, 1998). Differences in selective capabilities between other species of bivalves have been demonstrated in previous studies (e.g. Lesser et al. 1991; Bougrier et al. 1997; Bacon et al. 1998; Levinton et al. 2002; Beninger et al. 2007; Pales Espinosa et al. 2010b; Rosa et al. 2013). For example, Beninger and Decottignies (2005) found that live and dead cells of the diatom *Coscinodiscus perforatus*, whose epicellular frustules had been left intact, were handled similarly by the scallop, *Pecten maximus*, with no selection observed. Experiments repeated using the Pacific oyster, *Crassostrea gigas*, found that this bivalve was able to discriminate between the live and dead diatoms post-capture (Beninger et al. 2008). The underlying factors that caused differences in selection between the two bivalve species remain unknown, although they have somewhat different gill architectures and ciliary microstructures.

Results of the present study differed from those reported by Pales Espinosa et al. (2016), who modeled food selection in *M. edulis* and *C. virginica* using only lectin-binding profiles of the microalgae. For example, their logistic regression model indicated that binding affinity of *Arachis hypogaea* agglutinin (PNA) to the different microalgae is most important for selection in mussels and *Phaseolus vulgaris* agglutinin (PHA) and *Erythrina cristagalli* agglutinin (ECA) for selection in oysters, even though the majority of the microalgae bound to ConA and PEA. Differences between the present study and the study of Pales Espinosa et al. (2016) are likely a result of several methodological approaches. First, Pales Espinosa et al. (2016) used a static

system that exposed animals to a decreasing particle concentration over a 20 min period. Because the amount of pseudofeces produced by bivalves is partially dependent upon particle concentration (Bayne et al. 1977), rapidly declining particle concentrations in static systems result in small amounts of pseudofeces being produced. Lower pseudofeces production leads to difficulties in accurately quantifying the number of each particle type in the collected samples and a less reliable instantaneous assessment of particle selection. In contrast, a flow-through system delivers a constant concentration of particles over a longer period of time (1-2 hrs in the present study). Under constant conditions, a greater quantity of pseudofeces is produced, allowing for more accurate determination of the ratio of particles in food and pseudofeces, and more robust time-averaged assessment of selection. Such methods ultimately lead to stronger models and more consistent results than experiments that used a static system with decreasing particle concentration over a short period of time (e.g., Petersen et al. 2004; Rosa et al. 2015; Pales Espinosa et al. 2016). Additionally, and perhaps most importantly, Pales Espinosa et al. (2016) pooled data obtained for *M. edulis* and *C. virginica* to generate one classification model. Given the reports outlined above and the clear differences in the way in which mussels and oysters handle particles, such an approach is problematic and yields a model with questionable applicability to either species.

The physical and chemical surface properties characterized in this study represent a first step in determining factors that mediate preferential ingestion and rejection of particles by bivalves. These targeted characteristics are based upon previous studies that have identified various physicochemical properties which can act as mediators for particle discrimination (e.g. Taghon 1982; Solow and Gallagher 1990; Pales Espinosa et al. 2010b; Rosa et al. 2013). Given the array of adsorbed substances, e.g., sugar moieties, ions, bacterial communities, and organic

molecules, on suspended particles in the marine environment, it is possible that factors not characterized in the present study account for some of the observed selection. Further, as selection involves physiological responses, the differences in surface properties among particles may matter more than absolute surface characteristics. In the natural environment, particle selection always involves a choice, and the fate of a particle (ingested or rejected) may be dependent upon the types of particles that are encountered at the same time. Changes in the fates of some microalgal species reduced the strength of selection models. Nonetheless, the models suggest that certain characteristics (e.g. hydrophobicity) increase the *likelihood* of a particle being ingested when there is a choice. Because the surface chemistry of particles is often dominated by active groups, surface charge and hydrophobicity could be proxies for the bulk surface characteristics a bivalve will encounter. Results from the regression model using only the algae consistently ingested or rejected regardless of pairing (“endpoints data”) indicate that microalgae which had hydrophobic surfaces were significantly selected for ingestion. These algae also had the highest binding affinities to PEA. Microalgae with lower PEA binding affinity and hydrophilic surfaces were strongly rejected by both bivalve species. Interestingly, selection based on surface charge was not a linear relationship.

In conclusion, results of this study demonstrate that physical characteristics of particle surfaces, as well as chemical factors (e.g., lectins), must be included in any model to obtain a comprehensive assessment of particle selection. Together, the findings outlined in this study suggest that lectin profiles alone offer an incomplete picture of selection in bivalves. The types of sugar moieties present on the cell surface of microalgae need to be characterized further, although caution should be used in interpreting the data as both cellular phase and growth regime (nutrient availability) have been shown to affect these characteristics. The differences in the

physicochemical properties that affect particle discrimination in *M. edulis* and *C. virginica* demonstrate that these two species rely on different surface characteristics for particle-selection. This result emphasizes that future modeling work should take into account differences between species in selection. Finally, differences in the models generated using preferential rejection data (regression analyses) vs. those examining all particle fates (discriminant analyses) also indicate that the physicochemical factors that determine rejection may be decoupled from those that determine ingestion. This suggests the non-linear effects, wherein one characteristic may be more likely to result in rejection and not preferential ingestion, of all these properties on the selective capabilities of different bivalve species should be further explored.

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Figure 1. Binding of the FITC-conjugated lectins for each algal species characterized in this study. Data shown as fluorescence intensity (mean $\pm$ SD, n=3). Except for ConA with *R. salina*, significant binding of each lectin to the different algal species was found (K-S test, $P < 0.01$). Different letters (a, b, and c) indicate significant differences in binding affinity of the different lectins within a microalgal species (Tukey's HSD, $0.01 < P < 0.05$).

Figure 2. Electivity indices (EI) for the different algal pairings used in the selection experiments. Data are based on the percentage of algal cells in the pseudofeces versus the percentage of the same algal cells in the diet. Presented EI are for the first species in the x-axis paring label, with the second species having the opposite EI but with the same magnitude. A positive EI indicates the alga was rejected, and a negative EI indicates the alga was preferentially ingested. An EI of zero indicates no selection was taking place. *indicates the EI is significantly different from zero (Bonferroni adjusted $0.01 < P < 0.05$). EI data are presented as mean $\pm$ SD (n=8-13).

Figure 3. Single-factor regression plots of mussel endpoint data. EI was graphed against the two physical surface properties and PEA binding affinity. A) No significant relationship was found between EI and charge (EI=). B) Significant negative relationship was found between contact angle and EI, with higher angles (e.g., less wettable surfaces) resulting in preferential ingestion (EI= $-0.14\text{angle} + 0.04$, $P < 0.001$). C) Significant negative relationship was found between binding affinity of PEA and EI, with higher affinity resulting in preferential ingestion (EI = $-0.01\text{PEA} + 0.30$, $P < 0.001$).

Figure 4. Single-factor regression plots of oyster endpoint data. EI was graphed against the two physical surface properties and PEA binding affinity. A) No significant relationship was found between EI and charge. B) Significant negative relationship was found between contact angle and EI, with higher angles (e.g., less wettable surfaces) resulting in preferential ingestion (EI = $-0.03\text{angle} + 2.81$, $P < 0.001$). C) Significant negative relationship was found between binding affinity of PEA and EI, with higher affinity resulting in preferential ingestion (EI = $-0.02\text{PEA} + 0.71$, $P < 0.001$).

Figure 1

Figure 2

Figure 3

Figure 4

Table 1. Physical surface properties of the characterized microalgal species. Significant differences in surface properties were found among species, but no significant correlation was found between surface charge and contact angle across species. Data presented as means (SD), n=5-6. Algal sizes are based on the peak obtained using a Coulter Multisizer IIe© instrument. *denotes significant differences in surface properties (DA, $0.01 < P < 0.05$).

Algal species	Cell surface	Strain	Mean size (µm)	Surface charge (mV)	Contact angle (°)
<i>Tetraselmis chui</i>	Scales	PLY429	13	-7.5 (1.2)*	99.9 (1.8)
<i>Rhodomonas lens</i>	Membrane	Rhodo	12	-13.9 (0.6)	93.5 (2.4)
<i>Chlamydomonas sp.</i>	Glycoprotein wall	11/35	9	-13.6 (0.7)	102.9 (2.4)*
<i>Cricosphaera carterae</i>	Coccoliths	961	11	-13.8 (0.1)	106.0 (2.1)*
<i>Prorocentrum minimum</i>	Membrane	Exuv	21	-12.4 (1.1)	98.9 (7.8)
<i>Dunaliella salina</i>	Membrane	LB200	10	-11.0 (1.4)*	91.5 (2.8)
<i>Pavlova lutheri</i>	Membrane	MONO	3	-16.5 (0.7)*	66.4 (3.6)*
<i>Prasinocladus marinus</i>	Scales	163/1B	10	-24.1 (2.4)*	91.3 (2.5)
<i>Rhodomonas salina</i>	Membrane	F-3C	8	-13.2 (1.0)	93.7 (2.8)
<i>Spartina alterniflora</i>	Cellulose wall	N/A	2-6	-11.4 (0.5)	92.9 (6.2)

Table 2. FITC-conjugated lectins used in the algal characterization experiments, with carbohydrate specificity (Goldstein and Poretz, 1986).

Lectin	Abbreviation	Source	Carbohydrate specificity
Concanavalin agglutinin	ConA	<i>Canavalia ensiformis</i>	Methyl α -mannopyranoside; D-Mannose; D-Glucose
Wheat germ agglutinin	WGA	<i>Triticum vulgaris</i>	N-Acetyl-glucosamide
<i>Pisum sativum</i> agglutinin	PEA	<i>Pisum sativum</i>	α -Methyl mannoside; α -Methyl glucoside

Table 3. Algal pairings used in selection assays. Pairings were based on cells size and differences and similarities of characterized surface properties (see Table 1 for genus designations). Algal sizes are based on the peak obtained using an Accuri C6 flow cytometer during the feeding assays. *pf* = pseudofeces

Algal species		Measured size (μm)		N of animals producing <i>pf</i>	
A	B	A	B	<i>C. virginica</i>	<i>M. edulis</i>
<i>R. salina</i>	<i>P. minimum</i>	6	12.1	9	9
<i>R. salina</i>	<i>P. marinus</i>	6	8.6	9	9
<i>C. carterae</i>	<i>P. marinus</i>	10	8.6	8	9
<i>Chlamydomonas sp.</i>	<i>C. carterae</i>	6.2	10	10	10
<i>Chlamydomonas sp.</i>	<i>P. lutheri</i>	6	4.2	7	8
<i>R. lens</i>	<i>P. lutheri</i>	5.5	4.2	6	8
<i>T. chui</i>	<i>Chlamydomonas sp.</i>	8.1	6.2	9	10
<i>T. chui</i>	<i>P. marinus</i>	8.1	8.6	10	10
<i>T. chui</i>	<i>R. lens</i>	9.2	5.5	6	10
<i>D. salina</i>	<i>C. carterae</i>	8.1	10	13	10
<i>D. salina</i>	<i>P. minimum</i>	9.3	11.5	2	10

Table 4. Fluorescent intensity of FITC-binding of the three tested lectins to each of the microalgal species. Data shown as means (SD), n=3. Different letters represent significant differences in binding affinity for a given lectin among the algal species (Tukey's HSD, 0.01 < P < 0.05). ^{ns} indicates there was no significant lectin binding to the algal species and therefore data were not used in the mixed model ANOVA.

Algal species	ConA	WGA	PEA
<i>Tetraselmis chui</i>	3.5 (0.4) ^a	3.5 (0.1) ^a	10.2 (0.6) ^{a, g}
<i>Rhodomonas lens</i>	2.3 (0.1) ^{b, e}	3.6 (0.3) ^a	3.9 (0.5) ^{b, d, e, f}
<i>Chlamydomonas sp.</i>	2.0 (0.0) ^{b, e, f}	3.0 (0.1) ^a	11.6 (5.4) ^a
<i>Cricosphaera carterae</i>	4.1 (0.1) ^c	56.7 (3.9) ^b	60.0 (1.8) ^c
<i>Prorocentrum minimum</i>	2.2 (0.1) ^{b, e}	5.6 (0.4) ^{a, c}	2.6 (0.6) ^{d, e}
<i>Dunaliella salina</i>	1.4 (0.0) ^d	15.4 (10.0) ^{c, d, e}	3.7 (0.5) ^{e, f}
<i>Pavlova lutheri</i>	2.3 (0.1) ^{b, e}	5.3 (0.6) ^{a, d}	5.2 (0.5) ^f
<i>Prasinocladus marinus</i>	1.5 (0.0) ^{d, f}	7.2 (1.3) ^{a, c, d}	5.7 (0.6) ^{a, g}
<i>Rhodomonas salina</i>	1.7 (0.1) ^{ns}	5.0 (1.1) ^{a, e}	5.2 (0.8) ^g

Table 5. Capture efficiencies (CE) of microalgae paired with *Pavlova lutheri* in different feeding experiments. CE was calculated to ensure the differences in microalgae size did not result in lower captures rates, which would lead to erroneous electivity indices being calculated for the microalga. There was no significant difference in mean CE of the microalgae between each algal pairing ($0.01 < P > 0.05$). Data are presented as means ($\pm$ SD), N= 6-8.

	Microalgal Pairing			
	<i>P. lutheri</i> 4.2µm	<i>Chlamydomonas sp.</i> 6.2µm	<i>P. lutheri</i> 4.2µm	<i>R. lens</i> 5.5µm
<i>M. edulis</i>	0.35 (0.15)	0.36 (0.15)	0.30 (0.04)	0.37 (0.17)
<i>C. virginica</i>	0.52 (0.21)	0.53 (0.23)	0.70 (0.08)	0.72 (0.10)