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ORIGINAL PAPER

Modeling food choice in the two suspension-feeding bivalves, *Crassostrea virginica* and *Mytilus edulis*

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Abstract Suspension-feeding bivalves are able to sort and select food particles from a complex mixture. Recent reports have indicated that this selection is mediated by interactions between lectins present in mucus covering the feeding organs and carbohydrates associated with the surface of microalgae. In this study, several statistical methods were evaluated to predict the likelihood for a given microalga to be ingested or rejected based upon its cell surface carbohydrate signature. First, the carbohydrate signatures of 16 microalgae were characterized using 10 different lectins. In June 2014, a subset of microalgae (12 species) was then used in feeding experiments where different pairs of microalgae were presented to oysters (*Crassostrea virginica*) and mussels (*Mytilus edulis*) to evaluate selection. Results show that cell surface carbohydrates are good predictors for particle fate. Specifically, microalgae rich in glucose/mannose residues were preferentially selected by both oysters and mussels. Several statistical methods for predicting the likelihood of a given alga being ingested or rejected were evaluated, and a decision tree that accurately models selection in the two bivalves is proposed even though the model warrants further validation with different species or in various seasons. Overall, these findings provide **a** promising predictive tool that could be used to assess bivalve performance and benthic–pelagic coupling under ecological or aquaculture contexts.

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Introduction

In marine ecosystems, benthic invertebrates contribute to many ecological functions, including the regulation of energy fluxes. This is the case for suspension-feeding organisms that often dominate the macrobenthos in coastal areas. Dense populations of benthic filter-feeders interact strongly with nearshore waters, removing phytoplankton, producing biodeposits, and cycling dissolved nutrients (Prins et al. 1997; Newell 2004). Trophic position allows these organisms to influence and control energy and nutrient transfer between communities. These organisms are confronted with a wide range of suspended material, including live plankton of varying sizes and quality, and diverse particulate debris (Alldredge and Silver 1988); consequently, they have developed strategies to enhance the nutritive value of ingested material and optimize energy gain. Suspension-feeding bivalves are known to have evolved the ability to discriminate among particles (e.g., Allen 1921; Fox 1936; Ward and Shumway 2004; Pales Espinosa et al. 2008). Although some aspects of the selection process have been elucidated, the mechanism by which particles of poor quality are rejected as pseudofeces while those of higher quality are ingested remains unclear, thus limiting the understanding of energy fluxes through coastal ecosystems. As a matter of fact, ecophysiological models (e.g., dynamic energy budget or scope for growth) developed to understand the functioning of shellfish within ecosystems currently rely upon incomplete information regarding feeding processes as they do not take into account pre-ingestive selection (Barillé et al. 2011).

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Previous studies have demonstrated that bivalves preferentially reject mineral particles and detritic matter while live cells are selectively ingested (Newell and Jordan 1983; Pastoreaud et al. 1996; Ward et al. 1998). Furthermore, some bivalves are able to preferentially select one microalga among a mix of several species (Shumway et al. 1985; Cognie et al. 2001). Other studies have supported the idea that biochemical cues can mediate the selection process (Beninger and Decottignies 2005; Pales Espinosa et al. 2008, 2011), providing a novel conceptual framework for unraveling the mechanisms used by suspension-feeding bivalves to optimize foraging. In addition, the physicochemical surface properties of food particles (specifically wettability), which are determined by surface chemical landscape, have also been proposed as factors contributing to particle selection (Rosa et al. 2013). Nevertheless, the current information remains unable to respond to the intriguing fundamental question: "Which factors determine whether a particle would be ingested or rejected as pseudofeces?"

To answer this question and identify biochemical cues involved in prey recognition by suspension-feeding bivalves, we focused on cell surface carbohydrates as potential candidates. Cell surface carbohydrates play critical roles in many cellular recognition processes, including fertilization (Bolwell et al. 1979; Clark 2013), symbiosis (Reisser et al. 1981; Wood-Charlson et al. 2006), and immunity/defense (Sharon and Lis 2004, references within). Interestingly, these glycans also have been found to be involved in selective feeding mechanisms. Thus, marine grazers, such as members of the Dinophyceae, use carbohydrates present on the cell surfaces of microalgae prey as cues to select and capture food particles (Ucko et al. 1999; Wootton et al. 2007). This selection process is mediated by biochemical tools (e.g., carbohydrate-binding proteins or lectins) that recognize and adhere to prey cell surface glycans (Wootton et al. 2007; Martel 2009). Lectins are a diverse group of sugar-binding proteins highly specific for carbohydrate residues (Sharon and Lis 2004). They are found in viruses, bacteria, plants, and invertebrate and vertebrate cells and are involved in a wide spectrum of biological functions (reviewed by Vasta et al. 2004), including recognition of food particles. Previous investigations demonstrated the presence of sugar-binding lectins in mucus covering the feeding organs (e.g., gills, palps) of suspension-feeding bivalves (Pales Espinosa et al. 2010a). Further studies showed that these lectins bind glycans on the surfaces of microalgae and mediate selection (Pales Espinosa et al. 2010b). Further, our previous investigations also showed that these lectins are regulated to favor the selection of high-quality food (Pales Espinosa and Allam 2013).

The main goal of the present study was to test the hypothesis that some carbohydrates contribute more than others in directing particle selection in suspension-feeding bivalves and to predict the likelihood for a given microalga to be ingested or rejected based upon its cell surface carbohydrate signature. Several marine microalgae were investigated and used in feeding experiments with the eastern oyster *Crassostrea virginica* and the blue mussel *Mytilus edulis*. Statistical models were evaluated to provide a quantitative assessment of particle selection.

Materials and methods

Bivalves

The study used eastern oysters (*Crassostrea virginica*) and blue mussels (*Mytilus edulis*). These species are among the most important members of the benthic communities in coastal areas of eastern North America and display contrasting ctenidia architectures. Oysters (61.7 ± 3.7 mm shell length, umbo to margin) were obtained from Great Atlantic Shellfish Farm (East Islip, New York, USA) and mussels (55.0 ± 4.3 mm shell length) were collected from Long Island Sound (Port Jefferson, New York, USA) in June 2014, corresponding to the start of the spawning season. Bivalve shells were cleaned of all epibionts, acclimated in the laboratory for 1 week (salinity 28, 15 °C) and fed daily (15 % dry weight) using DT's Live Marine Phytoplankton (Sustainable Aquatics, Tennessee, Pales Espinosa and Allam 2006). Bivalves were held in filtered (0.45 µm) seawater for a day prior to being used in the feeding experiments.

Microalgae

Bacteria-free microalgal cultures were obtained from the Northeast Fisheries Science Center Milford Microalgal Culture Collection (Milford, Connecticut, Table 1). Species were selected upon several criteria: a size range optimal for bivalve feeding (2–40 µm); a number of classes; easy growing species. Strains were grown in triplicate in F/2-enriched, 50:50 Milford Harbor:deionized water (Guillard 1982) at 18 °C under a 12 h dark:12 h light photoperiod in a lighted bioincubator. Cultures were harvested in exponential phase and used in lectin-binding assays the following day. For feeding experiments, microalgae were produced in 3-L cultures using comparable conditions as described above. Growth rate was determined every day, and microalgae were harvested in exponential phase and used immediately.

Binding of FITC-labeled lectins to microalgae

Procedures used to bind lectins to microalgae were previously described in Pales Espinosa et al. (2010b). All lectins used in this study were fluorescently labeled with fluorescein isothiocyanate (FITC, EY Laboratories, Inc., California, Table 2). Briefly, subsamples of microalgal cultures were centrifuged

Table 1 List of 16 microalgae used in lectin-binding experiments

Class	Species	Strain	Cell length (μm)
Dinophyceae	*Alexandrium fundyense*[T]	38-3	30
Bacillariophyceae	*Amphora coffaeaformis*	A-ora	20
Bacillariophyceae	*Chaetoceros simplex*[A]	Chaet-G	5
Chlorophyceae	*Chlamydomonas* sp.	11/35	10
Chlorophyceae	*Chlorella autotrophica*	580	2
Prymnesiophyceae	*Cricosphaera carterae*	961	10
Chlorophyceae	*Dunaliella salina*	LB200	9
Eustigmatophyceae	*Nannochloropsis* sp.	UTEX-2341	2
Bacillariophyceae	*Nitzschia closterium*[A]	D-828	25
Bacillariophyceae	*Nitzschia pusilla*[A]	0–1	30
Prymnesiophyceae	*Pavlova lutheri*	MONO	4
Prasinophyceae	*Prasinocladus marinus*	163/1B	10
Dinophyceae	*Prorocentrum minimum*	Exuv	20
Cryptophyceae	*Rhodomonas lens*	Rhodo	12
Cryptophyceae	*Rhodomonas salina*	F-3C	8
Prasinophyceae	*Tetraselmis chui*	PLY429	9

Class to which microalga belong, cell size (length) and strain (Milford Microalgal Culture Collection) are indicated. Four species were not used in feeding experiments, either because they made aggregates (A) or might be toxic (T)

at 400×*g* for 10 min, washed once, and re-suspended in 0.22-μm-filtered seawater (FSW, salinity 28). FITC-conjugated lectins were diluted in FSW to 1 mg mL^{-1}. One 50-μL aliquot of lectin (or FSW control) was added to each microcentrifuge tube containing 1 mL of washed microalgae (10^6 cells). Microalgae were then incubated in the dark at room temperature for 1 h. Each assay was performed in three biological replicates and analyzed using flow cytometry (see details below).

Feeding experiments

Microalgae were used in a series of particle selection experiments to determine possible relationships between selection or rejection and cell surface characteristics (i.e., lectin-binding pattern). For each feeding experiment, a diet of equal concentrations of two microalgae (ca. 2×10^5 cells mL^{-1} final concentration) was delivered to 12 mussels, 12 oysters, and an empty shell (i.e., control tank), all maintained individually in 4-L tanks. Microalgae were kept in suspension using a micropipette every 10 min, and at the same time, water samples were taken to determine possible changes in the ratio between the two microalgae (possibly from sedimentation or different retention efficiencies). Pseudofeces were collected from each bivalve about 20 min after the start of production, vortexed to disrupt particle aggregates, passed through a 50-μm nylon-mesh sieve, and analyzed using flow cytometry. Overall, 27 unique experiments (i.e., composed of different pairs of microalgae) were performed 3 day $week^{-1}$ over 2 weeks in June 2014 using staggered, exponentially growing microalgal cultures to minimize changes in microalga cell surface characteristics and bivalve physiological status.

Table 2 FITC-lectins used to characterize microalgae cell surface carbohydrates

Name	Origin	Carbohydrate specificity	FITC ratio
PHA-L	*Phaseolus vulgaris*	Complex oligosaccharide	1.19
ECA	*Erythrina cristagalli*	*N*-acetyllactosamine, galactose	1.94
SBA	*Glycine maxima*	α and β-*N*-acetylgalactosamine > α and β-galactose	2.00
HPA	*Helix pomatia*	*N*-acetylgalactosamine	2.08
PWM	*Phytolacca americana*	Oligomers of β(1,4)-linked *N*-acetylglucosamine	1.30
ConA	*Canavalia ensiformis*	α-D-mannose, α-D-glucose, branched mannose	1.70
PEA	*Pisum sativum*	Methyl-D-mannopyranoside, D-mannose	2.20
PNA	*Arachis hypogaea*	Terminal β-galactose	2.35
WGA	*Triticum vulgaris*	Chitobiose, *N*-acetyl-glucosamine	2.00
UEA-I	*Ulex europaeus*	α-L-Fucose	2.07

Origins of lectin, carbohydrate specificity and FITC ratio are given

Flow cytometry analysis

Microalgae were analyzed using a FACSCalibur flow cytometer (BD BioSciences, San Jose, California, USA). A minimum of 2×10^4 events were analyzed. The 488-nm argon laser was used for excitation, and microalgae were characterized, discriminated, and counted based upon one or more of the following parameters: forward (FSC; particle size) and side (SSC; intracellular complexity) light scatter, FITC fluorescence (FL1, 535 nm), and photosynthetic pigment auto-fluorescence (FL2, 585 nm for phycoerythrin; FL3, 670 nm for chlorophyll *a*).

Data treatment and statistical analysis

All statistical analyses realized in this study were conducted using XLSTAT software (XLSTAT 2015.1.02) and Statgraphics (v5.1).

Results obtained for microalgae treated with FITC-labeled lectins are presented as differences between fluorescence intensities (geometric mean) of treated cells and baseline auto-fluorescence in FL1 (control incubated with seawater). Additionally, the geometric mean fluorescence values were normalized to FSC, FITC/protein ratio, and the mean of the final, relative fluorescence intensity. To identify homogenous groups of microalgae based upon lectin-binding pattern, a hierarchical cluster analysis (HCA) was completed in MeV software (v4.9) using Pearson correlation (complete linkage method, leaf order optimization). An HCA tree coupled with annotated heatmap was generated. Robustness of clusters was tested with bootstrap resampling ($n = 5000$).

Data obtained from the feeding experiments were analyzed using goodness-of-fit tests (G test) as described previously (Pales Espinosa et al. 2009). Two series of tests were performed comparing the proportions 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 the proportion of each particle type in the diet or in the pseudofeces was not different between treatments. 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:

$$\mathrm{SE} = 1 - (P/D)$$

where P and D represent the proportion of the particle of interest in the pseudofeces and diet, respectively. A positive SE for a given particle type indicates that it is preferentially ingested (particle type is depleted in the pseudofeces, compared to diet), a negative SE indicates rejection (particle type is enriched in the pseudofeces compared to diet), and zero indicates the absence of active selection. After checking for normality, calculated SE values obtained for each of the two particles in each treatment were compared to zero using a one-sample t test (two-tailed). The null hypothesis was that the selection efficiencies were equal to zero (i.e., no selection). An interaction network analysis (Fig. 1, Cytoscape, v3.0) summarizes the design of the 27 experiments using *C. virginica* and *M. edulis*.

The forecast of microalgal fate (ingestion vs. rejection), based upon lectin-binding pattern, was evaluated using logistic regressions and classification trees. Logistic regression is the most common method used to predict the probability of an outcome, especially in public health and medical studies (Austin 2007; Li et al. 2012). To complement logistic regressions, classification trees (Breiman et al. 1984) were used as these were shown to be well adapted to complex data or to data that contain missing values (De'ath and Fabricius 2000; Elith et al. 2008).

In this study, the main objective was to identify a model that fits and explains observations. First, logistic regression models were built to evaluate whether overall lectin-binding pattern is related to selection or rejection of each microalga. The dependent variable was continuous and calculated as the proportion of individuals in the experiment that behaved similarly to the calculated SE. Because each microalga was used in multiple feeding experiments and can be selected in one experiment and rejected in another, we were not able to use the normalized intensities of FITC-lectin (NIFL) as the independent variables. Instead, for each of the ten lectins (e.g., ConA), we calculated and used the difference between the NIFL of the selected microalga and the NIFL of the rejected microalga (i.e., Δ NIFL, for example Δ ConA). Variables displaying colinearity and nonsignificant variables were removed. The Akaike information criterion (AIC) was used to select final models. The robustness of the model was cross-validated by removing 10 % of the data (i.e., 3 observations) and calculating a new logistic model using the remaining data (Ripley 1996). The performance of the model, in other words how well the validation model predicted the removed data, was evaluated using an accuracy rate. The cross-validation step was performed 9 times, using each of the observations once.

In parallel, to further evaluate whether the 10 descriptive variables (Δ NIFL) explain whether a microalga is selected or rejected and to determine rules that would govern the model, classification trees were also built (Breiman et al. 1984; Ripley 1996). In this study, different algorithms were tested (CHAID, exh CHAID, C&RT, and Quest), but the complexity of each tree was kept as low as possible (maximum tree depth = 2) to facilitate interpretation. The performance of a model was examined by evaluating the area under the receiver-operating characteristic

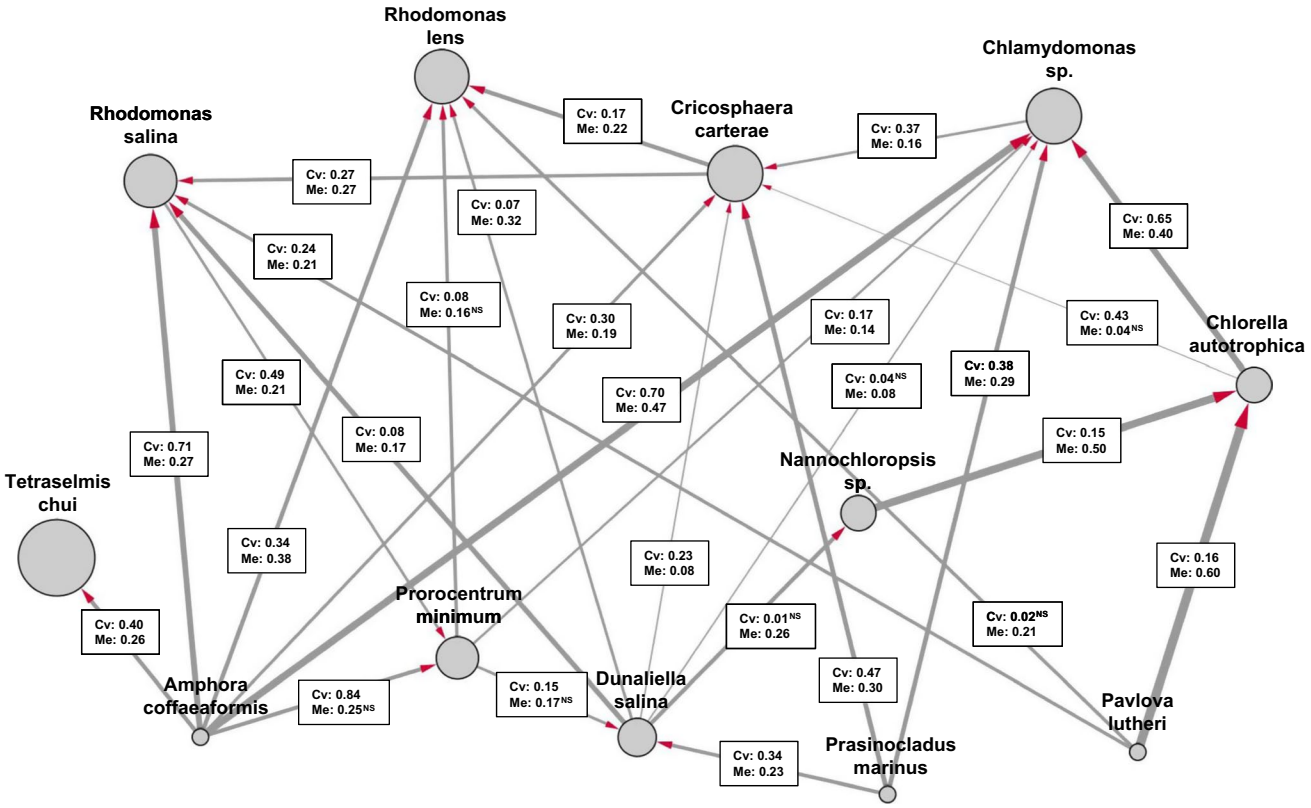

Fig. 1 Interaction network showing *Crassostrea virginica* (Cv) and *Mytilus edulis* (Me) feeding choices. Nodes associated with 12 species of microalgae are drawn as gray circles. Node size is proportional to closeness centrality (i.e., measurement that takes into account number of times a microalga was used in feeding experiments and number of time it was selected). Links represent pairs of microalgae used in feeding experiments and arrowheads point toward the microalga selected. Link width is proportional to sorting efficiency index indicated in the Cv/Me label. Sorting efficiencies were significant (G test, $n = 10–12$, $P < 0.05$) unless indicated (NS). Choice trends were similar for both bivalves

curve (AUC, Hanley and McNeil 1982). According to a scale established by Swets (1988), a model could be non-informative (AUC = 0.5), less accurate (0.5 < AUC < 0.7), moderately accurate (0.7 < AUC < 0.9), highly accurate (0.9 < AUC < 1), or perfect (AUC = 1).

Results

Binding of FITC-labeled lectins to microalgae

The lectin-binding responses of the microalgal species and the clustering are summarized in Fig. 2. Overall results showed that a majority of the tested microalgae bound the lectins ConA, PEA and WGA, even though intensity differed among species. The other lectins appeared to be more specific, binding only to certain microalgae. For example, *Amphora coffaeaformis* is one of the few species that strongly bound UEA. Additionally, the analysis allowed the clustering of two groups of microalgae based upon lectin-binding profiles (bootstrap confidence value, BC, 100 %). The first group (A) is composed of six species, among which are *Chaetoceros simplex* and *Chlorella autotrophica*, and is characterized by relatively high-intensity PWM and UEA binding, combined with low intensity of PHA, ECA, or WGA binding. The second group (B), which includes *Nitzschia closterium*, *Alexandrium fundyense*, and *Rhodomonas salina*, usually presents high-intensity SBA binding associated with low-intensity PWM binding. Following this first split into two groups, further clustering of microalgae was not supported by bootstrap confidence values (BC < 85 %), except for most of the biological replicates which generally clustered based upon lectin-binding profiles. Only *Tetraselmis chui* triplicates presented high dissimilarities in their binding profiles. It is also noted that the clustering of these 16 microalgae was not strictly correlated with taxonomic relationships. In contrast, the two Prymnesiophyceae, *Pavlova lutheri* (group B) and *Cricosphaera carterae* (group A) or the three Chlorophyceae, *C. autotrophica* (group A), *Chlamydomonas* sp. (group B) and *Dunaliella salina* (group B) had very different lectin-binding profiles. Overall, the carbohydrate pattern of each

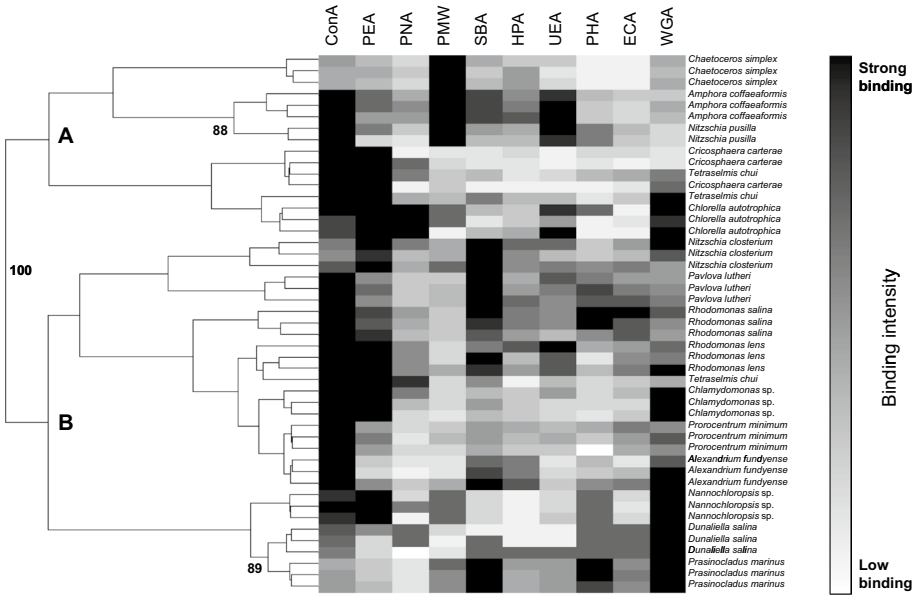

Fig. 2 Hierarchical cluster tree (HCL) of FITC-lectin-binding pattern of 16 microalgae (see Table 2 for lectin characteristics). HCL tree is coupled with an annotated heatmap (normalized intensity of FITC-lectin binding) indicating lectin-binding intensity. Dark color indicates high level of lectin binding, while light color indicates low lectin binding. In this way, *Prorocentrum minimum* and *Alexandrium fundyense* display high binding intensity for ConA and WGA but low intensity for PWM and PNA. Robustness of clusters was tested with bootstrap re-sampling ($n = 5000$). Bootstrap confidence values are displayed on tree if higher than 85 %. Groups *A* and *B* represent the 2 major clusters

of the 16 microalgae can be considered unique even though there are some similarities among them.

Feeding experiments

Microalgae were used in a series of 27 particle selection experiments to determine whether the selection or the rejection of a given microalga can be explained by cell surface characteristics. Among the 16 microalgal species characterized using FITC-lectin binding, the Bacillariophyceae *C. simplex*, *N. closterium* and *N. pusilla* (which all form dense aggregates), and the Dinophyceae *A. fundyense* (large, slow growing and potentially toxic) were not used in the feeding experiments (see "Discussion"). An interaction network analysis summarizes the design and the results of the 27 experiments using *C. virginica* and *M. edulis* (Fig. 1). First, the percentage of each microalga in the diet was carefully checked over the course of each feeding experiment. Particular attention was given when small microalgae were used (see Table 1; Fig. 1), and results indicated that the ratio between the two species in the diet did not change significantly (Chi-square goodness-of-fit tests, $P > 0.1$). In cases of significant changes from microalgal sedimentation (only observed when the pair *Cricosphaera carterae* vs. *Rhodomonas salina* was used), the feeding experiment was stopped and re-initiated.

Results indicated that both species of bivalves tended to selectively ingest or reject the same microalgae even though the sorting efficiency was sometimes not significant (7 nonsignificant of 54 experiments). Some microalgal species, including *R. salina*, *R. lens*, *Chlamydomonas* sp., *C. carterae*, and *T. chui*, tended to be selected more than others. In contrast, other species, such as *A. coffaeaformis*, *Prasinocladus marinus*, and *P. lutheri*, were more often rejected. As noted previously, each microalga was used in several feeding experiments and could either be selected or rejected, depending upon the second species composing the pair. Because of this constraint, a Δ NIFL (i.e., difference between the normalized intensities of FITC-lectin of the selected microalga and the NIFL of the rejected microalga) was calculated for each lectin and each microalgal pair. Interestingly, results showed that in 74.0, 77.8, and 81.5 % of the feeding experiments, the selected microalga was the one that more intensely bound ConA, PEA, and (PEA + ConA), respectively (Fig. 3). No other lectins or combination of lectins showed higher scores.

Logistic regression of sorting outcome (ingestion vs. rejection) with Δ NIFL as explanatory variables resulted in a minimum AIC (i.e., Akaike information criterion estimates the quality of a model) model with five variables for oysters and four variables for mussels (Table 3). In the model for oysters, Δ HPA was the most influential

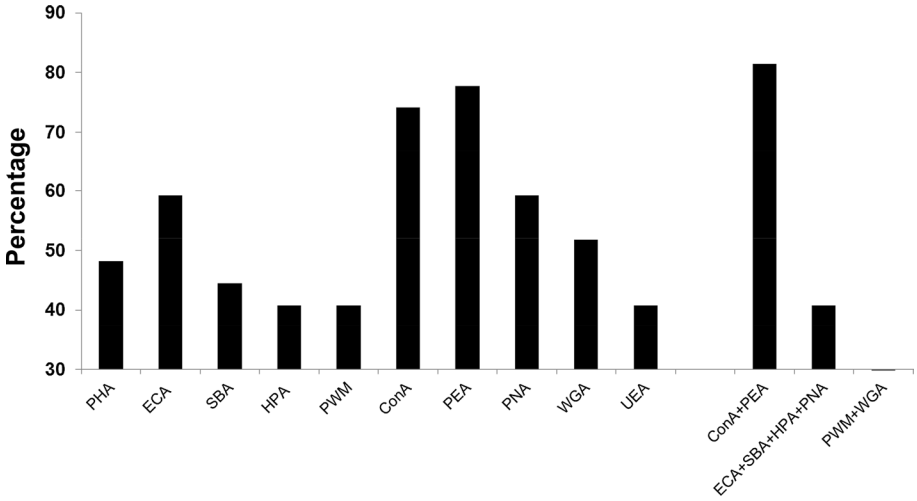

Fig. 3 Percentage of experiments with positive difference between normalized intensity of FITC-lectin of selected and rejected microalgae. In addition to single lectins, combinations of ConA and PEA (mannose–glucose-binding lectins), ECA, SBA, HPA and PNA (galactose or *N*-acetylgalactosamine-binding lectins), and PWM and WGA (*N*-acetylglucosamine-binding lectins) were also considered. Microalgae that bind ConA and PEA with higher intensity are selected in 81.5 % of cases

Table 3 Logistic regression summary and cross-validation for oysters and mussels

Goodness-of-fit statistics		Value
Oysters		
AIC		166.02
R^2		36.15
Mussels		
AIC		107.65
R^2		64.24
Model parameters	Coefficient	Pr > Chi^2
Oysters		
Intercept	2.52	<0.0001
Δ ECA	−2.52	0.031
Δ HPA	4.94	0.022
Δ PWM	−2.75	0.004
Δ ConA	−0.31	0.015
Δ PNA	−1.76	0.003
Mussels		
Intercept	4.47	<0.0001
Δ PHA	−11.08	0.000
Δ ECA	9.44	0.000
Δ SBA	2.78	0.003
Δ ConA	−0.55	0.003
Models		AUC
Oysters		
Model		0.75
Cross-validation		0.74–0.80
Mussels		
Model		0.86
Cross-validation		0.83–0.88

Variables presenting multi-colinearity and nonsignificant variables were removed from analysis. Cross-validation was evaluated by removing 10 % of observations and testing their fitting in the new model. Robustness of model is given by AUC value

(coefficient = 4.94). The variables Δ ECA, Δ PWM, Δ PNA, and Δ ConA had a negative effect on selection (i.e., negative coefficients). The area under the curve (i.e., AUC, 0.75) for the oyster model is considered to be moderately predictive (Swets 1988), and the AUC calculated for cross-validation varied within a narrow range of 0.74–0.80, indicating that the model was robust. The proposed model for mussels was quite different from that for oysters. The most influential variables were Δ ECA (coefficient 9.44), which had a positive effect, and Δ PHA, which had a strong negative effect upon selection (−11.08). The calculated AUC for this model was 0.86, with cross-validation ranges of 0.83–0.88, and can be considered moderately predictive.

Classification tree analysis yielded a model with approximately the same predictive characteristics as logistic regression. The dependent variable was qualitative, either "selected" or "rejected." One classification model was developed for both oysters and mussels because particle choice trends were similar in both bivalves. When single-lectin explanatory variables were used (Δ NIFL), the more robust model was obtained with the C&RT algorithm (Gini index, maximum tree depth of 2, Fig. 4). The AUC calculated for this model was 0.75, suggesting a moderately predictive model. In addition, cross-validation showed that the AUC obtained for the validation samples ranged of 0.77–0.82. The outcome of this analysis predicted that if Δ ConA is in the interval [−2.332, 4.958] and Δ PEA is in the interval [0.205, 4.79], then the corresponding microalga is selected for in 86.4 % of cases. In addition, a second rule indicated that if Δ ConA is in the interval [−4.958, 2.332] and Δ PEA is in the interval [−4.79, 0.205], then the particle is rejected in 84 % of cases. In other words, if Δ PEA is positive and Δ ConA > −2.332, then it is likely that the microalga will be selected for.

Based upon this result, Δ (PEA + ConA) was calculated and used with the other single-lectin explanatory variables

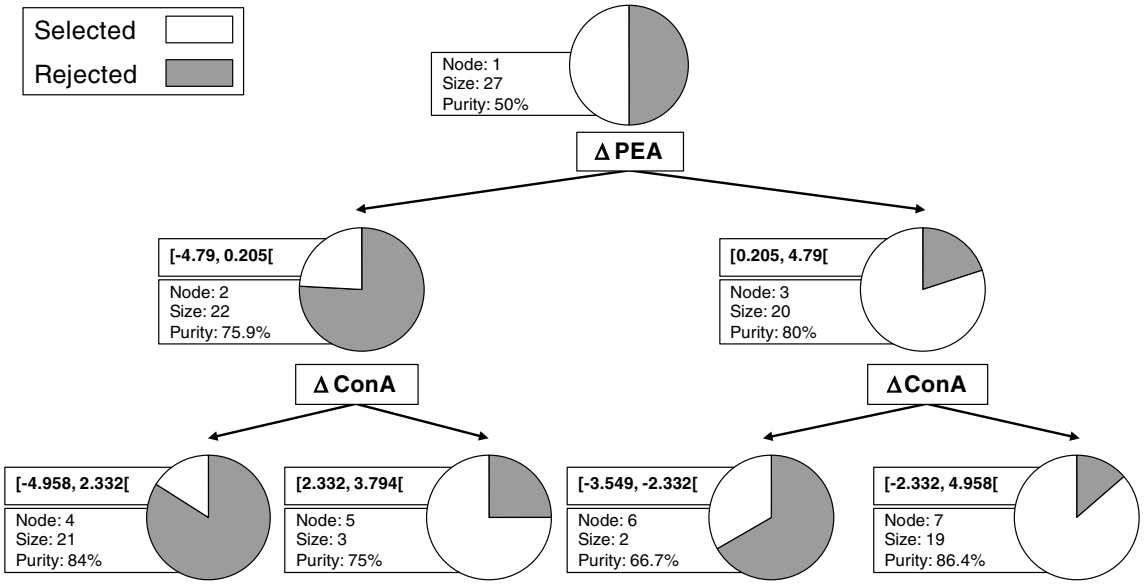

Fig. 4 Classification tree model describing selection or rejection of a pair of microalgae based on differences in their lectin-binding patterns. Rules were built using C&RT algorithm (maximum tree depth = 2). Intervals for explanatory variables are given in brackets. Size indicates number of objects at the node. Purity (also shown in pie graphs) is the percentage of objects corresponding to the dominating category of the dependent variable at the node. In this way, rule for Node 7 predicted that if Δ ConA is in interval [−2.332, 4.958] and Δ PEA in interval [0.205, 4.79], then corresponding microalga is selected in 86.4 % of cases. Robustness (AUC) of the model was 0.75 and AUC of validation samples ranged from 0.77 to 0.82

in another series of classification trees. The best model was obtained when using the C&RT method (Gini index, maximum tree depth of 2, Fig. 5). The AUC calculated for this model was 0.96, suggesting a highly predictive model. In addition, cross-validation showed that the AUC obtained for the validation samples ranged of 0.95–0.97. This tree was more complex than the first classification model using PEA and ConA independently, but it showed that the combination of PEA and ConA may be possible. The results indicated that when Δ (PEA + ConA) is positive, the corresponding microalga is likely to be selected (81.5 % of cases). In addition, Δ ECA may also play a role in the selection. In fact, a rule indicated that if Δ ECA is in the interval [−0.507, 1.311] and Δ (PEA + ConA) is positive, then the particle is selected in 90.5 % of cases. In other words, if Δ (PEA + ConA) is positive and Δ ECA > −0.507, then it is likely that the microalga will be selected for.

Discussion

Understanding particle selection in suspension-feeding bivalves is essential to improve existing models of their growth and, as a result, to better understand the dynamics of ecosystems they dominate. The mechanisms used by suspension-feeders to capture and ingest the most nutritious food particles from a mixture of particles of various sizes and chemical compositions have been a central question among bivalve ecophysiologists for several decades. To date, the mechanism that would best explain particle selection in suspension-feeding bivalves is the ligand-receptor concept we recently introduced, implying specific interactions between carbohydrates present on microalgal cell surfaces and lectins present in mucosal secretions covering bivalve feeding organs (Pales Espinosa et al. 2009; Pales Espinosa and Allam 2013). The characterization of glycans present on microalgal cell surfaces and the relationships between these glycan patterns and microalgal selection by bivalves was, therefore, a research priority.

The specificity of lectins makes them excellent tools to identify cell surface glycans and discriminate among microalgae. Using FITC-lectins, it is possible to differentiate microalgae with similar morphology such as *Gymnodinium*-like species, *Alexandrium tamarense* strains (Rhodes et al. 1995; Hou et al. 2008) or *Pseudo-nitzschia* species (Seob Cho et al. 2001). In the present study, the cell surface carbohydrates of 16 microalgae belonging to different taxonomic classes were characterized using 10 different FITC-lectins. Cluster analysis indicated that these microalgae clustered in only two groups which were independent of taxonomic class. Most of the microalgae bound ConA

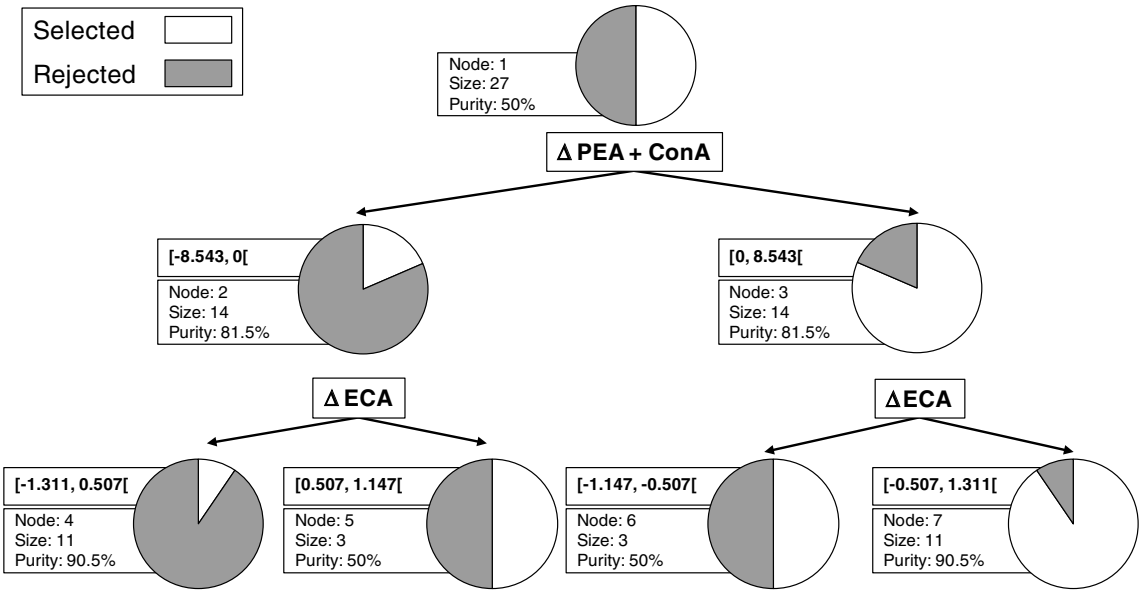

Fig. 5 Classification tree model describing selection or rejection of a pair of microalgae based on their differences in lectin-binding patterns. Rules were built using C&RT algorithm (maximum tree depth = 2). Intervals for explanatory variables are given in brackets. Size indicates number of objects at the node. Purity (also shown in pie graphs) is the percentage of objects corresponding to the dominating category of the dependent variable at the node. In this way, rule for Node 7 predicted that if Δ ECA is in interval [−0.507, 1.311] and Δ PEA + ConA in interval [0, 8.543], then corresponding microalga is selected in 90.5 % of cases. Robustness (AUC) of the model was 0.96 and AUC of validation samples ranged from 0.95 to 0.97

and PEA. The other lectins were more specific and bound to few species. Thus, each of these microalgae displayed a unique glycan signature, in terms of glycan nature and quantity, in agreement with previous work (Aguilera and Gonzalez-Gil 2001; Cho 2003; Hou et al. 2008).

Several previous studies have reported the use of FITC-lectins to evaluate changes on microalgal cell surfaces. For example, changes in cell surface glycan composition during cell cycle/growth were demonstrated in four Dinophyceae (Aguilera and Gonzalez-Gil 2001), in the cyanobacteria *Anabaena cylindrica* (Tien et al. 2005) and *Synechocystis* sp. (Panoff et al. 1988), and in several diatoms (Waite et al. 1995). In addition, change in lectin-binding profile was recently observed in the microalga *Tetraselmis maculata* following nutrient depletion, which may mimic conditions under the stationary phase of growth (Pales Espinosa and Allam 2013). In contrast, some studies have also shown that cell surface carbohydrates do not fluctuate in relation to microalgal growth stage (Cho 2003; Logan et al. 2010), which suggests that some species are more stable than others in terms of cell surface glycan quality and quantity.

In this study, the three biological replicates of each species tended to cluster together, even though some of the bootstrap values were <60 %, indicating variability among the triplicate cultures. This was the case for *T. chui*, *N. closterium* and the two *Rhodomonas* species. This variability among cultures may be attributable to either the labeling process or differences between stages of growth of cells from the 3 replicate cultures or within the same culture. For example, *Tetraselmis* sp. cell division can include delayed release of daughter cells from the mother cell wall, perhaps introducing variation in cell surface composition (Oakley and Dodge 1974). Thus, flow cytometry analysis showed that exponentially growing cultures of the two *Rhodomonas* species presented two subpopulations with different lectin-binding profiles (data not shown).

As previously noted, several species were not included in the feeding experiments. For example, the aggregate-forming microalgae were removed from feeding experiments because the physical characteristic of these aggregates (size, number of cells, shape) might interfere with both cell counting and gill architecture (Cognie et al. 2003). Similarly, some Dinophyceae and Pelagophyceae are known to produce toxins that significantly affect bivalve behavior and metabolism. Numerous studies have shown that toxic microalgae can induce extreme effects in a large range of bivalves, including valve closure, mantle retraction, inhibition of the beating of the gill cilia and decreased clearance rate (Shumway 1990; Matsuyama et al. 1997; Hegaret et al. 2007; Robbins et al. 2010). Accordingly, it was reasonable to assume that these microalgae may directly or indirectly affect particle selection and consequently mask/interfere with the role of cell surface carbohydrates in these processes. Regarding the toxic microalgae examined in

this study (i.e., *Prorocentrum minimum* and *Alexandrium fundyense*), Hégaret et al. (2007) showed that these two Dinophyceae are ingested and found in bivalve biodeposits, and only *A. fundyense* seems to significantly affect the valve closure of oysters. In this study, no specific behavioral changes were observed (naked eye observation) when *P. minimum* was used in feeding experiments.

Lectin-labeling profiles using 10 commercial lectins allowed the separation of the 16 microalgae into two significant clusters (groups A and B), whereas subgroups identified within each group were not significant. Selected and rejected microalgae clustered in both the A and B groups, confirming that this general classification is not sufficient to explain selection. Because microalgal clustering can depend upon the binding of "more specific" lectins (other than ConA and PEA), these lectins may not provide information on the major cell surface carbohydrates mediating particle selection. The 27 feeding experiments showed that the most selected microalgae were *R. salina*, *R. lens*, *Chlamydomonas* sp., *C. carterae* and *T. chui*; *A. coffaeaformis*, *P. marinus* and *P. lutheri* tended to be rejected. In the two-choice experiments, selection or rejection of a given microalga, however, was highly dependent upon the other particle composing the pair, and, thus, the same microalga could be either selected or rejected. For example, *C. autotrophica* or *Prorocentrum minimum* was selected or rejected at a similar frequency. It is possible that the intensity of the lectin binding, in other words not only the nature but also the quantity of carbohydrate present on the microalgal cell surface, influences particle selection. Interestingly, the difference in lectin binding between the selected and the rejected microalgae seems to support this hypothesis, especially for ConA and PEA. Thus, in more than 86 % of the feeding experiments, the selected microalga bound to ConA and PEA with a higher intensity than the rejected microalga. It is also intriguing to note that in the 5 feeding experiments (of 27) that did not follow this outcome, 2 used *P. minimum*, 2 used *Rhodomonas* sp., and 1 used both *Rhodomonas* sp. and *P. minimum*. In these cases, the Dinophyceae was rejected, even though its (PEA + ConA) was the highest. It is then reasonable to think that the putative presence of toxins may have affected the fate of *P. minimum*. Similarly, *Rhodomonas* sp. was selected, whereas its (PEA + ConA) was lower than that of the other microalga composing the pair. The microalga *Rhodomonas* sp. usually presents two subpopulations with different lectin binding. Because the labeling was not done just before the feeding experiments, an underestimation of the real level of lectin binding, and most specifically of ConA and PEA, may have occurred.

To test the hypothesis that some carbohydrates may influence particle selection in suspension-feeding bivalves more than others, thereby predicting the likelihood for a given microalga to be ingested or rejected based on its cell surface carbohydrate signature, several statistical models were evaluated. Logistic regression and classification or decision trees are commonly used for similar tasks and provide complementary information linking forcing factors to observed outcomes (Lemon et al. 2003; Austin 2007). These two methods, however, have different statistical assumptions. Thus, for the logistic regression, the influence of a variable upon the result is uniform across all observations, whereas, for classification trees, the effect of a variable on some observations is unrelated to its effect on another group of observations (Long et al. 1993). While logistic regression is limited to linear changes in explanatory variables, classification tress, and more generally both classification and regression trees, can model relationships with steps, breakpoints, nonlinearity, non-additivity, and a variety of other features difficult to represent with linear techniques (Hastie et al. 2009; Crawley 2012; Flanagan and Cerrato 2015). These two methods are presented as complementary (De'ath and Fabricius 2000), as each can "provide insights not available with the other" (Long et al. 1993). Classification trees have been used successfully in ecology. For example, De'ath and Fabricius (2010) have shown that the abundance and the health of coral species along the Great Barrier Reef are linked to water clarity. Similarly, Kolar and Lodge (2002) have developed a decision tree model to predict which alien fishes may cause damage in the Great Lakes if introduced.

The best model describing the observed data was obtained using the C&RT algorithm, and most specifically combining the two explanatory variables, Δ ConA and Δ PEA. The rules established by the model indicated that of two microalgae, the one richer in mannose/glucose residues on the cell surface would be more likely selected (90.5 % of cases). The AUC value calculated was 0.96, ranking this model as excellent. Associating ConA and PEA is biologically meaningful because they are both mannose-/glucose-binding lectins. Those two lectins, as well as ECA (*N*-acetyllactosamine-binding lectin), seem to be important determinants of the fate (i.e., selection vs. rejection) of a particle. This result is not entirely surprising because mannose/glucose residues on the cell surface have been shown to play major roles in interspecies recognition processes. In this way, mannose receptors are described as primordial molecules in immunity able to recognize pathogens and mediate phagocytosis (Aderem and Underhill 1999). Moreover, these receptors are involved in the recognition and the maintenance of mutualistic symbionts in corals (Wood-Charlson et al. 2006) and nematodes (Bulgheresi et al. 2006). Interestingly, they were also shown to mediate microalga capture/ingestion by members of the Dinophyceae (Ucko et al. 1999; Wootton et al. 2007). Finally, we have previously reported that a mucus lectin with affinity to mannose, glucose, or both, is likely involved in particle selection in *C. virginica* (Pales Espinosa et al. 2010b).

Results obtained in the current study strongly support the role of mannose/glucose receptors in particle selection. Interestingly, two mannose-binding lectins were detected in mucus covering pallial organs using proteomic techniques (Pales Espinosa et al. 2016). Furthermore, the RNA transcript levels of these lectins increased following starvation, supporting the role of these compounds in particle capture/sorting (Pales Espinosa and Allam, unpublished).

In summary, this study demonstrates the essential role of cell surface carbohydrates in microalgal selection by suspension-feeding bivalves. Microalgae rich in glucose/mannose residues were found to be preferentially selected. In addition, statistical analysis showed that decision tree (using the C&RT algorithm) combining the two explanatory variables, Δ (ConA + PEA), is the best model describing the observed data. It should be noted, however, that experiments reported in the current study were performed in June and consequently reflect specific physiological conditions for both bivalves. As first described by Bayne and Svensson (2006) in the Sydney rock oyster *Saccostrea glomerata* and more recently by Pales Espinosa and Allam (2013) for *M. edulis*, selection in suspension-feeding bivalves is highly correlated with both exogenous (seasonal differences in carbon and nitrogen availability) and endogenous (cycles of reproduction and growth) factors. Consequently, these models must be tested/validated in different seasons (different bivalve physiological status). Nevertheless, our findings represent a paradigm shift in our understanding of the mechanisms of particle selection and provide a predictive tool that could be used for assessing bivalve performance and benthic–pelagic coupling under ecological or aquaculture contexts.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics standards This study complies with the current laws of the USA, where it was performed.

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