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Mechanisms Of Particle Retention and Selection in Suspension-Feeding Bivalves

Maria Rosa

University of Connecticut - Storrs, maria.rosa@uconn.edu

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Mechanisms Of Particle Retention and Selection in Suspension-Feeding Bivalves

Maria Rosa, Ph.D.

University of Connecticut, 2016

Suspension-feeding bivalve molluscs are among the most important groups of benthic organisms in coastal ecosystems, and have evolved a highly effective mucociliary feeding mechanism to process the particulate material to which they are exposed. These selective capabilities are well documented, but the underlying mechanisms remain undefined. This thesis assessed pre- and post-capture particle processes to better understand passive and active selection mechanisms of bivalves. Several questions were posited, including: 1) How do physicochemical interactions affect particle capture and selection; 2) Why are some particles more likely to be rejected or ingested than others; and 3) Is a chemosensory response mechanism involved?

Using flow-through chambers and natural suspended matter, capture efficiency (CE) of blue mussels (*Mytilus edulis*) was determined seasonally over a one-year period. Polystyrene microspheres of known size were used to verify size-specific capture. Results demonstrate that particles $\geq 4\mu\text{m}$ are captured with ~100% efficiency, and no evidence was found of bivalves adjusting either feeding behavior or physiology over time. Flow-through chambers were also used to study post-capture selection of mussels and oysters (*Crassostrea virginica*) exposed to microalgae whose surface-property profiles (surface charge, wettability, and surface sugars) were determined. Algae were delivered to bivalves in pairs, and rejection and preferential ingestion outcomes used to develop statistical models and examine which surface properties

determined selection. Multiple regression models identified specific surface sugars and wettability as the strongest predictors of particle selection, explaining ~90% of the variability in selection for mussels and ~94% for oysters. *In vitro* studies with characterized microspheres and microalgae demonstrated that distinct surface properties interacted variably with bivalve mucus, resulting in differential adherence of particles and in some cases capture by mussels. Finally, effects of microalgae metabolites on particle capture and transport were examined using assays with excised gills and *in vivo* endoscopic examinations. Results demonstrated that addition of metabolites had no effect on transport, indicating an active chemosensory response does not affect selection. Findings outlined in this thesis further demonstrate that specific physicochemical properties of particles, and their interactions with mucus in the pallial organs mediate a passive particle selection mechanism in bivalves.

Mechanisms Of Particle Retention And Selection In Suspension-Feeding Bivalves

Maria Rosa

B.S., City College of the City University of New York, **2007**

M.S., University of Connecticut, **2011**

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

at the

University of Connecticut

2016

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Maria Rosa

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Mechanisms of Particle Retention and Selection in Suspension-Feeding Bivalves

Presented by

Maria Rosa, B.S., M.S.

Major Advisor

J. Evan Ward

Associate Advisor

Sandra E. Shumway

Associate Advisor

Gary H. Wikfors

Associate Advisor

Edward J. Catapane

University of Connecticut
2016

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Introduction

Suspension-feeding bivalves are one of the most important groups of organisms in near-shore marine ecosystems, often dominating respiration in the macro-benthos (Dame 1996), and playing a major role in nutrient cycling (Newell RIE 1988; Smaal and Prins 1993; Newell RIE et al. 2005), seston modification (Newell RIE 1988; Asmus and Asmus 1991, 2005), and macrobenthic food web structure (Tenore and Dunstan 1973; Asmus and Asmus 1991; Dame 1996; Wong et al. 2003). Bivalves are exposed to large amounts of particulate matter, which includes both nutritious and non-nutritious particles (Newell RC 1965, Owen 1966). As a way to process efficiently the bulk of material that they encounter, bivalves have evolved capabilities for selective feeding that allow them to reject some of the captured material. The presence of a highly selective, pre-ingestive sorting mechanism may serve as a way to optimize energy gain (Taghon et al. 1978; Kiørboe and Møhlenberg 1981; Newell CR et al. 1989; Iglesias et al. 1992; Grizzle et al. 2001; Ward and Shumway 2004) by enabling bivalves to ingest particles with a higher nutritive quality. Bivalves have been shown to ingest preferentially, for example, microalgal cells (*Rhodomonas lens*; *Phaeodactylum tricornutum*) over detrital particles (ground *Spartina* sp. and suspended bottom material, respectively) (Kiørboe and Møhlenberg 1981; Ward et al. 1997; Levinton et al. 2002). Other workers have shown selection between different microalgal species, with preferential ingestion of a cryptomonad flagellate over a diatom and a dinoflagellate (Shumway et al. 1985), and selection among algal species of the same size (Lesser et al. 1991; MacDonald and Ward 1994; Shumway et al. 1997). The exact mechanisms used by suspension-feeding bivalves to determine which particles are ingested and which are rejected as pseudofeces are, however, relatively unknown.

The process of pre-ingestive particle sorting by suspension-feeding bivalves has been described as either active or passive (see reviews by Jørgensen 1996; Ward and Shumway 2004). Active selection, if present, would be dependent upon an immediate physiological response by the cilia or feeding organs to feeding stimuli (Ward and Shumway 2004). Passive selection, on the other hand, is dependent upon the physicochemical interactions between the particles and the feeding organs, with factors such as particle size and shape possibly serving as bases for sorting (i.e., larger particles preferentially selected over smaller particles, see Bayne et al. 1977). Work by Pales-Espinosa et al. (2009) has provided evidence for another type of passive selection in bivalves, one based upon the specific chemical interaction between lectins in the mucus of pallial organs and carbohydrates present on the surfaces of microalgal cells. In several studies, these workers isolated mucus from the ctenidia (= gills) and labial palps of both oysters, *Crassostrea virginica* (Pales-Espinosa et al. 2009) and mussels, *Mytilus edulis* (Pales-Espinosa et al. 2010) and measured specific lectin activity. These studies confirmed that a carbohydrate-lectin interaction is involved in mediating particle sorting in both *C. virginica* and *M. edulis*. Although bivalves might have the capacity to alter lectin profiles in response to different microalgal species, a factor that can change spatially, this response is not always immediate and may shift seasonally (Pales-Espinosa and Allam, 2013).

Selection based upon the nonspecific physicochemical interactions between particles and the feeding organs is another example of passive selection. Physicochemical properties, such as wettability and electrostatic charge, have been suggested to play a role in selection (Newell RC et al. 1989; Beninger 1991). Wettability

is a weak force dependent upon hydrophobic-hydrophilic interactions between a surface and a liquid, and refers to the ability of a surface to be wetted as a function of hydrogen bonding. In some marine invertebrates, particle capture has been shown to be related to wettability, with wettable particles being retained at a higher proportion than non-wettable particles for example, by the crustacean *Daphnia magna* (Gerritsen and Porter 1982). Surface charge also has been implicated in particle selection, with charged particles being more readily captured than particles with a neutral charge by both the brittle star *Ophiopholis aculeate* (LaBarbera 1978) and larvae of the northern quahog (= hard clam) *Mercenaria mercenaria* (Sollow and Gallagher 1990). Hernroth et al. (2000) examined the influence of the surface charge of radioactive-labeled *Salmonella typhimurium* cells on 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. Combined, these findings suggest that the surface properties of cells may also play a role in particle capture and selection by suspension-feeding marine bivalves.

More recently, Rosa et al. (2013) demonstrated that the eastern oyster, *C. virginica*, and the blue mussel, *M. edulis*, can discriminate between 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. This conclusion also points to the need to characterize the surface properties (e.g., wettability and charge) of organic and inorganic particles that are used in selection experiments to understand fully the roles that physicochemical characteristics of particles play in the feeding process. In bivalves, both specific (e.g.,

lectin-sugar) and non-specific (e.g., surface-charge, wettability) interactions seem to contribute to particle discrimination. The type of mucus produced also may mediate these interactions, as may mucus constituents produced by the feeding organs, and these mechanisms may act in concert to produce a biologically significant selection response.

While some headway has been made recently regarding the mechanisms that underlay particle selection, there still is much to be explored. Some of the unknowns include whether or not there are baseline passive processes in place that may result in some particles being more likely than others to be ingested or rejected based upon chemical composition. If present, such a process could be linked to a basic default mechanism wherein most particles are accepted, and changes in seston quality and quantity induce rejection (MacDonald and Ward 1994). Alternatively, though less likely, the default mechanism could be all captured particles are rejected, and changes in seston quantity and quality induce ingestion. Further, it is unknown if there is an immediate response (= active component) that could trigger this shift in the sorting mechanism.

To understand more clearly the mechanisms that accomplish selection, a decoupling of the various components thought to be involved in particle discrimination is necessary. In other words, how bivalves use particle characteristics to discriminate at each level of the sorting process needs to be addressed. Reports of pre-capture, qualitative selection (Yahel et al. 2009) suggest that not all particles of the same size are retained equally, especially those at the lower limits of capture capabilities (e.g., 0.3 to 2 μm in size). Post-capture, even when there is a strong preference for a particular

type of particle over another, calculated sorting efficiencies of particles are not 100% (Shumway et al. 1985; MacDonald and Ward 1994; Ward et al. 1997), indicating that discrimination is not an absolute process. Accordingly, to assess the mechanisms that accomplish selective feeding, it is necessary to determine which characteristics make one particle more likely to be ingested over another. The current research is one of the first studies to take a holistic, multi-tiered approach to understand the mechanisms of particle selection in several species of bivalves.

The following chapters outline the projects designed to assess experimentally the mechanisms of particle discrimination at the pre-and post-capture level in bivalve molluscs. First, a literature review, encompassing what is known about particle selection, and the possible mechanisms controlling the sorting process, is included in this dissertation. Differences in particle capture efficiency (CE) were experimentally examined to determine if there are shifts in this process, and if so are they based on size or other factors? Surface characteristics (i.e. wettability and surface charge) of different particles, both nutritious and non-nutritious, were characterized, and how individual bivalve species handle these particles (i.e. rejection vs. preferential ingestion) was modeled in order to make any differences in selective capabilities among species more readily apparent. This approach was used to answer the question of why are some particles more likely to be selected preferentially (rejected or ingested) than others? Further, how these particle characteristics interact with the mucus covering the pallial organs and how this mucus may mediate a passive selection process were examined. Results will further elucidate the physicochemical interactions that mediate passive selection and address the question of how non-specific chemical interactions

affect particle capture and retention? Finally, no previous work has made a strong case that an immediate, active mechanism underlies particle selection. A response by the frontal cilia of the gill to phytoplankton metabolites, particularly one that leads to a change in the handling of a particle, would indicate that an immediate chemosensory response is taking place. Techniques were developed to address the questions of whether there is an immediate chemosensory response mechanism that plays a role in particle selection? Such a response would also further indicate that a qualitative selection based on chemical factors associated with the types of cell these organisms encounter in the wild underlays the selection mechanism.

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Chapter I

Particle capture, retention and pre-ingestive selection in suspension-feeding marine bivalve molluscs: A review

1. Introduction

Suspension-feeding bivalve molluscs are among the most important near-shore groups of organisms, often dominating the macrobenthos and contributing significantly to the benthic food web structure and benthic-pelagic coupling. These contributions are affected by the ability of the molluscs to ingest particles selectively, rejecting some particles and depositing them in the benthos as undigested material. This pre-ingestive sorting process has been extensively studied for the last 60+ years in an effort to determine both what types of particles, e.g. microalgae and detritus, these organisms select, and how the selective mechanism is controlled. Early work regarding particle selection by bivalves has been extensively reviewed (see Ward and Shumway, 2004), and the current review is focused on advances made in the last 15 years.

Feeding in bivalves is generally understood to be physiologically plastic, responding to changes in seston composition and particle loads (Bayne 1976, Bayne et al. 1976, 1977, Iglesias et al. 1992, Bacon et al. 1998). To process the bulk of particulate material they encounter, suspension-feeders can either reduce particle clearance rate, or select between particles and increase production of pseudofeces (captured material which is not ingested). The presence of this highly selective, pre-ingestive sorting mechanism serves as a way to optimize energy gain (Taghon et al. 1978, Kiørboe and Møhlenberg 1981, Newell CR et al. 1989, Iglesias et al. 1992, Grizzle et al. 2001, Ward and Shumway 2004) by enabling bivalves to ingest particles with a higher nutritive quality selectively. Bivalves have been shown to ingest microalgae preferentially (*Rhodomonas lens*; *Phaeodactylum tricornutum*) over detrital particles (ground *Spartina* sp.; suspended bottom material, respectively, Ward et al.

1997, Levinton et al. 2002) and select between different microalgal species (Shumway et al. 1985), including algae of the same size (Lesser et al. 1991, MacDonald and Ward 1994, Shumway et al. 1997). The process of pre-ingestive particle sorting by suspension-feeding bivalves has been described as either active or passive (see reviews by Jørgensen 1996; Ward and Shumway 2004). Active selection, if present, would be dependent upon an immediate physiological response by the cilia or feeding organs to feeding stimuli (Ward and Shumway 2004). Passive selection, on the other hand, would be dependent upon the physicochemical interactions between the particles and the feeding organs, with factors such as particle size and shape possibly serving as bases for sorting (i.e., larger particles preferentially selected over smaller particles, see Bayne et al. 1977).

While some headway has been made recently regarding the mechanisms that underlay particle selection, there still is much to be explored. Some of the unknowns include whether or not there are baseline passive processes in place that may result in some particles being more likely than others to be ingested or rejected based upon chemical composition. If present, such a process could be linked to a basic default mechanism wherein most particles are accepted, and changes in seston quality and quantity induce rejection (MacDonald and Ward 1994). Alternatively, though less likely, the default mechanism could be that all captured particles are rejected, and changes in seston quantity and quality induce ingestion. Further, it is unknown if there is an immediate response (= active component) that could trigger this shift in the sorting mechanism. Particle fate is dependent on its encounter with the gill and subsequent retention and discrimination by the pallial organs. Research on the selective processes

of suspension-feeding bivalves has demonstrated that particle discrimination can occur during each of these processes. Understanding the mechanisms that underlie selection at each of these steps would help to elucidate how they act in concert to determine the material ultimately ingested.

2. Particle capture

2.1 Pumping, Clearance, & Filtration Rates

Suspension-feeding bivalves filter water by capturing particles from the seston during feeding activities. The measurement of the water flow per unit time (L hr^{-1}) that passes through a bivalve is known as the pumping rate. This flow is a direct result of water currents produced by the lateral cilia located on the gill filaments. This rate scales with the size of the gill, and can be described using allometric equations. Clearance rate, also measured in volume per unit time, is an indication of the number of particles cleared by the bivalve. If all particles entering the bivalve are removed, then clearance rate is equivalent to pumping rate. If particles are not cleared with 100% efficiency, then pumping and clearance rates are not comparable. The clearance rate is sometimes used interchangeably with filtration rate, which is a measurement of the mass of particles cleared per unit time (e.g. mg hr^{-1}). It is not the intention of this review to go in depth into the specifics of pumping and filtration by bivalves. We refer readers interested in the physiological considerations and constraints on bivalve filter feeding to the in-depth review by Cranford et al. (2011).

Clearance rate has been posited as being physiologically plastic, with suspension-feeders being able to adjust this rate as a response to environmental factors (Bayne and Newell 1983, Cranford and Grant 1990, Bacon et al. 1998, Baker et al. 1998, Bayne 2004). The ability to adjust CR allows bivalves to optimize particle selection (Hawkins et al. 1999), with some bivalves increasing CR as seston loads increase. Experiments on the feeding behavior of filter-feeding zooplankton have shown that these animals can maximize their net energy intake if they control both the rate of filtration and the mechanical properties of the filter unit (Lehman 1976, Bayne et al. 1977, Jørgensen et al. 1986, Shimeta and Jumars 1991, Iglesias et al. 1992). If similar thinking is applied to suspension-feeding bivalves, selection can be a “mechanical property” of the filter, which, along with control over the clearance rate, can maximize their energy intake. Several studies have reported differences in clearance rate by bivalves depending on the seston composition. Bayne et al. (1988) reviewed the early literature on feeding and digestion in suspension-feeding bivalves, and discussed the available information within the scope of physiological compensations. They provided evidence of an immediate and active response of filtration and clearance rates to changes in seston by bivalves to compensate for lower food quantity and quality, or periods of non-submersion in the case of the intertidal blue mussel *Mytilus edulis*. Factors that can elicit a CR response in several bivalve species include variations in seston loads (Foster-Smith 1975a, Palmer and Williams 1980, Ward and MacDonald 1996, Iglesias 1996, Cranford et al. 2005, Strøhmeier et al. 2009), presence of phytoplankton metabolites (Bricelj and Malouf 1984, Birkbeck et al. 1987, Shumway and

Cucci 1987, Silverman et al. 1995), and temperature (Thompson and Richoux 2001, Kittner and Rissgard 2005).

It has been demonstrated that many bivalve species have physiological control over the lateral cilia, and consequently the rate at which water is pumped (Paparo 1972, Jørgensen 1976, 1982, Catapane 1983). Temperature also has an effect on ciliary activity and consequently the clearance rate of particles (Aiello 1960, Malanga et al. 1981, Richoux and Thompson 2001). Kittner & Riisgård (2005) studied the effects of temperature on filtration rates of *M. edulis*, and reported a linear relationship between temperature and filtration rate, with no evidence of temperature acclimation by the mussels. Results should be interpreted with caution, however, as the authors used several mussels in one tank and estimated how many were pumping based on valve gape, calculating the rate by dividing across by the number of animals filtering. Further, the rates are based on filtration using an unialgal diet, which can result in lower rates than using natural seston (e.g. Wright et al. 1982, MacDonald et al. 2011). Strøhmeier et al. (2009) demonstrated that both mussels (*M. edulis*) and scallops (*P. maximus*) continued to filter particles at low seston concentrations (0.15 mg L^{-1}), a finding that contradicts some previous reports of cessation of feeding (low CR) under low particle loads and indicating that current concepts of functional responses of bivalves in oligotrophic environments need re-examination. These workers also reported little short-term variability in mean CR of the mussels ($4.2 \pm 2.2 \text{ L hr}^{-1}$, $n = 144$) and scallops ($28.2 \pm 12.7 \text{ L hr}^{-1}$, $n = 132$). The CR of the scallops were negatively correlated with chlorophyll *a* concentrations, but not correlated with temperature, supporting previous findings by Macdonald and Ward (1994). Dionisio Pires et al. (2004) also examined

clearance rates *in situ* of zebra mussels (*Dreissena polymorpha*) and found that CR were higher for the 0 -1 μm - and 30 -100 μm -sized particle classes than other particles available in the seston. This size preference corresponded with a high clearance rate for cyanobacteria and other phytoplankton, with low CR for detrital particles. In a long-term *in situ* study, Li et al. (2009) examined CR of bay scallops *Argopecten irradians*. They used flow cytometry to distinguish between organic and inorganic particles, as well as to distinguish phytoplankton cells from detritus. These workers reported that for particles >20 μm , the CR was significantly greater for detrital particles than for phytoplankton. For particles smaller than 2 μm , the CR of phytoplankton (0.95 L hr⁻¹) was significantly higher than the CR of the similarly sized detrital particles (0.24 L hr⁻¹). Overall, the workers reported that clearance rates were low for particles ≤ 5 μm in size and postulated that these differences were indicative of selection by the scallops. The authors, however, did not collect pseudofeces or calculate a capture efficiency, therefore the clearance rates reported are not necessarily indicative of selectivity. The findings support the fact that CR is an important component of a feeding behavior response in scallops, and further demonstrate the interconnectivity of CR and particle selection. Taken together, the findings outlined above demonstrate a physiological control over CR as a response to the types of particles (quantity and quality) available in the seston.

2.2 Particle capture efficiency

Particle capture is the first step in the feeding process and is a consequence of particle encounter and retention on the gill. The processes of particle capture, retention, and transport are mediated by mucociliary action on the pallial organs (Foster-Smith

1975b, Beninger et al. 1992, 1993, Ward et al. 1998). Particles suspended in the water enter the inhalant aperture/siphon via currents produced by the movement of the lateral cilia on the gill filaments. Particles entering the pallial cavity are either directly intercepted by the frontal surface of the gill or trapped by currents created by the laterofrontal cilia (Atkins 1937, Jørgensen 1981, Ward 1996, Beninger et al. 1997, Ward et al 1998, Riisgård & Larsen 2010). Water, and particles not retained on the gill, are then pushed out through the exhalant aperture/siphon. In some bivalve species, particles can also be hydrodynamically entrained onto the principal filaments of the gills (Jørgensen 1976, Owen and McCrae 1976, Owen 1978, Ward et al. 1998).

Although post-capture selection has been well studied, much less is known about selective retention during particle capture. Particle encounter efficiency relates to the proportion of particles that come into contact with the the gill filaments, whereas retention efficiency is the proportion of encountered particles that are actually retained (see Shimeta and Jumars 1991). Although previous workers have used the term “retention efficiency” to describe particle capture efficiency in bivalves (Riisgård, 1988; MacDonald and Ward, 1994; Cranford and Hill, 1999; Strømmeier et al., 2012), unless *in vivo* techniques are employed to differentiate the number of particles that are encounter from those that are actually retained (Ward et al. 1998a), retention efficiency cannot be determined. Therefore, we use the term capture efficiency (CE) to accurately describe the process being measured in place of retention efficiency. In some cases, CR has been used interchangeably with capture efficiency (CE). Capture efficiency and clearance rate, while related, are not the same. Capture efficiency is not a rate, and is independent of volume of water filtered or time. Further, the CR of differently sized

particles can only be compared if these particles are captured with 100% efficiency. For the purposes of this review, and to avoid confusion, CR will be referred to as the individual publications cited used the term, with appropriate comments as to whether the findings reflect capture efficiency.

Early research on feeding processes by suspension-feeding molluscs reported that most bivalves capture particles $\geq 4 \mu\text{m}$ with close to 100% efficiency, with particle capture decreasing linearly with particle size (Vahl 1972, Palmer and Williams 1980, Riisgård 1988). Vahl (1972) examined CE in blue mussels (*M. edulis*) using a flow-through system with a common head tank and measured particle concentrations several times over the course of the experiment. He reported “negative” CE values at the lowest size class (1-2 μm), which he attributed to recirculation of water in the chambers. Further experiments to minimize recirculation also resulted in a few negative values at the smallest size classes, and the author concluded that the mussels themselves were releasing small particles, or breaking up larger aggregates, resulting in a higher number of small particles in the mussel chambers versus the “control” chambers. These findings demonstrated an effective CE of zero for smaller sized particles, though they may also be indicative of the methodological limitations of accurately enumerating smaller particles. Wilson (1983) examined the CE of the European oyster *O. edulis* fed suspensions of *Isochrysis galbana* (T-Iso strain, $\sim 4\mu\text{m}$), and using a rubber sleeve to capture all of the outgoing flow directly from the exhalent siphon. He found that as the *I. galbana* cell concentration was increased, the CE of the oysters decreases in a parabolic curve ($R^2=0.84$). Interestingly, for the long-term experiments (~ 56 hours),

there was variation in CE of algae for the different concentrations examined, though no pattern was found to explain the observed differences and trends in capture.

Studies examining the mechanisms of particle capture in suspension-feeders have demonstrated that qualitative factors, such as the surface properties of particles, can have an effect on CE. Particles with a charged surface, for example, were demonstrated to be more readily captured than particles with a neutral charge by both the brittle star *Ophiopholis aculeate* (LaBarbera 1978) and larvae of the northern quahog (= hard clam) *Mercenaria mercenaria* (Solow and Gallagher 1990). In other marine invertebrates, particle capture has been shown to be mediated by surface hydrophobicity. For example, hydrophilic particles are retained at a higher proportion than hydrophobic particles by the crustacean *Daphnia magna* (Gerritsen and Porter 1982). Characterization of the surface properties of bacterial species found that they tend to be more hydrophilic (Grasland et al. 2003) than several microalgal species (Ozkan and Bergeluou 2013a, Rosa et al. in review), which may account for the reported higher capture efficiencies of these particle types. Conova (1999) examined the role of hydrophobicity in particle capture by the suspension-feeding mole crab *Emerita talpodia*. She reported that as smaller sized particles (0.5 to 10 μm) were made more hydrophilic, their adhesion to the capture organ generally decreased. Interestingly, for particles 15 to 25 μm in size, particle hydrophobicity did not affect capture rates. Thus, hydrophobicity appears to play a role in particle capture in the smaller size ranges.

Capture efficiency (CE) at the lower size threshold of particles varies by species, and likely is dependent upon gill architecture and the cilia/cirri microstructure. Mussels,

for example, have a filibranchiate homorhabdic gill, with large compound laterofrontal cilia (=cirri, Atkins 1938, Owen 1974), that could account for the reported high CE of particles in the 4 to 10 μm -size range (Riisgård 1988, Rosa et al. 2015). Scallops have a filibranchiate heterorhabdic gill, with a single row of laterofrontal cilia (Atkins 1938, Owen and McCrae 1976, Beninger 1991) that seem to be inefficient at capturing particles not directly intercepted by the frontal surface. Generally, scallops have been reported to have low CE for 2 to 7 μm particles (Møhlenberg and Riisgård 1978, Riisgård 1988). Oysters have a pseudolamellibranchiate, heterorhabdic gill structure with developed laterofrontal cirri that are less complex than those of mytilids (Owen and McCrae 1976, Ribelin and Collier 1977) and have generally high CE for particles $>3 \mu\text{m}$.

Due to the known mechanical limitations of the bivalve gill on particle capture at the lower size threshold, feeding studies have generally focused on capture and ingestion of particles or phytoplankton larger than $\sim 5 \mu\text{m}$, with fewer papers examining the contributions of smaller particles to the bivalve diet. Palmer and Williams (1980) were some of the earliest workers to examine the effects of particle concentration on capture efficiency of different sized particles. These authors tried pre-conditioning scallops (*Argopecten irradians*) and oysters (*Crassostrea virginica*) by feeding them a mono diet of 4- μm - or 10- μm - sized algae. They found no effect of this pre-conditioning treatment on CE, suggesting that the size of particles available in the seston did not affect overall capture efficiency. Interestingly, for the scallops fed the 4- μm algae, the CE was found to increase as particle concentration increased, which the authors posited could be due to higher mucus production as a response to higher seston loads. The same effect was not true for oysters, and there was considerable variability in CE

for these animals throughout the experiments at the various seston loads. The authors suggested that these results indicated the need to determine whether bivalves could alter “efficiency of gill response” to any changes in the size class dominating the seston. Silverman et al. (1995) were among the few early investigators that examined the uptake of bacteria by bivalves. They studied freshwater mussels and found that the bivalves were able to uptake and utilize laboratory-cultured *E. coli* and with relatively high clearance rates. On a weight-specific basis, the zebra mussel *Dreissena polymorpha* was able to ingest the smaller bacteria at rates 30 -100 times faster than the other two mussel species (*Corbicula fluminea*, and *Carunculina texasensis*) studied. This higher capture rate was attributed to the larger gill and higher number of laterofrontal cirri in this mussel compared to the other two species tested, and which aided in the capture of the smaller particles. In a later study, Hernroth et al. (2000) manipulated the cell surface characteristics (=charge) of the bacterium *Salmonella typhimurium* cells (~1µm) and fed them to mussels (*M. edulis*). The bacteria with the manipulated surface charge were retained with the same efficiency as the larger control polystyrene particles (10 µm) and a higher efficiency than the non-manipulated bacteria.

In recent years, there has been a shift (increase) in research examining the contributions of picoplankton and other small particles (<4µm) to bivalve growth. Picoplankton are operationally defined as particles ranging from 0.2µm to 2µm in size, including the cyanobacteria and small eukaryotes that can be numerically dominate in the seston. Yahel et al. (2009) studied particle capture and selection by the burrowing bivalve *Lithophaga simplex* in a semi-oligotrophic environment. Particle loads are generally low in this study site (~0.1 mg L⁻¹ of POC) and ultraphytoplankton (< 8 µm)

dominate the seston. Despite the low seston loads, bivalves were found to preferentially ingest some bacteria (*Synechococcus* and eukaryotic algae; 0.5 and 0.3 Chesson alpha, respectively) over others (*Prochlorococcus* and non-photosynthetic bacteria; 0.2 and 0 Chesson alpha, respectively). More striking, the mean capture efficiencies for the small photosynthetic bacteria *Synechococcus* (~0.9 μm) and *Prochlorococcus* (~0.4 μm) were 69 % (\pm 14 SD) and 41% (\pm 19 SD), respectively. These findings demonstrated higher capture efficiencies for smaller particles than previously shown. LeBlanc et al. (2012) developed a method for quantifying isotopic-labeled proteins in the byssus threads of *M. edulis* using chromatography and tandem mass spectrophotometry. To determine isotope uptake in tissue, mussels were fed a diet with labeled *Nannochloropsis* sp. and the assimilation of this diet into the protein fibers studied. Mussels were found to uptake nutrients efficiently from the microalgae (~2 μm) and with a relatively high capture rate explaining the observed incorporation. Sonier et al. (2016) also examined the contribution of picoplankton to the growth of the blue mussel *Mytilus edulis* in field studies. The CE of the bivalves fed picoplankton ranged from 3 to 37%, with an average CE of 20% (2% SE). Estimates of the capture efficiencies of the 2- to 20- μm particles were higher, ranging from 19 to 81% with an average CE of 60% (3.5% SE). These findings demonstrate that some bivalve species have higher CE for smaller particles than previous reported, which were calculated as being effectively zero for particles less than ~3 μm (e.g., Vahl 1972, Riisgård 1988). The contribution of these smaller particles to the diet can be significant because even a 20% mean CE for a population could be important when the majority of available seston particles are within this smaller size range. Sonier et al. (2016) modeled the contribution

of smaller particles to the mussel diet, and, for the first time, showed that picoplankton could be a significant proportion of the total net intake and contribute 13-28% of the energy needed for tissue and shell growth in mussels. Strøhmeier et al. (2012) reported mean CE of 14-43% in *M. edulis* feeding on 1- μ m particles during *in situ* studies, with variations in mean CE of these smaller particles depending on season. Together, these findings suggest that the contribution of small organic particles to bivalve energetics is likely higher than previously reported. The importance of picoplankton to bivalve diets should be reassessed in light of new data, particularly in coastal areas and aquaculture sites with high bivalve biomass.

Particle aggregation (flocculation) scavenges smaller particles, such as picoplankton and bacteria (Waite et al. 2000), which increases their bioavailability to suspension-feeders. Marine aggregates (a.k.a. flocs) range widely in size, with the largest (marine snow) generally within the upper size limit of particles bivalves can effectively ingest (~500 μ m). Karlsson et al. (2003) carried out experiments on the cockle *Cerastoderma edule* using different flow speeds, and found that at high flow velocities, 500- μ m particles were captured, and at the lowest velocities the 200- μ m particles were ingested. The authors suggested these differences in capture were a matter of availability, i.e., the higher flow rates tested re-suspended the larger particles. Marine aggregates have also been shown to increase efficiency of picoplankton uptake by the scallops *P. magellanicus* (Cranford et al. 2005). Kach & Ward (2008) used picoplankton-sized particles (fluorescently labelled microspheres & *Escherichia coli*) in feeding studies with several suspension-feeding molluscs (*Mya mercenaria*, *M. edulis*, *C. virginica*, *A. irradians*, *C. fornicata*). Microspheres and cells were delivered to the

animals as free suspensions or incorporated into aggregates. Results indicated that except for the suspension-feeding snail *C. fornicata*, all animals ingested significantly more of the aggregate-bound particles than the freely suspended particles. Thus, aggregation and floc formation serves as a mechanism for the efficient uptake of picoplankton and bacteria that are generally captured with lower efficiencies. Further, results indicate bivalves can also capture and ingest large particles. This ability to capture larger particles means that bivalve grazing could also affect zooplankton communities, including on aquaculture farms where bivalves are suspended in the water column in high numbers and can further affect the pelagic zone. A study by Shumway et al. (1987) examined the food sources of near-shore and offshore populations of the scallop *Placopecten magellanicus*, and through gut content analysis demonstrated that the scallop is an opportunistic feeder that preys on available seston. Species found in the gut ranged in size from 8 μm to 250 μm , and included zooplankton tests and ciliates. The presence of some of the larger forms in the gut, however, may be indicative of their indigestibility and the authors suggested that the contribution of large zooplankton to the bivalve diet is minor. Most recently, Peharda et al. (2012) examined zooplankton grazing among four bivalve species (*Ostrea edulis*, *Mytilus galloprovincialis*, *Modiolus barbatus*, *Arca noae*) in the Adriatic Sea. Animals were collected monthly and stomach contents analyzed and compared to seston samples collected at the same time. Zooplankters were found in all bivalve species, with the cultured species (*O. edulis* and *M. galloprovincialis*) having the highest abundances of these organisms in their stomach than the native benthic species (*M. barbaratus* and *A. noae*). Bivalve larvae were the most abundant zooplankton in all samples, followed by

tintinnids and copepods. The methodology used in the study, which relied on gut contents for identification of ingested particles, was limiting because species that are more easily digested are not taken into consideration. The above findings indicate that the effective size range of particles that suspension-feeding bivalves can capture is large, and can be influenced by particle availability.

2.3 Pre-capture selection (differential capture)

As the number of studies assessing CE of suspension-feeding bivalve molluscs fed smaller particles has increased, it has become apparent that there are some species capable of pre-capture selection. These findings raise the question of whether observed post-capture selection patterns are a consequence of, at least to some extent, “pre-capture” selection. In other words, are some particles more likely to be retained than others, and is this differential retention responsible for the differences in particle ratios between biodeposits (i.e. pseudofeces and feces) and the water column? If differential capture occurs, based on size (mechanistic) or the physicochemical properties of the particles, it could result in over or under estimation of the post-capture selection response. The use of flow cytometric techniques (FCM) was an important advance in allowing scientists to examine capture of similarly sized particles such as microalgae. Shumway et al. (1985) applied FCM to study particle capture in the European oyster *Ostrea edulis* and demonstrated that this species preferentially captured the dinoflagellate *Prorocentrum minimum* over a similarly sized diatom and flagellate (*Phaeodactylum tricornutum* and *Chroomonas salina*, respectively). The authors suggested that properties other than cell size resulted in the differences in capture. Differential capture was also demonstrated in the blue mussel *Mytilus edulis*

(Cucci et al. 1985, Newell et al. 1989) and juvenile scallops *Placopecten magellanicus* (Shumway et al. 1997). More recently, Yahel et al. (2009) examined *in situ* feeding in the tropical bivalve *Lithophaga simplex* by using a direct technique (InEx system) to sample ambient water before it entered the inhalent aperture and as it exited the exhalent siphon. They then used flow cytometry to differentiate between particle types and calculate CE for the particles. They found that *L. simplex* preferentially retained the photosynthetic bacteria *Synechococcus* and larger eukaryotic algae. A small proportion of non-photosynthetic bacteria, sharing a size overlap with the retained photosynthetic bacteria, were not captured as efficiently. Similarly, Jacobs et al. (2015) reported differences in capture efficiency between particles of similar size. Picophytoplankton between 0.7 and 1 μm in diameter were found to be cleared at higher rates than bacteria ($\sim 0.6\mu\text{m}$ in diameter) by *Mytilus edulis*, further suggesting that factors other than size affected capture. In their study, the authors reported that “optimal retention” plateaued for particles larger than 6 μm in diameter, with nanophytoplankton ($\sim 6\ \mu\text{m}$) and ciliates (10-200 μm) being cleared at similar rates. These findings indicate a size-independent preferential capture, at least for particles $< 4\ \mu\text{m}$, and suggest that surface characteristics may contribute to particle CE. This evidence of pre-capture qualitative selection would be a form of passive selection, and an underlying mechanism of selection worth further exploration. It is important to note that evidence for differential capture is mostly for a small particle range, and so far evidence for differential capture of particles $\geq 5\mu\text{m}$ is mixed. Cranford & Grant (1990) for example, ran experiments where they fed scallops a mixed diet of *Isochrysis galbana* ($\sim 5\ \mu\text{m}$), *Chaetoceros gracilis* (4-10 μm), macroalgal detritus (kelp aged in seawater, 2-40 μm), and sediment

organic matter (2-40 μm) and calculated the CR and CE of the various size classes. They found no significant differences in CE when fed the various sediments versus the kelp particles as a function of class size. Due to the aerosol filtration system employed by suspension-feeding bivalves for particle capture and the efficient capture of particles greater than $\sim 5 \mu\text{m}$, any differential capture is most likely to be observed in the smaller size classes.

2.4 Shifts in capture efficiency (CE)

Reports of shifts in particle capture efficiency of bivalve molluscs as a response to changes in seston composition and concentration suggest a plasticity of this physiological trait. Field studies by Stenton-Dozey and Brown (1992) on the clam *Venerupis corrugatus* demonstrated an effect of tides on CE. The clams captured particles sized 5 to 9 μm with the highest efficiency during low tide, and particles 8 to 13 μm during high tide. Barille et al. (1993) conducted laboratory and field experiments on the oyster *C. gigas* to examine the effects of variable seston quality and quantity on CE. These workers found no effect of food quality on CE in laboratory or field experiments. They did, however, find an effect of seston loads on CE. At the lowest particle concentration, *C. gigas* demonstrated no change in capture of particles larger than $\sim 3 \mu\text{m}$ (70%). At the higher seston concentrations, CE was lower for particles $\sim 3 \mu\text{m}$ (20%) and highest for particles larger than 12 μm (ca. 100%). More recent efforts have focused on seasonal field experiments to examine variations in CE. Naddafi et al. (2007) used Delayed Fluorescence (DF) excitation spectroscopy to examine feeding selectivity by zebra mussels, *Dreissena polymorpha*, continuously over a period of several months (April-November). These workers calculated CR and used it as a proxy

for capture efficiency (see section 2.2). During the months when food concentrations were low they found that CR did not vary with different phytoplankton groups. When food concentrations were higher, mussels cleared dinoflagellates at significantly higher rates than the other available phytoplankton groups. The authors reported lower CR for cyanobacteria during summer months (Jul-Aug) than during the fall (Sept-Oct). Mussels preferentially cleared and ingested cryptophytes compared to chlorophytes and dinoflagellates. This study demonstrated a physiological shift by zebra mussels in the capture of certain cells, presumably in response to food availability. The authors argued that the mussels regulate selectivity as a response to food size. Based on their methods and study structure, however, it is not clear if they could differentiate the effects of cell size versus other particle characteristics on capture. The types of rejected microalgae (e.g. chlorophytes and dinoflagellates) were all similar in size; thus hypothetically if a different sized cell of the same algal Class were introduced and rejection were to occur, it would indicate that characteristics of the algae cell other than size were in fact responsible for the observed selection response. More than likely, the rejection was based on cell properties of the available algal species and not on size. More recently, Strømmeier et al. (2012) reported a seasonal variation in retention efficiency in the mussel *M. edulis*. They used a flow-through method to simulate *in situ* conditions and calculated RE and CR based on the size-distribution and concentration of available particles. Animals were re-used at two sampling sites, with 6 sampling times between May and August. They reported that later in the summer (August), the smaller sized particles (4 μm) dominated the seston. During this time, the workers reported a shift towards higher capture of smaller particles over larger particles. They concluded that

mussels have the capacity to control particle retention mechanisms in response to a shift in seston composition. These results are counter to current understandings of the hydrosol filtration system employed by bivalves. The authors did not provide an explanation proposed for the observed patterns in CE that would account for the capture of smaller particles in preference to larger sized particles. Work by Rosa et al. (2015) examined these apparent shifts in seasonal CE in *M. edulis*, and used different sized polystyrene microspheres of uniform shape as control. They reported that the capture of microspheres $\geq 4 \mu\text{m}$ in diameter was consistently high across all sampling months, with only the 2- μm particles being captured at a lower efficiency than particles of greater size. These trends in CE were different from those calculated using natural seston, which did demonstrate apparent shifts in CE seasonally. Based on these findings, the authors suggested alternate explanations for the purported changes in CE over time and shifts in the size of particles that are captured most efficiently, including a result of: 1) artifacts associated with the way in which many particle analyzers calculate particle diameter; 2) disaggregation of flocculent material collected from control chambers that leads to release of small particles; 3) qualitative factors of the particles that could affect capture. Rosa et al. (2015) concluded that CE of mussels is not physiologically plasticity at least for particles that are captured near 100% efficiency. In another study, Lopes-Lima et al. (2014) examined selective feeding by the freshwater uninoid *A. cygnea*. They found that in the winter months, cyanobacteria made up a large portion of the gut contents (in cells/g) even though these cells were less abundant in the seston. The authors suggested that seasonal and nutritional demands affect selectivity and inferred that *A. cygnea* elicits a CE response. In addition to the low

number of specimens collected at each sampling date ($n=6$), no data were presented on the surface properties of the cyanobacteria or whether these properties change with season. Therefore, a physiologically mediated change in CE cannot be conclusively demonstrated. Taken together, the reports outlined above suggest that any shifts in capture efficiency as a physiological response by bivalves to shifts in seston composition should be viewed with caution. In order to better determine if bivalves can physiologically regulate CE, studies should include appropriate controls to ensure methodological artifacts or other confounding factors are not responsible for the apparent patterns in particle capture and selection.

3. Post-capture particle selection

3.1 Functional morphology of pallial organs

Post-capture particle selection occurs on the gills and/or labial palps depending on the gill architecture of the species. Bivalves with homorhabdic gills have only one type of gill filament and generally unidirectional transport of captured particles (no selection in the gills). Some bivalves, such as those belonging to the genus *Arca*, have bi-directional transport on the gills (Atkins 1937), though selection on the gill by these species has not been conclusively demonstrated. Captured particles are transported ventrally, then to the labial palps where selection occurs. Bivalves with heterorhabdic gills have two types of filaments, and bidirectional transport of captured particles. Particles transported ventrally on the gill are generally rejected, whereas particles transported dorsally are moved to the labial palps where further selection is possible

(see Ward 1996, Ward et al. 1997). Particle size and shape can affect whether or not particle selection occurs on the gills of these species. The orientation of larger particles as they are captured by the gill can preclude entrance into the principal filaments for selection in *C. gigas* (Cognie et al. 2003). In this instance the larger particles are sent to the labial palps where they are processed. The types of particles that are selected have also been shown to be affected by the organs of selection (e.g. gills alone vs. gills and labial palps) (Kjørboe and Møhlenberg 1981, Lesser et al. 1991, Bougrier et al. 1997, Bacon et al. 1998, Beninger et al. 2007, Rosa et al. 2013). Several studies have demonstrated the ability of bivalves to alter the area of the pallial organs as a response to changes in seston composition and particle load concentration. Several bivalve species with populations living in turbid environments have been found to have larger gill and labial palp areas, including the blue mussel *M. edulis* (Theisen 1982), the oyster *C. gigas* (Barille et al. 2000), and the arcid bivalve *Scapharca kagoshimensis* (Yoshino et al. 2013). A study by Dutertre et al. (2007) further demonstrated that morphological differences between pallial organs (e.g. size) had a functional effect on selection and clearance rates by the pacific oyster, *Crassostrea gigas* using a combination of morphological biometrics and video endoscopy. They found a morphological plasticity in the surface area of the gills and labial palps for the oysters depending on the different regions where they were studied. At low seston loads, selection by this oyster was found to occur only on the gills and CR was positively related to gill area. Oysters with small gills were found to have CR that was positively correlated with labial palp size at the higher seston loads studied. These biometric measurements indicate that differences in gill and palp size are integrated with pre-ingestive selection, and thus the

functional morphology of pallial organs. Shifts in the ratio of gill to labial palp area of *C. gigas* individuals were correlated with turbidity levels to which they were exposed (Dutertre et al. 2009), with larger labial palp sizes corresponding to oyster populations from more turbid environments. Beninger et al. 2008 also examined the impact of seston quality on feeding selectivity by *C. gigas* and found that, for large particles that cannot enter the principal filaments between gill plicae, the labial palps are the main organ of particle selection. Selection efficiency on the gill was inversely related to seston quality and, as quality of available particles increased, the efficiency of selection on the gill decreased. Similar to other oysters, *C. gigas* is adapted to high turbidity environments and can control (increase) the quality of ingested food even when seston loads are high. The authors found that the capacity for selection on the palp can be overloaded in this oyster, particularly at high seston loads, and suggest it is probably due to the size of the organ. Garrido et al. (2012) carried out endoscopic examinations of two different bivalve species, *Mulinia edulis* and *Mytilus chilensis*, both with homorhabdic gills (unable to select on this organ) that occupy different littoral zones. The infaunal siphonate spissulid clam *Mulinia edulis* has a constriction at the tip of each labial palp, allowing articulation at the distal region and rotation about its axis. The motility of the palps allows it to manipulate rejected particles into a mucus-bound ball that is stored at the base of the inhalant siphon and expelled intermittently. Heavy ciliation of the mantle tract, which aids in pseudofeces elimination, was also found. The asiphonate intertidal bivalve *Mytilus chilensis* was found to have shorter labial palps, which are heavily folded in the face directly contacting the gill filaments. If seston load is high enough to result in pseudofeces production, it is eliminated continuously via the

exhalent aperture. The labial palp morphology of *M. edulis* allows for processing of the fine clays and particles this bivalve encounters. In *M. chilensis* on the other hand, the sandy and coarser sediments this bivalve encounters are more easily processed by the shorter palps. Together, the findings outlined in this section further demonstrate plasticity in the morphology of the pallial organs of bivalves as a function of their habitat, even in animals with similar gill architectures. Most importantly, this plasticity allows bivalve species to more efficiently process particles in environments with variable seston loads.

3.2 Mucocilliary processes

Beyond the morphological differences in pallial organs, the production and composition of the mucus covering the gills and labial palps can vary between species and within pallial organs. The role of mucus in particle capture and selection has been widely debated. Early work by Nelson (1927) described the presence of mucus on the gills of oysters, which served to “trap” food particles and transport them to the mouth. Later workers disregarded the role of mucus and suggested direct ciliary activity was responsible for particle capture, describing sieve-like properties of the bivalve gill (Jørgensen 1966), with the action of different types of gill cilia responsible for enhancing particle capture (Jørgensen 1975). Some of the early points of contention included how bivalves could select particles that would be imbedded in mucus during the capture and transport processes. The role of gill mucus in particle capture, transport, and selection was elucidated over several years via a series of experiments. Foster-Smith (1978) used direct and *in vivo* observations of the pallial organs to investigate the role of mucus

in feeding by *M. edulis*, *Cerastoderma edule*, and *Venerupis pullastra*. He noted that mucus traps particles almost immediately and moves them along the frontal surfaces of the gill 'ridges' (= filaments) towards marginal food grooves for ingestion or to the dorsal margin of the palps for rejection. Newell and Jordan (1983) were among the first researchers to hypothesize that the viscosity of mucus in feeding was reduced by mechanical action of the labial palps, allowing for the sorting of captured particles. With the development of *in vivo* observations of the pallial organs by means of video endoscopy (Ward et al. 1991), a better assessment of suspension-feeding processes was possible. Using video endoscopy, Beninger et al. (1992), Ward et al. (1993), and Ward (1996) examined particle feeding in a total of nine species of bivalves encompassing the three major gill architectures (filibranch, pseudolamellibranch, and eulamellibranch), and confirmed the role of mucus in the feeding process. They showed that particles on the ordinary filaments are transport by the frontal cilia either embedded within a mucus layer or in direct contact with cilia. Particles on the principal filaments are transported dorsally on current chiefly created by the beating of the cilia on the frontal surface of the ordinary filaments and the lateral cilia on the principal filaments. In the ventral groove, mucociliary transport mechanisms dominate, whereas in the dorsal tract hydrodynamic transport mechanisms dominate. Direct endoscopic observations by Ward et al. (1998) also demonstrated that particles are captured by direct interception with the gill filaments, and that retention of particles is likely enhanced by mucus present on the frontal surfaces or ordinary filaments. Further confirmation of mucociliary transport on the frontal surface of ordinary filaments (*M. edulis*) was reported by Beninger et al. (1997) using confocal laser microscopy. The presence of

mucus has also been demonstrated to aid in the uptake of viruses from the surrounding water. Di Girolamo et al. (1977) studied the adhesion of *Vibrio* species to mucus collected from *C. gigas*, *O. lurida*, and *M. mercenaria*. These workers found that ionic or hydrogen bonding are responsible for the binding and rapid adherence of different virus species to the bivalve mucus. Mucociliary processes have been demonstrated to mediate particle capture and transport by suspension-feeding bivalve molluscs (see Ward 1996). The mucus on the ordinary and principal gill filaments, as well as the two tracts/margins that transport captured particles, varies in cohesiveness. The mucus on the dorsal tract of the gill, for example, is composed of loose “slurry” that carries particles to the labial palps for further processing, and facilitates particle discrimination on this organ. Mucus on the ventral margin of the gill is composed of a compact string with tightly bound particles, and these particles are generally rejected as pseudofeces. Mucus covering the gills and labial palps has also been demonstrated to vary in composition and abundance depending on the species of bivalve and the loci of selection (Beninger et al. 1992, 1993). Beninger et al. (1993) examined the role of mucus in particle transport in two species of bivalves, the blue mussel *M. edulis* and the scallop *P. magellanicus* using whole-mount staining techniques on excised gill tissues. Acidic mucus polysaccharides have been found on the crests of the ordinary filament in *P. magellanicus*, which can select particles on the gill. In these bivalves, the ordinary filaments are mainly used in cleaning and bulk rejection of captured particles (Beninger et al., 1993). The gill filaments of mussels *M. edulis* were found to have mixed mucopolysaccharides, which the authors posited to be reflective of the non-separation of selection and cleaning processes of the gill. Captured particles are transported to the

ventral or dorsal margins of the gill via mucociliary transport (ordinary filaments) or hydrodynamic transport (principal filaments) and incorporated into either a compact mucus string (ventral margin) or a mucus-water 'slurry' (dorsal tract) before being transported toward the labial palps. On the palps, the viscous mucus string is pulled from the gill via ciliary action, and palp action reduces the cohesiveness of the mucus to release entrapped particles so selection of individual particles is possible (Ward 1996).. The combined results of *in vivo* examinations and mucocyte-distribution studies clearly demonstrated the roles of mucus in particle capture and transport in bivalves (Beninger et al. 1992, Ward et al. 1993).

Complementing the non-specific physicochemical aspects of mucus and their role in particle capture and processing, specific chemical constituents of mucus (e.g., lectin specificity) and conservation of activity between serum and mucus from different bivalve species (Fisher and DiNuzzo 1991) have furthered the possibility of mucus playing a role in particle discrimination. Recent work by Pales-Espinosa et al. (2010a) demonstrated some of the specific chemical interaction between lectins in the mucus of the pallial organs and carbohydrates present on the surfaces of microalgal cells. In several studies, these workers isolated mucus from the gills and labial palps of oysters, *Crassostrea virginica* (Pales-Espinosa et al. 2009) and mussels, *Mytilus edulis* (Pales-Espinosa et al. 2010b) and measured specific lectin activity. The lectins in the mucus were able to agglutinate different microalgal cells, indicating binding to the carbohydrates on the cell surfaces. Further, coating of algal cells with mucus from the pallial organs resulted in a disruption of the bivalve's ability to recognize and select between the algae, further demonstrating that lectins were able to reversibly bind to the

cell-surface carbohydrates. These studies strongly suggested that a carbohydrate-lectin interaction is involved in mediating particle sorting in both *C. virginica* and *M. edulis*. In a study examining the physiological control of mucus production, Pales Espinosa and Allam (2013) examined the transcript levels of a mucosal lectin (MeML), which were generally found to be similar in the gills and labial palps of *M. edulis*. The levels were found to vary seasonally in both pallial organs. In the gills, the lowest levels were found in May (ripening of gonads in gametogenic cycle) and highest levels found in November (associated with somatic growth). This trend was maintained regardless of the pre-conditioning diet to which mussels were exposed, suggesting endogenous factors in MeML transcript regulation. In the palps, the opposite was observed when the mussels were exposed to a high quality diet, with higher levels in May and lower levels in November. Poorly fed mussels did not exhibit this seasonal trend in transcript expression. Sorting efficiencies were significantly correlated to MeML expression in the labial palps, but not the gills. These findings indicate that although bivalves might have the capacity to alter lectin profiles in response to different microalgal species, a factor that can change spatially, this response is not always immediate and may shift seasonally.

3.3 Physicochemical properties of particles- a mechanism for particle selection

Once successfully captured, particles can be rejected prior to ingestion, or ingested and digested. Physicochemical surface properties of particles, such as electrostatic charge and hydrophobicity (=wettability), are a set of factors that have been suggested to play a role in particle sorting mechanism of bivalves (Newell CR et al. 1989; Beninger 1991). The physicochemical surface properties of organic and inorganic

marine particles have been well studied as a way to explain the aggregation and flux of materials to the benthos. Tangentially, these studies have identified factors that may be used by suspension-feeders in particle discrimination. Surface properties of phytoplankton have been reported to contain a relatively wide range of surface characteristics. Neihof and Loeb (1972) reported that organic marine seston particles (consisting of a mix of bacteria, algae, and detritus) generally have a negative charge. Inorganic particles tested (e.g. glass, resin, clay) had a lower range of measured surface charge, exhibiting what the authors called a homogenous mobility behavior. These charges were not correlated to particle size or aggregation. There are few reports of positively charged particles in seawater. Particles that have been reported to have a positive charge were typically inorganic marine sediments (Pravdic 1970) composed of clay, quartz, and iron in the 2 to 200 μm size range (+32 mV in seawater of salinity 36). The authors reported that the positive charge affects deposition and agglomeration, as surface charge reverses from a negative value in freshwater, to the measured positive value in estuarine water. The differences in charges between organic and inorganic particles have been partially explained by adsorbed organic constituents (Neihof and Loeb 1974). The surface chemistry of natural particles in seawater is controlled largely by the adsorption of organic matter (Abramson et al. 1942, Neihof and Loeb 1974, Hunter 1980), with carboxylic acid (-COOH) and phenolic (-OH) groups being some of the major ionizable functional groups identified in organic films (Hunter 1980). Most recently Ozkan & Berberoglu (2013a) characterized various physical and chemical properties of five species of freshwater and marine microalgae. They reported variations in zeta potential (a proxy for surface charge) among species, though no consistent

trends in properties among classes were found. In a follow-up study, Ozkan and Berberoglu (2013b) used 10 different freshwater and marine microalgal species, and six different inorganic substrate materials, to examine cell to cell- and cell-substrata interactions based on the previously characterized physicochemical properties. They examined total interactive energy as a linear sum of the Van der Waals interactions (attractive), electrostatic interactions (repulsive), and acid-base interactions (electron transfer between polar components, attractive in hydrophobic interactions, and repulsive in hydrophilic interactions). Results demonstrated that the total interactive energy is a function of the distance between the interacting surfaces, with a negative interactive energy indicating adhesion, and a positive interactive energy indicating repulsion. These workers also noted that cells with larger diameters experienced larger attractive or repulsive forces, provided everything else in the system was the same. All of these characteristics result in several physicochemical cues that can be utilized by marine species in feeding processes. Given the range of physicochemical surface properties of the numerous particles suspended in the seston, biologists began examining whether the surface characteristics of particles could be utilized to elicit a selection response by suspension-feeders (LaBarbera 1978, Gerritsen and Porter 1982, Ward and Targett 1989, Hernroth 2000, Rosa et al. 2013). These findings have implications for an aerosol filter system, as the interactions between particles (e.g., microalgae and detrital cells) and the collector unit (e.g., mucus covered pallial organ) can help explain some of the patterns of particle capture and selection observed to date in bivalve molluscs.

3.4 Particle discrimination - passive mechanisms -

Most of the early work on feeding selectivity focused on the types of particles that bivalves selected post-capture. Several studies clearly demonstrated that bivalves preferentially ingest some particles over other (Newell and Jordan 1983, Shumway et al. 1985). Work by Newell and Jordan (1983) clearly demonstrated that the oyster *C. virginica* preferentially ingested organic material over inorganic silt. In a study using video endoscopy to examine particle feeding *in vivo* by the eastern oyster *C. virginica*, Ward et al. (1997) definitively demonstrated that detrital cord grass was rejected, and cells of the microalga *Rhodomonas lens* were preferentially ingested. Most strikingly, the dry cord grass was also moved ventrally for rejection when provided as a sole particle, suggesting that choice alone is not necessary to trigger selection of particles. Further, this finding indicates a strong rejection response for some particle types over others. Results of Ward et al (1997) and others provide strong evidence for the rejection of detrital material. From an energetics point of view, this is advantageous as bivalves have generally been shown to have low absorption efficiency (AE) for detrital material, meaning that a lot more energy is spent consuming and digesting inorganics than what the animal receives as a net intake (Bricelj and Malouf 1984, Cranford and Grant 1990).

This process of pre-ingestive sorting by suspension-feeding bivalves can be described as either passive or active. Passive selection would be dependent upon the physicochemical interactions between the particles and the feeding organs, with factors such as particle size and surface charge possibly serving as bases for sorting. Active selection, on the other hand, would be dependent upon a physiological response by the cilia or feeding organs to feeding stimuli (see section 3.5, below). Observed differences

in selection responses among bivalve species suggest that the factors these organisms rely upon to reject or preferentially ingest particles can be different. Early work on feeding selectivity indicated that qualitative attributes of different particles affect selection by bivalves (Newell and Jordan 1983, Shumway et al. 1985, Ward and Targett 1989, Beninger 1991, Ward et al. 1997). To further examine some of the cellular properties bivalves could use to discriminate between particles, Brilliant & MacDonald (2003) fed both heat-killed and live cells of *Chlorella* to the sea scallop *Placopecten magellanicus*. They found that scallops were able to discriminate between the two particles, rejecting the heat-killed *Chlorella* cells and preferentially ingesting the live cells. Heat treatment, according to the authors, diminished the quality of the particles while retaining their physical integrity (cell-wall intact) and suggests that a quality cue is used to discriminate between particles. Beninger and Decottignies (2005) used live and dead cells of the diatom *Coscinodiscus perforatus*, whose epicellular frustules had been left intact and uncleaned, and found that all cells were handled similarly by the bivalve *Pecten maximus*. Because no selection was noted between the live and dead cells of this diatom, the authors suggested that the organic casing (= frustule) and any associated organic molecules were factors mediating selection by the scallop, perhaps by being an indication of food quality. When the same experiments were repeated with *C. gigas*, the oyster was able to select between the diatom cells, rejecting the heat-killed diatom cells (Beninger et al. 2008). These findings suggest that cellular status acts as a quality factor for selection in *C. gigas*. Similarly, Dutertre et al. (2007) found that *C. gigas* demonstrated preferential rejection of heat-killed *T. suecica* over the live cells, as well as over the chain diatom *S. costatum*. Kasai et al. (2004) used stable isotope

techniques to examine seston and tissues of the clams *Ruditapes philippinarum* and *Macra veneriformes* and to determine food intake. Based on C¹³ and N¹⁵ isotopic signatures, most of the diet (90%) of these estuarine bivalves was of marine origin, with only 10% being of terrestrial origin. In the tidal flat where the study took place, POM had generally higher contributions of terrestrial constituents; therefore differences in the tissue are due to selective feeding on both phytoplankton and marine detritus over terrestrial particles. Although many of these researchers suggest that particles have qualitative factors that act as “cues” to which bivalves elicit a selection response, physicochemical surface properties of the delivered particles were not determined and differences in these characteristics among particles were unknown. Based on these results, an active selection response cannot be conclusively implicated (see section 3.4 below).

In a study examining selection in the eastern oyster *C. virginica*, Urban and Kirchman (1992) found that adding kaolinite clay to the diets disrupted their ability to select between different algal species and starch. They found that more starch was ingested when the kaolinite was present in the diet, indicating the presence of the inorganic material disrupted the selection response in oysters. A recent mesocosm study by Frau et al. (2016) examining feeding selectivity in the mussel *Limnoperna fortunei* exposed to natural phytoplankton assemblages found that the addition of rotifers did not affect selection between the phytoplankton (i.e. species rejected versus selected). Selection patterns between the phytoplankton were maintained even though rotifers were highly selected by the mussels. The authors suggested that selection of the phytoplankton was due to a combination of cell shape and quality. Some cell types

(belonging to the Volvocales, Cyptophyceae, and *Trachelomonas* sp.) were strongly rejected. This and other studies (Rosa et al. 2013) show that patterns of selection are not fixed, and in some cases selection can be changed depending on the type of particle delivered to the animals.

Pales Espinosa et al. (2016) determined lectin-binding profiles of different species of microalgae, and fed them in pairs to *M. edulis* and *C. virginica* in order to model food selection in these species. They reported that some lectins (e.g. *Pisum sativum* agglutinin) strongly bound to the sugars on the cell surfaces of all tested microalgal species, and other lectins had high specificity for the sugars on the cell surfaces of one or two algal species only. Statistical models indicated that the microalgal species preferentially ingested by the bivalves had cell surfaces generally rich in glucose and mannose sugar residues. These workers then generated statistical models of particle selection by mussels and oysters, using lectin profiles to predict the likelihood of a particle being selected. Although predictive models were generated, the results of Pales Espinosa et al. (2016) should be interpreted with caution as these workers pooled data obtained for *M. edulis* and *C. virginica* to generate one classification model. Given 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. Results from Rosa et al. (*in review*, see Chapter 3) demonstrated that both physical and chemical surface properties of microalgae affect selection processes of *M. edulis* and *C. virginica*. These bivalves were demonstrated to use distinct 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. Data from microalgal characteristics and feeding experiments were used to generate significant statistical models for predicting selection in both bivalve species. There were also differences between the particle-selection models generated for mussels and oysters, which the authors attributed to the loci of selection, which are different in these two species of bivalve. Results of the various independent experiments outlined above all indicate that specific physicochemical properties of particles are factors that mediate selection, and suggest that specific chemical interactions are some as the mechanisms that underlay particle selection.

More recently, Rosa et al. (2013) experimentally assessed the effects of wettability and surface charge upon pre-ingestive particle selection by the eastern oyster (*C. virginica*) and blue mussel (*M. edulis*). The authors quantified the surface properties of several types of synthetic microspheres, composed of polystyrene, silica, and alumina. They delivered the microspheres in different pairings to individual animals, determined the number of each sphere type in collected biodeposits, and calculate a selection index. Results from Rosa et al. (2013) demonstrated that both bivalve species could discriminate between particles of the same size based upon the quantified surface properties. Specifically, the highly wettable (= hydrophilic surface) alumina microspheres were consistently and strongly rejected by both species of bivalve regardless of the specific particle pairing used. The authors also characterized the surface properties of several detrital particles and a microalga to determine if differences in surface properties were observed among particles regularly encountered by bivalves under natural seston conditions. The results indicated that further

characterization of microalgae and detrital particles were needed to explore fully the role of surface properties as a selective cue for suspension feeders.

3.5 Particle discrimination - active mechanisms

It has been demonstrated that bivalves can detect and respond to dissolved chemicals (Loosanoff and Engle 1947, Birckbeck et al. 1987, Shumway and Cucci 1987, Ward et al. 1992 Ganessan et al. 2012), and the presence of distance chemoreception in different bivalve species is well documented. The ability of bivalves to perceive chemical cues of particles once they are captured has not been demonstrated. Numerous histological studies have found that the gill cilia can be stimulated via mechanical and chemical means (Aiello 1960, 1970, Jørgensen 1975, Malagna 1975, Davenport and Fletcher 1978, Malagna et al. 1981, Catapane 1983, Carroll and Catapane 2007), though none have suggested that the cilia are innervated (Aiello 1970). One prerequisite for an active chemosensory response would be the “recognition” of particles of different quality followed by translocation from one transport tract (e.g., acceptance tract) to an adjacent one (e.g., rejection tract; see Ward and Shumway 2004). In some suspension-feeding bivalves, such a process could occur via ciliary mechanisms on the frontal surface of gill filaments. Endoscopic observations of particle movement demonstrate that, in most cases, particles which are transported by these cilia are within micrometers of the frontal surface ($< 5 \mu\text{m}$), so changes in beat angle or frequency would translate to changes in particle movement (Ward 1996).

For contact chemoreception response to be possible, bivalves would need both receptors and innervation of the cilia and epithelium. Hodgson and Fielden (1984) found three types of ciliary receptors in the siphons and mantle edge of two species of bivalve molluscs. The authors suggested that the cilia function as chemoreceptors. Morphological investigations of the mantle and siphons of several different bivalve species have demonstrated that these tissues have sensory organs, with the degree of ciliation varying between species (Fishelson 2000). To our knowledge, only one study has attempted to determine the presence of chemoreceptors in a bivalve species. Dwivedy (1973) examined the presence of chemoreceptors on the labial palps of *C. virginica* by using microelectrodes attached to the tissue and exposing the whole animal to chemicals known to stimulate taste receptors (NaCl, HCl, quinine sulfate, and sucrose). He reported that different concentrations of the substances resulted in a change in receptor potential, suggesting that oysters were probably able to discriminate between the different chemicals. Results of this experiment have been questioned, however, due to some of the methodological errors, mainly the fact that the size of the electrodes used (20µm) was larger than the receptor cells, with any physical movement of the receptor causing a pseudo-response. This means that the author could not definitively show that a signal was due to a chemosensory response, something Dwivedy also acknowledges. While the ability of marine bivalves to employ contact chemoreception to control an active selection mechanism is possible, to date there are few data that support or refute the involvement of an active, chemosensory mechanism in particle selection.

4. Methodological considerations

It has been suggested that the use of different methodologies and experimental designs are responsible for some of the differences observed in physiological measurements. Early on, Riisgård (1977) measured flow rates through animal-holding chambers used to assess clearance rates of mussels in flow-through systems. He reported that at lower flow rates the measured CR approached the flow rate of the system, indicating that the mussels were recirculating the water in the experimental chambers. This finding demonstrated that earlier reports of clearance rates increasing with increasing flow rates were in fact an artifact of the measurement technique (Haven and Morales-Alamo 1970, Vahl 1972, Walne 1972). Riisgård recommended that a minimum flow rate of 200ml/min be used to prevent this artifact, with a corrected equation to take into account the flow rate when measuring clearance rate. Petersen et al. (2004) examined the CR of *M. edulis* using three of the most commonly employed methods: the deposition method, the flow-through method, and an indirect method. Use of the deposition method to calculate CR resulted in significantly lower rates than those using a flow-through method, and results demonstrated that the experimental design and method used can affect the calculated rates. These authors pointed out that both the use of different techniques, as well as exclusion of natural seston assemblages during experiments can affect calculated rates. These finding makes comparison of CR from various studies, using different techniques, difficult. Because of the connectivity between CE and CR, the authors also found that calculating CR based on all particles $>2\ \mu\text{m}$ resulted in rates that were $\sim 10\%$ lower than rates using only particles $>4\ \mu\text{m}$. In a note, Bayne (2004) examined some of the assumptions made by Petersen et al. (2004)

and suggested that in some cases the biodeposition method does not underestimate CR compared to other techniques of measuring this rate. In particular, he noted that in experiments with mussels where all biodeposits are not collected immediately may result in the re-suspension of pseudofeces. This in turn means that not all egested material is collected, resulting in calculation errors that would also account for some of the differences observed by Petersen et al. (2004). In other words, the use of different species and different collection techniques within a specific methodological approach could also result in erroneous calculations of CR. To validate the use of flow-through chambers to calculate CR, Filgueira et al. (2006) examined reported rates in the mussel *Mytilus galloprovincialis* from experiments that used flow-through and static mesocosm methods. Most importantly, these workers developed a validation protocol that could be applied to studies using the flow-through method and correct for any differences in CR due to methodologies. Calculations also demonstrated that a flow-through method is more reliable, i.e. less likely to overestimate CR as a consequence of particles mixing in the experimental chambers, than using a static method. Recently, Rosa et al. (2015) demonstrated that the use of different particle counters can also result in differences in calculated clearance rates (see Chapter 2). In a seasonal study measuring CR and CE in *Mytilus edulis*, these workers analyzed the same water samples by means of both an electronic particle counter (Coulter Multisizer IIe) and a laser in-situ scattering transiometer (LISST-100x, Sequoia Inc.). They found that CR ranged between 0.67 L hr⁻¹ to 3.34 L hr⁻¹, and were similar to rates reported previously for *M. edulis* of similar size (MacDonald and Ward 2009, Cranford et al. 2011). More importantly, Rosa et al. (2015) reported that in two of the months (September and December) data collected by

the LISST-100x indicated a significant decrease in CE of particles within the 11 to 25 μm size class compared to smaller sizes (between 4-10 μm), but no significant differences in CR between these two size classes. Data collected using the Coulter Multisizer in September indicated no significant difference in CE between particles in the 11 to 25 μm and 4 to 10 size classes, but a significantly lower CR for the smaller size particles. Additionally, for many of the months studied (e.g., May, Sept, Dec, Mar 14), CR calculated from data generated by the LISST was significantly different than that generated by the Multisizer. These findings demonstrated that the instrument used to analyze samples may yield different results even when using the same experimental design and water samples. This in turn indicates that results based on data collected using different instruments can affect the conclusions reached. Field studies have also shown that the use of natural seston assemblages can lead to results that differ from those obtained using mixed microalgal diets in the laboratory. Data published by Barille et al. (1993) clearly shows that capture efficiencies (CE) calculated based on field experiments were lower than those calculated for concurrent laboratory experiments using the same seawater spiked with microalgae, even though the particle loads were similar. Capture efficiency ranged from 43 to 70% for the 3- μm particles in the laboratory experiments, whereas in the field experiments CE ranged from 10 to 27% for the same sized particles. Similar differences in clearance rates between field and laboratory studies have also been reported (Barille et al. 1993, Petersen et al 2004). In a study examining shifts in capture efficiency by *M. edulis*, Rosa et al. (2015) reported higher CE for uniform particle types in the 2- to 4 - μm size range than previously reported. These differences could be a result of methods used previously to measure

CE, which relied on indirect techniques (i.e. sampling water entering and exiting a chamber) and often used monoalgal cultures. Results from experiments using a direct delivery technique (e.g., Yahel et al. 2009, Strømmeier et al. 2012, Rosa et al. 2015) have reported that mean CE of 2 to 4 μm particles can range from 40-80% for mussels, and 20-60% for scallops. In contrast, previous experiments using indirect methods have reported CE for similar sized particles to be between 20 and 60% for mussels, and 0 and 20% for scallops (e.g. Møhlenberg and Riisgård 1978, Riisgård 1988, Palmer and Williams 1980). Some of these differences could be due to the fact that in indirect methods not all of the water passing out of the chamber is accessible by the animal, which could result in a dilution effect and affect the calculation of CE.

The use of monoalgal cultures (e.g. Vahl 1972, Møhlenberg and Riisgård 1978) could also account for the reported lower CE of some smaller particles. Several studies using bacterial cultures and natural seston assemblages have reported higher CE of smaller particles (e.g. Hernroth et al. 2000, Rosa et al. 2015). If bivalves indeed capture smaller natural particles (e.g., picoplankton) at higher efficiencies than previously reported, both the methods used to determine CE and the contribution of these particles to molluscan energetics may warrant re-examination. Combined, these findings show that a general consensus by scientists toward the use of standardized methods for physiological measurements (e.g. CR and CE) is needed, namely, the use of similar methods that utilize flow-through chambers and direct measurements of particle capture for accurate CE measurements. No such recommendations have been clearly outlined for standard techniques in selection experiments.

Differences in selection responses of bivalves between field and laboratory experiments have also been reported. One possible explanation for this discrepancy is the use of static versus flow-through systems to measure physiological traits. The amount of pseudofeces produced by bivalves is partially dependent upon particle concentration (Bayne et al. 1977), meaning rapidly declining particle concentrations in static systems can result in small amounts of pseudofeces being produced. Lower pseudofeces production in turn leads to difficulties in accurately quantifying the number of each particle type in the collected samples and a less reliable instantaneous assessment of selection. In contrast, a flow-through system delivers a constant concentration of particles over a longer period of time, and under constant conditions a greater quantity of pseudofeces is produced. In selection studies, a flow-through system allows for a 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. 2013, Pales Espinosa et al. 2016).

5. Conclusions and future directions

In the past 60+ years, particle selectivity in suspension-feeding bivalve molluscs has been extensively studied (see Ward and Shumway 2004 and references therein). Researchers have independently examined the types of particles generally captured and retained by these groups of organisms in an effort to understand the types of particles and cells used as food. The extensive literature has shown generally that

bivalves select organic “live” particles over inorganic detrital material (Bayne et al. 1977, Kiørboe et al. 1980, Iglesias et al. 1992, Ward et al. 1997, Safi et al. 2007, Beninger et al. 2008). Bivalves can discriminate between different microalgal species, preferentially ingesting some over others. This discrimination, however, is not 100% as particles that are less desirable are still ingested but in lower quantities (Shumway et al. 1985, Cucci et al. 1987, Ward et al. 1997, Baker et al. 1998). Further, at low particle concentrations, bivalve species appear to “dampen” their selection response and ingest most of the particulate material available (Newell et al. 1989, Iglesias et al. 1996, Hawkins et al. 1999, Beninger et al. 2008). Reports of bivalves that actively feed on toxic microalgae are also difficult to explain, as this behavior seems to counter the notion that particle selection results in the ingestion of higher quality material. The lack of general “rules” for particle selection has made it more difficult to design studies that elucidate the mechanisms behind particle selectivity. Differences in selective capabilities between species of bivalves have been demonstrated (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), suggesting that not all bivalve species rely on the same physicochemical factors for selection. Some of the differences between mussels and oysters, two of the most studied bivalve species, may be a result of the different gill architectures they possess and the loci of selection (Ward et al. 1997, 1998).

Most published studies suggest that a passive selection mechanism directs particle discrimination, with the physicochemical properties of different particles interacting with the mucus covering the pallial organs directing particle fate. To date, few studies support an active contact chemoreception component. The lack of

evidence, however, does not mean such a mechanism is non-existent, and more definitive studies are needed. One prerequisite for an active chemosensory response would be the “recognition” of particles of different quality followed by translocation from one transport tract (e.g., acceptance tract) to an adjacent one (e.g., rejection tract, see Ward and Shumway 2004). On the gill, active particle selection would likely be elicited by the frontal cilia due to their direct involvement in particle selection in some species. In particular, studies should seek to identify a mechanism for an active physiological response to different food types. Earlier studies have demonstrated that the presence of extracellular ectocrines can affect selection. Ward and Targett (1989) reported that when cellular metabolites from several microalgal species were absorbed onto different microspheres, it resulted in significant changes in selection depending on the microalga species and the microsphere type used. Based on the differences in selection response by *M. edulis*, the workers concluded that mussels use physicochemical cues to discriminate between particles. The surface properties of the microspheres before and after treatment, however, were not characterized. Without this information it is difficult to determine if the observed selection responses were due to passive interactions or contact chemoreception. In fact, it is difficult to separate an active versus a passive response to ectocrines on the particle surface. Methods would need to be developed that can distinguish if a paracrine response, a form of cell-to-cell communication where one cell produces a signal to induce a response in a nearby cell, is mediating a selection response.

As new technologies (e.g., FITC and Isotopic labeling, filed based particle counters) and methods for studying particle capture and selection in suspension-feeding

bivalves arise, older reports and assumptions need to be revisited. It has been demonstrated that physiological responses of bivalves under field conditions are different from those measured under laboratory setting. Further, recent studies have shown that the capture efficiency of smaller particles may be higher than previously reported. These higher CEs may indicate that experiments focusing on particles whose size is at the lower threshold of capture (e.g. picoplankton and nanoplankton, $< 4 \mu\text{m}$), and understanding the effects of particle surface properties on CE, is a fertile area for future research. Qualitative factors of particles, such as surface properties, could also contribute to the differential capture of natural seston, however only a few papers have convincingly demonstrated such an effect (Hernroth et al. 2000, Yahel et al. 2009). Such factors may be particularly important at the lower size class threshold (e.g., $<4 \mu\text{m}$), and if so, the contribution of smaller particles (e.g., picoplankton) as a food resource for bivalves may be greater than previously recognized.

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Chapter II

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

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Abstract

Historically, particle capture efficiency (CE) in suspension-feeding bivalve molluscs has been shown to be strongly dependent on particle size, increasing asymptotically to a maximum of about 100% for particles \geq ca. 4 μm in diameter. Recent advances in the analysis of the particulate matter of seston have allowed for more precise studies of bivalve feeding under natural conditions. Some studies have reported that the mechanisms associated with particle capture exhibit physiological plasticity, and under certain conditions smaller cells and particles are captured in preference to larger ones. For bivalves, however, there is no mechanistic explanation that would account for such fine-scale control of CE based on size. The current study experimentally assessed the seasonal control of CE by the blue mussel, *Mytilus edulis*, employing a flow-through system to examine particle capture of natural seston. The natural particle field was analyzed using two different types of particle analyzers, the LISST-100x and the Coulter Multisizer IIe. Mussels were simultaneously delivered synthetic microspheres of defined diameter (2 - 45 μm) to control for the effects of seasonal differences in the size and shape of natural particles. The capture of microspheres was quantified by means of flow cytometry (FCM), and results cross-checked with the multisizer. Additionally, gene expression of a mucosal lectin (MeML)

associated with the feeding organs of mussels was examined as a biomarker for physiological response to seasonal changes in the particle-food supply. Results demonstrated that for microspheres $\geq 4 \mu\text{m}$ CE of mussels was always near 100%, and did not change seasonally. In contrast, there was an apparent seasonal shift in CE of natural particles, with particles 17-to-35 μm in equivalent spherical diameter (ESD) occasionally being captured at lower efficiencies than particles 4-to-15 μm in ESD (e.g., during September and December). No relationship between MeML expression and seasonal CE was found. These findings call into question the physiological plasticity of CE in mussels and alternative hypotheses are presented. We suggest that the purported changes in CE are not a consequence of behavioral or physiological responses of mussels, but rather a result of one or more of the following confounding factors; 1) instrument artifacts that can arise as a result of the way in which laser and electronic particle counters calculate ESD to estimate particle size; 2) disaggregation of flocculent material collected from control chambers; 3) post-capture escape of highly motile phytoplankton cells from the infrabranchial chamber; 4) qualitative factors of the particles that could affect capture; or 5) mathematical happenstance of calculating CE on particle-size classes that contain widely different numbers of particles.

Keywords

Mussels, *Mytilus edulis*, capture efficiency, instrumental artifacts, LISST-100x, Coulter multisizer

1. Introduction

Suspension-feeding bivalve molluscs are one of the most important groups of animals in coastal ecosystems, often dominating the macrobenthos (Dame 1996). Bivalves are exposed to large amounts of suspended matter that include both nutritious and non-nutritious particles (Newell RC, 1965; Owen, 1966; Newell CR et al., 1989). As a way to process efficiently the complex mixture of material that they encounter, bivalves have evolved capabilities for selective feeding that allow them to reject some of the captured material in pseudofeces (Loosanoff, 1949; Foster-Smith, 1975; Shumway et al., 1985; Bacon et al., 1998). Selective feeding can be based on physical factors such as particle size and shape (Bayne et al., 1977; Shumway et al., 1985; Cognie et al., 2003; Mafra et al., 2009), as well as physicochemical interactions between the particles and the feeding organs (see reviews by Jørgensen, 1996; Ward and Shumway, 2004). Rosa et al. (2013) demonstrated that the eastern oyster, *Crassostrea virginica*, and the blue mussel, *Mytilus edulis*, can discriminate between particles of the same size based upon the surface charge and wettability of particles. More specific chemical interaction between lectins in the mucus of pallial organs and carbohydrates present on the surfaces of microalgal cells has also been demonstrated. In several studies, workers confirmed that carbohydrate-lectin interactions are involved in mediating particle sorting in both *C. virginica* and *M. edulis* (Pales Espinosa et al., 2009, 2010a). A mucosal C-type lectin (dubbed MeML) in the blue mussel, *Mytilus edulis*, whose expression could be involved in particle sorting was also identified (Pales Espinosa et al., 2010b). Most recently, Pales Espinosa and Allam (2013) demonstrated

that the expression of MeML is regulated in response to the quality and quantity of food offered, further suggesting a physiological basis for qualitative particle selection.

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

In general, capture efficiency has been reported to increase non-linearly with increasing particle diameter to a maximum of about 100%, with some species of bivalves being more efficient at capturing small particles than other species (see Ward and Shumway, 2004). If particles were differentially captured at the larger size threshold, this would be a form of particle selection. Preferential retention of particles could thus be an important discriminatory mechanism that alters the composition of

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

In a few cases, qualitative factors have also been shown to affect CE. The European oyster, *Ostrea edulis*, for example, was found to capture the dinoflagellate

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

Some studies have also reported that bivalves can capture smaller particles at a higher efficiency than larger particles (e.g., Strømmeier et al., 2012). Lesser et al. (1991), for example, reported the clearance of the toxic dinoflagellate *Alexandrium tamarensis* (30-45 µm) by scallops at significantly lower rates than smaller (~16 µm diameter) phytoplankton species. Using a Coulter counter to examine particle selection in *M. edulis*, Bayne et al. (1977) reported that cells of *Phaeodactylum tricornutum* (~6 µm) were captured in higher quantities than larger inorganic particles, a shift the authors suggested may be a result of the coulter counter using spherical equivalents to calculate diameter. Using flow cytometry, Pile and Young (1999) reported that the cold-seep mussel *Bathymodiolus childressi* captured bacteria at significantly higher proportions than larger protozoans. Although the above reports suggest that particle capture in bivalves may be physiologically plastic and responsive to changes in the

particle food supply, controls for the effects of seasonal changes in the relative abundance of different shaped particles, such as using microspheres of defined geometries, were not usually included in these studies. Apparent changes in CE could result from changes in the proportion of particles with different geometries, or other effects unrelated to physiological plasticity. To date, no mechanism has been proposed for bivalves that would account for the capture of smaller particles in preference to larger sized particles. Resolving results from field studies of CE with current models of particle capture mechanisms is important not only for a deeper knowledge of particle capture and selection processes, but also for a better understanding of how selective grazing by bivalves could affect phytoplankton species composition and impact food web dynamics in near-shore waters (see Dame, 1996).

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

2. Materials and Methods

2.1 Study animals

Blue mussels, *Mytilus edulis*, were collected locally from wild populations at the Avery Point Campus of the University of Connecticut in Groton, CT, USA. Animals were

maintained in lantern nets hung from a dock adjacent to the intake line of the flow-through experimental system (see below). Mussels were acclimated to these conditions for at least 2 weeks prior to start of the feeding experiments.

2.2 Feeding experiments

Studies were carried out seasonally on two separate days for a period of 1 year (March, May, July, September, December 2013 and March 2014). A flow-through experimental system (Galimany et al., 2011) was employed to simulate environmental conditions experienced by bivalves *in situ*. The system was set up on a floating dock, and water was pumped directly from Long Island Sound into a common aerated head tank, which minimized particle settling (data not shown). Water was distributed to individual rectangular chambers, measuring 45 x 180 x 60 mm (width x length x height). Mussels (mean shell length 48 ± 5 mm [SD]) were secured to each chamber and the flow rate was set to 100 ml min^{-1} to prevent water recirculation. Animals were acclimated for 1-hr prior to collection of water samples. The concentration of particles in the water exiting the control chambers (n=4, no mussels) and the experimental chambers (n=16, one mussel each) was measured using two different types of particle analyzers commonly used in bivalve mollusc feeding studies. First, a multi-parameter laser in situ scattering and transmissometry instrument (LISST-100x, Sequoia Science) was used to quantify particle size distribution and volume. The system was baffled against stray light that would interfere with the optics during measurements. Particle size distribution and their concentrations in the water column were determined by applying an inversion algorithm to the scatter data (Agrawal and Pottsmith, 2000).

Within an hour of the LISST-100x analysis, a 250 mL water sample was collected from each chamber. These samples were gently inverted (10x), passed through a 70 μ m mesh screen and analyzed by means of an electronic particle counter (Coulter multisizer IIe) fitted with a 100 μ m aperture. Both of these instruments measure particle volume, using light scattering or electrical impedance, and assume a spherical shape to calculate particle size. These sizes are reported as equivalent spherical diameters (ESD, μ m).

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

2.3 Environmental parameters

Seston samples were collected from each of the control chambers to calculate total particulate matter (TPM), particulate organic matter (POM), chlorophyll *a* concentration, and the distribution of phytoplankton species. To determine TPM and

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

$$\frac{\mu g - chlorophyll - a}{L} = \frac{CF \times \frac{AF}{(AF - 1)} \times (F_1 - F_2) \times 7mL}{V}$$

where CF is the calibration factor of the instrument, AF is the acidification factor, 7 mL is the volume of acetone used to extract the chlorophyll, V is the total volume of sample filtered (in L), F_1 is the fluorescence reading before acidification, and F_2 is the fluorescence reading after acidification. Water temperature and salinity, measured by

means of a refractometer and reported as parts per thousand, were recorded during each field season.

2.4 Phytoplankton distribution

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

2.5 MeML transcript expression

The relative expression of the mucosal lectin (MeML) transcripts associated with the pallial organs were determined by means of qPCR on samples taken from the labial palps of mussels used in the flow through experiments. Labial palps were used as a proxy for gills because previous studies have shown that MeML expression in mussel labial palp and gill tissue is correlated (Pales Espinosa and Allam, 2013). Secondly, the labial palps have been found to have a higher signal-to-noise ratio than the gill tissue. Following each experiment, mussels were placed on ice and taken to the laboratory where labial palps were separately collected using sterile techniques. Tissue samples were then placed in liquid nitrogen and maintained at -80°C until further processing. Total RNA of each sample was extracted using TRI-Reagent® RT (Molecular Research Center Inc., Cincinnati, Ohio) and reverse transcribed into cDNA using M-MLV RT (Promega, Madison, Wisconsin). MeML transcript abundances were measured in duplicate with a set of specific primers designed by Pales Espinosa et al. (2010b) (forward 5'-ATGCTCAATTGGCTGGCATCATGG-3' and reverse 5'-ATGCTCAATTGGCTGGCATCATGG-3') and using 18S ribosomal RNA as a housekeeping gene targeted with the primer set forward 5'-CTGGTTAATTCCGATAACGAACGAGACTCTA-3' and reverse 5'-TGCTCAATCTCGTGTGGCTAAACGCCACTTG-3'. Real-time PCR monitoring was performed using an Eppendorf RealPlex cyciler with 96 well plates. Relative quantification was carried out in 10-µl reactions, including 5 µL of 2X Brilliant II SYBR® Green qPCR Master Mix (Agilent), 1 µL of each primer at 10 µM concentration and 3 µL of cDNA template to obtain a final concentration of 5 ng/µL cDNA in the reaction

volume. The thermal profile for real-time PCR assay was an initial denaturation step at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 30 s, and annealing and extension at 60°C for 1 min. A melting curve was set up and analyzed after each run to guarantee a specific amplification. The relative MeML expression level of each sample was calculated using the $\Delta\Delta C(T)$ method (Livak and Schmittgen, 2001), using the 18S data for the first normalization and data from March 2014 as a calibration for the second normalization. March 2014 was selected as a calibration month because it had the lowest mean fold average $\Delta C(T)$, reducing the noise-to-signal ratio of the $\Delta\Delta C(T)$ values. Data are presented as a fold change, where the value indicates the change in the expression level of MeML for each season relative to March 2014.

2.6 Particle analysis and feeding calculations

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

Capture efficiency (CE) was calculated for each size class of particles using the formula:

$$CE = 1 - (PC_f / PC_c) \quad CE = 1 - \frac{PC_f}{PC_c}$$

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

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

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

2.7 Statistical analysis

Seasonal differences in environmental parameters (TPM, POM) were tested using analysis of variance. To examine the effects of season and particle size on

capture efficiencies, a mixed-model analysis of variance for repeated measures was used (ANOVAR; Systat 13), with particle size class as the within subject effect (repeated) and season as the between subject effect. Data were tested for normality and homogeneity prior to analysis. Following ANOVAR analyses, a Tukey's HSD post-hoc test was applied to examine differences between levels of the independent variables. Differences in expression of MeML were tested on $\Delta C(T)$ values ($C(T)$ s normalized with the housekeeping 18S gene) by means of a one-way analysis of variance by ranks, followed by a Dunn's post hoc, due to the heteroscedastic nature of the data (Kruskal-Wallis; SigmaStat). For all statistical tests, an alpha level of 0.05 was used.

3. Results

3.1 Environmental and seston parameters

As expected, environmental conditions varied seasonally during the one-year study (Table 1). Water temperature ranged from 3.5 to 24.5°C, and salinity ranged from 30 to 35. The total particulate matter (TPM) ranged from 4.04 mg/L in December to 7.50 mg/L in March 2014. There were no significant differences in TPM between each of the seasons sampled. The particulate organic matter (POM) fraction was significantly higher during March 2013 (3.09 mg/L) and significantly lower in December 2013 (0.55 mg/L) compared to values for other sampling months. The lowest concentration of chlorophyll *a* was recorded in December (0.06 µg/L), and was significantly different than values for all other months. The highest concentration of chlorophyll *a* was recorded in May (5.13 µg/L), but this value was not significantly different than values for other months (except December). The seston was dominated by small particles (1-3 µm) in all months

sampled (data not shown), with the number of particles analyzed by the LISST on the order of 10^8 particles mL^{-1} and by the multisizer on the order of 10^6 particles mL^{-1} . Particle concentrations rapidly dropped to 10^3 particles mL^{-1} for particles ranging from 4 to 10 μm , and to ca. 10^2 particles mL^{-1} for larger particles. This overall particle-size distribution (PSD) did not change seasonally. Additionally, the PSD of the seston did not change significantly over the sampling period, or between the two sampling days. Therefore, data obtained for each of the two days were pooled.

3.2 Capture efficiencies

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

For natural seston analyzed by means of the LISST-100x, data analysis demonstrated significant effects of both season and particle size on CE (ANOVAR, $P < 0.01$; Table 2, Figure 1B). There were also significant interaction effects between these two independent variables. For example, in May, July, and September particles ca. 3- μm in size were captured at significantly higher efficiencies than in March (2013 &

2014, Tukey's HSD, $P < 0.01$). In May, particles in the 22-31 μm range were captured at significantly higher efficiencies than in September and December (Tukey's HSD, $P < 0.01$; Table 2, Figure 1B). Other seasonal differences in CE of particles in the same size class were also evident (Table 2). Overall, CE were lower for particles smaller than 3 μm in size. In March and May, CE generally increased or stayed constant as particle size increased above 5 μm . In September, however, CE were significantly lower for particle size classes ranging from 31 μm to 44 μm , compared to size classes 6 μm to 13 μm . In December, a similar trend was observed, with significantly lower CE for particle size classes ranging from 22 μm to 31 μm (Tukey's HSD, $P < 0.05$), compared to smaller and larger size classes (Table 2). These findings indicate an apparent seasonal shift in the size spectrum of natural particles captured by mussels, including the capture of smaller particles in preference to larger particles (Table 3).

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

(Tukey's HSD, $P < 0.05$; Table 3). Generally, larger particles were captured at higher efficiencies than smaller particles.

3.3 Clearance rates

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

3.4 Phytoplankton diversity and abundance

A total of 30 genera of phytoplankton were identified during the seasonal study (Table 4). The plankton species, all common to Long Island Sound (Hoppenrath et al., 2009), ranged in size from 6 to $110\ \mu m$. Total phytoplankton abundance was highest during March ($70\ cells\ mL^{-1}$), which corresponded to the spring bloom (data not shown) and lowest during December ($20\ cells\ mL^{-1}$). Overall, diatoms dominated the seston, with only seven of the most common genera being dinoflagellates. The dinoflagellate

genera were the numerically dominant phytoplankton in July (*Prorocentrum* and *Peridinium*). During September and December, phytoplankton abundance was dominated by chain forming diatoms (*Cerataulina*, 15 µm diameter and ~50 µm length; *Skeletonema*, 11.5 µm diameter and 45 µm length) and pennate diatoms (*Navicula*, 14 µm width and ~70 µm length; *Nitzschia*, 6 µm width and ca. 50 µm length). Centric diatoms (e.g., *Thalassiosira*, 27 µm) were mainly abundant during the spring bloom (March). In none of the samples, however, were centric genera the numerically dominant phytoplankton group.

3.5 MeML expression

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

4. Discussion

Results reported here demonstrate several important points regarding the capture of particles by mussels and point out potential errors associated with use of

different instruments. First, the capture of the spherical particles $\geq 4 \mu\text{m}$ in diameter was consistently high across all sampling months, with only the $2\text{-}\mu\text{m}$ particles being captured at a lower efficiency than particles of greater size. These data are consistent with current understanding of hydrosol filtration mechanisms employed by bivalves (Shimeta and Jumars, 1991; Ward et al., 1998b; Riisgård and Larsen, 2010). Small particles encounter gill filaments at a lower rate, and thus are captured at a lower efficiency, than larger particles. As particle size increases, CE increases and approaches 100%. The particle size above which CE is maximum is species specific, and for *M. edulis* it has been reported as ca. $4\mu\text{m}$ (Møhlenberg and Riisgård, 1978). The results are also consistent with other studies that used uniform particles of known size (including phytoplankton cells) in which all particles above a threshold size were captured with high efficiency (see Ward and Shumway, 2004 for review). Second, because CE of microspheres $\geq 4 \mu\text{m}$ was not affected by season, there is no evidence that mussels adjusted either feeding behavior or physiology over time (e.g., changes in interfilamentar spaces, beat frequency of the laterofrontal cirri). If mussels were adjusting particle capture seasonally, and capturing smaller particles in preference to larger ones (e.g., as determined for natural seston in September and December), then CE of the $25\text{-}\mu\text{m}$ and $45\text{-}\mu\text{m}$ spheres should have declined. No decrease in CE of larger microspheres, however, was found in any season. The $2\text{-}\mu\text{m}$ spheres were captured at a significantly higher rate in December compared to the other months, though still not at a higher efficiency than larger particles. The reason for this finding is unclear. Temperature during December was at an intermediate level (9°C), POM and chlorophyll a concentrations were at their lowest level, and TPM concentration was not

significantly lower than during the other months (Table 1). Environmental factors, therefore, provide little insight as to why the 2- μm microspheres were captured at a higher efficiency in December compared to the other months. Work by Lucas et al. (1987), examining two populations of *M. edulis*, found that during times when bacterial numbers in the seston were high, animals were able to capture smaller-sized particles (0.5-1.58 μm) with up to 57% efficiency. Larger phytoplankton (>4 μm), however, were still captured with close to 100% efficiency. Such differences in the retention of particles at the lower threshold of capture warrants further research.

In contrast to the capture of microspheres, CE of mussels feeding on natural seston (measured on the same dates, with the same animals) appeared to change seasonally, and at times smaller particles were captured more efficiently than larger particles. These data are comparable to results obtained by previous workers using similar particle analyzers (e.g., Strøheim et al., 2012). For example, in March 2013 CE for particles $\leq 6 \mu\text{m}$ in size, calculated from data collected using the multisizer, was significantly lower than CE for the same size particles in September. In September and December CE of particles between ca. 4 to 6 μm in size, calculated from data collected by the LISST-100x, were captured with a higher efficiency than particles ca. 22 to 37 μm in size. Other apparent differences in CE within and between months were found in the data sets collected by each of the two instruments. Additionally, in several months CE of mussels calculated from data generated by the LISST-100x and multisizer were different. For example, in March CE calculated from multisizer data increased from ~30% for 3- μm particles to ~70% for 8- μm particles, whereas CE calculated from LISST-100x data for particles in the same size range only changed from ~45% to ~60%.

In September, the highest CE (91%) calculated from data collected by the LISST-100x was for particles 3 μm in size, with efficiency generally decreasing with increasing particle size to 50% for particles ca. 26 μm in size. Data collected at the same time using the multisizer showed a different trend, with CE generally increasing with increasing particle size to a maximum of 90% for particles 25 μm in size.

The range of mean clearance rates measured in this study using both instruments (0.67 L hr^{-1} to 3.34 L hr^{-1}) were similar to rates that have been reported previously for *Mytilus edulis* of similar size (MacDonald and Ward, 2009; Cranford et al., 2011). In all months except March (when water temperatures were lowest), particles $<4 \mu\text{m}$ were cleared at a lower rate than larger particles. Our results also allow us to investigate whether the apparent shifts in CE could affect CR calculated from the collected data. In September and December, for example, data collected by the LISST indicated a significant decrease in CE of particles within the 11 to 25 μm size class compared to smaller sizes (between 4-10 μm), but no significant differences in CR between these two size classes. Data collected by the multisizer in September indicated no significant difference in CE between particles in the 11 to 25 μm and 4 to 10 size classes, but a significantly lower CR for the smaller size particles. There also was no correlation in any month between clearance rates and CE of natural particles 25 μm in size (a particle size that showed some of the largest differences in CE) calculated from data collected by either of the two instruments. These findings demonstrate that an apparent shift in CE does not always translate to a change in CR. The most obvious result from this comparative analysis is that in many months (e.g., May, Sept, Dec, Mar 14), CR calculated from data generated by the LISST was significantly different than

that generated by the multisizer. Differences were found within specific size classes and for the full range of particles. These results are striking, as they could affect the conclusions reached in studies using these different instruments.

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

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

One likely explanation for differences in the calculated CE of mussels feeding on natural particles versus microspheres, relates to the way in which particle analyzers calculate an equivalent spherical diameter (ESD) to estimate particle size. Both the LISST-100x and multisizer calculate ESD from measurements of particle volume. The LISST-100x uses near-forward scattering measurements to obtain information on the volume and concentration of suspended particles. The observed scattering measurements are then inverted and a spherical particle shape is assumed in order to estimate particle size from the measured particle volume (Agrawal and Pottsmith, 2000). Electronic particle analyzers, such as the multisizer, use a similar approach, but

measure the increase in the impedance of an electrical current that is applied across an aperture. The change in impedance is caused by particles passing through the current, which displace their own volume of the conducting liquid. The amplitude of the current fluctuation is directly proportional to the volume of the particle, which is then converted to ESD (User's manual for Coulter® multisizer IIe. England: Coulter Electronics Limited (1989)). Both of these techniques work well for particle shapes that approximate a sphere, but produce increasing error in ESD as particle shape deviates from spherical (e.g., chain-forming and pennate diatoms). The limitations of these instruments in accurately measuring particle size have been discussed previously (Jonasz, 1987; Karp-Boss et al., 2007; Reynolds et al., 2010). In particular, the analysis of a single species of phytoplankton with a complex shape by means of the LISST-100x can produce ESD data with peaks in several size ranges separated by tens or hundreds of micrometers, or broad size-frequency distributions that span tens or hundreds of micrometers (Karp-Boss et al., 2007). The error associated with calculating ESD and estimating actual particle size, changes with both the size of the particles (smaller particles tend to be more spherical) and season as the seston is dominated by different types of organic and inorganic particles, and different species of phytoplankton (Jonasz, 1987). Considering the complex assemblage of particles in the seston - with different sizes, shapes and concentrations - it is unlikely that a size-frequency distribution could be obtained that accurately reflects the true size range of natural particles in water before and after being processed by a suspension feeder. Additionally, phytoplankton with narrow widths (e.g., $< 3 \mu\text{m}$) and length to width ratios > 3 could be captured at a low efficiency, depending on their orientation as they approach the gill filaments, but

would be recorded as particles with an ESD above the size that should be captured with ca. 100% efficiency. Bayne et al. (1977) previously reported that mussels apparently captured the non-spherical algae *Phaeodactylum tricornutum* at higher efficiencies than larger particles. They suggested that the data from their study, analyzed using a coulter counter, showed that the shape of diatom cells could result in higher CE than what would be expected for a spherical particle of similar volume. Data from the current study support these contentions. In the months of September and December, the dominant phytoplankton species were non-spherical diatoms (Table 5). During these months, CE calculated by means of the LISST-100x was significantly different than other months (e.g., May), and CE of large particles (ca. 20 and 40 μm) was significantly lower than small particles (ca. 6 μm). Particulate characteristics of the seston, therefore, could create considerable error in the size-frequency distributions generated by particle analyzers that lead to spurious calculations of CE and faulty conclusions regarding physiological plasticity of the capture process.

Another factor that could explain purported changes in CE, is the disruption of marine aggregates (e.g., marine snow) during sampling handling and processing. Marine aggregates are a common component of the seston, with large proportions (> 70%) of natural particulates being present in these aggregations during certain times of the year (Alldredge et al., 1993; Crocker and Passow, 1995; Syvitski et al., 1995). These loose agglomerations of organic and inorganic particles can be disrupted by shear (Kiorbøe and Hansen, 1993; Manning and Dyer, 1999), such as that produced by the stirring or shaking of collected samples. Particle analyzers that rely on a vacuum to pull particles through an aperture, such as the multisizer, can also form shear fields that

disrupt aggregates and release constituent particles. Bivalves can capture aggregates of various sizes and ingest the constituent particles (Newell et al., 2005; Kach and Ward, 2008; Ward and Kach, 2009). Therefore, there would be more aggregates present in water samples taken before (i.e., control) than those taken after flowing over a bivalve. Disruption of aggregates and the release of small particles would bias the size distribution toward smaller sizes in the control samples. The calculated CE from such data would lead to the interpretation that the bivalve was able to capture more individual small particles than to which it was actually exposed. Because efficiency of capture is normalized to the particle size that produces the highest CE, this situation would result in higher CE for smaller particles and artificially lower CE for larger particles.

Another situation that could affect the calculated efficiencies is the presence of dinoflagellates or other highly motile cells in the seston. In the European oyster, *Ostrea edulis*, cells of *Alexandrium tamarense* (a dinoflagellate with a mean length of 35 μm), can escape from the pallial organs after capture (Bricelj et al., 1998). The freely swimming cells congregate between the gill lamellae and are periodically expelled from the mantle cavity by the rapid adduction of the oyster shell valves. The consequence of such an expulsion of cells in a flow-through experiment would be the measurement of a high number of particles in the size range of those cells (e.g., 35 μm). Further, the calculated CE of particles in this size range would be low, with the interpretation that these particles passed through the gills and were not retained, when in fact they were retained but were expelled as unconsolidated pseudofeces. Although the specifics of the process of particle capture and transport by mussels differs from that of oysters,

highly motile cells might be able to evade capture by the gills or swim from loosely bound material in the ventral grooves of mussels. In the current study, less than a third of the identified phytoplankton groups were dinoflagellates. Two of these identified genera, *Prorocentrum* and *Peridinium* (35 μm and 50 μm , respectively), constituted a large portion of the phytoplankton composition during the July sampling date. Assuming that the above scenario occurred, the presence of these dinoflagellates could have contributed to the lower CE measured for particles > 35 μm compared to May.

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

Finally, larger size classes tend to have orders of magnitude fewer particles than smaller size classes. Each individual particle in the larger size classes, therefore, contributes a greater proportion to the difference in counts between control and

experimental samples, and thus the calculated efficiencies. For this reason, most studies pool data from size bins that do not meet some minimal particle concentration (e.g., 100 particles ml⁻¹) for the control samples. Little theoretical work has been done to examine the mathematical consequences of calculating CE on particle-size classes that contain widely different numbers of particles.

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

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

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

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

LISST-100x

Size (μm)	Mar 13	May 13	Jul 13	Sept 13	Dec 13	Mar 14
1	0.41 ^{a,b} (0.41)	0.34 ^{a,b} (0.44)	0.48 ^a (0.36)	0.15 ^{b,c} (0.30)	0.24 ^{a,c} (0.22)	0.01 ^c (0.02)
2	0.44 ^{a,c} (0.24)	0.75 ^b (0.23)	0.67 ^{a,b} (0.21)	0.63 ^{a,b} (0.14)	0.54 ^{a,b} (0.19)	0.18 ^c (0.23)
3	0.45 ^a (0.15)	0.94 ^{b,c} (0.07)	0.78 ^b (0.19)	0.91 ^{b,c} (0.14)	0.57 ^{a,b} (0.15)	0.36 ^a (0.40)
4	0.49 ^a (0.18)	0.90 ^b (0.08)	0.76 ^{a,b,c} (0.13)	0.72 ^{a,b} (0.13)	0.59 ^{a,c} (0.14)	0.34 ^c (0.22)
5	0.53 ^{a,c} (0.19)	0.88 ^b (0.09)	0.75 ^{a,b} (0.14)	0.58 ^{a,c} (0.25)	0.62 ^{a,b} (0.15)	0.32 ^c (0.20)
6	0.55 ^a (0.20)	0.91 ^b (0.09)	0.78 ^{a,b} (0.13)	0.70 ^{a,b} (0.17)	0.62 ^a (0.14)	0.44 ^a (0.24)
7	0.57 ^a (0.19)	0.91 ^b (0.09)	0.78 ^{a,b} (0.14)	0.73 ^{a,b} (0.17)	0.63 ^a (0.15)	0.57 ^a (0.21)
8	0.58 ^a (0.20)	0.90 ^b (0.09)	0.76 ^{a,b} (0.15)	0.68 ^{a,b} (0.20)	0.64 ^{a,b} (0.15)	0.53 ^a (0.23)
9	0.58 ^a (0.19)	0.91 ^b (0.10)	0.78 ^{a,b} (0.16)	0.75 ^{a,b} (0.17)	0.67 ^{a,b} (0.16)	0.58 ^a (0.23)
11	0.60 ^a (0.18)	0.90 ^b (0.11)	0.78 ^{a,b} (0.17)	0.71 ^{a,b} (0.20)	0.68 ^{a,b} (0.17)	0.55 ^a (0.21)
13	0.58 ^a (0.17)	0.89 ^b (0.11)	0.77 ^{a,b} (0.17)	0.66 ^{a,b} (0.21)	0.64 ^{a,b} (0.16)	0.51 ^a (0.21)
16	0.54 ^a (0.17)	0.88 ^b (0.10)	0.74 ^{a,b} (0.17)	0.61 ^{a,b} (0.21)	0.56 ^a (0.15)	0.50 ^a (0.21)

19	0.52 ^a (0.17)	0.88 ^b (0.10)	0.73 ^{a,b} (0.18)	0.57 ^a (0.23)	0.48 ^a (0.14)	0.52 ^a (0.20)
22	0.53 ^{a,c} (0.19)	0.89 ^b (0.11)	0.72 ^{b,c} (0.19)	0.55 ^{a,c} (0.27)	0.41 ^a (0.15)	0.53 ^{a,c} (0.19)
26	0.56 ^{a,c} (0.19)	0.88 ^b (0.12)	0.70 ^{b,c} (0.19)	0.49 ^{a,c} (0.30)	0.37 ^a (0.19)	0.52 ^{a,c} (0.20)
31	0.60 ^{a,b} (0.20)	0.87 ^b (0.14)	0.68 ^{a,b} (0.20)	0.43 ^a (0.31)	0.42 ^a (0.22)	0.54 ^a (0.15)
37	0.64 ^{a,b,c} (0.17)	0.85 ^b (0.16)	0.67 ^{a,b} (0.20)	0.37 ^c (0.32)	0.49 ^{a,c} (0.22)	0.55 ^{a,b,c} (0.19)
44	0.70 ^a (0.18)	0.86 ^a (0.17)	0.68 ^a (0.23)	0.40 ^b (0.33)	0.67 ^{a,b} (0.18)	0.73 ^a (0.11)
52	0.73 ^{a,b} (0.27)	0.87 ^a (0.18)	0.64 ^{a,b} (0.35)	0.50 ^b (0.35)	0.84 ^a (0.11)	0.83 ^a (0.08)
61	0.77 ^{a,b} (0.35)	0.88 ^a (0.22)	0.60 ^{a,b} (0.44)	0.52 ^b (0.41)	0.97 ^a (0.10)	0.94 ^a (0.08)

Coulter Multisizer IIe

Size (µm)	Mar 13	May 13	Jul 13	Sept 13	Dec 13	Mar 14
2	0.50 ^a (0.35)	0.59 ^a (0.28)	0.63 ^a (0.22)	0.56 ^a (0.19)	0.59 ^a (0.38)	0.37 ^a (0.22)
3	0.33 ^a (0.29)	0.78 ^b (0.10)	0.77 ^b (0.17)	0.75 ^b (0.16)	0.76 ^b (0.26)	0.46 ^a (0.17)
4	0.41 ^a (0.29)	0.87 ^b (0.09)	0.83 ^b (0.14)	0.79 ^b (0.13)	0.82 ^b (0.18)	0.49 ^a (0.19)
5	0.50 ^a (0.34)	0.89 ^b (0.11)	0.86 ^b (0.15)	0.79 ^b (0.14)	0.83 ^b (0.11)	0.52 ^a (0.25)
6	0.52 ^a (0.34)	0.89 ^{b,c} (0.13)	0.87 ^{b,c} (0.14)	0.82 ^c (0.15)	0.75 ^{a,b,c} (0.21)	0.59 ^{a,c} (0.27)
7	0.62 ^a (0.28)	0.91 ^b (0.15)	0.89 ^b (0.08)	0.73 ^{a,b} (0.16)	0.74 ^{a,b} (0.22)	0.51 ^a (0.33)
8	0.70 ^a (0.27)	0.90 ^a (0.18)	0.83 ^a (0.14)	0.69 ^a (0.26)	0.70 ^a (0.24)	0.66 ^a (0.19)
9	0.67 ^a (0.30)	0.87 ^a (0.16)	0.90 ^a (0.13)	0.77 ^a (0.21)	0.78 ^a (0.24)	0.85 ^a (0.26)
10	0.57 ^a (0.26)	0.92 ^b (0.11)	0.86 ^b (0.12)	0.81 ^{a,b} (0.14)	0.77 ^{a,b} (0.19)	0.61 ^a (0.28)
11	0.58 ^a (0.25)	0.91 ^b (0.10)	0.88 ^b (0.12)	0.81 ^{a,b} (0.18)	0.80 ^{a,b} (0.18)	0.69 ^{a,b} (0.25)

13	0.62 ^a (0.27)	0.90 ^{b,c} (0.12)	0.92 ^c (0.08)	0.80 ^{a,b,c} (0.18)	0.79 ^{a,b,c} (0.18)	0.67 ^{a,b} (0.24)
17	0.57 ^a (0.29)	0.83 ^b (0.19)	0.90 ^b (0.13)	0.85 ^{a,b} (0.16)	0.83 ^b (0.16)	0.78 ^{a,b} (0.17)
25	0.53 ^a (0.31)	0.84 ^b (0.20)	0.86 ^b (0.20)	0.90 ^b (0.21)	0.72 ^{a,b} (0.19)	0.66 ^{a,b} (0.14)

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

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

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

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

Figure 1. Normalized capture efficiencies (CE) of mussels for all sampling dates calculated for (A) spherical synthetic particles analyzed by mean of flow-cytometry; (B) natural seston analyzed by means of LISST-100X; and (C) natural seston analyzed by means of the Coulter Multisizer IIe. For clarity, standard deviations and designations of statistical differences are not presented and can be found in Table 2. No significant differences in CE were found for synthetic spherical particles ranging from 4- μm to 25- μm (A), although CE of 2- μm spheres were significantly lower than CE for other sizes in all months (Tukey's HSD, $P < 0.05$). For natural seston analyzed by means of the LISST-100X (B), CE were often significantly lower for particles less than 5- μm compared to other sizes (e.g. Mar 13, May, July; Tukey's HSD, $P < 0.05$). In September and December, particles 4-to-6- μm were captured at significantly higher rates than larger particles (31-to-44- μm in Sept and 26 in December; Tukey's HSD, $P < 0.05$). For natural seston analyzed by means of the Coulter Multisizer IIe (C), CE of particles less than 5- μm were significantly lower than for other sizes only in the March sampling dates (Tukey's HSD, $P < 0.05$). In March 14, CE for 10- μm particles were lower than for 9- μm particles (Tukey's HSD, $P < 0.05$). Data are presented as means, SD not included for clarity.

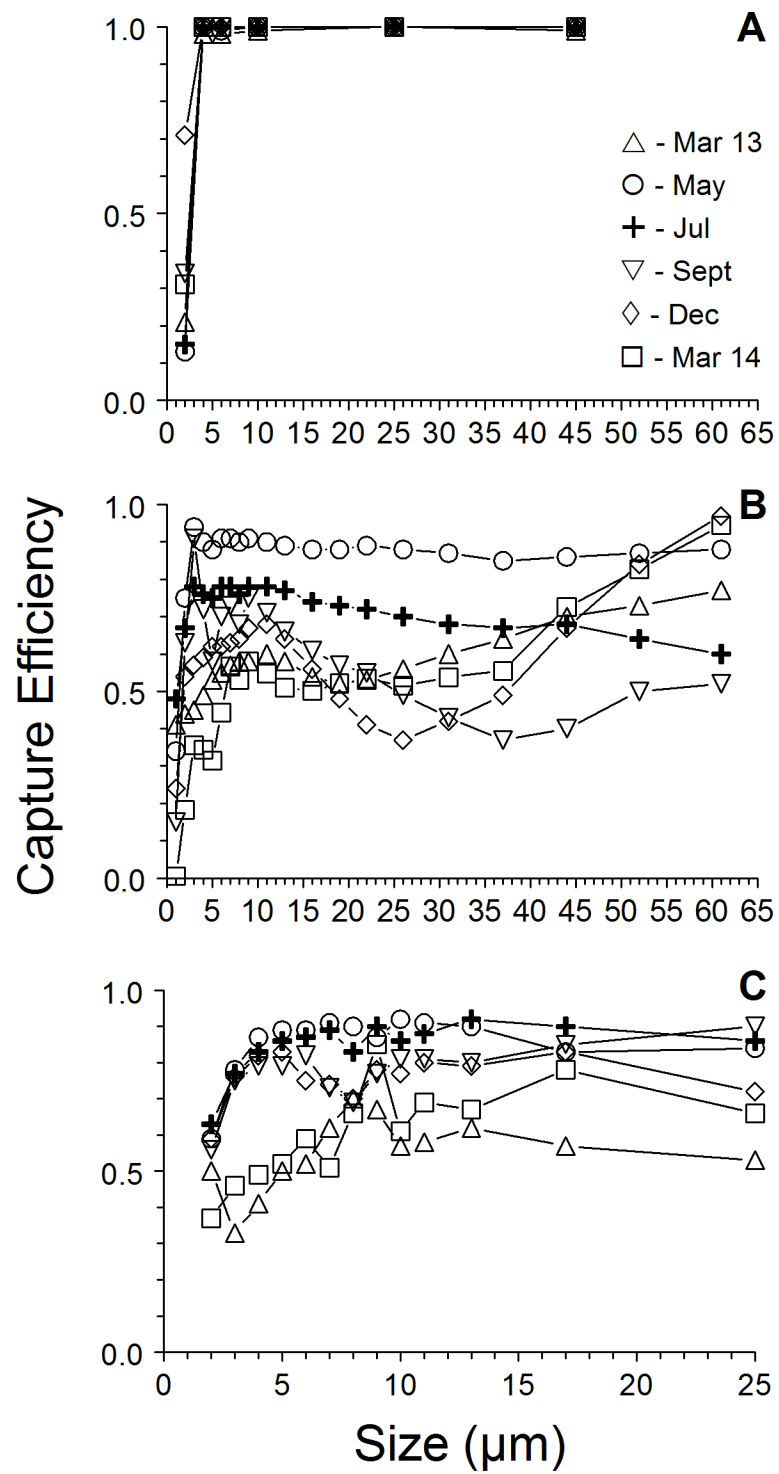


Figure 2. Comparison of clearance rates ($L\ hr^{-1}$) of mussels calculated by means of the LISST-100x and Multisizer particle analyzers. Clearance rates (CR) were calculated for 3 different size classes (particles $<4\mu m$, 4 to $10\mu m$, and 11 to $25\mu m$) and for the full range of sizes (LISST-100x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

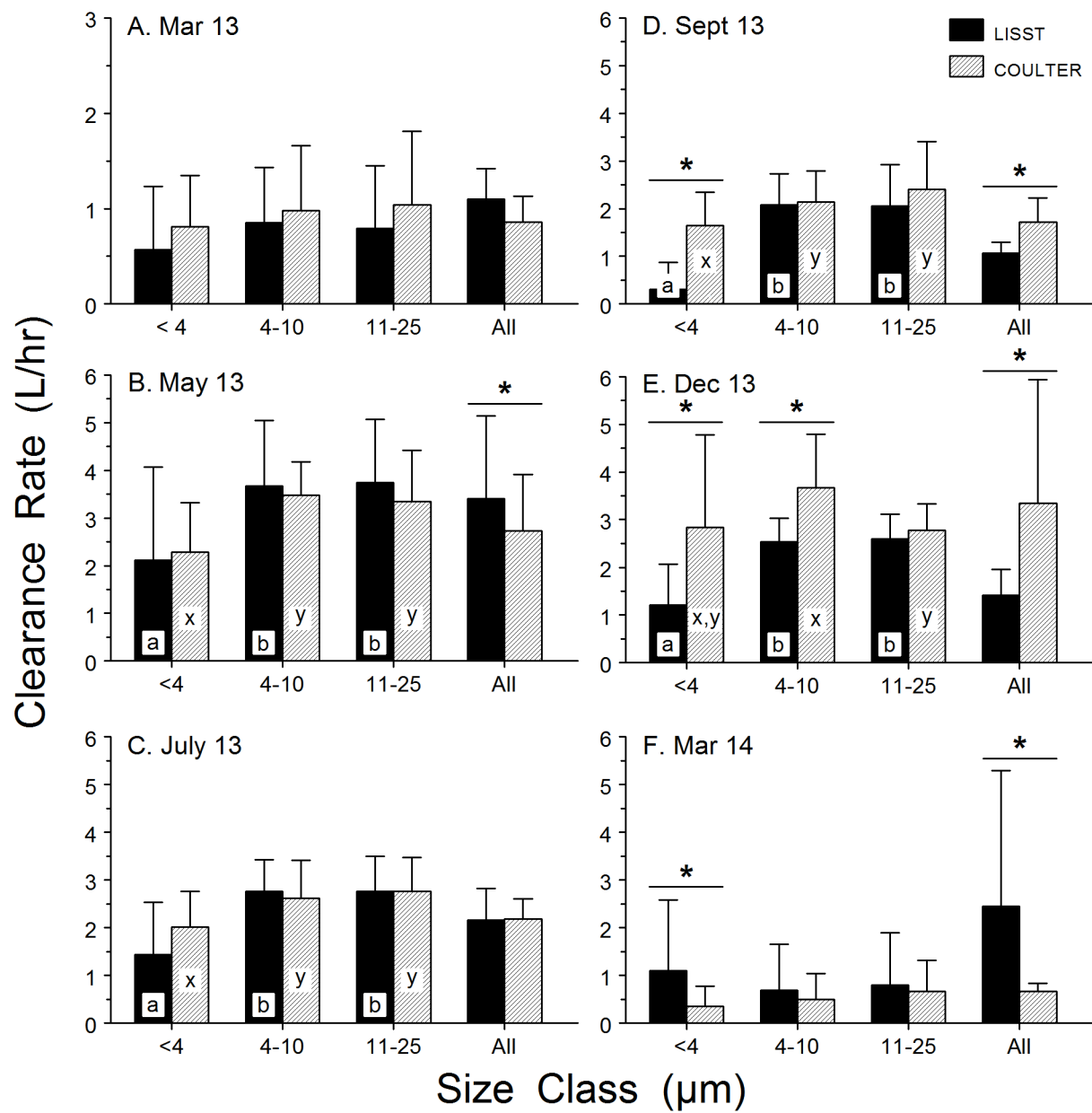
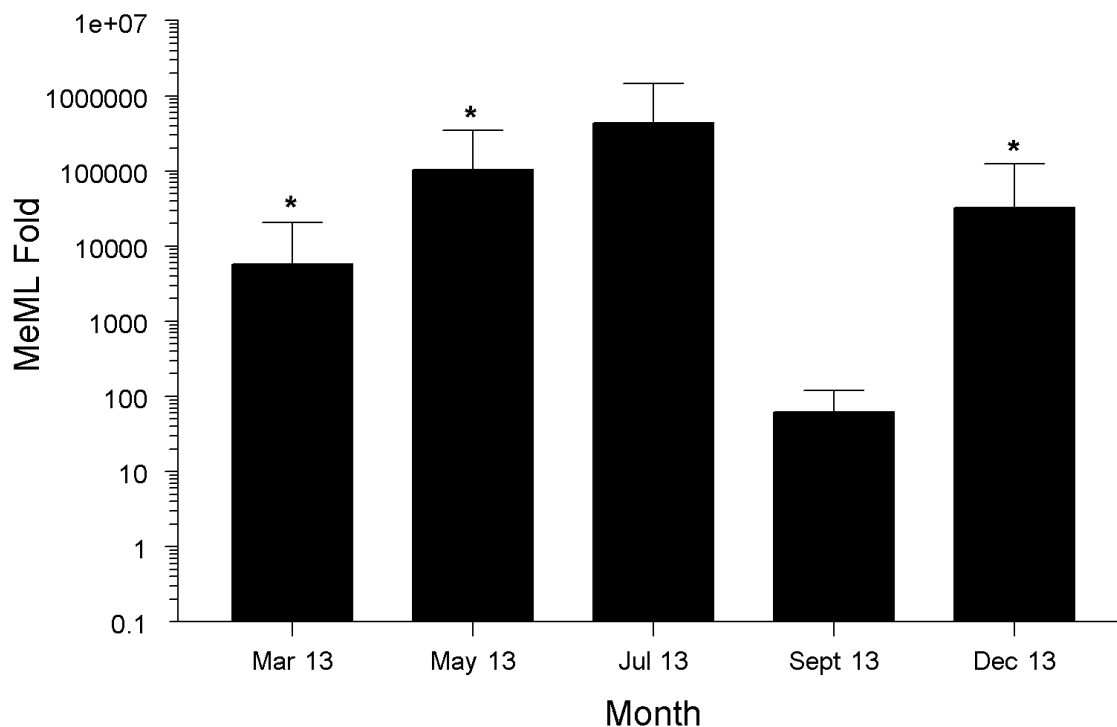


Figure 3. Seasonal variation of MeML transcripts levels in labial palps of mussels. Transcripts levels were determined by quantitative real-time PCR and normalized to 18S RNA. Expression levels are presented relative to March 2014 (which had the lowest expression levels). Data presented as means \pm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



Chapter III

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 discriminate 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 (i.e. *Pavlova lutheri*). These data were then used to develop DA and multiple regression models that considered the quantified surface properties and microalgal fates (rejected vs. ingested) to examine determinants of selection. The DA model correctly classified selection 58% of the time in mussels, and 57% of the time in oysters. Wettability was the most important factor in predicting selection in mussels, and surface charge was most important for oysters. The multiple regression model

demonstrated that 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. Data 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* (Sollow 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 is a weak force dependent upon hydrophobic-hydrophilic interactions between a surface and a liquid, and can act as a signaling cue 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 these studies examining the effects of physicochemical properties upon selection have used synthetic particles (e.g., polystyrene and silica). To understand fully the roles that physicochemical characteristics of natural particles play in the feeding process, the surface properties (e.g., wettability and charge) of organic and inorganic particles that are used in selection experiments need to be characterized.

In bivalves, both non-specific (e.g., surface-charge, wettability) and specific (e.g., lectin-sugar) interactions seem to contribute to particle discrimination. Workers have long recognized the role of lectins as signaling molecules in marine bivalves (e.g. Renwrantz and Stahmer 1983; Gauthier et al. 2004). Work by Pales Espinosa et al. (2009, 2010a) has provided evidence for selection based upon the specific chemical interaction between lectins in the mucus of pallial organs and carbohydrates present on the surfaces of particles and microalgal cells. These workers isolated mucus from the

ctenidia (= gills) and labial palps of oysters, *Crassostrea virginica* (Pales Espinosa et al. 2009) and mussels, *Mytilus edulis* (Pales Espinosa et al. 2010a) and measured specific lectin activity. These studies confirmed that a carbohydrate-lectin interaction is involved in mediating particle sorting in both *C. virginica* and *M. edulis*. Lectin binding to four different microalgal species demonstrated the presence of several different sugars, which varied in abundance and distribution (Pales Espinosa et al. 2010b). Coating the same microalgae in mucus extracted from the pallial organs (gills and labial palps) of *C. virginica* resulted in a decrease in the binding of the different lectins. These findings indicated that the lectins in mucus were binding to sugars on the microalgal cell surface. Thus, the authors demonstrated that sugars can act as recognition molecules and carbohydrate-lectin interactions are involved in particle selection. As with the studies examining physical surface properties, these findings were based upon use of mostly synthetic particles, with some work on a limited number of microalgae.

Different chemical substances (e.g., proteins, carbohydrates, humics) likely change the surface characteristics of organic and inorganic particles, affecting the aforementioned physicochemical characteristics (i.e., charge), 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 (Shresta 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 which can vary between species and among living and non-living particles (Waite et al. 1995). Understanding the differences in surface properties, and how they relate to the presence of carbohydrate moieties (= groups), can help to determine which differences serve as selective cues for various 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 suspension-feeding bivalves have been fed in aquaculture settings and in prior suspension-feeding studies. 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/hydrophobicity, 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 is designated as such from hereon.

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 (PEA) derived from *Pisum sativum*. 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 400Xg for 10 min, washed once with filtered seawater (0.22 μ m, FSW), and resuspended in FSW to a cell density of 10^6 cells/ml. FITC-conjugated lectins were diluted in artificial, filtered seawater (0.22 μ m, ASW) to a concentration of 1 mg ml⁻¹. A 50- μ l 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 algae 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 10 different pairings with 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 feeding chambers holding one bivalve each. The system provided a constant head pressure so that animals were exposed to a steady flow ($\sim 100 \text{ ml min}^{-1}$) 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 $0.45\mu\text{m}$ (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- μ m 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 μ m) of the other species used in selection assays, and below the threshold size (ca. 6 μ m; 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. To determine differences in fluorescence intensity among different microalgal species (null: There is no significant difference in fluorescence intensity of different lectins within algal species), a mixed model analysis of variance (ANOVAR) was run. A discriminant analysis (DA) was used on the characterized surface properties (surface charge, angle, lectin affinity) of the microalgae to determine if algal species had distinct characteristics, and if these could be used to distinguish among species. 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 run on the selection results with the absolute delta of characterized surface properties (surface charge, 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 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 variables, with surface charge, angle, and intensity of lectin binding (Con A, WGA, PEA) as the independent predictors. The predictors were standardized (converted to zero mean, unit variance) prior to use in the model. The dependent predictor model residuals were weighted by the inverse standard deviation of the mean electivity-index data. A

collinearity analysis was performed on the predictors prior to running the model (Belsley et al. 2013). This analysis indicated that PEA and WGA were highly collinear and inclusion of both in the model could be problematic. Therefore, WGA was not used in the regressions models; excluding PEA instead yielded nearly identical results to those reported below.

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 sub-set of data (endpoints). For these analyses the actual surface-property values were used as predictors to determine which surface characteristics (e.g. charge, angle, lectin binding 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 running 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 run using Systat 13 with a significance level of $\alpha = 0.05$.

3. Results

3.1 Microalgal physical surface properties

A range of surface characteristics was found for the individual algal species tested (Table 1). 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

(± 2.1 SD) for *C. carterae*. A scatterplot matrix (SPLOM) was generated for the biological replicates of each algal species. Using a confidence ellipse based upon the sample averages for each of the biological replicates, some species fall into groups within a 95% confidence interval (e.g., *C. carterae*, *P. marinus*). A discriminant analysis (DA) was run using the surface properties as the discriminant factors, with algae species as a classifying value. There was no correlation between surface charge and contact angle for any species characterized. The DA model was found to be statistically significant (Wilks' lambda $p < 0.001$). The model explained 96% of the data variability (canonical correlation of 0.966). In total, 78% of the algal species (7 of 9) were classified correctly using the model. Discriminant functions (with 1 being the highest) were 0.162 (angle) and 0.720 (charge), indicating that charge was the more important factor in classifying algae. A DA model using class instead of species to classify groups also was run (data not shown). This model was significant, but separation between groups and canonical correlations were not improved by the inclusion of classification by taxonomic Class, i.e., there was not evidence for consistency in surface charge or wettability within a taxonomic Class.

3.2 Microalgal FITC-conjugated lectin data

The mean fluorescence intensity for microalgae incubated with the lectins was significantly higher than background fluorescence of the microalgae alone in all cases but ConA coupled with *R. salina* (Figure 1; ANOVA, $P < 0.05$). 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. *C. carterae* also 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* also 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.

The level of binding affinity of each of the FITC-conjugated lectins to the nine different microalgal species was highly correlated (Pearson Correlation, $P < 0.001$). Five of the nine microalgal species characterized had similar binding affinities to each of the three lectins used. Most differences in binding affinity among the nine species of microalgae were found with the PEA lectin, and there were fewer significant differences in binding to WGA and ConA (Tukey's HSD $P < 0.05$; Table 4). Therefore, for the discriminant modeling analysis, only data for PEA lectin-binding intensity were used as an independent variable. For the regression analysis, WGA and PEA were strongly collinear, and thus WGA was dropped as a predictor variable.

3.3 Feeding experiments with microalgal pairings

Seven of the 11 feeding assays with mussels, and six of the 10 feeding assays with oysters resulted in significant preferential ingestion of one of the two microalgal species (Bonferroni adjustment $P < 0.05$; 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.

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, 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 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 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:

$$EI = 0.18 - 0.06\Delta angle - 0.10\Delta ConA + 0.13\Delta PEA.$$

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 run, 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 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 angle and PEA affinity between the algae. The regression model took the form:

$$EI = 0.46 + 0.20\Delta angle - 0.33\Delta ConA + 0.33\Delta PEA.$$

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 angle ($R^2 = 0.17$, $P < 0.05$) 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 ($R^2 = 0.38$, $P < 0.01$) and PEA binding affinity ($R^2 = 0.80$, $P < 0.01$), 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, for the first time, 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.

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.

Additionally, most microalgal species characterized in this study had contact angles (a measure of wettability) indicative of 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. In fact, 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 cartaræ*, 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, PEA and WGA 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 cue for 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 discriminant 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 discriminant 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 cues 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 and cues between species of bivalves also 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 the particle-selection cues upon which these bivalves rely the most are different. 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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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, $P < 0.05$).

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 algaal 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.

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 activity for a given lectin among the algal species (Tukey's HSD, $P < 0.05$). ^{ns} indicates there was no significant lectin binding to the algal species and therefore data were not used in the ANOVAR.

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 ($P > 0.05$). Data are presented as means (\pm SD), N= 6-8.

	Microalgal Pairing			
	<i>P. lutheri</i> 4.2µm	<i>Chlamydomonas</i> <i>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)

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, $P < 0.01$). Letters (a, b, and c) indicate significant differences in binding affinity of the different lectins to each of the microalgal species (Tukey's HSD, $P < 0.05$).

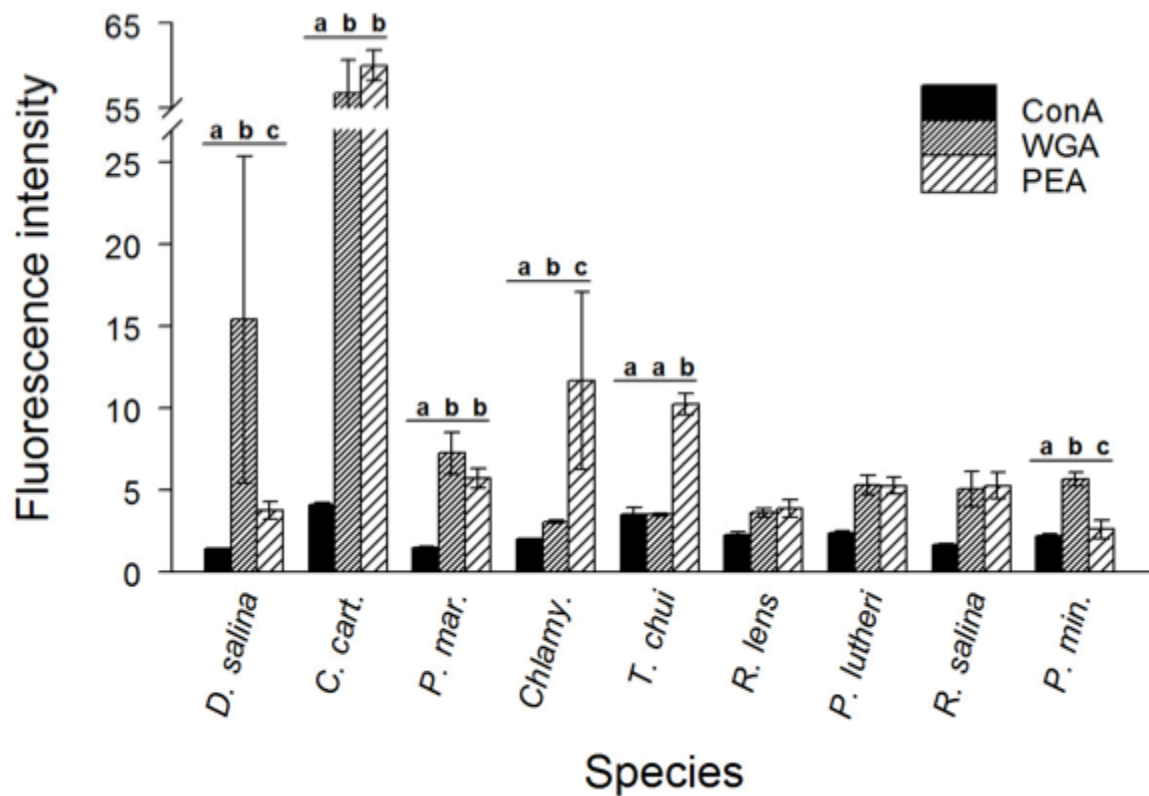


Figure 2. Electivity indices (EI) for the different algal pairings used in the selection experiments. Data are based on the percentage of algal cells in the pseudofeces versus the percentage of the same algal cells in the diet. Presented EI are for the first species in the x-axis paring label, with the 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 $P < 0.05$). EI data are presented as mean \pm SD (n=8-13).

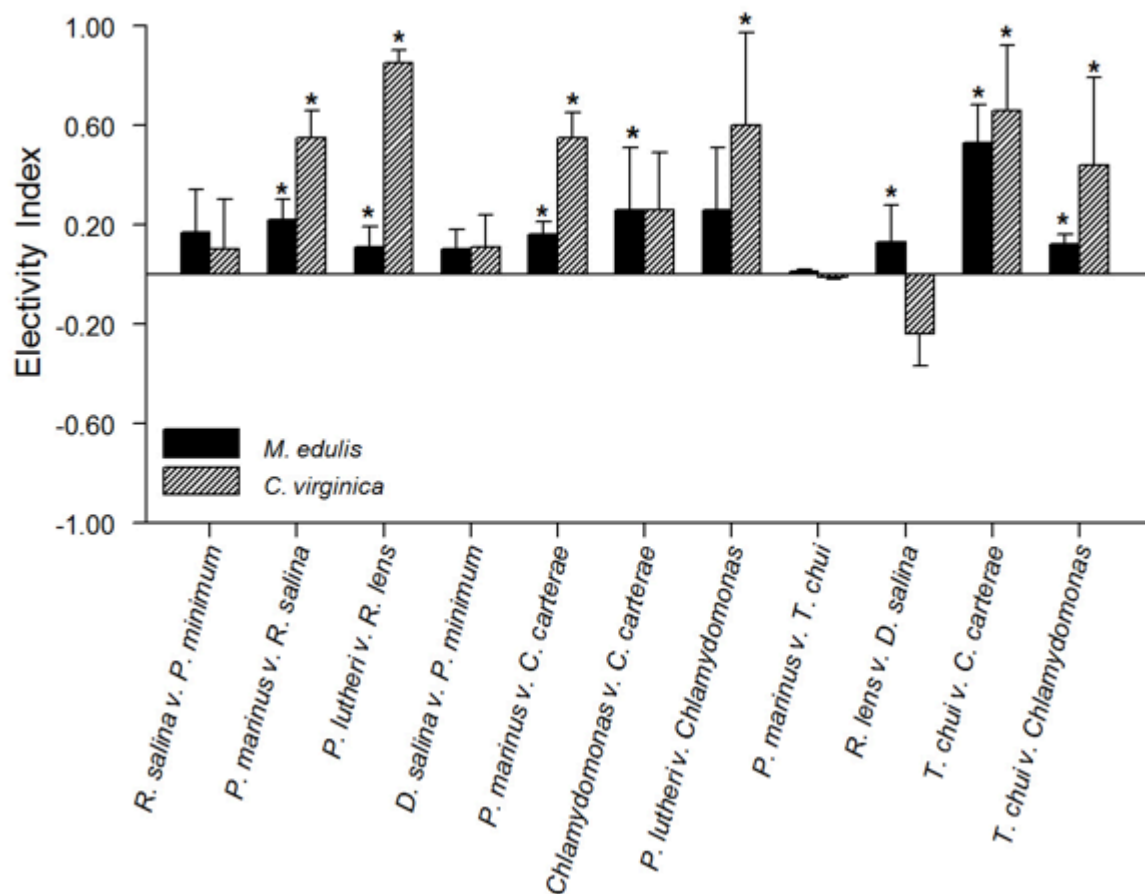


Figure 3. Single-factor regression plots of mussel endpoint data. EI was graphed against the two physical surface properties and PEA binding affinity. A) No significant relationship was found between EI and charge. B) Significant negative relationship was found between contact angle and EI, with higher angles (e.g., less wettable surfaces) resulting in preferential ingestion. C) Significant negative relationship was found between binding affinity of PEA and EI, with higher affinity resulting in preferential ingestion.

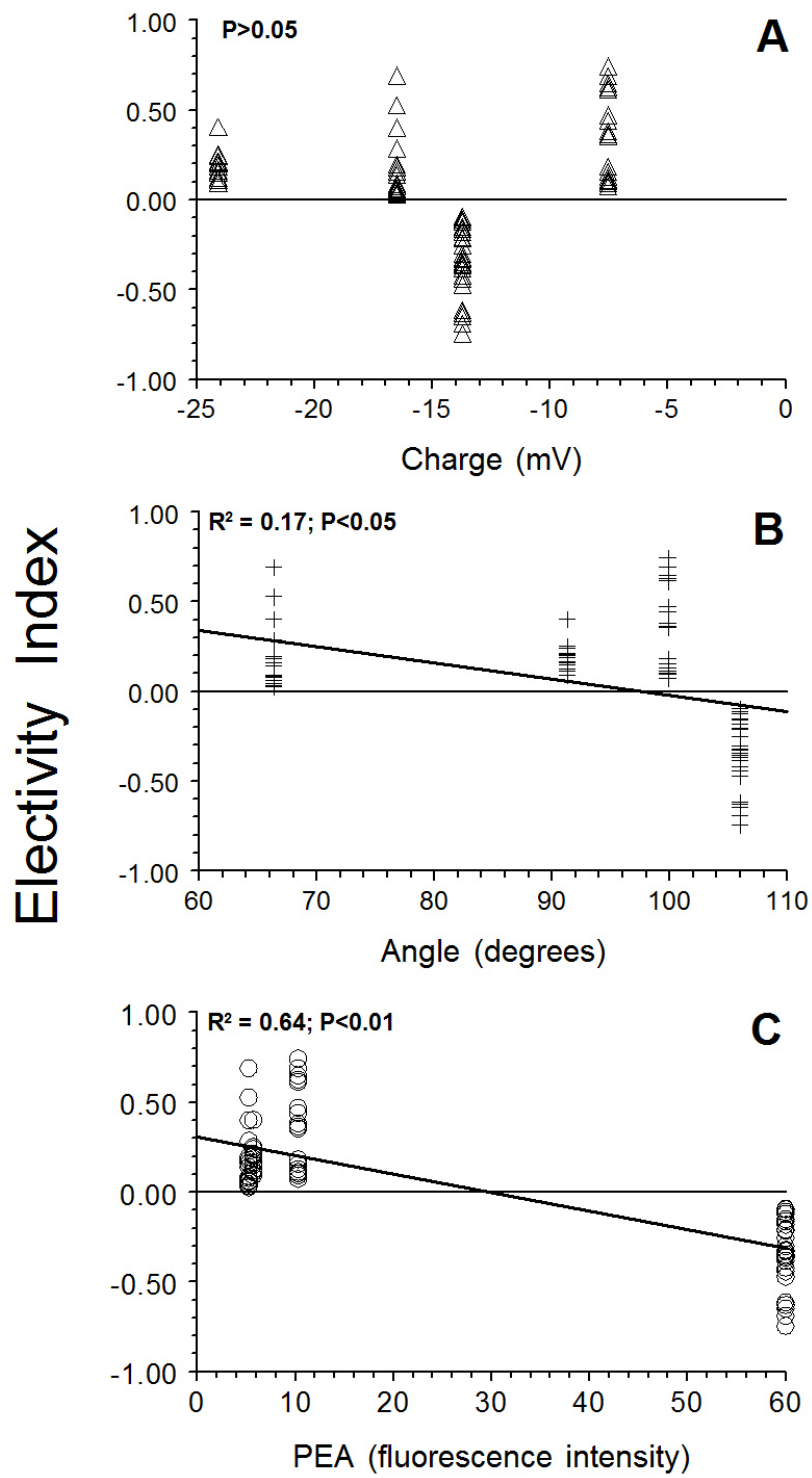
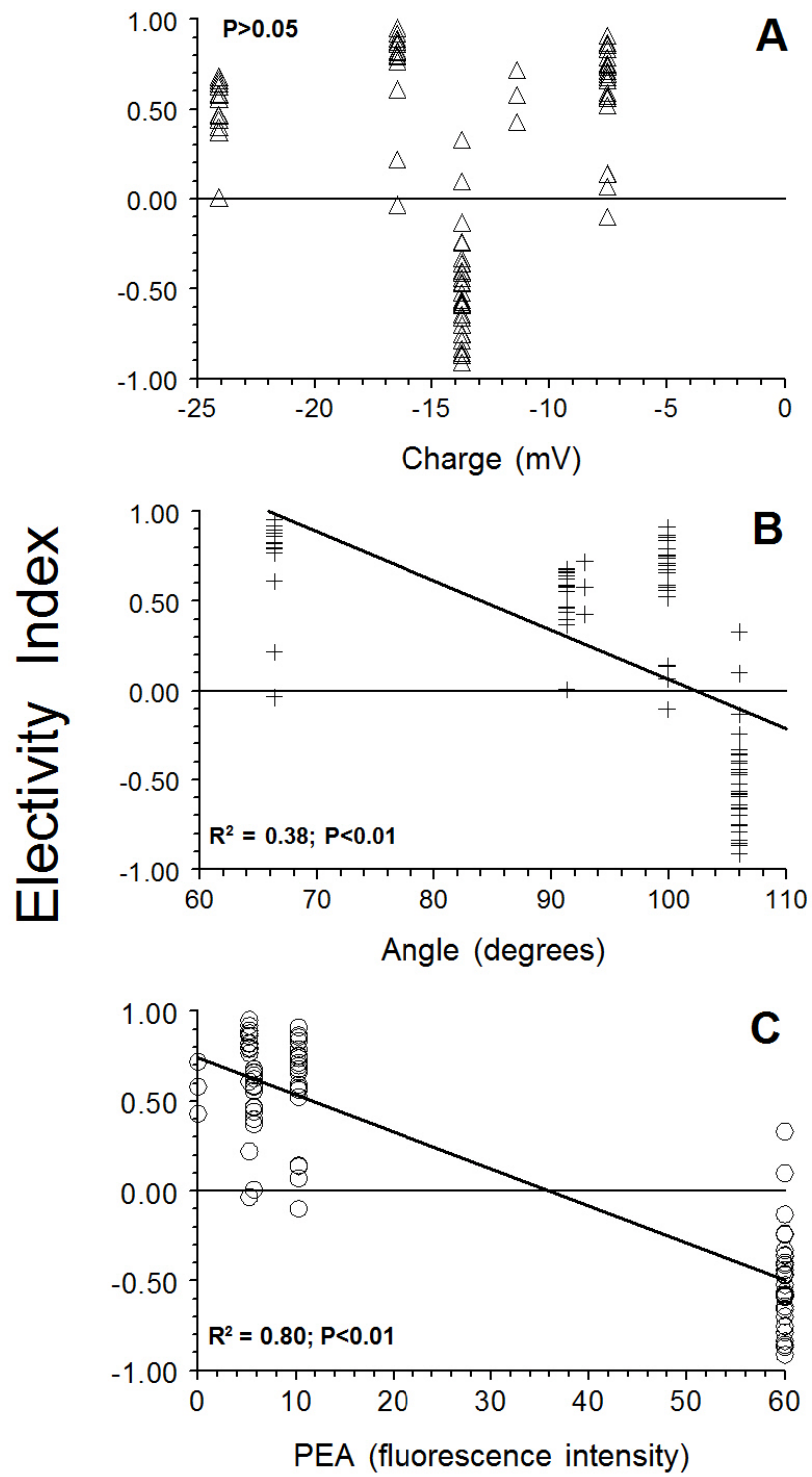


Figure 4. Single-factor regression plots of oyster endpoint data. EI was graphed against the two physical surface properties and PEA binding affinity. A) No significant relationship was found between EI and charge. B) Significant negative relationship was found between contact angle and EI, with higher angles (e.g., less wettable surfaces) resulting in preferential ingestion. C) Significant negative relationship was found between binding affinity of PEA and EI, with higher affinity resulting in preferential ingestion.



Chapter IV

Effects of particle-surface properties on particle capture by two species of suspension-feeding bivalve molluscs.

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Abstract

The capabilities of suspension-feeding bivalve molluscs to selectively ingest particles are well known. Physicochemical properties of particles have been shown to play a role in mediating post-capture selection in these animals. In particular, particle surface charge and wettability, a proxy for hydrophobic-hydrophilic forces, can be used by different bivalve species as selection criteria. How physicochemical factors of particles affect capture, however, has been little studied. To investigate such interactions, a series of adhesion assays and particle capture experiments was designed. First, mucus from the gills of the blue mussel, *Mytilus edulis*, and the bay scallop, *Argopecten irradians*, were isolated separately and fixed onto microscope slides. Slides then were incubated with polystyrene microspheres: uncoated or covalently-bound with individual neoglycoproteins (NGP; N-acetyl-glucosamine, D-mannose), or bovine serum albumin (BSA). Adhesion of each particle type to the mucus was quantified, and multivariate analyses were used to determine if differential adhesion was taking place. Secondly, untreated microspheres and spheres with covalently-bound NGP of different sizes (2, 3, 4.5, 6, 10- μ m) were delivered directly to the inhalant aperture of bivalves. The exhalent water was sampled, and capture efficiencies were calculated. Results demonstrate that the presence of epiparticulate NGP can change

the surface characteristics of the microspheres significantly, resulting in differences in adhesion and capture efficiency of particles in the smallest size classes.

1. Introduction

Suspension-feeding bivalve molluscs are exposed to large amounts of particulate matter which includes both nutritious and non-nutritious particles (Newell RC 1965, Owen 1966). As a way to process efficiently the bulk of material encountered, bivalves have evolved capabilities for selective feeding that allow rejection of some of the captured material. The presence of a highly-selective, pre-ingestive sorting mechanism may serve as a way to optimize energy gain (Taghon et al. 1978; Kiørboe and Møhlenberg 1981; Newell CR et al. 1989; Iglesias et al. 1992; Grizzle et al. 2001; Ward and Shumway 2004) by enabling bivalves to ingest particles with a higher nutritive quality preferentially.

The process of pre-ingestive sorting by bivalves can be described as either passive or active (see reviews by Jørgensen 1996; Ward and Shumway 2004). Passive selection is dependent upon the physicochemical interactions between the particles and the feeding organs, with factors such as organic content, wettability, and electrostatic charge serving as bases for sorting (Bayne et al. 1977, Newell RC et al. 1989, Ward and Targett 1989, Beninger 1991). Rosa et al. (2013) demonstrated that the eastern oyster, *Crassostrea virginica*, and the blue mussel, *Mytilus edulis*, could discriminate between particles of the same size based upon the surface charge and wettability of particles. In other studies, workers isolated mucus from the ctenidia (= gills) and labial palps of oysters, *C. virginica* (Pales-Espinosa et al. 2009), and mussels, *M. edulis*

(Pales-Espinosa et al. 2010), and measured specific lectin activity. Results of these studies confirmed that a carbohydrate-lectin interaction also is involved in mediating particle sorting in both *C. virginica* and *M. edulis*. Together these findings suggest that specific and non-specific physicochemical interactions play roles in mediating passive selection in bivalves. How these surface properties affect particle capture have been little studied.

Mucus produced by suspension-feeding bivalves is involved in many aspects of the feeding process (e.g., particle capture, selection, and transport) (Jørgensen 1990, Ward 1996), and composition of mucus covering the pallial organs of bivalves has been demonstrated to vary among species (Beninger 1991, Beninger et al. 1993). The sea scallop, *Placopecten magellanicus*, for example, possesses goblet cells on the crests of the ordinary filaments of the gill, which produce acidic mucopolysaccharides. These ordinary filaments are used mainly in rejection. The principal filaments of this bivalve, mainly used in particle acceptance, are covered with basic mucopolysaccharides (Beninger et al. 1993). The gill filaments of the mussel *M. edulis* produce mixed mucopolysaccharides, which the authors postulate is consistent with the non-separation of particle selection and cleaning functions of the mussel gill (Beninger et al. 1993). Differences in mucus composition between species may be related to whether or not the gill is involved in particle selection and could provide a possible mechanism for some of the inter-specific differences observed in selection.

Suspension-feeding bivalve molluscs employ an aerosol filtration system to trap particles from the seston using their gill, with particles $\geq 4\mu\text{m}$ historically reported to be captured with close to 100% efficiency (Riisgård 1988). Differential particle capture, if

present, would be a form of pre-ingestive selection. In particular, size-independent differential capture, with some particles of the same size being captured with higher rates than others, has been demonstrated in some marine suspension-feeders. *In situ* evidence of pre-capture, qualitative selection in bivalves (Yahel et al. 2009) indicates that not all particles that encounter the capture structures are retained. Further, the capture efficiency of *M. edulis* for the bacterium *Salmonella typhimurium* increased when the surface charge of these bacteria ($\sim 1\mu\text{m}$) was lowered experimentally (Hernroth et al. 2000).

The present study was designed to examine how both specific and non-specific physicochemical surface properties of particles may affect interactions with mucus and thus potentially capture, and if some properties are more likely to increase particle adhesion and capture than others. In particular, effects of various physicochemical properties on particle handling were examined experimentally in two species of bivalve molluscs: the blue mussel, *Mytilus edulis*, and the bay scallop, *Argopecten irradians*. These animals have filibranchiate gills, but with different gill architectures and ciliary microstructure. The mussel has well-developed laterofrontal cirri, capable of capturing $\geq 3.2\ \mu\text{m}$ particles with ca. 90% efficiency (Tammes and Dral 1955, Vahl 1972). In contrast, *A. irradians* has shorter laterofrontal cilia, and captures $3.3\ \mu\text{m}$ particles with an efficiency of only 50% (Palmer and Williams 1980). This study tested the null hypothesis that different physicochemical properties of particles have no effect upon particle adhesion and capture by these two species of bivalves.

2. Materials And Methods

2.1 Experimental Animals

Blue mussels (*M. edulis*) were collected from local populations in Long Island Sound, Groton CT. Bay scallops (*A. irradians*) were obtained from the NOAA/NMFS laboratory in Milford, CT. All animals were maintained at the University of Connecticut Avery Point Campus, Groton CT seawater facility for at least 2 weeks prior to the study, in accordance with local regulations for the transport and maintenance of aquaculture organisms. This ensured that animals were exposed to the same ambient conditions (e.g. temperature, salinity, dissolved oxygen, and pH) prior to all experiments.

2.2 Neoglycoproteins Coupled to Microspheres

To examine the influence of specific physicochemical properties upon particle capture, neoglycoproteins (NGP; sugars linked to bovine serum albumin (BSA)), were covalently bound to fluorescent, carboxylated polystyrene microspheres (Polysciences Inc.). Two types of NGP were used, one containing the sugar D-mannose and the other containing N-acetyl-glucosamine. These particular sugars were chosen because lectins in the pallial organ mucus of *M. edulis* and *C. virginica* have been shown to bind selectively to them and because they have been implicated in particle sorting (Pales Espinosa et al 2010). Further, N-acetyl-glucosamine and D-mannose have been found on cell surfaces of numerous algal species that commonly are used as food in bivalve culture (Pales Espinosa et al. 2016, Rosa et al. 2016 in review). Both types of NGP were bound covalently using a commercially available Polylink coupling kit (Polysciences, Inc.) at a concentration of ~500µg/mL. The protein BSA also was bound to microspheres as a control. For the adhesion assays, NGP and BSA were coupled individually to 10-µm carboxylate polystyrene microspheres. For the capture efficiency experiments, five sizes of microspheres were used: 2, 3, 4.5, 6, and 10 µm. A standard

Branford assay (Pierce™ Coomassie Protein Assay Kit) was used to confirm protein binding to the microspheres.

2.3 Characterization of Surface Properties

The physical surface properties of the uncoated and covalently-bound microspheres were characterized using methods published previously (Rosa et al. 2013). Briefly, in a standard solution such as that used in this analysis (i.e., pH 8.0, 15 ppt salinity) zeta potential is an indication of surface charge and is designated as such from hereon. Zeta potential was determined for each microsphere using a Zetasizer Nano ZS© (Malvern Instruments Inc., UK). Wettability of each type of microsphere was determined by measuring water contact angle (Hiemenz 1986). Pads of microspheres were produced by vacuum-filtering suspended particles onto 3-µm polycarbonate filters, and drying overnight at 70 °C (Rosa et al. 2013). A drop of MilliQ water (4 µl) was placed on the dried pad and photographed using a digital camera attached to a side-mounted dissecting microscope (Mohammadi et al. 2003). Image J® was used to measure the contact angle formed between the water droplet and the microsphere surface. A high contact angle (>90°) indicates a non-wettable surface (=hydrophobic). Conversely a low contact angle (<90°) indicates a wettable (=hydrophilic) surface (Volpe 2006).

2.4 Adhesion Assays

Mucus from the gills of mussels and scallops was isolated using methods adapted from Amaro et al. (1995) and optimized for bivalves (Rosa et al. unpublished). First, animals were cleaned of biofouling material, and a scalpel was used to sever the

adductor muscle(s). One valve then was removed carefully, and the exposed tissues were flushed with 60 ml of filtered seawater (0.22 μm ; FSW) to rinse off hemolymph and other debris. Animals were placed in FSW, and the gill was allowed to expand and resume mucociliary processes. Sterile, 50- μl inoculation loops then were used to remove 200 μl of mucus from the frontal surfaces of the gill and to transfer it to individual glass microscope slides, creating a thin and even surface on each slide. The collected mucus was fixed to the slide using absolute methanol and dried overnight at room temperature. Slides with the coating of mucus then were immersed in a solution containing the previously-characterized polystyrene particles and incubated on a shaker table at room temperature for 1 h. After incubation, slides were washed in distilled water (DI) to remove non-adhering particles and fixed with absolute methanol. A dissecting microscope with attached digital camera was used to record 60 haphazardly-selected images of the slide, and image J was used to count the total number of adhered microspheres. The numbers of particles adhering to the mucus-covered slides were compared to control slides that had been treated in the same manner, but did not contain mucus.

2.5 Capture Efficiency Assays

A suspension of each of the aforementioned microspheres was prepared in FSW at a density of 5×10^4 particles/mL (1×10^4 particles/mL for each size class). Suspensions of the combined size classes then were treated separately with either NGP or BSA as described above, or with FSW as an additional control, for a total of four treatments. Animals were cleaned of biofouling materials, placed in individual chambers with FSW (0.45 μm) and allowed to acclimate for up to 1hr. Microspheres were

delivered slowly to the inhalant aperture by means of a 1-mL glass pipette. Simultaneously, the exhalent water was collected through a sampling tube mounted on a micromanipulator and attached to a peristaltic pump (10 mL/min; Rosa et al. 2014). Number of microspheres of each size class in the samples was analyzed by means of a flow cytometer (Accuri C6) and an electronic particle counter (Multiziser IIe).

2.6 Data processing and statistical analysis

Differences in surface properties (charge, wettability) of the coated microspheres *versus* the control ('uncoated') microspheres were tested using an analysis of variance procedure (ANOVA). The number of microspheres adhering to the mucus-coated slides was compared to the number of particles adhering to the control slides using a Student's T-test (H_0 = means are equal). If there were significant differences in the number of microspheres adhering to the control slides *versus* the mucus-covered slides, then the null hypothesis (H_0 = physicochemical surface properties do not result in differential particle adhesion to bivalve gill mucus) was rejected. The total number of particles delivered to each animal ('in') and the total number of particles in the exhalent water ('out') was used to calculate capture efficiency (CE) for each microsphere size class (Rosa et al. 2015). Efficiency was calculated using the formula $CE = 1 - PC_{out}/PC_{in}$, where PC_{out} is the particle concentration in the exhalent water, and PC_{in} is the particle concentration delivered to each animal (Cranford and Grant 1990). Differences in the types and sizes of particles that were captured by each species of bivalve were analyzed using a mixed model ANOVA for repeated measures, which allowed for the determination of both size and treatment effects upon particle capture

efficiency. Statistical analyses were performed using Systat 13, and for all tests an alpha level of 0.05 was used.

3. Results

3.1 Microsphere Physicochemical Properties

The NGPs and BSA were successfully bound to the polystyrene microspheres at a concentration of ~500 µg/mL. Addition of coatings significantly altered some of the physical surface properties of the microspheres (Table 1). Binding of N-acetyl-glucosamine resulted in a significant decrease in surface charge (ANOVA, $P < 0.05$). Binding of the BSA and D-mannose resulted in a significant increase in contact angle (ANOVA, $P < 0.05$) indicating a more hydrophobic surface.

3.2 Adhesion Assays

Significantly fewer coated microspheres adhered to the mucus collected from *Mytilus edulis* gills compared to the uncoated microspheres (T-test, $P < 0.05$; Table 2). Microspheres coated with BSA demonstrated the largest decrease in adhesion. Significantly fewer microspheres coated with N-acetyl-glucosamine and BSA adhered to the mucus collected from *A. irradians* gills (T-test $P < 0.05$; Table 2). There was no significant decrease in adhesion of the D-mannose-bound microspheres to the mucus-covered slides.

3.3 Microsphere Capture Efficiencies

For mussels, data analyses revealed a significant effect of treatment upon capture efficiency of microspheres (ANOVAR, $P < 0.05$, Figure 1). CE for the D-

mannose-bound particles was significantly lower than the other microspheres (Tukey's HSD, $P < 0.05$). There was a significant effect of size on CE (ANOVAR, $P < 0.01$). As expected, the 2- μm and 3- μm microspheres were captured with similar efficiency, and at significantly lower CE than the 4.5 μm , 6 μm and 10 μm microspheres (Tukey's HSD $P < 0.05$). There was no significant interaction effect of treatment and size on capture efficiencies (ANOVAR, $P > 0.05$).

For the scallops, data analyses revealed no significant effect of treatment on CE of particles (ANOVAR, $P > 0.05$, Figure 2). As expected, there was a significant effect of size upon CE (ANOVAR, $P < 0.01$). The 2- μm , 3- μm , and 4.5- μm sized particles were captured at significantly lower efficiencies than the 6- μm and 10- μm particles, regardless of treatment (Tukey's HSD, $P < 0.01$). As with the mussels, there were no significant interaction effects of treatment and size upon capture efficiencies (ANOVAR, $P > 0.05$). Data analysis also revealed a significant difference in capture efficiency between the two bivalve species (ANOVAR, $P < 0.01$), with mussels capturing particles at higher efficiencies than scallops for each of the coatings. Mussels captured all but the 10- μm particles at significantly higher efficiencies than did scallops (Tukey's HSD, $P < 0.05$).

4. Discussion

This study experimentally assessed how specific and non-specific chemical interactions affect particle capture efficiency (CE) of mussels and scallops by using a series of adhesion and particle-capture assays. Results demonstrate that the addition of different coatings to microspheres, which decreased surface charge and increased hydrophobicity, significantly decreased adhesion of these particles to mucus extracted

from the gills of mussels. In scallops, only two of the treatments (N-acetyl-glucosamine and BSA) decreased particle adhesion to mucus extracted from the gill. Treatment of microspheres with D-mannose resulted in decreased particle CE by mussels. Findings from this study suggest that physicochemical surface characteristics can affect capture efficiency of bivalve species at the smaller particle size threshold.

Suspension-feeding molluscs rely upon mucus covering the pallial organs to aid in particle capture, transport, and processing. The type of mucus produced and chemical constituents may mediate physicochemical interactions with particles and act in concert to produce a biologically meaningful particle capture and selection response. Generally, polysaccharides of molluscan mucins contain acidic monosaccharides (Denny 1983), resulting in negatively charged subunits at physiological pH (6.8-7.3). Most binding to mucus occurs as ionic interactions (Di Girolamo et al. 1977, Decho 1990). Accordingly, particles with different surface characteristics (i.e., highly negative or positive charges) would be more likely to adhere. Other studies have shown that live bacterial cells adhere preferentially to mucus compared to other surfaces (Krovacek 1987), a property that serves to aid transmission of several infectious bacteria such as *Vibrio vulnificus* (Amaro et al. 1995) and *V. cholerae* (Boutonnier et al. 2003) in fish. Addition of different sugars and a protein to microspheres used in this study decreased particle adhesion to the mucus extracted from the gills of scallops and mussels. The N-acetyl-glucosamine coating significantly decreased the surface charge of polystyrene particles, resulting in lower adhesion of the microsphere to mucus extracted from both mussel and scallop gills. A significant increase in the contact angle (more hydrophobic surface) was found for the microspheres treated with D-mannose and BSA, although

only BSA-treated particles had significantly lower adhesion to the mucus extracted from either the mussel and scallop gills.

Although changes in the surface properties of NGP- and BSA-treated microspheres had effects upon adhesion to mucus, the changes did not have a clear effect upon particle capture. Only one of the coatings, D-mannose, resulted in a decrease in CE by mussels for the size classes tested. No effect of particle treatment upon CE was found for the scallops. Other studies have demonstrated that charged particles are more readily captured than particles with a neutral charge by both the brittle star *Ophiopholis aculeate* (LaBarbera 1978) and larvae of the northern quahog (= hard clam) *Mercenaria mercenaria* (Sollow and Gallagher 1990). Similarly, studies using particles or bacterial species with modified surface characteristics (e.g. Silverman 1995, Hernroth 2000) have demonstrated a higher CE of these smaller particles than reported for algae and other seston particles. No such effect of charge upon CE was found in this study. The particle that was captured at the lowest efficiency (D-mannose-coupled microspheres) by mussels was the most hydrophobic particle. Coupling with BSA also resulted in a more hydrophobic particle surface but did not have a similar effect upon CE. This result suggests that an interactive effect of the change in surface hydrophobicity (physical) plus the type of sugar (chemical) could be responsible for the observed decreases in particle capture efficiency.

In other marine invertebrates, particle capture has been shown to be mediated by surface hydrophobicity. For example, hydrophilic particles are retained at a higher proportion than hydrophobic particles by the crustacean *Daphnia magna* (Gerritsen and Porter 1982). Characterization of the surface properties of bacterial species found that

they tend to be more hydrophilic (Grasland et al. 2003) than several algae species (Ozkan and Bergeluou 2013, Rosa et al. in review), which may account for the reported higher capture efficiencies of bacteria. Conova (1999) examined the role of hydrophobicity in particle capture by the suspension-feeding mole crab *Emerita talpodia*. She reported that, as smaller sized particles (0.5 to 10 μm) were made more hydrophilic, adhesion to the capture organ generally decreased. Interestingly, for particles 15 to 25 μm in size, particle hydrophobicity did not affect capture rates. Thus, hydrophobicity appears to play a role in particle capture only in the smaller size ranges.

Our results indicate that, for certain, uniform particle types in the 2- to 4- μm size range, CE rates may be higher than previously reported. These differences could result from methods used previously to measure CE, which relied upon indirect techniques (i.e. sampling water entering and exiting a chamber) and often used monoalgal cultures. Results from experiments of this study as well as others using a direct delivery technique (Yahel et al. 2009, Strøhmeier et al. 2012) have reported that mean CE of 2 to 4 μm particles can range from 40-80% for mussels, and 20-60% for scallops. In contrast, previous experiments using indirect methods have reported CE for similar sized particles to be between 20-60% for mussels, and 0-20% for scallops (e.g. Møhlenberg and Riisgård 1978, Riisgård 1988, Palmer and Williams 1990). The use of monoalgal cultures (e.g. Vahl 1972, Møhlenberg and Riisgård 1978) could also account for the reported lower CE of some smaller particles. Several studies using bacterial cultures and natural seston assemblages have reported higher CE of smaller particles (Hernroth et al. 2002, Rosa et al. 2014). If CE of smaller natural particles (e.g.,

picoplankton) is, in fact, higher than has been reported for suspension-feeding bivalves, the contribution of these particles to molluscan energetics may warrant re-examination.

Although it has been demonstrated that different sugars can act as recognition molecules for lectins ('The sugar code', see Gabius 2000), in this study D-mannose and N-acetyl-glucosamine did not have clear effects upon particle adhesion or capture. Instead, coating with NGP altered the surface properties of the particles, resulting in differences in how the microspheres adhered to mucus and were captured by the two species of bivalves. An increase in hydrophobicity (higher contact angle) was associated with a significant decrease in adhesion of the microspheres to the mucus from mussels (D-mannose coating) and scallop gills (BSA coatings). These results suggest that sugar-lectin chemical interactions may have an effect upon post-capture selection, but for the initial particle capture step, surface charge and hydrophobicity may play larger roles. This finding does not support previous speculation by Pales Espinosa et al. (2010) that induction of mucosal lectin production of starved *M. edulis* could be a physiological response to increased particle adhesion and capture. The potential roles of other sugars in particle capture, however, should be further studied to determine the effect of chemical properties, alone or interactive, upon the particle capture process.

Finally, the observed differences in adhesion and capture efficiency of the treated microspheres by the two bivalves ('species effect') demonstrate the importance of considering each species, rather than generalizing across taxa. These different responses could be a consequence of differences in the gill architecture, laterofrontal cilia/cirri, and specific capture mechanisms (Ward et al. 1991, Beninger et al. 1992). Further, the mucus constituents have been demonstrated to be different in these two

species (Benninger et al. 1993), which could also affect adhesion. Although some insight has been acquired recently regarding the mechanisms that allow particle selection, there still is much to be explored. Findings outlined in this study suggest that there are baseline, passive selection processes utilized by suspension-feeding bivalves that may result in some particles being more likely than others to be captured. Such differential adhesion and capture processes may be based partly upon physicochemical properties, including potential interactive effects between surface charge, hydrophobicity, and chemical constituents. If differential capture of particles is confirmed, it will constitute the first discriminatory mechanism in the suspension-feeding process. Accordingly, the factors that mediate capture efficiency and preferential capture at smaller size ranges is a fertile area for future research.

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Figure 1. Capture efficiencies (CE) of microspheres delivered to blue mussels, *Mytilus edulis*. Microspheres were either uncoated (control) or treated with BSA, or one of two NGP (with D-mannose or N-acetyl-glucosamine). There was a significant treatment effect on CE, with the D-Mannose coupled microspheres being captured at the lowest efficiency (Tukey's HSD $P < 0.05$). Data presented as means \pm SE, N= 8-13.

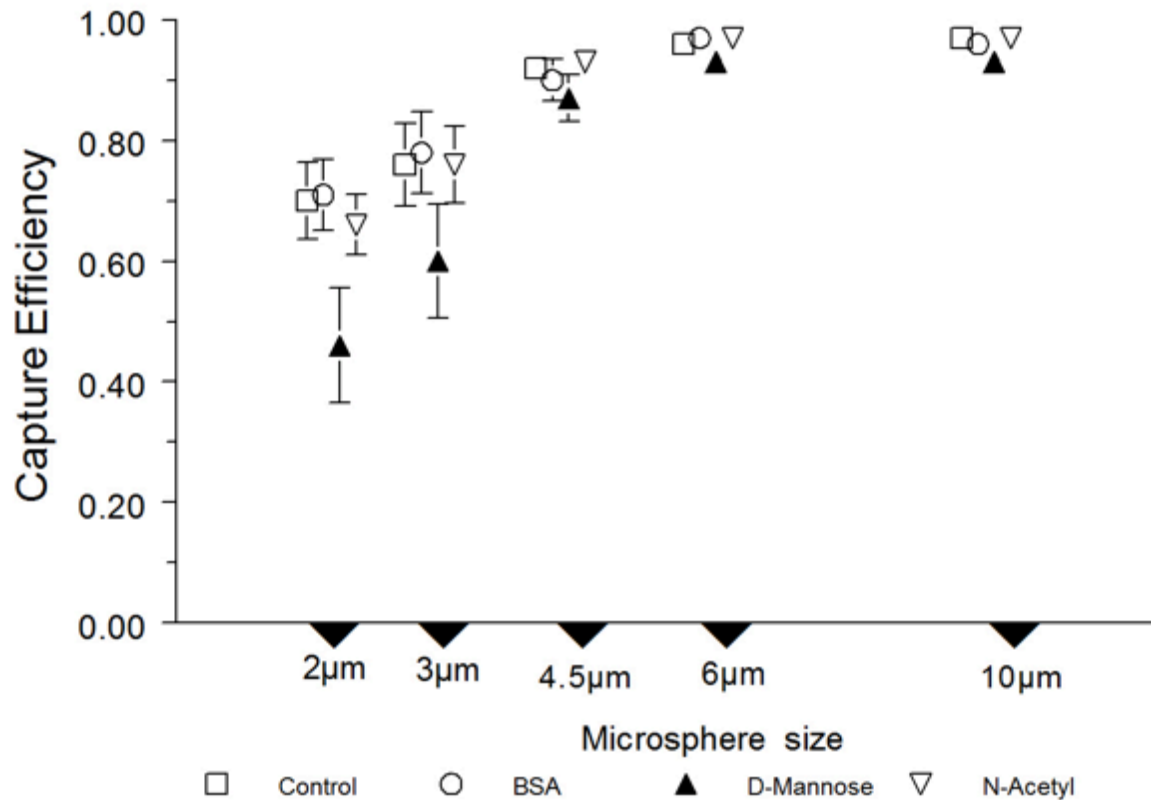


Figure 2. Capture efficiencies (CE) of microspheres delivered to scallops, *Argopecten irradians*. Microspheres were either uncoated (control) or treated with BSA, or one of two NGP (with D-mannose or N-acetyl-glucosamine). There was no significant effect of microsphere treatment on CE (ANOVAR $P > 0.05$). CE rates for the scallops were significantly lower than those calculated for the mussels (cf. Fig. 1) indicating a significant species effect on particle capture efficiency (ANOVAR, $P < 0.05$). Data presented as means \pm SE, N=6-10.

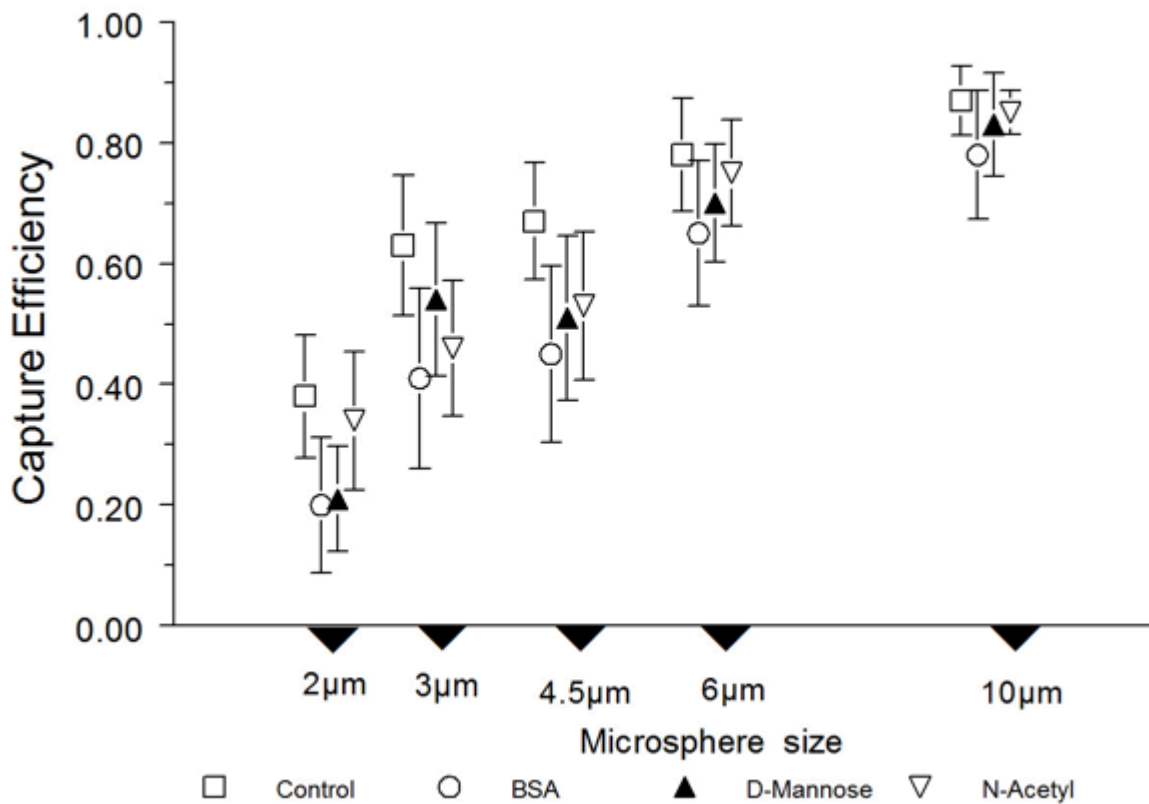


Table 1. Surface properties of the uncoated (control), BSA treated and NGP treated microspheres. Addition of the BSA and D-mannose had no significant effect on the surface charges of microspheres compared to the control, whereas N-acetyl-glucosamine significantly decreased the charge. Addition of BSA and D-mannose made the particles more hydrophobic (higher contact angle), whereas addition of the N-acetyl-glucosamine had no effect on the hydrophobicity of the particles compared to the control. Data presented as means (\pm SD), n=3-6. *denotes significantly different surface properties of treated microspheres from the uncoated microspheres.

Particle (treatment)	type	Surface charge (mV)	P-value	Contact angle (°)	P-value
Uncoated particle		-10.01 (1.77)	n/a	107.03 (0.96)	n/a
Bovine Albumin	Serum	-8.39 (0.63)	0.198	117.43 (4.75)*	0.020
N-acetyl- glucosamine		-5.60 (0.95)*	0.012	109.77 (2.67)	0.170
D-mannose		-7.79 (1.87)	0.356	118.03 (2.40)*	0.001

Table 2. Number of particles adhering to slides not coated with mucus (control slides) or coated with mucus extracted from the gills of mussels and scallops. Data presented as means (\pm SD), n=4-9. *denotes significantly different particle adhesion to mucus slides compared to control slides (T-test comparing means, $P < 0.05$).

Particle treatment	Control (no mucus)	Mucus from <i>edulis</i>	<i>M.</i> Mucus from <i>A. irradians</i>
Uncoated particle	337 (192)	270 (121)	199 (41)
Bovine Serum	361 (72)		
Albumin		178 (61)*	207 (70)*
N-acetyl- glucosamine	350 (64)	172 (23)*	216 (87)*
D-mannose	363(36)	146 (62)*	237 (148)

Chapter V

Effects of microalgal metabolites on ciliary activity of suspension-feeding bivalve molluscs

Abstract

Suspension-feeding bivalve molluscs have evolved a highly effective mechanism for particle discrimination to process the material to which they are exposed efficiently. The mechanisms controlling this pre-ingestive sorting process can be described as either passive or active. Recently, evidence of a passive selection mechanism, dependent upon the physicochemical (e.g., surface charge, wettability, lectins) interactions between particles and the feeding organs, has been demonstrated in several species of suspension feeding bivalves. In contrast, active selection, if it exists, would be dependent upon a physiological response by the cilia or feeding organs to food stimuli (e.g., chemoreception). To date, there has been no evidence that such a mechanism underlays particle selection in bivalves. The present study was designed to assess experimentally response by the frontal cilia of gill filaments to dissolved extracellular and intracellular substances from phytoplankton. Frontal ciliary tracts were chosen for investigation because they are responsible for directing material to marginal tracts for either acceptance or rejection and are therefore a likely locus for an active response to food metabolites. Assays on excised gills and gills *in vivo* were carried out to examine particle transport by the frontal cilia of the eastern oyster *Crassostrea virginica* and the blood ark *Anadara ovalis*. Results of the metabolite experiments demonstrated that the addition of exudates or extracts of *Tetraselmis chui* cells had no significant effect on the transport of particles captured on the frontal surface of the gill (chemoreception). There were no differences in the percentage of particles being transported dorsally or ventrally (i.e., likely ingested vs likely rejected, respectively) between control and experimental treatments. Results of follow-up experiments using neoglycoproteins covalently bound to microspheres demonstrated that particles coated

with D-mannose were rejected (transported to the ventral margin), whereas particles coated with N-acetyl-glucosamine were generally ingested. These sugars have been found on cell surfaces of numerous algal species that are commonly used as food in bivalve culture, and lectins in the pallial organ mucus of several bivalve species have been shown to selectively bind to them, indicating they may have a role in a passive selection response. Although contact chemoreception cannot be completely ruled out, these findings further demonstrate that physicochemical properties of particles, and not an active physiological response (i.e., chemoreception of dissolved metabolites), mediate particle selection in bivalves. The interactions of the particle surface with the mucus constituents on the feeding organs best determine particle fate.

1. Introduction

Suspension-feeding bivalve molluscs are some of the most important near-shore organisms, often dominating the macrobenthos and contributing significantly to the benthic food web structure (Newell 1988, Asmus and Asmus 1991, Dame 1993, Baker et al. 1998). These contributions to benthic-pelagic coupling and nutrient cycling are affected by their ability to ingest particles selectively, rejecting some matter and depositing in the benthos (Bayne et al. 1977, Newell 1988, Newell et al. 1989, Baker et al. 1998, Ward and Shumway 2004). Particle selection is a mechanism by which bivalves can optimize energy gain (Taghon et al. 1978, Kiørboe and Møhlenberg 1981, Newell et al. 1989, Iglesias et al. 1992) as these organisms are exposed to high quantities of both nutritious and non-nutritious particles (Newell 1965, Owen 1966). The selection process can be described as either passive or active. Passive selection would

be dependent upon the physicochemical interactions between the particles and the feeding organ, with factors such as particle size and surface characteristics (e.g., surface charge, wettability, lectins) serving as bases for sorting. Recently, evidence of such a process has been demonstrated in several species of suspension feeding bivalves (e.g. Beninger and Decottignies 2005, Pales Espinosa et al. 2009, 2010, Rosa et al. 2013). Active selection, on the other hand, would be dependent upon a physiological response by the cilia or feeding organs to feeding stimuli (i.e., chemoreception). To date, there has been no evidence that such a mechanism underlays particle selection in bivalves.

The function of gill cilia (lateral, latero-frontal, and frontal) in particle capture and transport in marine bivalves has been thoroughly described (Atkins 1937a, b, c, Tammes and Dral 1955, Dral 1967, Jørgensen 1982). Both their role in water pumping and particle processing, and the nervous system control of latero-frontal and lateral cilia, has also been extensively studied (e.g. Jørgensen 1975, Catapane 1983, Carroll and Catapane 2007, Frank et al. 2015). Much less is known about the regulation of frontal ciliary activity (Beninger et al. 1997, Silverman 1999). The frontal cilia of bivalves have been demonstrated to transport mucus and directly intercept and capture particles (Ward 1996). Endoscopic observations of particle movement demonstrate that, in most cases, the mucus layer is very thin and particles are within micrometers of the frontal surfaces ($< 5 \mu\text{m}$), so changes in beat angle or frequency would be translated to changes in particle movement (Ward et al. 1993, 1994). The gill filaments of some bivalves possess tracts of frontal cilia that beat in opposing directions (e.g., oysters, arks). Previous studies have shown that these tracts transport material both ventrally

and dorsally, and the role of such bi-directional transport in particle selection has long been a source of speculation (Allen 1921, Atkins 1937b, Beninger et al. 1992, Ward and Shumway, 2004). In fact, in several bivalve species, bi-directional transport of particles and selection on the gill has been demonstrated. Endoscopic examinations have demonstrated particle selection on the gill of the eastern oyster *Crassostrea virginica* (Ward et al. 1997, 1998), the European oyster *C. gigas* (Ward et al. 1998), and the king scallop *Pecten maximus* (Beninger et al. 2004). Given the ability of some bivalve species to select particles on the gill, and the relative ease of visualizing particle capture and transport on this organ, selection on the gill was the focus of this study. Active particle selection, if it occurs, would likely be elicited by the frontal cilia due to their direct involvement in the mucociliary transport of particles (Ward 1996, Beninger et al. 1997). Therefore, an immediate response by the frontal cilia to a cue that serves as a qualifier for selection could mediate the movement of particles to either acceptance and/or rejection tracts.

Chemical signals (= cues) are generally transduced, or converted into an action potential, by chemoreceptors. The presence of chemoreceptors on the pallial organs of bivalves would suggest that an active sorting mechanism could underlay particle selection. Hodgson and Fielden (1984) described three types of ciliary receptors in the siphons and mantle edge of two species of bivalves which they suggested function as chemoreceptors. Although attempts to identify chemosensory cells on the feeding organs of bivalves have been made, no conclusive evidence for such structures has been published. Experimental work by Dwivedy (1973) attempted to describe sensory cells on the labial palps of the eastern oyster *C. virginica*, though the methodology and

results of this study were later criticized (Beninger et al. 1990, Beninger 1991). To date, no such chemoreceptors have been reported for cilia on the gill of bivalve species. Nonetheless, the response of the lateral-frontal cilia of the gill to both environmental (e.g., low oxygen, high salinity; Davenport and Fletcher 1978) and chemical cues (e.g., dopamine, serotonin; Malanga 1975; Aiello 1970) is well documented. These data support a neurological, hormonal, or a paracrine (local response) control of ciliary activity, and suggest that an immediate response to different particle cues (e.g., epicellular moieties) is possible.

This project focused on the eastern oyster, *Crassostrea virginica*, and the blood ark, *Anadara ovalis* because both have bidirectional transport on the gill. The pseudolamellibranchiate gill of oysters is composed of ordinary and principal filaments with distinct ciliary tracts that allow for both capture and selection on this pallial organ. Particles conveyed to the dorsal margin of the gill are transported anteriorly to the labial palps, where further selection of particles takes place. Particles conveyed to the ventral margin of the gill are also transported anteriorly, but are usually rejected as pseudofeces. Endoscopic examinations of the gill of the eastern oyster *C. virginica* showed that redirection of particles on the ordinary filaments, from one ciliary tract to an opposing tract, can occur (Ward 1996). How this transfer is affected, and whether it is caused by a change in the rate or angle of ciliary beat, is unknown. The blood ark, *A. ovalis*, has a fillibranchiate gill composed of ordinary filaments with the ability to transport captured particles both dorsally and ventrally (Atkins 1937b). Consequently, this is an interesting specimen for examining if particle transport is affected by chemical cues. This study will test the null hypothesis that there is no response by the frontal cilia

of gill filaments to dissolved extracellular and intracellular substances from phytoplankton.

2. Methods

2.1 Preparation of metabolites

Phytoplankton extracts were used to determine if food compounds affect the movement of captured particles. Dissolved extracellular metabolites were prepared as per Ward and Targett (1989). Briefly, a 40-mL sample of *Tetraselmis chui* (PLY429) in the stationary phase (10^7) was centrifuged at 500 g for 5 min. The supernatant was decanted onto a fresh tube, and the pellet examined for cell lysis under a compound microscope. If no lysis occurred, the supernatant was centrifuged again to remove remaining particles, and then passed through a 0.8- μ m filter using gentle vacuum (as per Gainey and Shumway 1991). The filtrate was then designated as cell exudate. Intracellular metabolites (“extracts”) were prepared from the cells pelleted during centrifugation. First, the cells were washed by resuspending in ultrafiltered artificial seawater (ASW), centrifuged at 500 g for 5 min and then re-suspended in ASW. Cells were frozen overnight, and further lysed via treatment in a sonication bath for 30 min. All metabolites were prepared the same day of the assay or were frozen until use, though for no more than three days. Metabolites were maintained on ice during the experiments to prevent bacterial breakdown of the compounds.

2.2 Preparation of sugar-coupled microspheres

Two specific neoglycoproteins of interest (D-mannose and N-acetyl-glucosamine) were covalently coupled to carboxylated microspheres (10 μ m) using a commercially

available PolyLink Protein Coupling Kit (Polysciences, Inc.). These particular sugars were used because lectins in the pallial organ mucus of *M. edulis* and *C. virginica* have been shown to bind to them selectively, potentially playing a role in particle sorting (Pales Espinosa et al. 2010). These sugars have also been found on cell surfaces of numerous algal species that are commonly used as food in bivalve culture (Pales Espinosa et al. 2016, Rosa et al. 2016a *in review*). Further, characterization of the surface charge and wettability of these particles showed that the different sugar types result in the particles having distinct physicochemical properties (Rosa et al. 2016b *in review*). The use of these complementary methods (mixed metabolites vs. targeted sugars) allowed for a more precise measurement of the different cues that potentially control particle transport, and thus a selection response.

2.3 Isolated gill observations

The techniques of Silverman et al. (1999) were adapted to visualize the cilia of isolated gill using a compound microscope. Briefly, gills were isolated and pinned to strips of rubber glued to Stovall flow cell[®] (“chambers”) that were filled with isotonic ASW (Gainey & Shumway 1991). The chamber is deep enough (8 mm) so as not to interfere with the movement of the frontal cilia, and allowed for the separate addition of the different metabolites. Microalgal exudates and extracts were prepared as described above, and tested separately. Metabolites were added to one end of the rafter cell using a peristaltic pump on low speed and allowed to interact with the frontal cilia. Fluorescent polystyrene microspheres (YG) were used to track particle handling and to examine the effects of the metabolites on gill ciliary activity.

2.4 Endoscopic examinations

Fluorescent polystyrene particles (25µm) no hyphen were used to examine *in vivo* transport using endoscopic methods detailed in Ward et al. 1993. Briefly, the endoscope was connected to an optical zoom-adapted and attached to a CCD camera (Cohu, Inc.). The video camera and attached endoscope were then mounted on a micromanipulator to allow fine position within the bivalve pallial cavity. Video was recorded onto 8-mm videocassettes (Hi-8, Sony) for later analyses. Prior to examination, the ventral shell of each oyster was trimmed to accommodate the optical insertion tube (OIT) of the endoscope and prevent damage to the tube when the animal adducted its valves (Ward et al. 1991). Shell material was carefully trimmed without damaging the mantle, and animals were allowed to recover for at least 24 hours prior to use in endoscopic examinations. For observations, one oyster was placed in a 1-L aerated chamber filled with filtered seawater (0.2 µm nominal pore size, salinity 28). To determine if particle movement on the gill was affected by dissolved microalgal metabolites, two assays were developed. In the first assay, the endoscope was oriented to view the frontal surface of the gill ("frontal assay"). A peristaltic pump was used to deliver a soluble treatment (extract, exudates, or filtered seawater control) directly to the gill of an individual animal via a micropipette attached to a micromanipulator (Ward et al. 1993). The micropipette was carefully positioned to apply the soluble treatment, at a flow rate of 500 µl min⁻¹, onto the section of gill being visualized by the endoscope. Animals were exposed to each treatment in the chamber for 5 minutes. After the 5-minute exposure, an aliquot of 25-µm fluorescent polystyrene beads was delivered just posterior to the OIT. The number and direction of transport of captured microspheres during exposure to dissolved metabolites and control solution

was assessed. Immediately after addition of beads, the number of captured microspheres moving ventrally or dorsally was separately counted for 3 minutes. Only microspheres moving on the frontal surface of the ordinary and transitional filaments were counted. This is because particles that enter the plicae troughs all move dorsally on the gill via hydrodynamic action (Ward 1996), and the focus of this assay was on mucociliary transport on the ordinary filaments. After the particle counts, an additional 2 minutes was given to allow any captured particles to be cleared from the gill and a second aliquot of the microspheres was added. These steps were repeated at least 3x, more if less than 50 particles were counted within the timeframe, and an average count of the number and direction of captured particles was calculated. After the counts were finished, the chamber was flushed with FSW, the animal allowed to resume feeding and the next treatment added at least 15 min after flushing. Assays were repeated as described above for each of the three treatments, with the order of applied treatment being changed for each successive animal to control for residual effects, if any, of one treatment on the next. This assay tested the null hypothesis (H_0) that addition of extracellular metabolites had no effect on direction of captured particles, and hence ciliary activity. In the second assay, the endoscope was positioned to view the ventral groove of the gill. The animal was exposed to each treatment for five minutes as described above. Thirty seconds after the delivery of the YG beads (determined in preliminary experiments), the movement of particles on the ventral tract was recorded for 3 minutes. After the recording was stopped, 2 minutes were allowed to elapse and the trial repeated one more time. The total number of microspheres in the ventral groove for each of the treatments was counted. This assay tested the null hypothesis

(H₀) that metabolites had no effect on particle transport. Both assays were performed at 14°C and at 21°C. The two temperature regimes were tested because both temperature and seasonal differences in the sensitivity of gill tissue to different endocrine hormones have been reported (Catapane et al. 1979). Additionally, a choice experiment was carried out on the frontal surface by directly delivering the sugar-coupled microspheres with the untreated (control) YG microspheres. This particular assay utilized targeted microspheres and tested the null hypothesis (H₀) that physicochemical properties have no effect on particle transport.

2.5 Data handling and statistical analyses

In the *in vivo* endoscopic examinations, the average number of captured particles traveling ventrally and dorsally in each frontal assay trial was added, and the percentage of particles transported ventrally vs. dorsally on the frontal surface was calculated. In the ventral assays, the total number of particles on the ventral margin was counted. For each of the assays, the difference in means of particles being transported when exposed to metabolites versus the control (seawater treatment) was tested for normality (Shapiro-Wilk test), and values compared using a paired T-test.

For the neoglycoprotein-coupled microspheres, a modified electivity index (EI) was calculated (Jacobs 1974):

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

Where S is the proportion of neoglycoprotein-coupled microspheres in the ventral tract and W is the proportion of the same microsphere in the suspension delivered to the animal. All samples were counted on the ventral tract, thus a negative EI indicates this

gill tract is depleted of these microspheres, and most particles are being transported to the dorsal tract. The data were tested for normality, and an analysis of variance (ANOVA) was run to determine if there were any differences between the electivity indices calculated for each microsphere type. An alpha level of 0.05 was used for all tests.

3. Results

3.1 Isolated gill observations

Observations on the isolated gill segments of the ark *Anadara ovalis* using a compound microscope demonstrated a capacity for bidirectional transport of captured particles on the gill (Figure 1). Polystyrene microspheres delivered to the gill were transported ventrally, indicating a rejection of non-nutritious particles. In contrast, cells of *Tetraselmis chui* delivered to the gill were transported dorsally, from whence particles are generally ingested. This transport was the same regardless of which particles were delivered to the gill first. The depth of field available for use with the compound microscope using the gill chamber precluded measurements of particle velocity to measure the effects of the different metabolites on ciliary activity accurately. Thus, results of this assay are observational and not quantitative.

3.2 Endoscopic examinations

Direct observations of particles captured on the frontal surface of the gill filaments of the eastern oyster *C. virginica* revealed that the majority of the microspheres (~70%) were transported ventrally on the gill (Table 1). This was the case regardless of treatment or temperature regime tested. No significant difference was found in the percentage of particles moving ventrally or dorsally between the different

treatments (seawater, extract, or exudate; $p < 0.05$). For the ventral assays, under the 14°-temperature regime, the total number of particles on the ventral margin was significantly higher when the animal was exposed to the extracts than the filtered seawater (FSW) control (Table 2, $p < 0.05$). The number of particles on the ventral margin was significantly lower when the animal was exposed to the exudates than the FSW control ($p < 0.05$). In the 21°-temperature regime, there was no difference in the number of particles in the ventral margin between treatments ($p > 0.05$). In the ventral assays with the neoglycoproteins, there was no difference between the proportion of control microspheres and N-acetyl-glucosamine coupled microspheres in the ventral margin (Table 3; ANOVA, $p > 0.05$). The EI for the D-mannose was significantly different from both the control and N-acetyl microspheres (Tukey's HSD, $p < 0.05$), indicating a significantly higher number of the D-mannose coupled spheres were transported to the ventral margin of the gill.

4. Discussion

This project assessed experimentally the presence of active selection of particles in suspension-feeding marine bivalves by determining if soluble phytoplankton metabolites can elicit a chemosensory response of the gill cilia. Whereas the role of gill cilia in particle capture, transport, and sorting by bivalves, and its response to both environmental and chemical cues is well documented (Aiello 1960, Davenport and Fletcher 1978, Jørgensen 1982, Axiak and George 1987, Beninger et al. 1997, Silverman et al. 1999), thus far a mechanism for an active selection response to different food types has not been identified. Active particle selection would likely be elicited by the frontal cilia due to their direct involvement in particle transport. Results of

the metabolite experiments showed that the addition of the extracts or exudates of *T. chui* cells had no significant effect on particle transport on the frontal surface of the gill, indicating that metabolites did not affect ciliary movement. There were no differences in the number of particles moving dorsally or ventrally between the control assays and the experimental treatments. Results of the experiments with the targeted sugars demonstrated that particles coated with D-mannose were more likely to be rejected (transported to the ventral margin), whereas particles coated with N-acetyl-glucosamine were generally ingested.

Results from the ventral assays suggest an effect of temperature on particle transport in the ventral margin. In the colder temperature regime tested (14°C), addition of the extracts resulted in more particles appearing in the ventral tract. Addition of the exudates, on the other hand, resulted in a significantly lower number of particles in the ventral tract. These results are difficult to explain and are incongruent with results of the frontal surface assay described above. No such differences in the number of particles being transport in the ventral groove were observed when animals were exposed to different metabolites treatments at a warmer temperature (21°C). A possible explanation for the observed results could be that there are methodological problems with using the ventral assay. The ventral assay developed for this study had some inherent issues, i.e. a drawback of this assay is that only one demibranch and ventral margin could be viewed at a time. In some cases, it was noticed that the second demibranch was visible in the background, and in some cases more microspheres were noted in the second location than the one being counted. The differences were not quantified, but the observation is concerning as it raised the possibility that any differences in particle

counts between treatments could in fact be due to more particles being captured on the gill not being observed. If the observed results are not due to methodological issues, they indicate that the effects of metabolites on particle transport and selection in *C. virginica* may have a temperature dependence. A temperature sensitivity that could impact ciliary activity has been previously demonstrated in marine bivalves. Stefano and Catapane (1977) reported seasonal variations in levels of serotonin (5HT), dopamine (DA) and norepinephrine (NE) in the central nervous system of *M. edulis*. Summer levels of 5HT were found to be about twice those of wintertime, while levels of DA and NE also dropped dramatically in the colder months. The authors suggested that seasonal changes in monoamines could influence how sensitive the animals are to both endogenous and exogenous agents (e.g. chemical cues). Further, the extracellular metabolites of different microalgal species have been shown to affect feeding behavior of *M. edulis* (Ward and Targett 1989). Exudates from several microalgal species were tested separately, and two of the species tested (*Olisthodiscus luteus* and *Dunalliella tertiolecta*) resulted in reduced filtration rates. None of the species tested, however, was found to increase filtration rates as a response to the different dissolved metabolites.

The results from the frontal assay indicate that there is no direct effect of metabolites on active selection. No temperature effect was found on the sensitivity of the frontal cilia to the different metabolites tested, unlike the results of the ventral assay. The frontal assay provides more direct information than the ventral assay developed for this study. The assay offers a direct view of movement of captured microspheres, providing information on whether the experimental treatments can directly affect the directionality of particle movement. One drawback of this assay is that only a small

section of the gill (4-7 plicae) are visible in the field of view of the endoscope, thus if a particle is moving dorsally, and “flips” to be transported ventrally further down the field, or vice versa, this event could not be captured and recorded. Regardless, the percentage of captured particles that were transported dorsally on the frontal surface (~20% regardless of treatment) suggests that even if some events were missed, it would not have affected the overall conclusions. One prerequisite for an active chemosensory response would be the “recognition” of particles of different quality followed by translocation from one transport tract (e.g., acceptance tract) to an adjacent one (e.g., rejection tract, see Ward and Shumway 2004). Particles were rarely observed to change directionality mid-transport, the few instances where the tracer particles did shift from the frontal filaments to the transitional filaments occurred independently of the experimental treatments.

The discrepancies between the ventral assay and frontal assay at 14° C can be partly explained by the differences in how the types of gill cilia are controlled. The brachial nerve has an activating role in ciliary movement of the gill in *M. edulis* (Aiello 1960), while cilioexcitation of lateral cilia with direct electrical stimulation has been demonstrated in many bivalve species (Aiello 1970), only two species (*Tellina alternata*, *Trachycardium egmontianum*) had pronounced activity of the frontal cilia. Addition of serotonin was reported to increase the rate of beating in all cilia types, with lateral gill cilia being under reciprocal innervation by serotonergic and dopaminergic neurons originating in the central nervous system (Catapane 1983). The mechanisms controlling lateral vs. frontal cilia in marine bivalves in *Mytilus edulis* are independent, providing an advantage of more control of water currents and particle transport (Paparo 1972).

Similarities in the role of the central nervous system in controlling the activity of the lateral cilia to that of blue mussels have been found in the eastern oyster *Crassostrea virginica* (Carroll and Catapane 2007). While the lateral cilia are innervated (Catapane et al. 1979), no such evidence for the frontal cilia has been found (Aiello 1970). Differences in activity of lateral and frontal cilia exposed to different compounds and hormones have also been reported. Axiak & George (1987), for example, examined the effects of petroleum hydrocarbons on ciliary activity in the clam *Venus verrucosa* and found that addition of these compounds reduced the activity of the lateral and eulaterofrontal cilia, while increasing the activity of the frontal cilia. Taken together, previous reports and the findings outlined in the current study suggest that an effect by metabolites or other chemical cues on the activity of cilia and particle movement is not tied to a chemosensory selection response.

The ventral assays using the neoglycoprotein bound microspheres demonstrated that some sugar types (N-acetyl-glucosamine) were being transported dorsally at significantly higher rates than other sugar types (D-mannose). These sugars have different physicochemical properties (Table 3) that can be used by bivalves to discriminate between the microsphere types. Earlier studies have also demonstrated that the presence of extracellular ectocrines can affect selection. Ward and Targett (1989) reported that when cellular metabolites from several algal species were absorbed onto different microspheres, it resulted in significant changes in selection depending on the microalga species and the microsphere type used. Based on the differences in selection response by *M. edulis*, the workers concluded that mussels use physicochemical cues to discriminate between particles. Once the compounds were

absorbed, the workers did not characterize the surface properties of the particles, which would likely have changed with the treatment. Without this information it is difficult to determine if the observed selection responses were due to passive interactions or contact chemoreception. Further, microscopic observations of the excised gills indicated a rejection of non-nutritious particles, and ingestion of live *Tetraselmis chui* cells. This bulk transport was the same regardless of which particles were delivered to the gill first. This observation would be a further indication that physicochemical properties of the particles affect the direction of transport. The differences, however, could also be a result of contact chemoreception. The inability to visualize the frontal cilia using this method precludes more precise conclusions to explain the results.

While the results outlined here support the notion that an active selection response is not the mechanism behind particle selection, more work is needed to provide definitive evidence. One aspect of the mechanism that this study was unable to determine is whether there is indeed contact chemoreception. It is very difficult to separate an active versus a passive response to the ectocrines on the particle surface. A paracrine response, a form of cell-to-cell communication where one cell produces a signal to induce a response in a nearby cell, will be nearly impossible to narrow down using these methods. Further, the assays developed for the endoscopic work are limited to suspension-feeding species that can select on the gill. Bivalves that only select on the labial palps (e.g. *Mytilus edulis*) should be examined to determine if this organ has an inducible chemosensory selection response.

In conclusion, the results outlined in this study demonstrated that dissolved metabolites from a known food source (*T. chui*) do not elicit a consistent selection

response on the gill of oysters. Whereas the metabolites affected particle transport in cold temperature regime, differences were not observed on the frontal surface of the gill. The dissolved metabolites had no effect on particle transport on the frontal surface, so in essence did not induce a selection response. Findings outlined in this study further demonstrate that interactions between the physicochemical properties of particles and the mucus covering the feeding organs, and not an active ciliary response, mediate particle selection in bivalves. This supports a model in which a passive mechanism mediates particle selection in suspension-feeding bivalves.

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Table 1. Percentage of particles moving ventrally or dorsally on the frontal surface of oyster gills. Polystyrene particles (YG) were delivered to the gill *in vivo*, after the animal was exposed to one of three treatments (seawater, exudates, or extracts). Significantly more of the particles captured on the frontal side of the filaments were transported ventrally, with very few transported dorsally (ANOVA, $p < 0.05$), denoted by *. There were no significant differences in the percentage of particles moving ventrally or dorsally between the treatments tested (ANOVA, $p < 0.05$). There was also no effect of temperature of particle movement. Data presented as means (\pm SD), $n=5-6$.

<i>Crassostrea virginica</i>		Treatment		
Temperature	Particle direction	Seawater (control)	Exudates	Extracts
14°	Ventrally	70 (23)*	75 (25)*	71 (27)*
	Dorsally	30 (23)	25 (25)	29 (27)
		Seawater (control)	Exudates	Extracts
21°	Ventrally	75 (19)*	77(29)*	78 (19)*
	Dorsally	25 (19)	23 (29)	22 (19)

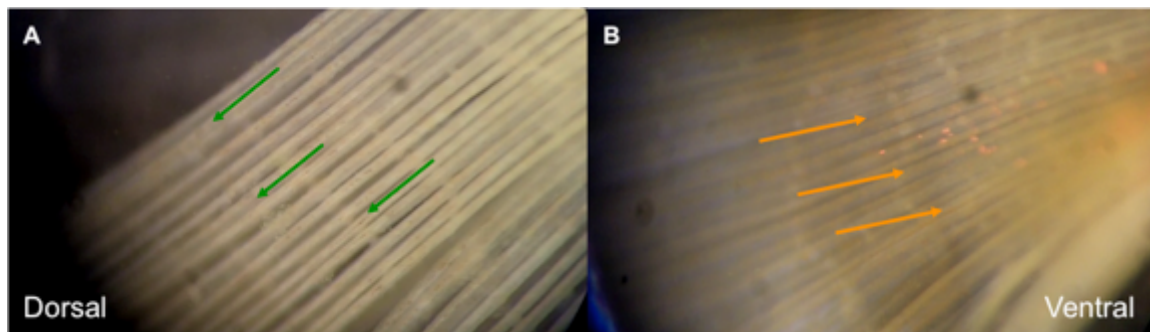
Table 2. Number of particle transported on the ventral margin of oyster gills. Polystyrene particles (YG) were delivered to the oyster gill *in vivo*, after the animal was exposed to one of three treatments (seawater, exudates, or extracts). In the colder temperature regime (14°), addition of extracts resulted in significantly more YG particles in the ventral tract, while addition of the exudates resulted in significantly less YG particles ($p < 0.05$). In the warmer temperature regime (21°), there was no difference in the number of particles in the ventral tract for each of the treatments tested ($p > 0.05$). Data presented as means (\pm SD), $n=7$.

<i>Crassostrea virginica</i>		Treatment	
Temperature	Seawater (control)	Exudates	Extracts
14°	100 (35)	67 (22)*	254 (135)*
21°	113 (71)	110 (36)	102 (63)

Table 3. Electivity indices for neoglycoprotein-coupled microspheres in the ventral tract of oyster gill. Particles were delivered with an uncoated non-carboxylated microsphere (YG) as a reference. Surface property values from Rosa et al. 2016 (in review) included. Data presented as means (\pm SD), n =7. * Indicates mean is significantly different from the control and N-acetyl-glucosamine treated particle (Tukey's HSD, $p < 0.05$), ⁺indicates surface property of significantly different from the control particle

Particle treatment	Electivity Index	Angle	Charge
Polystyrene reference (YG)	n/a	113	-11.3
Control YG-C (untreated)	-0.20 (0.14)	107	-10.0
N-acetyl-glucosamine	-0.29 (0.10)	110	-5.6 ⁺
D-mannose	0.02 (0.16)*	118 ⁺	-7.8

Figure 1. Video micrograph of the frontal surface of the gill of the ark *Anadara ovalis* exposed to whole cells of the chlorophyte *Tetraselmis chui* (A) and synthetic particles (B). Arrows indicate the bulk movement of captured particles. Observations further demonstrate bi-directional movement of captured particles on the *A. ovalis* gill in response to different particle types.



Synthesis

The goal of this dissertation was to experimentally assess the mechanism(s) underlying particle selection in marine suspension-feeding bivalve molluscs. As a consequence of their role as foundation species in coastal systems, the selective capabilities of these animals can have a large influence upon seston composition. For example, knowing the types of cells more likely to be retained may aid in understanding some of the factors affecting shifts in phytoplankton community composition. Additionally, because of their role as pelagic-benthic couplers, information on the types of particles that are more likely to be rejected can also contribute to a better understanding of the quality of material that is being made available to the benthic communities associated with bivalve species. To better understand the mechanisms of selection, this thesis assessed experimentally pre- and post- capture particle discrimination processes in bivalve molluscs to gain insight into the underlying mechanisms. In particular, I addressed three questions: 1) Are there intrinsic (physiological plasticity) and extrinsic (physicochemical particle surface properties) factors that mediate particle capture 2) Why are some particles more likely to be rejected or ingested than others; and 3) Is an immediate chemosensory response mechanism involved? The main dissertation chapters outline the empirical findings, and this section provides a synthesis of the results and answers to the main questions.

1. Are there intrinsic (physiological plasticity) and extrinsic (physicochemical particle surface properties) factors that mediate particle capture?

It is generally understood that suspension-feeding bivalves employ hydrosol filtration mechanisms to capture particles > 4 μ m in size with an efficiency of close to

100% (Møhlenberg and Riisgård 1978, Shimeta and Jumars 1991, Ward et al. 1998, Riisgård and Larsen 2010). Findings outlined in this thesis confirm that capture of spherical particles $> 4 \mu\text{m}$ in diameter by mussels is consistently high regardless of season, with only the $2\text{-}\mu\text{m}$ particles being captured at a lower efficiency than particles of greater size. The results are also consistent with other studies that used uniform particles of known size (including phytoplankton cells) in which all particles above the $4\text{-}\mu\text{m}$ threshold size were captured with high efficiency (see Ward and Shumway 2004 for review). Capture efficiency by mussels for particles $>4\mu\text{m}$ did not shift in response to seasonal changes in the size and composition of the particle field or other environmental parameters (e.g. temperature, salinity, etc.). These results provide good evidence that capture efficiency in mussels is not physiologically regulated over time or in response to changing particle fields. Results outlined in Appendix A also demonstrate that different microalgal species 5 to $7 \mu\text{m}$ in size were captured with the same mean efficiency (60 to 80%), and similar efficiency to synthetic polystyrene microspheres of the same size. Together, these findings demonstrate that above a given particle-size threshold there is no effect of physicochemical properties on capture. Therefore, since larger particles are captured with the same efficiency, any differences in ingestion and rejection are due to post-capture discrimination processes.

Effects of physicochemical properties on capture were further examined in mussels (*M. edulis*) and scallops (*A. irradians*). Results demonstrate that treatment of microspheres with the sugar D-mannose resulted in significant decrease in particle CE by mussels. The addition of other sugar types, which also changed the surface properties of the particles did not translate to a decrease in capture efficiency. These

results suggested an interaction effect of physical and chemical properties on particle capture. While there was no significant effect of the different sugar types on CE by the scallops, there was still an overall decrease in CE with the manipulated microspheres. Further, the effects of physicochemical properties on particles was most noticeable for particles $< 6\mu\text{m}$, indicating that surface characteristics have the largest effect on CE at the smaller particle size threshold. This demonstrated differential capture of small particles, combined with results showing differential adhesion, suggest a mechanism by which physicochemical surface properties affect particle discrimination at the pre-capture level in suspension-feeding bivalve molluscs. These findings advance what is currently understood regarding selective feeding in bivalves, demonstrating particle surface properties might be involved in mediating CE at particle size ranges that are below the 100% capture-efficiency threshold.

Calculated capture efficiencies for some particles sizes (those 2 to 4 μm) also deviated from previous reports. One such difference was the calculated CE of some microalgal species. Mussels fed the microalgae *Pavlova lutheri* (4 μm), for example, had a mean CE of $\sim 30\%$, the same as larger microalgae also used (5-6 μm) (Chapter 3, table 5). The mean CE for both microalgae, however, was significantly lower than would be expected for its size, with previous laboratory experiments showing mussels had a mean CE closer to 100% when exposed to the same microalgal species (Møhlenberg and Riisgård 1978). Data from one of the field studies showed that 2- μm spheres were captured at a significantly higher rate in December (mean CE 60%, $n=36$) compared to the other months. The reason for this finding is unclear, and environmental factors provided little insight into this observed effect. Reports of such shifts in CE of smaller

particles are not unprecedented. A study by Lucas et al. (1987), for example, found that during times when bacterial numbers in the seston were high, populations of *M. edulis* were able to capture 0.5-1.58 μm particles with up to 57% efficiency. As demonstrated in several experiments in this thesis, the CE of smaller particles was also higher than previous reports. Such differences in the CE at the lower threshold of particle size may be indicative of the role of physicochemical properties on capture when hydrodynamic effects are not as strong, and warrants further research. These findings provide strong evidence that CE of particles $<4\text{ }\mu\text{m}$ might be higher than previously thought and that the physicochemical properties that may affect this capture is a fertile area for future research.

2. Why are some particles more likely to be rejected or ingested than others?

Physicochemical properties of particles have been suggested as possible factors that mediate food selection in various marine invertebrates (LaBarbera 1978, Gerritsen and Porter 1982, Solow and Gallagher 1990, Shimeta and Jumars 1991, Hernroth et al. 2000). Inherent particle properties are determined by both intracellular and extracellular chemical and physical factors, with surface chemistry being dominated by ionizable functional groups (Hunter 1980). These properties vary among living and non-living particles, and can affect particle interactions such as aggregation (Abrahamson et al. 1942, Hunter and Liss 1982, Loder and Liss 1985, Waite et al. 1995). Some recent work has suggested that interactions between the physicochemical properties of particles (Beninger and Decoittignies 2005, Pales Espinosa et al. 2007, 2008, Rosa et al. 2013), and the mucus constituents produced by the pallial organs (Pales-Espinosa et al. 2009,

2010a, 2010b) play roles in selection by different bivalve species. Results of my thesis extend this conceptual framework and demonstrate that both synthetic microspheres and different species of microalgae have distinctive physicochemical surface properties, varying in the degree of hydrophobicity, negative zeta potential (surface charge), and surface sugar composition (see Chapter 3).

Mucus covering the pallial organs of suspension-feeding bivalves is involved in many aspects of feeding, such as particle capture, selection, and transport (Jørgensen 1996, Ward 1996), and its composition demonstrated to vary among species (Beninger 1991, Beninger et al. 1993). Results obtained in the current work demonstrate that the adherence of particles to mucus was dependent on the species and site of collection (labial palp vs. gill) and the surface properties of particles. Particle adherence was not linearly related to the surface charge of the particles. Instead, non-linear relationships were explored, with 3rd order polynomials best explaining the data. This finding matched what has been previously reported for the effects of surface charge on selection (Rosa et al. 2013), namely that end-point surface charges resulted in a selection response (rejection or ingestion), and mid-range surface charges resulted in no selection. The changes could be due to the rheological properties of bivalve mucus, which tend to have a slight negative charge in seawater (Denny 1983), but can have small variations depending on the osmolality of the water. To date, the exact charge of bivalve mucus has not been determined, but if it were in the -5 to -10mV range would explain the observed patterns. Namely, particles with a surface charge in this range are less likely to adhere to the mucus, and particles with a higher or lower surface charge than this mid-range are more likely to adhere to mucus. Unlike particle surface charge,

a strong and linear relationship was found between the hydrophobicity (wettability) of the particles and how well they adhered to the different mucus types. Generally, particles with the most hydrophobic surfaces (least wettable) were least likely to adhere to the mucus. This trend was true whether the particle was a synthetic microsphere (e.g. polystyrene) or a live microalgae. Together, these findings demonstrate that different particle surface properties can result in differential adherence to mucus covering the bivalve pallial organs.

The physicochemical properties of particles, demonstrated to affect adherence and capture, could also play a role in post-capture discrimination. While the mechanisms of particle capture and selection are related (e.g. affected by mucociliary processes), it shouldn't be assumed they are controlled in the same manner. Research outlined in the thesis demonstrated, for the first time, that both physical and chemical surface properties of microalgae affect selection processes in mussels (*Mytilus edulis*) and oysters (*Crassostrea virginica*). These bivalves can use physicochemical properties of microalgae to discriminate between microalgae, with differences in a particular surface property among different algal species resulting in strong selection, i.e., preferential ingestion or rejection. Statistical models demonstrated that hydrophobic microalgae were most likely to be ingested. This finding is surprising because the patterns observed for particle adherence and capture indicate that smaller-sized hydrophobic particles were less likely to bind to the mucus and be captured. Together these findings are intriguing because they may be indicative of a de-coupled mechanism by which below a certain size threshold the most hydrophobic particles are less likely to be captured via adhesion and mucus interactions, and at the same time

are more likely to be ingested when there is a choice. Although counterintuitive, these findings but may be explained by the general trends regarding the surface properties of particles in marine systems. Characteristics outlined in the main thesis chapters and appendices demonstrate that organic particles (e.g. microalgae) were more hydrophobic, whereas the low-organic particles (e.g. clays and particles from dead, ground up marsh grass) were more hydrophilic. These findings are similar to what has previously been reported for other marine particles (e.g. Ozkan and Berberoglu 2013). Thus it appears that mechanisms evolved to increase capture of the more abundant particles in the seston ($< 4\mu\text{m}$, hydrophilic and generally non-organic), but serve as a cue for selection (particles to reject in order to increase bulk of nutritious material ingested). If true, such a mechanism would explain why some particles are more likely to be selected than others. These findings provide a possible explanation for observed patterns of selection, where bivalves generally ingest organic particles over inorganic seston. It also provides solid evidence for the types of particles that are ingested versus rejected back into the environment, advancing current understanding of suspension feeding. Finally, the characterization of surface properties of microalgal species has added to a relatively unknown aspect to what has been documented for these particles. These data could be a powerful tool in further understanding the physiology of phytoplankton and how they interact with their environment (e.g., aggregate formation).

Modeling of the selection data suggested that the characterized surface properties do not offer a full picture of particle discrimination. While both physical and chemical property profiles were needed to develop meaningful models, some of the variability was not explained. This indicates another property, or an interactive effect not

yet tested, also plays a role in selection. Most strikingly, differences in the models generated using preferential rejection data vs. those examining all particle fates also indicate that the physicochemical factors that determine rejection may be decoupled from those that determine ingestion. Therefore, non-linear effects of particle surface properties on selection may be involved, wherein one characteristic may be more likely to result in rejection and not preferential ingestion. Non-linear effects of surface characteristics were also observed in the capture efficiency experiments, indicating that the process of particle selection are de-coupled from capture, where a surface property that would increase ingestion does not result in the likelihood of particle capture of smaller particles. Differences in the models also demonstrated that the physicochemical properties that affect particle discrimination in *M. edulis* and *C. virginica* are different. This, and other findings outlined in the thesis, emphasizes that future modeling work should take into account differences in the process of selection between bivalve species. These findings advance our understanding of the factors that mediate particle capture and selection in suspension-feeding bivalves, and build on 30 plus years of research to elucidate the mechanisms that enable particle discrimination.

The results presented in my thesis advance the field in confirming a passive mechanism as the most likely mediator of particle discrimination. This mechanism relies on the interactions between the physicochemical properties of particles and the mucus composition of the pallial organs, with some particle properties increasing the likelihood that a particle will be ingested or rejected. Hydrophobicity in particular appears to be an important factor in discrimination, with strong linear relationships being shown between selection and particle wettability (i.e. hydrophilic particles most likely to be rejected). It is

most likely that this surface property is an indicator of the bulk characteristics of a surface, related to sugar composition and ionizable components. Data from the particles characterized show that inorganic particles are hydrophilic, while organic particles are mainly hydrophobic, supporting the assertion that this property could be an indication of bulk organic content.

3. Is an immediate chemosensory response mechanism involved?

Results of the bulk metabolite experiments have shown that the addition of phytoplankton cellular exudates or extracts had no significant effect on particle transport on the frontal surface of the gill, indicating that metabolites did not affect ciliary movement. Therefore, any response to food metabolites is not manifested as an effect on selection. Results further suggest that there is no immediate active selection in determining particle fate. Follow-up experiments using previously characterized particles, whose surface properties had been manipulated by adding different sugars, resulted in changes in particle transport. These data further demonstrate that specific physicochemical properties of particles, and not an active physiological response, mediate particle selection in bivalves. As no chemosensory response mechanism was found, it appears that passive selection, where the interaction between particle surface and the mucus constituents of the pallial organs determine particle fate, is the mechanism of selection in suspension-feeding bivalves.

While the results outlined in this thesis demonstrate a passive mechanism most likely underlays particle selection, the role of an active mechanism based on contact

chemoreception could not be definitively ruled out. Data from the microspheres with the neoglycoprotein coupled sugars (Chapter 5) which demonstrated a selection response to the manipulates surfaces, could have been mediated by a change in ciliary activity to the addition of the sugars. Based on the available methods used, it is imposible to differentiate between a passive response and an active contact chemoreception response. While particles on the frontal surface were not affected by the addition of different metabolites, some follow-up experiments should be carried out to provide definitive answers.

4. Future directions

Empirical data outlined in this thesis has provided valuable data regarding the process of particle selection in suspension-feeding bivalve molluscs and allows for the development of new questions to better define the mechanisms involved in this process. Thesis results demonstrated that going forward a consensus on the methodologies and instrumentation used in selection studies should be reached. Some of the problems associated with estimating physiological parameters such as clearance rate (CR) and capture efficiency (CE), issues that others have previously pointed out (Bayne et al. 1977, Bayne 2004, Petersen et al. 2004, Filgueira et al. 2006), became clear during this work. The rates and capture efficiencies of animals feeding on natural seston were calculated based on the flow-through method, with a flow rate set to 100 mL min⁻¹ to prevent recirculation (Riisgård 1977). Calculations of CE under these conditions assume that all water and suspended particles can be accessed by the animals (e.g. no “bypass”). Deviation from this assumption can have a significant impact on CE

calculations, as shown mathematically by Shimeta and Jumars (1991). Both local particle concentration and velocity can directly affect whether all water is accessible by the bivalve, and thus calculated capture efficiencies. Normalizing CE to the particle size that has the highest efficiency helps minimize the effects of this bypass on calculated efficiencies. The lower CE indicate that the indirect method of calculating efficiencies, even using the flow-through method recommended for more precise physiological measurements, can lead to an underestimation of these values. Such factors should be taken into consideration when planning future feeding and selection experiments.

Capture efficiencies of small particles ($< 4\mu\text{m}$) calculated using direct methods were higher than previously reported. This result may be explained by mean CE being generally higher in field experiments than in laboratory settings. Alternatively, they could be evidence that CE of some smaller particles are higher depending on the particle type used. The findings outlined in the thesis also provided evidence for differential capture of smaller particles (pico- and nanoplankton, $< 4\mu\text{m}$) based on their physicochemical properties, when the hydrodynamics of the aerosol filtration system are not as efficient at particle capture. This qualitative differential capture would be a form of passive selection and in accordance with the major findings outlined. Together, these results highlight capture efficiency, particularly at the lower size threshold, as a fertile area for future research.

Most of the empirical data outlined in the thesis strongly suggest a passive mechanism underlies particle selection. The findings, however, were unable to conclusively rule out an active contact-chemoreception in mediating selection. Based on the inability to fully visualize the frontal cilia, and the lack of technology to precisely

measure contact chemoreception effects, the role of an active mechanism could not be definitively dismissed. Future studies and experiments should be designed to better identify chemosensory responses and conclusively demonstrate a lack of an active selection response. In particular, animals could be provided with particles that are known to be rejected (i.e. inorganic, such as clay or dry marsh grass) and attempt to reverse movement on frontal surface with metabolites of microalgae. Any changes in the movement of a captured particle on the frontal surface would be a definite indication of a chemosensory response affecting particle selection.

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Appendices

Appendix A

Characterization of surface properties of natural and inorganic particles

1. Introduction

The physical surface properties (charge and wettability) of seven different microalgae species were characterized using previously reported methods (Chapter II). These microalgae were not used in the feeding experiments, thus results of the characterization are presented below. Further, to determine effects of adsorbed constituents and bacterial adhesions on surface properties, particles were incubated in raw seawater and the surface properties were characterized. Finally, the surface properties of the same strain of *Tetraselmis chui* (PLY429), grown in the Milford laboratory and at the Avery Point campus, were characterized and the effects of growth conditions on these parameters compared.

2. Methods

Two types of previously characterized materials (kaolin clay and particles from dry, dead cordgrass *Spartina alterniflora*) were incubated in seawater for two weeks and characterized. A 1g sample of kaolin clay (Aldrich Chemical Inc.) was placed in 300mL of raw seawater. A 2-g sample of the cordgrass was ground up in 250mL of seawater and blended for homogenization (5 mins on high). The ground sample then was passed through 100- μ m mesh, and more seawater was added to increase the volume to 300mL giving a final concentration of 10^5 particles/mL for the incubations. Particles were incubated with vigorous aeration (=bubbling) for 2 weeks at 18°C with a 12:12 light:dark

photoperiod. Contact angle ($^{\circ}$) and charge (mV) were calculated using methods previously published (Rosa et al. 2013).

3. Results and conclusions

A relatively wide range of surface characteristics was found for the microalgal species, matching previous reports (Ozkan & Bergazolou 2013, Rosa et al. *in review*, Pales Espinosa et al. 2016). The surface charges ranged from -9.58mV for *A. fundyense* to -12.68 mV for *N. pusilla* (Table 1). As with other microalgae characterized previously, all surface charges were negative. Incubations in raw seawater, and subsequent organic coating and/or bacterial degradation of the particles, resulted in changes to the particle surface properties (Table 2). Incubations resulted in a significantly lower surface charge (mV) for Kaolin clay, and a significantly higher charge for *S. alterniflora*. The differences in culture growth methods resulted in no significant differences in surface charge for the samples of *Tetraselmis chui* cultures.

The characterized microalgal species had both wettable and non-wettable surfaces, with *N. pusilla* being the most hydrophilic (72.8° contact angle) and *A. fundyense* being the most hydrophobic (122.0° contact angle). Incubation of the Kaolin clay resulted in a significantly higher contact angle (Table 2). Both angles, though different, were still under 90° and still considered wettable (=hydrophilic). Incubation of the cord grass resulted in significantly higher contact angle (=hydrophobic). There was a significantly higher contact angle for the algal strains grown at Avery Point. Further, no

correlations were found between the characterized surface properties (Pearson correlation, $p < 0.05$).

These findings indicate that adsorbed constituents, as well as growth conditions, can affect the physical surface properties of particles as has been suggested previously. Carboxylic acid (-COOH) and phenolic (-OH) groups are some of the major ionizable functional groups identified in organic films, which can dominate the chemistry of marine particle surfaces (Hunter 1980).

Table 1. Physical surface properties of the characterized algal species. No correlation was found between surface properties within species. There were significant differences in surface properties between species. Data presented as means (\pm SD), n=5-6. Algal sizes are based on modes obtained using a Coulter Multisizer IIe[®] instrument.

Algae species	Strain	Mean size (μ m)	Surface charge (mV)	Contact angle ($^{\circ}$)
<i>N. closterium</i>	D-828	25	-10.88 (0.23)	106.75 (0.45)
<i>C. simplex</i>	Chaet-G	5.1	-10.55 (0.48)	73.44 (2.62)
<i>A. fundyense</i>	38-3	30	-9.58 (1.79)	122.03 (1.48)
<i>A. coffeaeformis</i>	A-ora	19	-12.13 (0.64)	81.97 (5.09)
<i>Nannochloropsis sp.</i>	UTEX-2341	2.4	-12.39 (1.33)	92.79 (1.05)
<i>C. autotrophica</i>	580	2	-10.58 (1.73)	90.35 (9.60)
<i>N. pusilla</i>	0-1	3.3	-12.68 (1.17)	72.82 (2.10)

Table 2. Surface characteristics of natural particles pre- and post-incubation in seawater. Incubation resulted in significant differences in surface characteristics as determined by a paired two-sample t-test. *p < 0.05, ** p<0.01

Particle type	Pre-incubation		Post-incubation	
	Charge (mV)	Contact angle (°)	Charge (mV)	Contact angle (°)
Kaolin clay	-14.66 (0.55)	9.46 (0.94)	-12.90 (0.40)*	25 (3.51)*
<i>Spartina alterniflora</i>	-11.40 (0.50)	92.85 (6.17)	-17.40 (3.96)*	117.57 (4.70)**
<i>Tetraselmis chui</i>	-7.54 (1.47)	100.15 (3.35)	-11.14 (3.75)	105.39 (4.51)*

Appendix B

Results of capture efficiency field study in Norway

1. Introduction

This project was conducted to further evaluate the possibility of shifts in particle capture efficiency (CE) by the blue mussel *Mytilus edulis*. Previous work (Chapter II) demonstrated that apparent seasonal shifts in CE by *M. edulis* were not related to particle size or a physiological compensation by the mussels, and most likely due to instrumental artifacts. Several hypotheses were presented to help explained observed shifts in CE when different particle counters were used. These included the way in which particle size is determined by field instruments; namely by estimating spherical diameter based on volume, the aggregation of particles which result in a higher number of smaller particles exiting animal chambers, and lack of appropriate controls. The field experiment was designed to examine CE of different sized particles by *M. edulis*, by directly delivering microspheres to examine capture based on size. Secondly, similarly sized algae, each having distinct physicochemical surface properties, were also directly delivered to further examine capture of natural particles. This study tested the null hypothesis that mussels do not have shifts in particle CE based on size.

2. Methods

2.1 Capture efficiency experiments

Experiments were carried out during the summer of 2014 in Lysefjord (County of Rogaland), Norway. Individual blue mussels *Mytilus edulis* were maintained in flowing

seawater in sampling chambers for 1 hr prior to microsphere delivery. Methods developed previously (Rosa et al. 2014; chapter I) were used to deliver directly equal concentrations of 2-, 3-, 6-, 10-, 20- and 25- μm spherical polystyrene microspheres to the inhalant aperture of each mussel using a pipette, and water leaving the exhalent aperture was collected using a sampling tube attached to a peristaltic pump (adapted from Yahel et al. 2009). An electronic particle counter (Coulter Multisizer IIe) was used to measure particles in the two samples, and CE was calculated for each size class by the formula $CE = 1 - (PC_{\text{ex}}/PC_{\text{in}})$, where PC_{ex} is the total particle count exiting the exhalent, and PC_{in} is the particle concentration directly delivered to the inhalant.

Additionally, a sub-set of mussels was exposed separately to five different microalgal species (*Chaetoceros calcitrans* – 5 μm ; *Isochrysis galbana*– 5 μm ; *T. pseudonana*– 6.78 μm ; *Rhinomonas* sp. – 7.42 μm ; or *Tetraselmis* sp. – 7.56 μm) to determine differences, if any, in CE of these algae. Microalgae were delivered using the modified In/Ex system as described above. Cultures were each diluted 50% with filtered seawater (FSW), and 1.5mL of the 2- μm size polystyrene standard was added as a tracer particle. A total of 3mL of this suspension (algae + polystyrene) was delivered directly to the inhalant aperture of 5 individual mussels, the exhalent siphon subsequently was sampled, and CE was calculated.

2.2 Algal characterization

Starter cultures from the algae used in the CE experiments were shipped to the University of Connecticut, Avery Point campus, and grown using sterilized seawater from Norway (salinity 30-34) to determine surface characteristics (Table 1). Surface

charge (zeta) and wettability (contact angle) were measured for each of the algae using methods described previously (Rosa et al. 2014).

2.3 Quantification of surface charge (zeta):

Limitations of the zetasizer instrument required that the algae be transferred to diluted seawater (dSW; salinity 15) prior to analysis. To prevent cell lysis, 10 mL of each algae was centrifuged for 5 min at 500x g, the supernatant was discarded and the pellet re-suspended in dSW. Cells were checked under a microscope to confirm that centrifugation and re-suspension did not affect viability (e.g. cells remained intact and motile). Each biological duplicate ("replicate") was measured at least 3x on the Zetasizer. Data were compared by Student's t-test to determine if values for each biological replicate were statistically different ($\alpha = 0.05$). Values for biological replicates that were similar were averaged. All but *C. calcitrans* had similar zeta values within biological replicates.

2.4 Quantification of wettability (contact angle):

Particle wettability is related to hydrophobic forces acting on the surface of the cell, with highly wettable cell surfaces being more hydrophilic. A contact angle $>90^\circ$ is a non-wettable surface (hydrophobic), conversely a contact angle $<90^\circ$ is a wettable surface (hydrophilic; Volpe et al. 2006). To calculate contact angle, the algae were filtered onto a 3- μm GC-C filter using gentle vacuum to create a surface pad, and 10mL of isotonic ammonium formate was then passed through the filter to remove salts. Pads

were placed in a 70°C oven overnight to dry. A goniometer was used to image a 4- μ l drop of Milli-Q water, and Image J (NIH Image) was used to measure the formed contact angle. Values for biological replicates that were similar were averaged. All but *Tetraselmis* sp. and *T. pseudonana* had similar contact angle values within biological replicates.

2.5 Statistical analyses

A discriminant analysis (DA) was run on the characterized surface properties, with the algal species as the grouping variable. An analysis of variance procedure (ANOVA) was run on the results of the capture efficiency assays to determine differences, if any, of particle and algae size on this metric. An alpha value of 0.05 was used for all analyses.

3. Results and Conclusions

3.1 Capture efficiency (CE) results

Microspheres >6 μ m were captured with closed to 100% efficiency. The smaller particles tested (2 and 3- μ m) were captured at significantly lower efficiencies (ANOVA, $P < 0.05$). There were no differences in mean CE of the 2- and 3- μ m sized particles. These findings are within what has been previously reported for these types of particles (Rosa et al. 2014), and within what is expected for particle CE based on what is known

about the bivalve aerosol filtration system. There were no significant differences in the CE of the different microalgae directly delivered to the mussels (ANOVA, $P < 0.05$). Results demonstrate that there is no differential capture of particles $\geq 4\mu\text{m}$ in size, whether they are synthetic microspheres or live microalgae.

3.2 Algal characterization

The characterized surface properties were not correlated for the algae species examined (i.e., properties were independent of each other). The discriminant analysis (DA) model was significant and could discriminate between species, with *I. galbana* being the most different (Table 2; Figure 3); however, there was overlap between several of the species. Both a classical and robust DA were run, resulting in similar p-values. The robust model with a quadratic equation gave the most accurate classification matrix. Canonical correlations indicate that the model explained 96% of variability, and contact angle was the most important surface characteristic in classifying the algae. Cultures also were imaged to inspect for any physiological abnormalities. In previous characterizations of different microalgal species (Rosa et al. *in review*, Appendix A), all measured values were similar within biological replicates. These findings indicate that differences in physicochemical properties of particles have no effect on capture (i.e. no differential capture) for particles larger than 4 μm . Further, results indicate that growth regime (i.e. media and nutrients), as well as algal growth stage (stationary vs. log), can affect surface properties.

Figure 1. Capture efficiency (CE) of spherical polystyrene beads directly delivered to mussels (*Mytilus edulis*). Counts were made using a Coulter Multisizer IIe. Data are presented as mean CE (\pm SD; n=24) for each of the three days sampled. The 2- and 3- μ m sized spheres were captured at significantly lower efficiencies than the larger size classes (ANOVA, $P < 0.05$). There was no significant difference in CE of 6-to-25 μ m sized microspheres.

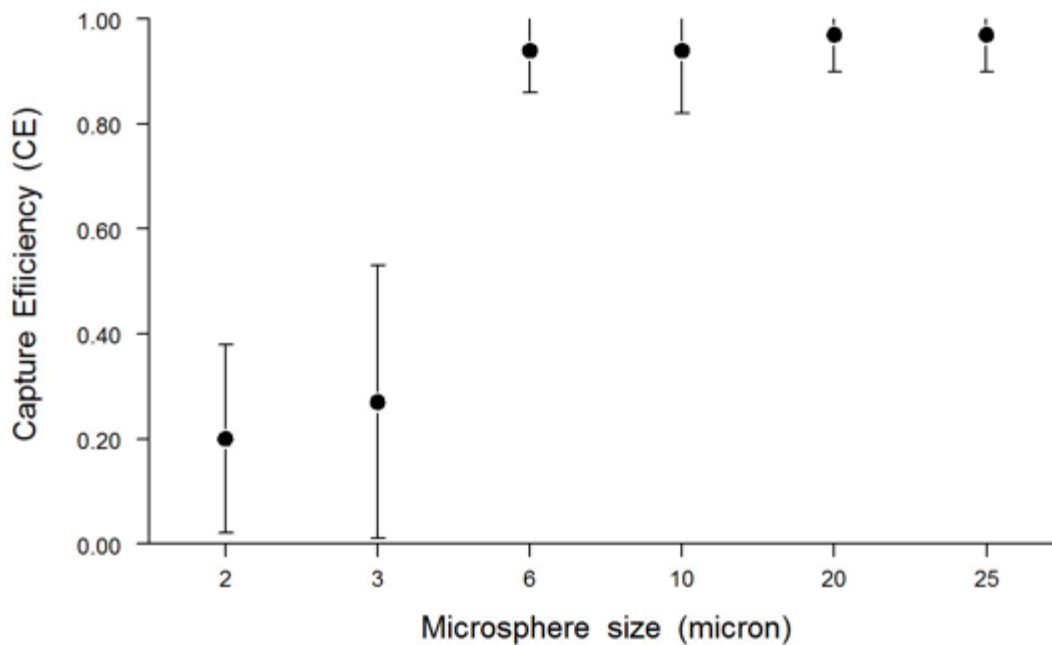


Figure 2. CEs of individual algal species delivered directly to mussels' inhalant siphon. Data are presented as mean CE (\pm SD; n=5). Algal sizes (equivalent spherical diameter; ESD) are based on modes obtained using a Coulter Multisizer IIe[®] instrument and were: *C. calcitrans* – 5 μ m; *I. galbana* – 5 μ m; *T. pseudonana* – 6.78 μ m; *Rhinomonas* sp. – 7.42 μ m; *Tetraselmis* sp. – 7.56 μ m. There was no significant difference in the CE of the different microalgae species (ANOVA, $P > 0.05$).

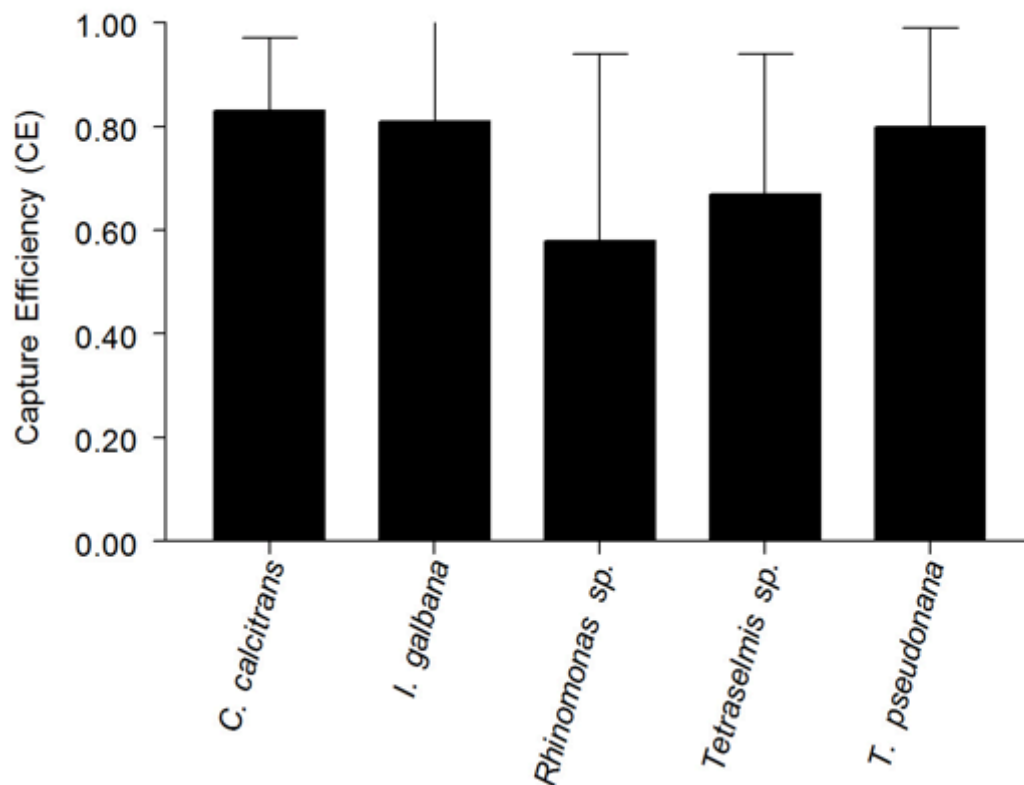


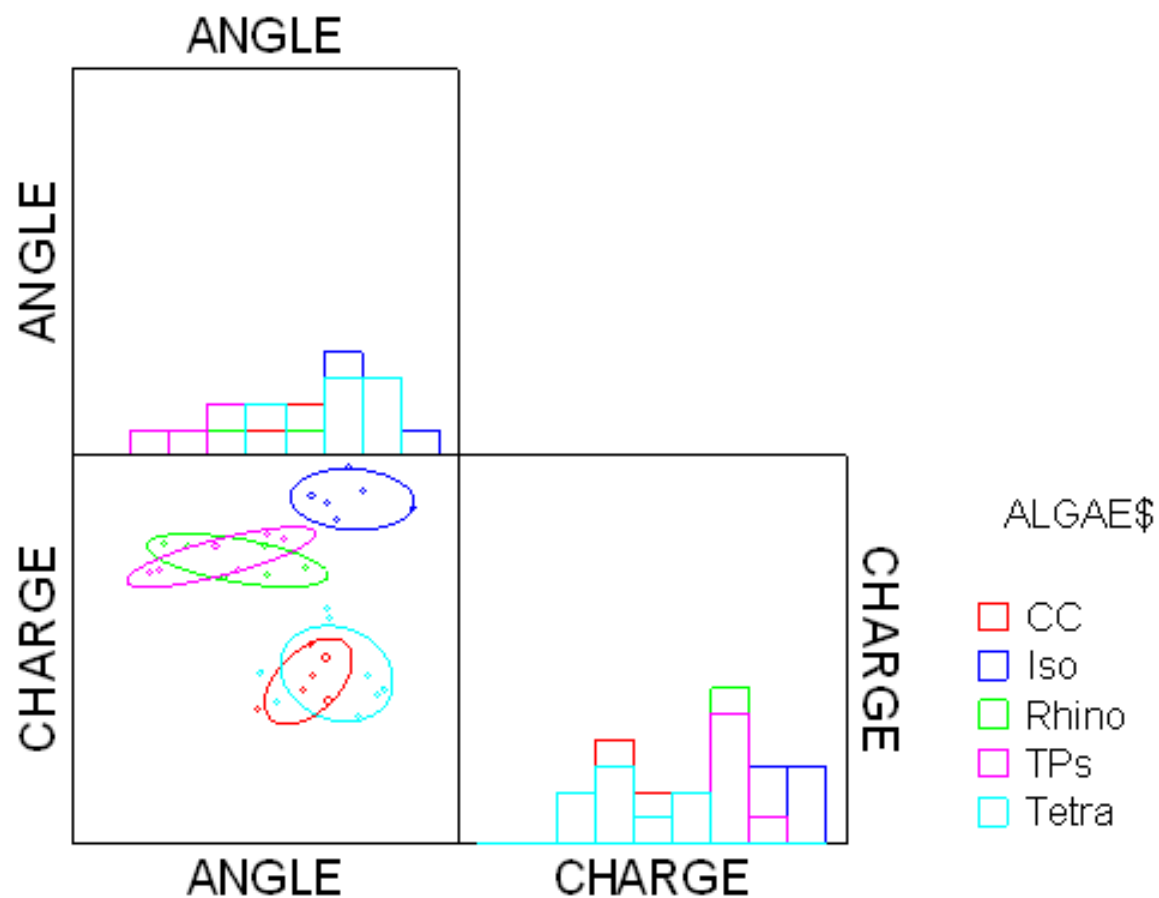
Table 1. Summary of measurements and surface characteristics for each microalgal species. Values for replicates with statistically significant surface properties (student t-test, $p < 0.05$) are shown separately.

Algae species	Growth regime (light:dark, temp)	Concentration (cells/mL)	Size (μm)	Surface charge	Contact angle ($^{\circ}$)
<i>Isochrysis galbana</i>	14:10, 22°C	1.3×10^7	4.4	-6.3 ± 0.6	96.5 ± 5.8
<i>Tetraselmis</i> sp. (rep 1)	14:10, 18°C	7.5×10^5	7.8	-13.5 ± 1.9	93.6 ± 6.4
(replicate 2)		5.6×10^5	7.2		102.3 ± 2.6
<i>Chaetoceros calcitrans</i> (rep 1)	14:10, 22°C	1.5×10^6	5.3	-14.5 ± 0.5	92.9 ± 4.3
(replicate 2)		1.8×10^6	5.4	-13.1 ± 0.3	
<i>Thalassiosira pseudonana</i> (rep 1)	14:10, 18°C	4.7×10^6	6.3	-8.7 ± 0.6	82.3 ± 3.9
(replicate 2)		4.7×10^6	5.1		89.4 ± 3.4
<i>Rhinomonas</i> sp.	14:10, 22°C	1.5×10^6	7.3	-9.0 ± 0.6	86.4 ± 6.0

Table 2. Classification matrix for algae from the robust discriminant analysis. The left column indicates the assigned algal species for each of the two characteristics. The top rows indicates the algae species grouping classified by the model based on similarities and differences between surface properties, with a percentage given for the “correctly” classified values based on the model’s predictive capabilities. The model was unable to correctly classify all of the *Tetraselmis* sp. and *T. pseudonana* replicates based upon input values.

Classification Matrix (Cases in row categories classified into columns)						
	CC	Iso	Rhino	TPs	Tetra	% Correct
CC	6	0	0	0	0	100
Iso	0	5	0	0	1	83
Rhino	0	0	5	1	0	83
TPs	1	0	1	4	0	67
Tetra	3	0	0	0	5	63
Total	10	5	6	5	6	78

Figure 3. Scatterplot matrix (SPLOM) of surface properties for algal species. Bottom left panel indicates the confidence ellipses for predicted algae classification based upon surface property values. The histograms indicate the spread and frequency of values within a particular surface characteristic.



Appendix C

Algal agglutination assay protocol

1. Introduction

To examine further how particle surface properties may mediate selection, a series of algal agglutination assays was carried out (adapted from Pales Espinosa et al., 2010) to examine the interactions between algal cell surfaces and the mucus from the pallial organs of different bivalve species. This protocol was designed to determine if the mucus collected from the pallial organs of bivalves agglutinate microalgal cells differently depending upon 1) site of mucus production (i.e. gill versus labial palp) and 2) species of microalgae?

2. Methods

2.1 Mucus collections

To collect mucus from the pallial organs of the eastern oyster *Crassostrea virginica* and the blue mussel *Mytilus edulis*, the shell was carefully opened using a scalpel to sever the pedal retractor muscles. Special attention was given to not cut into the major tissues to avoid cross contamination of mucus lectins with the hemolymph. Using a 60-mL syringe, the tissues were flushed with filtered, artificial seawater (ASW) to rinse off hemolymph and debris. Using a sterilized laboratory swab, the mucus from the gill was collected by gently wiping the swab over the filaments. The swab was used

to collect mucus from the frontal surface and from between the gill lamellae. The tip of the swab was cut off and placed in a Falcon tube with 60mL of filtered ASW. The Falcon tubes were maintained on ice to prevent degradation of the mucus proteins. This process was repeated for both sets of gills. Swabs from 3-10 animals were pooled into one sample. The procedure was repeated for the labial palps. Forceps were used to separate the palps and expose the lateral oral groove, and the swab was run along it to collect the mucus from between the palps. The cotton swabs with the collected samples were agitated gently in a shaker table to help separate mucus from swab tips. If needed, samples were frozen until used. Before the agglutination assays, each mucus collection was poured into a new falcon tube, and a mixing rod was used to squeeze excess water from the swab tips. Once squeezed, the tips were discarded. The collected samples then were centrifuged at 1000 X g for 10 minutes at 4°C. The supernatant was filtered through a 0.22-µm sterile Millipore filter and placed in a new falcon tube. The pellet was discarded. The collected samples were kept cold by submersing them in ice until ready for use in the agglutination assays.

2.2 Protein measurements

In order to confirm concentration of mucins collected, a standard curve was prepared following the instructions in the Pierce BCA protein assay kit (Thermo Scientific). Samples were also prepared following kit instructions, and incubated at 35°C for 30 min. 25 µL of each known concentration and the collected mucus samples (unknowns) were collected, placed in cuvettes and read on a Turner Systems

Spectrophotometer at 520 nm. The sample absorptions were plotted against the corresponding known concentrations, and standard curve and regression equation were calculated. The regression equation was used to calculate the protein concentrations of the extracted mucus (Table 1). Generally a concentration greater than 0.1 mg/ml is enough to agglutinate red blood cells (RBCs).

2.3 Agglutination assays

A 250- μ L aliquot of one microalgal species (at a concentration of 10^6 cells/mL) was added to a clean falcon tube. The algal sample was centrifuged at 500 X g for 5 min at 4°C. The supernatant was discarded, and the pellet re-suspended in 1.25ml of filtered seawater (0.22 μ m). The cells were checked under a light microscope for viability (i.e., no lysis following centrifugation and re-suspension). If cells were viable, 30 μ L of the algae and 30 μ L of reactant (mucus) were added to a micro plate well. This process was repeated in triplicate for each extraction. For a negative control, 30 μ L of filtered ASW was used as a reactant instead of the extracted mucus. Mixtures were incubated for 1 hr at room temperature. After the hour, an inverted microscope (Olympus IX70) was used to check for agglutination of the microalgal cells (20x). A micrograph was taken of each well for classification of agglutination (Figure 1). An index of 0 to 5 was used to classify agglutination. An index of 0 meant no agglutination took place (e.g. mucus sample matched the ASW control) and an index of 5 meant most of the algal cells were agglutinated.

3. Results and conclusions

Mucus collections generally resulted in low protein concentrations, regardless of species or pallial organs sampled (Table 1). More animal pooled for the mucus collections resulted in higher protein concentration counts, but there was no relationship between animal size and amounts of protein in the mucus collected. This meant a high number of animals (~10 individuals) had to be sacrificed for each sample replicate. Due to the low protein concentrations in the collected mucus samples, levels of agglutination for the tested microalgal species were low (Table 2). Samples collected from *M. edulis* resulted in no agglutination for some of the microalgal species tested (*Dunaliella salina* and *Pavlova lutheri*). Mussel mucus resulting in the highest agglutination was the gill samples incubated with *Rhodomonas salina* cells and the labial palp samples incubated with the *Prasinocladus marinus* cells. Mucus samples collected from *C. virginica* pallial organs generally resulted in lower agglutination of the algal cells than the mucus collected from the mussels. Gill mucus samples resulted in agglutination for only one of the microalgae tested (*P. lutheri*), and this was low (index of 0.5) compared to the mussel samples. Mucus collected from the labial palps also resulted in low agglutination levels for *P. lutheri* and *R. salina* (index of 0.9 and 0.3, respectively) and no agglutination for the other microalgal species tested. Due to the low levels of agglutination, it is difficult to reach accurate conclusions regarding the results of the assays. The differences in agglutination between bivalves, and among the site of mucus collection and microalgal species, do indicate the surface carbohydrates of the microalgae are differentially recognized. Lastly, the materials and efforts required to obtain quantifiable results suggest these methods are too crude as a means to gain

insight into the mechanisms of particle selection. These findings indicate that other, more targeted methods (e.g. lectin profiles, see Pales Espinosa et al. 2010) are a better option for characterizing chemical surface properties of the types of particles typically selected by suspension-feeding bivalve molluscs.

Figure 1. Sample agglutination assay images of microalgae incubated with mucus from the pallial organ of *Mytilus edulis*. Cells of *Prasinocladus marinus* were exposed to A) seawater control, B) mucus extracted from the gill, or C) mucus extracted from the labial palp of blue mussels, *M. edulis*. Agglutinated algae cells (arrow) in each mucus sample were compared to the seawater control sample (no agglutination).

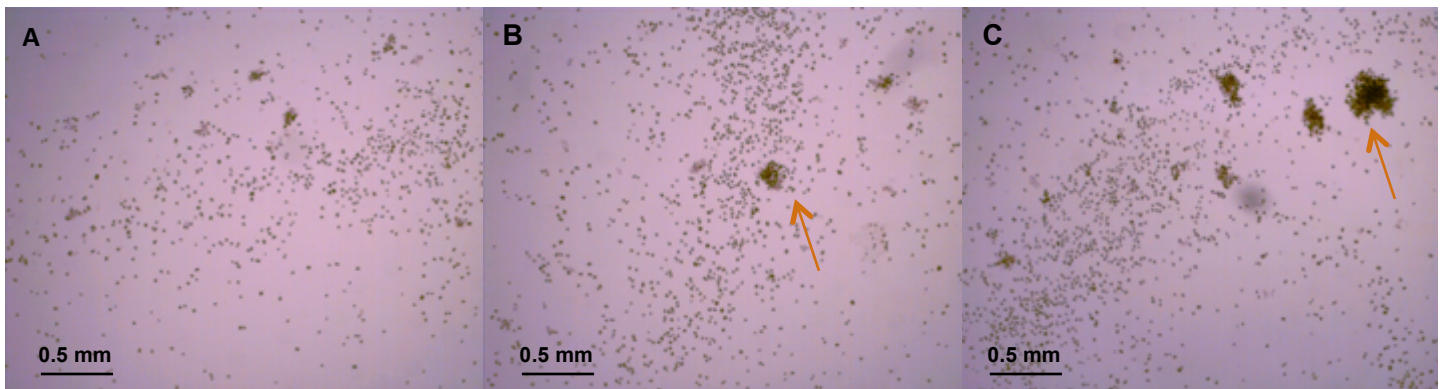


Table 1. Summary of mucus extractions for agglutination assays. Individual animals were pooled to increase the concentration of lectins in sample extract. The protein concentration in each extract was determined using the Pierce BCA protein assay kit. None of the pooled samples reached the 1 mg/ml protein concentration needed for RBC agglutination.

Bivalve	Pallial-organ sample	No. of animals pooled	Shell length (mm; mean \pm SD)	Protein concentration (μ g/ml)
<i>Mytilus edulis</i>	Gill #1	6	61.3 (3.0)	0
	Gill #2	6	56.2 (2.8)	0.5
	Gill #3	12	58.5 (2.6)	117.2
	Labial palp #1	6	61.3 (3.0)	0
	Labial palp #2	6	56.2 (2.8)	0
	Labial palp #3	12	58.5 (2.6)	35.9
<i>Crassostrea virginica</i>	Gill #1	4	83.5 (11.0)	17.1
	Gill #2	4	79.2 (4.7)	39.1
	Gill #3	4	84.3 (8.0)	17.9
	Labial palp #1	4	83.5 (11.0)	42.3
	Labial palp #2	4	79.2 (4.7)	28.3
	Labial palp #3	4	84.3 (8.0)	26.3

Table 2. Summary of agglutination assay results. Five different microalgae were incubated separately with mucus collected from either the gill or labial palps of two species of bivalves. Agglutination in the presence of mucus was compared against a control incubation of filtered seawater. An index of zero indicates no agglutination of the microalgal cells, and an index of 5 indicates all cells in the sample were agglutinated. Samples were run in triplicate, and the numbers represent the average of four biological replicates.

	<i>Mytilus edulis</i>			<i>Crassostrea virginica</i>		
	Gill mucus	Labial mucus	palp	Gill mucus	Labial mucus	palp
<i>Dunaliella salina</i>	0	0		0	0	
<i>Prasinocladus marinus</i>	0.5	1.5		n/a	n/a	
<i>Rhodomonas salina</i>	1.5	0.8		0	0.3	
<i>Pavlova lutheri</i>	0	0		0.5	0.9	
<i>Tetraselmis chui</i>	0.5	1		0	0	

Appendix D

Effects of surface properties on the adherence of particles to mucus produced by suspension-feeding bivalve molluscs

1. Introduction

Mucociliary processes have been demonstrated to mediate particle capture and transport by suspension-feeding bivalve molluscs (see Ward 1996). Many bivalve molluscs capable of selecting particulate material on the gill have two types of gill filaments, ordinary and principal, as well as two tracts/grooves to transport captured particles. The mucus on these tracts varies in cohesiveness (Beninger et al. 1997). The mucus on the dorsal tract of the gill, for example, produces loose “slurry” that carries particles to the labial palps for further processing, and facilitates action on this organ to loosen and discriminate between individual particles. Mucus on the ventral margin of the gill is more cohesive and produces a compact string with tightly-bound particles, and these particles generally are rejected as pseudofeces. Mucus covering the gills and labial palps also has been demonstrated to vary in composition and abundance depending upon the species of bivalve and the loci of selection (Beninger et al. 1992, 1993). The sea scallop *Placopecten magellanicus*, for example, possesses acidic mucopolysaccharide producing goblet cells on the crests of the gill ordinary filaments which function mainly in particle rejection. The principal filaments of this bivalve’s gills function mainly in particle acceptance and possess goblet cells that produce basic mucopolysaccharides (Beninger et al. 1993). The gills of the mussel *Mytilus edulis*, on the

other hand, possess goblet cells that produced mixed muco-polysaccharides, a mucus with medium cohesiveness that is consistent with the non-separation of particle selection and cleaning functions of the gill (Beninger et al. 1993). These differences in mucus type may be reflective of the role the gill plays in particle discrimination in these two species, with the gill being able to discriminate between particles in scallops but not in mussels.

Non-specific physicochemical interactions between particles and the mucus covering the pallial organs have been shown to mediate a passive selection mechanism in both *Crassostrea virginica* and *Mytilus edulis* (Rosa et al. 2013). Combined with findings on the role of specific mucus-associated lectins in particle selection (Pales-Espinosa et al. 2009, 2010), these studies suggest an expanded role of mucus in the feeding process (e.g., capture, selection, and differential transport). Other studies have demonstrated that mucus interacts with different prokaryotic cells. Live bacterial cells, for example, have been found to adhere preferentially to mucus over other surfaces (Krovacek et al. 1987), a property that serves as an aid to transmission for several infectious bacteria such as *Vibrio vulnificus* (Amaro et al. 1995) and *V. cholerae* (Boutonnier et al. 2003) to fish. The chemotactic response of bacterial cells to mucus also has been reported to be a characteristic of the mucus components (LaFrentz and Klesius 2009).

The goal of this project was to gain further insight into the mechanistic aspects of particle selection and determine how different surface properties affect particle capture and selection. In particular, adhesion assays were optimized to examine experimentally how the surface properties of a given particle can affect its interactions with bivalve

mucus, and whether some properties are more likely to affect particle adhesion than others. This study tested the null hypothesis that different particle surface properties do not result in differential adhesion to mucus produced by the bivalve pallial organs. Findings of this study could aid in the development of a rapid assay for screening and modeling particle selection capabilities in bivalve molluscs.

2. Materials and methods

2.1 Mucus adhesion assay protocol

To determine how different particles adhere to mucus collected from the pallial organs of suspension feeding bivalve molluscs, methods were adapted from Amaro et al. (1995) which was originally used for encapsulated bacteria. These methods could be utilized readily to examine adhesion of synthetic microspheres with differing surface properties. Microalgae cells, however, will lyse under this protocol, so alternate methods were developed to examine the adhesion properties of different microalgal species. For the adhesion studies, mussels (*Mytilus edulis*) and oysters (*Crassostrea virginica*) were cleaned and all biofouling, and encrusting organisms were removed. A sterilized (with 70% ethanol bath) scalpel was used to sever the adductor muscle and remove one of the valves. The animal then was rinsed with ultra-filtered seawater (FSW, 0.22µm), and placed in FSW to allow the gill to expand and mucociliary movement to resume. A sterile, 50-µl loop was used to remove mucus from the pallial organ of interest (gill or labial palp) gently, and the mucus was transferred to a clean and pre-labeled slide. Mucus collection was done carefully so as not to damage the tissue, particularly the

filibranchiate gill of mussels. If tissue was noted on the collecting loop, the sample was discarded. The process was repeated 4 times on the frontal surface of the two gill lamellae, for a total of 200 μL per slide. For the oyster assays, mucus was collected from both gill and labial palp tissues separately. After obtaining a thin layer over most of the slide surface, the mucus covered slides were allowed to dry overnight at room temperature. After drying, the slides were submerged in absolute methyl alcohol (HPLC grade) for 20 minutes, which fixed the mucus to the slide. Fixed slides then were dried for at least 1 hr at room temperature.

2.2 Microsphere adhesion

Previously-characterized particles of interest (Table 1) were prepared within 24 hr of being used in the adhesion assays. Microspheres in suspension (e.g. YG, YG-carboxyl) were sonicated for 30 min. Microspheres were washed in ultrapure water (Milli-Q; MQ) and centrifuged for 10 min at 1,500rpm. The supernatant was decanted, and particles were resuspended in MQ (3x) and stored in MQ. A suspension of the microspheres was prepared in artificial seawater (0.22- μm filter, salinity 28) at a density of 10^4 particles mL^{-1} . Mucus-covered slides and control slides (no mucus) then were incubated with the suspension in glass watch bowls (100mL of suspension in each, each bowl held 4 slides). Slides were incubated for 1 hr at room temperature with gentle mixing on a shaker table. After incubation, slides were rinsed in distilled water (DI) three times to remove non-adhering particles and dried overnight at room temperature. After drying, slides were fixed with methyl alcohol as described above. For imaging, 60

images were taken systematically of the dried slide using a camera attached to a Nikon microscope (10X mag.). The number of particles attached was counted using Image J. Amaro et al. (1995) used staining techniques prior to imaging their slides. In the current study, some counts were performed after staining with Alcian blue, but staining did not enhance the images. Given the image contrast and size of the microspheres, accurate counts could be obtained without staining. The number of particles in all of the images from one slide was added, and total number of adhering particles was compared between slides (mucus vs no mucus).

2.3 Microalgal adhesion

For the adhesion work using live microalgae, the study was repeated with the following modifications. Algae were washed of media by centrifuging the cultures at 500x g for 5 min and then re-suspending in filtered isotonic seawater (FSW). After centrifugation, algae were checked under the microscope to ensure cells remained viable and intact prior to use. A suspension of 10^5 per mL was prepared, which provided for robust counts of adhering numbers of cells, and used the same day. Slides (mucus-covered and control) were incubated with the algal suspension as described above. After incubation, slides were washed with ammonium formate (30 ppt) to remove salts and any non-adhering algal cells. Once the slide dried for a few minutes, it was fixed by submersion in glutaraldehyde (1% concentration, buffered to a pH of 7.8 with sodium hydroxide) for 20 min. Slides then were dried under a chemical hood for ~2 hrs and imaged as described above.

2.4 Statistical analyses

The numbers of microspheres adhering to the mucus-coated slides were compared to the control slides using a one-way ANOVA (H_0 = means are equal). Treatments were done in triplicate for each mucus type (gill or labial palp collection). Significant differences in the types of microspheres that adhered to the mucus resulted in rejection of the null hypothesis (H_0 = physicochemical surface properties did not result in differential particle adhesion to bivalve gill mucus). For all statistical analyses, an alpha level of 0.05 was used.

3. Results and Discussion

3.1 Microsphere adhesion assays

For the mussel assays, mucus collections were only done on the gill tissue. Collection of the labial-palp mucus was attempted, but the size of the pallial organ, generally very small and thin in this species, precluded reliable collections. Accordingly, all data are for adhesion of particles to the gill mucus. Silica, silica-cyano, and silica-amino microspheres adhered at significantly higher numbers to the mucus covered slide than to the control slide (Figure 1). The numbers of silica-cyano and silica-amino particles adhering, however, were approximately two orders of magnitude (100-fold) lower than for the silica microspheres. The silica microspheres adhered in lower numbers to the gill mucus collected from oysters compared to mussels, and there was significantly higher adhesion to mucus collected from the labial palp (Figure 2). The

mean number of silica-cyano spheres adhering to the mucus-covered slides was significantly higher than the number adhering to control slides, although no difference in adhesion between the two mucus types was found. The silica-amino microspheres in contrast, adhered in significantly higher numbers to the mucus collected from the gills than mucus from the labial palps. For the polystyrene particles, adhesion to the mucus slides was significantly lower than for the control (no mucus) slide. For the carboxylated polystyrene particles there was no significant difference in the mean number of particles adhering to the mucus slides vs. the control slides (Figure 2).

3.2 Microalgal adhesion assays

Overall adhesion of microalgal cells to both the control and mucus-covered slides was lower than the adhesion of synthetic particles. For mucus collected from mussels, the mean number of *Tetraselmis chui* cells adhering to the control slides was significantly higher than to slides with gill mucus (Figure 3). This high incidence of adherence is likely a result of the attachment and stalk (“pad”) of *T. chui* that is present in certain stages of growth. The “pad” allows the microalga to remain adhered to various surfaces during tide changes, and increases the cell’s adhesion to glass (Grant and Vadas 1976). There was no significant difference in adhesion of *Pavlova lutheri* cells between the control and mucus-covered slides. Cells of *Dunaliella salina* and *Rhodomonas salina* adhered in significantly higher quantities to the gill mucus than to the control slides. For mucus collected from oysters, *T. chui* also adhered to control

slides at significantly higher numbers than to slides with gill or labial palp mucus (Figure 4). There was no significant difference in adhesion of the algal species to mucus from the gill vs. labial palp (note: for *R. salina* no labial-palp sample was collected). The numbers of *D. salina* and *R. salina* cells adhering to mucus-covered slides was higher than to the control slides. There was no significant difference in the adhesion of *P. lutheri* cells to the control slides versus the mucus-covered slides.

3.3 Surface properties versus particle adhesion

Of all the microparticles or algal cells tested, silica microspheres demonstrated the highest adhesion to gill mucus from the mussel, and labial-palp mucus from the oyster. This microsphere was the most hydrophilic (contact angle = 7.2°) and had the highest surface charge (-11.6 mV) of all the particles tested (Table 1). For control slides, the highest particle adhesions occurred with the polystyrene and carboxylated polystyrene particles, both of which were the most hydrophobic (contact angle = ca. 112° - 113°) microspheres used in the study. Compared to other microalgal cells tested, *T. chui* adhered to all surfaces, especially control slides, in the highest numbers. This species was the most hydrophobic alga (contact angle = 105°) and had the lowest surface charge (-7.5 mV) of any microalgal species tested. As described above, the stalk of this species also could provide a direct contact point to enhance adhesion to surfaces. The fact these cells did not adhere to the mucus in higher numbers, however, suggests this morphological feature does not make it more adhesive to the mucosal components. There was no significant difference in adhesion of *P. lutheri* between the control and the mucus-coated slides, and this microalgae had the most hydrophilic of

the surfaces (contact angle = 66.4°) and had the most negative surface charge (-16.5 mV).

An adhesion index was calculated by dividing the average number of particles that adhered to the mucus slides by the average number of particles that adhered to the control (no mucus) slide for each microsphere and cell type. Calculated indices for each particle type were plotted against the surface charge and contact angle for each particle. An adhesion index for *T. chui* was not included in this analysis, as the presence of the pad made it more adhesive to the glass slide than the other microalgae tested. Data from Chapter 4, in which neoglycoprotein-coupled sugars were covalently bound to microspheres and characterized for use in adhesion and capture assays, were also included in this analysis (Table 1). A weak linear relationship was found between adhesion index and particle/cell surface charge for mucus collected from the mussel gill ($R^2 = 0.13$), the oyster gill ($R^2 = 0.18$), or the oyster labial palps ($R^2 = 0.11$), and nonlinear relationships were explored. It was found that a 3rd order polynomial best-explained particle adherence to mucus collected from the mussel gill ($R^2 = 0.36$), the oyster gill ($R^2 = 0.41$), or the oyster labial palps ($R^2 = 0.39$). These fits were not significant ($P > 0.05$), but the R-squared values were higher than those obtained from linear and 2nd order polynomial relationships, indicating that the non-linear relationship should be further explored. For contact angle measurements, a linear relationship best described the data. Particle adherence to mucus collected from the oyster gill and labial palp was not significant ($P > 0.05$). Particle adherence to the mucus collected from the mussel gill had a significant and negative correlation with the surface hydrophobicity (R^2

= 0.44, $P < 0.05$). Generally, particles with the more hydrophobic surfaces had higher adherence to the mucus-covered slides.

Figure 1. Numbers of microspheres adhering to mucus collected from the gills of the mussel *Mytilus edulis*. Number of particles is based on 60 images from each of the slides. * indicates significant difference in particle adherence between the control slide (no mucus) and the mucus covered slide. Data are presented as means \pm SD, n = 5-6.

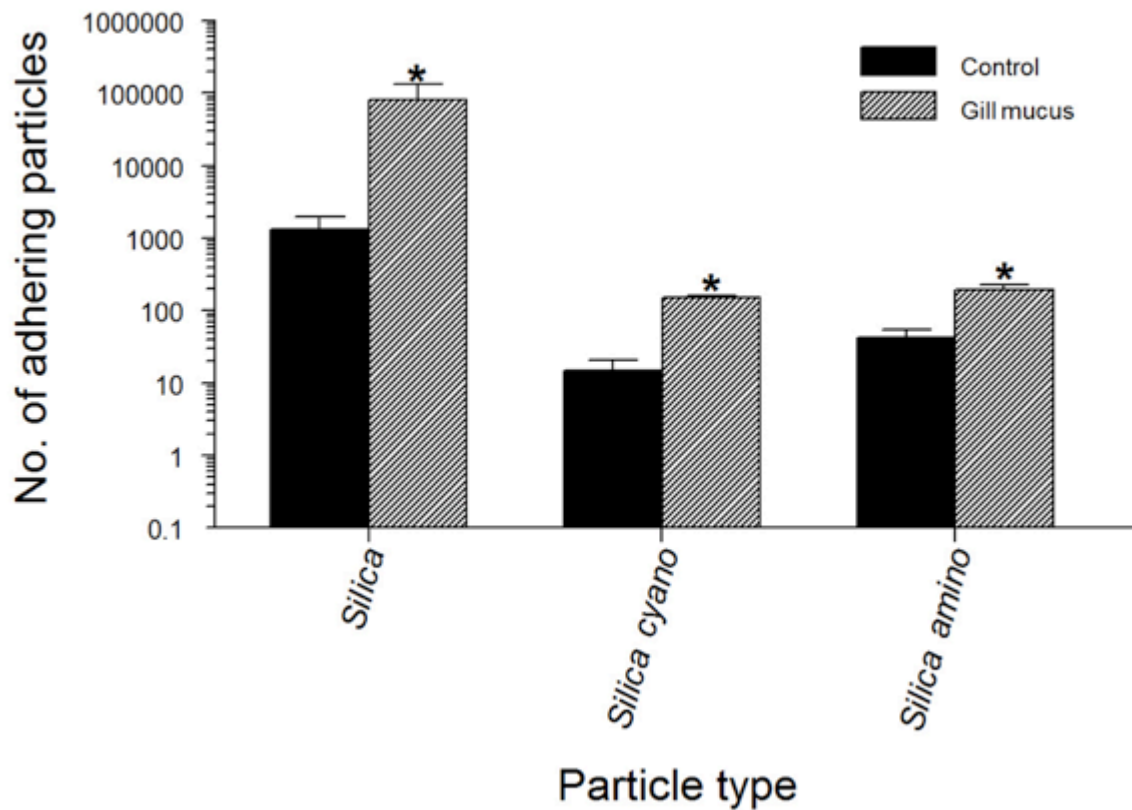


Figure 2. Numbers of microspheres adhering to mucus collected from the pallial organs of the eastern oyster *Crassostrea virginica*. Number of particles is based on 60 images from each of the slides. * indicates significant difference in adherence of particles between the control slide (no mucus) and the mucus covered slide. Data are presented as means \pm SD, n = 5-6.

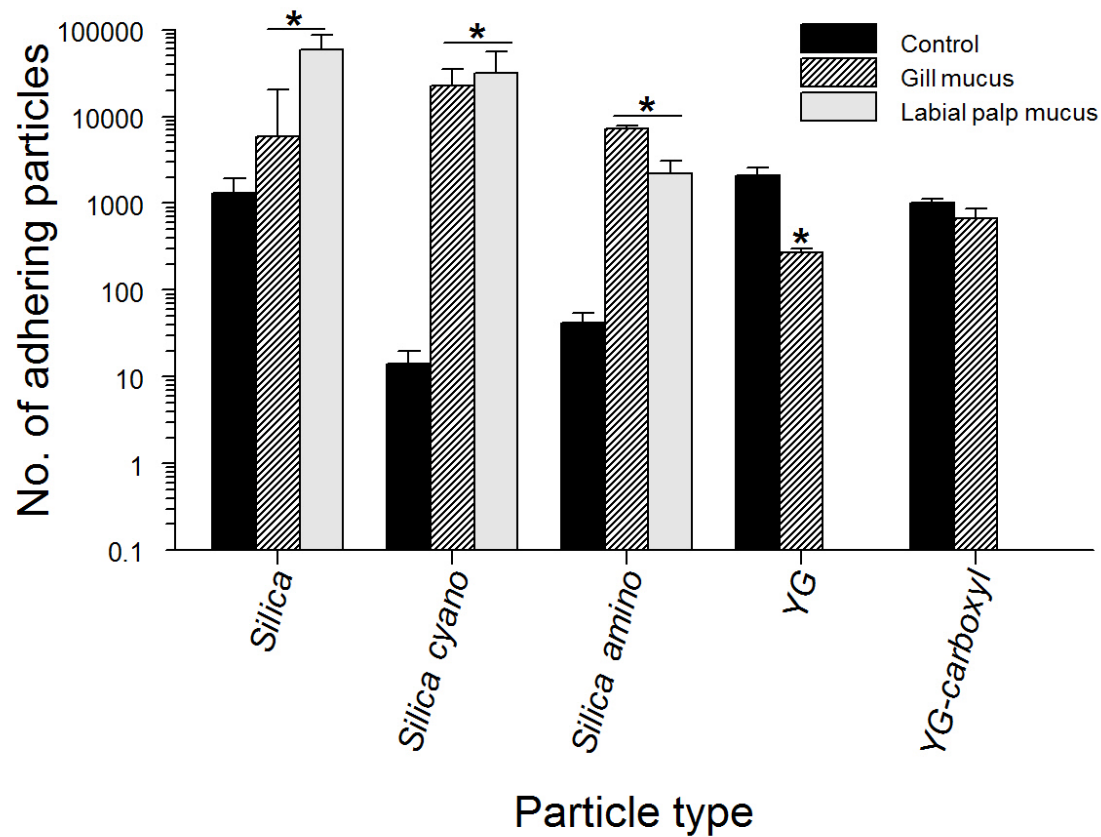


Figure 3. Numbers of algal cells adhering to mucus collected from the gills of the blue mussel *Mytilus edulis*. Number of particles is based on 60 images from each of the slides. * indicates significant difference in adherence of cells between the control slide (no mucus) and the mucus covered slide. Data are presented as means \pm SD, n = 3-5.

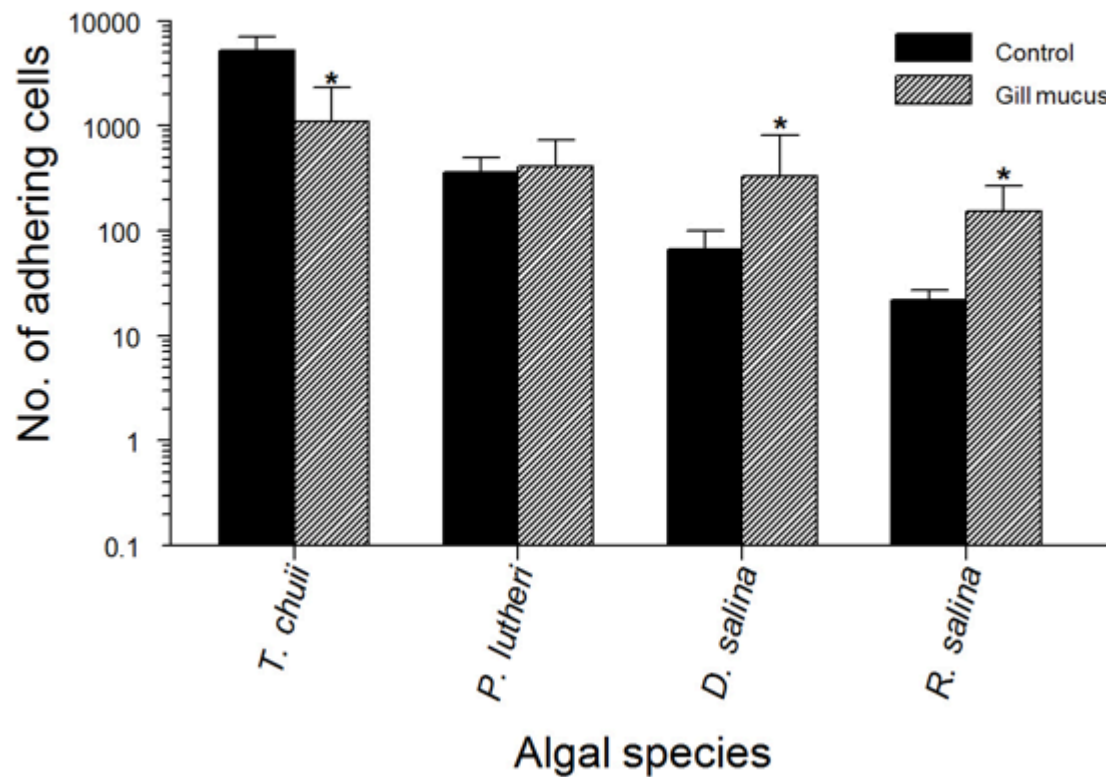


Figure 4. Numbers of algal cells adhering to mucus collected from the pallial organs of the eastern oyster *Crassostrea virginica*. Number of cells is based on 60 images from each of the slides. * indicates significant difference in cell adherence between the control slide (no mucus) and the mucus covered slide. Data are presented as means \pm SD, n = 4-7.

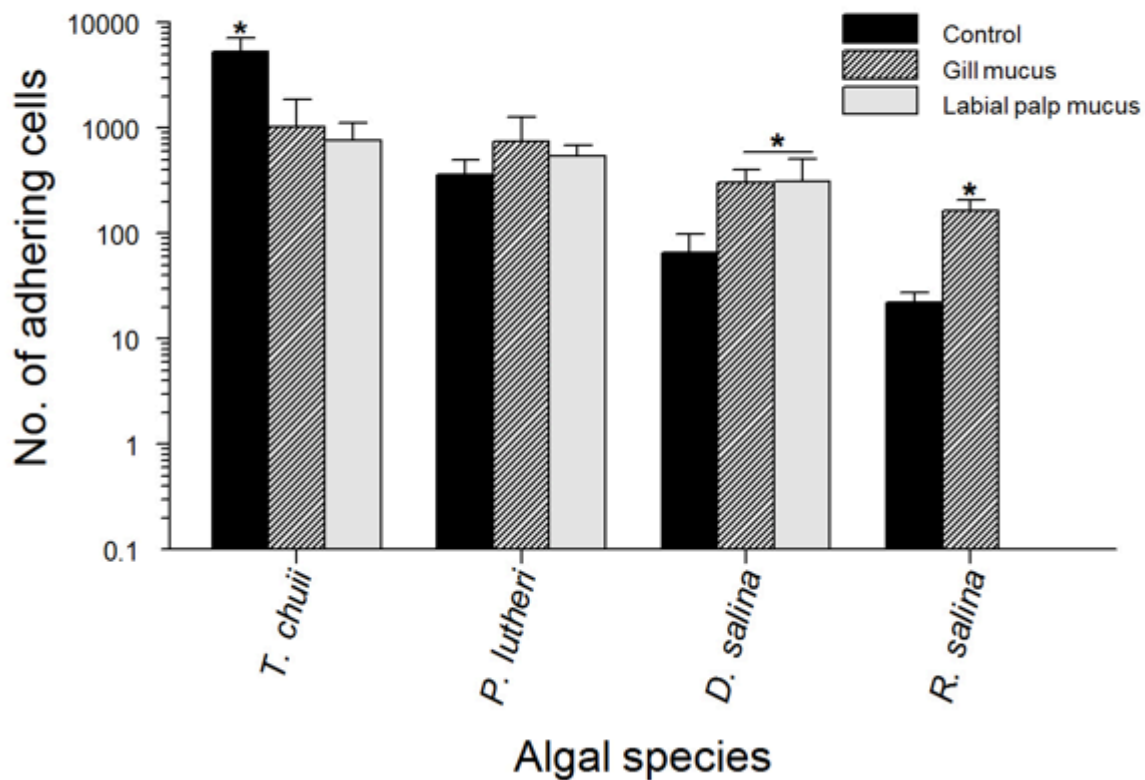


Figure 5. Adhesion index for each particle type used in the adhesion assays as a function of surface charge. A third-order polynomial was found to best explain the relationship between particles adherence and charge for each of the mucus samples (mussel gill, oyster gill, or oyster palp). The polynomial regression was not statistically significant, though the R-square values were improved over a linear best-fit line and 2nd order polynomial. Roughly, particles with the higher (< -10 mV) and lower (> -2 mV) surface charges adhered in greater numbers than particles within a mid-range of surface charge (-4 mV to -10 mV). Data are individual points calculated as a mean number of particles adhered to the mucus over the mean number of particles adhered to the control (no mucus) slide, n= 3-7.

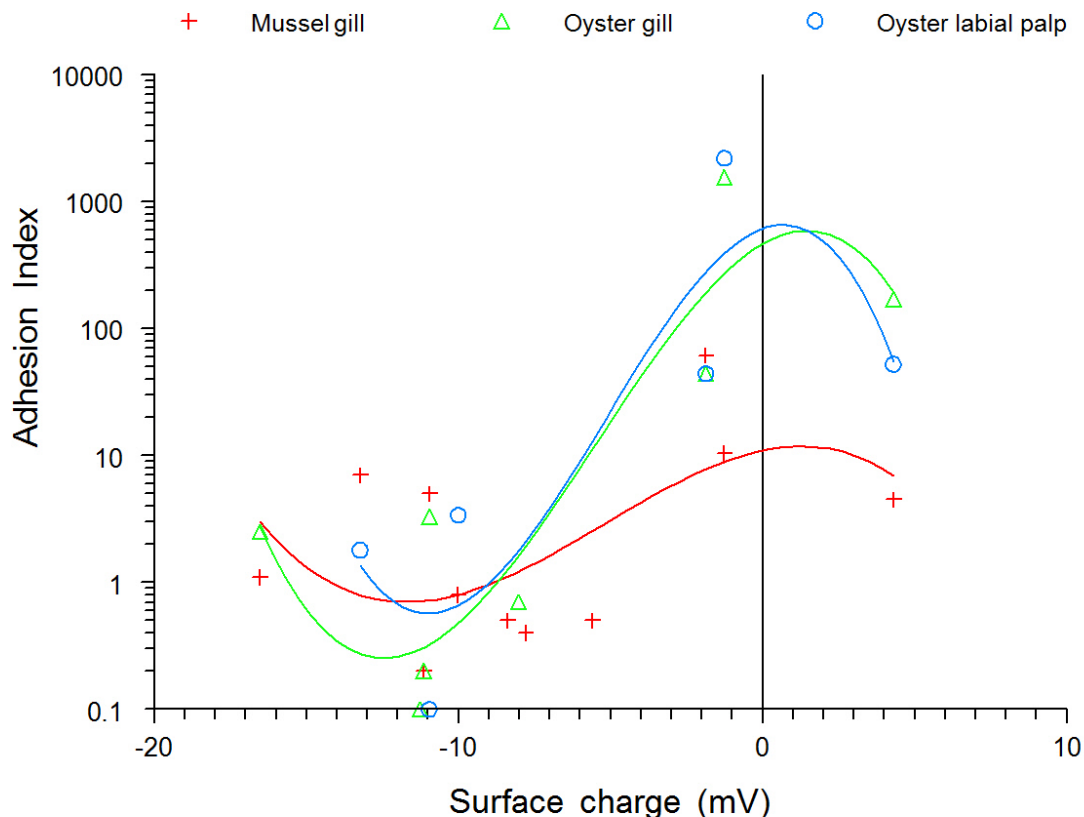


Figure 6. Adhesion index for each particle type used in the adhesion assays as a function of the contact angle. A higher contact angle ($> 90^\circ$) indicates a hydrophobic particle surface. A best-fit line was used to explain the relationship between particle adherence and each of the mucus samples (mussel gill, oyster gill, or oyster palp). Particle adhesion to the mucus collected from the mussel had a significant and negative relationship ($P < 0.05$). Roughly, as the particle surface becomes more hydrophobic, the adherence of the different particle types (both synthetic and microalgae) decreases. Data are individual points calculated as a mean number of particles adhered to the mucus over the mean number of particles adhered to the control (no mucus) slide, $n = 3-7$.

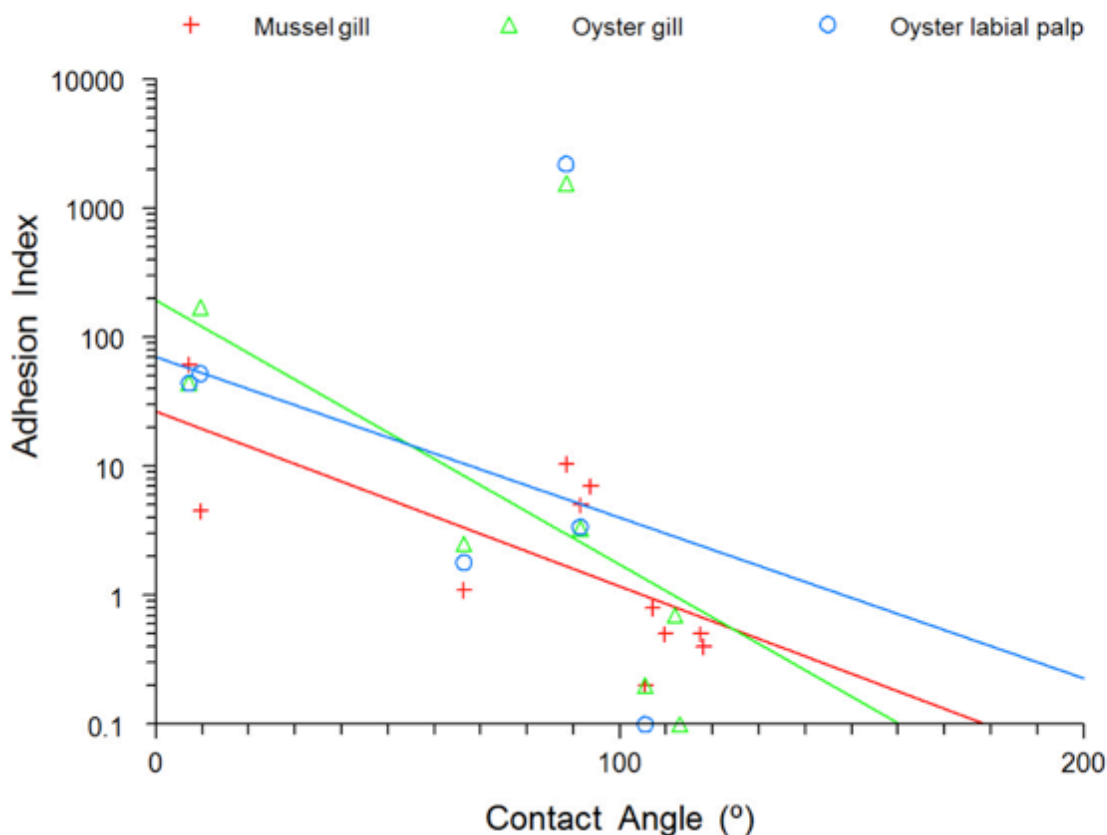


Table 1. Surface properties of the particles used in adhesion assays. Data are presented as means (\pm SD), n=3-7. Particles used in the different adhesion assays include synthetic microspheres, microspheres treated with a coupling kit wash and neoglycoproteins and sugars, or live microalgae cells. All particles used in the adhesion assays were also used in subsequent feeding studies.

Particle type	Contact (°)	Angle	Surface (mV)	charge
Silica	7.2 (1.3)		-1.9 (0.5)	
Silica cyano	88.4 (4.4)		-1.3 (0.4)	
Silica amino	9.6 (0.8)		4.3 (1.3)	
YG (polystyrene)	112.9 (3.6)		-11.3 (1.1)	
YG-carboxyl (YG-C)	111.9 (3.3)		-8.0 (0.9)	
YG-C washed	107.0 (1.0)		-10.0 (1.8)	
YG-C (Bovine serum albumin)	117.4 (4.8)		-8.4 (0.6)	
YG-C (N-Acteyl-glucosamine)	109.8 (2.7)		-5.6 (1.0)	
YG-C (D-mannose)	118.0 (2.4)		-7.8 (1.9)	
<i>Tetraselmis chui</i>	105.5 (4.5)		-11.1 (3.7)	
<i>Pavlova lutheri</i>	66.4 (3.6)		-16.5 (0.7)	
<i>Dunaliella salina</i>	91.5 (2.8)		-11.0 (1.4)	
<i>Rhodomonas salina</i>	93.7 (2.8)		-13.2 (1.0)	

Appendix E

Confocal microscopic examination of particle transport

1. Introduction

The frontal cilia of the gills in suspension-feeding bivalves transport mucus and directly intercept and capture particles (Beninger et al. 1992, Ward 1996, Beninger et al. 1997, Ward et al. 1998). Endoscopic observations of particle movement demonstrate that, in most cases, the mucus layer is very thin and particles are within micrometers of the frontal surfaces ($< 5 \mu\text{m}$), so changes in beat angle or frequency would be translated to changes in particle movement (Ward et al. 1993, Ward et al. 1994). Video endoscopy has proven useful in examining the movement of particles on the gill and palps of bivalves; however, it does not have sufficient resolution to examine movement of cilia. Therefore, confocal microscopy was used to study the movement of the frontal ciliary tracts, and to test the null hypothesis that rate and angle of beat of the frontal cilia do not change when exposed to dissolved food compounds (metabolites).

2. Methods

2.1 Gill preparations

The techniques of Silverman et al. (1999) were adapted to visualize the cilia of isolated gill tissue using a compound microscope. Briefly, gills were isolated and pinned to strips of rubber glued to a Stovall flow cell[®] ("chamber") filled with isotonic ASW (Gainey & Shumway 1991). The chamber was deep enough (8 mm) so as not to

interfere with the movement of the frontal cilia, and allowed for the separate addition of the different metabolites. Microalgal exudates and extracts were prepared and tested separately (see Chapter 5). Metabolites were added to one end of the chamber using a peristaltic pump (300µl per min) and allowed to interact with the frontal cilia. Fluorescent polystyrene microspheres (YG) were used to track particle handling and to examine the effects of the metabolites on gill ciliary activity.

2.2 Confocal microscopic observations

A spectral scanning confocal microscope (Nikon A1R) equipped with an incubation chamber for temperature control and 32-channel spectral detector was used. Changes in ciliary beat and angle were analyzed directly (frame by frame), or by changes in peak brightness (or fluorescence) as the ciliary tips pass through the focal plane (Silverman et al. 1999). Activity of the frontal cilia when exposed to dissolved material was then compared within animals and between species.

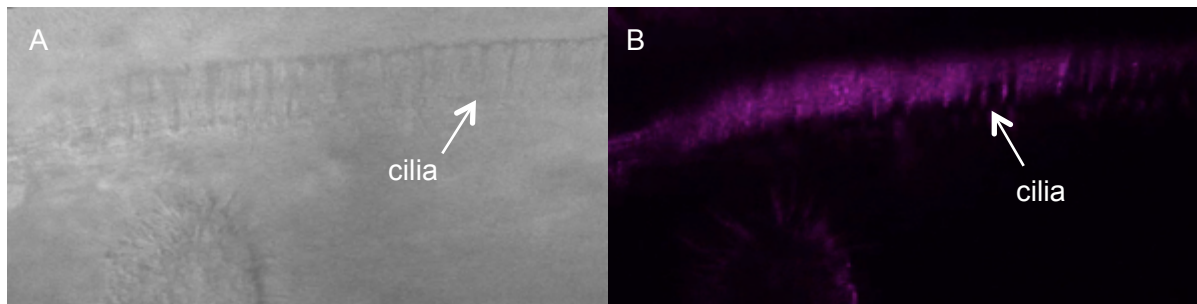
2.3 Statistical analyses

Repeated measures tests were performed to compare differences in the ciliary velocity and direction when the isolated gills were exposed to different dissolved metabolites and filtered seawater controls. If there were significant differences in ciliary movement based upon frame-by-frame comparisons, then the null hypothesis (H_0 =There is no response by the frontal cilia of gill filaments to extracellular metabolites and cell extracts) was rejected.

3. Results and Conclusions

Confocal observations were not carried out as planned, as the delivered microspheres and cilia could not be visualized clearly using this microscope (Figure 1). This was most likely due to the refractive index of both seawater and particles. Observations using the fluorescence filter, even with the high speed of the confocal scope ($\sim 420 \text{ frames sec}^{-1}$), could not accurately measure speed of beating cilia. While the method can be used to measure other aspects of particle capture and transport on the gill, at this time it could not be used to examine the effects of metabolites on the frontal cilia of the gill filaments. To determine effects of metabolites upon ciliary activity, methods which relied on endoscopic observations were used.

Figure 1. Freeze frame of the ark (*Anadara ovalis*) gill visualized using confocal microscopy. Gill sections were visualized using both (A) transparent light and (B) fluorescent filters. Due to interference from water refraction, it was not possible to directly observe particle movement and ciliary activity using the confocal microscope and the Stovall^(C) chambers.



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