

Use of encapsulated live microalgae to investigate pre-ingestive selection in the oyster *Crassostrea gigas*

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Abstract

The involvement of algal chemical cues in the pre-ingestive selection of food particles in *Crassostrea gigas* was studied using a new approach. Live cells of two microalgal species, *Nitzschia closterium* and *Tetraselmis suecica*, were separately entrapped in small alginate microcapsules using an emulsification/internal gelation method. Microcapsule size was adjusted to be within the range of particles ingested by oysters. Using this technique, about 80% of microcapsules had a diameter ranging from 21 to 100 µm. The monitoring of entrapped algae showed that phytoplankton cells remained alive and maintained an active growth for at least 24 days. In particle selection bioassays, adult *C. gigas* were fed a mixture of microcapsules containing the above algae species as well as control empty alginate microcapsules. The comparison of the proportions of each microcapsule type in the diet and in pseudofeces revealed that those containing *T. suecica* were significantly ingested while those containing *N. closterium* were preferentially rejected. Since microcapsule material (alginate matrix) prevented physical contacts between algae cells and oyster feeding organs, this study clearly demonstrates that extracellular metabolites produced by microalgae play a crucial role in the pre-ingestive selection of particles in suspension-feeding bivalves.

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1. Introduction

Suspension-feeding bivalves have developed various strategies for controlling their food intake process. These mechanisms allow bivalves to manage the amount of ingested food, to enhance the nutritive value of particles consumed, and to optimize energy gain. Thus, suspension-feeding bivalves are able to sort and preferentially ingest particles of interest (Loosanoff and Engle, 1947; Morton, 1960). Undesirable particles are then rejected in

pseudofeces. Numerous studies have demonstrated that bivalves can preferentially reject mineral particles (Kjørboe and Møhlenberg, 1981; Newell and Jordan, 1983; Iglesias et al., 1992; Barillé et al., 1997) and detritic matter (Pastoureaud et al., 1996; Ward et al., 1998). Moreover, among mineral matter, large particles (in size or density) are preferentially eliminated in pseudofeces (Defossez and Daguzan, 1996). In contrast, organic matter and, most particularly, intact and viable cells are selectively ingested (Ward et al., 1998). Furthermore, previous studies have demonstrated that several bivalves are able to select one or more microalgal species among a mix composed of several species (Shumway

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et al., 1985; Newell et al., 1989; Bougrier et al., 1997; Cognie et al., 2001). Other studies have supported the idea that bivalves could use chemical factors to discriminate particles (Newell and Jordan, 1983; Shumway et al., 1985; Levinton et al., 2002), but to our knowledge there is only one study that has suggested the role of microalgal ectocrines in pre-ingestive selection (Ward and Targett, 1989). These authors demonstrated that *M. edulis* ingests or rejects uniform microcapsules coated with extracellular phytoplankton metabolites depending upon the microalgal species used to produce these ectocrines. Despite the fact that ectocrine collection may have caused microalgal cells to break leading to the liberation of intracellular compounds, these findings raised several questions regarding the nature of these metabolites, and also about their potential association with the surface of microalgal cells (Ward and Targett, 1989). A good alternative for the investigation of phytoplankton extracellular products is the use of live microalgae entrapped within a permeable matrix allowing the continual production of ectocrines.

Different types of cells have been immobilized in large bioreactors or inside small microcapsules using hydrogel matrices such as alginate, carrageenan, gellan or agar (see review by Groboillot et al., 1994). Entrapped animal cells (pancreatic islet cells) have been optimized and transplanted (Soonshiong et al., 1993; Garfinkel et al., 1998) to treat patients with insulin-dependent diabetes. In biotechnological fields such as the food industry, immobilized cells (bacteria, yeast, algae, fungi) have been widely used to produce secondary metabolites enhanced by immobilization (review from Smidsrød and Skjåk-bræk, 1990; Groboillot et al., 1994). Entrapped algae have been used for wastewater treatment (Garbisu et al., 1991; Khattar et al., 1999; Mallick, 2002; Chen, 2003; Jimenez-Perez et al., 2004), for phytoplankton long-term storage (Romo and Perez-Martinez, 1997; Chen, 2003) and for continuous microalgal production for bivalve feeding (Chen, 2001, 2003). Procedures and techniques of cell immobilization are numerous and have different degrees of cost and technical difficulties (Groboillot et al., 1994).

This study investigates the role of microalgal metabolites in the pre-ingestive selection of the oyster *Crassostrea gigas* using encapsulated living algae. We developed a simple and non-destructive emulsification/internal gelation method initially used by Larisch et al. (1994) to produce small (<100 µm) alginate microcapsules that are able to preserve cell metabolic activity and that are suitable in feeding experiments with oysters. Microcapsules containing two distinct algae species were used to investigate pre-ingestive selection by oysters.

2. Materials and methods

2.1. Microalgal cultures

Investigated strains were supplied by Dr. G. Wikfors (Milford Marine Laboratory, Milford, CT, USA) and consisted of a Pennatae diatom, *Nitzschia closterium* Wm Smith, and a Prasinophyceae, *Tetraselmis suecica* (Kylin) Butcher. Algae were separately grown in F/2 medium (Guillard, 1982) at 15 °C under 14-h light/10-h dark cycle with 50 µE m⁻² s⁻¹ light intensity. Cells were harvested in the exponential phase and concentrated by gentle centrifugation (300 g, 15 min, 14 °C).

2.2. Production of alginate microcapsules

The microcapsules were prepared by an emulsification/internal gelation method adapted from Larisch et al. (1994). Forty milliliters of a UV sterilized sodium alginate solution (2% w/v, prepared with seawater enriched with F/2, pH 7.5, 28‰) were intensively mixed with 2 ml of a calcium carbonate solution (10% w/v) and 20 ml of a concentrated mono-algal suspension. The alginate/calcium/algae solution was dispersed in 200 ml of olive oil by stirring for 15 min at 500 rpm to obtain a homogenous emulsion. While maintaining the agitation, 40 ml of olive oil containing 180 µl of glacial acetic acid were then added to the emulsion. After 15 min gelation, water (500 ml) and hexane (80 ml) were added with gentle mixing, allowing the microcapsules to migrate to the aqueous phase. The clear oil was decanted and the microcapsules filtered off, washed several times with a calcium chloride solution (2% w/v in distilled water), and then resuspended in seawater enriched with F/2 medium. Using this technique, microcapsules containing either *N. closterium* or *T. suecica* were produced. Microcapsules without algae were also produced using 20 ml of sterile enriched seawater (F/2) instead of microalgal suspension.

2.3. Microscopic characterization of microcapsules

Microcapsules were microscopically examined using an Olympus Provis microscope equipped with a digital camera. Since the two microalgae have distinct characteristics (shape, color), their identification within the translucent microcapsules was easy. Digital images of each sample were captured and processed using an image acquisition software (Image grabber). The size of a minimum of 600 microcapsules for each preparation was determined.

2.4. Algal growth

Cells entrapped in alginate microcapsules were grown in three replicate Erlenmeyer flasks (250 ml) containing 150 ml of enriched seawater (F/2) under the conditions described above. To determine algal growth, flasks were sampled every other day for a period of 24 days. At each sampling interval, entrapped algae were microscopically monitored. Microcapsules were then dissolved with a sodium citrate solution (0.1 M). The released algal cells were counted using a Newbauer haemocytometer and chlorophyll *a* contents were determined by the method of Lorenzen (1967).

2.5. Feeding experiment

Oysters, *C. gigas*, were collected in Bourgneuf Bay (Atlantic coast of France). Bivalves were scrubbed to remove all epiphytes and encrusting organisms from their shells. They were maintained in static aerated seawater (at 20 °C ± 1) that was replaced every other day. All animals were purged in filtered seawater for a week prior to use in feeding experiments. The bivalves were placed in individual trays supplied with 1 l filtered seawater containing a mixture of microcapsules previously maintained in F/2 for 8 days. A control tray with empty oyster shell was used to measure microcapsule sedimentation. The mixture was composed of about 33% of empty microcapsules, 33% of encapsulated *N. closterium*, and 33% of encapsulated *T. suecica*. Microcapsules were kept in suspension using a magnetic stirrer (gentle to avoid spreading pseudofeces). Total concentration of microcapsules was about $9 \times 10^3 \pm 2.5 \times 10^3$ (mean ± SD) microcapsules per ml. Pseudofeces from 11 oysters were collected 3 h after the beginning of their production. For each pseudofeces sample, over 300 microcapsules were counted totaling about 3300 microcapsules.

2.6. Data analysis

A series of goodness-of-fit tests (*G* test) was performed using raw counts to compare the proportion of each type of microcapsules in diet and pseudofeces samples. The null hypothesis was that the proportion of each type of microcapsules was the same in diet and pseudofeces. In addition to the comparison of raw data, a sorting efficiency (SE) was calculated in order to examine particle selection (Iglesias et al., 1992). This index was defined as:

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

where *P* and *D* represent the proportion of the particle of interest in the pseudofeces and diet, respectively. A positive SE for a given particle type indicates that it is preferentially ingested (particle type is depleted in the pseudofeces, compared to diet), a negative SE indicates rejection (particle type is enriched in the pseudofeces compared to diet), and zero indicates the absence of active selection. After confirming their normal distributions, calculated SEs were compared to zero using a one sample *t*-test (two-tailed). The null hypothesis was that the selectivity efficiencies were equal to zero (i.e. no selection). In all statistical tests, results were considered significant if $p < 0.05$.

3. Results

3.1. Microscopic characterization of microcapsules

Observed under an optical microscope, the microcapsule appeared intact and spherical (Fig. 1). The smallest ones were attracted and agglutinated to each

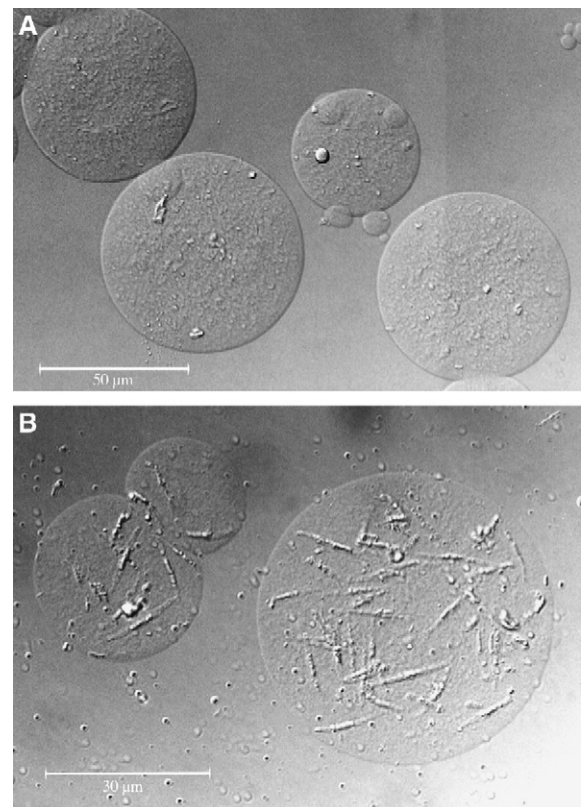


Fig. 1. Empty alginate microcapsules (A) and alginate microcapsules containing *Nitzschia closterium* (B) prepared with the emulsification–gelation method.

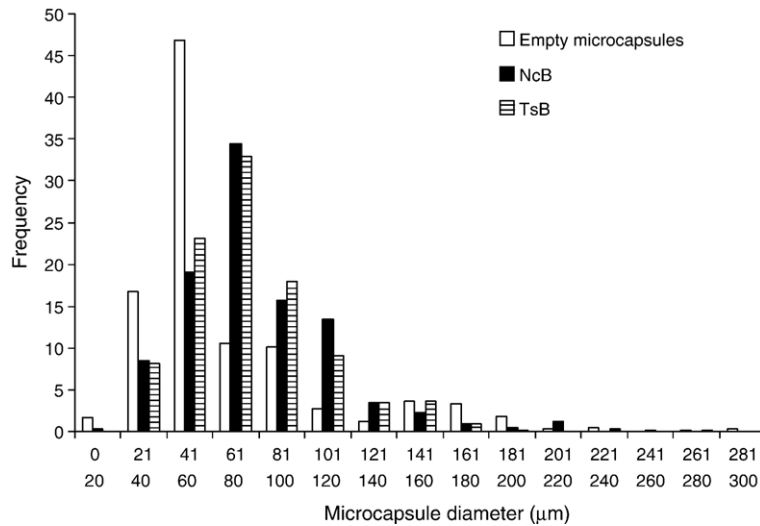


Fig. 2. Size frequency distribution for empty microcapsules, microcapsules containing *Nitzschia closterium* (NcB) and microcapsules containing *Tetraselmis suecica* (TsB).

other, forming small aggregates that were easily disrupted with gentle agitation. Size-distribution analysis demonstrated that 46.8% of empty microcapsules had a diameter ranging from 41 to 60 µm, and that over 75% of these microcapsules had a diameter ranging from 21 to 80 µm (Fig. 2). Microcapsules containing algae were slightly larger than empty ones: 34.5% of microcapsules containing *N. closterium* and 33% of microcapsules containing *T. suecica* had a diameter ranging from 61 to 80 µm. For both algae species, the 41 to 100 µm range contained over 75% of all microcapsules.

3.2. Algal growth

The growth of microalgal cells was determined using microalgal cell count and chlorophyll *a* content. For both microalgal species, the microscopic monitoring of the cultures showed an excellent stability of microcapsules (spherical pattern, no sign of degradation) and a clear increase in cell numbers within each microcapsule. At the beginning of the test, *N. closterium* abundance per microcapsule was equal to 15.5 ± 2.1 (mean \pm SD, Fig. 3). After a short lag phase (2 days), the exponential phase led to an abundance of 64.6 ± 2.4 cells

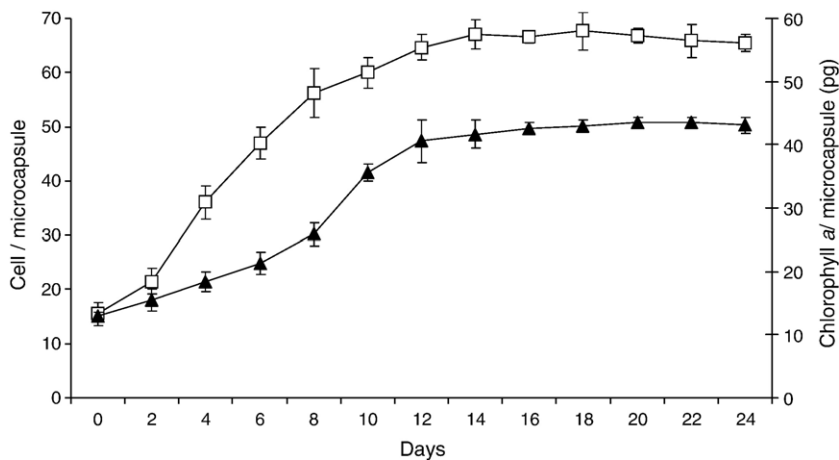


Fig. 3. Growth of *Nitzschia closterium* cells entrapped in alginate microcapsules expressed as cell counts (□) or chlorophyll *a* (▲) per microcapsule (mean \pm SD).

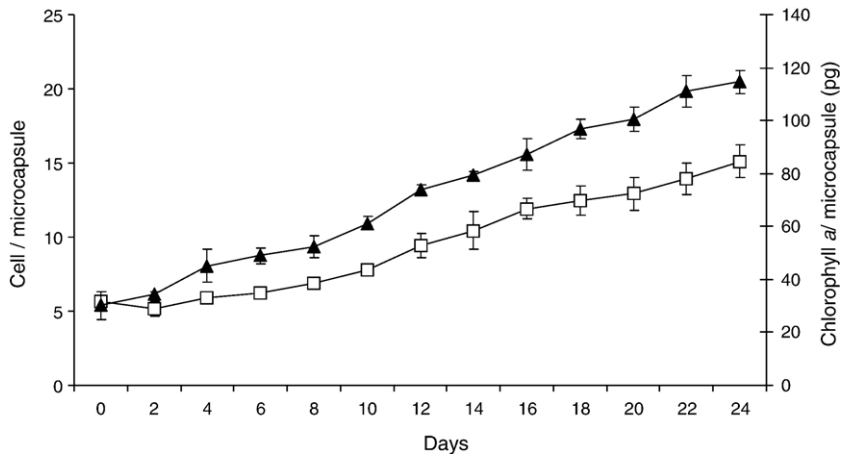


Fig. 4. Growth of *Tetraselmis suecica* cells entrapped in alginate microcapsules expressed as cell counts (□) or chlorophyll *a* (▲) per microcapsule (mean±SD).

per microcapsule (after 12 days) followed by the stationary phase where the abundance was 65.5 ± 1.3 cells per microcapsule at the end of the experiment (24 days). The amount of chlorophyll *a* per microcapsule increased from 13.0 ± 0.6 pg per microcapsule at the beginning of the test to 40.6 ± 3.4 pg per microcapsule at the end of the exponential phase (12 days). This content averaged 43.1 ± 1.3 pg per microcapsule at Day 24.

The mean abundance of entrapped *T. suecica* was equal to 5.6 ± 0.4 cells per microcapsule at the beginning of the experiment (Fig. 4). After a 2-day lag phase, cell number increased to 15.1 ± 1.1 cells per microcapsule at Day 24. No stationary phase was observed in the final stage of the experiment. The amount of Chlorophyll *a*

per microcapsule showed a similar pattern. Chlorophyll *a* averaged 30.2 ± 5.2 pg per microcapsule at the beginning of the experiment, and increased to 114.6 ± 4.3 pg per microcapsule at Day 24.

3.3. Feeding experiments

Although the targeted proportion of each microcapsule type was 33%, an *a posteriori* count revealed the following proportions in the feeding blend: $25.8 \pm 6.3\%$ (mean±SD) of empty microcapsules, $35.1 \pm 7.4\%$ of microcapsules containing *N. closterium* and $39.1 \pm 6.0\%$ of microcapsules containing *T. suecica* (Fig. 5). These proportions remained stable over the entire duration of the experiment indicating that differential settling of different types of microcapsules did not occur. The

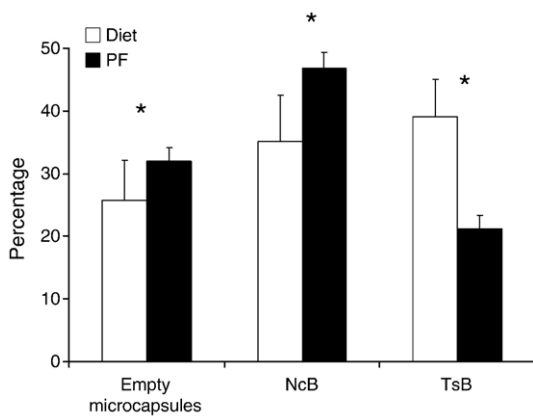


Fig. 5. Percentage (mean±SD) of each type of microcapsules in food supply (Diet) and pseudofeces (PF) of *Crassostrea gigas*. NcB and TsB: microcapsules containing *Nitzschia closterium* or *Tetraselmis suecica*, respectively. * indicates significant difference for each type of microcapsule between diet and pseudofeces (goodness-of-fit G_{test} , $p < 0.001$).

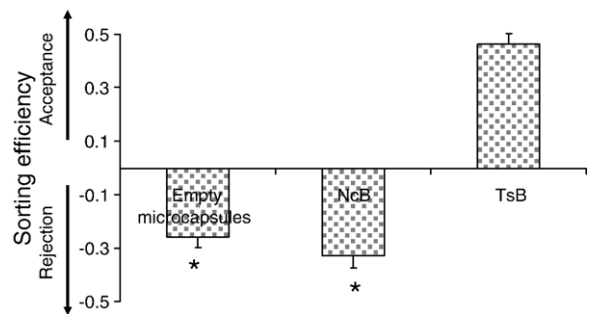


Fig. 6. Sorting efficiency (SE, mean±SD) of empty microcapsules, microcapsules containing *Nitzschia closterium* (NcB) and microcapsules containing *Tetraselmis suecica* (TsB). TsB were significantly depleted in pseudofeces while NcB and empty microcapsules were significantly rejected (* indicates significant ingestion or rejection, $p < 0.001$, two-tailed *t*-test).

proportion of empty microcapsules and microcapsules containing *N. closterium* significantly increased in oyster pseudofeces (32.0 ± 2.1 and $46.9 \pm 2.5\%$, respectively) compared to the diet (Fig. 5). In contrast, the proportion of microcapsules containing *T. suesica* significantly decreased from $39.1 \pm 6.0\%$ in diet to $21.2 \pm 2.2\%$ in pseudofeces. Statistical analyses performed on selection indices (Fig. 6) showed that oysters preferentially ingested microcapsules made with *T. suesica*, while they rejected those entrapping *N. closterium* or empty microcapsules ($p < 0.001$, *t*-test).

4. Discussion

In order to be ingested, microcapsules must be within the size range of particles ingested by oysters. Most bivalve suspension-feeders, including oysters, are able to capture particles greater than $5 \mu\text{m}$ in diameter with near 100% efficiency (Møhlenberg and Riisgård, 1978; Riisgård, 1988). The maximum size of ingested particles is not clearly established but it can reach 1 mm if the particle has a small width (Paulmier, 1972). However, considering a spherical particle, several studies reported an efficient uptake of particles with diameters as high as $200\text{--}300 \mu\text{m}$ (Paulmier, 1972). It has been demonstrated that *C. gigas* is able to efficiently ingest the large ($250 \times 150 \mu\text{m}$) diatom *Coscinodiscus perforatus* (Cognie et al., 2003). The entrapment technique used in our study is well adapted to the investigation of pre-ingestive selection because it produces microcapsules that are within the range of ingested particles. According to Larisch et al. (1994), this method produces microcapsules having diameters ranging from $50 \mu\text{m}$ to $1000 \mu\text{m}$ with a dominance of those in the range of $500 \mu\text{m}$ (Poncelet et al., 1995). One of the solutions to reduce microcapsule size is to increase the viscosity of oil used during emulsion (Esquisabel et al., 1997). The olive oil used in this study present a viscosity of 77 mPaS, which is one of the highest among vegetal oils (Fountain et al., 1997) and allow the production of smaller microcapsules. Over 75% of the microcapsules obtained in this experiment had diameters ranging from 21 to $100 \mu\text{m}$, independently of the algal species they contained.

Additionally, the technique we used is not toxic or destructive for algae as witnessed by the significant growth (cell abundance and chlorophyll *a* content) of entrapped *N. closterium* and *T. suesica*. In numerous studies, authors have shown that algae immobilized in hydrogels (alginate, agar) maintain ultrastructural integrity and physiological activities (Tamponnet et al., 1985; Hertzberg and Jensen, 1989). Metabolism is

generally activated by encapsulation and secondary compounds are highly produced (Tamponnet et al., 1985; Groboillot et al., 1994; Pane et al., 1998; Lebeau et al., 1999; Chen, 2003). Another advantage of the use of alginate gel is that this natural biopolymer is porous allowing the diffusion of nutrients and metabolites (Gombotz and Wee, 1998). Using electron microscopy, it has been determined that microcapsules made with alginate have small pores ranging from 5 to 200 nm in diameter. These pores allow the diffusion of small molecules while diffusion of larger molecules is affected but not stopped (Gombotz and Wee, 1998).

Similarly-sized spherical alginate microcapsules were obtained for both *N. closterium* and *T. suesica* despite difference in the shape and size of these two microalgal species. All of the above characteristics, including spherical shapes with sizes in the range of particles ingested by bivalve suspension-feeders, activated metabolism of entrapped microalgal cells, and microcapsule porosity, make microcapsules an attractive approach to investigate the role of extracellular metabolites in pre-ingestive selection. Our results clearly demonstrated that, compared to the diet supply, *C. gigas* pseudofeces were significantly depleted in alginate microcapsules containing *T. suesica* while enhanced in alginate microcapsules containing *N. closterium*. That is, *T. suesica* is preferentially ingested and *N. closterium* is preferentially rejected. These results are supported by previous studies, describing *T. suesica* as a good diet for *Ostrea edulis*, *C. gigas* and *Ruditapes decussatus*, especially in combination with other algal species (Walne, 1970; Helm, 1977; Helm and Millican, 1977; Langdon and Waldock, 1981; Albertosa et al., 1996; Robert et al., 2001). In contrary, the diatom *N. closterium* is known to produce a higher rate of biodeposition in *Crassostrea virginica* (Tenore and Dunstan, 1973) and in *C. gigas* (Barillé et al., 2003) compared to other tested algae. Bougrier et al. (1997) have demonstrated that *N. closterium* was clearly rejected in *Mytilus edulis* and *C. gigas* pseudofeces.

Although several studies have described the ability of bivalve suspension-feeders to select food particles before ingestion and to reject undesirable particles as pseudofeces (Pastoureaud et al., 1996; Ward et al., 1998; Ward and Shumway, 2004), the mechanisms of particle selection are not well understood. Some authors emphasize the importance of particle nature (mineral or organic), particle size or shape, or particle quality (live versus dead algae for example). Similarly, although anatomical location of particle selection has been established in several bivalve species (Ward et al., 1998; Cognie et al., 2003; Beninger et al., 2004), chemosensory

cells on these feeding organs have not clearly been identified (Beninger et al., 1990) and the specific mechanism allowing bivalves to sort desirable from the undesirable algae species is still unknown. Metabolites released by algae, including polysaccharides, nitrogenous substances, amino acids, fatty acids and vitamins (Shimizu, 1996) can be recognized by grazers and influence their feeding behavior (Shaw et al., 1995; Leising et al., 2005 and numerous references therein). Several studies concerning alimentation in marine animals have shown that these metabolites, which are able to trigger the reflex of feeding are sometimes simple molecules. In *Hydra* sp., a tri-peptide (glutathione) specifically activates feeding response (Lenhoff, 1961; Pierobon et al., 1997). Importance of amino acids in this process is supported by the studies of Baldwin (1995) which demonstrated that the ingestion rate of larval *C. virginica* were higher for algae cells with a low carbon:nitrogen ratio (*Thalassiosira pseudonana* for example). The study by Ward and Targett (1989) demonstrated that microalgal metabolites (obtained by filtering algal cultures) are involved in particle selection. Thus, *M. edulis* preferentially ingest or reject commercial synthetic microcapsules impregnated with algal metabolites depending upon the algae species used. Because alginate gel isolates microalgae and avoids direct contact with bivalve tissue, our results suggests that microalgae produce substances that are able to cross the matrix layer surrounding microalgal cells through alginate pores, inducing significant feeding response in *C. gigas*. These substances do not seem to diffuse to neighboring microcapsules (empty microcapsules for example), or at least, remain sequestered in the boundary layer during the feeding experiment as significant differences were observed in the uptake of different microcapsule types. If compounds responsible for the selective feeding have a hydrophobic nature, they would have a tendency to remain associated with the microcapsules. Similarly, such compounds will also remain associated with the microcapsules if they are anchored to the surface of microalgal cells. Our hypothesis supports the speculations made by Ward and Targett (1989) suggesting that ectocrines on the surface of microalgae or in a boundary layer surrounding cells are more important than dissolved ectocrines in particle selection by bivalves. Recently, Beninger and Decottignies (2005) demonstrated that the perifrutular envelope of *C. perforatus* plays a role in the pre-ingestive selection of scallops *Pecten maximus*. Molecules associated with microalgal cell envelopes can reach the surface of alginate microcapsules triggering the selection process observed in our study.

5. Conclusion

In conclusion, we devised a technique allowing the production of alginate microcapsules that contained viable and metabolically active microalgal cells. These microcapsules were used to investigate pre-ingestive selection in the oyster *C. gigas*. Results demonstrated the ability of oysters to select their food particles without direct contact between food processing organs and microalgal cells, suggesting the involvement of chemical cues in the selection process. Active metabolites produced by microalgal cells are able to cross the alginate matrix and induce a particle selection by oysters. This approach provides a new way to investigate molecules involved in particle selection processes in suspension-feeders.

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