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Wanted: alive and not dead: functioning diatom status is a quality cue for the suspension-feeder *Crassostrea gigas*

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Understanding the quality cues which govern the grazing of phytoplankton by suspension feeders is essential to understanding seston dynamics and benthic–pelagic coupling. We studied the effect of functioning cellular status on selection of two common coastal diatoms, Coscinodiscus perforatus var. pavillardii and Actinoptychus senarius, grazed upon by the commercially farmed oyster, Crassostrea gigas Thunberg. Two contexts were investigated, corresponding to those naturally encountered by oysters and their prey: (i) diatoms (C. perforatus var. pavillardii) larger than the size allowing selection on the gill (hence subject to selection on the labial palps only) and (ii) diatoms (A. senarius) within the size range allowing selection on the gill (hence subject to selection on both the gills and labial palps). Both intact and naturally dead cells possessed a perifrustular envelope, but the naturally dead cells lacked intracellular organic content. Oysters were able to discriminate between intact and naturally dead (empty) cells, both in the absence of prior selection on the gill and subsequent to prior selection on the gill. In contrast to previous findings in scallops, functioning cellular status is thus an important determinant of diatom selection and ingestion by oysters. This fine degree of discrimination may have evolved as an adaptation to the high turbidity, low food-quality estuarine environment typically colonized by oysters. The reduced organic content of rejected diatom cells in oyster pseudofaeces may affect the organic content of biodeposits, and subsequently the dynamics of nutrient release to the water column during mixing and resuspension.

INTRODUCTION

The interplay between primary production and primary consumers, especially suspension-feeders, is a dominant, structuring feature of coastal marine systems (Kautsky and Evans, 1987; Asmus and Asmus, 1991; Dame, 1993; Gili and Coma, 1998; Riisgård, 1998; Porter *et al.*, 2004; Widdows *et al.*, 2004; Cranford *et al.*, 2005; Giles and Pilditch, 2006). Many suspension-feeders selectively ingest certain phytoplankton, based upon size (Hugues, 1975; Shumway *et al.*, 1985; Defossez and Hawkins, 1997) and presence of "ectocrines" (Ward and Targett, 1989). Indirect evidence of selection based on organic content of phytoplankton has been brought forward for several suspension-feeding species (Newell and Jordan, 1983; Bayne *et al.*, 1993; Bacon *et al.*, 1998), and more direct evidence of qualitative selection has been advanced using endoscope-directed sampling from processing structures within the bivalve mantle cavity (Ward *et al.*, 1998; Cognie *et al.*, 2003; Beninger *et al.*, 2004).

Recently, it has been shown that it is the perifrustular envelope of diatoms, and not their functioning cellular status, which acts as a quality cue for selection in the scallop *Pecten maximus* (Beninger and Decottignies, 2005). The principal feeding structure of scallops is the gill, which is particularly complex in these species (Beninger and Le Pennec, 2006). Exclusive reliance on

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the perifrustular envelope as a quality cue in this species leads to the inability to discriminate between intact diatoms, and empty, dead diatoms with a vestigial perifrustular envelope. Although theoretical explanations of this phenomenon have been advanced (Beninger and Decottignies, 2005), it is important to verify whether other algal quality cues evoke the selective response in bivalves. While diatom functioning cellular status did not act as a quality cue in *P. maximus* (Beninger and Decottignies, 2005), such a situation might be found in bivalves with an even more complex gill structure. Recently, it has been shown that like *P. maximus*, the oyster *Crassostrea gigas* selectively ingests intact diatoms, as opposed to cleaned frustules of the same species, and that the characteristics of this selection are influenced by seston quantity and quality (Beninger *et al.*, in press), suggesting a highly refined selection mechanism. It is possible to discern the influence of functioning cellular status on the oyster selection response by using cultures of naturally occurring diatoms known to be part of wild oyster diets, since such cultures contain various proportions of intact cells and empty, dead cells with a persistent perifrustular envelope. Such an approach was used to rule out cell content as a quality cue with respect to *P. maximus* (Beninger and Decottignies, 2005).

In the present study, we examine the role of functioning cellular status as a quality cue for suspension-feeding in oysters, *C. gigas*, using two species of naturally occurring coastal diatoms.

METHOD

As pointed out in recent studies on selection in bivalves (Cognie *et al.*, 2003; Beninger *et al.*, 2004; Beninger and Decottignies, 2005), it is important to use algal species representative of the types of algae present or dominant in the natural habitats of the bivalves tested. For the present study, two naturally occurring, coastal, temperate diatom species were sed to test oyster discrimination between dead and live algae, because (i) diatoms are a major constituent of oyster diets, notably at the study site, Bourgneuf Bay (Riera *et al.*, 2002; Decottignies *et al.*, 2007b) and (ii) these species are locally abundant (Paulmier, 1972; Rincé, 1993).

An initial experiment (Experiment 1) was performed using diatoms larger than the critical size for entry to the oyster principal gill filaments. This allows a simple conclusion to be drawn with respect to the discrimination capacity of the labial palps alone. Following a positive result in this experiment, a second experiment was performed (Experiment 2), using a diatom small enough to enter the gill principal filaments. Since in this context, two selection sites may operate (gills and labial palps), it was necessary to use endoscope-directed sampling in order to recover mucus-particle masses independently from each site.

Experiment 1: large (>critical size) diatoms, test for discrimination at the labial palps alone

Test microalgae

A centric diatom of the genus *Coscinodiscus* was isolated from a plankton tow in December 2004 off Noirmoutier Island (47° 04′N, 2° 20′W), and cultured as per Beninger *et al.* (Beninger *et al.*, 2004). The species was identified as *Coscinodiscus perforatus* var. *pavillardii* (Forti) Hustedt (Okuno, 1970), using scanning electron microscopy (SEM) of cleaned frustules (30% hydrogen peroxide incubation at 85–95°C, reaction visually controlled and stopped with several drops of 1 M hydrochloric acid, frustules rinsed several times with distilled water). *Crassostrea gigas* is known to ingest this species (Cognie *et al.*, 2003). A biometric study of the cultured *C. perforatus* var. *pavillardii* was performed on 145 specimens examined under light microscopy, using LUCIA® G 4.80 (for Nikon® France) software, revealing a relatively uniform size distribution (mean sizes ± SD: diameter = 169 ± 5 μm, pervalvar axis = 61 ± 7 μm).

Under light microscopy, intact and naturally dead cells are readily distinguished because the latter appear empty (Beninger and Decottignies, 2005). The persistence of the perifrustular envelope in naturally dead cells has been documented previously (Beninger and Decottignies, 2005).

Specimen sampling and maintenance

Twelve *C. gigas* (mean ± SD: longest shell axis = 8.8 ± 1.7 cm, dry weight = 1.4 ± 0.5 g) were sampled in March 2005 in Bourgneuf Bay, France (47° 1′ 34.7″ N, 2° 1′ 55.9″ W), transported to the laboratory and cleaned of attached organisms and debris. They were maintained under conditions close to those of the sampling site (salinity = 33 ± 0.3, T = 16°C) in an aerated, recirculating seawater system, using 0.22 μm Millipore®-filtered seawater and ultra-violet sterilization. The specimens were fasted at least 1 week prior to experimentation, in order to purge gut contents and ensure feeding responses under experimental conditions.

Experimental protocol

Twelve *C. gigas* were placed in individual flow-through chambers as described in Palmer and Williams (Palmer

and Williams, 1980), with partial separations designed to produce a laminar flow set at $3-4\ \mathrm{L\,h^{-1}}$. The *C. gigas* specimens were affixed to the chamber floors using Futex® food cement.

The *C. perforatus* var. *pavillardii* stationary phase culture was added to the reservoir providing the inflow to the test chambers; temperature and salinity were close to those of the maintenance conditions and the original collection site (mean $\pm$ SD: $T = 17 \pm 1°\mathrm{C}$, $S = 33 \pm 0.1$). Experiment 1 cell concentration was constrained by the concentrations achieved in laboratory large-volume cultures. To allow comparisons with most previous studies, which used test species of different size, cell concentrations were determined both as cells $\mathrm{mL^{-1}}$ and on the basis of comparable organic matter (particulate organic matter, POM). A calibration of cell numbers and organic matter (loss on ignition, 250 mL triplicates: 48 h drying time at 60°C and 4 h ignition time at 450°C; Cognie *et al.*, 2001) was established using the *C. perforatus* var. *pavillardii* culture. Cell concentrations and types were determined in water samples of the experimental chamber inflow at intervals of 30 min, up to termination of the experiment at 120 min, and the mean ($\pm$SD) calculated for the experiment: 90 ± 20 cells $\mathrm{mL^{-1}}$ ($7.2 \pm 0.6\ \mathrm{mg\,L^{-1}}$, loss on ignition method as above), $36 \pm 4\%$ naturally dead cells and $64 \pm 4\%$ intact cells.

It should be noted that the large size of *C. perforatus* var. *pavillardii* translates to low numerical particle concentrations in the experimental medium (90 cells $\mathrm{mL^{-1}}$), but to normally occurring POM concentrations ($7.2 \pm 0.6\ \mathrm{mg\,L^{-1}}$) for the sampling site of Bourgneuf Bay (Haure and Baud, 1995).

Pseudofaeces were allowed to accumulate throughout the period of 120 min, at which time the pseudofaeces were recovered using a micropipette. Pseudofaeces samples were fixed in Lugol's solution for later particle typing and Sedgewick-Rafter counting, allowing the differentiation of live and empty cells (as for the water samples above). Live and dead cells were readily distinguished under light microscopy in the pseudofaeces (Fig. 1). The specimen's faeces were also examined under light microscopy to verify that *C. perforatus* var. *pavillardii* was indeed ingested over the course of the experiment.

Data analysis

SigmaStat 3.1 (Systat®) software was used for all statistical computing. The proportions of each cell type were arcsine-transformed to render the means independent of the variances [$\arcsin\sqrt{(p)}$] (Sokal and Rohlf, 1995) and the resulting normally distributed, homoscedastic data were analysed using a *t*-test, comparing live cell proportions in the pseudofeces and in the chamber inflow. For each specimen, a selection index (SI; Ward *et al.*, 1998; Cognie *et al.*, 2003; Beninger *et al.*, 2004; Beninger and Decottignies, 2005) was calculated as follows:

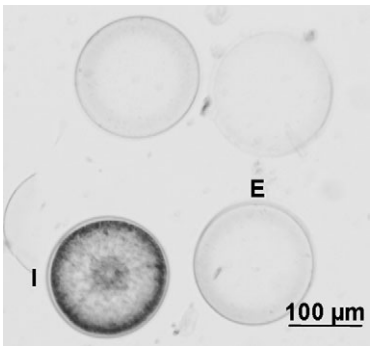

Fig. 1. Intact (I) and empty, dead (E) *C. perforatus* var. *pavillardii* cells from oyster's pseudofaeces, as viewed under light microscope.

$$\mathrm{SI} = \left(\frac{\mathrm{P}}{\mathrm{W}} - 1\right) \times 100$$

where P is the percent of live cells present in the pseudofeces and W the percent of live cells present in the chamber inflow. A mean SI ($\pm$SD) was then determined for the experiment.

Previous work has shown that empty, cleaned cells are preferentially rejected by *C. gigas*, both by the labial palps in the absence of initial selection by the gills (particles > critical size; Cognie *et al.*, 2003), and by both the gills and the labial palps successively (particles < critical size; Beninger *et al.*, in press), so it is clear that *C. gigas* can detect when both intracellular content and perifrustular envelope are absent. In the present study, we used naturally dead cells which are devoid of intracellular content, but which retain the perifrustular envelope, in order to determine the influence of functioning cellular status as a quality cue for *C. gigas*. The null hypothesis was that no distinction would be made between intact and dead cells, as has previously been shown in the scallop *P. maximus*. In this event, no significant difference in the percentages of live and dead cells would be observed between the chamber inflow and the pseudofaeces. The experimental hypothesis was that oysters could distinguish between intact and dead cells, and hence that functioning cellular status is an important diatom quality cue. In this event, the proportion of dead cells would be higher in the pseudofaeces than in the chamber inflow.

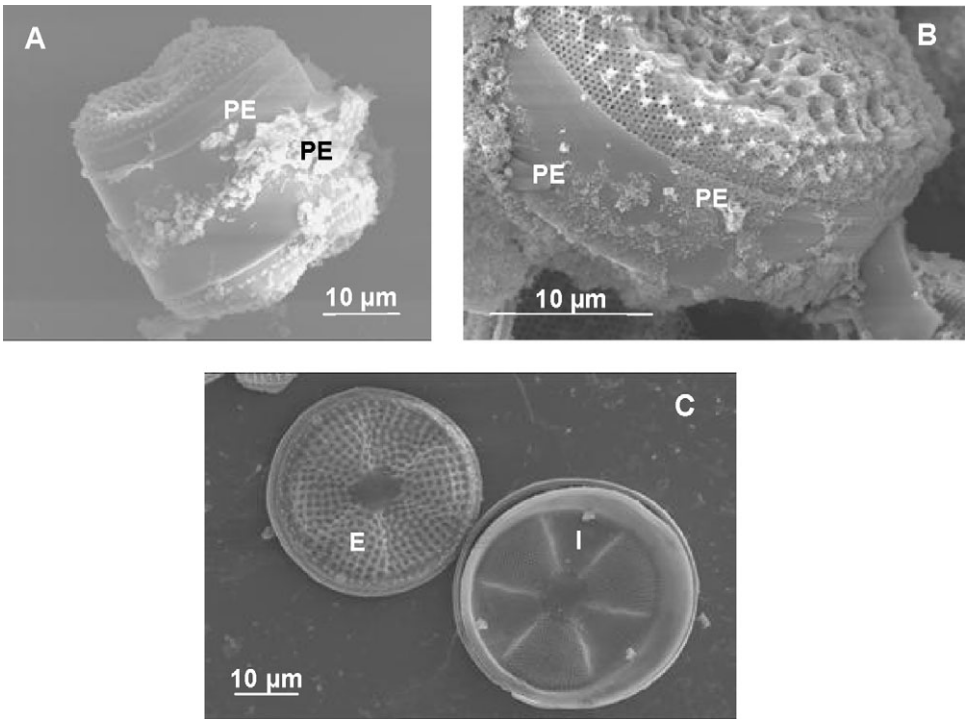

Fig. 2. SEM of *A. senarius* intact (**A**) and dead, empty cells (**B**). PE, dehydrated remnants of perifrustular envelope and associated organic matter after SEM preparation. The specimen in (A) is in division (exponential-phase culture). (**C**) Empty, cleaned frustules [prepared as in Cognie *et al.* (Cognie *et al.*, 2003); Beninger *et al.* (Beninger *et al.*, 2004; Beninger *et al.*, in press)] for comparison, showing the absence of organic matter from both the exterior (E) and interior (I) of the valves. Separation of the valves can be avoided under visually controlled conditions.

Experiment 2: small (<critical size) diatoms, test for discrimination at both gills and labial palps

Test algae culture and characteristics

Actinoptychus senarius was isolated from a plankton net tow off Noirmoutier Island in December 2004 (47° 04′N, 2° 20′W) and cultured as above. Taxonomic identification was performed using SEM as above. Exponential-phase cultures were used in this experiment, providing a greater proportion of live cells. All cells of this culture (intact and naturally dead) possessed a perifrustular envelope, confirmed by SEM observations (Fig. 2). As was previously postulated for *C. perforatus* var. *pavillardii* (Beninger and Decottignies, 2005), the persistence of the perifrustular envelope in naturally dead cells may be due to the presence of stabilizing sulphated mucopolysaccharides (Duke and Reimann, 1977; Volcani, 1981; Bhosle *et al.*, 1995), which also possess bacteriostatic properties (Sasikala and Subramoniam, 1987; Ritchie, 2006). As with the *C. perforatus* var. *pavillardii* cells from Experiment 1, intact and naturally dead cells (mean sizes ± SD: diameter = 49 ± 2 µm, pervalvar axis = 25 ± 27 µm) were readily distinguished under light microscopy, where the latter appeared empty.

Oyster sampling and maintenance

The five *C. gigas* (size range 9.2–13.7 cm longest shell axis) used in Experiment 2 were collected from Bourgneuf Bay in March 2006. The shells were cleaned of epibionts and maintained in a 400 L recirculating seawater tank, at a mean temperature and salinity close to that recorded in the sampling habitat ($T = 15 \pm 0.5$°C; $S = 34 \pm 0.4$). The specimens were fed every other day with a culture of *Skeletonema costatum* (Grev.) Cleve for a 4-week stabilization period and starved for 1 week prior to experimentation.

Experimental conditions

A small aperture was milled in the oyster valves, to prevent breakage of the endoscope optical insertion tube (OIT) when the valves closed periodically. Specimens were used for observation 24–48 h following this operation. They were attached in experimental chambers as above. Experimental chamber algal concentrations were determined as in Experiment 1.

Endoscopic observations and particle sampling

Individuals were allowed to acclimate to the presence of the OIT for 1 h prior to beginning observations. Only individuals feeding normally (i.e. with relaxed velum, deployed tentacles and particles drawn into pallial cavity) were used for observations. The endoscopy protocol has been detailed previously (Cognie *et al.*, 2003; Beninger *et al.*, 2004; Beninger and Decottignies, 2005).

Water from a reservoir containing the *A. senarius* culture was added continually to the experimental chamber, at 8 L h^{-1}, throughout the 2 h experimental period. Mean chamber concentration was 2667 $\pm$ 223 cells mL^{-1} (17.7 $\pm$ 0.9 mg L^{-1}, loss on ignition method as above), similar to the natural concentrations from the sampling site in the wild (Haure and Baud, 1995; Haure *et al.*, 1996). The composition was determined to be 18 $\pm$ 4% (mean $\pm$ SD) naturally dead cells and 82 $\pm$ 4% intact cells in chamber inflows. Endoscopy-directed sampling was performed with a micropipette every 15 min in the chamber water column, the gill dorsal (initial acceptance) tract and the gill ventral (initial rejection) tract. Pseudofaeces were also recovered at the end of each experimental period. All samples were fixed in Lugol's solution for subsequent counts using light microscopy as above.

The proportions of each cell type were determined for a minimum of 300 cells of the total volume sampled over the 2 h course of the experiment for each oyster. The ingestion of *A. senarius* cells by *C. gigas* specimens was verified by microscopic examination of faeces.

Data analysis

SigmaStat 3.1 (Systat®) software was used for all statistical computing. Arcsine transformations of the proportions were performed as above, and prior to each analysis, assumptions of normality and homoscedasticity were tested. Proportions of intact to empty cells in water, dorsal tract, ventral tract and pseudofaeces were compared using one-way parametric ANOVA, followed by SNK a posteriori tests. An SI based on that of Beninger *et al.* (Beninger *et al.*, 2004) was calculated in order to express the degree and direction of selection at each site:

$$SI = \left(\frac{P}{W} - 1\right) \times 100$$

where P is the proportion of intact cells sampled at a given site (ventral tracts, dorsal tracts and pseudofaeces) and W the proportion of intact cells sampled in the ambient water. The selection indices obtained for ventral tracts, dorsal tracts and pseudofaeces were statistically treated in the same manner as the proportions.

The null hypothesis was that no distinction would be made between intact and dead cells, at any of the three processing sites (dorsal tracts, ventral tracts, pseudofeces/labial palps), as has previously been shown in the scallop *P. maximus*. In this event, no significant difference in the percentages of live and dead cells would be observed at any of the processing sites. The experimental hypothesis was that oysters could distinguish between intact and dead cells at one or more of these sites, and hence that functioning cellular status is an important diatom quality cue, with exact significance depending on the number and order of sites at which such distinction is observed. In this event, the proportion of live cells would be higher in the dorsal tracts than in the ventral tracts or experimental chamber water.

RESULTS

Experiment 1: large (>critical size) diatoms, test for discrimination at the labial palps alone

Oyster pseudofaeces from the experimental specimens were significantly depleted in live *C. perforatus* cells, compared to the chamber inflow (Fig. 3, *t*-test, $P \leq 0.001$). The SI indicated strong negative selection with respect to dead cells: -34.4 ± 7.0 (mean $\pm$ SD). Microscopic observation of oyster faeces at the conclusion of the experiment revealed both intact and empty *C. perforatus* cells, confirming that this microalgae was ingested.

Experiment 2: small (<critical size) diatoms, test for discrimination at both gills and labial palps

Compared with the water in the feeding chamber, the oyster ventral (initial rejection) gill tract was significantly depleted in intact *A. senarius* cells, whereas the dorsal (initial acceptance) tract was enriched in intact cells. Furthermore, the oyster pseudofaeces were significantly depleted in intact *A. senarius* cells, even when compared with the ventral gill tract (Fig. 4A).

The selection indices show the extent of these selection trends (Fig. 4B). The ventral and dorsal gill tracts present virtually reciprocal selection indices with respect to intact *A. senarius* cells, whereas the pseudofaeces show a further depletion in intact *A. senarius* cells, compared with the ventral (initial rejection) gill tract. It should be noted that in this setup, all pseudofaeces were deposited in the experimental chamber, since ejection of particle-mucus

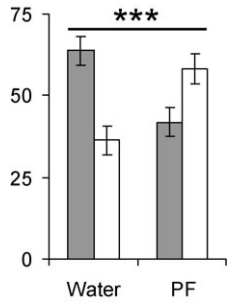

Fig. 3. *Coscinodiscus perforatus* var. *pavillardii* intact and naturally dead, empty cells. Mean percentages of intact (black bars) and naturally dead, empty cells (white bars) from water and pseudofaeces (PF). Error bars represent the standard deviation; ***mean percent of intact cells in water and in pseudofaeces significantly different, $P < 0.001$.

masses from the gill ventral tract was precluded due to the elimination of valve clapping movements by the clamp installed to allow optical tube and micropipette insertion. It was thus possible to separate rejecta from the gills (VT) and labial palps (PF).

Both intact and dead *A. senarius* cells were observed in the specimen's faeces at the conclusion of the experiments, confirming that this microalgae was ingested (Fig. 5).

DISCUSSION

The results of Experiment 1 lead to the rejection of the null hypothesis and acceptance of the experimental hypothesis: *C. gigas* labial palps can distinguish between intact and dead *C. perforatus* var. *pavillardii* cells, in mixtures not subjected to initial selection by the gills. This extends the previous observations of Cognie *et al.* (Cognie *et al.*, 2003), who demonstrated the ability of *C. gigas* labial palps to distinguish between intact cells and empty, cleaned frustules in the absence of initial selection by the gills. Specifically, it shows that functioning cellular status is an important diatom quality cue which may be detected by the labial palps alone, in the absence of initial selection by the gills.

It has been proposed that detection of particle quality cues from particles arriving on the labial palps, bound in relatively high-viscosity mucus, may be contingent upon fluidization of the mucus matrix by physical action of the palps (Newell and Jordan, 1983). Disengagement of particles from the mucus matrix has been reported for the labial palps of the Eastern oyster, *C. virginica* (Ward *et al.*, 1994), and the mechanism has been shown to include both mechanical fluidization and addition of low-viscosity mucus in the mussel *Mytilus edulis* (Beninger and St-Jean, 1997).

The finding that the perifrustular envelope is a crucial diatom quality cue for scallops (Beninger and Decottignies, 2005) is not surprising, since these outermost molecules are most likely to make contact with the bivalve feeding surfaces. The importance of functioning cellular status as a diatom quality cue for oysters may be due to differences between live and dead cells in the molecular composition of the envelope and associated molecular "halo". It may be that the complex perifrustular envelope of intact cells (Volcani, 1981) is qualitatively different from that of dead cells and that this difference may be detected by oysters, but not by scallops. Alternatively, organic production of small molecules such as sugars and amino acids leaking out of living cells (Granum *et al.*, 2002; Wetz and Wheeler, 2007) may constitute positive signals for suspension-feeders (Taghon, 1982; Ward and Targett, 1989). In any event, these results show that, contrary to the situation in the scallop *P. maximus*, functioning cellular status is a key determinant of diatom quality for the oyster *C. gigas*.

The results of Experiment 2 clearly lead to the rejection of the null hypothesis and acceptance of the experimental hypothesis at both the gill and labial palp

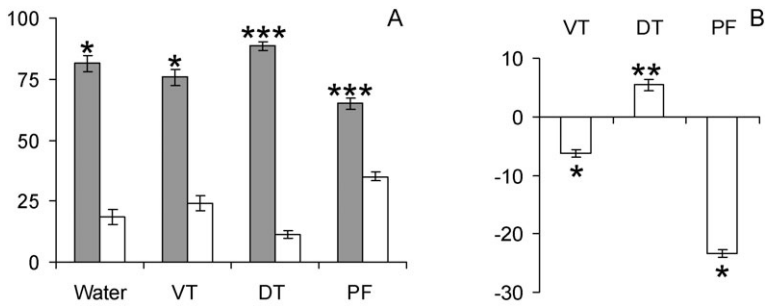

Fig. 4. *Actinoptychus senarius* intact and naturally dead, empty cells. (**A**) Mean percentages of intact (black bars) and naturally dead, empty cells (white bars) from the different sampling sites: water, dorsal tracts (DT), ventral tracts (VT) and pseudofaeces (PF). (**B**) Mean SI at the different sampling sites: ventral tracts (VT), dorsal tracts (DT) and pseudofaeces (PF). Error bars represent the standard deviation; bars with an asterisk are significantly different from all others: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

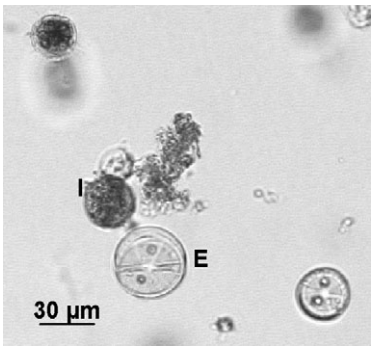

Fig. 5. Intact (I) and empty, dead (E) *A. senarius* cells from oyster's faeces, as viewed under light microscope.

selection sites. The *C. gigas* gill is thus capable of recognizing the functioning cellular status of diatoms, and positively selecting for it, and the labial palps are also capable of doing so, hence further enriching the ingested mixture. It should be noted that the labial palp enrichment is more significant than that of the gill (pseudofaeces compared with ventral gill tract), suggesting a multiplicative selection effect when the mixture is first processed by the gill.

The finding that functioning cellular status influences selection in *C. gigas* extends our understanding of the quality cues which suspension-feeding bivalves may use when processing the highly heterogeneous seston mixtures in the wild. *Crassostrea gigas* has been shown to be capable of selective dietary shifts, which allow it to maximize energy intake under highly variable regimes of seston quality and quantity (Decottignies *et al.*, 2007a). The ability to distinguish between intact (energy-rich) and dead (energy-poor) diatoms, may constitute one mechanism of maximization of energy intake in this species.

Enhanced rejection of dead diatoms will result in oyster pseudofaeces which is energy-poor, compared to that of scallops, whose pseudofaeces contains the same proportions of intact and dead cells as the ambient water and the various particle processing sites (Beninger and Decottignies, 2005), and may thus result in a qualitative difference in biodeposits from these two types of bivalves. In light of the consequences and influence of bivalve biodeposits on the benthic ecosystem (Kautsky and Evans, 1987; Asmus and Asmus, 1991; Giles and Pilditch, 2006), it would be interesting to compare these biodeposits in the field, as has been done for the bivalves *M. edulis* and *Placopecten magellanicus* (Cranford and Hill, 1999). In particular, oysters and scallops inhabit different bottom types, with scallops being totally subtidal, soft-bottom suspension-feeders and oysters predominantly hard-substrate, intertidal suspension-feeders. We might thus expect the differences in particle quality selection cues between these species, both in the wild and in culture operations, to result in qualitative and quantitative differences in benthic–pelagic coupling, especially in the face of cyclic diatom blooms which are characterized by low concentrations of dead cells in the exponential phase, and high concentrations of dead cells in the senescent phase.

Quality-based selection is presumed to be disadvantageous when high-quality particles are in relatively small proportion to overall seston content (Sierszen and Frost, 1992). The fine quality-based discrimination demonstrated by *C. gigas*, under conditions of both relatively low (Experiment 1) and relatively high (Experiment 2) intact cell proportions, has been corroborated by field studies (Decottignies *et al.*, 2007a), and is at variance with this theoretical construct of qualitative selection in suspension-feeders. It may be that the typically turbid habitat of oysters has led to a selective advantage in fine discrimination of algal quality cues.

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