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ANALYSIS OF LARVAL OYSTER GRAZING BY FLOW CYTOMETRY[1]

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ABSTRACT The ingestion of 8 algal species by oyster larvae (*Crassostrea gigas* Thunberg, 1793) was measured by flow cytometry (FCM). In a preliminary experiment, cell size (estimated by light scatter) and chlorophyll fluorescence of 30 algal species were evaluated to select those species which could be mixed together and still be easily discriminated by FCM. Grazing experiments were carried out over 48 h with 6 and 15-day old larvae fed on 3 algal mixtures, each containing 3 different algal species. The concentration of each species was estimated at 0, 6, 12, 24 and 48 h by FCM. Grazing pressure on a given algal species was dependent upon the age of the larvae, the time of the day and the composition of the mixture. Grazing rates of older larvae were about twice those of younger ones after 48 h (mean value of 102 and 57 cells/larva/hour respectively). Almost no grazing activity was observed during the time interval 12–24 h for the 6-day larvae. Significant differences between mixtures were observed after 48 h and the selective filtration of one *Chaetoceros* strain is of importance as this alga also proved to be of better nutritional value for oyster larvae. Data on *Tetraselmis* were difficult to interpret because of tigmotactic reactions of the cells.

KEY WORDS: oyster larvae, *Crassostrea gigas*, grazing, algae, selective feeding, flow cytometry

INTRODUCTION

The type and amount of food used during the rearing process are among the most important factors necessary to bring large numbers of oyster larvae to metamorphosis (Rhodes and Landers 1973, Gerdes 1983). Cole (1937) was the first to demonstrate that the use of marine unicellular algae could produce significant growth of *Ostrea edulis* L. larvae under laboratory conditions and cultures of unicellular algae are still used commonly in hatcheries and nurseries rearing young bivalve molluscs (Ukeles 1976, Chrétiennot-Dinet et al. 1986). Three criteria need to be fulfilled for an alga to be used in a bivalve nursery: adequate size, good food quality and ease of mass culturing. Different species have been tested for their food quality (see for example Walne 1970, Nascimento 1980, Wikfors et al. 1984, Enright et al. 1986, Whyte 1987), and *Isochrysis galbana* is often used as a reference standard for the growth response of bivalves (Gerdes 1983). It has also been demonstrated that mixtures of several algal species generally improve larval growth (Guillard 1975, Epifanio 1979, Gerdes 1983). On the other hand, Reid (1982) suggested that suspension feeders in culture may have specific requirements and that their feeding behavior must be assessed species by species. Ukeles (1976) pointed out that oyster larvae seem to have more specific dietary requirements than clam larvae and that juveniles are less demanding than veligers. Recent studies have examined the capacity of bivalves to select nutritive particles from inorganic material of similar size (Kiørboe et al. 1980, Kiørboe and Møhlenberg 1981, Newell and Jordan 1983, Newell et al. 1989). One important criterion for studying such selection is the ability to recognize particles and cells of similar size but different quality. A major advantage of flow cytometric analysis (FCM) is the ability to distinguish simultaneously between cells and particles of nearly equal dimensions but of different optical properties. For these reasons, FCM has been introduced recently to study the grazing of filter-feeding organisms (Cucci et al. 1985, 1989, Shumway et al. 1985, 1990). Experiments on feeding selectivity have been performed mostly on adults (Shumway et al. 1985) and little information is available for oyster larvae (Korringa 1952, Crisp et al. 1985). Mackie's results (1969) suggest that species selectivity is effective within a given size range but according to Crisp et al. (1985), oyster veliger larvae (mean size over 200 μm) do not discriminate between algal species or algal sizes when fed on mixtures of *Nannochloris*, *Isochrysis* and *Tetraselmis*. Fritz et al. (1989), working with natural assemblages of phytoplankton, concluded that larvae select small cells (<10 μm) but that little selection occurs within this size range. In the present paper, we describe the use of FCM to detect possible particle selection by oyster larvae grazing up on mixtures of various algal species.

MATERIAL AND METHODS

Flow Cytometry

In order to choose phytoplankton species suitable for grazing experiments, 30 cultures (Table 1) were first analysed in Roscoff with an EPICS 541 flow cytometer (Coulter, Hialeah, Florida).

[1]This work was part of the fourth GAP (Group for Aquatic Productivity) meeting in L'Houmeau (France): 19–22 April 1988.

TABLE 1.

List of the species screened by flow cytometry with their origin, their average size and the symbol used in Fig. 1. The last column indicates the mixture in which they have been used during the grazing experiment.

Species	Source	Size (μm)	Symbol (Fig. 1)	Mixture where used
Synechococcus sp. (ROSO4)	Roscoff (F)	1–2	1	
Micromonas pusilla (ROSO9)	Roscoff (F)	1–2	2	
Flagellate 1	Argenton (F)	2–3	3	
Flagellate 2	Argenton (F)	2–3	4	
Microcystis sp.	Arcachon (F)	2–3	5	
Stichococcus bacillaris	Arcachon (F)	3–6	6	
Pseudoisochrysis paradoxa	USA	4–6	7	
Nannochloris atomus	Arcachon (F)	2–3	8	
Nannochloropsis salina	Marseille (F)	2–3	9	
Chaetoceros "minus"	Tahiti (F)	3–4	10	A
Chaetoceros pumilum	Conway (U.K.)	3–8	11	B
Flagellate 3 (ROSO7)	Roscoff (F)	3–4	12	
Flagellate 4	Argenton (F)	3–4	13	
Chroomonas sp.	Arcachon (F)	10–13	14	
Isochrysis "tahiti"	Tahiti (F)	5–6	15	C
Hemiselmis sp.	L'Houmeau (F)	4–5	16	
Thalassiosira pseudonana	Arcachon (F)	3–5	17	
Chlamydomonas sp.	Arcachon (F)	6–10	18	
Chaetoceros calcitrans	Brest (F)	5–16	19	
Pavolva lutheri	Brest (F)	7–9	20	
Isochrysis galbana	Conway (U.K.)	5–6	21	A,B
Chaetoceros gracilis	Tahiti (F)	6–10	22	
Dunaliella primolecta	Roscoff (F)	7–9	23	
Tetraselmis incisa	Arcachon (F)	8–10	24	A
Tetraselmis tetrathele	Arcachon (F)	10–16	25	B
Pleurochrysis carterae	USA	9–12	26	
Cryptomonas maculata	Roscoff (F)	9–14	27	
Tetaselmis suecica	Conway (U.K.)	9–11	28	C
Gymnodinium cf. *nagasakiense*	Roscoff (F)	25–30	29	
Pyramimonas disomata	Argenton (F)	4–6	30	C

Excitation light was provided by an argon laser tuned at 488 nm (100 mW). From 1000 to 10000 cells were analysed for the following parameters: (i) forward angle light scatter (FALS), (ii) right angle light scatter (RALS), (iii) orange fluorescence (OFL: between 530 and 590 nm) which is proportional to phycoerythrin, (iv) red fluorescence (RFL: above 690 nm) which is proportional to chlorophyll. For each species the average value of each parameter was computed and standardized to that of fluorescent beads added to the sample. FALS and RALS were standardized to 1 μm beads (Polysciences) while OFL and RFL were standardized to 10 μm beads (Coulter: 2% of fullbright).

Among the analyzed species (Table 1), some were easily discriminated by FCM because of specific features such as the orange fluorescence of phycoerythrin for cyanobacteria and cryptophytes or the high ration of RALS to FALS for cocco lithophorids (Olson et al. 1989); others had sufficiently different scatter and chlorophyll signatures to be distinguished (Fig. 1). When two species were too similar, however, there was an overlap between the two populations and it was not possible to assign those cells with intermediate properties to one or the other species. For a given difference in average cell properties between two species, the population overlap is lower when individual cells differ little from the average cell, i.e. if there is little spread in the population. A practical rule would be that the population means + 2 population standard deviations do not overlap:

$$x_1 + 2\sigma_1 < x_2 - 2\sigma_2 \qquad (1)$$

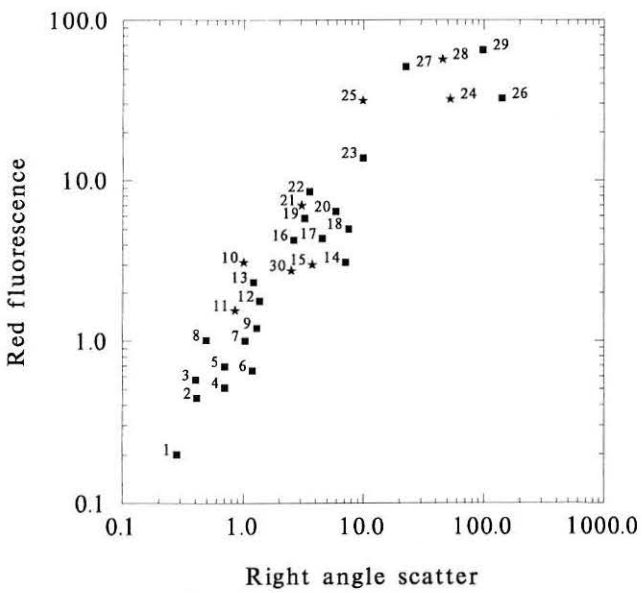

Figure 1. Flow cytometric measurements of the average right angle scatter (related to 1 μm beads) and red fluorescence above 690 nm (related to 10 μm 2% beads) for 30 phytoplankton species. Stars correspond to selected species used in mixture (see Table 1 for the list of species).

where x_1 and x_2 are population averages of a given cellular property for two species (1 and 2) and σ_1 and σ_2, their population standard deviations. Assuming that the two species have the same population coefficient of variation (C), this would give:

$$x_1 + 2Cx_1 < x_2 - 2Cx_2 \quad (2)$$

or

$$x_1/x_2 < (1 - 2C)/(1 + 2C) \quad (3)$$

Although population spread varied widely depending on species and culture status (exponentially growing cultures were in general more tightly distributed than stationary phase ones), the population coefficient of variation was, in general, of the order of 30%. Therefore two species could usually be discriminated if their ratio for at least one of their average properties was larger than 4 (equation 3).

In view of this analysis and of practical experimental constraints (resistance of the cells to handling, adaptability to mass culture), three mixtures of three species were used for the grazing experiments (Table 1, Figs. 1 and 2); a last change had to be made in mixture C as a small cryptophyte culture collapsed during handling just at the beginning of the experiments and had to be replaced by *Pyramimonas disomata*. Unfortunately the latter species could not be discriminated from *Isochrysis* "*tahiti*" and the two species had to be counted together. However the results of mixture C can be compared to those of mixtures A and B for total grazing activity.

During the grazing experiments, a 0.1 ml volume was analysed for each sample with an EPICS Profile (Coulter). Excitation was provided by an argon laser (488 nm, 20 mW). Measured parameters were identical to those for the selection of species except for orange fluorescence which was detected between 515 and 640 nm. Each parameter was recorded on a 3 decade logarithmic scale mapped onto 1024 channels. Data acquisition was done in list mode. The Profile software supplied the volume analyzed and the concentration of each species in the sample.

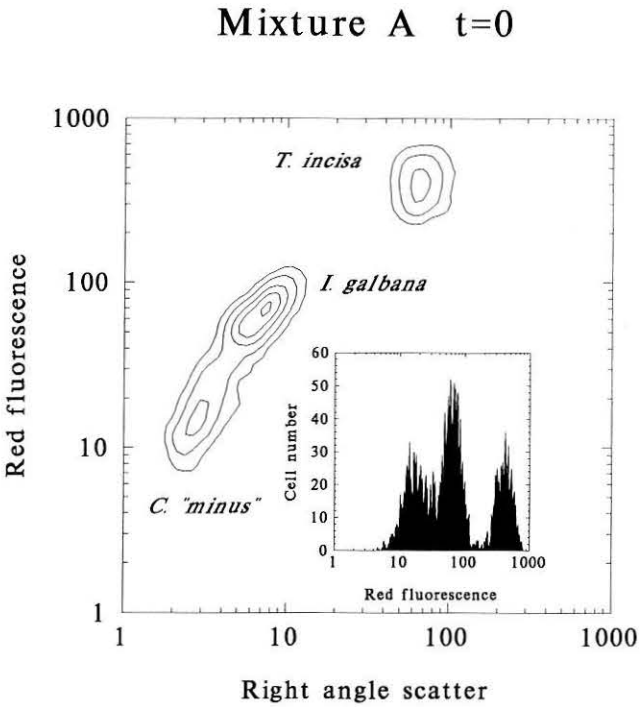

Figure 2. Cytogram of right angle scatter vs red fluorescence (above 690 nm) for mixture A at time t = 0 of the grazing experiment. Axes are scaled in arbitrary units. Contours correspond to 2, 5, 10, 15 and 20 cells. The inset represents the one-dimensional histogram of red fluorescence for this sample. It reveals that *Chaetoceros "minus"* and *Isochrysis galbana* are less well separated than *Tetraselmis incisa*.

Algal Cultures and Oyster Larvae

The eight algal strains used in the experiments (Table 1) were chosen not only according to their flow cytometric properties, but also to test their potential food value, i.e., their ability to be grazed. They belong to different taxonomic groups and some are well known as food sources for bivalve larvae (Walne 1970, Nascimento 1980, Whyte 1987). Among them *Isochrysis galbana* is considered to be among the best foods for oyster larvae (Guillard 1958, Webb and Chu 1982), but many are local isolates and need testing. Each culture was grown at 18°C in Conway's medium (Walne 1966) and illuminated by fluorescent tubes giving a mean surface irradiance of 50 μEins $\cdot$ $m^{-2} \cdot s^{-1}$ with a light-dark cycle of 14:10. Cultures were in exponential phase at the beginning of the experiments.

Larvae of the Japanese oyster, *Crassostrea gigas* Gmelin (6 and 15-day old) were provided by IFREMER-LA TREMBLADE (France) and kept unfed in the dark at 22°C for 24 hours prior to the grazing experiments. Their mean size was 94.4 $\pm$ 0.9 μm and 125.8 $\pm$ 3.2 μm respectively.

Three algal mixtures (A, B, C), each containing 3 different algal species (Table 1 & Fig. 3A, B, C), were fed to the larvae. Each mixture was tested in duplicate on both 6 and 15 day-old larvae, as well as in the absence of larvae (control). For each test, 5000 larvae were placed in 2 litre spherical flasks filled with 1 litre of 0.2 μg filtered seawater (salinity = 28‰). Algal mixtures were added to these flasks at the beginning of the experiment. The final concentration of each algal species was 10^4 cells $\cdot$ ml^{-1}. Flasks were kept in the dark at 22°C. Samples of 10 ml, prefiltered on a 100 μm mesh inox grid to remove the larvae, were taken at times t = 0, 6, 12, 24 and 48 hours.

Data Analysis

The grazing pressure is expressed either as the number of cells of each species remaining in the medium at the different sampling times (on the basis of 10000 cells $\cdot$ ml^{-1} at t = 0) (Fig. 3); as the number of cells cleared per larva per hour for each species (Fig. 4); or as the number of cells cleared per larva in the different mixtures (Fig. 5). The use of a control rather than t = 0 densities accounted for any change in cell density from t = 0 which occurred in the absence of larvae. The grazing activity between times t and t' (G: number of cells cleared per larva per hour) was computed as:

$$G = (X'_n(t) - X'_n(t'))/L \cdot (t - t')$$

where,

$$X'_n(t) = 10000 \cdot X_n(t)/Y_n(t)$$

with $X_n(t)$ the number of cells per ml of species n at time t in the grazed sample counted by FCM, $Y_n(t)$ the number of cells in the control and L the number of larvae per ml.

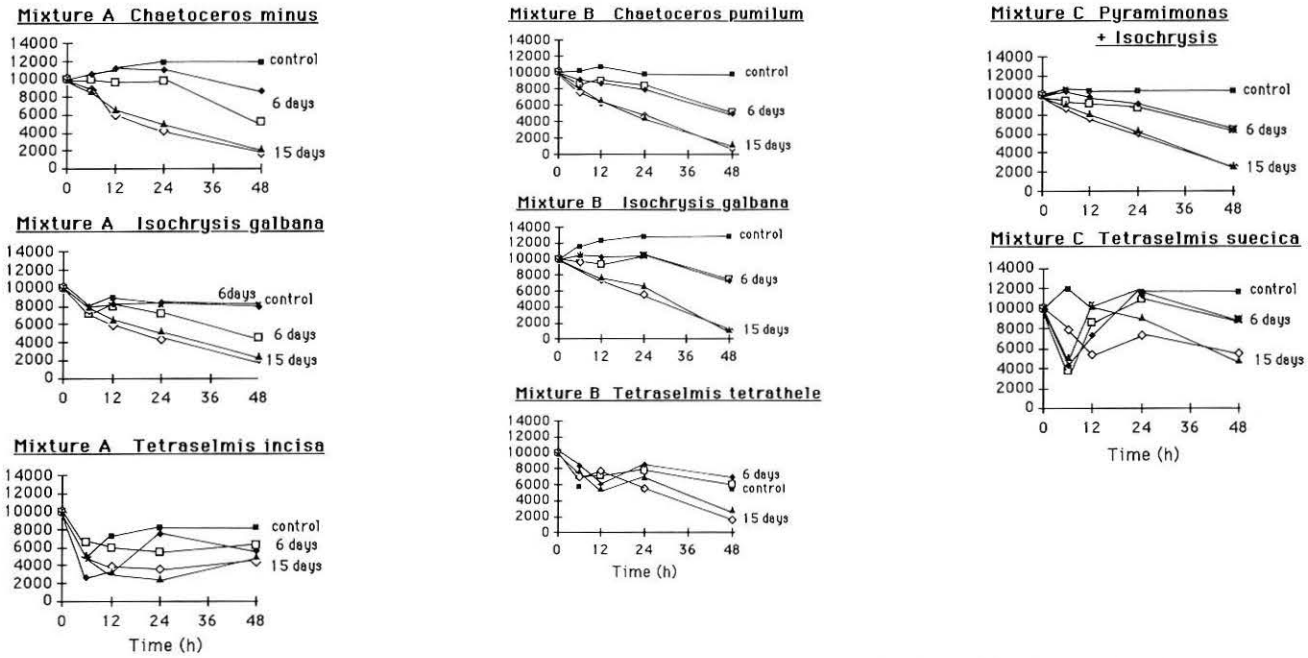

Figure 3. Evolution of cell number of each species vs time, for both types of larvae (6 and 15 day old) and for the control (different symbols denote replicate experiments).

RESULTS AND DISCUSSION

Selection of food species is critical for rearing experiments and for successful hatchery culture (Guillard 1958). According to Webb and Chu (1982), and our own experience, all species used in the present experiment were potential food sources for oyster larvae. *Tetraselmis* spp. were introduced in the mixtures because of their relatively large size as compared to the mouth diameter of the youngest larvae (Ukeles and Sweeney 1969, Robert and His 1987). Cell concentration as well as larval densities are factors affecting feeding activity (Schulte 1975, Ukeles 1976) and care was taken to keep larval densities low enough (5 larva per ml) to ensure high individual feeding activity, but high enough to expect significant variations with an initial cell concentration of $3 \cdot 10^4$ cells $\cdot$ ml^{-1} at the beginning of the experiments (Nascimento, 1980, gives 20–30 cells $\cdot$ μl^{-1} as an adequate concentration for *Crassostrea gigas* larvae of 70–80 μm in length). The larvae generally grazed actively as large numbers of cells were cleared from the medium after 48 h for both types of larvae (Fig. 3). The response of both replicates is quite similar for the 6 and 15 day-old larvae, except for *Tetraselmis* (see explanation below) and for mixture A with 6 day-old larvae which were less active in one sample. However, grazing rates varied among the different species, depending on larval age, time, and mixture composition (Fig. 4).

From a quantitative point of view, larval age (related to size) is most important. After 48 h, (Fig. 4G and 5C), older larvae (15-day) had cleared roughly twice as many cells as younger ones: on average 102 and 57 cells in a mixture per larva, per hour, respectively. This result is in agreement with the fact that the amount of food ingested increases with larval size (Rhodes and Landers 1973, Lucas and Rangel 1981, Gerdes 1983). The difference between both ages of larvae was most evident during the time interval 6–12 h for mixture A, as the amount of cells cleared by the 15-day larvae was 6 times higher than for the 6-day larvae (Fig. 4B) and greater still in the three mixtures after 12 h (Fig. 4E and 5B).

The time sequence of the number of cells cleared (Fig. 3, 4 and 5) shows clearly that the grazing pressure was not constant during the experiment. Both groups of larvae grazed actively during the first 12 h, except for the 6-day old larvae with mixture A (Fig. 4A

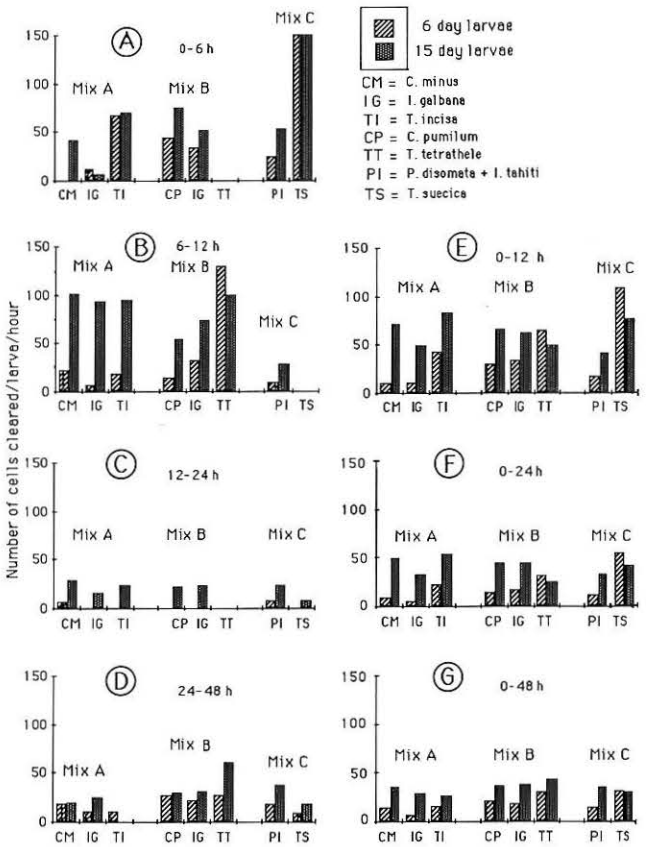

Figure 4. Number of cells cleared per larva and per hour for the different species at various time intervals: mean value of the duplicates.

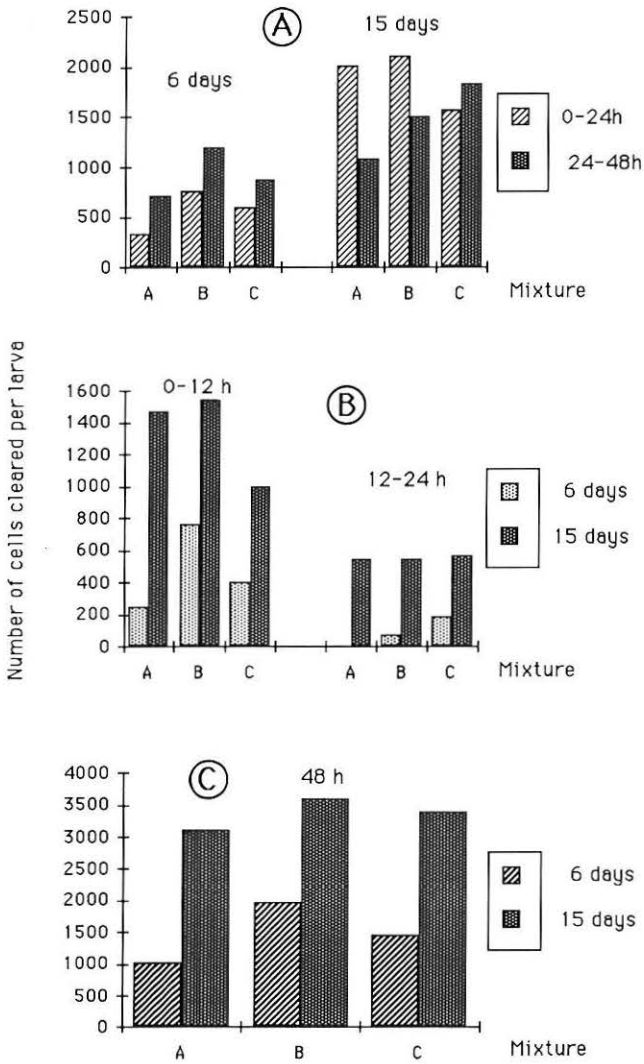

Figure 5. Number of cells cleared per larva in the different mixtures (*Tetraselmis* values discarded): mean value of the duplicates. A: comparison between the first (0–24 h) and second day (24–48 h) for the 6 and 15 day-old larvae; B: comparison between the 6 and 15 day-old larvae during the time intervals 0–12 and 12–24 h; C: comparison after 48 h between the 6 and 15 day-old larvae.

and B, Fig. 5B). Between 12 and 24 h, the grazing pressure was much lower; reduced to a third of the 0–12 h values for the 15-day larvae and almost none for the 6-day ones (Fig. 4C and Fig. 5B). It seems that the larvae filled their stomachs within the first 12 hours, and then reduced their grazing activity considerably. This type of behavior has already been observed in prosobranch veligers (Fretter and Montgomery 1968) and rhythmic activity has been demonstrated in adults of *Ostrea edulis* (Morton 1971) or *Crassostrea virginica* (Palmer 1980).

Under natural conditions, information on larval feeding behavior is rather scarce (His et al. 1985, Robert and His 1988) but experimental studies usually indicate a continuous feeding strategy (Korringa 1955, Ukeles 1976, Fritz et al. 1984). A comparison between the 1st and 2nd day (Fig. 4F, D and Fig. 5A) shows that the 6-day larvae grazed more on the 2nd day, (1.5 times more on average), but that older ones were less active, except for mixture C. A two-way analysis of variance (ANOVA) after 48 h (*Tetraselmis* values excluded) shows significant differences between the 6 and 15-day old larvae ($p = 0.999$) but also between species ($p = 0.988$).

A comparison between the different mixtures after 48 h (Fig. 5C) shows that grazing activity is highest with mixture B and lowest with mixture A for both types of larvae. During the time interval 0-6 h, *Chaetoceros* "*minus*" (mixture A) was not grazed at all by the 6-day larvae, while *Chaetoceros pumilum* (mixture B), of a similar size, was apparently consumed readily (Fig. 4A). *Isochrysis galbana* was also less grazed in mixture A than in mixture B (Fig. 4A, B and D). It is of interest to note that mixtures A and B contained two different species of *Chaetoceros* whose effect on the growth and survival of oyster larvae has been tested with special emphasis on their lipid contents (Robert et al. 1989). The best growth, obtained with *C. pumilum*, seems to be explained by the optimal balance of the different lipid classes and fatty acids as the lipid content of larvae is clearly related to the diet. *Chaetoceros* "*minus*" is then considered to be of rather poor food value and the lack of feeding by the 6 day larvae on this alga might be related to its chemical composition. It is also clear that larvae ingest what is available and without other alternative, ingested mixture A on the 2nd day. Feeding behavior is a complex phenomenon with possible adaptations relating to environmental conditions, but younger larvae are more sensitive to the mixture composition and different species belonging to the same genus may not prove to be equivalent. The demonstration of a relationship between particle selection and biochemical nutritional value for *Chaetoceros pumilum* is of great interest for larval bivalve feeding studies as growth is tightly related to food ingestion and FCM is a promising tool from this point of view. The case of *Tetraselmis* has to be considered separately since after 6 h, cells were observed to attach to the vessel wall at the air-water interface. As a result of this tigmotactic behaviour, the cell density had drastically decreased in the 6 h samples, giving a high apparent grazing activity on the species of this genus. It was then decided to agitate the flasks before sampling. The phenomenon probably biased some grazing estimates as the results were somewhat erratic (Fig. 3) and *Tetraselmis* data were discarded for the ANOVA and in values given in Fig. 5. However, after 48 h, the mean number of each type of cell (all species included) cleared per larva and per hour did not differ significantly whether *Tetraselmis* data were included or not: 19 vs 15 for the 6 day larvae and 34 vs 35 for the 15 day larvae (Fig. 4). Because of these problems, it was not possible to discuss the influence of the cell size of *Tetraselmis* on the grazing activity of the youngest larvae.

CONCLUSIONS

FCM is useful for the study of selective grazing by bivalve larvae fed on mixed algal populations, provided suitable species are available in culture. The first 12 hours are particularly sensitive for selectivity, and mixture composition is more critical for smaller larvae. Care must be taken under experimental conditions using algal cultures to select phytoplankton cells which can be distinguished by FCM, but it is also important to avoid cells which tend to clump or stick, sink or aggregate. In this respect, *Tetraselmis* is not recommended for FCM analysis. The selected algae must also be easy to grow, resistant to handling (fragile species cannot be used) and nontoxic. For young larvae, algal cells must be of a suitable size, usually less than 10 μm, therefore the range of species becomes rather restricted. About 50 species belonging to 35 genera have been tested under experimental conditions on bivalve larvae (Chrétiennot-Dinet et al. 1986). However, marine

phytoplankton comprises more than 50 known genera with representatives within the size range 1–5 μm and almost 100 within 5–10 μm. Only 3 algal genera in the size range considered are reported to have toxic effects in aquaculture (Shumway 1990). It would be very interesting in the future to try to apply FCM to *in situ* analysis, as the natural diet of bivalve larvae is still poorly known and our knowledge of the natural food content of young bivalve larvae very limited (His et al. 1985, Chrétiennot-Dinet and Guillocheau 1987, Robert and His 1988).

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