Vol. 24: 201–204 | MARINE ECOLOGY – PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published July 11

NOTE

Flow cytometry: a new method for characterization of differential ingestion, digestion and egestion by suspension feeders

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ABSTRACT: Whether filter-feeding organisms can (1) select, (2) preferentially ingest, and/or (3) preferentially digest suspended particles, is of major importance to our understanding of material flow through marine systems. Investigation has until recently been limited by lack of techniques that can distinguish quantitatively between different particles of the same size. We demonstrate here the ability to distinguish algal food particles quantitatively, even when they are of a similar size, by the detection of their fluorescent photosynthetic pigments using a flow cytometer. The usefulness of this technique is illustrated by experiments on the mussel *Mytilus edulis* fed a mixed algal diet. In these experiments, a cryptomonad was digested in preference to a dinoflagellate and a diatom.

The study of particle selection by organisms, ranging in size from small heterotrophic flagellates and ciliates of a few micrometers to adult bivalve molluscs, has been hampered in the past by technical difficulties. It was originally inferred from morphology and direct observation of intact animals, that suspension-feeding bivalves were capable of particle selection (Jørgensen 1966, Owen 1966). Subsequent studies were greatly assisted by use of the Coulter Counter which allowed quantitative estimates of filtration rate and of size selective particle retention (Sheldon & Parsons 1967, Stuart & Klumpp 1984). Such work suggested that many bivalves select particles on the basis of size alone (Foster-Smith 1975a, b, Winter 1978). Recent studies suggest, however, that at least 11 species of bivalves can select algae preferentially from a mixture of algae and silt particles (Kiørboe et al. 1980, Kiørboe & Møhlenberg 1981, Newell & Jordan 1983).

Most of these studies examine the question of selection of nutritive particles from inorganic particles of a similar size. Major limitations of the Coulter Counter technique are: (1) particles occupying the same volume range cannot be distinguished simultaneously; (2) even though only 0.5 ml is actually sampled per analysis, relatively large volumes (~ 10 to 20 ml) of culture liquid are required to maintain the orifice within the sample. A standard protocol is described here in which algae from several different chromatic groups (i.e. having different photosynthetic pigment complements) can be detected simultaneously during flow cytometry analyses (FCM) (Yentsch et al. 1985) using small volumes of liquid for the measurement. As a result, particles of similar size but of different optical properties can be analyzed quantitatively.

Fluorescence derived from 1 particle is split by a 590 nm dichromic mirror and is received by 2 photomultiplier tubes. One receives a wavelength spectral region > 630 nm such as would result from chlorophyll emission; the other, shorter wavelengths (530 to 560 nm) as would result from phycoerythrin and phycocyanin emission. Several thousand particles can be analyzed per minute, yet a sample volume of only 1 to 2 ml is required for an analysis of as many as 10^4 particles.

Analysis of bivariate 3-decade log scale plots of fluorescence intensity shows that there are at least 4 useful regions within the plots in which various microalgae can be distinguished by their fluorescence properties. These include (1) green unicells with chl *a* and chl *b*, and diatoms with chl *a* and chl *c* plus fucoxanthin; (2) dinoflagellates, with chl *a* and chl *c* plus peridinin and which give more fluorescence than 1; (3) cryptomonads which have both phycoerythrin or phycocyanin plus chlorophyll fluorescence; (4) cyanobacteria which have predominantly phycoerythrin and phycocyanin fluorescence (although they also have chl *a*). Detailed discussion of the usefulness of algal fluorescence spectral signatures is given elsewhere (Yentsch & Yentsch 1979). Clones having identi-

0171-8630/85/0024/0201/$ 02.50

cal fluorescence spectra and intensity responses cannot be utilized simultaneously. In addition, bacteria can be stained and incorporated into mixed-resource grazing experiments.

Mytilus edulis from the same spatfall and of approximately 60 mm shell length were maintained at 8 °C in running seawater at West Boothbay Harbor. They were held in 0.7 µm filtered seawater for 24 h prior to use to purge their digestive tracts, and were then placed in 3 l glass vessels with 2 l of algal culture mixtures. Control algal cultures were left without mussels to correct for algal cell division during the course of the experiment. Clonal phytoplankton cultures were grown at 15 °C under a 14/10 h light-dark cycle on F/2 medium. Three clones were equally mixed to obtain a final cell density approximating natural phytoplankton levels of 10^4 cells ml^{-1} and were analyzed in a Coulter EPICS V Flow Cytometer/Sorter with a single argon ion 5 W laser using an excitation wavelength of 514 nm with a power of 1,000 mW. The events (number of cells) registered met gate criteria based on red fluorescence (chlorophyll), therefore only algal cells were analyzed. Based solely on pigment fluorescence intensity, a total of 10,000 cells were analyzed for each sample, with the total number partitioned among the 3 clones. All bivariate histograms are on the same scale and show number of events (cells analyzed) plotted with increasing relative phycoerythrin fluorescence (X-axis) and increasing relative chlorophyll fluorescence (Y-axis).

A major advantage of the FCM approach is the ability to distinguish between cells of nearly equal dimensions. This is shown in the experimental results summarized in Fig. 1. Three different species were presented to mussels. These were the spindle-shaped diatom *Phaeodactylum tricornutum* (clone Phaeo), which is ca 2.5 to 3.5 × 12 to 23 µm in size; the dinoflagellate *Prorocentrum* sp. (clone Exuv.), ca 5 to 6.15 × 8.75 to 12.5 × 15 µm; and the cryptomonad flagellate *Chroomonas salina* (clone 3C), 6.25 to 7.5 × 8.75 to 12.5 µm.

Fig. 1 shows the relative populations for the 3 components at times zero (A), 15 min control (B), and 15 min experimental (C) vessels. The mean time required to analyze 10^4 cells from the 15 min experimental vessel was 478 s compared with only 346 s in the control, showing that after 15 min, there were fewer algal cells in suspension within the experimental vessels and, therefore, food was consumed during the experiment. In this and other (unpubl.) experiments there was no differential clearance of cells ranging in size from those used here to cyanobacterial cells (*Synechococcus* sp.) of ca 1 µm size.

An important advantage of the FCM technique is that differential digestion and egestion can be detected. True feces represent material which has been ingested and passed through the gut. Pseudofeces are formed by mussels when filtered particles are consolidated with mucus and rejected prior to ingestion. Examination of feces of mussels in our experiments, using epifluorescence microscopy, showed intact algal cells suggesting that at least some components of the ingested food pass through the gut intact.

After completion of the feeding experiments, mussels were transferred to 2 l vessels containing filtered seawater (0.7 µm Gelman glass fiber) and left for 4 h, after which feces were collected. The material was gently sonicated for 1 min and passed through a 55 µm Nitex net to remove any remaining clumps prior to FCM analysis. The results of such analyses are summarized in Fig. 2. Cryptomonads were absent from the feces, while the diatom and dinoflagellate evidently passed through the gut without significant digestion. In contrast, while having similar and higher propor-

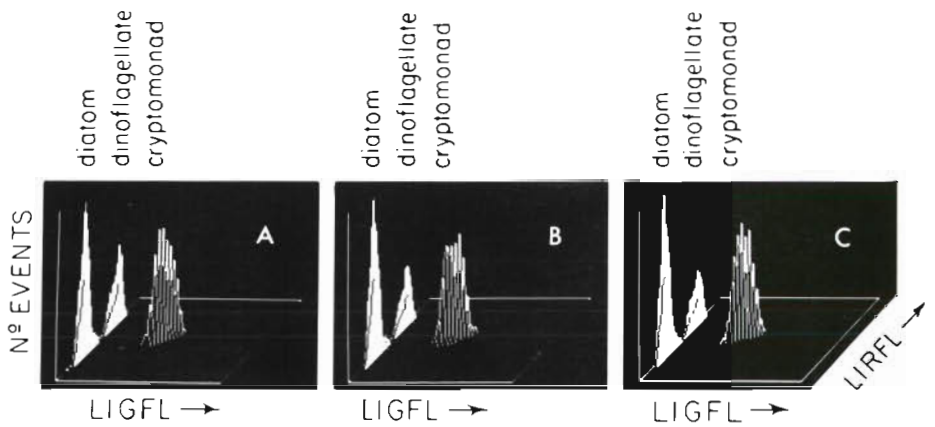

Fig. 1. Bivariate histogram plots of number of events (cells analyzed) and X = log integrated green fluorescence (LIGFL) (fluorescence due to phycoerythrin) *vs* Y = log integrated red fluorescence (LIRFL) (fluorescence due to chlorophyll). *Mytilus edulis* grazing on algal mixture of a diatom (clone Phaeo), a dinoflagellate (Exuv), and a cryptomonad (3C) over 15 min time course. (A) initial; (B) control at 15 min; (C) experiment at 15 min

tions of the dinoflagellate and diatom, respectively, than the initial algal mixture, the pseudofeces also contained the cryptomonad species.

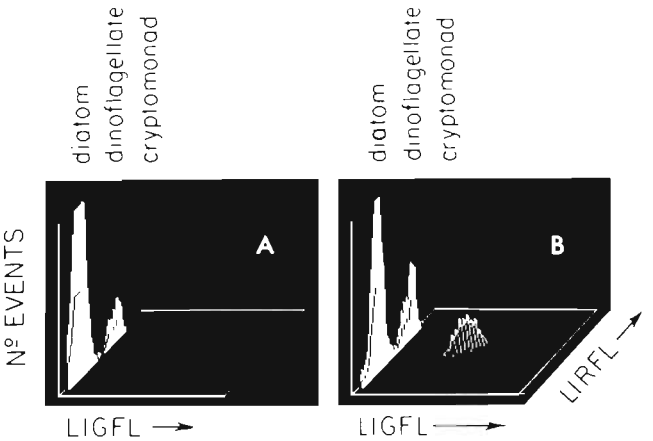

Fig. 2. Bivariate histogram plots of number of events (cells analyzed) and X = log integrated green fluorescence (LIGFL) *vs* Y = log integrated red fluorescence (LIRFL) of (A) feces and (B) pseudofeces from *Mytilus edulis* following 15 min feeding on clones Phaeo, Exuv and 3C. In (A) the 10,000 events analyzed are partitioned basically between the diatom and dinoflagellate, thus the diatom peak is off scale

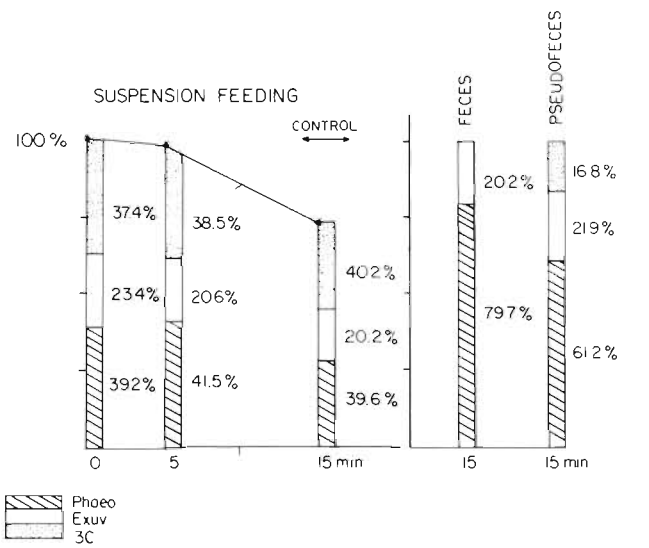

Fig. 3. Summary of suspension-feeding experiment using clones Phaeo (the diatom *Phaeodactlyum tricornutum*), Exuv (the dinoflagellate *Prorocentrum* sp.) and 3C (the cryptomonad *Chroomonas salina*) as food particles and *Mytilus edulis* as suspension feeder. Analysis of culture mixtures were run at zero time (n = 5; total 5.39×10^4 cells ml^{-1}), corresponds to Fig. 1A; 5 min (n = 5; total of 5.3×10^4 cells ml^{-1}); and 15 min (n = 6; total of 3.92×10^4 cells ml^{-1}), corresponds to Fig. 1C. The feces (n = 4) and pseudofeces (n = 1) were analyzed following egestion subsequent to a 15 min feeding exposure time. These correspond to Fig. 2A and B, respectively. Count at time zero is taken as 100 %. Controls remained the same after the 15 min experimental time

The cell numbers obtained by direct cell counts and the relative numbers of cells of each clone obtained by FCM are summarized in Fig. 3. This shows the important distinction between cell clearance, which in this cell size range was similar for the diatom, the dinoflagellate, and the cryptomonad, and the differential absorption or utilization of one component or another of a mixed food supply during passage through the gut.

The complex size overlap presented by natural particles in the sea once limited the scope and design of experiments on the differential utilization of particulate matter. We can now take advantage of fluorescence and light-scatter characteristics of particles to examine differential use by consumer organisms of food resources comprised of groups having similar-sized cells with different chromatic signatures. Even bacteria can be used experimentally, provided they have been stained with fluorescent materials prior to use so that bacterial fluorescence can be distinguished from algal fluorescence. Flow cytometry thus presents a potentially manageable, sensitive and unified approach to the study of interrelated problems of particle selection, food preferences, material flow, and marine organisms.

Acknowledgements. We thank Carter Newell at Great Eastern Mussel Farms, Damariscotta, Maine, for mussels. This work was supported in part by NSF grants OCE-82-13567 and NA-82-FA-C-00043, ONR grant N00014-81-C-0043, The Maine Department of Marine Resources and the State of Maine. This is Bigelow Laboratory for Ocean Sciences contribution number 84008.

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Accepted for printing on April 6, 1985