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The influence of three algal filtrates on the grazing rate of larval oysters (*Crassostrea gigas*), determined by fluorescent microspheres

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ABSTRACT

Fluorescently labelled microspheres (FLMs) were used to measure the grazing rates of 6- to 12-day-old larval oysters. FLMs were mixed in a 1:1 ratio with algal cells and fed to the oysters at different concentrations. Ingested FLMs were enumerated using epifluorescent microscopy. FLMs were good tracers of algal grazing rates and experiments with FLMs were easy and rapid to conduct and required very little apparatus.

Tests were conducted to determine if filtrates from three microalgae species inhibited feeding. Filtrates from dense cultures were tested against controls (containing no filtrates) for their impact on the grazing rates of larvae. The grazing rates of *Crassostrea gigas* larvae were significantly reduced when the animals were exposed to filtrates from *Phaeodactylum tricornutum* and *Gonyaulax grindleyi*. Filtrate from dense cultures of *Thalassiosira pseudonana* did not reduce grazing rates. Grazing rate (G) versus particle concentration (c, microspheres and *T. pseudonana* in a 1:1 mixture) was fitted to the equation $G=(G_{max}\cdot c)/(K_c+c)$. The concentration required to reach half the saturating rate of particle grazing (K_c) was 24 particles μl^{-1} and the maximum grazing rate (G_{max}) was 0.415 particles min^{-1}.

INTRODUCTION

Aquaculturists and government agencies are concerned with the negative impacts of algae on the production of cultured animals. Such impacts are typically assessed by measuring the behavioral or physiological responses of animals to algae. Although there are several responses that can be measured (e.g. respiration, growth, grazing, swimming behavior), grazing rate is one of the quickest and easiest of these to obtain. Thus, we have used the grazing rate of oyster larvae to examined the impact of extracellular products (fil-

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trates) from three commonly encountered microalgae. To do this we have used fluorescently labelled microspheres (FLMs) as tracers of grazing rate since they are simple and rapid to use (Pace and Bailiff, 1987). Typically, FLMs have been applied to the study of protozoa, but they appear to be equally good for larval oysters (Widdows et al., 1989), which are of a similar size to protozoa.

If ingested, phytoplankton can be toxic to aquaculture species (Taylor, 1990), but they may also affect animals if extracellular products are released; then the toxicity may reside in the water itself (Huntley et al., 1986; Ward and Targett, 1989). Like many higher plants, phytoplankton release allelopathic compounds which inhibit growth of competitor species (Maestrini and Bonin, 1981) or inhibit grazing by herbivorous zooplankton (Huntley et al., 1986). Such allelopathic compounds are not only of interest ecologically but also because of their possible impact on aquaculture. Bivalve larvae are sensitive to naturally occurring chemical stimuli (Fitt et al., 1990), and the feeding of adult animals is influenced by the presence of various phytoplankton species (Shumway and Cucci, 1987). For example, Ali (1970) demonstrated that the medium in which the prymnesiophyte *Isochrysis galbana* had been grown was inhibitory to the filtering activity of the bivalve *Hiatella artica* (L). Also the filtration rate of the blue mussel, *Mytilus edulis* (L.), was reduced by dissolved extracellular materials in filtrates from the rhaphidophyte *Heterosigma akashiwo* and the chlorophyte *Dunalliela tertiolecta* (Ward and Targett, 1989).

Dissolved organic matterial (DOM) can pose a difficult problem for the aquaculture industry in that the removal of the particulate fraction may not remove the compounds from the water. Although some DOM, particularly free amino acids, may be beneficial to larval bivalves (Manahan and Crisp, 1982, Manahan and Stephens, 1983) other compounds excreted by algae may not (Ward and Targett, 1989). However, prior to developing methods of coping with these compounds the producers should be identified, since knowledge of the source might allow early action to be taken and thus prevent the problem.

In this study we have examined three possible sources of toxic compounds: senescent cultures of the food species *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* and the toxic bloom species *Gonyaulax grindleyi*. Change in the grazing rate of 9-day-old *Crassostrea gigas* larvae was used to determine the impact of filtrates derived from these species. To measure grazing rate we used fluorescently labelled microspheres and have also evaluated their applicability to future studies.

MATERIALS AND METHODS

Algal cultures

Cultures of the two diatoms *Thalassiosira pseudonana* (Hustedt) Hasle and Heimdal, clone 3H (NEPCC# 58) and *Phaeodactylum tricornutum* Bohlin

(NEPCC# 640) and the dinoflagellate *Gonyaulax grindleyi* (NEPCC# 535) were obtained from the Northeast Pacific Culture Collection (NEPCC), Department of Oceanography, University of British Columbia. Algal cultures were grown in enriched artificial or natural seawater. Artificial seawater (ESAW) was based on the recipe by Harrison et al. (1980) as modified by Thompson et al. (1991). Natural seawater was treated with activated charcoal (to remove organic compounds), gravity filtered through a Whatman #1 filter paper, refiltered through a 0.8 μm Millipore filter, autoclaved and then enriched with ES levels of nutrients, trace metals and vitamins (Harrison et al., 1980). Cultures were exposed to continuous light (provided by Vita-lite fluorescent tubes) at 19°C and 35 μmol photons m^{-2} s^{-1} for *P. tricornutum* and *G. grindleyi* and at 22°C and 110 μmol photons m^{-2} s^{-1} for *T. pseudonana*. Growth rates were measured by changes in in vivo fluorescence using a Turner Designs Model 10 fluorometer and/or cell counts using a Coulter Counter (model TAII).

Filtrates containing extracellular metabolites were prepared from cultures of *T. pseudonana* in exponential phase at 10^9 cells l^{-1}, *P. tricornutum* in late exponential phase at 1.8×10^9 cells l^{-1} and *G. grindleyi* 2 days into senescence at 6.2×10^6 cells l^{-1}. To minimize the possibility of cell disruption, filtrates of *T. pseudonana* were prepared via gravity, or low vacuum (<50 mm Hg) filtration through GF/F Whatman filters, while filtrates of *P. tricornutum* and *G. grindleyi* were screened through 33 μm Nitex netting followed by filtration through a 0.8 μm Millipore filter.

Oyster larvae

Approximately 10^6 5-day-old (90–100 μm long) larvae were placed in a 200-l polypropylene tank and maintained at 22°C. The larvae were fed *T. pseudonana* at a density of 100 cells μl^{-1}; this density was monitored and adjusted daily to 100 cells μl^{-1}. Experiments were conducted on larvae ranging in age from 6 to 12 days, with filtrate experiments conducted on 9-day-old animals, averaging 120 μm in length.

Experimental protocol

Fluorescently labelled microspheres (FLMs, Seradyn Particle Technology Div., Indianapolis, IN, 46225, USA) were used as prey analogs to estimate grazing rates. Microspheres and *T. pseudonana* cells were 5.0 and 5.1 μm in diameter, respectively, and were assumed to be grazed equally without preference by the oyster larvae. Prior to experiments, the microspheres were stored in 0.5% bovine serum albumin (Pace and Baliff, 1987) for >24 h. To make the various concentrations needed, microspheres were sonicated (Bronsonic 220, bath sonicator at 60 Hz), counted with a Coulter Counter (Model TAII), then diluted with either pasteurized (75°C for 12 h) natural seawater or algal filtrates, resonicated and then recounted to ensure the spheres were mono-

dispersed. Except where noted, microspheres were mixed 1:1 with *T. pseudonana* cells.

Three different experiments were conducted with microspheres:
(1) We measured the time required for the oysters to saturate their consumption of microspheres; a time below the saturating time was then used to determine grazing rate in future incubations. In this experiment the larvae were incubated with 200 particles μl^{-1} (1:1, microspheres:*T. pseudonana*) for 10, 20, 30, 40, 50, 60, 80, or 120 min.
(2) We measured microsphere ingestion as a function of bead concentration to determine saturating food concentrations. Future grazing rate experiments were then conducted at saturating concentrations to ensure that food was not limiting and thus did not influence grazing rates. This experiment was run at 2, 20, 60, 100, 140, 180, 200, 300 and 600 particles μl^{-1} (1:1, microspheres:*T. pseudonana*).
(3) We measured the influence of two "controls" and three algal filtrates on grazing rates of the larvae. This experiment was conducted over 40 min and at 200 particles μl^{-1}.

In all three experiments, larvae were withdrawn from the 200-l tank (after mixing), collected onto 100 μm Nitex netting, concentrated into pasteurized natural seawater, repeatedly mixed and then separated into subsamples that were placed in 20 ml glass scintillation vials. After 20 min (direct observation indicated most animals were swimming), mixtures of microspheres and *T. pseudonana* cells and/or filtrates were added and the vials mixed. The total volume of fluid in a vial was 16 ml, containing an average of 150 ± 100 oysters. Experiments were terminated by adding buffered formalin (final concentration of 5% v/v). Ingested microspheres were counted, post-fixation, by direct observation with a Zeiss epifluorescent inverted microscope.

The following treatments were used to examine the effect of filtrates on grazing rate: (1) a "sterile" control containing only microspheres (200 μl^{-1}) suspended in pasteurized seawater; (2) a simulated in situ control containing microspheres and *T. pseudonana* cells (1:1 at 200 μl^{-1}) suspended in water from the tank that the oysters were grown in; and (3) three treatments containing microspheres and the *T. pseudonana* cells (1:1 at 200 μl^{-1}) suspended in filtrates. The filtrates were prepared from dense algal cultures (as described above). Treatments and controls received the same handling and were conducted in triplicate at 18°C.

Unless otherwise stated, data are presented in terms of microspheres consumed. Since the larvae were fed microspheres and *T. pseudonana* cells in a 1:1 ratio, 2·(microsphere consumption) = total consumption. Data were fitted to curves using the Marquardt–Levenberg least squares algorithm (Sigmaplot 4.0, Jandel Scientific, CA). Analysis of variance and least-significant-

difference (ANOVA, LSD) statistics were calculated (on square root transformed data) using the Systat statistical program (Wilkinson, 1990).

RESULTS

Feeding rate versus time

The mean number of ingested microspheres increased with time (Fig. 1). These data were fitted to the function:

$$I = I_{max}(1 - \exp^{-bt})$$

where I = the number of ingested microspheres, I_{max} = the maximum number of microspheres ingested, t = time (min), and b is a constant. For our data, the value of b was 0.020, I_{max} was 3.5 (for microspheres only), requiring > 120 min for ingestion to equilibrate with egestion (become asymptotic; Fig. 1, broken line). The curve appeared to be linear between 0 and 60 min.

When the data from 0 to 60 min were fitted by a least squares linear regression (forced through the origin), the slope was 0.0458 microspheres oyster^{-1} min^{-1}, with an R^2 of 0.69 and a standard error of the slope of 0.0025 ($n = 18$) (Fig. 1, solid line). This indicates that ingestion rate was nearly linear over the first 60 min. Filtrate experiments were therefore run for 40 min to ensure that we measured grazing rate over a period where it was constant.

Grazing rates versus particle density

The change in grazing rate with food concentration was fitted to the equation:

$$G = (G_{max} \cdot c)/(K_c + c)$$

where G = the number of ingested microspheres (min^{-1}), G_{max} = the maximum number of particles ingested, c = the concentration of microspheres and K_c = the concentration of microspheres required to reach the half-saturation ingestion rate (Fig. 2). The average value of K_c was 6 microspheres μl^{-1}, indicating that about 24 particles (microspheres plus algal cells) μl^{-1} were required to saturate the grazing ability of 120 μm (9-day-old) oyster larvae. At saturating concentrations, the larvae grazed at a rate of 0.21 microspheres oyster^{-1} min^{-1}. The filtration rate of the larvae (calculated at K_c) was 0.0175 μl oyster^{-1} min^{-1}. Filtrate experiments were conducted at 100 microspheres (200 particles) μl^{-1}, a concentration that produced a fairly stable response in terms of microsphere ingestion (Fig. 2).

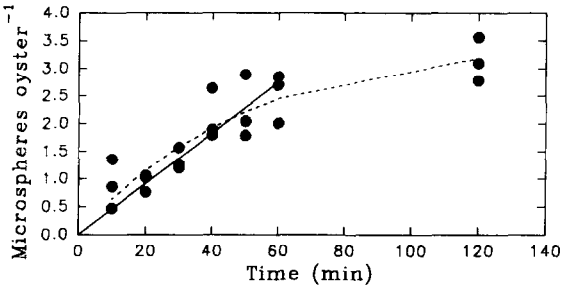

Fig. 1. Mean number of microspheres ingested by larval *Crassostrea gigas* versus time (note that number of total particles consumed would be twice the number of microspheres, since microspheres and *T. pseudonana* cells were provided in a 1:1 ratio). Each point represents an average determined from the observation of 62 ± 17 animals. The solid line represents a linear fit through the origin for data from 0 to 60 min. The dotted line represents the data fitted to the function $I = I_{max}(1 - \exp^{-bt})$, see Results for an explanation of the terms.

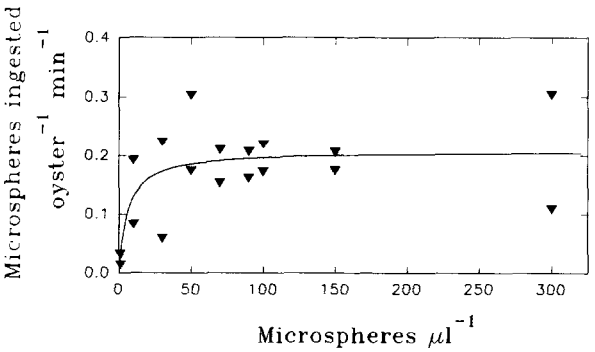

Fig. 2. Number of microspheres ingested oyster^{-1} min^{-1} versus concentration of microspheres in solution (note same assumption as Fig. 1). The line is a fit of the data from experiments run for 40 min at 200 particles μl^{-1} to the function $G = (G_{max} \cdot [c])/(K_c + [c])$, see Results for an explanation of the terms.

Filtrate experiments

For analysis purposes, the values of grazing rate from the sterile treatment were divided by two; the sterile treatment contained only microspheres while the other treatments contained 1:1 microspheres: *T. pseudonana* (Fig. 3). After this manipulation was made, significant differences in the grazing rates of larval oysters existed between treatments (filtrates) (ANOVA, $P < 0.05$). Further analysis by Tukey's multiple range test indicated where differences occurred. Some differences existed at $\alpha = 0.050$, but differences were more pronounced at a slightly higher probability level of 0.053 (Fig. 3).

There was no difference between the sterile treatment (divided by two) and the in situ or *T. pseudonana* filtrate treatments ($\alpha = 0.05$), suggesting that there was no preference between microspheres or *T. pseudonana* cells. At

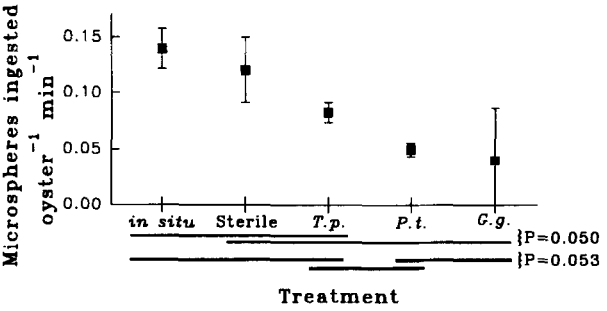

Fig. 3. Number of microspheres ingested oyster^{-1} min^{-1} as influenced by treatments ($n=3$). Treatments were as follows: the control containing pasturized seawater and beads only at 200 μl^{-1} (divided by 2), "Sterile"; the control containing microspheres and *Thalassiosira pseudonana* cells (1:1 at 200 μl^{-1}) suspended in water from the tank the oysters were grown in, "in situ"; and "*T.p.*", "*P.t.*" and "*G.g.*" were treatments containing microspheres and *T. pseudonana* cells (1:1 at 200 μl^{-1}) suspended in filtrates from cultures of *T. pseudonana*, *Phaeodactylum tricornutum* and *Gonyaulax grindleyi*, respectively. The filtrates were prepared from dense algal cultures (as described in the text). Lines under treatments indicate treatments which do not differ significantly (LSD, P as indicated). Error bars are one standard deviation

$\alpha=0.053$ the grazing rates of larvae in the sterile and in situ treatments were significantly higher than those of larvae exposed to *P. tricornutum* and *G. grindleyi* filtrates (Fig. 3). We conclude that filtrates of these two phytoplankton species are likely to reduce the grazing rate of larval oysters.

DISCUSSION

Choice of method

Fluorescently labeled microsphere (FLM) ingestion by larval oysters was linear from 0 to 60 min, and the microspheres were easily seen inside the oyster larvae. Further, the microspheres were ingested approximately twice as fast in samples with 200 FLMs μl^{-1} as in samples with 100 FLMs μl^{-1} and 100 *T. pseudonana* cells μl^{-1}, suggesting that there was no discrimination between FLMs and diatoms. Thus, it appears that microspheres act as suitable prey analogs. However, it has been shown that microzooplankton (organisms 20–200 μm in size) may reject microspheres (Stoecker, 1988), and, in recent years, a detailed body of literature has examined and, in some instances, criticized the use of prey analogs, primarily for protozoa (see McManus and Okubo, 1991).

Use of microspheres as indicators of relative, rather than absolute, ingestion obviates many of the problems associated with prey analogs (see McManus and Okubo, 1991). We have used microspheres as tracers of relative food ingestion and have only done so at a single concentration (for the filtrate experiments). Thus, this technique and our assessment of the filtrate effect

on grazing can be considered accurate regardless of the absolute filtration rates. Further, since microspheres and algae were grazed equally (Widdows et al., 1989; this study), our measurements should also be accurate estimates of absolute grazing rates. However our estimates of filtration rate (~ 1.05 μl oyster^{-1} h^{-1}) are at the low end of the range (0.5 to 3.6 μl oyster^{-1} h^{-1}) given by Gerdes (1983) for similarly sized larvae of *C. gigas*, using the prey depletion approach (see below).

Previous studies have typically estimated larval oyster grazing by examining the depletion of food from a culture vessel (e.g. Gerdes, 1983; Crisp et al., 1985). Experiments using microspheres to determine grazing have some advantages over those using the depletion approach: (1) they can be conducted in smaller containers over relatively short time periods; (2) they eliminate problems associated with change in prey concentration by means other than grazing (e.g. sedimentation, decomposition and prey growth); (3) they allow a rapid and direct assessment of ingestion; (4) they need not be immediately analyzed, as preserved samples may be stored indefinitely; and (5) once the initial cost of a fluorescent microscope is overcome, the experimental cost is small. Widdows et al. (1989) have already demonstrated that microspheres can be used to determine the effects of environmental stress associated with variation in the oxygen content of the water on bivalve grazing; we have demonstrated that microspheres are also suitable to assess the effects of algal filtrates. Shumway (1990) suggests that a bioassay (described by Thain and Whatts, 1987), using embryonic development of the oyster *Crassostrea gigas*, "shows real promise as a monitoring tool" of water quality. Possibly, uptake of microspheres by oyster larvae could also serve as a physiological index to estimate the effects of water quality (Widdows et al., 1989).

Filtrates

Extracellular exudates from several algae have been shown to affect bivalve feeding: the medium in which the prymnesiophyte *Isochrysis galbana* was grown inhibited filtering of *Hiatella artica* (Ali, 1970), and dissolved extracellular materials in filtrates from two flagellates, *Olisthodiscus luteus* and *Dunaliella tertiolecta*, reduced the filtration rate of the blue mussel *Mytilus edulis* (Ward and Targett, 1989). Other organisms are also affected by algal filtrates: clearance rates of the copepod *Calanus pacificus* were reduced by filtrate from the dinoflagellate *Protoceratium reticulatum* (= *Gonyaulax grindleyi*) (Huntley et al., 1986), and exudates from the red tide dinoflagellate *Alexandrium tamarense* induced ciliary reversals and eventual lysis of the tintinnid *Favella ehrenbergii* (Hansen, 1989). Thus, various classes of phytoplankton are capable of producing exudates which may inhibit grazing in a wide variety of organisms.

Thalassiosira pseudonana filtrate did not influence the grazing rate of *C. gigas* larvae (this study), nor did it have an impact on the feeding behavior

of the blue mussel, *Mytilus edulis* (Ward and Targett, 1989). However, we found that filtrates from both *P. tricornutum* and *G. grindleyi* significantly inhibited grazing rates of larval *C. gigas*.

Toxic filtrates from exponentially growing *G. grindleyi* reduced feeding of the copepod *Calanus pacificus* upon the dinoflagellate *Gymnodium resplendens* (Huntley et al., 1986). Thus, it seems likely that exponentially growing or senescent cultures of *G. grindleyi* contain a dissolved substance capable of reducing the grazing activity of at least two herbivores. Since *G. grindleyi* forms blooms in coastal waters these dissolved substances may be introduced into aquaculture operations. Potentially grazing and hence growth of larval oysters could be reduced by > 50% (Fig. 3).

Phaeodactylum tricornutum is a species commonly used in mariculture of filter feeding organisms. Some filter feeders preferentially select *P. tricornutum* (S.E. Shumway, pers. commun.). However, the nutritional value of *P. tricornutum* to *C. gigas* is variable (Wilson, 1978; Epifanio et al., 1981) and is probably affected by growth conditions (Wilson, 1979; Thompson and Harrison, 1992). These studies suggest that growth conditions may affect the suitability of *P. tricornutum* as a food source for bivalves by influencing its biochemical composition (Wilson, 1979; Thompson and Harrison, 1992). Our findings suggest a different aspect of growth conditions: older cultures of two microalgal species, which *C. gigas* could encounter, produce extracellular compounds that inhibit feeding of larval oysters by > 50% (Fig. 3). Low levels (1% v/v) of medium from older cultures of *P. tricornutum* did not influence the grazing rate of *Ostrea edulis* (Wilson, 1979). However, our results indicate that 100% filtrates from *P. tricornutum* cultures inhibit grazing in *C. gigas*. Our data are insufficient to resolve whether it is the age of the culture or the cell density and concentration of the filtrate that was important in elucidating this response. It is known that exponentially growing *P. tricornutum* produces extracellular compounds which induce spawning in the sea urchin *Strogylocentrotus droebachiensis* (Starr et al., 1990). The chemical structure of these substances produced by *P. tricornutum* and their impact is under further investigation (Starr et al., 1992).

CONCLUSIONS

On the basis of this work, filtrates from some microalgae can reduce the grazing rate of larval oysters. Earlier work by Wilson (1981) demonstrated that half of the 29 phytoplankton species examined produced exudates which affected the development of some marine invertebrates. Aquaculturists should therefore be concerned with not only the particulate matter in intake water but also the dissolved substances contained in it. The magnitude of this potential problem is largely undetermined and future work should examine not only different microalgae but also how the various environmental factors in-

fluence the quantity and quality of algal exudates (Bayne, 1965; Fogg, 1966; Myklestad et al., 1989). For the working aquaculturalist there is some indication that the effect of inhibitory substances in seawater can be minimized by charcoal treatment (Craigie and McLachlan, 1963) or DOM removed by sand filtration (Manahan and Stephens, 1983) but this too should be further investigated. Finally, we recommend fluorescent microspheres as a useful tool to examine these questions.

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