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Growth and grazing rates of *Protoperidinium hirobis* Abé, a thecate heterotrophic dinoflagellate

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Abstract. Growth and feeding rates of a laboratory-reared small thecate heterotrophic dinoflagellate, *Protoperidinium hirobis* Abé, grown on the diatom *Leptocylindrus danicus*, were measured in batch cultures. Ingestion rates were determined directly by the enumeration of empty diatom frustules produced by dinoflagellate feeding. Both growth and feeding rates saturated at diatom concentrations of $\sim 10^4$ cells ml^{-1}, and reached maximum values of 1.7 divisions day^{-1} and 23 diatoms $grazer^{-1}$ day^{-1}, respectively. This rate of cell division is notably high compared to photosynthetic dinoflagellates, which seldom grow faster than 1 division day^{-1}. A maximal clearance rate of 0.5 μl h^{-1} was measured. Mean cell size varied proportionally with food abundance, with food-saturated cells having double the mean volume of food-depleted cells. Timing of cell division and grazing rate patterns were also examined; while mitosis occurred chiefly during the dark period, no diel variations in feeding rate were detected. These rates represent the first direct growth and ingestion measurements to be made for a thecate heterotrophic dinoflagellate. They serve to underscore one function these dinoflagellates perform within the microzooplanktonic food web: that of transforming large diatoms into particles more easily ingested by microzooplankters.

Introduction

Until recently, the nature of feeding in thecate heterotrophic dinoflagellates was unknown. These colorless, non-photosynthetic dinoflagellates are typified by *Protoperidinium*, a large and ubiquitous genus whose members have never been found to contain visible food vacuoles. Now it is clear that a unique pseudopodal mechanism is employed by *Protoperidinium* and other related genera to feed on phytoplankton, especially on diatoms, whereby food is digested in an 'extracellular' food vacuole (Gaines and Taylor, 1984; Jacobson and Anderson, 1986). The only pertinent data on feeding rates in these organisms are those of Lessard and Swift (1985) who, before the mechanism of ingestion was understood, used a single-cell isolation radioisotope technique to measure feeding rates of field-captured thecate heterotrophic dinoflagellates that were fed using the small, <20 μm size range of wild phytoplankton. They reported rather high clearance rates, with most species assimilating ^{14}C-labeled material (i.e. algae), while a few species incorporated ^{3}H-thymidine-labeled material (i.e. bacteria). Such data are useful, but there is a clear need for direct measurements of ingestion rates by techniques which are not subject to elemental recycling or other uncertainties associated with mixed assemblages. There is also a need to examine the growth rate of these microheterotrophs when supplied with different food concentrations. To these ends, we report the direct, simultaneous measurement of feeding and growth in one of the first thecate heterotrophic dinoflagellate species to be successfully maintained in culture.

Method

Culture conditions and organism

Protoperidinium hirobis Abé was isolated by D.M.J. from Vineyard Sound, MA (31‰ salinity). This uni-'algal' but non-clonal strain was identical to the original description of *P.hirobis* in all but one respect: the two antapical (posterior) spines were consistently 40% shorter in length than reported by Abé (1936). Small cells with diameters ranging from 11 to 15 μm (compared to a normal mean diameter of 23 μm) appeared in cultures, but never exceeded 10% of the population. *P.hirobis* was fed a diatom (*Leptocylindrus danicus*, which was also isolated by D.M.J. from Vineyard Sound) which was found to support both long-term growth and high rates of growth; no other diatom food supported better growth. This system was maintained in completely full, capped 16 ml polypropylene centrifuge tubes which were slowly rotated end over end on a plankton wheel apparatus. The dinoflagellates were maintained by diluting aliquots of dinoflagellates and diatoms into either filtered Vineyard Sound seawater or fresh f/2 media (Guillard and Ryther, 1976), depending on the rate of diatom depletion. Additional diatoms were added when cultures became food depleted. All culturing and experiments were carried out in the same incubator (20°C, light:dark 14:10 at ~100 μE m^{-2} s^{-1}).

Ingestion rates

Ingestion data were derived from a novel technique which involved the enumeration of empty diatom walls (frustules) created by dinoflagellate pseudopodal feeding. This delicate feeding mechanism precludes the destruction of frustules. With unconventional optics (i.e. an off-centered bright-field stage condenser on an inverted Zeiss microscope), both the nearly empty thin silica frustule walls and a small characteristic golden-brown residual inclusion constituting the remains of the diatom cytoplasm were visible at low magnifications. (The cylindrical frustules are inconspicuous under bright-field illumination and, although the frustule wall is well defined under phase optics, the color of the residual particle is less distinct.) The golden-brown residue was never seen in non-grazed diatom cultures, even when lysed, deteriorating cells were present; therefore the identification of grazed diatom cells was unambiguous. Ingestion rates (I) as diatom cells l^{-1} dinoflagellate^{-1} day^{-1} were calculated as follows:

$$I = \frac{f_t - f_o}{X \cdot t}$$

where f_t and f_o are final and initial frustule concentrations, respectively, t is the time interval and X is the average grazer concentration during this time interval. The average number of grazers (i.e. dinoflagellates) was estimated according to Heinbokel (1978) as follows:

$$X = \frac{d_1 - d_0}{[\ln(d_1) - \ln(d_0)]}$$

where d_1 and d_0 are the final and initial dinoflagellate concentrations, respectively. Clearance rates were calculated by dividing ingestion rate by food concentration.

Experimental protocol

Two sets of experiments were run: one designed to measure grazing and growth over several days and at several food densities (the grazing/growth experiments); another to detect diel variations in feeding and cell division at a single optimal food density over a 30 h period (the diel time course experiment).

Grazing/growth experiments

Two consecutive experiments (hereafter termed grazing/growth experiments) were performed using dinoflagellate/diatom suspensions in order to simultaneously measure both the rate of grazing and growth as a function of food concentration. A series of equivalent dinoflagellate inocula was exposed to seven food densities (with targeted levels of 100, 500, 1000, 2000, 3000, 6000 and 10000 diatom cells ml^{-1}) in 32 ml polycarbonate centrifuge tubes; each treatment was performed in triplicate. Fortuitously, the strain of *L.danicus* used grew as single or paired cells, ranging in length from 30 to 60 μm, instead of producing, as is usual, long, highly variable colonies. While *P.hirobis* can also graze on longer colonies, these non-colonial diatoms simplified the interpretation of grazing data. Prior to experiment initiation, excess empty diatom frustules were removed from the stock dinoflagellate culture by allowing the frustules and living diatoms to settle out within a graduated cylinder, after which the swimming dinoflagellates were collected by decanting. In the first of two experiments, an initial dinoflagellate concentration of 100 cells ml^{-1} was used, but this resulted in rapid depletion of the diatom standing crop, making data interpretation difficult; therefore, feeding rate analysis was abandoned and the experiment was repeated using an initial dinoflagellate concentration of 25 cells ml^{-1}. (The first experiment is included here to help demonstrate the reproducibility of the growth rate data.) Replicate samples of dinoflagellate inocula were preserved for enumeration of initial empty frustules and replicate diatom control suspensions (i.e. without dinoflagellates) of 3000 cells l^{-1} were similarly sampled to measure spontaneous (non-grazed) empty frustules. After 24 h, the three lowest diatom treatments had become diatom depleted and additional diatoms were introduced to restore the target concentration. The food concentrations in the high-diatom treatments (>3000 cells l^{-1}), however, had increased; neutral density screens were attached to these tubes in order to limit diatom growth. We assume that grazing rates are influenced to a greater extent by food concentration than by light intensity, an assumption which is supported by a diel experiment (see below).

Tubes were sampled once every 24 h for 3 days. The sampling procedure

involved gently pouring the media back and forth between two small beakers to ensure thorough mixing, followed by the transfer of a 2 ml aliquot to a vial holding 100 μl Lugol's iodine solution. The colorless dinoflagellates were rendered more conspicuous by the iodine stain, but the color characteristics of grazed diatoms were not affected. Samples were subsequently transferred to settling slides and enumerated on a Zeiss inverted microscope. The entire sample was examined for dinoflagellates and empty frustules, except when frustule density was excessively high, whereupon random fields were examined at 400× until >200 frustules had been encountered. Diatom growth during the experiment is indicated by horizontal error bars around growth and grazing rate data points. Grazing rate calculations were based on the first 48 h of the experiment, while growth rates were based on the full 72 h period except where declining abundances (an apparent consequence of depleted food) occurred in the last day of the experiment—in these cases growth rate has been derived from the first 48 h period. Grazing and growth rate data were graphically rather than mathematically analyzed to determine half-saturation constants.

Diel time course experiment

A second experimental protocol was designed to examine short-term variation of growth and feeding on a diel cycle under conditions of optimal food abundance. This time course experiment consisted of taking 2 ml samples from a single 300 ml centrifuge tube (with approximate cell densities of 300 dinoflagellates ml^{-1} and 10^4 diatoms ml^{-1}) every 3 h over a 30 h period. In order to enumerate dividing nuclei, aliquots were fixed with formalin (3%) and stained with 4,6-diamidino-2-phenylindole (DAPI: Sigma Chemical Co.) at a final concentration of 10 μg ml^{-1} for 30 min without washing and observed by epifluorescence microscopy with Zeiss filter set no. 487702. Doublet cells (i.e. daughter cells having completed cytokinesis, but still attached) were most easily detected when viewed with another fluorescence filter set (no. 487706) in which the unstained cytoplasm of *P.hirobis* exhibited an intense green autofluorescence. Therefore, the samples were counted twice, first for dividing nuclei (while observing DAPI fluorescence) and again for doublet cells and total cell number (while observing green autofluorescence). The time course of total cell number was used to compute the instantaneous specific cell division rate as a function of time. The following equation was used with 2 h time steps to obtain a finite-difference approximation of μ (h^{-1}), according to Nelson and Brand (1979):

$$\mu_t = \frac{N_{t+2h} - N_{t-2h}}{4 \cdot N_t}$$

where N_t is the dinoflagellate abundance at sampling time t, and N_{t-2h} and N_{t+2h} are the dinoflagellate abundances of the immediately preceding and immediately following sampling times, respectively. Ingestion rates were determined as for the previous experiment.

The frequency of feeding cells as a function of time was also measured. Because all fixatives tested failed to quantitatively preserve the fragile pseudopodal dinoflagellate–diatom connection intact, a live count technique was employed, as follows: 100–200 μl samples were transferred in a series of drops onto the surface of a small sheet of plexiglass and quickly examined under a dissecting microscope. This volume was chosen to limit the number of rapidly swimming dinoflagellates per drop to 5–10. All counts were made within the walk-in culture chamber; red-filtered illumination was used during the dark phase. Between 50 and 100 cells were examined for each calculation of feeding frequency. Cell size was determined using an ocular micrometer. Duration of feeding was determined by transferring cells that had just initiated feeding to 2 ml depression wells, where they were monitored once every 5 min until they released their diatom prey. The volume of *P.hirobis* was estimated using a geometric model consisting of a cone atop a solid hemisphere.

Results

Grazing/growth experiments

The grazing/growth experiment was run twice; the first experiment resulted in rapid diatom depletion, so it was repeated with a reduced grazer inoculum size. The first grazing/growth experiment yielded an average dinoflagellate specific growth rate of 1.15 day^{-1} for the highest diatom treatments; in treatments with <2000 cells ml^{-1} growth appeared to be food-limited (Figure 1a) and grazing rates were not calculated due to the significant food depletion that had occurred. Both growth and grazing rates as a function of food concentration were measured in the second grazing/growth experiment. Growth curves were again exponential for food-replete cultures (Figure 1b). Both the growth and the grazing curves have the form of a hyperbolic Michaelis-Menten saturation equation: the ingestion rate reaches a maximum of 23 diatoms $grazer^{-1}$ day^{-1} (Figure 2a) and the growth rate curve plateaus at $\mu = 1.23$ day^{-1} (corresponding to 1.7 divisions day^{-1} and a generation time of 15 h) (Figure 2b). Clearance rates ranged from 0.5 μl $grazer^{-1}$ h^{-1} to 0.1 μl $grazer^{-1}$ h^{-1} in conditions of low and high food abundance, respectively (Figure 3). Half-saturation food concentration (K_m) values were derived from the growth and grazing rate graphs (Figure 2); given experimental variation, these values were similar, the half-maximal grazing rate having occurred at a food concentration of 1400 ± 700 diatoms ml^{-1}, while the corresponding growth rate occurred at 800 ± 400 diatoms ml^{-1}. Mean dinoflagellate size decreased with decreasing food abundance (Figure 4). Food-replete dinoflagellates had a mean diameter of 22 μm, while food depleted cells had a mean diameter of 18 μm, a decrease in volume of ~45%.

Diel time course experiment

The dinoflagellate growth curve obtained during the diel time course experiment exhibited a form which deviated slightly from a smooth exponentially increasing

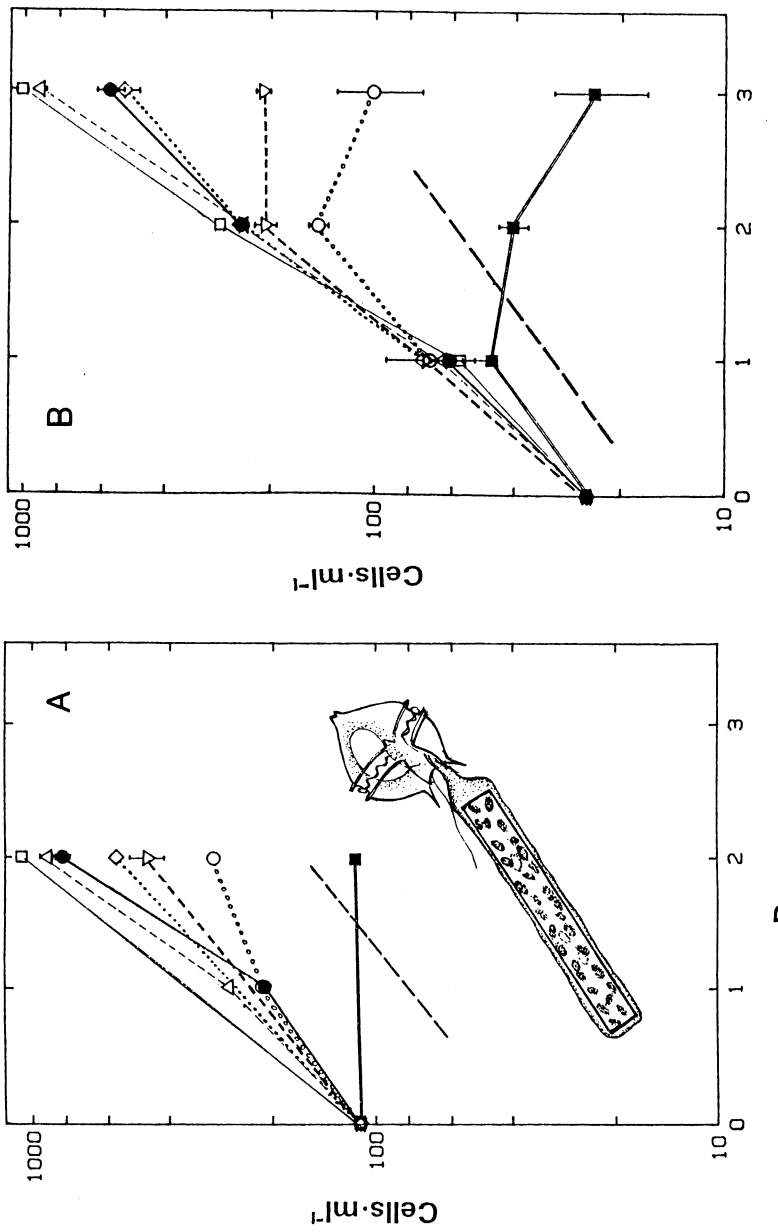

Fig. 1. *Protoperidinium hirobis* growth curves with varying food concentrations (the right-most data symbols in each case fall in an ordered sequence of food concentrations, from top to bottom: 10000, 6000, 3000, 2000, 1000, 500 and 100 diatom cells ml^{-1}) from two grazing/growth experiments (A and B). Dashed line represents one division day^{-1}, error bars represent ±1 s.e. Drawing shows configuration of feeding cell.

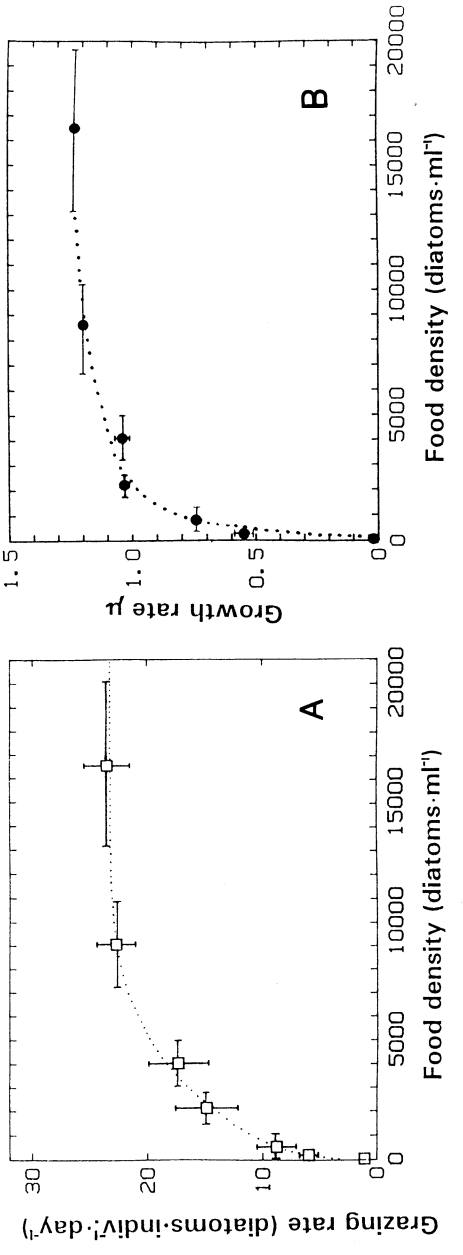

Fig. 2. Ingestion rate (**A**) and growth rate (**B**) of *Protoperidinium hirobis* as a function of food concentration, based in part on data of Figure 1b. Arrow indicates $\mu = 0.69$ or one doubling per day. Horizontal error bars = empirical range of diatom concentration. Vertical error bars = ±1 s.e.

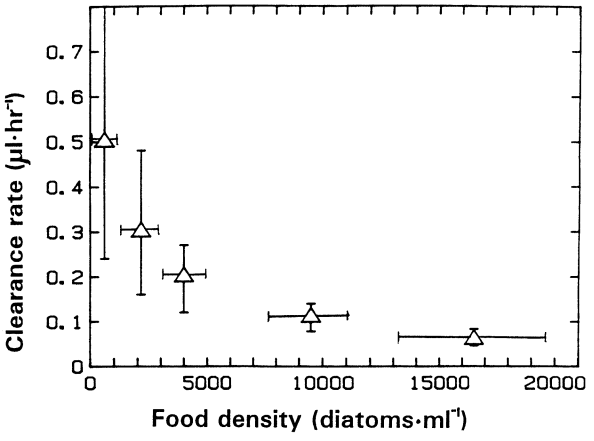

Fig. 3. Clearance rates of *Protoperidinium hirobis* calculated from the data shown in Figure 2a.

function. There was an initial lag phase lasting several hours followed by growth at a specific rate of 1.08 day^{-1} (or 1.56 divisions day^{-1}) (Figure 5). The frequency of dividing nuclei and doublet cells is shown in Figure 6, together with an instantaneous growth rate function derived from Figure 5; mitotic cells were found throughout the dark phase with a major peak at 04.00 h, 1.5 h prior to the end of the dark phase. Two peaks are apparent in the growth rate function, which has maximal values at 23.00 and 06.00 h. During this experiment an ingestion rate of 20.2 diatoms grazer^{-1} day^{-1} was measured (compared to a maximum of 23 diatoms grazer^{-1} day^{-1}). The time course of empty frustule accumulation, which records the progress of feeding, is shown in Figure 7. Since the actual dinoflagellate concentration through time is known (Figure 5) a null hypothesis of constant cell specific grazing activity through time can be assessed by plotting a hypothetical frustule accumulation curve using the assumption of a constant ingestion rate of 20.2 diatoms grazer^{-1} day^{-1}. This curve, also plotted in Figure 7, fits well with the empirical data, but displays deviations from the hypothetical curve consistent with a depressed grazing rate between 00:00 and 08:00 h.

Finally, feeding frequency (the proportion of cells attached to diatoms at any given time) through time remained fairly constant at 25–35% (Figure 8). The duration of feeding measured in seven cells ranged from 40 to 78 min, with a mean of 63 min.

Discussion

The present study provides the first direct measurements of growth and grazing in a thecate heterotrophic dinoflagellate under defined culture conditions. The unique pseudopodal grazing mechanism found in *Protoperidinium* and other related genera provides an opportunity to measure grazing rates with a novel technique that is both unambiguous and precise. Conventional grazing procedures consist of measuring the disappearance of food, taking the difference

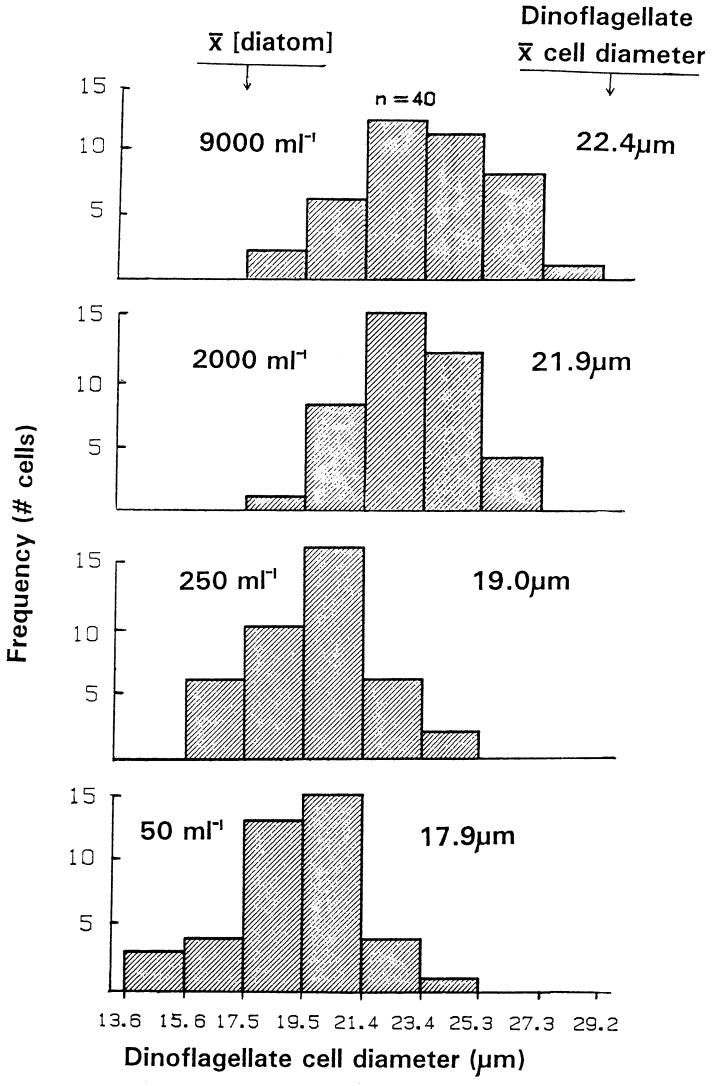

Fig. 4. *Protoperidinium hirobis* cell diameter-frequency histogram from four of the seven food treatments of the second grazing/growth experiment. The unconventional *x* axis reflects the conversion of optical micrometer units.

between food abundance in separate grazed and control food treatments. Such studies necessarily require the assumption of identical growth characteristics of phytoplankton with and without grazers, even though nutrient regeneration and related factors are not identical in the two treatments. Grazing rates calculated in this study avoid both this assumption and the errors inherent in calculating the difference between two large numbers by counting the appearance of empty diatom frustules as a direct consequence of grazing.

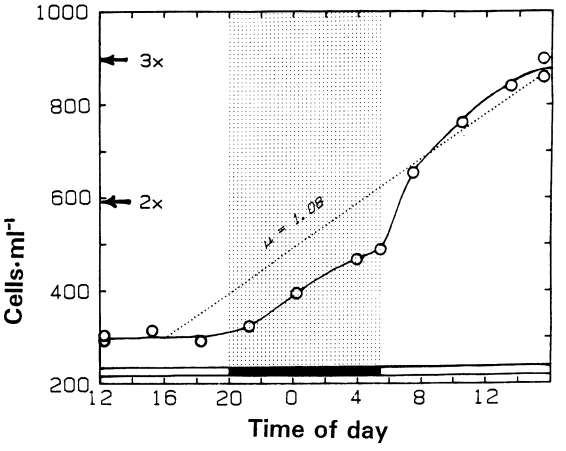

Fig. 5. Growth curve of *Protoperidinium hirobis* from the diel time course experiment, L:D cycle as shown. Arrows denote multiples of inoculum cell concentration. Dotted line shows 24 h interval over which growth rate was calculated.

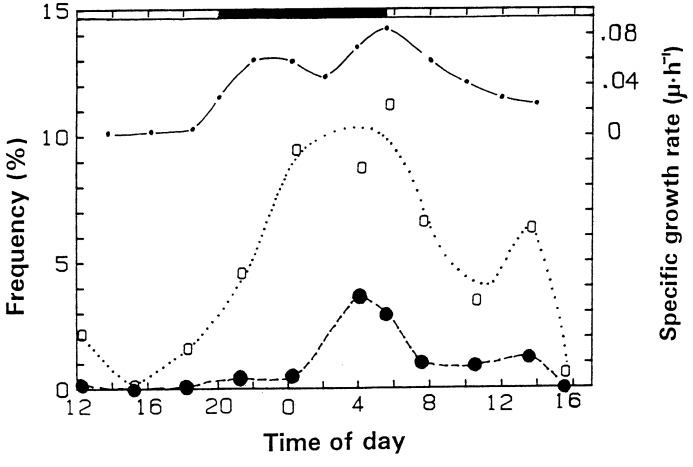

Fig. 6. Frequency of *Protoperidinium hirobis* elongated or dividing nuclei (open circles) and doublet cells (closed circles) and instantaneous growth rate (at top) from the diel time course experiment.

While the enumeration of empty frustules is a strength of this study, one weakness involves changes in diatom concentrations, especially at the lowest food densities, that occurred in the course of the experiment. (This problem, of course, plagues most grazing studies.) Further, the dinoflagellate density data for these low food density treatments is erratic, and may be influenced by the history of the dinoflagellate's previous food conditions. Therefore, the maximal clearance rate measurement, which is derived from data collected at the low food levels, may lack rigor.

The growth rates of *Protoperidinium hirobis* grazing on the diatom *Leptocylindrus danicus* are surprisingly high. Most dinoflagellates have inherently

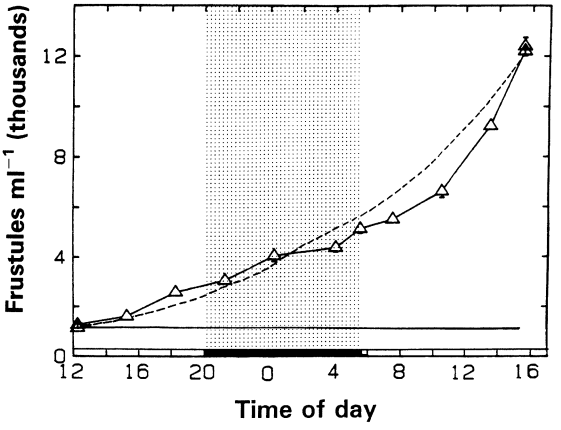

Fig. 7. Abundance of empty diatom frustules through time from the diel time course experiment. Dashed line = 'predicted' frustule curve (see text).

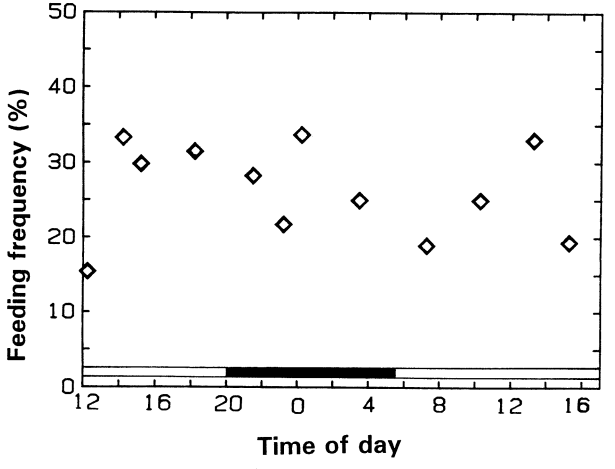

Fig. 8. Frequency of feeding *P.hirobis* cells through time from the diel time course experiment.

slow growth rates with few reports of division rates in excess of one division day^{-1} or $\mu = 0.69$ day^{-1} (Loeblich, 1967). Growth rates measured in these three experiments range from 1.1 to 1.2 day^{-1}. The higher value corresponds to 1.7 divisions day^{-1} or a generation time of 15 h. This rate of growth is among the highest ever recorded for a dinoflagellate. *P.hirobis* is smaller than most species of *Protoperidinium*. A more typically sized, 45 μm species, *Protoperidinium spiniferum*, never exceeded 0.3 divisions day^{-1} ($\mu = 0.23$ day^{-1}) in culture (unpublished observations). The double-peaked form of the μ vs t function (Figure 6) may be a direct consequence of this 'ultradian' or greater than daily division mode of growth. Recently Bjørnesen and Kuparinen (1991) measured a growth rate of 0.31 day^{-1} in a wild population of a small Antarctic species of *Gymnodinium*, although this was at 1°C. A larger naked heterotrophic dinoflagellate, the 30 × 60 μm *Gyrodinium dominans* grew at a rate of 0.9 day^{-1}

in warm (25°C) conditions on a diet of a large *Chattonella* species (Nakamura *et al.*, 1992).

This study is the first to document the diel pattern of cell division in a thecate heterotrophic dinoflagellate. Phototrophic dinoflagellates typically divide in the hours near dawn with a 'division gate' duration of 5–7 h (Chisholm, 1981). Division within the *P.hirobis* population persists through a rather lengthy period of at least 16 h, beginning at the start of the dark phase and extending well into the light phase. The period of cell division coincides with an apparent depression of feeding activity, as might be expected. A close examination of the growth of *G.dominans* showed no such diel pattern of cell division (Nakamura *et al.*, 1992).

Ingestion rates reached a maximum of 23 diatoms grazer^{-1} day^{-1}; this datum, together with the corresponding doubling time of 17 h (during which 13.4 diatoms are ingested) can be used to estimate the gross growth yield or efficiency of *P.hirobis*. Although cell carbon measurements have not yet been made for the species involved, cell volumes of the dinoflagellate and diatom are easily calculated. The plasma volume of the diatom is harder to estimate, however. If one assumes a plasma volume:cell volume ratio of 0.25, it follows that one grazer ingests ~2.5 times its own plasma volume prior to cell division. This corresponds to a gross growth yield of 40%, a high but typical protozoan value (Heinbokel, 1978; Fenchel, 1982; Andersen, 1989; Caron *et al.*, 1985). In the case of *Protoperidinium*, this important parameter needs to be determined in a more rigorous fashion.

The measured clearance rates of *P.hirobis*, ranging from 0.1 to 0.5 μl grazer^{-1} h^{-1}, are considerably lower than those obtained by Lessard and Swift (1985), who reported clearance rates ranging from 1 to 28 μl grazer^{-1} h^{-1} for species of *Protoperidinium*. However, it is difficult to compare their clearance rates with the ingestion rates obtained in this study. Food abundance has a strong influence on clearance rate measurements, and the food densities used by Lessard and Swift (who obtained their food assemblage from an open ocean net tow, which was then diluted) were undoubtedly lower than that used in the present study, possibly resulting in higher clearance rates. It should be noted that Lessard and Swift used only the <20 μm size fraction of their plankton samples for food. It is now known that *Protoperidinium* spp. feed, at least in coastal waters, almost exclusively on particles larger than 20 μm (Jacobson and Anderson, 1986). Furthermore, *P.hirobis* is smaller than the large protoperidinioid dinoflagellates used in the Lessard and Swift study.

The rates, frequencies and durations measured in the diel time course experiment can be interrelated to test for internal consistency. Since diatom particles range from one to two cells in length, an average meal size of 1.5 diatom cells can be assumed. Thus an ingestion rate of 20.2 diatoms day^{-1} corresponds to 13.5 meals day^{-1}. Given a feeding frequency of 25% (Figure 8), a feeding duration can be calculated using the following relationship:

$$\text{Feeding duration (h)} = \frac{f \times 24}{n \times 100}$$

where f = % feeding frequency and n = number of meals day^{-1}. This result of 0.44 h or 27 min is shorter than the directly measured mean feeding duration of 60 min, but this latter value may be overestimated due to suboptimal conditions imposed upon the isolated cells. The feeding frequency, on the other hand, may be underestimated since these dinoflagellates very readily release their tethered diatom when disturbed. Although there are some discrepancies among these data, a reasonable conclusion is that mean feeding durations fall between 0.5 and 1 h. The above mentioned meal frequency of 13.5 meals day^{-1} indicates that the interval between meal cycles is somewhat less than two hours. Given a meal duration of up to one hour, roughly an hour would still be available for the completion of cytoplasmic processes that would allow the repeated deployment of the pallium [the pseudopodal apparatus described by Jacobson and Anderson (1986)].

In general, the growth and feeding rates for *P.hirobis* determined in this study seem internally consistent. What ramifications do these measurements have on the *Protoperidinium*/diatom communities? Since the majority of *Protoperidinium* spp. whose feeding behavior has been observed feed on a wide variety of diatom species (Jacobson and Anderson, 1986) it is reasonable to attempt an extrapolation of these data. On occasions of especially high *Protoperidinium* abundance (i.e. 10^4 l^{-1}) a significant portion of diatom standing crop (2×10^5 diatom cells l$^{-1} \times$ day^{-1}) may be grazed, which suggests that this species and others like it may represent an important trophic link in the marine food web. Theoretically, the *Protoperidinium* community can respond far more rapidly to abundant diatom resources than can metazoan grazers of diatoms. Episodes of peak *Protoperidinium* abundances are rather brief (Hasle and Smayda, 1960; Jacobson, 1987) and at 'off-peak' times the *Protoperidinium* community is not likely to have a large impact on diatom standing stocks. However, the remarkable ability of these heterotrophs to transfer biomass from large diatom particles to smaller, more compact and perhaps more nutritious particles may be important to some ciliates and other protists can only ingest small (<40 μm) particles. The very brevity of peak *Protoperidinium* abundances may reflect their own desirability as food to metazoans as well as protistan predators. The food value of thecate heterotrophic dinoflagellates compared to that of diatoms and photosynthetic dinoflagellates deserves investigation.

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