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A Study of Reproduction and Other Life Cycle Phenomena in Planktonic Protists Using an Acridine Orange Fluorescence Technique*

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Abstract

The percentage of dividing individuals and temporal reproductive patterns were determined for natural populations of several planktonic protists including five species of tintinnids, a dinoflagellate, and a diatom. To obtain these data, a method was used in which the nuclei of planktonic ciliates and phytoplankters can be fluorescently stained with acridine orange at the time of collection and fixation. The technique is simple and can be used routinely in studies of reproduction or other life cycle phenomena of natural protistan populations. For the tintinnids, often more than half of the individuals were in some recognizable stage of fission; periodicity in the division process was only observed once and apparently followed a pulse of conjugation in the population. With the diatom *Ditylum brightwellii* the fluorescent staining technique yielded data on the extent and timing of division which were consistent with, but more complete than, previous enumerations of paired cells.

Introduction

One of the major problems resisting solution in the study of pelagic food chains is that of measuring the growth rate or secondary production of zooplankton. Traditional efforts to estimate the growth of natural zooplankton populations have required extrapolation from laboratory measurements and/or monitoring of single populations for relatively extended periods of time (Mullin, 1969). Both methods are limited. The relevance of laboratory measurements to the natural situation is almost always suspect, and it is generally exceedingly difficult to sample repeatedly a single, well defined, zooplanktonic population for a sufficient period of time to derive the needed demographic statistics. A compromise between the two approaches, the use of large volume containers (e.g. Mullin and Evans, 1974; Beers *et al.*, 1977), has recently shown promise as a partial solution to the problem but is not applicable to the full range of environments and populations of interest.

Edmondson (1960) introduced another technique in which the number of eggs being carried by the females of a population and the length of time required for the eggs to hatch are used to calculate the birth rate of the natural population from a single preserved sample. The Edmondson "egg ratio" technique, and variants thereof, have only occasionally been applied to problems concerning zooplankton growth or production (Edmondson *et al.*, 1962; Hall, 1964; Checkley, 1980). A slight modification of the technique, however, has been successfully applied in defining species-specific growth rates and temporal division patterns in natural phytoplankton populations (Swift and Durbin, 1972; Weiler and Chisholm, 1976; Weiler and Eppley, 1979; Weiler, 1980). In these studies actively dividing and/or recently divided cells were identified to obtain an index of the population's reproductive ("birth") rate.

Edmondson (1971) suggested that such a technique could be used with populations of protozoa if a suitable means could be found to identify dividing individuals. In this study we examined reproduction in natural populations of several planktonic protists. Tintinnids, a loricated suborder of the Ciliophora, received particular attention, since they are ubiquitous in marine and estuarine waters, are conveniently sampled, and have been shown to play a significant role in the dynamics of the planktonic food web (Heinbokel and Beers, 1979). In addition we anticipate that the assemblage of tintinnids and other ciliates, with their potential for rapid growth (Heinbokel, 1978) and response to changing conditions, will provide a very valuable model for investigating general features of zooplankton dynamics such as their response to patchy phytoplankton (food) distributions or to pollutants. An

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important part of this work was to develop a technique which could be easily and routinely carried out and which would allow enumeration of dividing organisms with little or no additional effort over that presently required for routine microscopic examination of micro-zooplankton assemblages. While the major emphasis in this paper is on the reproduction of tintinnids, we wish to stress that the technique employed here can be applied easily and simultaneously to other ciliate taxa and to the great majority of phytoplankton species which co-occur in these samples, and that asexual reproduction is not the only life cycle phenomenon that can be profitably studied with this technique.

Materials and Methods

The frequencies of division for five tintinnids, one dinoflagellate, and one diatom from captured planktonic populations were investigated during several experiments undertaken between April 1979 and September 1980. Experiments were conducted for 24- to 36-h periods at various locations within the Chesapeake Bay (USA) and its subestuaries. The specific organism(s) observed, location and starting time for each experiment are as follows: Experiment I, *Tintinnopsis acuminata* (Daday) (see Kofoid and Campbell, 1929; Gold and Morales, 1976), York River (37°14′N lat.; 76°29′W long.), 1 700 hrs April 24, 1979; Experiment II, *Eutintinnus pectinis* (Kofoid) and *Ditylum brightwellii* (West), central Chesapeake Bay (38°23′N lat.; 76°20′W long.), 1 700 hrs August 24, 1979; Experiment III, *Tintinnopsis levigata* (Kofoid and Campbell), York River (37°17′N lat.; 76°34′W long.), 1 000 hrs March 18, 1980; Experiment IV, *Eutintinnus pectinis* (Kofoid), central Chesapeake Bay (38°34′N lat.; 76°27′W long.), 1 000 hrs July 23, 1980; Experiment V, *Ceratium furca* (Ehrenberg), central Chesapeake Bay (38°04′N lat.; 76°12′W long.), 1 930 hrs August 18, 1980; Experiment VI, *Stylicauda platensis* (Cunha and Fonseca) (see Cosper, 1972) and *Amphorellopsis acuta* (Schmidt), central Chesapeake Bay (37°24′N lat.; 76°05′W long.), 1 300 hrs September 24, 1980.

For all experiments, a 200-l Nalgene cylindrical tank equipped with a stirring paddle and aeration line was filled with surface water. The tank was maintained at ambient surface water temperatures by continuously bathing the outside of the container with bay water. A two-liter sample was taken from the tank each hour, the organisms concentrated on 20 μm Nitex netting and preserved with either an acridine orange-formaldehyde mixture or a modified Bouin's fixative. For acridine orange-formaldehyde fixation, a working solution of 200 μg ml^{-1} acridine orange in concentrated 37% (w/v) calcium carbonate buffered formaldehyde was diluted 1:19 with sample, resulting in final concentrations of 10 μg ml^{-1} acridine orange and ~2% (w/v) formaldehyde. The modified Bouin's fixative, comprised of picric acid saturated in concentrated calcium carbonate buffered formaldehyde containing 5% (v/v) acetic acid, was also diluted 1:19 with sample.

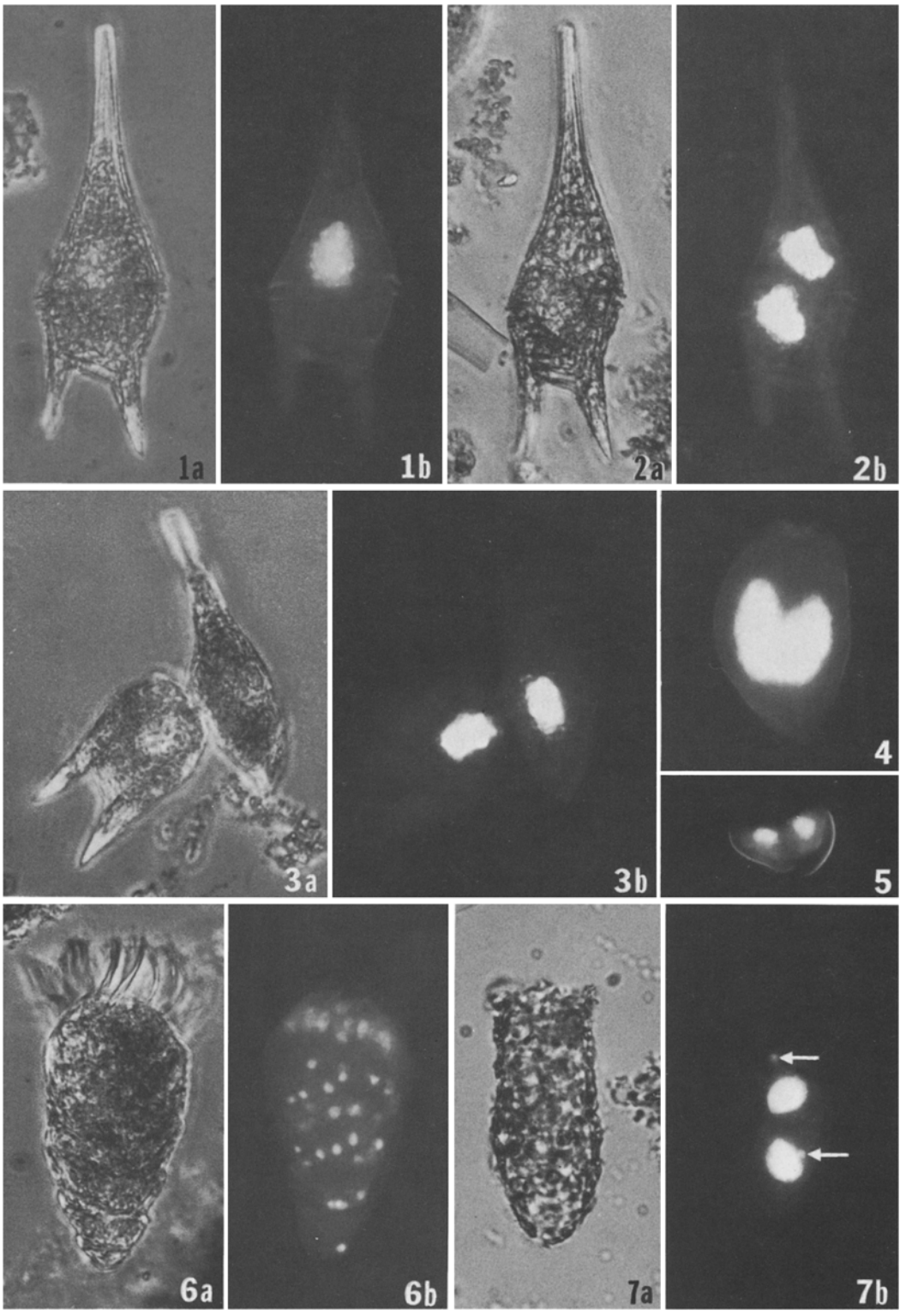
Observations on the nuclear events during the asexual reproductive cycle of *Eutintinnus pectinis* were made from organisms in an agnotobiotic culture established September 27, 1979 and maintained for one week at 23 °C. The culture was started in a glass carboy containing one-liter cultures each of the microflagellates *Isochrysis galbana, Monochrysis lutheri,* and *Dunaliella tertiolecta* and 17 l of 20 μm Nitex filtered Chesapeake Bay water (38°45′N lat.; 76°25′W long.). To this mixture approximately two thousand *E. pectinis* individually isolated from net tows taken at the same location were added. Once the tintinnids reached sufficient numbers (> 1 ml^{-1}), subsamples of the culture were taken periodically and preserved in either the modified Bouin's fixative or in acridine orange-formaldehyde.

Samples preserved in acridine orange-formaldehyde were concentrated onto 20 μm Nitex netting, washed 2–5 min with tap water, transferred to a Zeiss settling chamber, and examined with a Leitz Divert inverted microscope equipped with a 100 W mercury lamp for epifluorescence (exciting wavelength of 455–490 nm) and a 6 V 15 W tungsten lamp for transmitted brightfield/phase contrast illumination. Bouin's fixed samples were washed onto 20 μm Nitex netting, stained with acidulated alum hematoxylin (Galigher and Kozloff, 1971), dehydrated through 100% ethanol, and transferred to a type HA Millipore filter. This filter was placed face down and affixed onto a coverslip by exposure to boiling acetone vapors, cleared in xylene, and mounted. Hematoxylin stained specimens were examined with a Zeiss WL brightfield/phase microscope. An Olympus OM-2N was used for photography.

Results and Discussion

Classical cytological staining techniques for nuclei (e.g. Feulgen and hematoxylin stains) have been used to investigate protistan growth dynamics but are much too tedious to be employed in the routine examination of plankton samples. Even the relatively simple acetocarmine stain used by Weiler (1980) requires extensive manipulation of the sample. Acridine orange-formaldehyde, however, has the significant advantage of functioning as a combined fixative, preservative, and fluorescent stain in which samples can be stored (with no special precautions) for a year or longer without deterioration in stain quality. Subsequent handling is minimal, since specimens need only be washed and examined. Furthermore, if screening of appropriate mesh or pore size is used for concentrating and/or washing samples, then quantitative cell counts and classifications of nuclear morphologies of many plankters can be obtained simultaneously.

We generally employ a working solution of 200 μg of acridine orange per milliliter of full strength formaldehyde. This solution is quite stable and after several



Figs. 1–7. Specimens preserved and stained in acridine orange-formaldehyde fixative. Figures presented as paired photographs picture the same individual viewed with (a) phase contrast and (b) epifluorescent illuminations. Fig. 1. Non-dividing *Ceratium furca* containing a single nucleus. ×425. Fig. 2. Dividing *C. furca* with paired nuclei following the completion of karyokinesis. ×425. Fig. 3. Late division in *C. furca* with one nucleus segregated into each of the nearly separated daughter cells. ×425. Fig. 4. *Prorocentrum micans,* a dinoflagellate possessing a prominent bilobed trophic nucleus. ×750. Fig. 5. Late division in *P. mariae-lebouriae* showing paired nuclei and dislocated thecal valves. ×570. Fig. 6. Non-dividing *Strombidium strobila,* a ciliate with multiple macronuclei. ×400. Fig. 7. Trophic *Tintinnopsis acuminata* exhibiting the typical complement of two micronuclei (arrows) and two macronuclei. ×750

months storage in the dark at room temperature (ca. 21 °C) continues to perform well. The length of time that the fixative-stain is applied is relatively unimportant since staining occurs within 2–5 min and the extent of staining is dependent upon stain concentration rather than time. Final acridine orange concentrations between 1 and 10 μg ml^{-1} yield excellent nuclear staining for most unicellular plankters. Solutions stronger than 10 μg ml^{-1} acridine orange generally obscure nuclei by overstaining the cell cytoplasm. Stain concentrations below 1 μg ml^{-1} result in weak or no nuclear fluorescence. Acridine orange can also be added to samples previously preserved in formaldehyde, although the quality of the nuclear stain is slightly inferior to that obtained by simultaneous fixation and staining. Formaldehyde, which can cause some distortion of soft bodied organisms, may be replaced by glutaraldehyde. A working solution of 100 μg acridine orange per ml of 25% glutaraldehyde, when diluted 1:9 with sample, usually improves organism preservation and yields nicely stained nuclei. Care should be taken to refrigerate glutaraldehyde working solutions and samples, since the fixative may deteriorate at room temperature (see Hayat, 1970, for a discussion on the use of glutaraldehyde).

Some of the larger protistan plankters (e.g. the ciliates *Prorodon marinum* and *Cyclotrichium sphaericum*) accumulate excess cytoplasmic stain regardless of fluorochrome concentrations. In such instances, soaking the organisms in tap water for 24–48 h following the short standard wash often removes enough excess stain to permit nuclear observation. Wash times in excess of 48 h tend to diminish nuclear fluorescence and are not recommended. In well stained specimens, the nuclei typically stain an intense yellow-green, while cytoplasm exhibits a weak yellow to red fluorescence. The nuclear stain fades very gradually when exposed to intense blue-violet illumination but is sufficiently stable, lasting for several minutes, to permit routine microscopic examination and photography. Photographs of stained specimens representing a number of taxa are presented in Figs. 1–7 to demonstrate the wide utility of this technique.

The morphological events accompanying asexual reproduction in the tintinnids studied here are similar and differ little from those previously described for other tintinnid species (Biernacka, 1952). For clarity, a brief account of the division process is presented (see Figs. 8–17). In the early stages of division, a replication band appears in each of the macronuclei and the developing oral apparatus of the posterior daughter cell is visible midventrally. The replication bands migrate through and are associated with an elongation of the macronuclei. Accompanying passage of the replication bands, the macronuclei move toward each other and eventually fuse to form a single, condensed nucleus. The condensed nucleus undergoes successive divisions, two in *Tintinnopsis acuminata, T. levigata,* and *Stylicauda platensis,* three in *Eutintinnus pectinis,* and four in *Amphorellopsis acuta,* providing each daughter cell with the normal trophic complement of two, four, or eight macronuclei in these species, respectively. Micronuclear division either precedes or accompanies the first fission of the condensed nucleus and cytokinesis occurs concurrently with the last macronuclear division. In preparations employed here, tintinnid micronuclei are often obscured by macronuclei and other cellular inclusions and thus division frequencies are based solely on macronuclear division patterns.

During Experiment I, *Tintinnopsis acuminata* reproduced by typical asexual division and the observed frequencies of dividing cells exhibited no apparent daily periodicity (Fig. 21). The frequencies from acridine orange stained material are consistently lower, by an average of 13%, than those derived from hematoxylin preparations. Based on hematoxylin stains, an average of 50.0% of the *T. acuminata* population was in division at any given time throughout the experiment, with 47.0% having replication bands and 3.0% in later stages.

The grosser aspects of nuclear morphology (i.e., size and shape) are easily observed following acridine orange

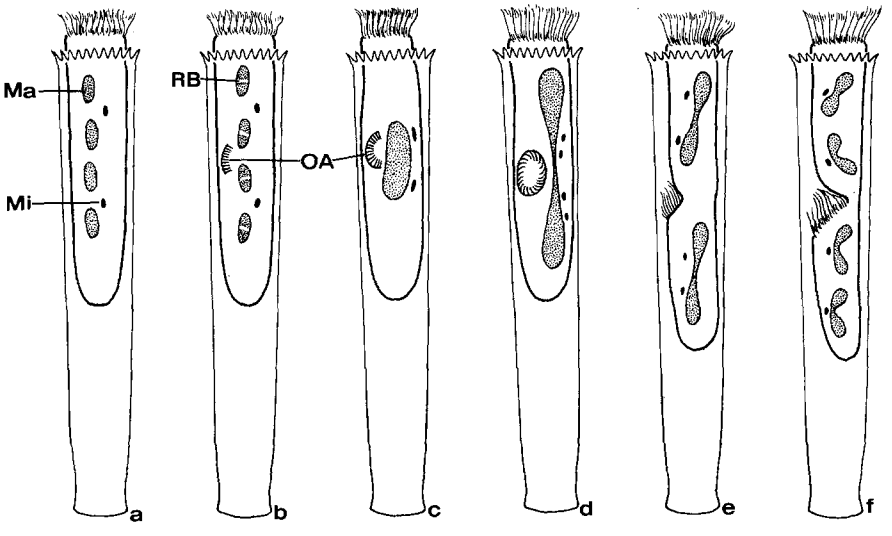
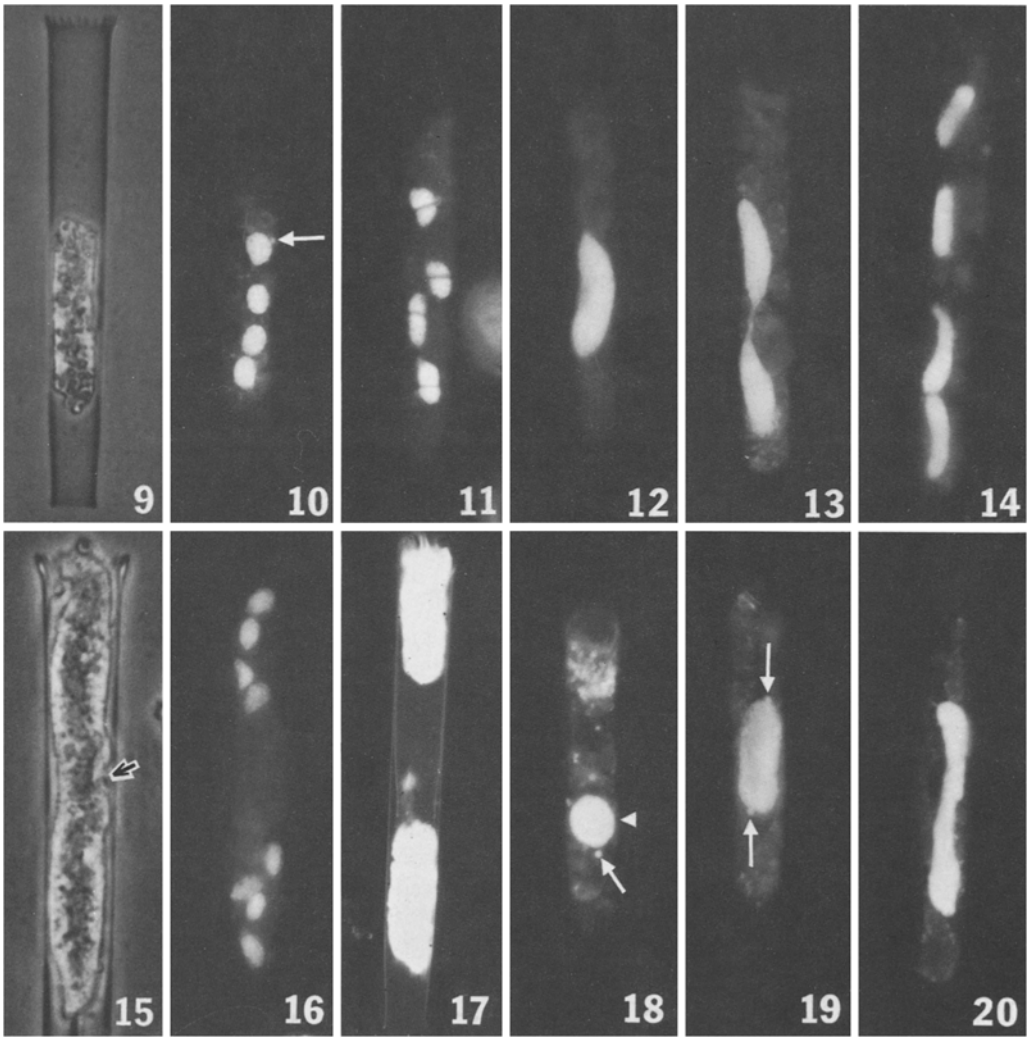


Fig. 8. *Eutintinnus pectinis.* Sequential stages in the asexual division. (a) trophic organism, (b) replication band stage, (c) condensed nucleus stage, (d, e, and f) first, second, and third macronuclear fissions. Abbreviations: Ma, macronucleus; Mi, micronucleus; RB, replication band; OA, developing oral apparatus of the posterior daughter cell



Figs. 9–17. *Eutintinnus pectinis.* Typical asexual division sequence as revealed by acridine orange-formaldehyde preparations. Fig. 9. Phase contrast of a non-dividing individual. ×400. Fig. 10. Fluorescence of the same individual as Fig. 9, showing the normal complement of four macronuclei and one of the usually two micronuclei (arrow). ×510. Fig. 11. A cell in midreplication band stage with one replication band present in each macronucleus. ×510. Fig. 12. Condensed nucleus produced by the fusion of the four macronuclei. ×510. Fig. 13. An organism nearing the completion of the first macronuclear division. ×510. Fig. 14. The nuclei of a cell having completed the second macronuclear division. ×510. Fig. 15. A very late stage of division showing an indentation (arrow) at the formative site of the posterior daughter's oral apparatus. Phase contrast. ×510. Fig. 16. The same cell as Fig. 15 viewed by fluorescence microscopy with four macronuclei in each of the forming daughter cells. ×510. Fig. 17. Two recently divided cells showing the formation of a new lorica prior to the migration of the anterior daughter from the parental lorica. The apparently intense cytoplasmic fluorescence is not the result of poor staining, but of deliberate overexposure to enhance visualization of the lorica. ×510. Figs. 18–20. Sequential stages of macronuclear anlage development in *E. pectinis* as seen by fluorescence microscopy. **Fig. 18.** An early phase in the maturation of the macronuclear anlage (arrow head); micronucleus (arrow). ×510. **Fig. 19.** Late stage in anlage development closely resembling the condensed nucleus of asexual division; micronuclei (arrows). ×510. **Fig. 20.** A highly elongated anlage undergoing the first of three successive divisions. ×510

staining. However, some of the finer nuclear details occasionally become obscured in heavily loricated or thick specimens. The lorica of *Tintinnopsis acuminata* is constructed of agglomerated non-biogenic flakes and fragments of diatom frustules (Gold and Morales, 1976). These particles diffract light emitted by the fluorescing nuclei and make identification of early replication band stages difficult. Similar complications are not encountered with staining techniques in which specimens are cleared and mounted. Thus, the data obtained by the two staining methods used in Experiment I show marked differences in the percent of cells with replication bands, but yield nearly identical values for the more obvious postreplication band stages.

Another problem that can arise when examining stained and settled samples results from cell geometry. For example, the centric diatom *Coscinodiscus* sp. commonly settles with the pervalvar axis, and thus the axis of nuclear elongation and fission, parallel to the optical axis of the microscope. In this situation, dividing and non-dividing cells may become difficult to distinguish. A similar situation exists for the dinoflagellate *Prorocentrum mariae-*

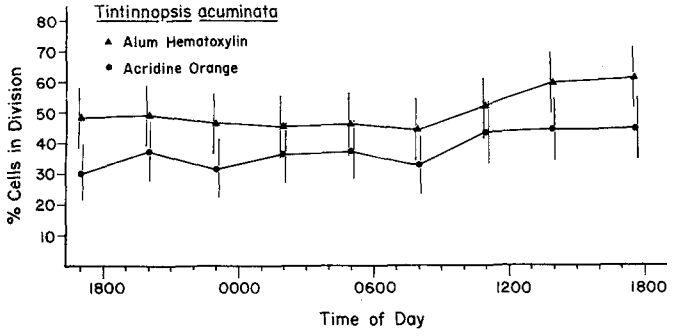


Fig. 21. *Tintinnopsis acuminata.* Experiment I: division frequencies. Means and 95% confidence intervals are given for each sample ($n = 100$)

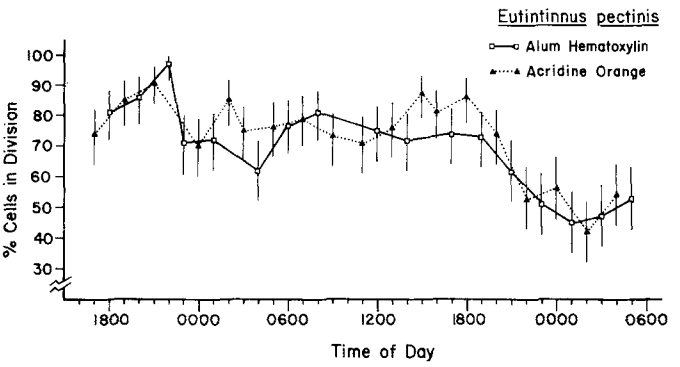


Fig. 22. *Eutintinnus pectinis.* Experiment II: division frequencies. Means and 95% confidence intervals for each sample ($n = 100$)

lebouriae which occurs in many of our samples. Whether the enumeration of only those cells in appropriate orientation for easy characterization of nuclear condition will produce biased data must be determined in each individual case.

For much of Experiment II, a sizable proportion of the *Eutintinnus pectinis* population contained macronuclei which were morphologically unlike any stage observed in the asexually reproducing culture or in *Tintinnopsis acuminata* during Experiment I. Rather than the typical complement of four macronuclei, these cells possessed a single macronuclear anlage (Fig. 18), a stage observed in the sexual phenomena of ciliates. Initially the macronuclear anlage was only slightly larger than a trophic macronucleus but gradually increased in size and temporarily developed polytene chromosomes. Morphogenesis of the polytene chromosomes closely resembled the sequence reported in exconjugants of hypotrichs (Ammermann, 1971; Ammermann *et al.,* 1974). The macronuclear anlage eventually became indistinguishable from the condensed nucleus of asexually reproducing organisms (Figs. 19 and 20), and the remainder of the sexual process was analagous to the latter stages of asexual division (Figs. 13–17).

The data for division frequencies in *Eutintinnus pectinis* are presented in two ways: total percentage of cells in division (Fig. 22) and percent cells in specific division stages (Fig. 23). The percent of cells in division varied during the experiment but showed no discernible diel pattern (Fig. 22). The hyaline loricae of *E. pectinis* did not interfere with identifying early division stages and so the data obtained with the two staining techniques were generally in close agreement. Therefore, in Fig. 23, no distinction was made as to type of staining. For the first 18 h of the experiment, an average of 54% of the cells contained macronuclear anlagen. This value fell sharply to 8% over the next 3 h and held steady for the remainder of the experiment. The decline in occurrence of the macronuclear anlage stage was accompanied by an equally abrupt increase in condensed nuclei and nuclear fission stages. These nuclear configurations, plotted in Fig. 23, reached a peak frequency of just over 50%. The peak persisted for only 3–4 h before quickly dropping to a low level.

While sexuality has often been observed in tintinnids (Apstein, 1893; Entz, 1909; Hofker, 1931; Gold, 1969; and Gold and Pollingher, 1971), the nuclear and cortical processes occurring during conjugation have received little attention. Similarly, nothing is known of the factors leading to sexuality in tintinnids or of the effects of sexuality on population dynamics and vitality. The only detailed account of the nuclear events accompanying conjugation in tintinnids (Laackmann, 1908), although incomplete, strongly indicates that these phenomena proceed as have been described for other ciliates (see Raikov, 1972). Macronuclear anlagen form in ciliates which have undergone either conjugation or autogamy. The anlagen observed in *Eutintinnus pectinis* could have arisen from either of these two sexual processes. However, the occurrence in these samples of several fused pairs of *E. pectinis* suggests that conjugation was the probable mode of sexuality.

Those *Eutintinnus pectinis* in Experiment II which had apparently undergone conjugation passed in unison through the macronuclear anlage and subsequent developmental stages. Presumably then, the sexual process was

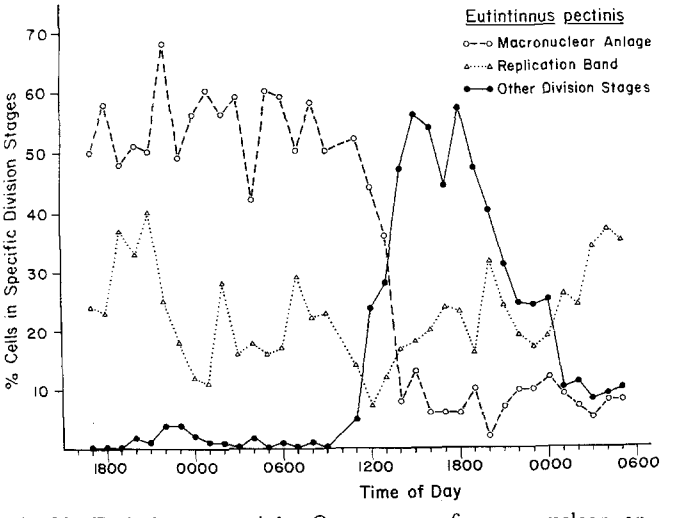
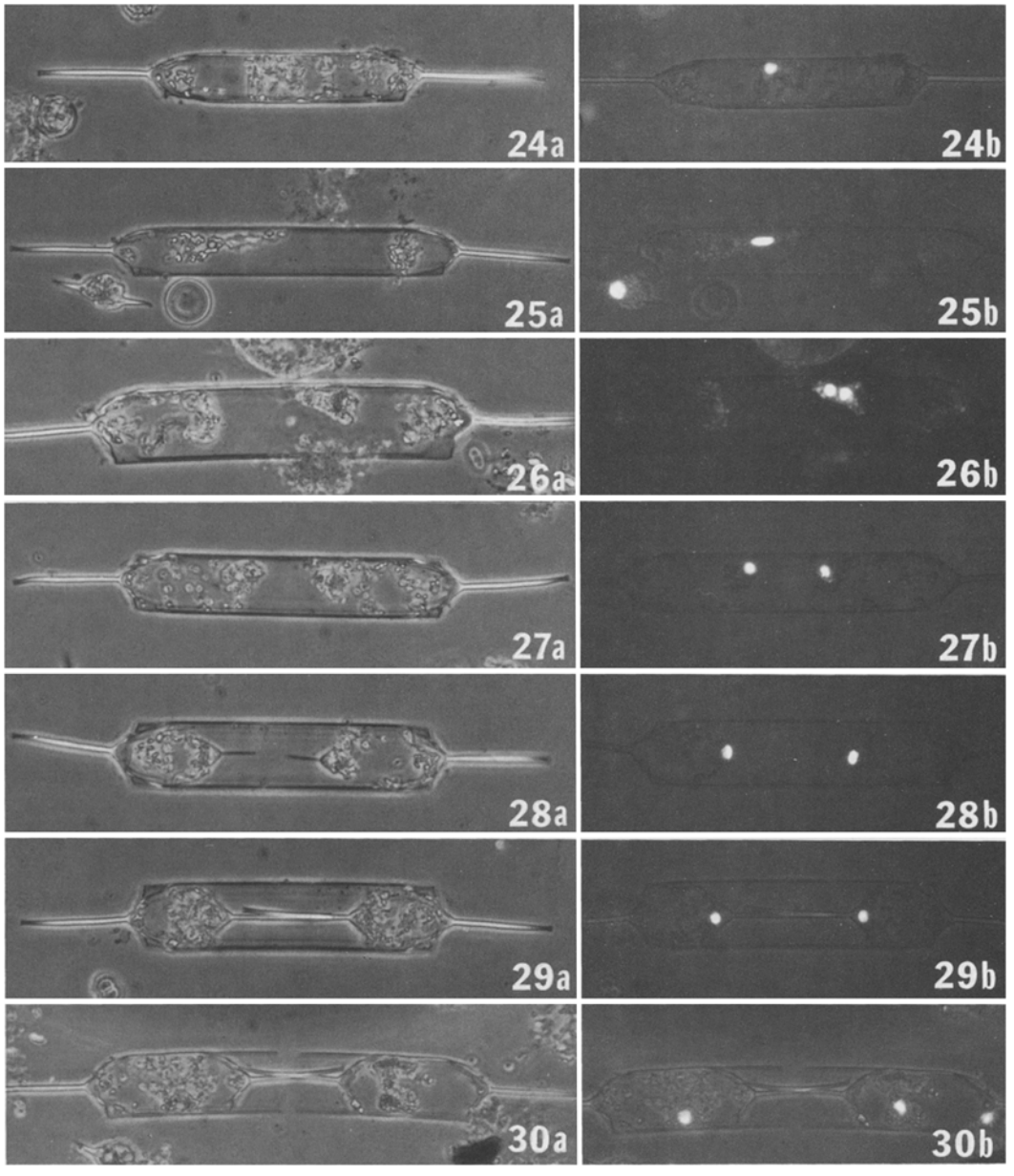


Fig. 23. *Eutintinnus pectinis.* Occurrence of macronuclear anlagen, replication bands, and other division stages (i.e., condensed nucleus and first, second, and third nuclear fissions) from Experiment II



Figs. 24–30. *Ditylum brightwellii.* Division pattern. Each cell is presented as seen with phase contrast illumination (a) and combined phase contrast and acridine orange fluorescence (b). Magnifications are all ×230. Fig. 24. Non-dividing individual. Fig. 25. An elongated nucleus, as observed in this cell, is the first indication of division. Fig. 26. A specimen, which appears in phase contrast to be non-dividing, is revealed by fluorescence to possess paired nuclei. Fig. 27. Separation of the nuclei and cytoplasm precedes assembly of new hypovalves. Fig. 28. An early stage in hypovalve elaboration. Fig. 29. Typical doublet cells. Fig. 30. Separating doublet cells

initiated synchronously, and, as shown in Fig. 23, involved some 50–60% of the population. Conjugation in *E. pectinis* may be seasonally modulated, as suggested for some ciliates (see Raikov, 1972). Alternately, synchronous conjugation might result from the hydrographically mediated concentration of individuals or mixing of two distinct populations of *E. pectinis* which have complementary mating types. Such a concentration of individuals and subsequent pulse of sexual activity has been reported by Tyler (1980) for *Gymnodinium nelsoni* in the Chesapeake Bay.

The division sequence for *Ditylum brightwellii* is illustrated in Figs. 24–30. The small spherical nucleus of the trophic organism increases in size and elongates early in the reproductive process. The elongated nucleus divides to produce two spherical nuclei which migrate toward opposite ends of the cell. Following separation of the nuclei, the hypovalve for each daughter cell begins to form and is progressively elaborated. Dividing organisms can be segregated into "predoublets" (those cells in division stages preceding the earliest detectable formation of new hypovalves, Figs. 25–27) and "doublets" (Figs. 28–30). The data for division frequencies of *D. brightwellii* presented in Fig. 31 show a pronounced diel periodicity resembling patterns described in other diatoms (Smayda, 1975; Chisholm *et al.,* 1978; Nelson and Brand, 1979; William-

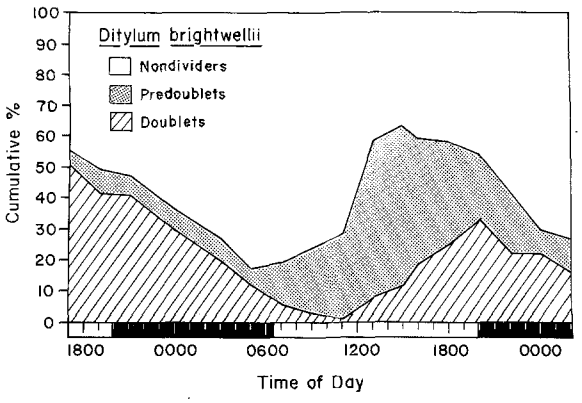


Fig. 31. *Ditylum brightwellii.* Abundance of dividing (predoublets and doublets) and non-dividing *D. brightwellii* during Experiment II. A total of one hundred cells was examined for each sample. Black areas indicate the interval between sunset and sunrise

son, 1980). Also apparent, is the similarity in division frequencies obtained with the fluorescence technique described here (predoublets plus doublets) and those obtainable by more standard microscopy (doublets).

Data for the four tintinnids studied in Experiments III through VI are summarized in Fig. 32. Each of these tintinnid species was reproducing asexually and the proportion of dividing organisms remained relatively constant throughout the sampling periods. In all cases replication band stages far outnumbered other division stages with the latter never exceeding 12% of any sample. In Experiment V, the dinoflagellate *Ceratium furca* showed a pronounced diel periodicity in division with the highest number of dividing cells (46% of the population, $n = 100$) appearing shortly after dawn. The data for acridine orange stained *C. furca* are quite comparable to those obtained by other investigators using more tedious staining techniques (Weiler, 1980 and included references).

The ability to routinely identify and enumerate protists which are reproducing or exhibiting other life cycle phenomena provides a powerful tool for investigating the dynamics of planktonic systems. A number of questions of general ecological interest can be asked about these organisms. Examples include investigation of the response of the zooplankton to environmental changes or variability such as chemical or thermal perturbations (e.g. Reeve *et al.*, 1977) or the well documented patchiness of the phytoplankton (e.g. Platt *et al.*, 1970; Lasker, 1975; Mullin and Brooks, 1976; Wrobleski and O'Brien, 1976). Another potential use for this technique will be to apply it to defining the instantaneous growth rates of the natural protistan populations (e.g. Weiler and Chisholm, 1976; Weiler, 1980). This latter goal, however, will require two additonal determinations: defining any diel periodicity in the division process and the duration of the recognizable division stages. In addition to being of known duration, the division stages must also be of relatively fixed length for a given temperature, regardless of generation time. In ciliates, the duration of nuclear fission appears fairly conservative, whereas the DNA synthesis phase of the cell cycle (comparable to replication band stages) is somewhat more variable (Prescott and Stone, 1967; Suhr-Jessen *et al.*, 1977).

Campbell (1926) and Biernacka (1952) reported an increase in the abundance of *Tintinnopsis* sp. division stages during the early morning hours and suggested that tintinnids exhibit a diel pattern in cell division. The results of the experiments conducted here on five species of tintinnids do not support the existence of a regular periodicity in tintinnid division. In all experiments where tintinnids were reproducing asexually, organisms divided with even frequency throughout the sampling period. While a slight rise in percent cells in division occurred toward the end of some experiments there was no distinct peak indicative of phased cell division. In contrast, the *Eutintinnus pectinis* population of Experiment II underwent a synchronized sexual phenomenon which resulted in a pronounced peak of late division stages. A sexually induced synchrony in cell division may persist for some time in natural populations following such an event but would probably deteriorate within several generations.

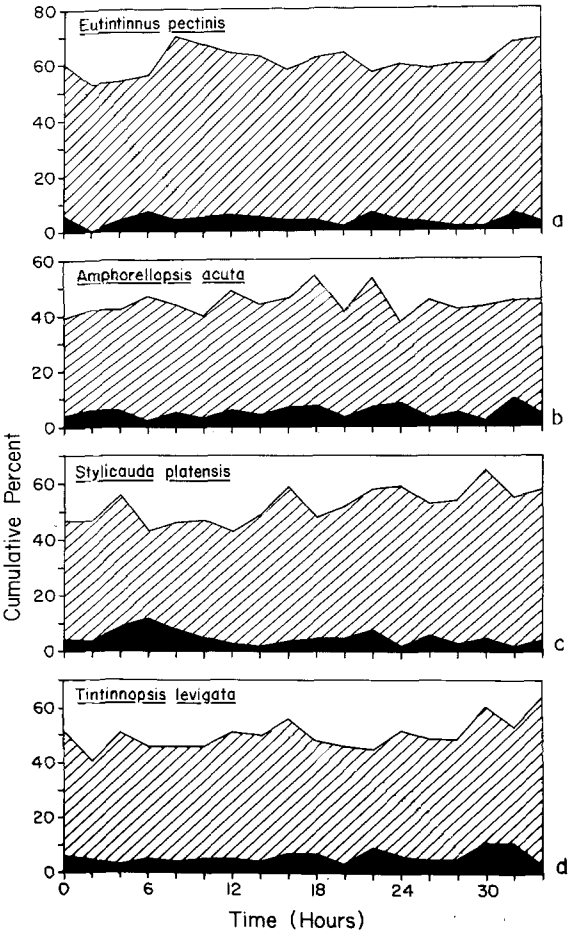


Fig. 32. Division frequencies for the tintinnids (a) *Eutintinnus pectinis,* (b) *Amphorellopsis acuta,* (c) *Stylicauda platensis,* and (d) *Tintinnopsis levigata* ($n = 100$ for each sample). □ non-dividing, ▨ replication band stage, ■ other division stages. Data for *E. pectinis* and *A. acuta* are from acridine orange-formaldehyde preparations; those for *S. platensis* and *T. levigata* are from alum hematoxylin stains

Clearly, phased cell division in tintinnids, whether sexually induced or diel, is a distinct possibility and must be carefully considered in any experiments designed to define tintinnid reproductive rates.

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