# Morphology of the Toxin-Producing Diatom *Nitzschia pungens* Grunow forma *multiseries* Hasle

Daniel J. MacPhee[1] and Louis A. Hanic

*Department of Biology, University of Prince Edward Island, University Avenue, Charlottetown, P.E.I. C1A 4P3, Canada*

Dianne L. Friesen

*Department of Pathology and Microbiology, University of Prince Edward Island, University Avenue, Charlottetown, P.E.I. C1A 4P3, Canada*

and David E. Sims

*Department of Anatomy and Physiology, University of Prince Edward Island, University Avenue, Charlottetown, P.E.I. C1A 4P3, Canada*

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The toxin-producing diatom *Nitzschia pungens* Grunow forma *multiseries* Hasle from three toxic blooms in two Prince Edward Island estuaries, spanning 1987–89, was studied using light and electron microscopy. Cell ultrastructure of *N. pungens* is, in general, similar to that of other species of *Nitzschia* and other diatoms. Important features include prominent peripheral, polarized nucleoli (numbering one or two) and imperforate poroids, present on inner valves and girdle bands. Cell division is usually synchronous for all cells in a filament with respect to polarity and time. Postdivisional elongation of the filament appears to involve a "slide-by" process whereby sibling cells slide by each other along their opposed valve faces and then stop, becoming fused by their overlapping tips. The raphe is probably involved in this, as well as in filament and cell motility. Observations of particle motion along the cell raphe suggest the existence of a motility apparatus such as microcilia which would facilitate locomotion, intercellular coordination, and the postdivisional slide-by process. No bacteria or other organisms were observed associated with field samples of toxic *N. pungens* f. *multiseries*. This supports a view that domoic acid production is autonomous.

La présente étude porte sur la diatomée *Nitzschia pungens* Grunow forma *multiseries* Hasle productrice de toxine. Des échantillons en ont été recueillis de 1987 à 1989 lors de trois poussées phytoplanctoniques dans deux estuaires de l'Île-du-Prince-Édouard, puis étudiés par microscopie optique et électronique. En général, l'ultrastructure cellulaire de *N. pungens* ressemble à celle d'autres espèces du genre et d'autres espèces de diatomées. Les principales structures comprennent des nucléoles proéminentes périphériques et polarisées (au nombre de un ou deux) et des pores imperforés présents sur l'hypovalve et la ceinture. La division cellulaire est généralement asynchrone chez toutes les cellules d'un filament pour ce qui est de la polarité et du moment. L'élongation du filament après la division semble comporter un processus de "glissade" par lequel les cellules filles, suivant un plan parallèle aux deux valves, s'éloignent l'une de l'autre, puis s'arrêtent lorsqu'elles se fusionnent à leurs pôles superposés. Le raphé joue probablement un rôle dans ce processus, tout comme dans le mouvement du filament et de la cellule. Des observations du déplacement de particules le long du raphé portent à croire à la présence d'un appareil locomoteur comme des micro-cils qui faciliteraient le mouvement, la coordination entre les cellules et le processus de glissade après la division. Aucune bactérie ni autres organismes ont été observés dans les échantillons de *N. pungens* f. *multiseries* toxiques recueillis sur le terrain. Cette observation étaye la notion que la production d'acide domoïque est autonome.

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*Nitzschia pungens* Grunow forma *multiseries* Hasle is a pennate, chain-forming, motile, planktonic marine diatom widely distributed in coastal waters (Hasle 1965). It is the suspected source of toxin in three successive years of mussel poisoning in Prince Edward Island (P.E.I.), Canada (Bates et al. 1989). The first and most serious episode occurred in the autumn of 1987, when three deaths and over 100 illnesses followed consumption of toxic mussels (Perl et al. 1990). Domoic acid (DA), a potent neurotoxin isolated from mussels, was determined to be the cause of the poisonings (Wright et al. 1989). Sustained, almost unialgal blooms of *N. pungens* occurred at several sites during all three episodes. Plankton hauls of blooms tested positive for DA, leading to the conclusion that *N. pungens* f. *multiseries* was the source of DA. Supporting evidence came when cultures of f. *multiseries* were found to produce DA (Subba Rao et al. 1988, 1990; Bates et al. 1989, 1991). However, none of the cultures studied was axenic, allowing the possibility that DA production in *N. pungens* is due to an infection (i.e. bacterial, fungal, viral).

Infective agents including viruses (Hoffman 1978) and eubacteria (Paerl and Kellar 1978; Nozaki et al. 1989) have

[1]Author to whom reprint requests should be directed. Current address: Department of Zoology, University of Western Ontario, 1151 Richmond St., London, Ont. N6A 5B7, Canada.

been recorded in algae. Cyanobacteria have been reported in several diatoms (Drum and Pankratz 1965; Heinbokel 1986) as ecto- or endosymbionts. However, none of these has been associated with toxin production.

The present study provides additional light microscopical and ultrastructural information on *N. pungens* f. *multiseries* morphology. No evidence was found for the presence of an infective agent in this species.

## Materials and Methods

Plankton samples were taken with a 10- to 20-μm-mesh plankton net from the Cardigan River and the Brudenell River (Table 1). Sample 1 was fixed in 3% glutaraldehyde in filtered seawater and stored at 5°C for 10 mo prior to osmication and embedding. Sample 2 was transported to the laboratory at ambient seawater temperature (0°C) where it was concentrated with a 10-μm-mesh screen and fixed for 2 h at 5°C in an excess of freshly prepared 3% glutaraldehyde in filtered seawater. Sample 2 was then filtered, rinsed with seawater, and postfixed for 12 h in 1% osmium tetroxide in seawater. The cells were concentrated with a 0.4-μm filter and placed in 3% agar cylinders with an internal cavity (approximately 2–3 mm in diameter, 3 mm long). The cylinders were capped with agar, heat sealed, dehydrated in ethanol, cleared in propylene oxide, and infiltrated with an epon–araldite mixture. They were cut into pieces, placed in moulds, and polymerized for 12 h at 65–75°C.

Samples 3 and 4 were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, in 21 ppt saline water with or without 0.1% ruthenium red. After 12 mo of storage at 5°C, they were concentrated in agar blocks and processed as with Sample 2.

Thin sections were cut with a diamond knife, mounted on bare grids or formvar-carbon coated grids, and poststained with uranyl acetate and lead citrate. Serial sections were viewed for studying certain structures (e.g. pyrenoid membranes, raphe canal, joining of silica frustule and girdle bands).

For scanning electron microscopy (SEM), cells were fixed in 3% glutaraldehyde in seawater or cacodylate buffer as above. Samples were centrifuged lightly, washed in distilled water, and rinsed in ethanol. Drop-samples were placed on SEM stubs, air-dried, and then sputter coated with gold. For frustule morphology and species determination, cells were acid cleaned either by the method of Hasle and Fryxell (1970) or a modification where, following the addition of sulfuric acid, the solution was heated to boiling. In either case, three passes through the procedures were used to assure removal of all organics. (The remaining silica frustules, when placed on a slide and treated with 1% hydrofluoric (HF) acid for a few minutes, completely dissolved, indicating that organics, including a cuticle if present, were removed.) Drops of the diatom solution were placed on SEM stubs, air-dried, and coated with gold. Thirty cells from each collection were taken at random for photography and measured for key features (number of costae and poroids per unit length).

Thirty cells from whole mounts were examined by light microscopy (LM) for cell length, width, and general structure. Whole mounts of glutaraldehyde and osmium fixed samples were stained with toluidine blue for nucleolar number or were treated with 1% HF acid for several minutes for removal of the silica wall component for studying the nature of cell-tip overlap.

Cell division and movement were studied from living material of all four samples and cultures of clone NPARL (a DA-producing clone of *N. pungens* f. *multiseries* obtained from Dr. S. Bates, Department of Fisheries and Oceans, Gulf Fisheries Centre, Moncton, N.B.). Cultures were grown at 15–20°C in 27 ppt salinity Instant Ocean (Aquarium Systems, 8141 Tyler Blvd., Mentor, OH 44060, USA), made with P.E.I. well water supplemented with Fritz ($f_2$) Algae Food (Fritz Chemical Co., Dallas, TX 75217, USA).

## Results and Discussion

### Field Data

Two forms of *N. pungens* were encountered in this study: f. *multiseries* was present in all samples and f. *pungens* was present in Samples 3 and 4 (Table 1). F. *pungens* has also been reported from the Cardigan River in 1989 (Smith et al. 1990), where it was found to predominate during the early bloom period. Cultures of f. *pungens* taken from the September 20, 1989, sample and from the Cardigan River (I. Novaczek, P.E.I. Food Tech. Centre, 550 University Ave, Charlottetown, P.E.I., C1A 4P3, pers. comm., and Smith et al. 1990, respectively) were found to produce no DA. The two forms are morphologically very similar. The distinguishing characteristic is that f. *pungens* has large poroids which occur in two rows; in f. *multiseries* the poroids are small and occur in three to five rows (Hasle 1965). The poroids of both forms in our samples were not resolvable by LM. They were readily seen by SEM, although only on the inside of the valves.

The occurrence of f. *pungens* in eastern P.E.I. has two important implications. As pointed out by Smith et al. (1990), using *Nitzschia* as an indicator species for DA levels is only valid where the species forms and their frequencies are known. This means that identifications must be supported by SEM data. Earlier distributional studies of *Nitzschia* in eastern P.E.I. were based mainly on LM data (i.e. Hanic and Cormier 1989; Bates et al. 1989). It is probable that f. *pungens* was occasionally present, which limits interpretation of the data unless archived samples can be reexamined by SEM.

*Nitzschia pungens* f. *multiseries* was almost the sole phytoplankter in Sample 2. At the time of sampling, the bloom

TABLE 1. *Nitzschia* sampling sites and cell and DA concentrations.

| Sample No. | Sample site | Date (d/mo/yr) | Bloom period | Concentration (cells·L$^{-1}$) | Abundance (% by number)[a] | % f. *pungens* | % f. *multiseries* | DA in plankton (μg dry weight$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 1 | Cardigan | 10/12/87 | End | $2 \times 10^7$ | >95 | ~0 | ~100 | —[b] |
| 2 | Cardigan | 19/12/88 | End | $1 \times 10^5$ | >95 | ~0 | ~100 | 673 |
| 3 | Brudenell | 20/09/89 | Start | — | ? | 81 | 19 | 0 |
| 4 | Brudenell | 02/11/89 | End | — | >95 | 8 | 92 | 1060 |

[a]Abundance of *N. pungens* f. multiseries plus f. *pungens* in the phytoplankton, given as a percentage of total cells (all species).
[b]Sample was taken 1 d later but at the same site as in Sample 1. Sample tested positive for DA by mouse bioassay (E. Todd, Health and Welfare Canada, Ottawa, Ont., Canada).

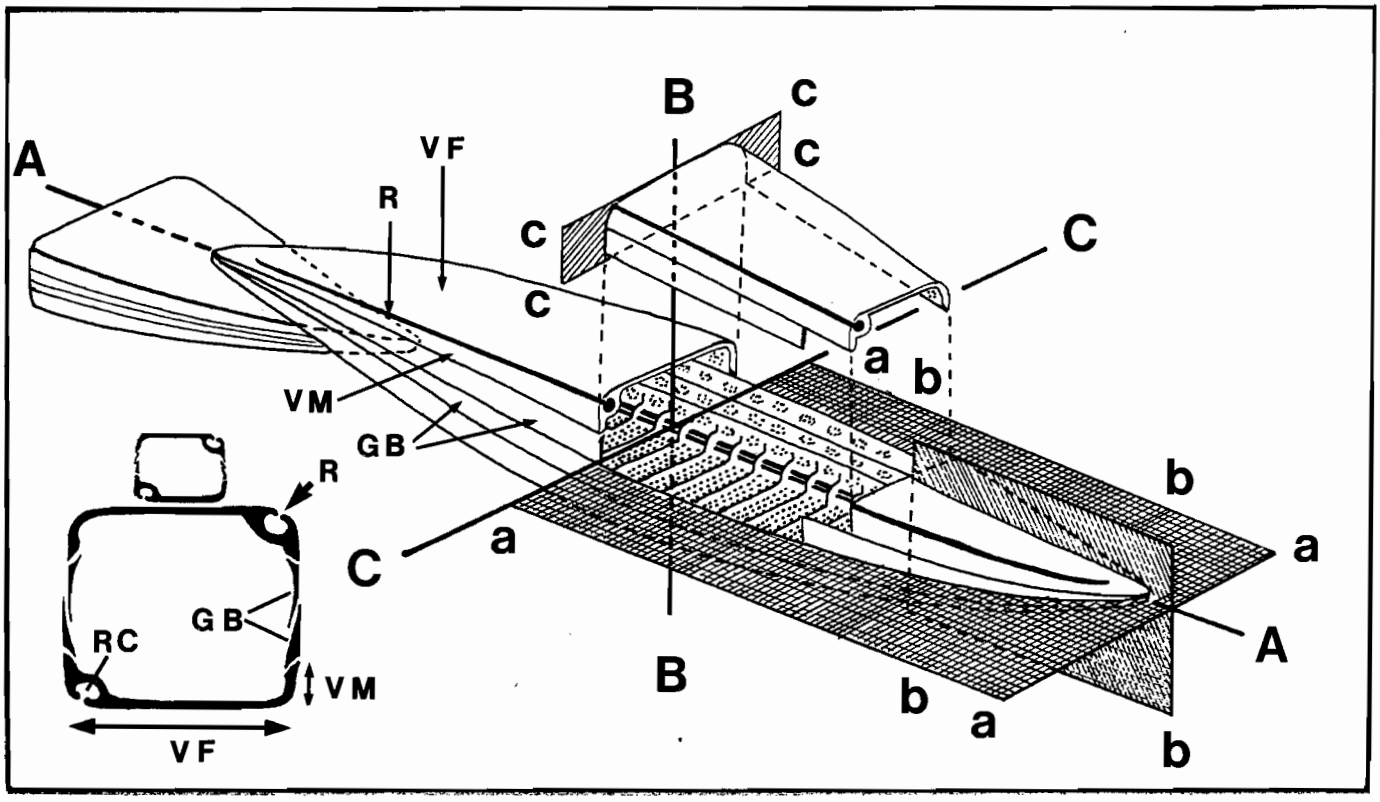


FIG. 1. Silica frustule of *N. pungens* f. *multiseries* shown diagramatically, with cell length much reduced. Reference axes and planes: A, apical axis; B, principle or pervalvar axis; C, transapical axis; a, valvar plane; b, apical plane; c, transapical plane. Raphe (R) on edge of upper valve face (VF) and valve mantle (VM). Section of upper valve removed to show raphe canal (RC), costae, three to five rows of poroids in the intercostal areas and rectangular depressions, containing several poroids on the inner side of girdle bands (GB). Diagram in left-hand corner shows cross-section of two overlapping cells with the raphes in the normal diagonally opposite position.

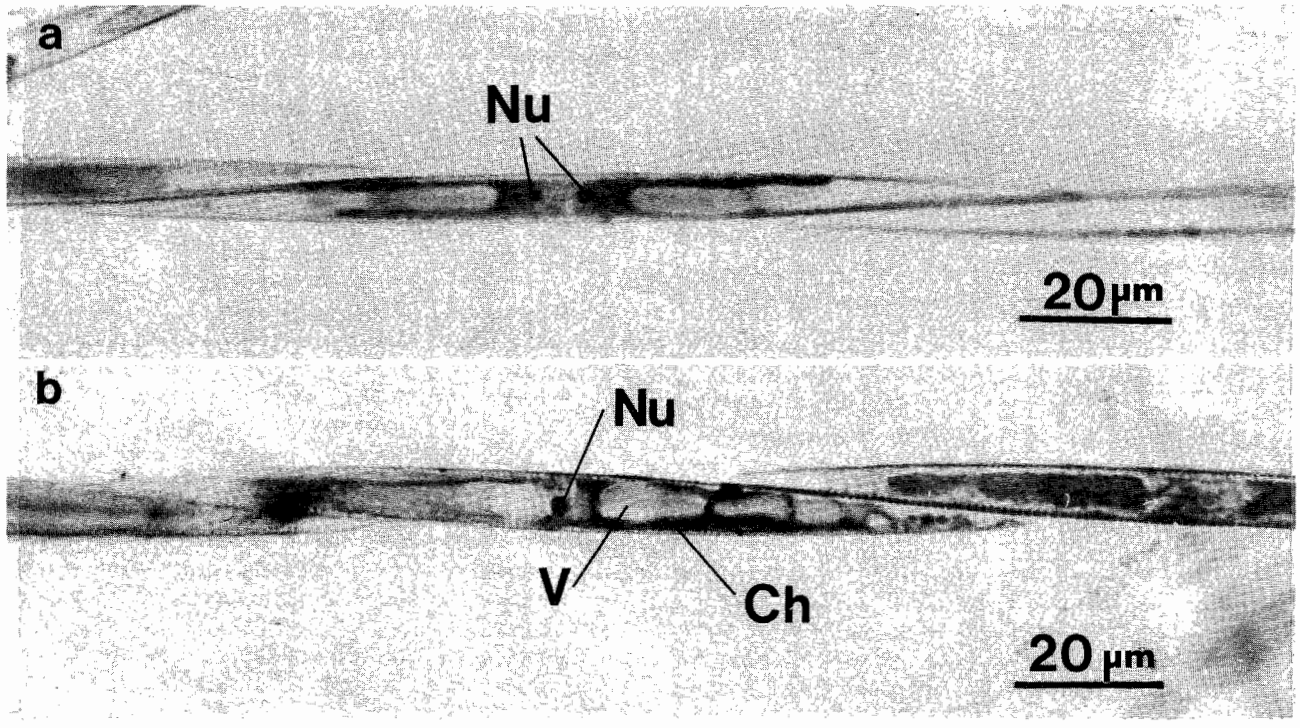


FIG. 2. Filaments from Sample 4 in optical apical section fixed for TEM stained 10 mo later with 1% toluidine blue. Girdle view, central cell showing chloroplasts (Ch), vacuoles (V), and nuclear area, with two nucleoli (Nu) in Fig. 2a and one nucleolus in Fig. 2b.

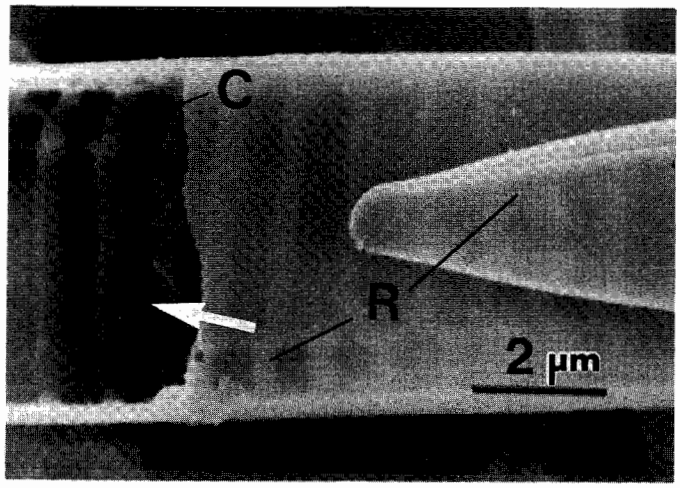


FIG. 3. Sample 2, sulfuric acid cleaned preparation showing overlapping cells, lower cell missing part of upper valve, inner part of lower cell showing costae (C), and intercostal areas (arrow), with well-defined inner openings to the poroids. Note upper cell (right) is attached to lower cell, indicating cementing material was not removed by acid treatment. Note also that raphe of upper valve of this cell is on diagonally opposite side of cell (normal position), compared with that of cell below. In bottom cell, raphes are opposite in mirror image (the abnormal) position.

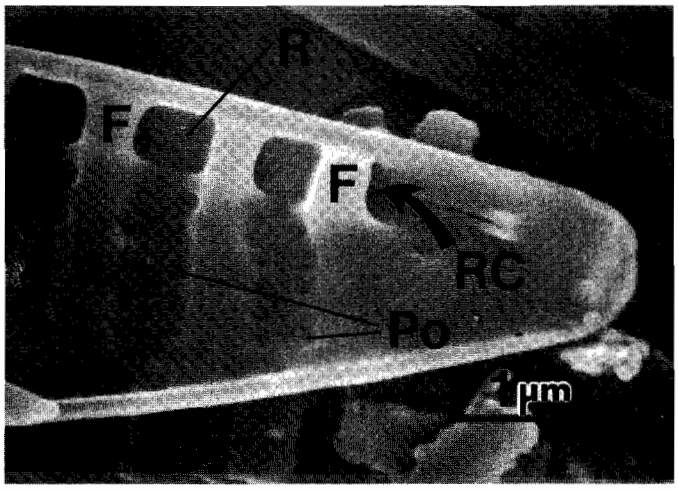


FIG. 4. Sample 2, sulfuric acid cleaned cells showing tip of inner valve with poroids (Po), raphe (R) with terminus, and fibulae (F) bridging raphe canal (RC).

had declined in concentration to $0.1 \times 10^6$ cells·L$^{-1}$ from a maximum level of $1.2 \times 10^6$ cells·L$^{-1}$ reached earlier on November 14, 1988. Sample 2 yielded a DA content of 673 μg·g dry weight$^{-1}$ which is significant, although considerably lower than the peak of 11 900 μg·g$^{-1}$ recorded from a plankton sample from the Cardigan River, January 1, 1988 (Bates et al. 1989).

All four samples were viewed alive by LM within an hour of collection. Sample 2, which was extensively used for our ultrastructural studies, was critically examined by both bright-field and phase-contrast microscopy. In all samples, *Nitzschia* occurred in long chains and appeared healthy and actively dividing. Filaments exhibited motility when placed on a glass slide and illuminated. Outside surfaces appeared clean and free of epiphytic bacteria or other organisms.

## Morphology

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Girdle bands appear to encircle the cell without a break (Fig. 1). Bands are deepest in the midregion of the cell and taper gradually to almost nothing at both poles. On the inside, they have a single row of regularly spaced rectangular depressions (Fig. 1 and 5) lined with several conspicuous poroids. During cell enlargement, additional girdle bands are formed which appear to be incorporated in the sibling cells following division. The valve mantle is shallow (Fig. 1 and 5), allowing for flexing during cell enlargement. The edge of the mantle is bluntly and convexly rounded. The abutting edge of the girdle band is also blunt but concave, fitting into the mantle in the manner of a hinged flap (Fig. 5). This construction allows slight outward flexing of the girdle band, which enables expansion during division and maintenance of cell width.

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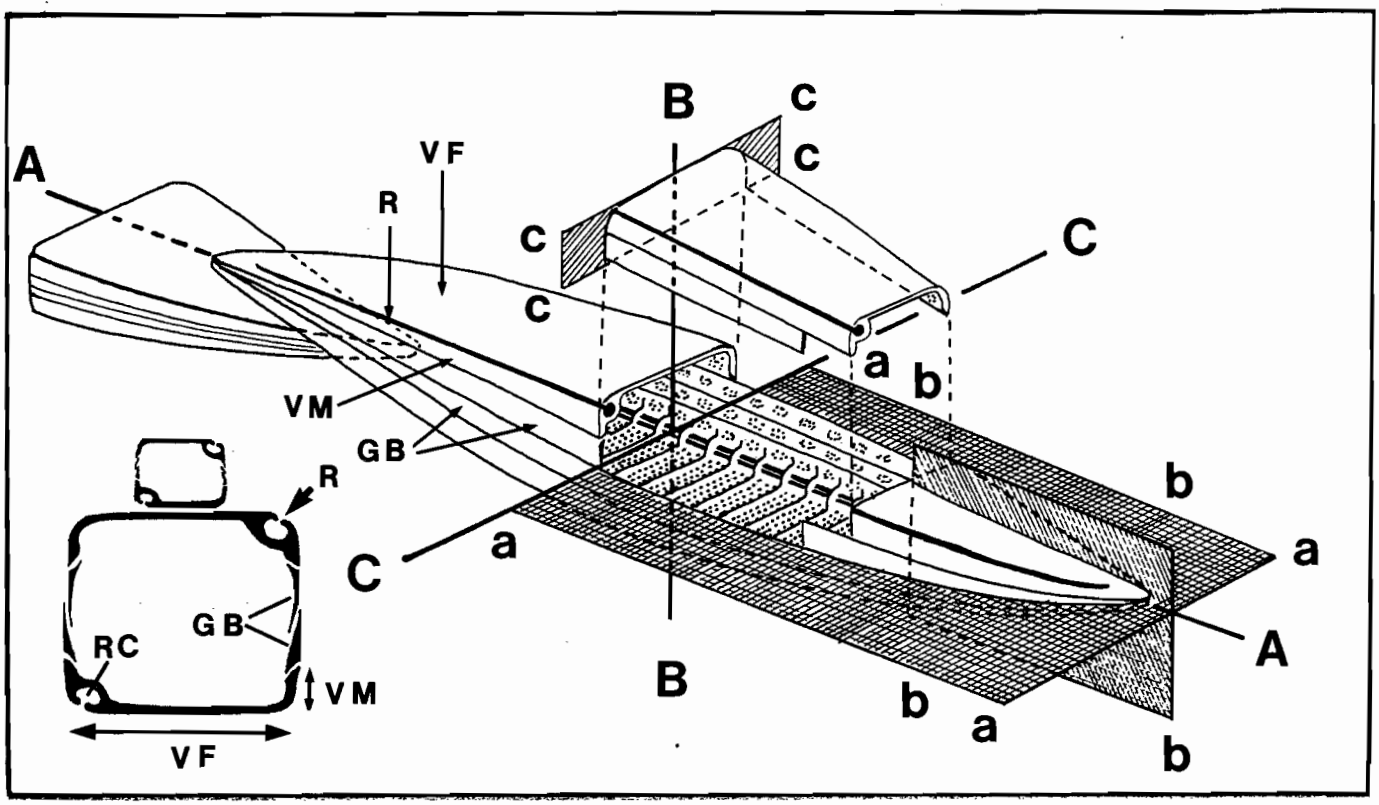


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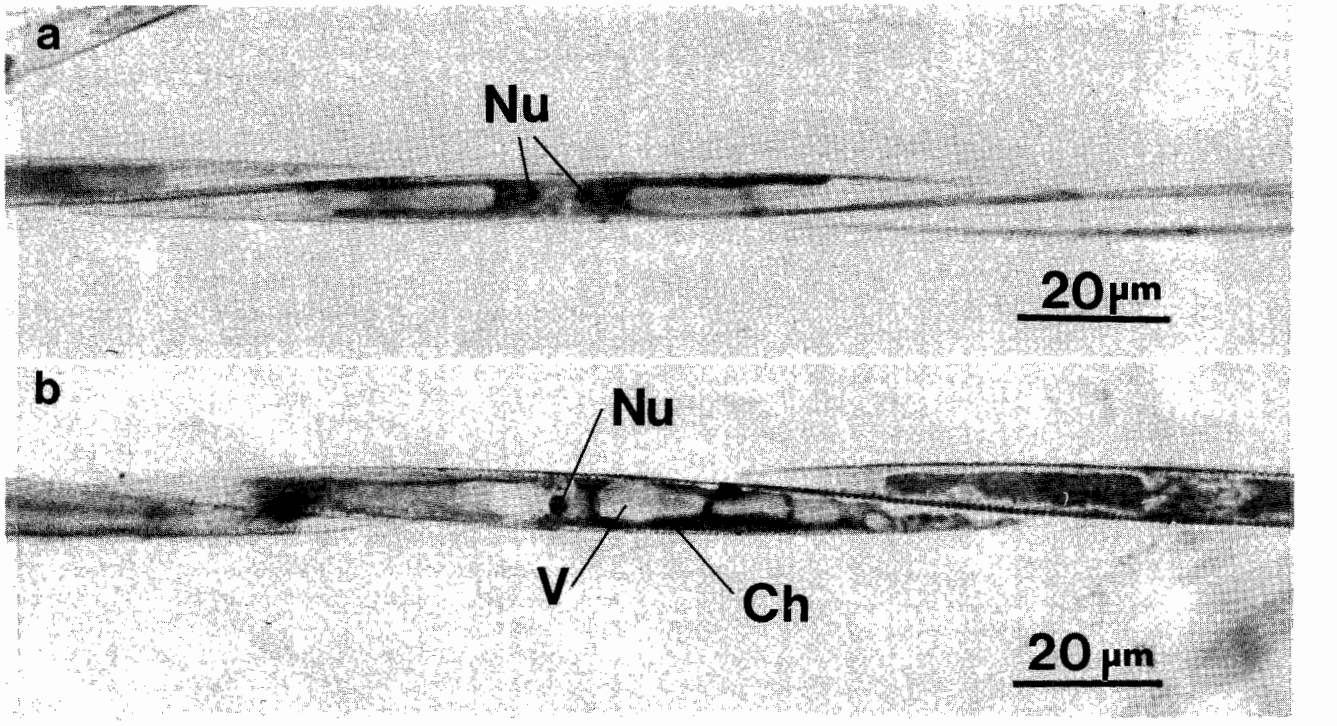


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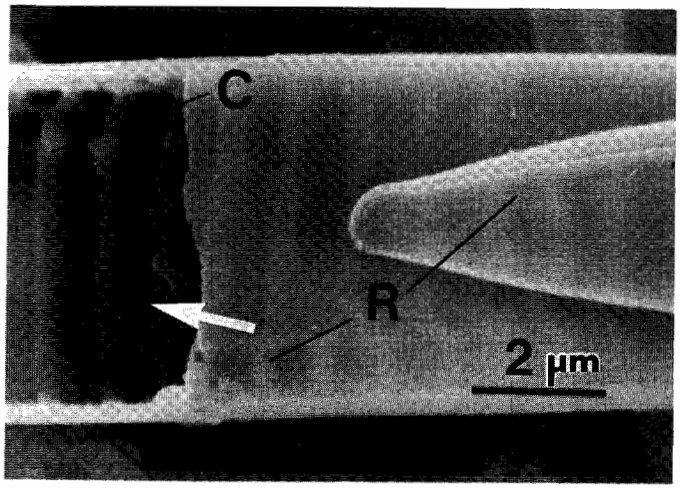


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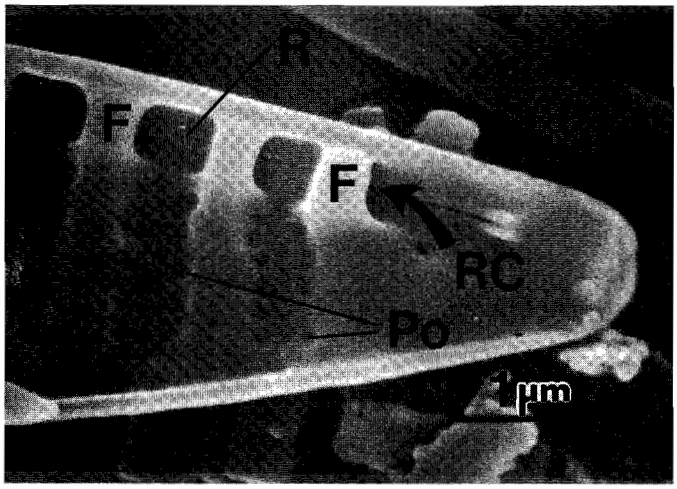


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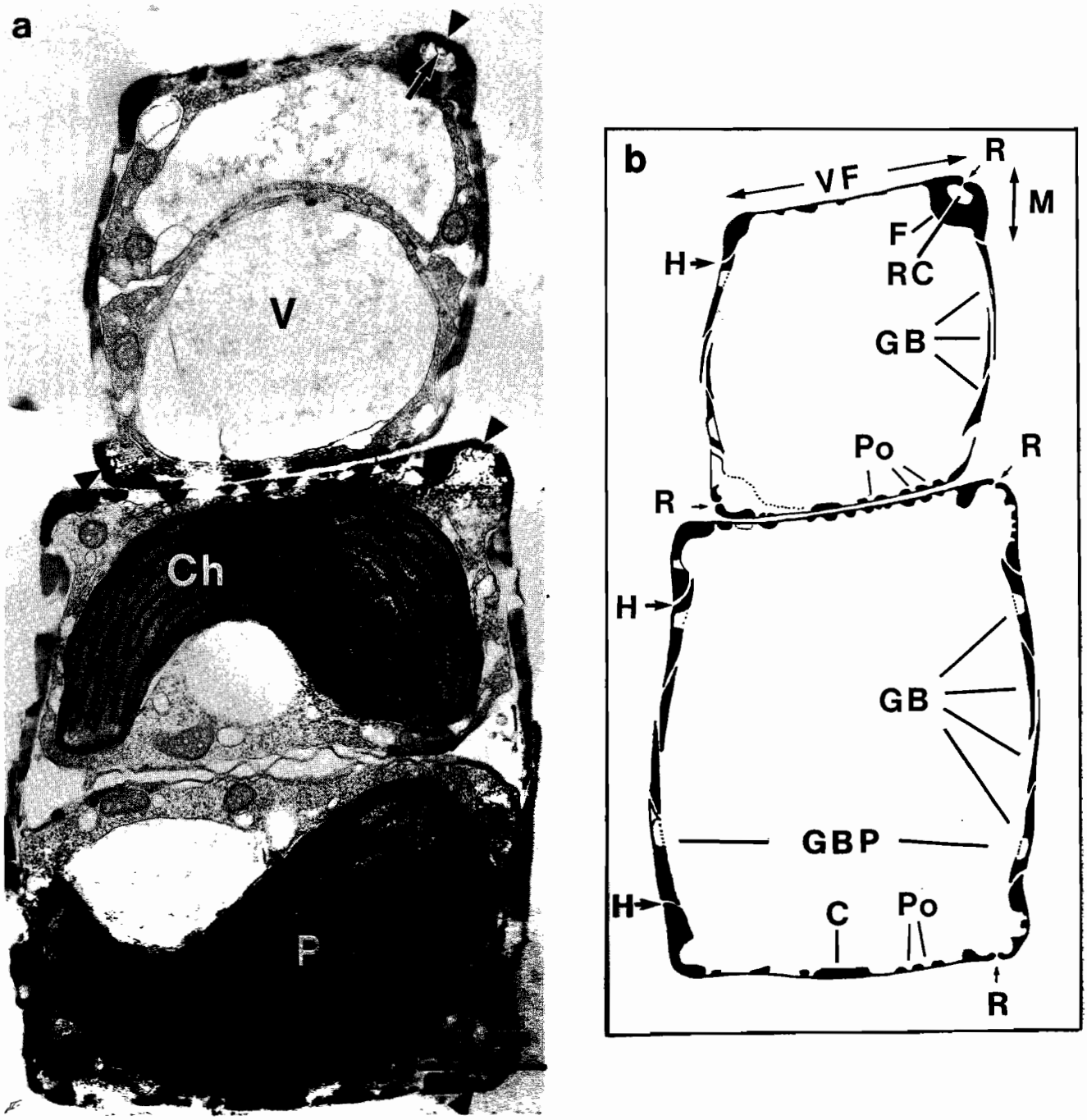


FIG. 5. (a) TEM and (b) diagram of paratransapical plane section from Sample 2 through overlapping tips of two cells in synchronous division. (a) Upper cell sectioned closer to tip showing large vacuoles (V) and cytoplasm extending into raphe canal but drawn back from raphe to form cylinder-like structure (arrows). Lower cell section containing chloroplast (Ch) in each of two sibling cells. Pyrenoid (P) enclosed in membrane and transected by ribbon- or tube-like thylakoids. Raphes (arrowheads) in normal diagonal position in upper cell and in abnormal adjacent (*Hantzschia* species) position in lower cell. Note absence of silica connections between adjacent valve faces. (b) Interpretation of silica wall component from three serial sections (including section in Fig. 5a). Note hinge-like joint (H) between edges of valve and adjacent girdle band allowing for swelling and maintenance of valve width. Valve face (VF), mantle (M), raphe (R), raphe canal (RC), fibulae (F), girdle bands (GB), girdle band poroid areas (GBP), costae (C), and poroids (Po).

being peripheral and polarized, could indicate that the cell is in active protein synthesis (Ghadially 1988).

Golgi stacks (Fig. 7) are prominent and perinuclear, indicating secretory activity. They are often more concentrated adjacent to nucleoli (Fig. 7) and contain several cisternae from which arise numerous vesicles.

The chloroplasts (Fig. 7–10) are long flattened structures appressed to the cell wall. Centrally they press up to, and often extend into, the nucleus (Fig. 7). Distally they sometimes extend just to the point of cell overlap but may also extend into the cell tip. The chloroplast contains thylakoids stacked in layers of three or four (Fig. 8) with the outer thylakoid enveloping

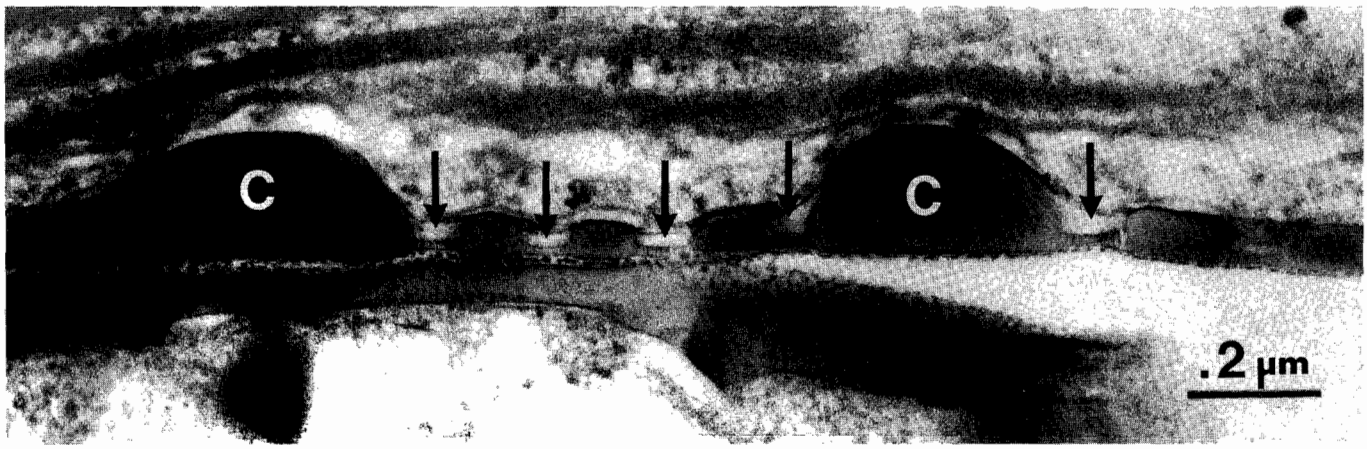


FIG. 6. Sample 4, paratransapical section through overlapping valves showing silica component of costae (C) and imperforate poroids (arrows) of intercostal areas.

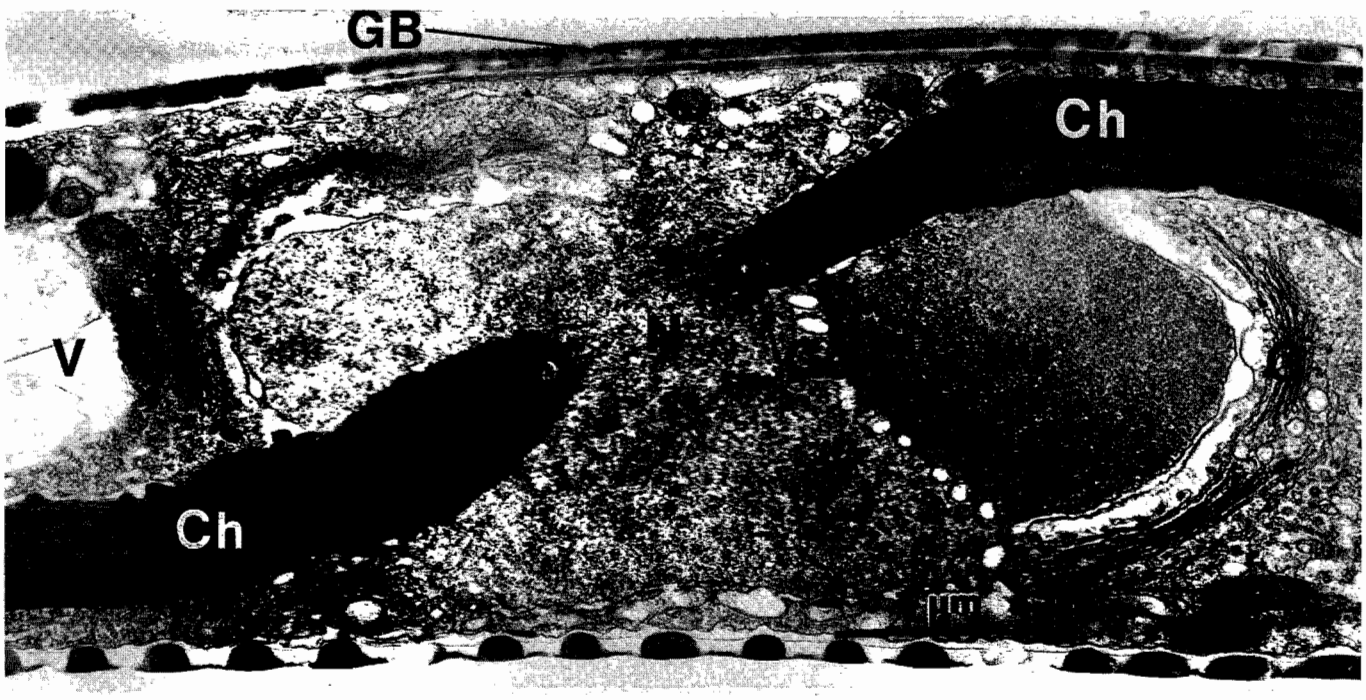


FIG. 7. Paravalvar plane section through overlapping girdle bands (GB). Nuclear area showing oval nucleus (N), polar nucleolus (Nu), perinuclear Golgi (G) with associated vesicles, chloroplasts (Ch) pressing into the nucleus, genophore (Ge), vacuole (V), and mitochondria (M).

the whole chloroplast. A clear area (Fig. 7) is found at one edge of the chloroplast, probably the site of the genophore. Internally the chloroplast contains one or more long membrane bound pyrenoids (Fig. 5, 9, and 10) which are transected by one to three narrow ribbon- or tube-like thylakoids.

The vacuole (Fig. 2, 5, and 7) is large and often vesiculated. It lies central to the chloroplast, extending from the nuclear area to the cell tip.

Mitochondria are numerous and occur throughout the cell (Fig. 5, 7, and 10). They contain tubular cristae and are about 0.3 µm in diameter and up to 20 µm long (Fig. 10).

The cytoplasm extends into the spaces between costae (Fig. 9), appressing the underside of the intercostal areas and poroids of the valves. The cytoplasm also extends into the raphe canal sometimes forming a cylinder-like structure (Fig. 5). The raphe in *N. pungens* in cross-section (described earlier as resembling two inward puckering lips) differs from nonchain-forming, motile *Nitzschia* species. In *N. palea*, one lip projects into the other, tongue and groove fashion (Drum 1963). In *N. sigmoides* the lips pucker outward markedly (Pickett-Heaps 1983).

## Motility

Suspended cells and filaments of *N. pungens* do not exhibit motility. Cells and filaments exhibit motility, however, when placed on a glass slide with a coverslip and illuminated. The difference is that under a coverslip, cells and filaments are forced into contact with each other, and with glass surfaces. A single filament placed in a hanging drop also does not exhibit motility while in suspension or following contact with the surface water film. Likewise, two or more filaments do not exhibit

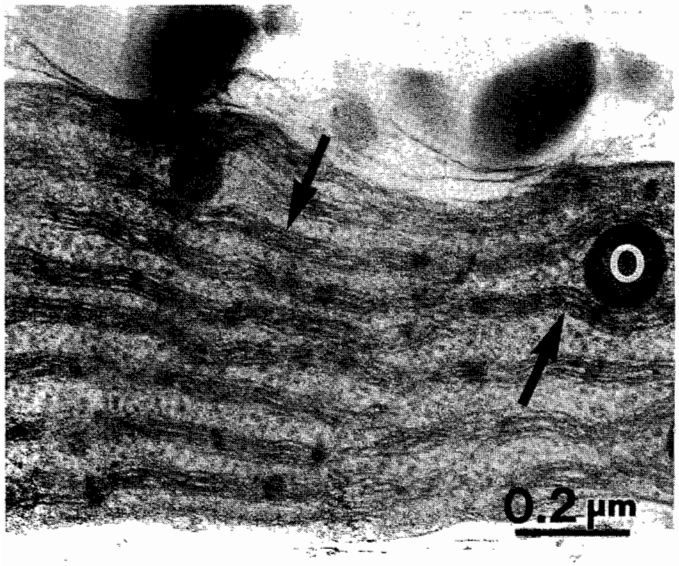


FIG. 8. Chloroplasts showing thylakoids containing stacks of three or four lamellae (arrows) and osmiophillic granules (O).

motility while separated. However, as the filaments fall to the bottom of the drop and make contact, motility occurs. In this situation, one filament is serving as a substrate for the other. While there may be more than one purpose for motility, one function could be for dispersal in the water column, to maximize access to light and nutrients.

Motility is expressed by filaments (or individual cells) gliding along their long axis for a period, stopping, and then reversing direction. The gliding speed is rapid (25 $\mu m \cdot s^{-1}$), and uniform, with motion lasting in any one direction from seconds to minutes. The constant speed suggests coordination of movement between cells. However, considering that we observed no connecting pores between valves of adjacent cells and that the raphes of the valve of one cell do not make contact with the raphes of the valve of the adjoining cell, it would seem that coordination by direct cytoplasmic contact can be ruled out. A mechanism involving indirect cytoplasmic contact, however, may be involved. The following observations bear on these two points.

When fine particles of pumice are added to a hanging-drop preparation of *N. pungens*, one or more particles attach to the cells along the raphes. They move along the cell(s) at the same speed exhibited by filaments moving on glass. Particles on adjacent cells move in the same or in opposite directions and have independent starting or stopping times. Particles on the same cell move in the same direction. Elongate pointed fragments of pumice, when affixed by the pointed end, trail by the other end, swinging about the sharp point (of contact) during directional changes. Single particles (especially large ones) and clusters of particles passed from cell to cell and often moved over the length of several cells. Particles were also seen to drop off when reaching the tip of the last cell of the filament. These observations suggest that motile, sticky cytoplasmic elements project from the raphe and have the ability to hold onto a substrate and, through some unknown manner, (perhaps ciliary-like action?) move the cell along. Besides providing for motility, microcilia could also provide a sensory mechanism for intercellular coordination. Movement of one cell within a filament could entrain movement in the other cells, resulting in all cells (of the filament) moving in the same direction at the same speed as that of one cell. However, we found no ultrastructural evidence of motile microciliary elements.

## Cell Division

The raphe and associated motility apparatus may also be involved in filament elongation following cell division. In field and culture samples, all cells in a filament divide synchro-

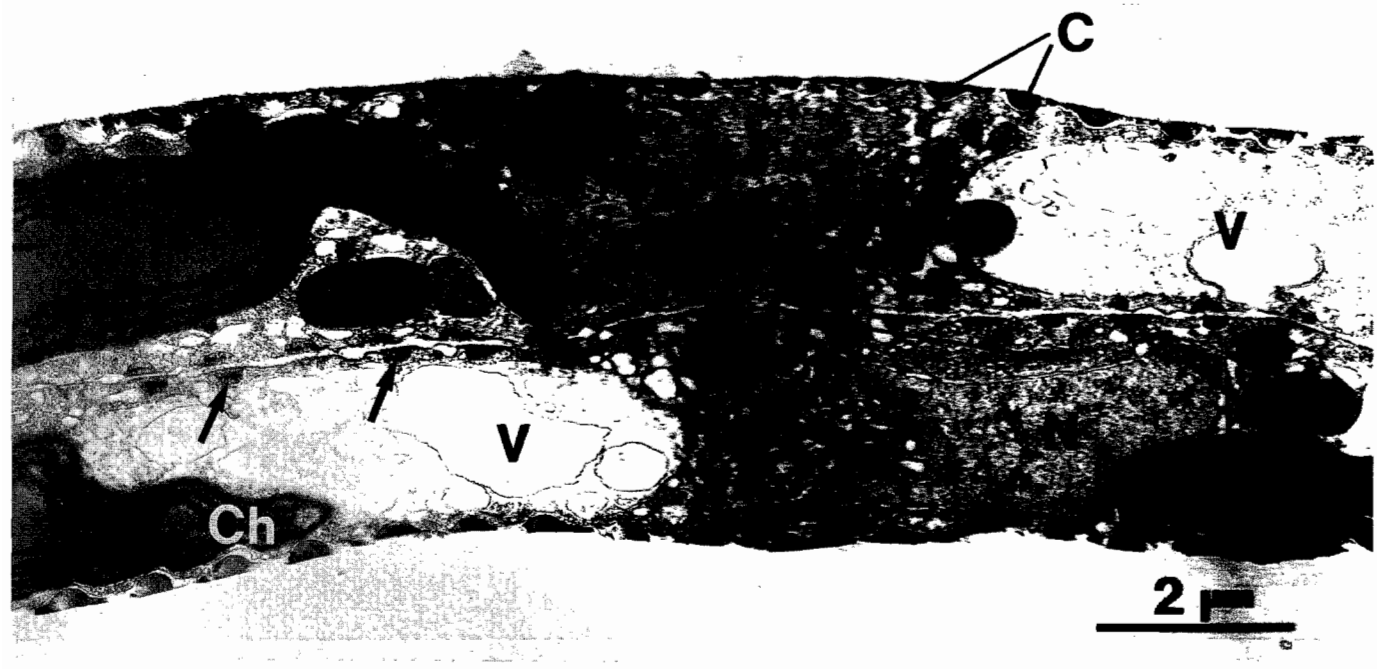


FIG. 9. Paraapical plane section of dividing cell through nuclear area showing nuclei (N), vesiculated vacuole (V), scattered lipid droplets (L), Golgi (G), and chloroplasts (Ch) with pyrenoid (P). Silica walls seen in apical plane showing costae (C) and costal initials (arrows).

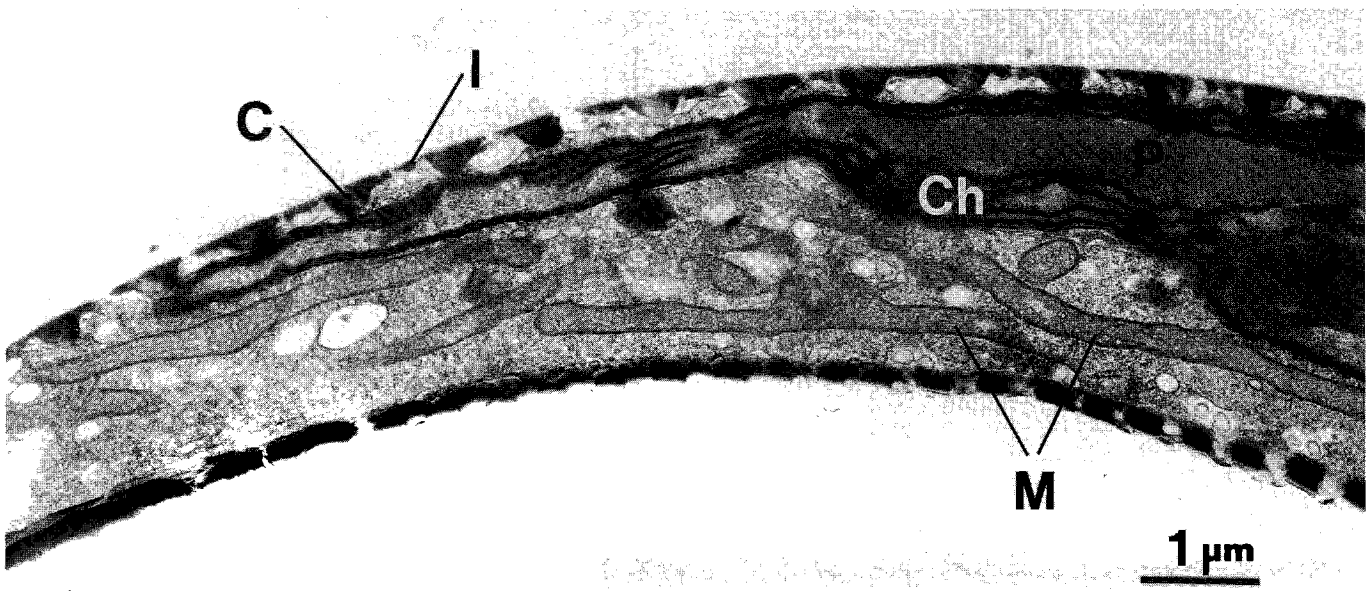


FIG. 10. Paraapical plane section of cell showing long mitochondria (M), chloroplast (Ch) with pyrenoid (P), costae (C), intercostal area (I), and poroids.

nously, or nearly so. Furthermore, separation of sibling cells is synchronously polarized. During division, the first new valve components laid down are the costal initials (Fig. 9), followed by the intercostal areas. Following this, we infer that the two sibling cells slide past each other along the plane of their newly formed and opposed valve faces, at the same time staying in line with the axis of the filament. The actual sliding was not seen. However, filaments in various stages of the "slide-by" process were seen. The post divisional slide-by process is probably the same as that involved in cell motility. When the valve surfaces of the sibling cells are one half to three quarters exposed, the remaining opposed tips become rigidly fused. The joint does not dissolve in HF acid and resists considerable manipulation (i.e. the cells remain in filaments). We were not able to determine if silica deposition was completed before or after the slide-by and fusion process. If deposition was incomplete, the newly formed valves would be more flexible and allow closer valve contact. There would also be an avenue through the cell wall (other than through the raphe) for extrusion of cementing material.

A comparable slide-by process also operates in filament extension and motility in the chain-forming pennate diatom *Bacillaria paradoxa* (Drum and Pankratz 1966). As in *N. pungens*, sibling cells of *B. paradoxa* slide along their valve faces following division. However, in *Bacillaria*, this is not followed by fusion of the tips. Rather, the cells remain free to move independently and continuously along their faces, held together by the opposed raphes which fit into each other like two interlocking rails (Drum and Pankratz 1966). When *Bacillaria* filaments are fully extended, they bear a very close resemblance to filaments of *N. pungens*. It should be pointed out that should *B. paradoxa* be present in a fixed plankton sample, some filaments would likely be in the fully extended state. These, if viewed by LM alone, could be falsely identified as *N. pungens*.

Endo- or Exosymbionts?

In approximately 100 cells from Sample 2 examined by TEM, no evidence was seen of external or internal bacteria, fungi, or encapsulated viruses. We cannot rule out the complete absence of bacteria or viruses because adherent agents may have been washed off during sample preparation, but we are confident that large concentrations (as would presumably be needed to affect cell physiology) would have been encountered. These results lend support to a view that DA production in *N. pungens* f. *multiseries* is autonomous.

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