

# Autogamic reproductive behavior and sex cell structure in *Thalassiosira angulata* (Bacillariophyta)

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## Abstract

Our understanding of diatom reproduction is limited, especially for marine planktonic centric diatoms. This project aimed to elucidate sexual behavior and gametogenesis in the mediophycean diatom, *Thalassiosira angulata*, and to determine the fine structure of its reproductive cells. A monoclonal culture of *T. angulata* was successfully induced and enumerated, and all stages of auxosporulation documented. Spermatogenesis was absent in all inductions. Nuclear behavior of the auxospore mother cell (AMC) during auxosporulation indicated that reproduction was autogamic. In our clone, reproduction began with a differentiating mitosis and subsequent perivalvar expansion of the AMC, a meiocyte. The smaller cell often continued normal vegetative growth. In the AMC, meiosis I was acytokinetic and resulted in one viable haploid and one pycnotic nucleus. Meiosis II resulted in the production of two viable haploid nuclei, which we postulate later fused to produce the zygotic nucleus. Cell expansion was observed in very young AMCs both prior to and following autosyngamy. The initial cell valves were produced after at least two acytokinetic and pycnotic mitotic divisions, after which the cell was capable of undergoing its first normal mitosis involving production of two sibling hypovalves.

**Keywords:** autogamy; auxosporulation; diatoms; reproduction; *Thalassiosira angulata*.

## Introduction

Diatom life histories are primarily diploid, with gametes being the only transient haploid stage. Sexual reproduction is thought to be an infrequent event, normally involving a relatively small fraction of the population. Multiplication is primarily asexual, and in most species a decrease in cell size occurs until the cell becomes non-viable. To prevent extinction, the population must employ strategies to restore large cell size classes. During the cell size reduction, there is a “window of opportunity”, or a size range when cells become responsive to sexual induction cues. In the majority of species investigated thus far, sexuality is used to introduce a new generation of large cells (and gene combinations) into the resident population (Round et al. 1990).

The factors triggering induction of sexuality in centric diatoms are poorly understood. Most successful induction attempts have focused on the manipulation of light:dark cycles (Armbrust et al. 1990, Nagai et al. 1995), nutrient availability and medium changes (Iyengar and Subrahmanyam 1944, Schultz and Trainor 1968, Heath and Darley 1972, Jewson 1992a), pH (Steele 1967), light intensity (Manton and von Stosch 1966, Roshchin 1972, Schmid 1994) or the presence of bacteria (Nagai et al. 1996). Induction of sexuality may also be linked to season (Drebes 1974, Hiltz et al. 2001). The unpredictable nature of sexual induction in the laboratory has undoubtedly been one of the most challenging obstacles to the advancement of knowledge on the reproductive biology of centric diatoms.

Two types of sexual reproduction have been reported among diatoms: allogamy and autogamy. Allogamy refers to mixing of gametes produced by two parental cells (even if parent cells are derived from the same clone) and autogamy refers to the combining of gametic nuclei from a single parental cell, an auxospore mother cell. All allogamous centric species examined are reported to reproduce oogamously, with a non-motile “female” gamete nucleus fusing with a motile “male” gamete nucleus (Drebes 1977, Roshchin and Chepurnov 1999, Kooistra et al. 2003, 2006). Expansion of the resulting diploid zygote to the species-specific maximum size takes place via production of the auxospore and an initial cell, which is many times larger than its parent cell (Drebes 1977, Round et al. 1990). However, the entire process of oogamy has been observed in only a handful of cases (von Stosch 1951, Migita 1967, Steele 1967, Manton et al. 1970, Schmid 1994).

Autogamy has been fully documented in very few centric diatom species, but is suspected to occur in more. It has been examined most intensively in *Cyclotella meneghiniana* Kützing (Iyengar and Subrahmanyam 1944) and *Melosira nummuloides* C.A. Agardh (Erben 1959). More frequently the sexual processes are inferred as autogamy because no signs of spermatogenesis were found in the auxosporulating populations. In most of these cases, nuclear behavior in sexualized cells was not ascertained because available material did not lend itself to such investigation. In these and other non-oogamous diatoms (von Stosch 1967, Drebes 1977), autogamy is regarded as a “deficiency phenomenon and compensational event of an originally-existing oogamous reproduction” (Drebes 1977).

Much of the present understanding of centric diatom autogamy is inferred from examination of allogamy. Indeed, the later stages of auxospore development and initial cell production appear similar regardless of the type of sexual process leading to auxospore development. In most allogamous species examined, one cell (the female parent) transforms into a tri-nucleated oogo-

nium with one egg-nucleus and acts as the parent cell for the zygote and auxospore. Less frequently, oogonia produce two bi-nucleated cells, each containing an egg nucleus (e.g., *Attheya decora* West, Drebes 1977), or one binucleated cell with an egg nucleus and a polar body [e.g., *Biddulphia rhombus* (Ehrenberg) W. Smith, Drebes 1977]. Another sexually compatible cell (the male parent) becomes a spermatogonangium where spermatogenesis results in a production of four or more uni-flagellated sperms per cell. Auxospore expansion in examined centric species with radial valve symmetry is isometric (von Stosch 1982, Kaczmarska et al. 2001). In some species, the initial cell liberates itself from the auxospore shell following osmotic swelling and resumes propagation by vegetative division (Schmid 1994).

In autogamous species, two normal meioses are reported in the auxospore mother cell (AMC), which results in a cell with four haploid nuclei, two of which act as the gametic nuclei (Iyengar and Subrahmanyam 1944, Erben 1959). Following autosyngamy of the functional nuclei, the diploid zygote grows in size as an auxospore, apparently driven by an expanding, central vacuole. We are not aware of a more recent report on the behavior of the meiotic nucleus in autogamously reproducing centric diatoms. Auxospore expansion and production of initial valves in autogamous auxospores appears to proceed as in the sexual auxospores.

In general, our understanding of sexual processes in centric diatoms remains limited, in contrast to significant advances made in the past decade among the pennate species (Mann 1993, Roshchin 1994, Mann 1999, Davidovich et al. 2006). It has been estimated that auxosporulation and sexualization are known for only approximately 0.1% or less of extant species (Mann 1999). Since the pioneering work on centric diatom reproduction several decades ago (von Stosch 1950, 1951, 1965, Drebes 1977, von Stosch 1982), which has been subsequently and repeatedly reviewed (e.g., Edlund and Stoermer 1997), few new insights have been added to this field, leaving many questions about marine, centric planktonic diatom reproduction unanswered (Mann 1993).

The primary goal of this project was examination of the structures and behavior of sexual cells and conditions that facilitate sexual reproduction in *Thalassiosira angulata* (Gregory) Hasle (Hasle 1978). A native clone of this species was subjected to experimental induction of sexuality to gain a better understanding of the range of sexualization successes and the range of intracolon variability in the sexualized and vegetative cell morphology.

## Materials and methods

### Stock cultures

A monoclonal culture of *Thalassiosira angulata* (PB001) was isolated by pipetting from spring plankton samples collected from Passamaquoddy Bay, New Brunswick, Canada in summer 2003. Stock was grown in either f/2 (Guillard 1975) or Carolina Alga-Gro™ (Burlington, USA) medium at 6–8°C, at 14:10 h light:dark cycles and at illumination levels of 20  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ . Light levels

and culture temperatures were monitored weekly using a quantum/radiometer/photometer with both an LI-250 fibre optic probe and a cosine sensor (LI-COR, Lincoln, USA).

### Sexual induction protocol

The clone was subjected to the following sexualizing protocol: 1 ml of stock culture (grown as above) was added to 4 ml Carolina Alga-Gro™ in 3–4 sterile 10 ml polystyrene dishes in July, August and November 2004. The induction-dish culture was exposed to 48 h of continuous low light intensity (20  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), at 6–8°C followed by 48 h darkness. Temperature was elevated to 12°C after the first 24 h in darkness. Next, the culture was exposed to 48 h of high light intensity (100  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) at 12°C. The cultures were examined for the presence of sexual cells and enumerated on day one, 48 h after darkness, and cell types were enumerated every three days until auxosporulation was complete. Following each dish enumeration, the culture was sampled for epifluorescence light microscopy (ELM) and scanning electron microscopy (SEM).

### Cell type enumeration

Cells were enumerated following Hiltz et al. (2001) in induction dishes using a Zeiss (Carl Zeiss, Oberkochen, Germany) inverted light microscope (LM) with a 10 $\times$  objective. Cells were classified into four categories: (1) square cells with a ratio of pervalvar axis:diameter of approximately 1:1 were considered vegetative (non-reproducing or non-inducible) cells; (2) elongated cells with a ratio of pervalvar axis:diameter of about 2:1 or more were considered as AMCs or gametangia; (3) cells showing pervalvar expansion and spherical cells were considered auxospores; and (4) cells that were significantly larger in diameter than the pre-induction vegetative population were considered as initial and post-sexual cells. In each recording, cells were counted across a transect in 10 adjacent fields of view, 100–400 cells per induction dish, every three days, until no signs of sexual reproduction were seen. Dishes were examined and enumerated at different times of the day (light phase) and the position of the dish on the microscope stage was randomized.

The first two inductions on 16 July 2004 and 5 August 2004 were performed in duplicate, and these counts were tested for similarity in the relative proportions of the four cell types using a paired t-test following arcsine transformation. The two data sets were not found to be significantly different ( $p=0.985$ ) and so were pooled in the results presented below. The third induction was performed in triplicate.

### ELM and SEM

Nuclear behavior was examined using 4', 6-diamidino-2-phenylindole (DAPI) stain (BIOMOL Research Laboratories Inc., Plymouth Meeting, USA) which was chosen for its selective and proportional staining of nuclear DNA. The protocol used was based on Strüder-Kypke (personal communication 2004). One ml of diatom sample in 9 ml

of double filtered seawater was treated with Lugol's solution and bleached with 150  $\mu\text{l}$  of saturated  $\text{Na}_2\text{SO}_3$  solution for approximately 1 min. DAPI stain was diluted to 3.3  $\mu\text{g ml}^{-1}$  in distilled water and was used to stain the sample before it was incubated in darkness for 24 h with refrigeration. The sample was then concentrated and examined using ELM (UV filter: 365 nm excitation, 420 nm emission) on a Zeiss Axioskop2 Plus epifluorescence microscope with FluoArc fluorescent source, AxioCam color camera and Axiovision 3.1 software (Carl Zeiss, Oberkochen, Germany) and a Dell Precision 330 host computer (Dell, Round Rock, USA) using a 40 $\times$  objective. Automatic exposure time was set to 212–500 ms. Unprocessed images were preferred when fluorescence and exposure were sufficient to display structures important in the process. When unprocessed images were insufficient, composite images were formed by extracting the blue channel from the epifluorescence image and combining it with the regular brightfield image using QCapture Pro software (QImaging, Burnaby, Canada).

For SEM examination, samples were prepared as described in Kaczmarek et al. (2000). All morphometric analyses were performed using Carnoy v. 2.0 measurement software (Schols and Smets 2001) on SEM images.

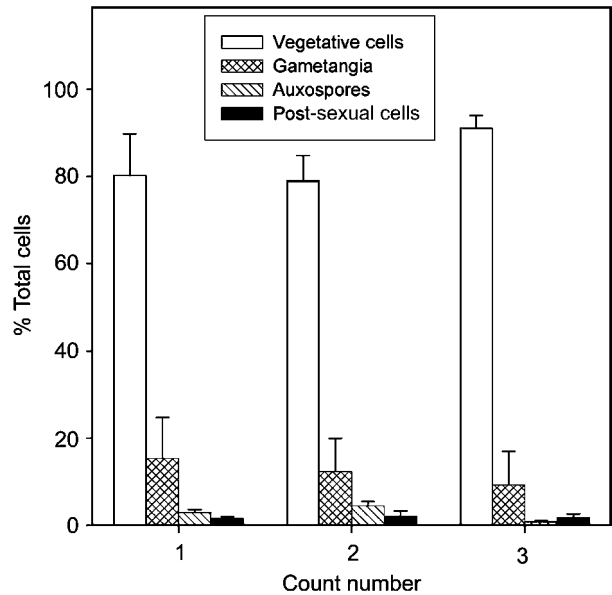
## Results

### Sexual induction success

*Thalassiosira angulata* clone PB001 was successfully induced in three separate experiments. Vegetative cells were the most numerous cell types in all experiments (Figures 1 and 2). The abundance of sexual cells declined through the duration of the experiments, illustrating the effectiveness of vegetative multiplication in maintaining population size. Possible seasonal differences in the sexualization success were observed (comparing summer and fall inductions). In the summer induction, small, asexual cells strongly dominated induction dishes throughout the experiment. The contribution of sexualized and post-sexual cells to the population was highest at the beginning of the experiment. Summer inductions produced relatively few initial cells compared to the fall induction, suggesting that there is seasonality in reproductive success of this clonal isolate.

### Morphometric characteristics

Selected morphometric characters of *Thalassiosira angulata* clone PB001 are summarized in Table 1 and plotted in Figures 3 and 4, to visualize the range of differences in pre- and post-sexual cells relative to cells that did not respond to our induction. Frustule morphometry conforms to the species description detailed in Hasle (1978) and Harris et al. (1995), but not Gleser et al. (1988). Our specimens of this clone and other clones of this species currently in culture in our laboratory do not show areolation as strongly eccentric as often reported for small valves of *T. angulata*. Our coarser specimens are somewhat similar to *T. nordenskiöldii* Cleve in valve ornamentation, valve diameter and number of areolae and

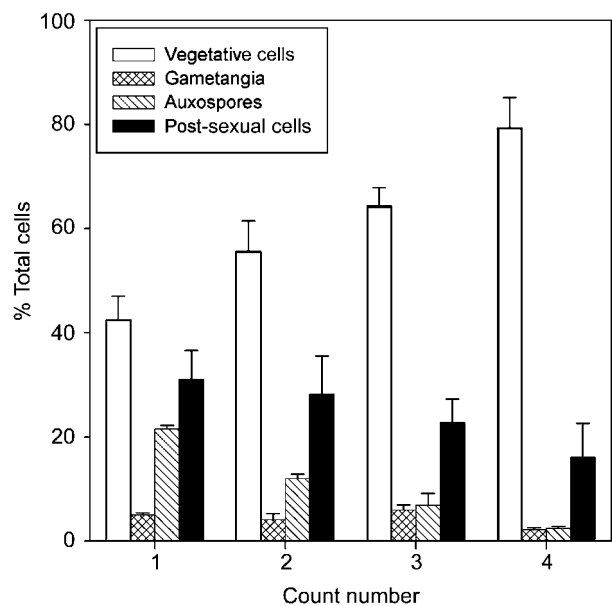


**Figure 1** *Thalassiosira angulata*: average relative abundance (%) of four cell types (+1 SE) in three replicates of combined 20 July and 13 August 2004 inductions of clone PB001.

fultoportulae in 10  $\mu\text{m}$ , as noted by Hasle (1978) and Harris et al. (1995).

The valve diameters of vegetative cells in clone PB001 ranged from 11.2–14.7  $\mu\text{m}$ , while the length of the perivalvar axis ranged from 8.5–14.9  $\mu\text{m}$ . The areolae were of the same size and numbers on the valve face and the mantle, and the number of areolate striae ranged from 14.7–22.6 in 10  $\mu\text{m}$ , while the number of fultoportulae ranged from 2.1–3.5 in 10  $\mu\text{m}$ . Vegetative cells normally had 3–7 girdle bands per epivalve.

Cells responsive to the initiation of sexualization (AMCs) were slightly smaller than asexual cells. Despite small differences in their means (Table 1, Figures 3 and

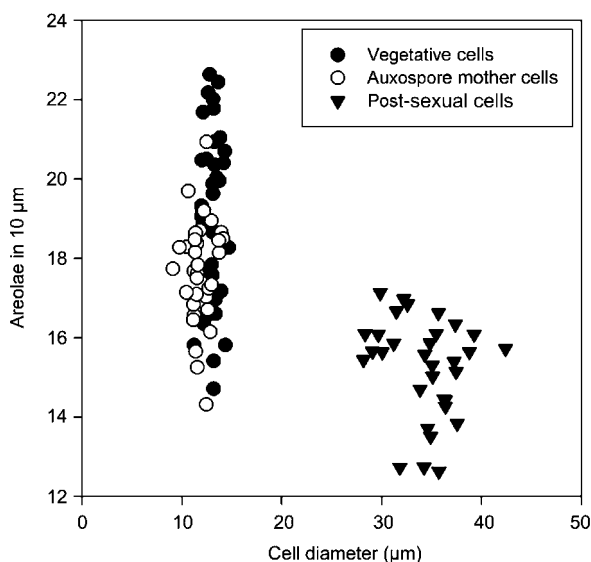


**Figure 2** *Thalassiosira angulata*: average relative abundance (%) of four cell types (+1 SE) in four replicates of 25 November 2004 inductions of clone PB001.

**Table 1** *Thalassiosira angulata*: mean morphometric data for vegetative cells, autogamous auxospore mother cells (AAMCs) and post-sexual cells of clone PB001.

Stage	Diameter ( $\mu\text{m}$ )	Pervalvar axis ( $\mu\text{m}$ )	Areolae (in 10 $\mu\text{m}$ )	Fultoportulae (in 10 $\mu\text{m}$ )	Number of girdle bands
Vegetative					
n	38	38	38	37	31
Mean	13	11.7	19.1	2.6	5.7
SD	0.8	1.4	2.1	0.3	1
AAMC					
n	34	26	34	26	19
Mean	11.8	19.4	17.7	2.7	8
SD	1.1	4.8	1.3	0.3	2.3
Post-sexual					
n	32	n/a	32	31	n/a
Mean	34.3	n/a	15.3	2	n/a
SD	3.4	n/a	1.3	0.4	n/a

Post-sexual cells were observed most frequently in valve view, therefore, peralvar axis and number of girdle bands were not measured. n: number of specimens observed; SD: one standard deviation; n/a: not applicable.

**Figure 3** *Thalassiosira angulata*: scatter plot showing the number of areolae in relation to cell diameter for vegetative cells, auxospore mother cells and post-sexual cells of clone PB001.

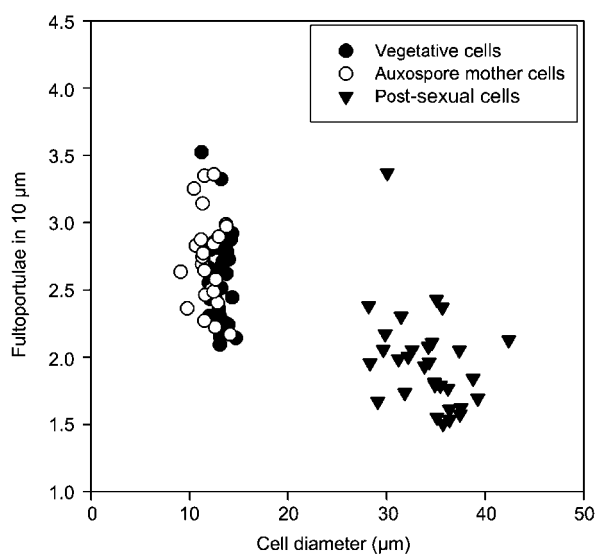
4), sexualized cells were statistically significantly different from asexual cells in several characters: the diameter of the valve in sexualized cells ranged from 9.1–13.9  $\mu\text{m}$ , the peralvar axis from 14.9–30.9  $\mu\text{m}$ , the number of striae from 14.3–20.9 in 10  $\mu\text{m}$ , and the number of fultoportulae from 2.2–3.4 in 10  $\mu\text{m}$ . The AMC elongated in the peralvar direction by adding 6–13 additional copulae over the course of development. The results of the peralvar expansion are shown in Figure 5 (compare proportions to the smaller cell in Figure 6) and the image reflects the relatively light silicification of these girdle bands (they often collapsed in SEM preparations).

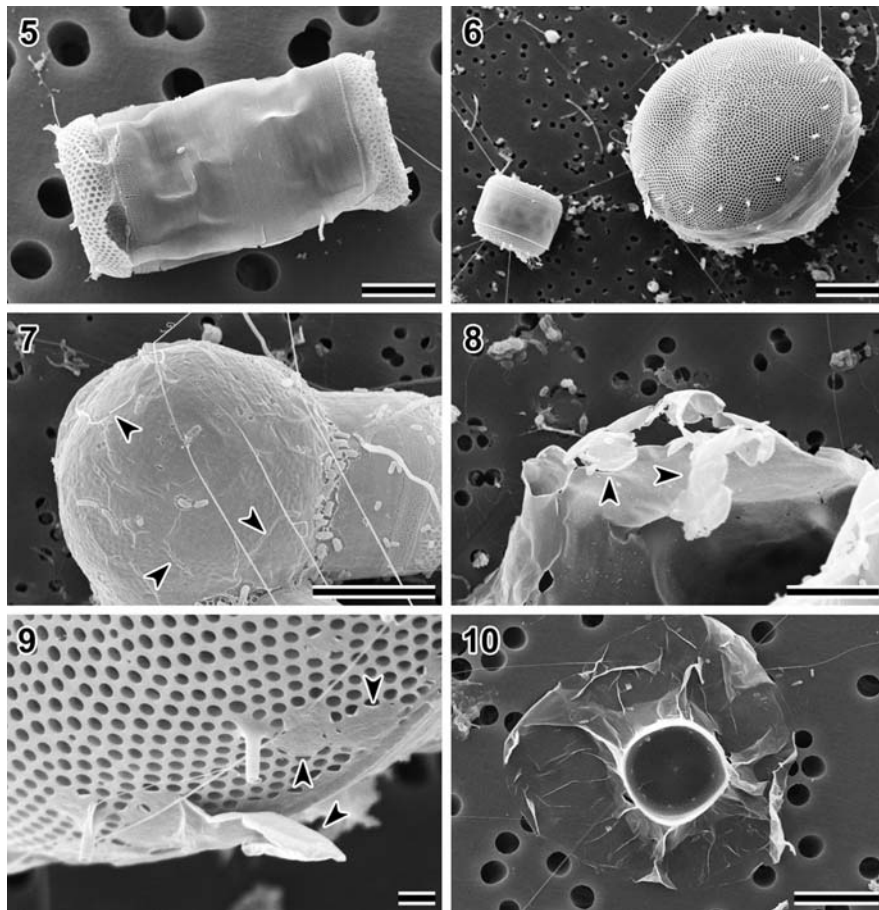
Auxospores were very fragile prior to the production of the initial frustule and most of our cells prepared for SEM collapsed, despite using a number of preparation techniques. Some small and/or less damaged auxospores had scales (Figures 7–9, arrowheads), though these were sometimes very thin and lightly silicified (Figure 9 arrowhead). Most of the walls of larger, older auxospores had

organic envelopes, suggesting that the wall was non-siliceous at this stage of development (Figure 10).

Initial and post-sexual cell diameters ranged from 28.3–42.4  $\mu\text{m}$ , with the means and range given in Table 1 and Figure 3, respectively. These cells were slightly larger than attributed to the species (Hasle 1978), showed coarser areolation (12.6–17.1 areolate striae in 10  $\mu\text{m}$ ) and fewer fultoportulae (1.5–3.4 in 10  $\mu\text{m}$ ) compared to both sexualized and asexual cells (Figures 3 and 4). Initial valves showed imperfect processes and areolation on the valve face (Figures 11–13). At times, AMC frustules remain attached to the initial cell even through the first and second mitotic divisions (Figures 11–13).

In the majority of mature auxospores examined, when both parental thecae were still attached, only two initial valves were evident. However, it appears that three valves may have been produced (Figures 14–18) by

**Figure 4** *Thalassiosira angulata*: scatter plot showing the number of fultoportulae in relation to cell diameter for vegetative cells, auxospore mother cells and post-sexual cells of clone PB001.



**Figures 5–10** *Thalassiosira angulata*: clone PB001, SEM.

(5) Autogamous auxospore mother cell; note numerous copulae compared to next Figure. Scale bar=5  $\mu\text{m}$ . (6) Small vegetative propagating non-induced cell in girdle view and large post-sexual cell in valve view. Scale bar=10  $\mu\text{m}$ . (7) A young auxospore showing scales in the auxospore wall (arrowheads). Scale bar=10  $\mu\text{m}$ . (8) A ruptured auxospore wall showing thin, small and delicate scales hanging from the broken edge (arrowheads). Scale bar=5  $\mu\text{m}$ . (9) Fragment of an initial valve with thin, small and probably organic scales still attached to the valve mantle region (arrowheads). Scale bar=1  $\mu\text{m}$ . (10) Fragment of a broken auxospore wall over a parental valve; note the absence of scales. Scale bar=10  $\mu\text{m}$ .

some auxospores. In the normally very delicate, chiefly organic, scale-less and collapsible walls of older auxospores, a stronger, dome-shaped structure supporting this wall may be noted, even in LM in some specimens (Figures 17, 18). In SEM preparations, we also found unusually areolated initial epivalves (compare “areolae” in Figures 14 and 15 to those in Figures 11–13), indicating that these valves were not interrupted in the process of formation. These valves were complete, as indicated by deposition of a number of cingulae (Figure 16, arrowheads). In such auxospores the initial cell does not contain an initial epivalve.

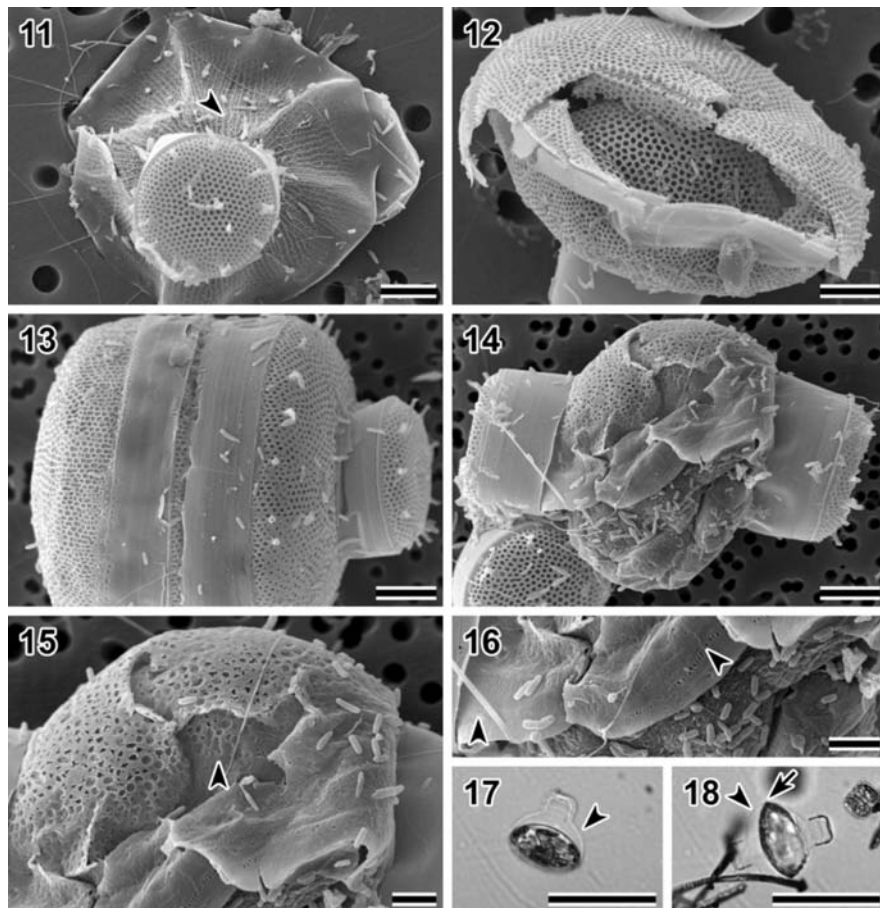
#### Behavior of asexual cells

Vegetative cells investigated consisted of those that did not sexualize, autonomous post-sexual cells and their descendants. In *Thalassiosira angulata* the process of mitotic division in these cells began with the migration of the nucleus from close to the valve face center (in larger cells) or valve face mantle junction (in smaller cells) to about half the distance between the valves, hence close to the girdle (Figure 19). After the division began, the sibling nuclei either remained close to the girdle position, or,

in synchrony, moved into a position closer to the cell center (Figure 20). Cytokinesis and the formation of the new hypothecae occurred next, and centripetal cytokinesis had often begun before karyokinesis was completed. The cells were often observed undergoing cytokinesis via pinching in of the protoplast before the nuclei were fully autonomous. New hypothecae were deposited while sibling nuclei took up a more central position with respect to new hypothecae (Figure 21). Following completion of hypothecae, nuclei moved away from them, towards the epivalves (Figure 22). At the time of division, chloroplasts were observed dispersed along the parent frustule.

#### Auxosporulation

**Initiation** Sexualization was first evident in cells undergoing an unequal mitotic division (a differentiating mitosis). Figure 23 shows these division products, one normal vegetative cell and one auxospore-fated elongated cell (meiocyte or AMC). The vegetative sibling cell of the AMC continued normal asexual divisions, sometimes while remaining connected to the developing auxospore by a thread between the sibling valve’s central fulcrotortulae.



**Figures 11–18** *Thalassiosira angulata*: clone PB001, SEM (Figures 11–16), LM (Figures 17, 18).

(11) Large initial epivalve under a parental valve; note different areolation between the two valves. Scale bar=5  $\mu\text{m}$ . (12) Initial frustule with central fuloportula lacking. Scale bar=5  $\mu\text{m}$ . (13) Sibling cells from the progeny of the first mitotic division of an initial cell. Scale bar=5  $\mu\text{m}$ . (14) Auxospore with displaced auxospore mother cell valves. Scale bar=5  $\mu\text{m}$ . (15) Close-up of the dome-shaped structure from the previous Figure. Note small cavities (arrowhead) on the top of this structure and nearly structureless rim. Scale bar=2  $\mu\text{m}$ . (16) Close-up of the rim part of the dome-shaped structure, note several copulae and poroid perforations (arrowheads) on some bands. Scale bar=2  $\mu\text{m}$ . (17) Mature auxospore showing the dome-shaped structure (arrowhead) between the initial valve and the auxospore mother cell valve. Scale bar=50  $\mu\text{m}$ . (18) Complete auxospore showing the dome-shaped structure (arrowhead) above an initial valve (arrow) with both auxospore mother cell valves still attached to the auxospore. Scale bar=50  $\mu\text{m}$ .

**Early development** The AMC underwent perivalvar elongation by the addition of copulae (Figure 5). In ELM, meiotic nuclei in the AMC often appeared notably different from vegetative nuclei, due to their swelling, which resulted in greater DAPI stain density and distension (Figure 24). The diploid nucleus of the AMC then underwent the first meiotic acytokinetic division, meiosis I, which resulted in the bi-nucleated younger AMC with two haploid nuclei located approximately in the center of the AMC (Figure 25). One of the haploid products of meiosis I then aborted and became pycnotic (degraded until reabsorbed by the cell, Figures 26 and 27), but could persist in a pycnotic state through early stages of auxospore development (Figures 28–31). The functional nucleus proceeded to meiosis II, which resulted in two nuclei still visibly associated with one pycnotic nucleus (from meiosis I) when positioned in an orientation where all three nuclei were clearly distinct from each other (Figures 28–30). We found this stage short-lived, as it was one of the least frequently found, even in the most sexualized

cultures. In addition, all three nuclei were located close to each other, limiting the range of orientations in which they could be seen as separate entities. More frequently they were observed partially superimposed, rendering image recording unconvincing, but could be distinguished visually.

There was considerable variability in the magnitude of expansion in the AMC/auxospore during the meioses. Some cells had meiosis II products within an elongated, but still straight-cylindrical AMC (Figures 28 and 29). Other AMC in meiosis II had the three nuclei, while the cell was beginning to expand (starting the auxospore stage) symmetrically, or by asymmetrically bulging more on one side. Maximum diameters of these tri-nucleate, expanding cells normally did not exceed AMC valve diameter by more than a factor of 2.

**Later development** The young auxospore quickly expanded further (Figures 31–35); small and medium size stages were encountered relatively infrequently. Degrad-

duction of the pycnotic nucleus from meiosis I was normally completed by these early stages of expansion as even the very young, small, spherical auxospores normally contained only one nucleus located in the equatorial region of the AMC, as it did during meiosis. The bi-nucleated stages (one viable plus one pycnotic) were normally not seen after the auxospore expanded to more than twice the AMC diameter. In these auxospores the nucleus was found underneath the parental theca and remained at that location throughout the expansion (Figures 32–35). Interestingly, the nucleus was not found to be preferentially located under specific parental thecae (epi- or hypovalve). Plastids were generally restricted to the periphery, close to the auxospore wall (Figures 32 and 35). As the auxospore expanded, the protoplast retracted from underneath the parental valves. Occasionally, a nucleus was found close to the equatorial position in the auxospore after it reached maximum size. Mature auxospores are more ellipsoidal rather than spherical in shape.

**Initial cell development** Most often two, but perhaps, in some cases, three valves were produced in the auxospore before the initial cell appeared. The diploid auxospore nucleus left its position of under or close to a parental valve but remained close to the auxospore wall. The initial epivalve was produced following acytokinetic mitosis (Figures 36 and 37). Nuclei resulting from this division did not remain in close proximity (as the meiotic nuclei did) but separated distinctly, sometimes leaving a small globule of DAPI staining material in between (Figure 37). Pycnotic mitotic nuclei associated with the initial valve production must have disintegrated very rapidly because only very few auxospores contained them under the initial epivalve. Following the production of the initial epivalve, there may have been a considerable localized retraction of the protoplast from against the auxospore wall (Figure 38). The functional nucleus was seen located closer to this retracting protoplast prior to new valve production. This second division (Figure 39) was also acytokinetic and led to pycnosis of one of the nuclei (Figure 40). Initial cells (cells capable of normal cytokinetic mitosis, *sensu* Kaczmarska et al. 2001; Figure 41) may or may not have contained initial valves, as suggested in the previous section and illustrated in Figures 14–18. In all cases, valves were laid down parallel to the orientation of the parental AMC valve plane. Some initial cells maintained connection with the AMC valves, presumably by retaining some portions of the auxospore wall past the few normal mitotic divisions of the initial cell and its progeny.

### Putative oogonium

Among thousands of cells examined, a single cell with a nuclear constitution consistent with a three-nucleated oogonium was encountered and is shown in Figure 42. It contains one larger, presumably gametic nucleus and two smaller nuclei.

A schematic summary of *Thalassiosira angulata* clone PB001 life history is shown in Figure 43, while meiocytes

and early zygotes in autogamous modes of reproduction known among diatoms are compared in Figure 44.

## Discussion

### Sexualization

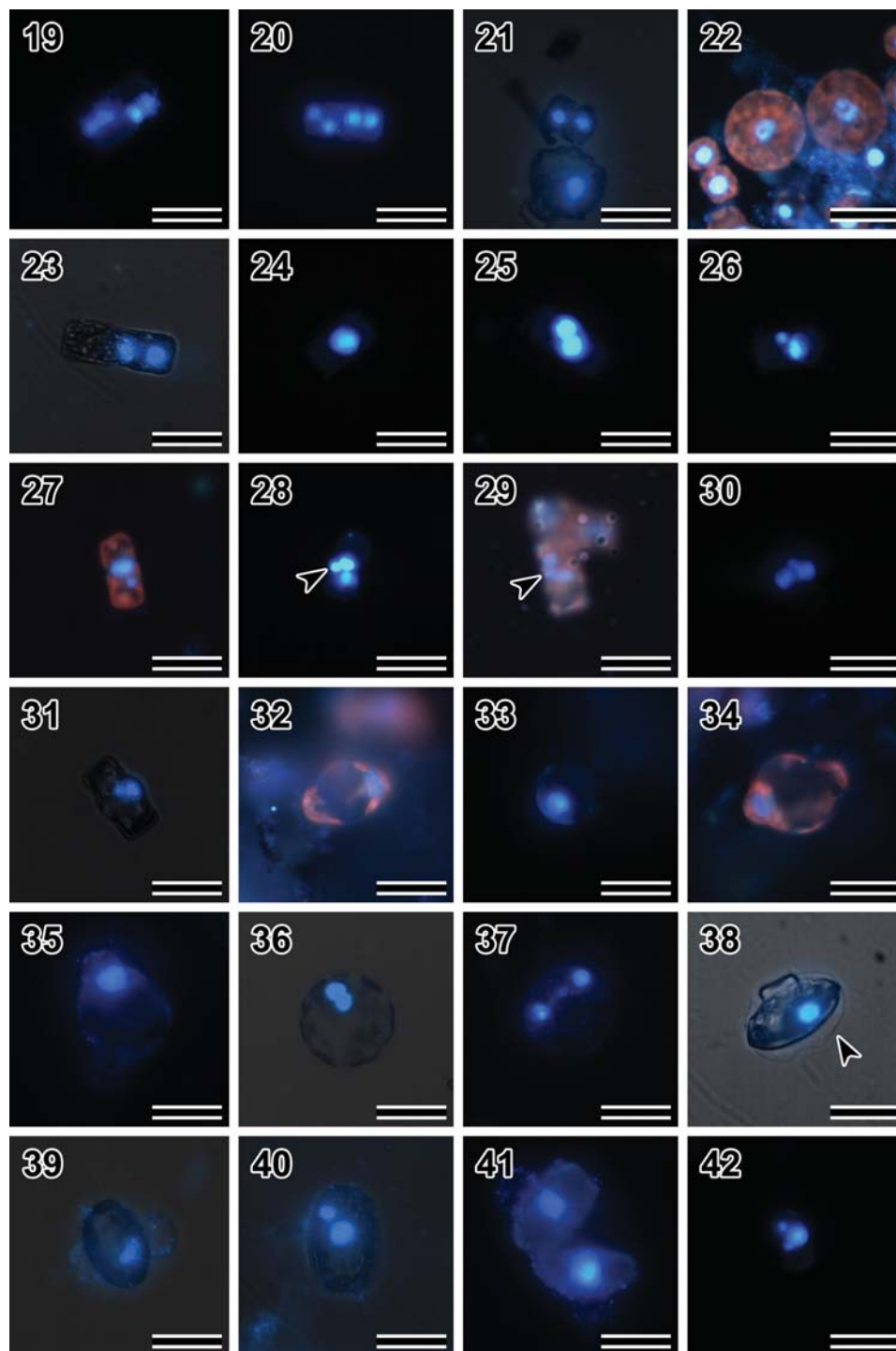
*Thalassiosira angulata* clone PB001 responded to sharp changes in the light and temperature combination. Both factors were found necessary, as initial trials with only light changes did not result in auxosporulation. A combination of adequate temperature and irradiance may mimic the conditions of the spring plankton bloom in cold temperate waters, where *T. angulata* is reported with highest abundances. We also report intraclonal, possibly seasonal differences in the success in sexualization of the clone. The mechanisms responsible for the expression of seasonality in culture are currently unknown. Nonetheless, fall induction success was significantly higher than summer induction in our experiments. This is in accordance with reports on the timing of auxosporulation in a number of coastal marine diatoms from Helgoland (Drebes 1974). Stronger fall induction and reproductive success of our clone is surprising, because in the Quoddy Region of the Bay of Fundy the majority of *Thalassiosira* species bloom in the spring and are normally scarce in the fall. *T. angulata* occurs in the Bay of Fundy plankton (Hasle 1978), but seasonal dynamics of local populations are unknown.

The frequency and magnitude of sexual episodes in natural waters is a poorly understood aspect of diatom biology and only a few reports are available for natural populations (Jewson 1992a,b, Waite and Harrison 1992, Jewson et al. 1993, Crawford 1995). Compared to those studies, our inductions, in particular the fall induction of the clone PB001, are higher.

### Sexualized and asexual cell morphology and development

As expected, pervalvar length, areolation and number of processes differed significantly between the pre- and post-auxosporulation cells. Valve diameter differences between the non-induced cells and AMCs were also statistically significant; even though means of valve diameters differed by only about 1  $\mu\text{m}$ . If indeed this difference is biologically significant, it would indicate a rather tight relationship between the size and sex-inducibility in this clone under the conditions of our experiment. Similarly, a small difference between inducible and asexual cells was also found in *Aulacoseira herzogii* (Lemmermann) Simonsen (Jewson et al. 1993).

The mitotic nuclear behavior observed in our clones follows the steps in *Melosira varians* C.A. Agardh (Tippit et al. 1975) and *Coscinodiscus wailesii* Gran (Schmid and Volcani 1983). Similarly, auxospore expansion and the development of the initial cell in our clones follows a well-established pattern for radial centric species (Kaczmarska et al. 2001, Medlin and Kaczmarska 2004). Auxospore expansion is mostly isometric. The siliceous scales abundant in *Thalassiosira eccentrica* (Ehrenberg) Cleve (Schmid 1984) are scarce in our clone and detect-



able only in young auxospores. These scales appear thin and non-rigid (lightly silicified) and most likely undetectable in LM. In older auxospores, the auxospore walls appear scaleless and mainly organic in SEM. The apparent absence of scales in auxospore walls of three *Stephanodiscus* species (Round 1982, Edlund and Stoermer 1991, Jewson 1992b) may also be due to their presence in only the early stages of auxospore development. The initial valves are produced following acytokinetic mitoses, similar to some other (von Stosch and Kowallik 1969, von Stosch et al. 1973, Hoops and Floyd 1979), but not all species investigated (Iyengar and Subrahmanyam 1944, Chiappino and Volcani 1977). It is possible that in our

clone some auxospores produce three (not two) initial valves before initial cells arise, similar to observations by Drebes (1972), Hori (1993), Edlund and Stoermer (1997) and Kaczmarska et al. (2000).

#### **Type of sexuality in *Thalassiosira angulata* clone PB001**

The most significant difference in the auxosporulation of our clone compared to published accounts for centric diatom species occurs in the progress of meiosis. In particular, the fate of the nuclei resulting from meiosis II in our clone is undocumented in autogamous centric (Iyen-

gar and Subrahmanyam 1944, Erben 1959) or pennate diatoms (Geitler 1973, 1979, 1985, Idei in Hori 1993, Mann 1993, 1994). These studies reported normal meiotic tetrads of nuclei, either with (Figure 44IIIA), or without cytokinesis (Figure 44IIIB). This will be discussed in detail below in the context of allogamous and autogamous auxosporogenesis reported for centric diatoms.

It is a widely held consensus that the vast majority of centric diatoms reproduce by monoecious oogamy, with a few species able to undergo sex-compensatory or purely vegetative cell enlargement (Drebes 1977, Roshchin 1994, Edlund and Stoermer 1997, Roshchin and Chepurinov 1999, Kooistra et al. 2003, 2006). Indeed, since von Stosch (1951) documented the existence of flagellate sperm cells in *Melosira varians*, the vast majority of literature reports oogamous centric diatom sexual reproduction (Drebes 1974, 1977, Edlund and Stoermer 1991, Idei in Hori 1993, Roshchin and Chepurinov 1999). This reinforces the impression that oogamy is the sole means of reproduction in centric diatoms.

Diatom egg cells come in three forms (Drebes 1977, Round et al. 1990). The most common form of mature oogonium with a gametic egg nucleus is a tri-nucleated cell before fertilization takes place, or a bi-, tri- or tetra-nucleated oogonium in species where the sperm penetrates the oogonium before oogenesis is completed and fertilization may occur (von Stosch et al. 1973). A few species produce two eggs per gametangium and fewer still undergo oogenesis with the production of a residual cell, a polar body (Drebes 1977). Spermatogonangium development is more frequently varied among centric diatoms and may be a very (or exceedingly) rare type of cell in oogamously reproducing cultures (Steele 1967, Armbrust 1990, Jewson 1992a,b). Among thalassiosiroid diatoms, oogamy has been reported or inferred in *Thalassiosira eccentrica* (Drebes 1979, Schmid 1994), *T. lacustris* (Grunow) Hasle (Idei in Hori 1993), *T. weissflogii* (Grunow) Fryxell et Hasle (Armbrust et al. 1990), two

*Cyclotella* species (Geitler 1952, Hoops and Floyd 1979), and three species from the genus *Stephanodiscus* (Round 1982, Edlund and Stoermer 1991, Jewson 1992b).

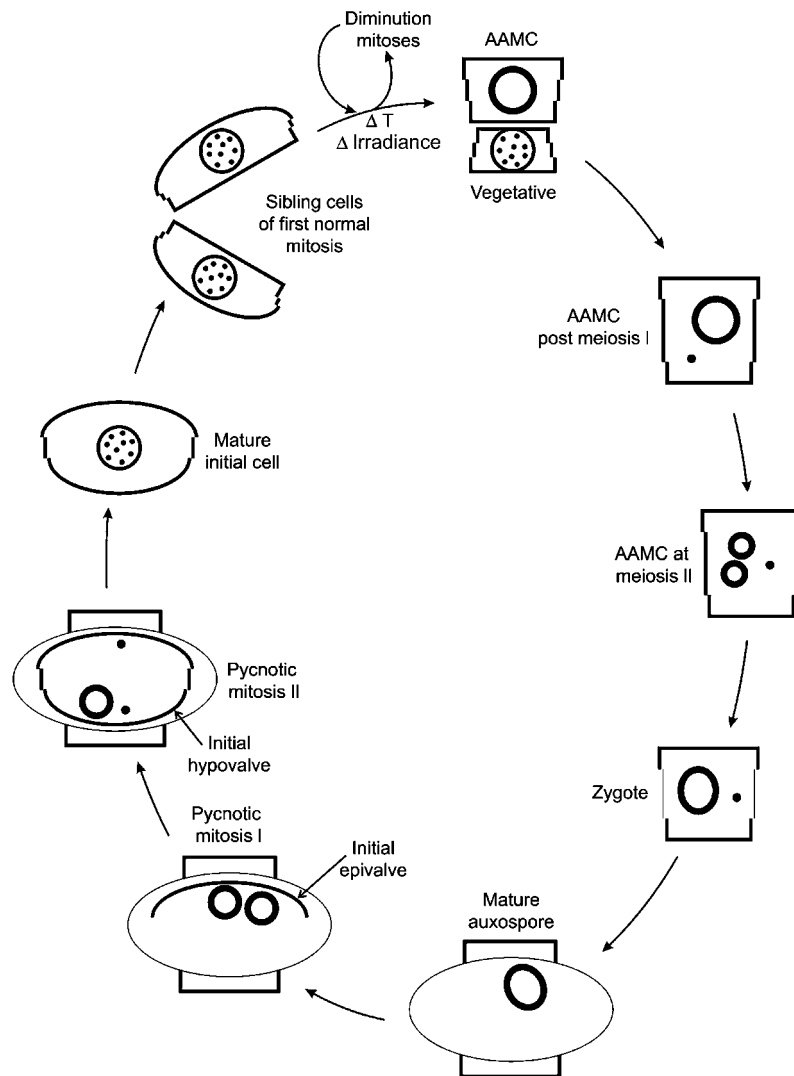
None of the sex cell types discussed above and consistent with spermatogonangia, sperm or oogonia with egg(s) reported for oogamous diatoms were observed in our material (with one possible exception). Instead, our clone consistently demonstrated bi- and tri-nucleated cells of a different type. In AMCs and very young auxospores, the nuclear complement was either bi-nucleated with both nuclei of equal size, bi-nucleated with one normal nucleus and one pycnotic, or tri-nucleated, with two nuclei of equal size and one pycnotic nucleus. The types of nuclei, their number and arrangement suggests autogamous origin of auxospores. The single exception, a possible egg-containing gametangium was observed (Figure 42), but due to its rarity and absence of sperm cells, it could not account for the abundance of auxospores observed in our inductions.

We therefore postulate that in order to restore the diploid state of the tri-nucleated AMC observed in our inductions (Figures 29–31), in the absence of male gametangia and gametes, the two functional sister haploid nuclei from meiosis II must fuse to form the diploid zygotic nucleus (autosyngamy). We propose that autogamy in *Thalassiosira angulata* PB001 (Figure 43) begins with one round of differentiating mitosis, producing two cells of unequal size: a small vegetative cell and a larger sibling, the meiocyte. The meiocyte is termed here the “autogamous auxospore mother cell” (AAMC) to differentiate it from its analog in allogamy when the auxospore develops from the oogonium. In our clone the autogamous AMC carries three nuclei, with acytokinetic meiosis I producing only one viable haploid nucleus and meiosis II with two sister, equal sized, gametic nuclei.

Following the autosyngamy of these two gametic sister nuclei, the cell has either already undergone cellular

**Figures 19–42** *Thalassiosira angulata* in epifluorescence light microscopy, DAPI stained; all scale bars=25  $\mu$ m.

(19) Two small sibling cells undergoing a typical mitosis in near girdle position. (20) Two small sibling cells in the latest stages of mitosis, one pair farther ahead, with sibling nuclei positioned at the opposite sides of the frustules. (21) Fully autonomous sibling cells, note location of the nuclei close to the central position with respect to the cell epivalves. (22) Two large, post-sexual cells. Note nuclei positioned near the valve face center. The lower of the two small cells in girdle view shows the nucleus located in the position for mitosis. (23) A differentiating mitosis, with a larger auxospore mother cell and a smaller asexual cell. (24) A young pre-meiotic auxospore mother cell nucleus. (25) Auxospore mother cell in meiosis I. (26) Auxospore mother cell after meiosis I, with one pycnotic nucleus. (27) Another, longer auxospore mother cell after meiosis I, with an enlarged functional nucleus, product of meiosis I. (28) Tri-nucleated auxospore mother cell in meiosis II, with one small pycnotic nucleus and two nuclei of a larger size. Note the pycnotic nucleus (arrowhead) at the same focal plane and closer to one of the two sibling nuclei. The second larger nucleus is positioned slightly below the two others and is not in sharp focus. (29) Another auxospore mother cell in the tri-nucleated stage with a smaller pycnotic nucleus (arrowhead) and two gametic nuclei in process of separation, one of them partially obscured by a chloroplast, two small asexual cells pressed to the top of the auxospore mother cell. (30) Another auxospore mother cell with two gametic nuclei close to each other and a small pycnotic nucleus from meiosis I slightly behind them. (31) Very young bi-nucleated auxospore with slightly expanded girdle region, opposite to the larger nucleus. One nucleus is pycnotic. (32–35) Mono-nucleated auxospores in various stages of expansion. Note the position of the nucleus under or near to one of the auxospore mother cell valves. (36) Auxospore in the early stage of the first acytokinetic division prior to the production of initial epivalve. (37) Somewhat older bi-nucleated auxospore following the first acytokinetic division. Two nuclei are clearly separated and a DAPI-staining small “dot” appears between the nuclei (compare with von Stosch et al. 1973). (38) Auxospore with an initial epivalve completed in the area opposite to the auxospore mother cell valve. Note softly folding auxospore wall above the initial epivalve (arrowhead). Epivalve is evident by a smooth, clearly defined outline that contrasts with the ill-defined protoplast boundary underneath the auxospore mother cell valve. (39) Auxospore in the second acytokinetic division, prior to formation of the initial hypovalve. (40) Bi-nucleated initial cell with one pycnotic nucleus. (41) Initial cell after the first typical cytokinetic mitosis, note the more convex profile of the initial valves relative to the two sibling hypovalves. (42) Tri-nucleated putative oogonium, with one large nucleus and two small pycnotic nuclei, see text for explanation.



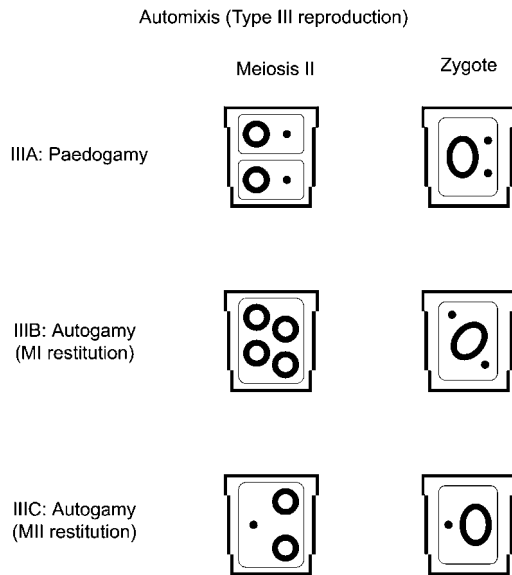
**Figure 43** *Thalassiosira angulata*: schematic representation of the life cycle in clone PB001 with emphasis on nuclei and initial cell. Vegetative nuclei are stippled, meiotic and intra-auxospore reproductive nuclei are drawn with thick lines, pycnotic nuclei are indicated by filled circles. AAMC: autogamous auxospore mother cell. Not to scale.

expansion or does so following syngamy. This finding may be of significance as it shows that syngamy does not act as the trigger for the initiation of the expansion in *Thalassiosira angulata*. This is an interesting difference, since Iyengar and Subrahmanyam (1944) suggested that in another thalassiosiroid diatom, *Cyclotella meneghiniana*, syngamy is the cue for the initiation of auxospore development. After syngamy, auxospore expansion and production of the initial valves and initial cell in *T. angulata* follows the general pattern observed in radial centric diatoms.

We choose to use the term autogamy to describe the observed nuclear behavior. It is a logical expansion of autogamy as defined by Geitler (1973) in his type III diatom reproduction (Figure 44). In type IIIB, fully acytokinetic meiosis results in a tetrad of viable nuclei, two of which function as gametic nuclei and fuse (first-division restitution) while two other undergo pycnosis. In our observations, a further simplified acytokinetic meiosis results in only three nuclei, because one product of meiosis I undergoes pycnosis before the remaining one divides. Therefore, in *Thalassiosira angulata* clone PB001

both gametic nuclei derive from the same haploid nucleus from meiosis I. "Mating" in our diatom brings together sister nuclei of meiosis II (second-division restitution). We propose that this kind of autogamy might be designated type IIIC (Figure 44IIIC).

Therefore, two different types of autogamous AMCs exist among diatoms, tri- (this study, Figure 44IIIC) and tetra-nucleated AMCs (Figure 44IIIB; Iyengar and Subrahmanyam 1944, Erben 1959), each with different consequences for genetic structure of populations resulting from their progeny. Due to random intra-tetradal mating, two-thirds of progeny from tetra-nucleated AMC automixis (type IIIB) will bring together a pair of homologous chromosomes separated in the first meiotic division, while the remaining one-third will pair with a sister chromosome. All progeny resulting from type IIIC autogamy will pair with a sister chromosome, because this "mating" will bring together only sister chromatids of meiosis II. Type IIIC autogamous progeny therefore loses half of the parental genetic complement, leading to high genomic homozygosity. Although heterozygosity is considered evolutionarily advantageous, a great many algae



**Figure 44** *Thalassiosira angulata*: schematic comparison of meiotic and zygotic stages in three types of automixis, types A and B modified from Drebes 1977.

Functional nuclei represented by large open circles and ovals, pyncnotic nuclei shown as small filled circles. Not to scale.

have integrated haploid (*de facto* homozygous) stages into their life histories, an indication that there must be an evolutionary advantage in maintaining a homozygous stage as well.

#### Autogamy in other radial centric diatom species

Several centric diatoms are often cited as capable of autogamous reproduction. Two species investigated in depth are *Cyclotella meneghiniana* (Iyengar and Subrahmanyam 1944) and *Melosira nummuloides* (Erben 1959). Both species were also reported to produce male gametes in culture in later studies (von Stosch 1967, Schultz and Trainor 1970, Hoops and Floyd 1979).

In contrast to *Thalassiosira angulata*, in *Cyclotella meneghiniana*, *Melosira nummuloides*, and *M. varians* (Persidsky in Geitler 1935) meiotic division of the AMC produce four nuclei and therefore would conform to type IIIB (Figure 44IIIB). Several other species are capable of autogamy, at least on some occasions: *Coscinodiscus granii* Gough (Roshchin 1975), *Melosira moniliformis* (O. Müller) C.A. Agardh *sensu lato* (von Stosch 1967, Drebes 1977, Stosch 1982), *M. moniliformis* var. *subglobosa* (Grunow) Hustedt (Roshchin and Chepurnov 1999), *Ellerbeckia arenaria* (Moore ex Ralfs) Crawford (Schmid and Crawford 2001), *Detonula confervacea* (Cleve) Gran [as *Schroederella schroederi* (Bergon) Pavillard, Drebes 1974] and possibly *Actinopterychus undulatus* (Bailey) Ralfs and *Paralia sulcata* (Ehrenberg) Cleve [as *Melosira sulcata* (Ehrenberg) Kützing, von Stosch 1967]. The number of nuclei, nuclear behavior during meiosis and initial cell production could not be determined in these studies.

In the absence of spermatogenesis, Schmid and Crawford (2001) also speculated that *Ellerbeckia arenaria* auxospores were formed via autogamy or vegetative enlargement. Alternative life history patterns consisting of

extrusion of cell contents into a pre-auxospore structure have been observed in *Leptocylindrus danicus* (Ehrenberg) Gran (French and Hargraves 1986) and was postulated to represent a modified form of autogamy. In most of the species listed above, autogamy is suggested, because spermatogonangia and sperm are absent or too infrequent to account for the abundance of auxospores present. Consequently, some of the accounts of autogamy remain inconclusive.

Persidsky also reported autogamy (with tetra-nucleated AMC stages) in *Chaetoceros boreale* Bailey and *C. densum* Cleve in a privately published manuscript summarized by Kolbe (1929). Geitler (1935) later suggested that the two seemingly incompatible accounts of autogamy, one by Persidsky (in Kolbe 1929) and the other by Cholnoky (1933) might be reconcilable. He suggested that the cells considered "AMCs" in those studies may be considered instead as tri-nucleated unfertilized eggs in *Ellerbeckia arenaria* (as *Melosira arenaria* Moore ex Ralfs, Cholnoky 1933) and as a tri-nucleated egg with a fertilizing nucleus in two *Chaetoceros* species (Persidsky in Kolbe 1929). If Geitler's (1935) interpretation is correct, all these species would simply have been observed in various stages of oogamous reproduction, reinforcing the impression that oogamy is the normal (preferred) mode of centric diatom reproduction. More recent work remains silent on the issue of number of autogamous nuclei in AMCs (Drebes 1977, Round et al. 1990, Edlund and Stoermer 1997).

In the context outlined above, it should not be surprising that even current reviews continue to consider sexuality in centric diatoms as purely or primarily oogamous. In their excellent review of studies on diatom life histories, Edlund and Stoermer (1997) stated that "all members of the Coscinodiscophyceae (centric diatoms) that have been studied utilize oogamy". More recently, Kooistra et al. (2003, 2006) write that while "meiosis and gamete formation vary considerably among diatoms [...] all centric diatoms studied so far produce motile flagellated micro-gametes (sperm) with one anterior flagellum and nonmotile naked macro-gametes (eggs)". Although correct in the context of scarcity of data on centric diatom sexuality, such pronouncements seem to have successfully discouraged systematic investigations of alternative modes of reproduction in centric diatoms despite their important effect on the genetic structure of resulting diatom populations.

It is hypothesized here, and earlier (Iyengar and Subrahmanyam 1944, Schmid and Crawford 2001), that autogamy among centric diatoms occurs more often than has been documented. It is widely believed that centric diatoms are monoecious, although not necessarily at the same time or by the same size-classes (von Stosch 1951, Roshchin 1975, Roshchin and Chepurnov 1999). We observe that oogamy in monoecious organisms is genetically quite similar to automictic sexuality. Both processes may be considered as two different kinds of selfing, with the type IIIC automixis representing the most extreme case (Figure 44), theoretically, more rapidly leading to homozygosity.

A more frequent occurrence of autogamy than is currently recognized may also be supported, at least in part,

by the findings where spermatogonangia were very rare (enumerated at less than 0.5%, Armbrust et al. 1990, Jewson 1992a,b) while auxospores were more frequent, or findings that "eggs" were formed in the absence of observed spermatogonangia (Steele 1967). Others reported that even when sperm and eggs are produced, auxospores developed from eggs that underwent incomplete meiosis while the mature eggs remained unfertilized and degenerated (von Stosch 1967, Drebes 1977).

We postulate that autogamy was the mode of size restoration in the *Thalassiosira angulata* culture studied in this paper. While this study shows the potential of this species to reproduce autogamously in repeated trials, no interclonal crossings of greater size range classes were performed and thus we do not suggest that the clones are obligatorily autogamous. We also do not know at this time whether this form of reproduction occurs in nature, or how often it takes place. Future studies may find that *T. angulata* also includes oogamy in its reproductive repertoire. For example, larger cells may practice oogamy, while cells of the smallest size classes may reproduce autogamously. Indeed our clone cell size approaches the specific minimum.

Nevertheless, our results highlight the diversity of strategies that centric diatoms can employ to achieve the highest reproductive success. It has been suggested that species may select their reproductive strategies based on environmental considerations. For example, *Ellerbeckia arenaria* has been postulated to auxosporulate autogamously or through vegetative enlargement due to the unpredictability of its semi-aquatic habitat. Similarly, recent work by Edlund and Spaulding (2006) on selfing in two pennate diatom genera suggests this strategy. Other diatom species, including *Thalassiosira angulata*, may practice a similar wide range of reproductive strategies.

There is mounting evidence that vegetative enlargement (von Stosch 1965, Nagai and Manabe 1993), parthenogenesis (von Stosch 1967, Drebes 1977), and automixis are practiced in all types of diatoms. Many of these studies elegantly illustrate that the centric diatoms, along with the pennate diatoms, are not as limited and predictable in their size restoration strategies as is so often assumed.

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