

## OOGAMOUS REPRODUCTION, WITH TWO-STEP AUXOSPORULATION, IN THE CENTRIC DIATOM *THALASSIOSIRA PUNCTIGERA* (BACILLARIOPHYTA)<sup>1</sup>

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*Thalassiosira* species are common components of marine planktonic communities worldwide and are used intensively as model experimental organisms. However, data on life cycles and sexuality within the genus are fragmentary. A clone of the cosmopolitan marine diatom *Thalassiosira punctigera* Cleve emend. Hasle was isolated from the North Sea and oogamous sexual reproduction was observed in culture. Cells approximately 45 µm and smaller became sexualized. Oogonia were produced preferentially and spermatogenesis was infrequent. Unfertilized oogonia always aborted and their development was apparently arrested at prophase of meiosis I. Further progression through meiosis and auxospore formation occurred only after a sperm had penetrated into the oocyte. Many cells of the new large-celled generation (approximately 90–120 µm in size) immediately became sexualized again but only oogonia were produced. A few of the large oogonia became auxospores and produced initial cells 132–153 µm in diameter. The second step of auxosporulation probably involved fertilization of large-celled oocytes by the sperm of the small-celled spermatogonangia that were still present in the culture. An F<sub>1</sub> clone obtained after selfing within the small-celled auxosporulation size range was investigated. Like the parent clone, the F<sub>1</sub> clone was homothallic but no auxosporulation was observed: spermatogonangia were unable to produce viable sperm, apparently because of inbreeding depression. Aggregation and interaction of oogonia were documented, and may be relevant for understanding the mechanisms of signaling and recognition between sexualized cells and the evolution of sexuality in pennate diatoms.

**Key index words:** auxosporulation; centric diatoms; inbreeding; life cycle; mating; oogamy; sexual reproduction; *Thalassiosira*

**Abbreviations:** DAPI, 4,6-diamidino-2-phenylindole

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*Thalassiosira* Cleve emend. Hasle is a large genus of centric diatoms containing over 100 species, mainly from marine and brackish habitats (Hasle and Syvertsen 1996). *Thalassiosira* species are very common in planktonic communities worldwide and some are used intensively in experimental studies of cell physiology and biochemistry (Peers and Price 2004, Zhukova 2004), including advanced methods of molecular genetics (Armbrust 1999, 2000, Armbrust and Galindo 2001). Cell wall formation has been studied in detail in *Thalassiosira eccentrica* (Ehrenb.) Cleve (Schmid and Schulz 1979, Schmid 1984a, b) and recently the first whole diatom genome was sequenced in *T. pseudonana* Hasle and Heimdal (Armbrust et al. 2004).

Isolating new cultures and providing conditions that favor vegetative multiplication are generally not problematic in diatoms, but there are often difficulties in the long-term maintenance of clones (von Stosch 1965, Chepurnov et al. 2004, Mann and Chepurnov 2004). The main problem is that most diatoms studied exhibit a gradual cell size reduction during vegetative cell division, according to the principle known as the MacDonald–Pfitzer rule (Crawford 1981, Pickett-Heaps et al. 1990). There is overwhelming experimental evidence that, due to size reduction, cells sooner or later become critically small and die in cultures that exhibit purely vegetative growth (Geitler 1932, Roshchin 1994, Mann et al. 1999, Chepurnov et al. 2004). The principal mechanism of cell size restitution is through formation of specialized cells called auxospores. These quickly expand in volume (hours, less often days) and afterwards a new large cell (the initial

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cell) is formed inside the auxospore envelope; the initial cell can then resume vegetative multiplication. Each auxospore typically results from a sexual reproduction event and represents the cell formed after fusion of two haploid gametes: it is essentially a special type of zygote, although the gametic nuclei often remain unfused during the early stages of auxospore development (Round et al. 1990). Exceptions in which auxosporulation is asexual, or where size is restored via a morphogenetically different process called vegetative cell enlargement, seem to be rare (Chepurnov et al. 2004). Thus, sexuality and auxosporulation are central to understanding diatom cell size reduction–restitution life cycles, with profound consequences for interpretations of population dynamics in nature.

Very little is yet known about the biochemical and genetic mechanisms underpinning the sexual behavior of diatoms. The first information on cell cycle regulation of diatom sexuality was obtained from a *Thalassiosira* species, *T. weissflogii* (Grun.) Fryxell and Hasle. Cells of *T. weissflogii* are sensitive to induction of spermatogenesis in the early G1 phase of the cell cycle (Armbrust et al. 1990) and molecular studies (Armbrust 1999, 2000) have revealed a novel gene family (the Sig-genes) that is suggested to be specific to sexual reproduction. The SIG polypeptides may play a role in sperm–egg recognition and could therefore be potential molecular markers for sexual reproduction in natural populations of centric diatoms (Armbrust 1999). The DNA sequences of Sig1 genes have since been obtained for three more *Thalassiosira* species (*T. oceanica* Hasle, *T. guillardii* Hasle, and *T. pseudonana*), and some geographically distant isolates of *T. weissflogii* (Armbrust and Galindo 2001).

Despite the importance of *Thalassiosira* species as models for studying aspects of diatom sexuality, there is a surprising absence of basic information concerning gametogenesis, fertilization, and auxospore formation, which have been documented in some detail in only two *Thalassiosira* species, namely *T. eccentrica* (Drebes 1979, Schmid 1984a) and *T. lacustris* (Grunow) Hasle (Idei 1993). In *T. weissflogii*, abundant evidence of vigorous induction of spermatogenesis has been provided in experimental cultures (Vaulot and Chisholm 1987, Armbrust et al. 1990, Armbrust 1999). However, no evidence has yet been provided on the occurrence of oogenesis and auxospores in this species, either in culture or in nature. Unfortunately, spermatogenesis and reports of periodic increases and decreases in mean cell size in cultures of *T. weissflogii* (Armbrust and Chisholm 1992) are not sufficient in themselves to prove that oogenesis, fertilization, and auxosporulation have also occurred in culture, because of the possibility that size may have been restored by vegetative enlargement or through non-sexual auxosporulation. For example, in two species, the freshwater *Cyclotella meneghiniana* Kützing (Schultz and Trainor 1968, von Stosch and Drebes in Drebes 1977a) and the marine *Melosira nummuloides* C. Agardh (Erben 1959), auxospore formation has been reported to be preceded by autogamous sex-

ual reproduction in the auxospore mother cell. We have confirmed the accuracy of these reports in isolates of the same species collected from different parts of the world with those from which the original reports were made during the course of studies for Håkansson and Chepurnov (1999) and Chepurnov et al. (2004). All of the clones isolated behaved identically, in that auxosporulation was completed successfully without any signs of spermatogenesis. Spermatogenesis did occur at other times, but normally appeared only in small cells and, as reported previously (Erben 1959, Schultz and Trainor 1968, Drebes 1977a), fertilization of auxospore mother cells by sperm was never seen. Nagai et al. (1995) found simultaneous auxosporulation and spermatogenesis in multiple strains of the planktonic marine centric diatom *Coscinodiscus wailesii* Gran. They did not exclude the possibility that oogamous sexual reproduction might occur in natural populations of this diatom but in culture, auxospores developed exclusively by asexual processes and again fertilization by sperm did not occur. Finally, in several clones of *Actinopterychus senarius* (Ehrenberg) Ehrenberg [syn. *A. undulatus* (Bailey) Ralfs], meiosis was circumvented in most of the cells predetermined to become oogonia and these cells developed into auxospores parthenogenetically (Behre and von Stosch in Drebes 1977a). Thus, the formation of sperm and auxospores within a culture does not prove that oogamy occurs; direct observation of fertilization is needed.

We selected *T. punctigera* (Castracane) Hasle for study because it is large-celled, morphologically distinctive, and very common worldwide in marine coastal plankton (Hasle 1983), where it sometimes produces blooms (Horner 2002). Additionally, *T. punctigera* grows well in culture and has already been used as an experimental organism representative of the diatoms as a whole (Hamm et al. 2003, Vrieling et al. 2005). Although *T. punctigera* has already been shown to auxosporulate in monoclonal cultures (Hasle and Syvertsen 1996, p. 49, Fig. 12), details of this process have not been documented. We demonstrate sexual reproduction in *T. punctigera*, providing a basis for using this species for studies of the genetic and physiological control of sexuality in centric diatoms.

#### MATERIALS AND METHODS

A planktonic sample containing *T. punctigera* cells was collected on May 24, 2002 in the Westerschelde (Zeeland, the Netherlands) at Terneuzen (51°20'N, 3°50'E). The following day, a single *Thalassiosira* cell was isolated and grown as a monoclonal culture in F2 culture medium (Guillard 1975) based on filtered and sterilized seawater (32 psu) from the North Sea. When the culture was to be observed intensively and documented photographically, it was maintained in polystyrene 50 mm Petri dishes with approximately 15 mL of culture medium at 18° C in a growth room with 12:12 light:dark (L:D) and 25–30  $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  from cool-white fluorescent lights. Cells were reinoculated into fresh medium every 2 weeks, before the culture had reached a stationary phase. At other times, before sexualization was first detected and also for maintaining a stock culture, the clone was kept in a

plastic tube with approximately 12 mL of culture medium in a chamber at 7°C, with an 8:16 L:D period and approximately  $5 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . Under these conditions, the cells divided less often and hence were reinoculated every 1.5–2 months.

Subcultures grown in Petri dishes were regularly monitored under a Zeiss Axiovert 135 inverted microscope (Zeiss Gruppe, Jena, Germany). Details of cell cycle and sexual behavior were observed and documented photographically with a Zeiss Axio-plan 2 Universal microscope (Zeiss Gruppe) equipped with a digital camera (VIP III, Hamamatsu Photonics Deutschland, Herrsching, Germany); an aliquot of the culture was mounted in a Buerker counter chamber (Paul Marienfeld GmbH & Co., KG, Lauda-Koenigshofen, Germany, cat. No. 06 402 30; depth of chamber 0.1 mm) and covered with a coverslip. Preparations were observed for 1–2 h and, to compensate for evaporation, a small amount of culture medium was regularly added by micropipette to the chamber (precisely to a groove between the ridges). Single cells could be rotated by gently moving the coverslip, allowing them to be observed in both girdle and valve view.

For DAPI (4'-6-diamidino-2-phenylindole) staining of nuclei, cultures were fixed with Lugol's iodine, formalin, and sodium thiosulfate solutions, according to Rassoulzagedan's method (Sherr and Sherr 1993). After a few hours' fixation, cultures were stained with DAPI (0.5  $\mu\text{g}/\text{mL}$ ) for 20 min and filtered gently onto black polycarbonate filters (pore size, 0.2  $\mu\text{m}$ ; Isopore GTBP membranes; Millipore, MA, USA) at low vacuum (<10 kPa). The filters were then mounted in a drop of low-fluorescence (halogen-free) immersion oil (Zeiss).

Frustules were cleaned by oxidation with hydrogen peroxide and glacial acetic acid and washed repeatedly with distilled water before being mounted in Naphrax (PhycoTech, St. Joseph, MI, USA). Voucher specimens of cleaned material have been deposited in the Laboratory of Protistology and Aquatic Ecology, Ghent University, Belgium. Light microscopical (LM) observations of cleaned frustules and preparations stained with DAPI were carried out using the Zeiss Axio-plan 2 Universal microscope. The morphometric measurements presented in Table 1 were made with the aid of ImageJ software version  $\times 1.29$  (<http://rsb.info.nih.gov/ij/>). Scanning electron microscopy (SEM) was performed using a JEOL JSM5600LV (JEOL, Tokyo, Japan).

## RESULTS

*Morphology and identity of the diatom.* The initial isolate grew vigorously in culture and had morphological characters typical of the genus *Thalassiosira* (Round et al. 1990, Hasle and Syvertsen 1996). Cells were drum-shaped with circular valves and contained numerous discoid chloroplasts (Fig. 1, B–I). Numerous long organic threads were extruded from the small marginal fultoportulae (strutted processes). Normally, the cells were solitary, but occasionally they became linked in short chains of up to four cells held together by a single thin central connecting thread. The frequency of chain formation increased as the cells became smaller.

LM and SEM examination of cleaned frustules (available as supplementary material) revealed a variable number of long external tubular processes (occluded processes). The marginal fultoportulae bore urn-shaped external tubes and had four satellite pores internally. There was only one central fultoportula and a single marginal rimoportula (labiate process). The

valve margin was extended out into a narrow flange, which was ribbed. The cingulum of a complete theca typically consisted of six bands: the first band (valvocopula) was wide and porous, bearing many rows of round areolae, the second band was narrower and possessed a single row of areolae, and the remaining four bands were plain but similar in width to the second. Sometimes, an extra plain band was observed; this was always much narrower than any of the other bands.

Based on the characteristics described above and the morphometric data presented in Table 1, the Westerschelde clone was unambiguously identified as *T. punctigera* (Hasle 1983, Mahood et al. 1986). *T. punctigera* has previously been reported in natural samples from the same region (Kat 1982, Muylaert and Sabbe 1996).

*Nuclear dynamics over the mitotic cell cycle.* During interphase, the nucleus was ellipsoidal and lay in a bridge of cytoplasm connecting the centers of the valves (Fig. 1A). As the cell cycle progressed, a few cytoplasmic strands were extruded from the central cytoplasmic bridge in various directions (Fig. 1, B and C). One of these became attached to one side of the cell (unfortunately, we could not determine exactly where attachment occurred in relation to unique features such as the rimoportula) and became distinctly thicker than the rest (Fig. 1D). Then, the nucleus migrated toward the side of the cell, in the direction of the thickened strand. Finally, the nucleus rounded off and became tightly appressed to the interior of the girdle (Fig. 1, E and F). Karyokinesis occurred (Fig. 1G) and both daughter nuclei quickly returned to the cell center (Fig. 1, H and I) as the cleavage furrow cut the cell in two, before re-assuming an ellipsoidal shape (Fig. 1, J and K).

*Oogamy and auxospore formation.* For nearly a year after isolation of the clone in May 2002 (the first measurements of valvar diameter were performed at the beginning of June, see Table 1), only vegetative multiplication of cells occurred, accompanied by a gradual cell size reduction. On April 12, 2003, when the cells had reduced from approximately 67 to 37–45  $\mu\text{m}$  in diameter (mean = 41.8, SD = 1.87;  $n = 23$ ), we detected the onset of gametogenesis, and auxospore formation followed soon after. Sexual reproduction was oogamous, involving production of large eggs and small motile uniflagellate spermatozooids. Over the whole of the sexually inducible size range, the frequency of oogenesis dramatically exceeded the frequency of spermatogenesis (Table 2).

The first sign of the differentiation of cells into oogonia was the separation of the central cytoplasmic bridge (containing the nucleus) from one of the valves and its contraction toward the other (Fig. 2, A and B). Simultaneously, the nucleus became spherical. Normally, the cytoplasm surrounding the nucleus remained connected to the opposite theca by a few cytoplasmic strands (Fig. 2, E and F). The oogonial nucleus (Fig. 2, C–G) was always visibly larger than the

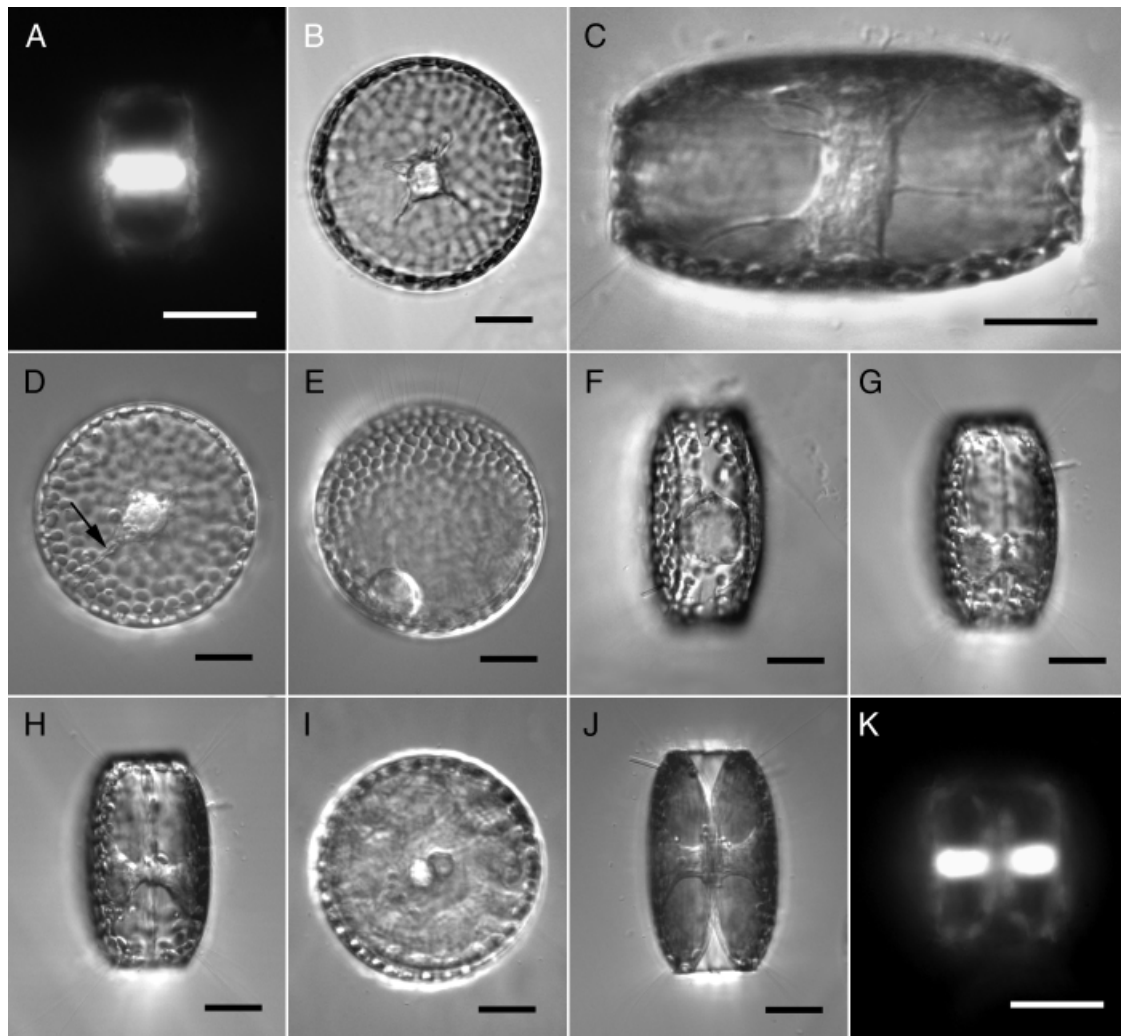


FIG. 1. *Thalassiosira punctigera* mitotic cell cycle in live (B–J) or DAPI-stained (A and K) material. (A) Early interphase cell in girdle view with a single central nucleus. (B and C) Formation of cytoplasmic strands extending from the central bridge of the cytoplasm: valve (B) and girdle (C) views. (D) Thickening of cytoplasmic strand (arrow), valve view. (E and F) The nucleus has moved to the girdle and become spherical, in valve (E) and girdle (F) views. (G) Dividing nucleus in girdle view. (H and I) After karyokinesis, in girdle (H) and valve (I) views. The daughter nuclei are moving back to the cell center. (J and K) The nuclei have returned to the cell center and their ellipsoidal shape is restored; girdle view. Scale bars, 20  $\mu\text{m}$ .

TABLE 1. *Thalassiosira punctigera*: morphometric data on valves from the natural clone, the  $F_1$  progeny of the clone, and two published reports.<sup>a</sup>

Material studied	Number of observations	Diameter ( $\mu\text{m}$ )	Areolae per 10 $\mu\text{m}$	Striae per 10 $\mu\text{m}$	Marginal strutted processes per 10 $\mu\text{m}$	Marginal ribs per 10 $\mu\text{m}$
Clone 06.06.2002	10	64.7–69.9 (67.2 $\pm$ 1.55)	18–23 (20.3 $\pm$ 1.57)	23–26 (24.7 $\pm$ 1.16)	4	15–17 (16 $\pm$ 0.67)
Clone 01.05.2003	10	31.2–39.4 (33.9 $\pm$ 2.78)	18–22 (20.0 $\pm$ 1.16)	20–26 (22.5 $\pm$ 1.65)	4–5 (4.9 $\pm$ 0.32)	14–18 (16.1 $\pm$ 1.45)
$F_1$ -cells	10	87.6–119.5 (107.7 $\pm$ 12.19)	15–22 (18.4 $\pm$ 2.77)	17–26 (20.5 $\pm$ 2.8)	4–6 (5.1 $\pm$ 0.57)	12–19 (15 $\pm$ 2.22)
Hasle (1983)	138 <sup>b</sup>	40–186	10–23	15–30	3–11 mostly 4–5	10–16
Muylaert and Sabbe (1996)	ND	45–100 (88 $\pm$ 7)	10–16 (12 $\pm$ 2.3)	ND	4.5–6 (5.1 $\pm$ 0.5)	ND

<sup>a</sup>Values are ranges (mean  $\pm$  SD).

<sup>b</sup>Data were taken from Tables 1 and 2 of the article.

ND, no data.

TABLE 2. Percentage of vegetative and sexualized cells (433 randomly selected cells) in the original culture during exponential phase of growth (May 15, 2003).

Cell type	Original clone <sup>a</sup> (%)	F <sub>1</sub> cells formed after the first auxosporulation (%)
Vegetative	5.0	6.0
Oogonia	82.7	3.7
Spermatogonangia	2.6	0
Total	90.3	9.7

<sup>a</sup>Cell size range 26.3–39.6  $\mu\text{m}$  (mean = 35.9, SD = 3.21,  $n = 30$ ).

nucleus of vegetative cells but at this stage no nuclear division occurred and the oogonia themselves were never seen to divide. The further development of the oogonia involved elongation of the cell in the perivalvar direction, followed by gradual separation of the thecae and partial exposure with the protoplast. The position of the cleft between the thecae did not bear a constant relationship to the position of the enlarged nucleus. Sometimes, in open oogonia, the nucleus became positioned more or less arbitrarily, without obvious association with either of the parental thecae (Fig. 2G).

Those *T. punctigera* cells that differentiated to produce sperm (the spermatogonangia) passed first through a series of special mitotic divisions without intervening cell growth (“depauperating” sensu Drebes 1977b). In our clone, there were always three depauperating mitoses per spermatogonangium, producing eight spermatogonia. The first of the spermatogonial divisions occurred in the median

valvar plane (Fig. 3A), as during ordinary mitotic division of vegetative cells, and each daughter cell (half-spermatogonangium sensu Drebes 1977a) then deposited a rudimentary, weakly silicified theca (Fig. 3, B and F). The rudimentary thecae were narrow in girdle view and seemed to have few or no girdle bands. The second and third depauperating divisions took place in radial planes (Fig. 3, C–E) and no deposition of any siliceous elements was detected. Because of the rarity of spermatogenesis in our clone, some details of sperm formation remain unknown. However, spermatozooids lacked chloroplasts and after their release from the spermatogonial frustule (Fig. 3G), residual bodies containing chloroplasts were always left behind. Spermatogenesis was thus merogenous.

In Buerker counter chamber preparations, we observed a few instances of fertilization. Sperm approached the oogonium (Fig. 3H) and attached themselves either to the girdle (if the oogonium was still closed: Fig. 3I), or to the exposed surface of the oocyte (Fig. 3J). Physical contact between the male and female gametes was not immediately followed by plasmogamy. For example, in one case it took approximately 10 min for the sperm to penetrate into the egg cell and effect plasmogamy, after attachment between the cells. All the oogonia examined that were observed to interact with the sperm, including those into which sperm had penetrated, contained a single large nucleus, suggesting that meiosis was incomplete at fertilization.

A subculture in which gametogenesis was taking place (producing many oogonia and a few spermatogonangia) was fixed and stained with DAPI

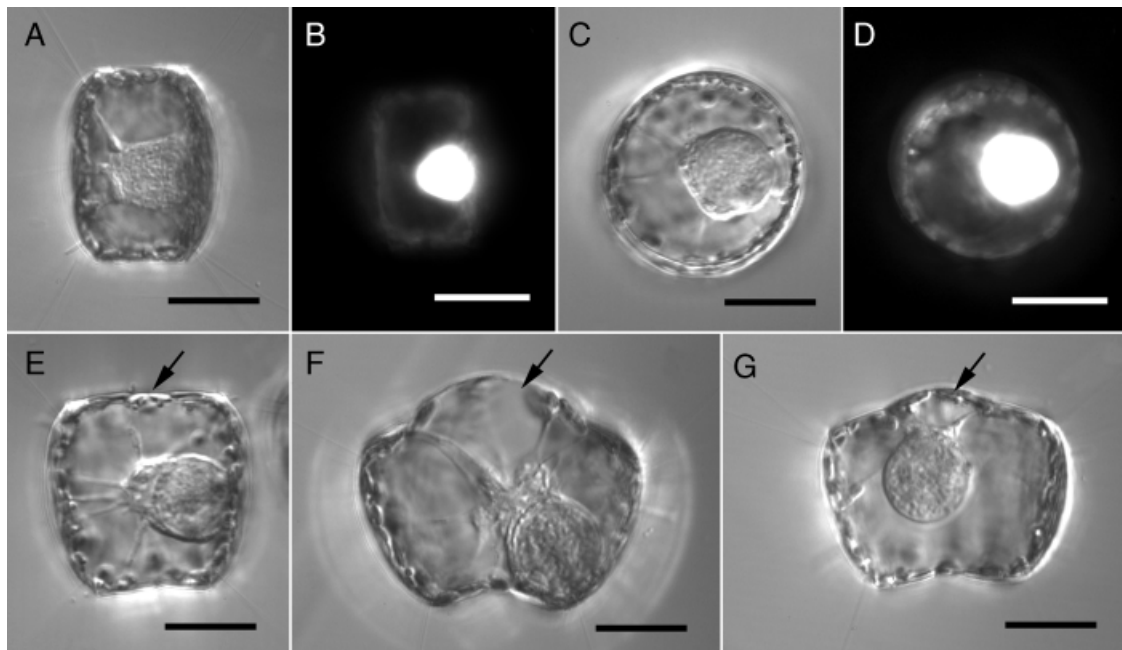


FIG. 2. *Thalassiosira punctigera* oogonia, either live (A, C, E–G) or stained with 4,6-diamidino-2-phenylindole (DAPI) (B and D). (A–D) In early oogonia, the nucleus rounds off and enlarges in volume; girdle view (A and B) and valve view (C and D). (E–G) are girdle views. Note that the membrane of oocyte is partly exposed (arrows) by partial separation of oogonial thecae. Scale bars, 20  $\mu\text{m}$ .

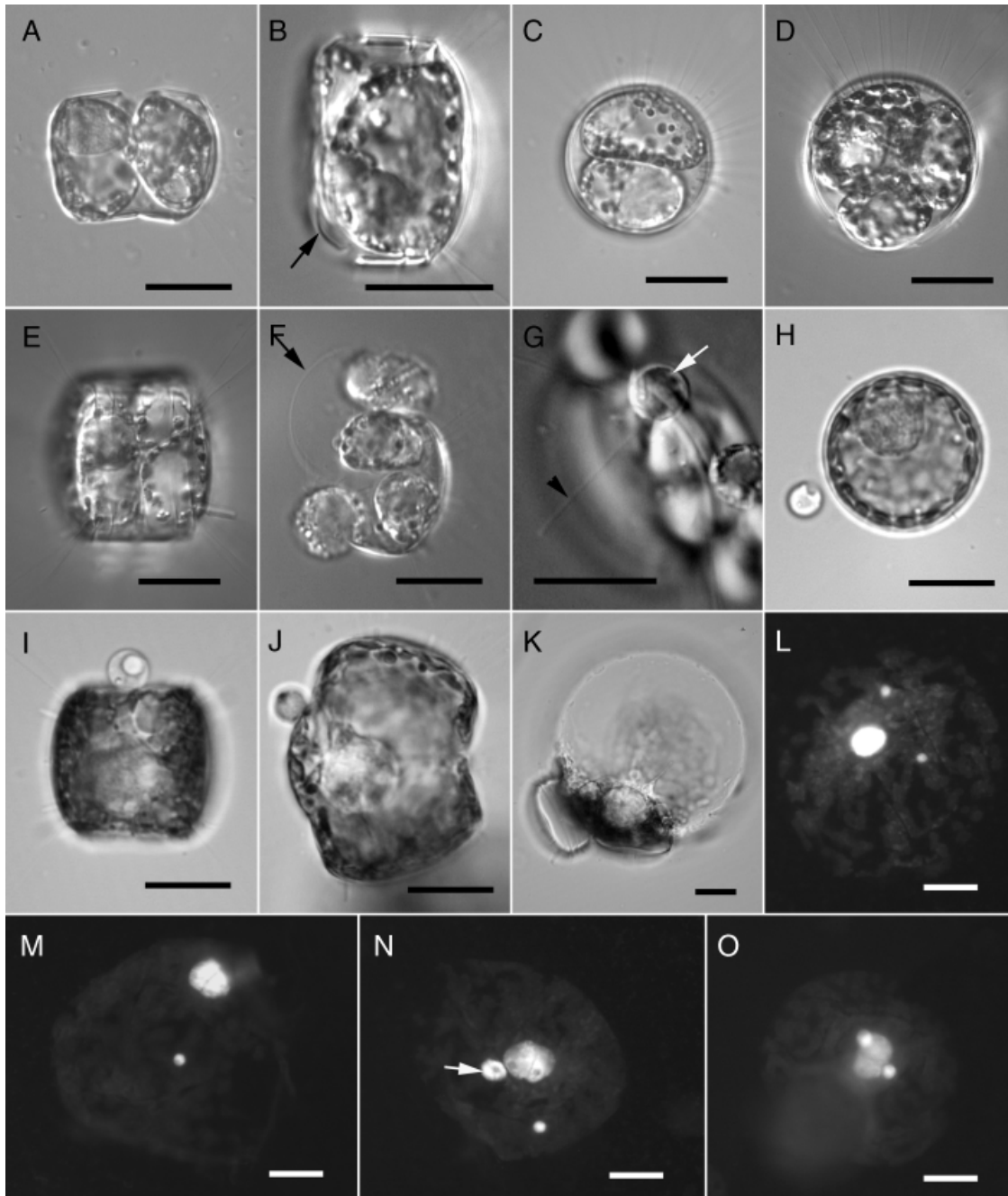


FIG. 3. *Thalassiosira punctigera* depauperating mitoses, sperm, and fertilization. (A) Spermatogonangium after the first mitosis, in girdle view. (B) Half-spermatogonangium in girdle view. Note the rudimentary theca (arrow) deposited after the first depauperating mitosis. (C) After completion of the second depauperating mitosis, valve view. (D and E) After the third depauperating mitosis, in valve (D) and girdle (E) views. (F) Open half-spermatogonangium, with four spermatogonia and rudimentary theca (arrow). (G) Uniflagellate spermatozoid (arrow), with anterior flagellum (arrowhead), just released from the spermatogonangial frustule. (H) Spermatozoid approaching the oogonium. (I and J) The spermatozoid has attached to the oogonium. (K) Aborted oocyte (apparently unfertilized). (L–O) Oocytes stained with DAPI (the cells are flattened by a cover slip): the large nuclei are oocytal and small nuclei are derived from the sperm. In (M), the large nucleus has just divided at meiosis I. In (N), the division of the large nucleus represents meiosis II; the superfluous nucleus that aborted after meiosis I is arrowed. In (O), either the two small nuclei are both from sperm or one of the two represents the aborted superfluous product of meiosis I in the oocyte. Scale bars, 20  $\mu$ m.

(Fig. 3, L–O). Most of the oogonial cells contained a single large nucleus, as observed in living cells and in earlier stages of oogenesis (Fig. 2, B and D). In a few oogonia, however, there was an extra small nucleus

(less often two), which was often distant from the large nucleus and appeared very compact, suggesting that the chromatin was highly condensed (Fig. 3, L–N). Because at this stage the oocyte nucleus had not yet

divided meiotically, the small nuclei must have been derived from sperm.

A few oogonia were discovered in which the oogonium nucleus had divided once (Fig. 3M) or twice (Fig. 3N), i.e., it had apparently passed through meiosis I or meiosis II, respectively. Remarkably, in both cases, the oocytes simultaneously contained at least one sperm nucleus (Fig. 3, M–O). The sperm nuclei were smaller than any of the oogonial nuclei. For example, Fig. 3N shows three larger nuclei, all with a single nucleolus. Two of these are very closely appressed to each other and almost equal in size; these seem to represent sister nuclei from meiosis II. The third nucleus (arrowed) is slightly smaller and more condensed; this we interpret as the superfluous nucleus from meiosis I. In addition to the oogonium in Fig. 3N contains a small, dense sperm nucleus. Thus, stained cells indicate that oocyte development in *T. punctigera* was arrested early in meiosis, probably in prophase of meiosis I, judging by similarities to the swollen nuclei of oocytes in meiotic prophase in other centric species, e.g., *Stephanopyxis palmeriana* (Grev.) Grun. (Drebes 1966) and *Chaetoceros didymum* Ehrenberg (von Stosch et al. 1973); further progression through meiosis is triggered by interaction with sperm.

It was impossible to prove that all of the eggs that were observed to develop into auxospores had been fertilized before expansion started, but indirect evidence suggests that fertilization is obligatory. For example, we examined a subculture that originally (just after re-inoculation of cells into fresh medium) contained exclusively vegetative cells. In a few days, numerous oogonia were detected but there were still no signs of spermatogenesis or auxosporulation. At this stage, 25 young oogonia were isolated (each in a separate well of a 96-well Repli plate) and then monitored daily. None developed into auxospores and all finally died (Fig. 3K). However, shortly after spermatogenesis started in the subculture from which the oogonia had been taken, auxospores also appeared.

*Auxospore development and initial cell formation.* The expansion of auxospores was initially isometric and chloroplasts were evenly distributed over the spherical cell (Fig. 4, A and B). We could not determine the exact timing of karyogamy, but in expanding auxospores we could detect only one nucleus, which was presumably diploid. During expansion, the thecae of the oogonium remained attached to the auxospore and the auxospore gradually changed in shape from spherical (Fig. 4A) to a slightly compressed ellipsoid, with its widest axis at right angles to the peralvar axis of the oogonium (Fig. 4, B and C). Once the auxospore had reached its final size, the auxospore contents gradually contracted away from the auxospore wall at the side where the nucleus was located, typically in front of one of the parental thecae (Fig. 4C). Contraction produced an evenly curved free surface lying approximately half way between the auxospore wall and the mid-plane of the auxospore; the initial epivalve was deposited beneath this

surface, with the nucleus remaining appressed to its center (Fig. 4C). Later, there was a corresponding contraction on the opposite side of the auxospore, the nucleus migrated to this side, and the initial hypovalve was deposited. Then, the nucleus regained the elongate shape (Fig. 4D) typical of vegetative cells (e.g., Fig. 1A). As a general rule, the deposition of each diatom initial valve is preceded by an acytokinetic mitosis, with rapid abortion of one of the products (Geitler 1963). Unfortunately, we could not obtain unambiguous evidence of such mitoses in *T. punctigera*.

The SEM examination of oxidized culture material containing numerous auxospores did not reveal any silica scales in the auxospore wall, which appears to be purely organic. The diameters of 34 initial cells still associated with the parental oogonia (Fig. 4D) were 82.7–123.6  $\mu\text{m}$  (mean = 103.8, SD = 8.59); the parental oogonia from which they were derived were 26.3–40.6  $\mu\text{m}$  (mean = 36.4, SD = 3.25). No correlation was found between the sizes of the oogonia and those of the initial cells.

*Frequency of gametogenesis.* Once sexual reproduction had been initiated in the experimental culture kept at 18°C with a 12:12 L:D and 25–30  $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , the frequency of oogenesis increased continually but spermatogenesis remained comparatively rare. In late May 2003, a month after the first gametangia were seen, the vast majority of cells were sexualized, mostly becoming oogonia, and only 5% of the cells were vegetative, judging by their appearance in LM (Table 2). During the next 2 weeks, the last vegetative cells of the original clone became converted into gametangia as well.

On August 1, 2003, we transferred some cells from the stock culture, which had been kept at low light and temperature (see Materials and Methods), to Petri dishes kept under experimental culture conditions (18°C, 12:12 L:D, 25–30  $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ). Initially, the new subculture looked purely vegetative, although the cells were within the sexually inducible size range (with diameters of 37–45  $\mu\text{m}$ , mean = 42.6, SD = 2.21,  $n = 20$ ). During the next few days, however, vigorous gametogenesis occurred, with oogenesis absolutely predominant over spermatogenesis. In 10 days, all cells of the subculture had been lost to gametogenesis and auxosporulation, although the latter was infrequent.

*Second-step auxosporulation.* Initial cells that were formed by auxosporulation in the parental clone multiplied successfully by mitotic cell division without any loss of vigor, despite their inbred origin (Fig. 4, E and F). Soon after their formation, some of the  $F_1$  cells (but not the initial cells themselves) became sexualized and produced oogonia (Fig. 4, G and H) but not sperm. A few of the large female cells of the  $F_1$  developed into auxospores and yielded viable initial cells (Fig. 4I). This second step of auxosporulation probably resulted from fertilization of the large oogonia by sperm from the original parent clone,

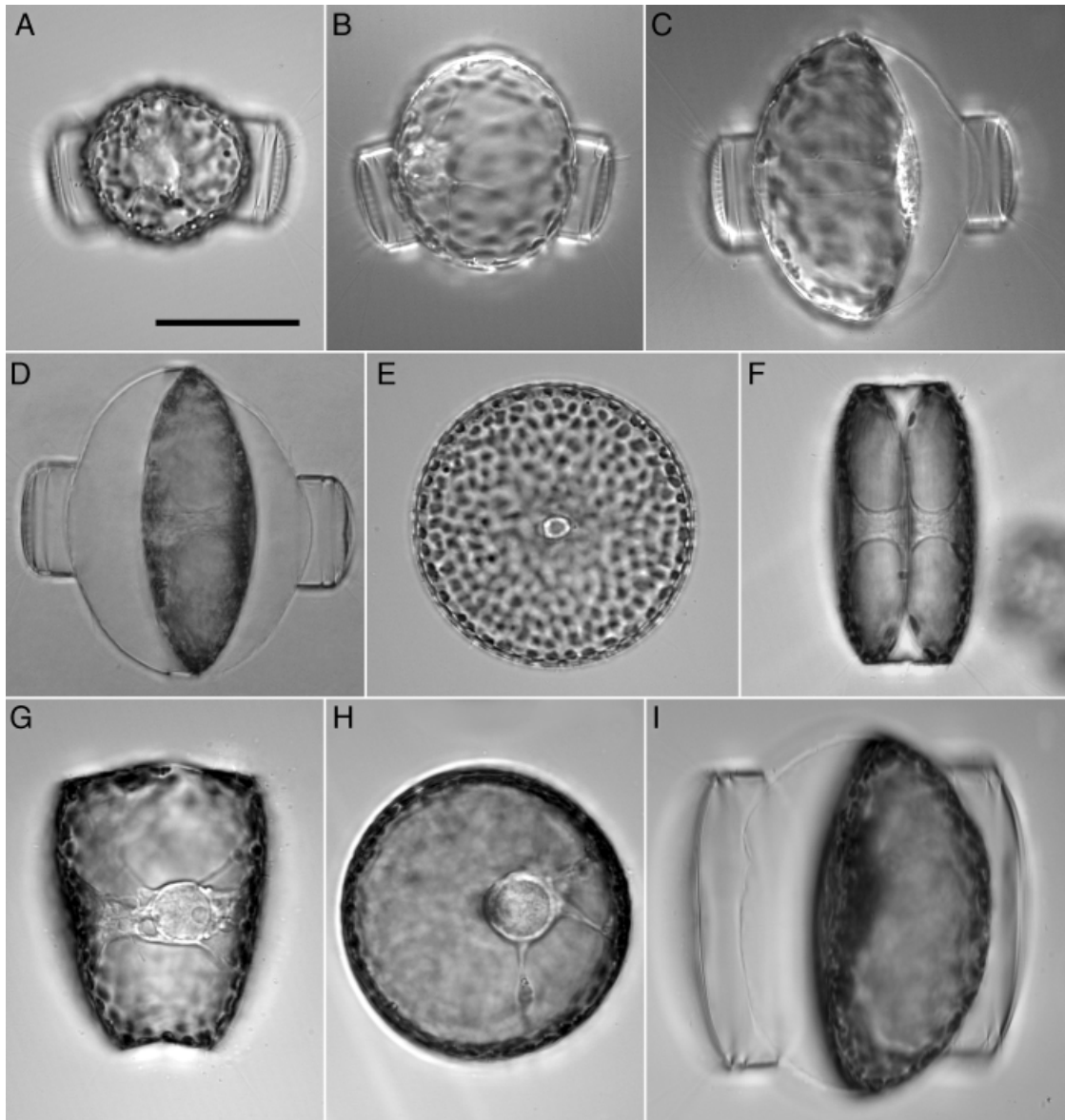


FIG. 4. *Thalassiosira punctigera* auxospore development, initial cell formation, and second step of auxosporulation. (A and B) Expanding auxospores. (C and D) Initial cell formation. Deposition of the epivalve (C) and hypovalve (D). (E and F) Vegetative cells of the  $F_1$  generation in valve (E) and girdle (F) views. (G and H) Large oogonia of the  $F_1$  generation in girdle (G) and valve views (H). (I) Deposition of initial epivalve in the auxospore derived from the large oogonium. Scale bar, 50  $\mu\text{m}$ .

because sperm were still being produced in small numbers in the same culture by the remaining small cells of the parental generation. We did not directly observe sperm penetration into  $F_1$  oogonia. However, when we isolated large female  $F_1$  cells and grew them, the oogonia never transformed into auxospores; sperm-producing cells of the parental clone had to be present for auxospore formation. Hence, fertilization was apparently an obligate condition for oogonia to produce auxospores, during both first- and second-step auxosporulation.

The frequency of oogenesis during second-step auxosporulation was estimated soon after the  $F_1$  cells had been formed and when they comprised approximately

10% of all cells (Table 2). Approximately one-third of the  $F_1$  cells had differentiated as oogonia, which varied in diameter from 91.8 to 120.2  $\mu\text{m}$  (mean = 102.4, SD = 6.02,  $n = 25$ ). As fertilization was rare, only six initial cells were measured. These (with the parental oogonia in parentheses) had diameters of 131.6 (106.1), 134.1 (102.6), 142 (99.5), 149.9 (100), 152.0 (108.7), and 152.5 (99)  $\mu\text{m}$ .

*Clone of the  $F_1$  generation.* Soon after the first initial cells were formed in the original clone, one initial cell was isolated and grown as the " $F_1$  clone." After 10 days, cells of the new clone were 103.1–110.9  $\mu\text{m}$  in diameter (mean = 106.4, SD = 2.36,  $n = 10$ ). A few of the  $F_1$  cells differentiated into oogonia but none

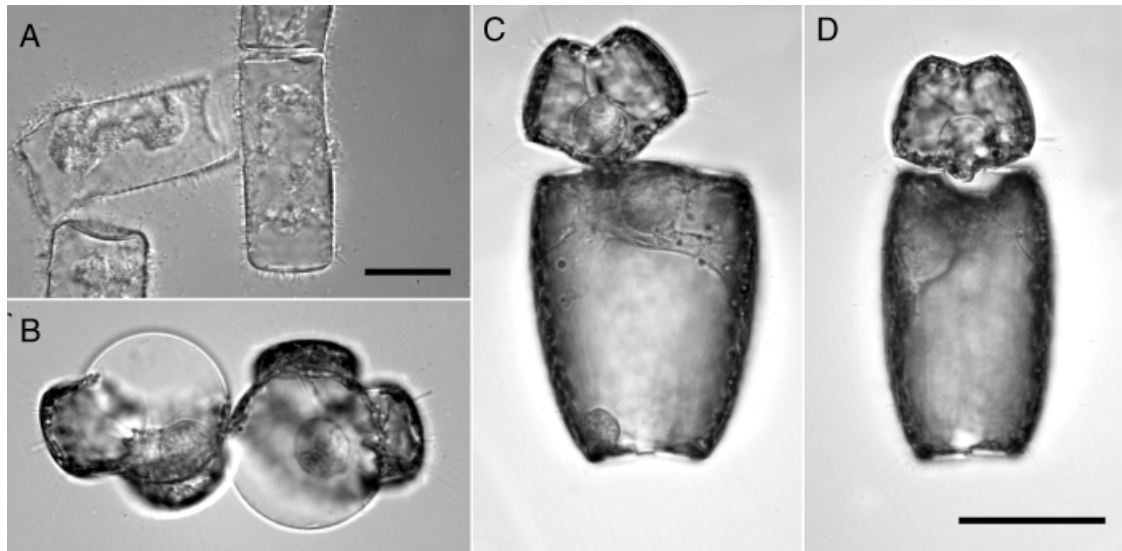


FIG. 5. *Thalassiosira punctigera* unusual morphology and sexual behavior: (A) Critically small vegetative cells that are already dead, girdle view. (B–D) Aggregated oogonia. (B) Two equal-sized oogonia of the natural clones. (C and D) The same pair of oogonia (which differ markedly in size) at time 0 (C) and c. 30 min later (D). Scale bars, 20  $\mu\text{m}$  (A) or 50  $\mu\text{m}$  (B–D, see bar in D).

produced auxospores. The culture was maintained at a higher light and temperature condition (see Material and Methods) for 2 months and then examined in detail, by which time the cells had declined to 77.3–84.5  $\mu\text{m}$  (mean = 80.22, SD = 2.32;  $n = 20$ ). No oogonia were found, despite their occurrence when the cells were larger; all cells were vegetative.

During the next 4 months, the  $F_1$  clone continued to be purely vegetative. Oogonia were not found again until  $F_1$  cells had declined to 43.3–46.2  $\mu\text{m}$  (mean = 44.8, SD = 1.05,  $n = 10$ ); the largest oogonium was 45.5  $\mu\text{m}$  in diameter. Three weeks later, when the cells were 29.3–34.6  $\mu\text{m}$  in diameter (mean = 32.5, SD = 1.48,  $n = 10$ ), the first spermatogonangia were found, the largest being 34.5  $\mu\text{m}$ . Thereafter, oogonia and spermatogonangia became increasingly common but no auxospores were produced. Detailed examination showed that, although the contents of the spermatogonangia generally passed successfully through three depauperating mitoses, further development of spermatogonia did not occur and they all eventually aborted. Thus, the failure of auxosporulation may be attributable to lack of sperm. In the month after spermatogonangia were first detected, morphologically abnormal vegetative cells began to appear, with disproportionately broad girdles. Typically, these cells did not divide (or exceptionally rarely) and eventually died (Fig. 5A); they can be interpreted to have reached the critical minimum size. In a further month, the whole culture became extinct, when the smallest cells were 21.4–24.3  $\mu\text{m}$  in diameter (mean = 22.9, SD = 1.12;  $n = 10$ ).

*Aberrant behavior of oogonia.* During vigorous oogenesis in the parental clone (Table 2), oogonia often stuck together in pairs or larger aggregates, especially in dense cultures. Attachment was robust,

as shaking the culture did not usually destroy the clusters. Oogonia of first-step gametogenesis adhered to each other (Fig. 5B) or to large oogonia in second-step gametogenesis (Fig. 5, C and D). Clumped oogonia developed like solitary oogonia and progressed to the stage at which the thecae opened wide, allowing the contents to bulge out of the parental frustule (Fig. 5B). Usually, there was no visible interaction between oogonia. However, in a few cases where young oogonia (i.e., oogonia in which the cells were only slightly bent, exposing only a small part of the oocyte) touched each other, there appeared to be an attempt of the oocytes to fuse, in which one of them (the smaller cell in (Fig. 5, C and D) behaved more actively than the other. Within 30 min, a cytoplasmic projection from one oogonium could be seen pushing inside the frustule of the neighboring oogonium, although this never resulted in plasmogamy.

#### DISCUSSION

*Gametogenesis, fertilization, and auxospores.* Several aspects of oogamous auxosporulation in *T. punctigera* resemble those in *T. eccentrica* (Drebes 1979) and *T. lacustris* (Idei 1993). All three species produce one egg per oogonium, spermatogenesis is merogenous, and the oogonial thecae remain attached to opposite ends of the auxospore while it expands (intercalary auxospores: Drebes 1974, 1977a). Intercalary auxospores are found in a wide variety of centric diatoms (Drebes 1974). Among *Thalassiosira* species, they have been illustrated previously in *T. punctigera* by Horner (2002, p. 31) and they also occur in *T. rotula* Meunier and *T. angulata* (Greg.) Hasle (as “*T. decipiens*”: Drebes 1974), *T. gravida* Cleve (Lebour 1930, Drebes 1974), *T. decipiens* (Grun.) Jørgensen (Hasle 1979),

and *T. eccentrica* (Drebes 1979). In some other genera and species of Thalassiosirales, the auxospores are again intercalary, e.g., in *Detonula pumila* (Castracane) Gran (Drebes 1974, as *Schroederella schroederi* (Bergon) Pavillard) and *Porosira glacialis* (Grun.) Jørgensen (Drebes 1974). In *Cyclotella* sp. (Geitler 1952), the auxospore is intercalary and the oogonial thecae remain attached to the auxospore at least during the early stages of expansion. By contrast, in *Detonula confervacea* (Cleve) Gran the auxospores are lateral (Drebes 1974) and in *Skeletonema*, the auxospore expands to one side of the mother cell, so that the mother cell thecae are displaced to lie close together on one side of the expanded auxospore, at an acute angle to each other (Schütt 1893, Drebes 1974). Despite this similarity, *Detonula* and *Skeletonema* are not sister taxa in recent molecular phylogenies of Thalassiosirales (Medlin and Kaczmarska 2004, Kaczmarska et al. 2006). In *Stephanodiscus* sp. (Round 1982), *S. neoastraea* Håkansson and Hickel (Jewson 1992), and *S. niagarae* Ehrenberg (Edlund and Stoermer 1997), the mother cell thecae seem to lose their association with the auxospores very early.

There is some variation within *Thalassiosira* in the number and orientation of the depauperating mitoses that precede formation of the spermatocytes. Judging by published illustrations (Drebes 1979, Fig. 6a), both the first and the second depauperating mitoses in *T. eccentrica* occur in the valvar plane. In *T. punctigera* and *T. lacustris* (Idei 1993), however, the second depauperating mitosis is radial, i.e., perpendicular to the plane of the first division. Again, illustrations by Drebes and Idei suggest that there are two rounds of depauperating mitoses in *T. eccentrica* and *T. lacustris*, whereas we found three in *T. punctigera* (Fig. 3, C–E), with the third being radially orientated, like the second. Such differences need to be interpreted cautiously, because the number can vary not only among species but within a single species (Chepurnov et al. 2004) as the cells decrease in size during the life cycle. However, in *T. punctigera* the number of depauperating mitoses was constant, despite size reduction. In the *Cyclotella* species studied by Geitler (1952), there were no depauperating mitoses, each spermatogonangium giving rise to just four sperm.

Our data show that the sperm can penetrate the oocyte before karyokinesis of meiosis I. By contrast, oogonia complete meiosis II before fertilization in *Stephanopyxis*, *Lithodesmium*, *Streptotheca*, and *Odontella* (von Stosch 1954, 1956, von Stosch and Drebes 1964, Drebes 1966). Fertilization of oogonia before completion of meiosis, as in *T. punctigera*, has been reported in *Chaetoceros didymum* (von Stosch et al. 1973) and *Cyclotella* (Geitler 1952), where it occurs during meiotic prophase as in *T. punctigera*, and in *Melosira varians* (von Stosch 1951), where it occurs at anaphase I. These diatoms represent widely separated clades in recent molecular phylogenies (Medlin and Kaczmarska 2004) and there is clearly much homoplasy in sexual development.

Among numerous oogonia of *T. punctigera*, both live and stained with DAPI, we never saw an unfertilized oocyte developed into an auxospore, and later stages of meiosis in oogonia were always in the presence of a sperm nucleus. Therefore, unfertilized oocytes seem to be arrested during early or mid-prophase of meiosis I, when the nucleus is markedly enlarged; sperm penetration is apparently a prerequisite for completion of meiosis. In the related genus *Cyclotella*, Geitler (1952) found that oocytes in diplotene and diakinesis contained sperm nuclei (which were usually peripheral), whereas those in pachytene did not, suggesting stimulation of meiosis by sperm penetration like that in *T. punctigera*.

Some centric species seem to possess mechanisms to protect oogonia from multiple penetration of sperms (polyspermy). In *S. palmeriana* (Drebes 1966) and *M. moniliformis* (O. F. Müll.) C. Agardh var. *octogona* (Grun.) Hust. (Idei and Chihara 1992), for instance, in which part of the egg surface is exposed through a crack between oogonial thecae, the oogonium closes almost immediately after the sperm has penetrated into the egg to create a rapid physical block to polyspermy. By contrast, the oogonial thecae of *T. punctigera* do not close after sperm penetration and sperm are slow to penetrate into the exposed protoplast of the oogonia. In this, they resemble *Chaetoceros didymum* (von Stosch et al. 1973) but differ from *M. moniliformis* var. *octogona* (Idei and Chihara 1992). As a consequence, polyspermy may be possible in *T. punctigera*, producing oogonia like those we occasionally observed containing two sperm nuclei. However, without thin-section transmission electron microscopy or confocal microscopy, we cannot guarantee that both spermatozooids had passed through the plasmalemma of the oocyte.

*Multi-step auxosporulation.* *T. punctigera* is the first species of Thalassiosirales shown to exhibit two-step auxosporulation. The initial cells formed by small-celled oogonia are not of the maximum size possible for *T. punctigera*, nor are they outside the sexual size range. They therefore have one of two fates: either they can become vegetative and begin mitotic cell division, or they can become re-sexualized and function as oogonia of a larger size; in the latter case, they produce initial cells of maximal size. The possibility of two-step auxosporulation in centric diatoms is rarely acknowledged (it is not mentioned by Round et al. 1990) but has been documented in *M. nummuloides* (Schreiber 1931), *M. moniliformis* (Kustenko 1978), and *Coscinodiscus janischii* A. Schmidt (Roshchin 1975, 1994). In cultures of *Coscinodiscus granii* Gough from the Black Sea, auxosporulation has been reported to occur in as many as three steps (Roshchin 1994). For the two species of *Melosira* mentioned above, details of auxosporulation were not documented. In *Coscinodiscus granii*, multi-step auxosporulation may be linked with an alternation between oogamous and asexual patterns of development (Roshchin and Chepurnov 1999) but confirma-

tion is needed. In *Coscinodiscus janischii*, however, auxosporulation has been clearly shown to be associated with oogamous reproduction and this species shows striking similarities to *T. punctigera* in some details of its reproductive behavior. Two clones of *Coscinodiscus janischii* isolated by Roshchin from the Black Sea in different years behaved identically in that cultures switched to oogamous sexual reproduction when the cells were 150–170  $\mu\text{m}$  in diameter. The initial cells they produced were up to 327  $\mu\text{m}$ . When cells of the new generation were 230–260  $\mu\text{m}$  in diameter, some became sexualized again but only oogonia were produced, as in *T. punctigera*. The oogonia did not develop parthenogenetically and so, because no sperm were present, no auxospores were produced. During size reduction from 230 to 170  $\mu\text{m}$ , the cultures exhibited only vegetative growth. Below 170  $\mu\text{m}$ , on the other hand, both oogenesis and spermatogenesis took place and auxospores were formed, as in similar-sized cultures of natural clones. Although the development of large oogonia (230–260  $\mu\text{m}$ ) into auxospores was never observed in culture, Roshchin suggested that this could occur in natural populations of *Coscinodiscus janischii*, through fertilization by the sperm of small-celled spermatogonangia. In samples from natural populations in the Black Sea, he regularly found *Coscinodiscus janischii* cells that were 300–400  $\mu\text{m}$  (maximum 427  $\mu\text{m}$ ) in diameter. The similarity between *Coscinodiscus janischii* and *T. punctigera* is particularly interesting because *Thalassiosira* and *Coscinodiscus* belong to different major clades of centric diatoms (Kooistra et al. 2003, Medlin and Kaczmarska 2004), suggesting either that multi-step auxosporulation has originated independently in different lineages, or that it is actually very widespread in centric diatoms but unreported.

The largest auxospore we observed from second-step auxosporulation was 152.5  $\mu\text{m}$ , whereas Hasle (1983) indicates that the maximum size for *T. punctigera* is 186  $\mu\text{m}$  (Table 1). This could indicate that a third step could sometimes occur, but we did not test for it. However, the recent discovery of cryptic speciation in centric diatoms (Sarno et al. 2005) cautions against assuming that all diatoms identified as *T. punctigera* will necessarily belong to the same biological species and have the same size ranges.

**Mating system.** The discovery of homothallic (monoecious) reproduction in clonal cultures of *T. punctigera* is not surprising, because homothally is widespread among centrals (Drebes 1977a, von Stosch 1982); indeed, strict heterothally has never been reported within the centric group. In contrast, heterothally seems to be widely distributed among pennate diatoms (Roshchin 1994, Mann et al. 1999, Chepurnov and Mann 2004, Chepurnov et al. 2004, Sabbe et al. 2004, Mann and Chepurnov 2005). However, some centric diatoms do possess some mechanisms that are likely to promote outbreeding and the principal variation documented so far in centric mating systems relates to synchrony (or the lack of it) in

the production of gametes of opposite sex. Some centric species, e.g., *Stephanopyxis* (von Stosch and Drebes 1964, Drebes 1966), are *simultaneous* hermaphrodites: male and female gametes are produced at the same time in monoclonal culture, from cells of similar size. More often, however, bisexuality is preferentially *consecutive* (or *subsequent*): the size ranges for production of eggs and sperm only partially overlap (von Stosch 1956, Drebes 1977a). Interestingly, consecutive hermaphroditism has been reported so far only in the form of protogyny, i.e., the formation of oogonia starts first.

The breeding system in *T. punctigera* could perhaps be classified as monoecy alternating with dioecy, with transitions between the two occurring via size reduction (in one direction) or multi-step auxosporulation (in the other direction). Roshchin has made a similar characterization of the complex life cycle of *Coscinodiscus janischii*, which involves two sexually inducible cell size ranges. Roshchin found that the large-celled size range was apparently associated with dioecious mating behavior: clones were able to produce either exclusively eggs, or only sperm (within the range 230–260  $\mu\text{m}$ , he reported spermatogonangia in natural collections: Roshchin 1994). Small cells, on the other hand, were simultaneous hermaphrodites. In smaller cells of the two clones of *T. punctigera* involved in the present investigation, the size ranges for producing female and male gametangia largely overlapped but with a slight tendency toward protogyny. However, many more clonal isolates of *T. punctigera* and *Coscinodiscus janischii* need to be studied to confirm the idea that there are two discrete sexual size ranges with different characteristics and possibly different triggers. There is considerable evidence that gametogenesis in centric diatoms is often controlled by the environment, as well as internal factors related to cell size (Drebes 1977a, Chepurnov et al. 2004). For instance, von Stosch (1954) reported in *Lithodesmium undulatum* Ehrenberg that subcultures of a single clone placed in continuous light almost exclusively produced eggs, but in alternating light and dark, the subcultures could be induced to produce either a mixture of eggs and sperm or sperm alone. In *T. punctigera*, we did not detect any signs of sexualization in “stock” cultures kept in dim light and at a low temperature. Our observation that oogenesis was much more frequent than spermatogenesis in both the natural clone (see Table 2) and the F<sub>1</sub> clone is curious and needs further evaluation using several isolates and more variation in experimental conditions than we were able to supply. The “polygamous” status of our clones may be environmentally induced or determined genetically; other clones may be preferentially “male.”

**Effect of inbreeding.** The principal difference in sexual behavior between the natural clone of *T. punctigera* and its daughter clone obtained experimentally after self-fertilization was that the F<sub>1</sub> clone was incapable of producing sperm, even though it produced spermatogonangia. There have been several

previous reports that self-fertilization affects the viability and fertility of the offspring in centric diatoms, e.g., in *Stephanopyxis turris* (von Stosch 1965), *Chaetoceros didymum* (von Stosch et al. 1973), and *M. moniliformis* (A. M. Roshchin and V. A. Chepurinov, unpublished data). A plausible explanation of these reductions in fitness in the F<sub>1</sub> progeny is inbreeding depression (Carr and Dudash 2003), caused apparently by homozygous unmasking of deleterious recessive alleles. If so, however, these centric diatoms must possess such alleles at many unlinked loci for the effect to be as drastic as we observed within a single generation in *T. punctigera*. Aggressive decline in the viability or fertility of inbred progeny would also be indirect evidence that natural populations of centric diatoms are largely outbreeding, even though their clones are hermaphrodite, i.e., their eggs are normally fertilized by sperm from a different clone. This agrees with the conclusions of Rynearson and Armbrust (2004, 2005) and Evans et al. (2005), following population genetic studies of the centric diatom *Ditylum* and the pennate *Pseudo-nitzschia*. In habitual inbreeders, on the other hand, the effect of inbreeding should be softened, as the fitness is expected to rebound during the selective decrease in frequency of deleterious alleles in successive inbred generations (“purging the genetic load,” Crnokrak and Barrett 2002).

*Interaction between oogonia.* The interaction between the oogonia of *T. punctigera* observed in the natural clone (Fig. 5, B–D) is intriguing, although further characterization of this phenomenon—e.g., by examining the biochemical and genetic basis of signaling and recognition (Chepurinov et al. 2004)—will be needed before it can be interpreted fully. It is very likely that the oogonia of centric diatoms produce pheromones that guide the sperm toward the eggs to effect fertilization, as in other algal groups (Coleman et al. 2001, Sekimoto 2000). The example of *T. punctigera* indicates that it is conceivable that the pheromone may generate a reaction in oogonia as well. Alternatively, the interaction between the oogonia may be mediated purely by surface interactions that would not normally occur but are made possible by the artificially dense crowding in culture. Oogonia and eggs may be naturally adhesive and this may partly explain the interaction that we observed, but there must also be stimulation of cytoskeletal activity in some cases, because we observed extension of an oocyte toward and into a neighboring oogonium. Such cases, in which morphologically and physiologically identical gametes interact in centric diatoms, may throw light on one of the most intriguing and momentous, but still completely unexplored events in the evolution of diatoms, namely how the oogamy of centric diatoms has undergone evolutionary transformation into the physiologically different, but morphologically alike and equal-sized, non-flagellate gametes of araphid pennates (Chepurinov and Mann 2004, the exception is *Rhabdonema*, where the

gametes are non-flagellate but unequal in size). Remarkably, *Thalassiosira* belongs to the major clade of centric diatoms from which the araphids are believed to have evolved (Kooistra et al. 2003).

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### Supplementary Material

The following supplementary material is available as part of the online article from <http://www.blackwell-synergy.com>:

**Fig. S1.** *Thalassiosira punctigera* vegetative cells: LM (A–C) and SEM (D–H). (A) Optical section of cell in valve view, showing the central nucleus and multiple chloroplasts. (B) Valve face and radiating chitin fibrils. (C) Girdle view of two cells connected by a central thread. (D) Theca with five occluded processes (one of the five is arrowed) on the slightly convex valve surface. Note also the marginal ring of fulcra (arrowhead). (E) Three marginal fulcra on the external valve face, with intact urn-shaped extensions. (F) Ribbed flattened valve margin (arrowhead) and the cingulum. Ligulae are arrowed. (G) Internal view of three marginal fulcra and a rimoportula (located closer to the cell centre). (H) Central fulcra. Scale bars: 20  $\mu\text{m}$  (A–C), 5  $\mu\text{m}$  (D and F) or 1  $\mu\text{m}$  (E, G, and H).