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Auxosporulation, mating system, and reproductive isolation in *Neidium* (Bacillariophyta)

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Three allogamous *Neidium* demes, referrable to *N. ampliatum sensu lato,* coexist without intergradation in Blackford Pond, Edinburgh, and some other lakes. A slight change in valve shape has occurred in one deme during the last 20 years. Morphological deme traits persist in healthy clonal cultures, but with time or during senescence, aberrant morphologies and sizes of cell can be produced that have no parallel in nature. Simplification of valve outline occurs as cells get smaller, but the initial cells also have a simplified morphology. The rostrate apices of some *N. ampliatum* demes develop rapidly after auxosporulation, during the first divisions of the initial cells. The 'major' and 'minor' demes of *N. ampliatum* are heterothallic, although some inbreeding occurs. Very rarely, mixed pairs of 'major' × 'minor' are formed, but hybrid auxospores are apparently never produced, so the demes are reproductively isolated. The 'maternal' gametangium has a nongenetic influence on initial cell size; however, the effect is slight and control of initial cell size is very well buffered to variation in gametangium size, so the concept of 'cardinal points' is valid for this species complex. The characteristics of sexual reproduction in clones and seminatural populations (including the effective, though incomplete, suppression of triplets and larger groups during copulation) prompt hypotheses about pairing mechanisms, in particular that a chemoattractant is involved. Preferential polyandry in *Sellaphora* and theoretical considerations indicate that the chemoattractant is produced by only one mating type. Size selection of mates in the 'minor' deme probably reflects progressively easier and more rapid sexualization as cells become smaller.

INTRODUCTION

The genus *Neidium* Pfitzer is a well-defined group of freshwater raphid diatoms, easily recognized by the presence of longitudinal canals in the cell wall (Round *et al.* 1990), which are located near the margins of the valve face and appear in the light microscope (LM) as conspicuous longitudinal bands of modified stria structure. With very few exceptions, the striae are conspicuously punctate in LM, and the external central raphe endings are prolonged into hook-like, curved, or straight external fissures, which point in opposite directions (cf. Figs 1–26, 41, 42, 45, 46). The interphase chloroplast arrangement is also highly characteristic among freshwater diatoms, with four chloroplasts, one in each quadrant of the cell (e.g. Mann 1996, fig. 14). The one known exception, *Neidium binodeforme* Krammer, in which two valve-appressed chloroplasts are present during interphase, has apparently evolved through rephasing of chloroplast division relative to cell division (Mann 1996). Finally, the characteristics of sexual reproduction and auxosporulation also appear to be constant and involve girdle–girdle pairing, plasmogamy via narrow copulation apertures, behavioural anisogamy, and the formation of silicified caps over the ends of the auxospores (Mann 1984a, this paper, and unpublished observations). The *Neidium* studied by Drum *et al.* (1966) also had this type of auxosporulation. So far, however, nothing has been published on the mating system of *Neidium* species, because previous observations of sexual reproduction were made using heterogeneous natural populations.

The context for studies of the mating system is the growing realization that many raphid diatoms are not homothallic, as previously thought (Drebes 1977: see Chepurnov *et al.* 2004). Some *Sellaphora* Mereschkowsky and *Eunotia* Ehrenberg species, for example, exhibit a simple form of heterothally, with two mating types (Mann *et al.* 1999, 2003, 2004). *Achnanthes longipes* C. Agardh has a more complex mating system, in which four types of clone can be distinguished on the basis of the clone's behaviour in interclonal crosses and its ability to reproduce intraclonally (Chepurnov & Mann 1997, 1999, 2000). Heterothallism can be accompanied by behavioural differences between the gametes produced by the different mating types, as in *Sellaphora capitata* D.G. Mann & S.M. McDonald, where the cells of one mating type produce active 'male' gametes, whereas the other produces passive 'female' gametes (Mann *et al.* 1999, 2004). Alternatively, there may be no obvious difference, either in structure or behaviour, between the gametes and gametangia produced by different mating types, as in *Seminavis* D.G. Mann in Round *et al.* or *Eunotia* (Chepurnov *et al.* 2002; Mann *et al.* 2003). However, although we have argued that heterothallism is probably primitive within pennate diatoms (Roshchin 1994; Chepurnov & Mann 2004; Chepurnov *et al.* 2004), there is insufficient evidence to be sure, and in the raphid group (the largest of the main lineages of extant diatoms, containing the majority of species and genera), the mating system, and even sexual reproduction itself, are unknown in most taxa.

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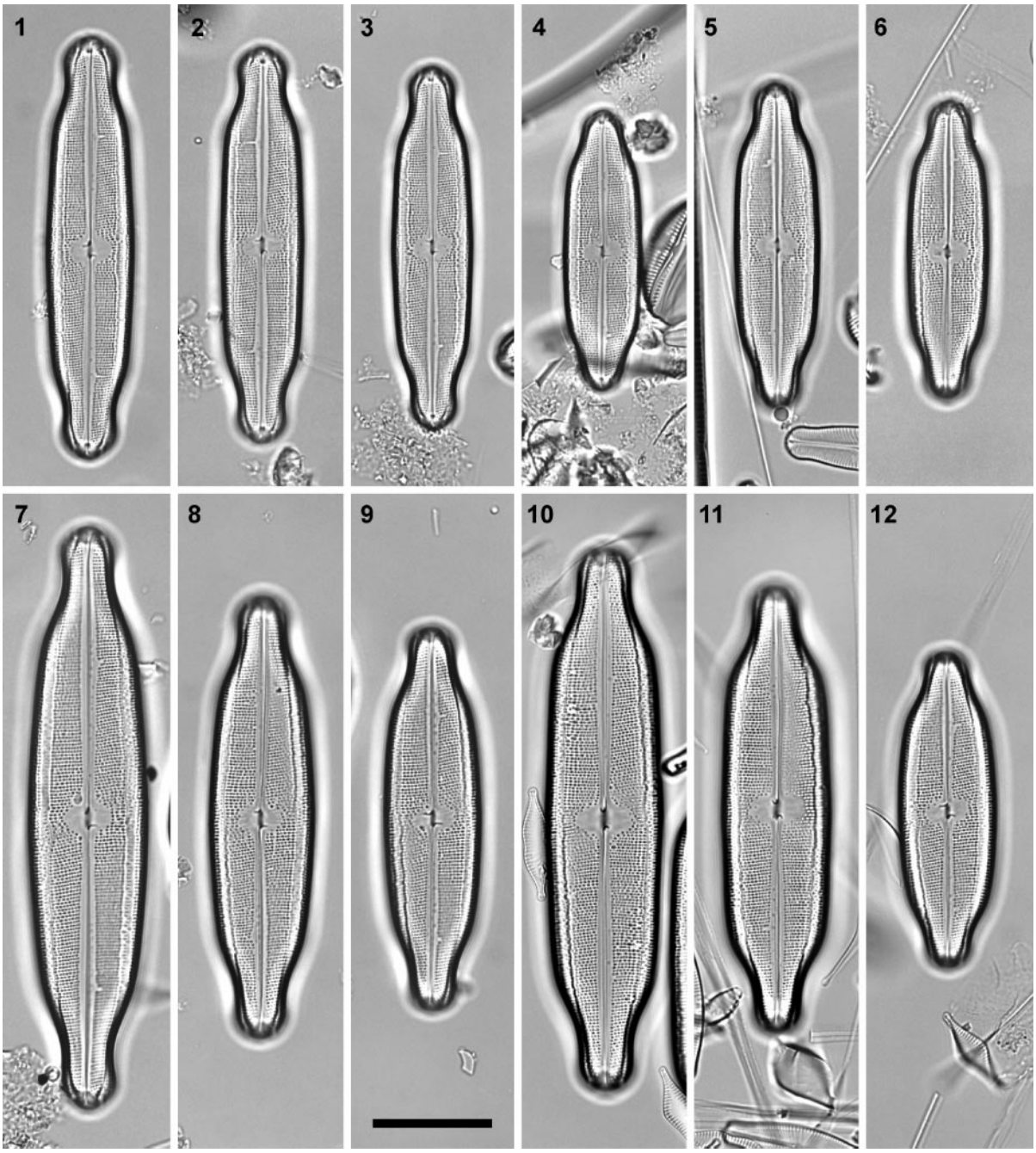


Figs 1–12. *Neidium ampliatum:* valves from Blackford Pond populations. Bright field optics. Scale bar = 20 μm.
Figs 1–6. 'minor' deme. Stages in size reduction in natural populations sampled on 18 December 1985 (Figs 1–4) and 14 January 1996 (Figs 5, 6).
Figs 7–12. 'major' deme. Stages in size reduction in natural populations sampled on 18 December 1985 (Figs 7–9) and 14 January 1996 (Figs 10–12); note the more angular shape in Figs 10–12.

We have now been able to maintain *Neidium ampliatum* (Ehrenberg) Krammer in culture and have taken the opportunity to determine aspects of the mating system and to add to the knowledge of life history and speciation provided by Mann (1984a, 1989b, 1999). The behaviour of mating cells and comparisons with sexual reproduction in *Sellaphora* lead to the hypothesis that cells of opposite mating types differ in their ability to produce a chemoattractant.

MATERIAL AND METHODS

Neidium cells, identifiable as *N. ampliatum* as defined by Krammer & Lange-Bertalot (1986), were obtained from the epipelon of two Edinburgh lakes: Blackford Pond (UK National Grid Reference NT 253709, 70 m alt.) and Figgate Loch (Grid Reference NT 298736), under 0.5–1 m water. Neither lake has been studied in detail limnologically. They are small

(~100 m diameter) artificial lakes fed by groundwater seepage and underwater springs (Blackford Pond) or by a small stream (Figgate Loch) and lie in parks in suburban Edinburgh. Both lakes support large populations of waterfowl and are highly eutrophic. The bottom sediments are soft muds, with some areas of gravel in Figgate Loch. Another Edinburgh lake, Dunsapie Loch (Grid Reference NT 281731), was also found to contain *Neidium* demes corresponding to those in Blackford and Figgate, and an old mill water-course at Millgate, Balerno, near Edinburgh (Grid Reference NT 168656), contained the 'major' deme.

Sediment samples were obtained by using a glass tube as described by Round (1953). The mud–water slurry was allowed to stand for several hours or overnight and then the water was removed. The surface of the mud was covered by a layer of lens tissue (as a filter, allowing the passage of diatoms but not mud particles) and epipelic algae were harvested using cover-slips or lens tissue placed on top. If kept in continuous light at a moderate temperature (*c.* 15°C), many epipelic algae survived in the mud for several days, even beneath cover-slips, and any *Neidium* present often became sexual 4 or 5 days after transfer to the laboratory. Blackford Pond epipelon has been sampled in this way since the early 1980s; between 1987 and early 1989, samples were taken every 2–4 weeks. Harvested epipelon was surveyed for species 2–3 days after collection from nature by scanning most or all of a single 24 × 50 mm cover-slip at ×100 or ×250; such cover-slips generally bore many thousands of cells. After four or more days, a second 24 × 50 mm cover-slip was scanned for the presence of sexual reproduction. Voucher specimens of contemporary cover-slip preparations are kept in the Edinburgh herbarium.

Living 'seminatural' diatoms were studied while still attached to the cover-slips used to harvest them. Cultures were established by streaking harvested epipelon on agar plates of freshwater medium or filtered lake water, incubation for a few weeks at 15°C in dim light (*c.* 5 μmol photons m^{-2} s^{-1}), and isolation of small clonal colonies into liquid WC medium with silicate (Guillard & Lorenzen 1972). Cultures were maintained thereafter in similar conditions with 12 : 12 or 14 : 10 h light–dark cycles. For mating experiments, small aliquots of cultures in exponential growth were inoculated together into fresh WC medium in the wells of Repli dishes and kept at 15 or 20°C and 5–20 μmol photons m^{-2} s^{-1} conditions, with 12 : 12 or 14 : 10 h light–dark cycles. These light and temperature regimes are our standard culture conditions for benthic species, and we did not examine the effects of different conditions on *Neidium* growth and auxosporulation. Other light and temperature regimes might perhaps have been even more favourable to sexual reproduction, but pairing was often intense in our experiments (cf. Fig. 51), and initial cells developed and divided successfully.

Photomicrographs of mating in cultures were made by inoculating large aliquots together into WC medium in 90 mm diameter Petri dishes, in which clean 24 × 50 mm cover-slips had already been placed. The diatoms settled onto the cover-slips and mated there. The cover-slips were inspected using an inverted microscope and were removed as appropriate for further study with a Reichert Polyvar photomicroscope, when they were dried on one side and sealed onto a microscope slide using petroleum jelly. Cleaned material of natural populations and cultures was prepared by oxidation with 30 volume hydrogen peroxide or a 1 : 1 mixture of concentrated nitric and sulphuric acids and mounted in Naphrax. In order to preserve associations of gametangia within their pairs, or gametangia with initial cells, some populations, still attached to the cover-slips used to harvest them, were incinerated at 550°C in a muffle furnace and then mounted in Naphrax.

Photographs were taken with ×100 bright field or interference contrast optics (Numerical Aperture 1.32), using Kodak Technical Pan film. Negatives were subsequently digitized and the images manipulated using Adobe Photoshop (http://www.adobe.com/). Striation densities were measured by striatometer (Droop 1993), using a Polyvar drawing attachment.

RESULTS

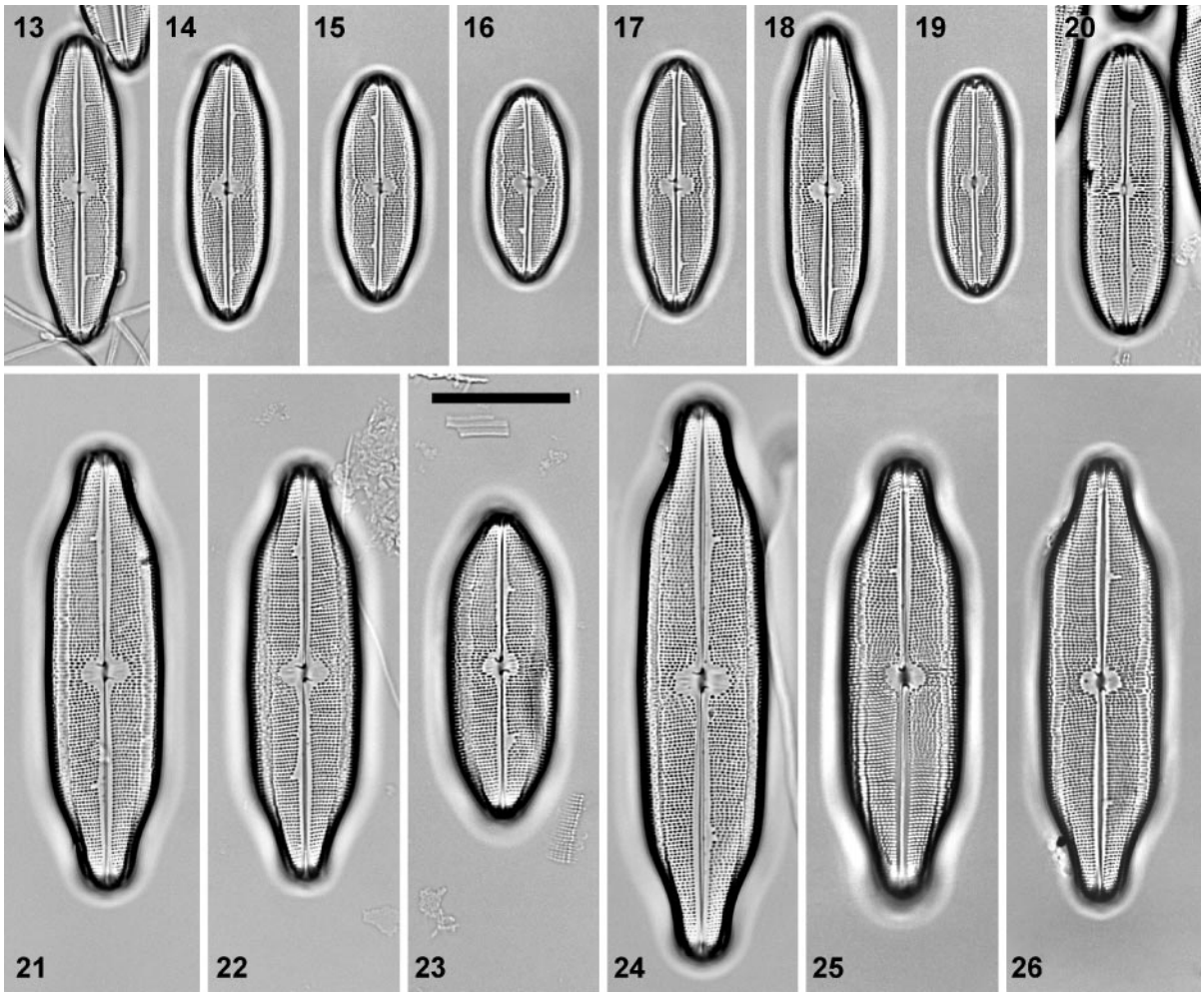
Taxonomy and morphology

Three *Neidium* demes have been present in Blackford Pond during the last 25 years. They have rarely been abundant, and routine surveys of ≫ 1000 cells on cover-slips used to harvest epipelon frequently failed to reveal a single cell of one or more demes (Table 1). The frequent rarity of the demes and the sporadic occurrence of sexual reproduction in our seminatural populations (Table 1) is the principal reason why this study has taken so long.

One *Neidium* deme (not illustrated) is a large-celled linear form with rounded cuneate apices that probably falls within *N. iridis* (Ehrenberg) Cleve, as treated by Hustedt (1930). The other two are very similar to each other in the range of shapes they exhibit during the life cycle (Figs 1–12). Like the *iridis* deme, they are linear, but they have rostrate or subcapitate apices, which are especially well developed in the upper (though not the uppermost) and middle parts of the size reduction cycle. As size reduction proceeds, pole morphology simplifies, and in the smallest cells (cf. Figs 15, 16, 23, from cultured material) the poles are scarcely rostrate at all. The two 'rostrate' demes can be separated on the basis of size, one (the 'major' deme: Figs 7–12) being consistently wider than the other (the 'minor' deme: Figs 1–6) at equivalent stages in the life cycle, and also by striation density (17–18 striae in 10 μm in 'major', as opposed to 19–21 in 'minor'). The 'major' valves are often more abruptly narrowed toward the pole, producing a sharp shoulder subapically, but the degree of development of this feature varies within populations and among populations sampled at different times. Generally, 'major' valves collected since 1990 have been more linear, with more angular shoulders, than those collected earlier (contrast Figs 10–12, from 1996, with Figs 7–9, from 1985). No trend has been obvious in 'minor' (Figs 1–6).

The external central raphe endings were particularly variable in 'major' and can be bent (e.g. Fig. 9), curved, or recurved (e.g. Figs 10, 11). Different morphologies can even be combined in the same valve (Figs 8, 24). Occasionally one of the raphe slits lacks an external central fissure (Fig. 7). The 'major' and 'minor' demes are both referable to *N. ampliatum* as circumscribed by Krammer & Lange-Bertalot (1986, figs 105/4 and 106/7, respectively).

Each of the three *Neidium* demes in Blackford Pond has remained discrete during more than 20 years, and no obvi-



Figs 13–26. *Neidium ampliatum:* valves from cultured material. Bright field optics. Scale bar = 20 μm.
Figs 13–19. 'minor' deme, clones from Blackford Pond (except Figs 15, 16).
Fig. 13. Clone BS32.
Fig. 14. Clone 11.
Figs 15, 16. Clone 44 (from Figgate Loch).
Fig. 17. Clone BLA2.
Fig. 18. Clone BLA13.
Fig. 19. Clone 11: small linear valve formed in senescent cultures.
Figs 20–26. 'major' deme, clones from Blackford Pond.
Fig. 20. Clone BS59: small linear valve formed in senescent cultures (contrast Figs 22, 23, from the same clone, and compare Fig. 19, of the 'minor' deme).
Fig. 21. Clone BM26.
Figs 22, 23. Clone BS59: stages in size reduction.
Fig. 24. Clone BLA8.
Figs 25, 26. Clone BLA9: note the inward deflection of the marginal canals at the centre in Fig. 26.

ously intermediate morphologies have been detected. The *iridis* and 'minor' demes have also been found in Figgate Loch (Figs 15, 16), and the *iridis* and 'major' demes [together with a deme belonging to the *Neidium dubium* (Ehrenberg) Cleve complex] in Dunsapie Loch; both lakes are within a few kilometres of Blackford Pond. Again, no intermediate morphologies have been detected. At Balerno Millgate, cells apparently identical to the Blackford 'major' deme, with 18 striae in 10 μm, were present in 1989; no other *Neidium* species were seen.

Since 1997, several sets of *N. ampliatum* clones have been isolated from Blackford Pond (Figs 13, 14, 17–26) and one clone from Figgate Loch (Figs 15, 16). Both demes were easily isolated via agar into liquid medium and subsequently grew rapidly in WC medium. In culture, the 'major' and 'minor' clones reduce in size (e.g. Figs 15, 16, 22, 23), but, when maintained in exponential phase, both keep the morphological characteristics of wild-collected cells, e.g. 'major' deme clones continue to exhibit greater width, slightly coarser striation, and more accentuated shouldering than 'minor' clones (Figs 13–20, 21–26). The more sharply angled morphology characteristic of post-1990 natural populations of the 'major' deme (Figs 10–12) is retained in culture (Figs 24–26).

In senescent material, aberrant cells were produced (not illustrated), with very pronounced pattern discontinuities at the points where the secondary side of the valve is completed

(Voigt discontinuities: Mann 1981; Round *et al.* 1990) or disorientated striae; another common change was a slight inward arching of the longitudinal canals at the centre (Fig. 26). After prolonged growth in unfavourable conditions in culture (overcrowding, exhaustion of the medium), linear valves with rounded apices were found in both demes (Figs 19, 20). These were narrower than normal rostrate valves and were probably formed as internal valves (Round *et al.* 1990, p. 49). Cells are produced in culture that are smaller than any yet found in nature (36 μm in 'major' clone BS59; 26 μm in 'minor' clone 44).

Sexual reproduction, auxosporulation and postauxospore shape modification

Many aspects of sexual reproduction and auxospore development in *N. ampliatum* were described by Mann (1984a) from observations of seminatural material of the same Blackford populations that we study here [Mann used the taxonomy then current and referred the populations to *Neidium affine* (Ehrenberg) Pfitzer *sensu* Hustedt (1930)]. Briefly, cells pair actively and bond to each other, girdle to girdle (Figs 27–33). Then they enter meiotic prophase, when the nucleus expands, becomes spherical, and moves slightly toward the side adjacent to the other gametangium (Figs 27–29). After meiosis I, cytokinesis produces two protoplasts that lie on either side of the median valvar plane, as at mitosis, but no new valves are produced (Fig. 30). Meiosis II is followed by the abortion of one haploid nucleus in each protoplast, so that only two gametes are produced per gametangium. These detach from their parental thecae and reposition themselves within the gametangium to lie on either side of the median transapical plane (Figs 31 left, 34). One gamete from each gametangium then moves through a narrow copulation aperture (illustrated by Mann 1984a, figs 9–15, 28) into the other gametangium to fuse with the passive gamete remaining there. Consequently, the two ellipsoidal zygotes lie entirely within the two gametangia (Figs 31–33, 35). They then expand parallel to the long axes of the gametangia, becoming cylinders with more or less hemispherical ends (Figs 38, 39). The auxospore diameter is apparently kept constant, and hence the mature auxospore is parallel-sided, at least in part because of the presence of hemiellipsoidal, silicified caps covering the tips of the expanding cell, which overlap the forming edge of the transverse perizonium (Figs 39, 40; Mann 1994a).

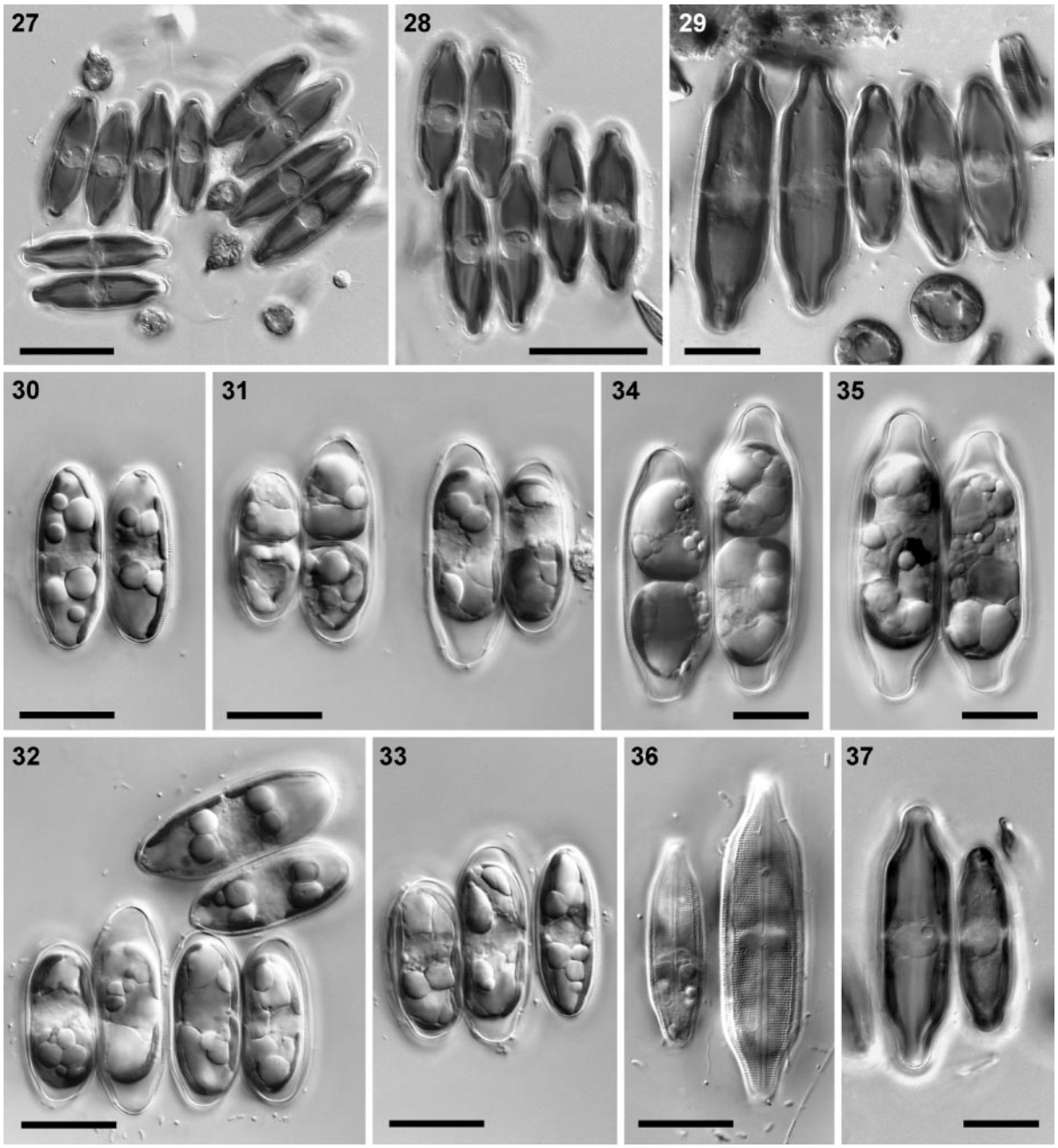
The process summarized above has been observed many times since 1984, and no revision of the basic account is necessary. However, some additions can be made. Occasionally, instead of pairs, unpaired ('singlet') cells have been found. We did not observe directly how such cells become sexualized. However, the pattern of bacterial colonization around such cells indicated that there had originally been a second cell alongside the remaining one, i.e. there had originally been normal copulation between two cells, but one of them subsequently reverted to the vegetative state and moved away. Triplets (Fig. 29) and larger groups of up to six bonded gametangia also occur, but only rarely, as shown by counts made in early 1996 (Table 2), when 97.4% of 6768 sexualized 'major' cells formed pairs, and 98.5% of 2085 'minor' cells. However, although triplets, quadruplets, and larger groupings are rare, there is evidence of mutual attraction among groups containing many sexualized cells, since pairs are frequently clustered. In Fig. 28, for example, the cells clearly form three pairs, but the apices of one pair interdigitate with those of another. It is also not unusual to find mixed clusters, containing both 'minor' pairs and 'major' pairs. Fig. 29 shows a case in which a single sexualized 'minor' cell (in meiotic prophase) lies between a 'major' pair in the earliest stages of differentiation and a 'minor' pair in meiotic prophase. Whereas the 'minor' pair have developed normally, with their nuclei adjacent, the superfluous 'minor' cell, sandwiched between the 'major' and 'minor' pairs, has a central nucleus. However, in such cases, although the 'major' and 'minor' pairs are closely associated, no bonding appears to have occurred between them. Triplets have never been observed to produce more than two auxospores. The third gametangium either reverts to the vegetative state (cf. Fig. 33) or produces gametes that subsequently abort. No cases were found of multiple fusion of gametes to form triploids or tetraploids.

In the 4384 pairs, triplets, and larger groups of gametangia listed in Table 2, pairing was exclusively 'major'–'major' or 'minor'–'minor'. Several hundred further 'legitimate' pairs were counted in January and February 1996 and thousands more have been seen during periodic surveys of Blackford epipelon at intervals over 25 years. Observation was particularly intense in 1987–1989 (part of this sequence is given in Table 1) and in early 1998. At various times, all three demes were observed pairing and often two were sexual at the same time (e.g. Table 1). Sometimes one deme was extremely rare and greatly outnumbered by one or both of the other demes. Nevertheless, no interbreeding was found. In early 1996, a thorough search was made for mixed pairs ('minor' × 'major') during a period when both *N. ampliatum* demes were unusually common in Blackford epipelon. Three such pairs were found among over 4700 studied. In one, the cells disengaged before proceeding to meiosis. In the other two (Figs 36, 37), the gametangia underwent meiosis and formed gametes, but no hybrid zygote was produced.

The initial cells of both *ampliatum* demes have linear valves (sometimes very slightly expanded at the centre) with rounded poles, contrasting with the broadly rostrate poles of shorter *ampliatum* cells (Figs 41–43, contrast Figs 1–12). We followed subsequent shape changes in cultured material. Rostrate poles (Figs 45, 46) develop very quickly, during the first divisions of the initial cell, even during the first division (Fig. 45), through strong contraction of the subapical region (Fig. 44).

Life cycle metrics and mate choice in seminatural populations

Mann (1989b) gave the size of 'major' gametangia as 62–84 μm (44 measurements), for 'minor' as 34–66 μm (510 measurements), and for *iridis*, 89–120 μm (6 measurements); all measurements were from Blackford Pond material. A more extensive survey of 'major' gametangia from Blackford on 19 January 1996 (294 observations) gave a range of 59–89 μm and the proportion of 'major' cells becoming sexualized was greater among small cells (Fig. 47). No larger 'major' gametangia have been found at any time. The vast majority of cells on 19 January 1996 (Fig. 47) were clearly below the sexual size threshold, but those above 90 μm were probably



Figs 27–37. *Neidium ampliatum:* sexual reproduction. Differential interference contrast (DIC) optics. Scale bars = 50 μm (Figs 27, 28) or 20 μm (Figs 29–37).

Figs 27, 28. Clusters of five and three pairs of the 'major' deme in a seminatural population from Blackford Pond. The gametangia are at various stages of meiotic prophase, mostly with expanded spherical nuclei.

Fig. 29. Pairs of the 'major' (left) and 'minor' demes, with a superfluous 'minor' cell between; from mixed seminatural populations.

Figs 30–33. Mixed cultures of 'minor' clones 11 and BLA13.

Fig. 30. Clone 11 × 11 pair at meiosis II.

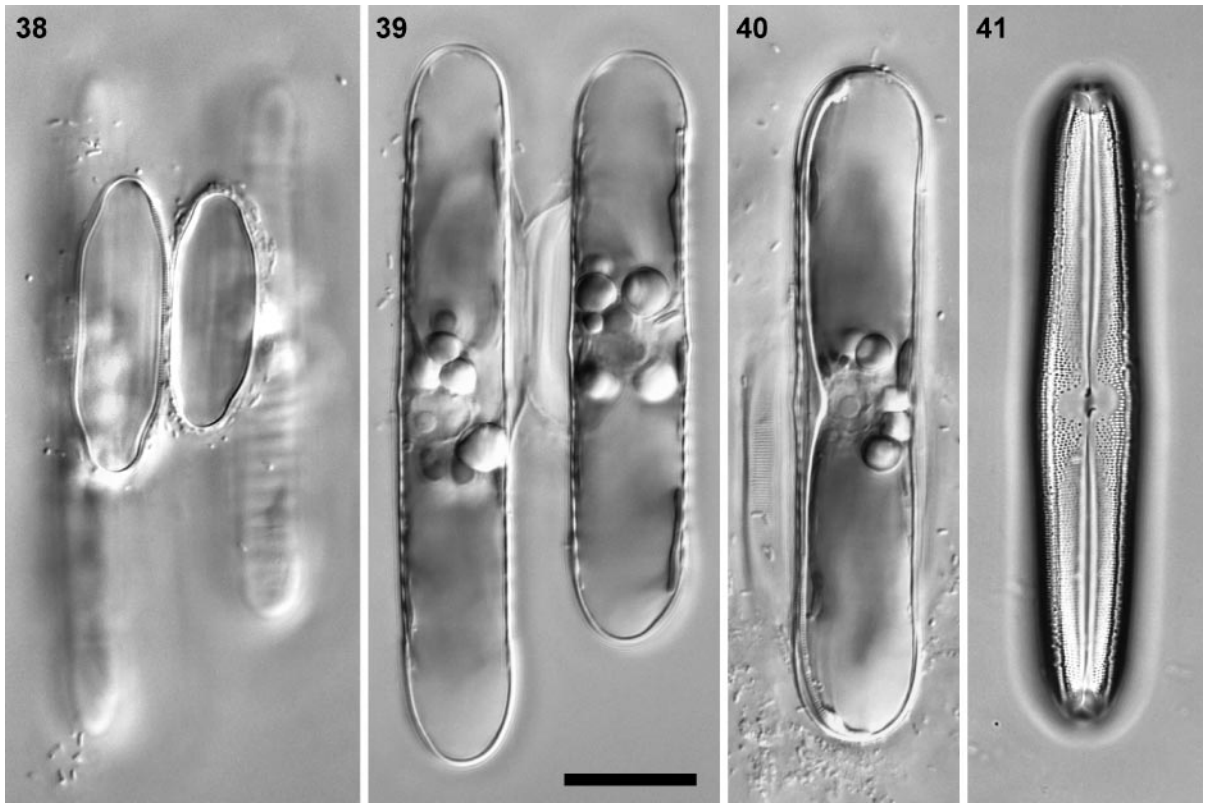
Fig. 31. Two adjacent 11 × BLA13 pairs: gametangia with rearranged gametes (left) and ellipsoidal zygotes (right, following plasmogamy).

Fig. 32. Cluster of two 11 × BLA13 pairs and one 11 × 11 pair (bottom right). The two lower pairs have already formed zygotes; the other is at meiosis II.

Fig. 33. Triplet containing two clone 11 cells and one BLA13 cell (centre). The right-hand 11 cell has reverted to being vegetative; the other two cells have completed plasmogamy and contain ellipsoidal zygotes.

Figs 34, 35. Mixed cultures of 'major' clones BLA8 (larger cells) and BLA9: BLA8 × BLA9 pair with rearranged gametes (Fig. 34) and pair after plasmogamy, with ellipsoidal zygotes (Fig. 35).

Figs 36, 37. Interdeme pairing in seminatural populations in January 1996: a focus on the gametangial valves during late gametogenesis (Fig. 36) [a lower focus, showing the rearranged gametes, was published by Mann (1999), fig. 67], and a pair in meiotic prophase (Fig. 37).



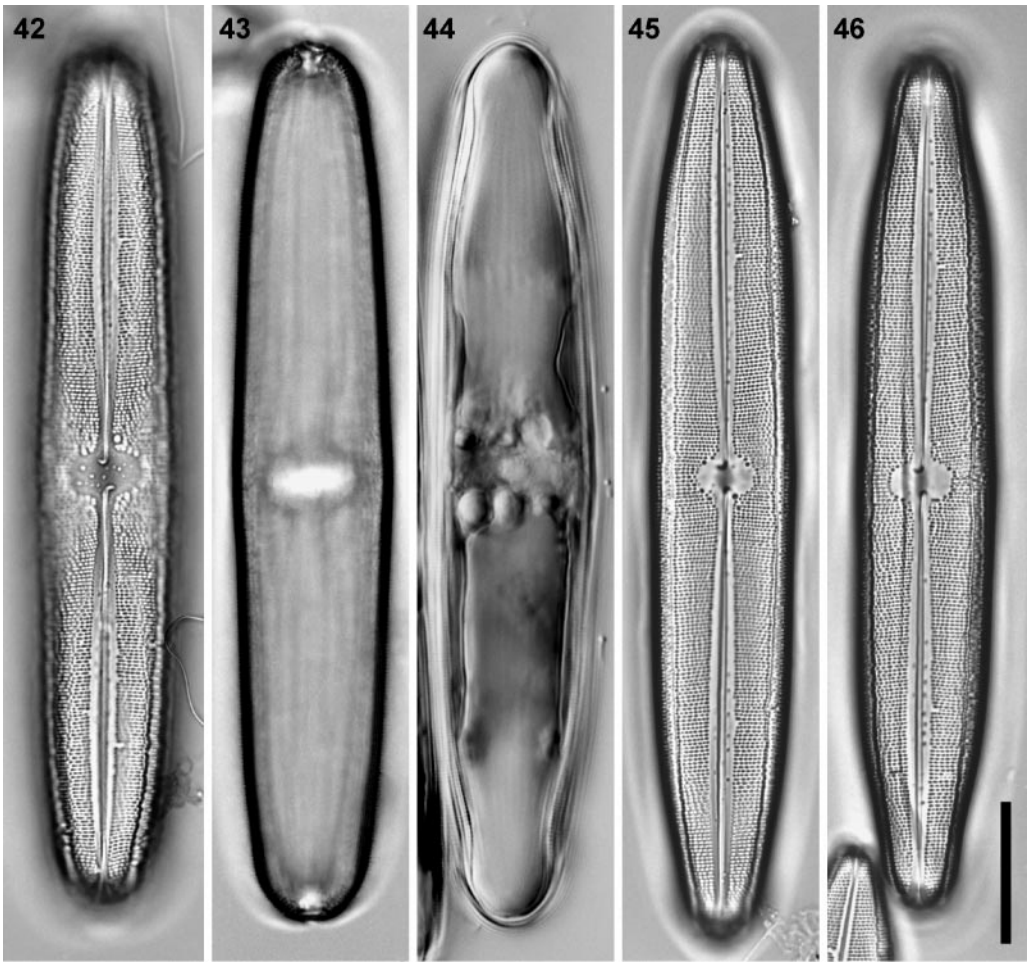
Figs 38–41. *Neidium ampliatum*, 'minor' deme. Scale bar = 20 μm.
Figs 38, 39. Mixed culture of clones 11 and BLA13. A pair during auxospore expansion, showing the gametangial thecae (Fig. 38: the clone 11 cells are smaller) and an optical section of the auxospores (Fig. 39). In the left auxospore, the large fusion nucleus is visible. DIC optics.
Fig. 40. Fully expanded auxospore from a clone 11 × BLA13 cross; the initial epivalve is present (in section at left). DIC optics.
Fig. 41. Initial valve from December 1983 seminatural population. Bright field optics.

not. The sexual size threshold for the 'major' deme is therefore probably *c*. 90 μm. The smallest 'major' gametangium observed so far was 41 μm (preserved material from 18 March 1988). Initial cells of 'major' were 121–148 μm (N = 64), of minor 94–111 μm (N = 233); *iridis* initial cells were not measured. Cells similar to 'major' from Blackford, but from Balerno Millgate, had gametangia of 54–78 μm (N = 44) and initial cells of 123–139 μm (N = 25).

Slides of preserved material from 24 September 1984 allowed investigation of mating choice within the 'minor' deme in a seminatural population and also the relationship between initial cell length and gametangium length. The distribution of gametangium lengths was normal (Fig. 48), with no evidence of size classes resulting from episodic auxosporulation [and tests for kurtosis revealed none: Snedecor & Cochran (1980)]. Analysis of variance among and within 249 pairs showed that gametangia had apparently exhibited size preference during mating: cells tended to mate with cells of similar size ($F_{248,249}$ = 2.44, $P \ll 0.001$). In much smaller data sets for 'minor' from 19 January 1996 (18 pairs) and 25 March 1996 (13 pairs), any preference was not significant ($F_{17,18}$ = 1.75, P = 0.12; $F_{12,13}$ = 2.20, P = 0.09, respectively).

In the 24 September 1984 material, there was a weak correlation (r^2 = 0.167, N = 126; Fig. 49) between the length of the initial cell and the length of the gametangium in which it was formed (the 'mother' gametangium) [initial cell length (μm) = 89.5 + (0.24 × mother gametangium length)]. Because *N. ampliatum* is allogamous, both gametangia contribute to each zygote and so we also investigated the influence on initial cell length of the ('paternal') gametangium that provided the active gamete. Multiple linear regression revealed a significant effect of the 'mother' gametangium but not of the 'paternal' gametangium [initial cell length (μm) = 91.3 + (0.257 × mother gametangium length) − (0.056 × father gametangium length); $F_{2,123}$ for regression vs error = 13.06, $P \ll 0.001$; for the contribution of the paternal gametangium, $F_{1,123}$ = 1.16, P = 0.72; for the contribution of the maternal gametangium, $F_{1,123}$ = 24.96, $P \ll 0.001$)]. Finally, the 24 September 1984 data set was partitioned into those pairs in which at least one initial cell had already formed and those that were at earlier stages of auxospore development, with no initial cells. Analysis of variance of gametangium length showed a clear difference between these two categories ($F_{1,496}$ = 84.14, $P \ll 0.001$). Mean gametangium length was lower in pairs that had formed at least one initial cell than in as yet unproductive pairs (48.97, as against 54.03 μm); variance of gametangium length was higher in unproductive pairs, though perhaps not significantly ($F_{246,252}$ = 0.061, P = 0.94).

In a population of the 'major' deme studied in January 1996, there was no evidence of mating preference: no significant difference was found in gametangium length among and within 143 pairs, triplets, and larger groups of gametangia ($F_{142,150}$ = 1.06, P = 0.36).



Figs 42–46. *Neidium ampliatum,* 'major' deme. Bright field optics (except Fig. 44). Scale bar, 20 μm.
Figs 42, 43. Initial valve produced intraclonally in BLA9 culture: valve face (Fig. 42) and outline (Fig. 43).
Fig. 44. Cell formed by the first division of the initial cell, in median focus, in a BLA8 × BLA9 cross. Note that the new theca, seen in section within the linear–elliptical initial cell, has rostrate poles.
Figs 45, 46. Early (Fig. 45) and later (Fig. 46) postauxospore valves with increasingly rostrate poles, produced following homothallic reproduction in BLA9 cultures.

Crossing experiments

Seven clones of each of the 'minor' and 'major' demes were used for crossing experiments (Table 3; voucher specimens exist for most clones and see Figs 13–26), though only a minority of the mating combinations were possible because the clones were not all contemporary.

No auxosporulation occurred in any 'minor' clone when grown alone (Table 4). Five of the 'major' clones remained vegetative in monoclonal culture, but in BLA7 there was rare intraclonal auxosporulation and BLA9 (Figs 25, 26) exhibited prolific intraclonal auxosporulation and produced viable initial cells, which measured 121.5–143.5 μm (mean 134.65, s = 5.63, n = 20); this agrees with the range of measurements from seminatural populations (see above). Judging by data from seminatural populations (above), BLA8 was close to the sexual size limit when crossed (*c.* 89 μm). In 2003, we grew one *iridis* clone that was within the sexual size range. This showed no intraclonal auxosporulation.

Interclonal crosses among five 'minor' clones revealed two mating types, one represented by clone 11 (Fig. 14), the other by clones 4, 44 (Figs 15, 16), BLA2 (Fig. 17), and BLA13 (Fig. 18). There was no obvious difference in morphology between clone 11 and the four clones of opposite mating type. When clones of the same mating type were grown together, cells did not interact and there was a more or less homogeneous spread of single and recently divided cells (Fig. 50). In mixtures of roughly equal numbers of cells of two compatible clones, however, almost all cells were paired within 2–3 days (Fig. 51) and thereafter formed auxospores. Where two compatible clones were distinguishable on the basis of size, e.g. in 11 × 44 or 11 × BLA13 (Table 3), each pair of gametangia usually contained one cell of each clone (e.g. Figs 31, 32). However, in 11 × BLA13, some pairs were found with both cells *c.* 40 μm long (Figs 30, 32), which must represent 11 × 11 (Table 3). In September 1998, the 'minor' clone 44 was at the lower limit of gametangium size seen in natural populations (34 μm). Nevertheless, sexual reproduction was vigorous in crosses with clone 11.

The 'major' deme behaves similarly. In a first set of crosses, BM 26 (Fig. 21) and BS 59 (Figs 22, 23) had the same mating type, whereas BS53 had another (Table 4). In a second set, BLA7 and BLA8 (Fig. 24) were unable to mate with each

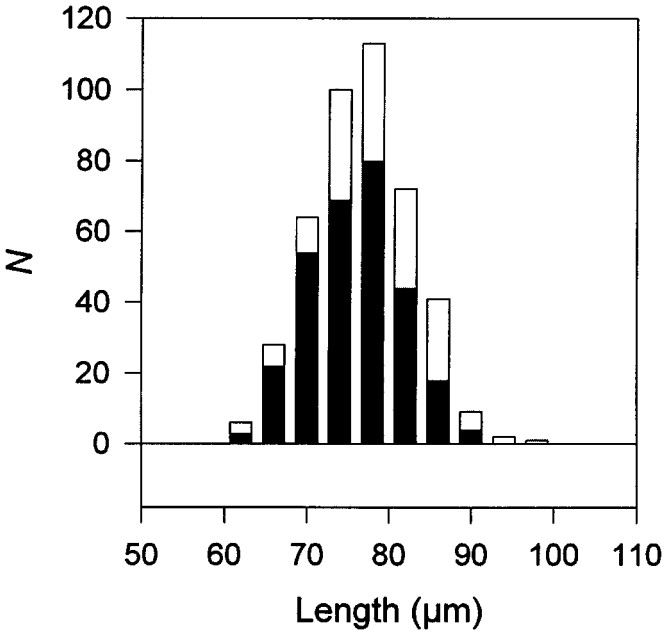


Fig. 47. Distribution of cell lengths among 436 gametangia (black) and vegetative cells (white) in a seminatural populations sampled on 19 January 1996. Note the increasing proportion of sexualized cells among smaller cells.

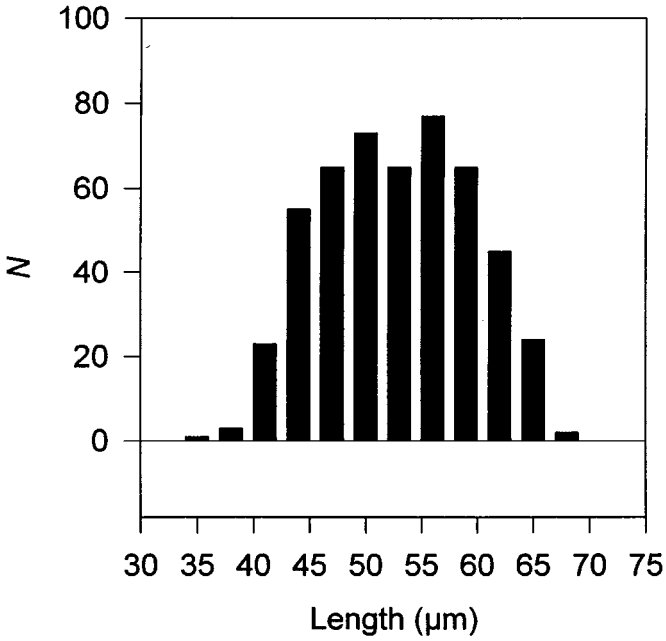


Fig. 48. Distribution of gametangium lengths among 249 pairs (498 gametangia) in a seminatural population fixed and preserved on 24 September 1984.

other, but BLA8 could mate with BLA9 (Figs 25, 26) (Table 4) and in this cross cells could be distinguished on the basis of size (Table 3): pairs were either BLA8 × BLA9 (Figs 34, 35) or BLA9 × BLA9 (intraclonal mating occurred in BLA9 whether or not another compatible clone was present). Because of similarities in cell size between BLA7 and BLA9 (Table 3) and the presence of intraclonal mating in BLA9, the results of the BLA7 × BLA9 cross could not be interpreted.

Attempts were made to cross 'minor' and 'major' clones. The BS32 'minor' clone would not interbreed with either mating type of the 'major' deme, and no mating was found between any of the 'minor' clones 4, 11, 44 BLA2, and BLA13 and any of the 'major' clones BLA7, BLA8, and BLA9 (Table 4).

DISCUSSION

Life cycle of *Neidium*

All three Blackford demes exhibit a size reduction–restitution cycle, as in most diatoms (Edlund & Stoermer 1997; Chepurnov *et al.* 2004), though not all (e.g. Wiedling 1943, 1948). All are also allogamous, producing two gametes per gametangium and hence (apart from infrequent cases of abortion) two auxospores per pair. In the 'minor' and 'major' demes, as in many other pennate diatoms (e.g. Geitler 1932; Tropper 1975), valve shape changes during the life cycle as size decreases. It is well known that (e.g. as summarized by Round *et al.* 1990, p. 84) in the middle and late stages of size reduction in pennate diatoms, valve shape generally simplifies and approaches elliptical (particularly well illustrated by Tropper 1975, figs 2–16, 21). The earliest stages of shape change (immediately after auxosporulation), on the other hand, are less well documented, because auxosporulation and postauxospore cells are rarely observed. However, it seems to be not uncommon that the largest cells also have a simplified morphology, like the smallest. Thus, initial cells may lack features characteristic of the vegetative cells, like rostrate or capitate apices or undulate margins. We have demonstrated this in the 'major' and 'minor' demes of *N. ampliatum*, and it is also evident in, e.g. *Eunotia tropica* Hustedt (Idei 1993: the undulate margins are not initially present) and *Cymatopleura solea* (Brébisson & Godey) W. Smith (Mann 1987: the central waist is poorly developed in the initial cell). We were surprised, however, by the rapidity with which rostrate apices are developed in *Neidium* (appearing within a few cell generations from the initial cell), because most shape changes in diatoms are gradual (e.g. Mann 1994a). How the transformation is achieved is unclear. Contraction of the protoplast may occur during valve formation (cf. contraction of the gametes within the gametangial thecae: Fig. 34), or perhaps there is localized secretion of polysaccharide within the frustule at the poles. Besides their simpler outline, initial cells often differ from the normal vegetative cells in other respects, such as having a curved cross-section, or a deviant polarity or striation pattern (e.g. Mann 1984b); *Cymatopleura* initial valves lack undulations (Mann 1987) and *Caloneis* and *Amphora* initial epivalves lack a raphe (Geitler 1969; Mann 1989a).

As elsewhere, e.g. in the *Sellaphora pupula* (Kützing) Mereschkowsky complex (Mann *et al.* 1999), *Neidium* produces smaller cells in culture than have been observed in nature and also 'unnatural' shapes. This anomaly presumably arises because sexual reproduction usually intervenes before the smallest viable cells can be produced in natural populations. However, there is also an anomaly with respect to the early part of the life cycle, in that large cells – the inevitable consequence of obligatory sexual reproduction and auxosporulation – are also rare or apparently absent in natural populations. Our new data expand the size ranges for gametangia and ini-

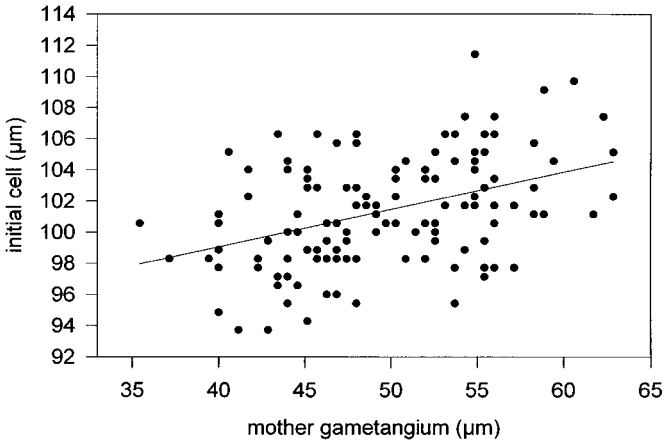
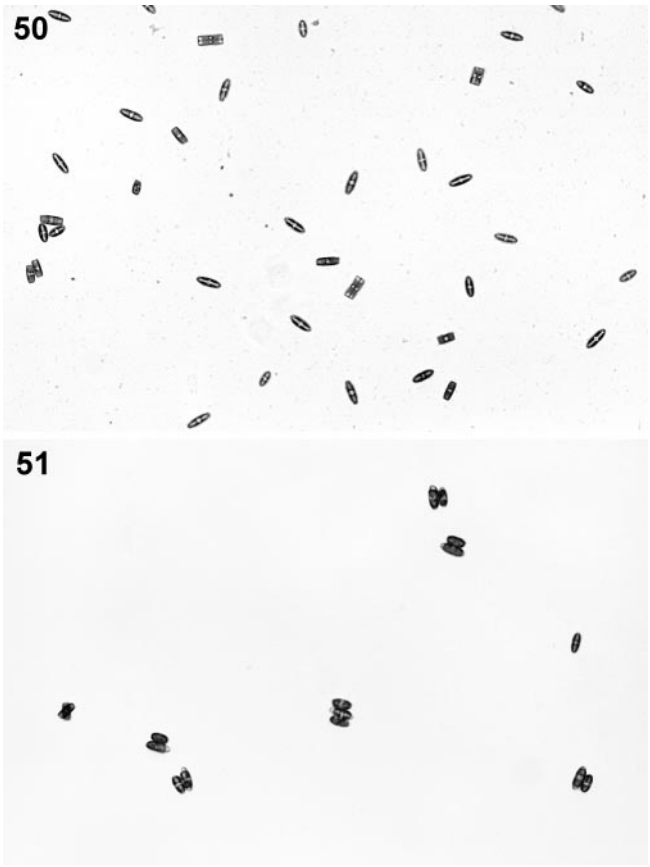


Fig. 49. Plot of initial cell length *vs* the length of the gametangium in which the initial cell was formed: seminatural population fixed and preserved on 24 September 1984. Note that the range of length is far less among initial cells than among gametangia.

tial cells to 34–66 and 94–111 µm, respectively, for the 'minor' deme and 41–89 and 121–148 µm for the 'major' deme, but for both demes, the largest vegetative cells (> 80 µm in 'minor' and > 100 µm in 'major') have scarcely ever been observed in nature during more than 20 years' observations. Possible reasons for this dearth of large cells have been given by Mann (1988, pp. 404–5) and involve the effects of the costs of sex on population structure. These include the cost of signalling and active pairing, interruption of synthesis and cell division during meiosis, plasmogamy and auxospore development (Lewis 1983), the extra costs of auxosporulation (e.g. in perizonium development, acytokinetic mitoses, and formation of new thecae), and wastage and abortion of gametes and gametangia. All of these will tend to reduce the growth of a sexual, auxosporulating population relative to any contemporary, sympatric population – of the same or different species – that remains asexual and does not auxosporulate, and such costs must be set against any advantage of sexual reproduction. Now that the mating system and sexual size thresholds are known for *N. ampliatum*, members of the *S. pupula* complex, and several other diatom species, it should be possible to quantify these costs directly, by experiments involving mixtures of clones within and outwith the sexual size range. The current study shows, however, that one of these costs – wastage through superfluous gametangia – is quite small. Thus, if we assume that quadruplets and sextuplets usually resolve into two or three pairs, as observed, and that wastage therefore occurs only in triplets and quintuplets, the data in Table 2 show that only a tiny proportion of gametangia – 34 out of 6768 'major' gametangia and 9 out of 2085 'minor' gametangia (0.50% and 0.43%, respectively) – will be lost.

In cultures, a nongenetic dependence of initial cell size on parental cell size has been shown to occur in some centric (e.g. Migita 1967; Roshchin 1973, 1976; Nagai *et al.* 1995) and pennate (e.g. Roshchin 1990, 1994; Davidovich 1994; Mann *et al.* 1999) diatoms. The seminatural population of *N. ampliatum* 'minor' that we analysed in detail showed a very weak correlation between the sizes of gametangia and initial cells, which was unexpected because in this case (in contrast to the culture-based studies of clones mentioned above) there



Figs 50, 51. *Neidium ampliatum,* 'minor' deme: mixed cultures of two clones, in which the clones are distinguishable on the basis of cell length.

Fig. 50. Incompatible combination: clone 4 (smaller cells) × BLA13, 3 days after mixing clones. The cells of both clones are irregularly distributed and unpaired.

Fig. 51. Compatible combination: clone 11 (smaller cells) × BLA13, 2 days after mixing clones. Almost all cells were paired.

is the possibility of heritable variation as well as life cycle influence. The regression relation for the 'minor' deme suggests that a near doubling of gametangium length, from 35 to 65 µm, is accompanied on average by an increase in initial cell length only from 98 to 105 µm. Thus, initial cell size is well, though not fully, buffered against variation in gametangium size. Consequently, Geitler's (1932) idea that the 'cardinal points' within the life cycle (initial cell size, upper sexual size threshold, minimum viable size) can be used to characterize species or races is valid for this species complex. Elsewhere, however, more caution is necessary, as noted by Edlund & Bixby (2001), who have recently reviewed most of the relevant data, including several sets of their own new observations. They found that the sizes of gametangia and initial cells are generally correlated, making interpretation of the 'cardinal points' difficult if they are determined from natural populations with no information about genetic variation. Only in a few cases, e.g. one population of *Aulacoseira islandica* ssp. *helvetica* (O. Müller) Simonsen (Edlund & Bixby 2001, data of Bethge) and *Coscinodiscus granii* Gough (Schmid 1995), was there apparently no relationship between the sizes of gametangia and initial cells.

Each zygote results from the fusion of a passive gamete

Table 1. *Neidium* in Blackford Pond: observations of vegetative and sexualized cells in seminatural populations incubated during 1.5 years, from autumn 1987.[1,2]

	Vegetative				Sexualized			
Date	Undifferentiated *ampliatum*	'major'	'minor'	*iridis*	Undifferentiated *ampliatum*	'major'	'minor'	*iridis*
5 Oct. 1987	+	n/a	n/a	−	+	n/a	n/a	−
19 Oct. 1987	n/a	+	+	−	n/a	+	+	−
2 Nov. 1987	+	n/a	n/a	−	n/a	+	+	−
17 Nov. 1987	n/a	+	+	−	n/a	+	+	−
27 Nov. 1987	n/a	−	−	−	n/a	−	−	−
16 Dec. 1987	+	n/a	n/a	−	−	n/a	n/a	+[3]
22 Dec. 1987	−	n/a	n/a	−	−	n/a	n/a	−
27 Jan. 1988	−	n/a	n/a	−	n/a	+	−	−
5 Feb. 1988	+	n/a	n/a	+	−	n/a	n/a	−
22 Feb. 1988	−	n/a	n/a	−	−	n/a	n/a	−
4 Mar. 1988	−	n/a	n/a	−	−	n/a	n/a	−
17 Mar. 1988	+	n/a	n/a	+	n/a	+	−	−
18 Apr. 1988	n/a	+	+	−	n/a	+	−	−
13 May 1988	+	n/a	n/a	−	n/a	+	−	−
6 May 1988	n/a	+	−	−	n/a	−	−	−
24 June 1988	n/a	+	+	−	n/a	−	−	−
5 Aug. 1988	n/a	+	+	+	n/a	−	−	−
19 Aug. 1988	n/a	+	+	+	n/a	−	−	−
12 Sep. 1988	n/a	+	−	−	n/a	−	−	−
26 Sep. 1988	n/a	+	+	+	n/a	+	−	−
10 Oct. 1988	n/a	+	+	−	n/a	+	−	+[3]
7 Nov. 1988	n/a	+	+	+	n/a	+	−	−
29 Nov. 1988	n/a	+	−	+	n/a	+	−	−
11 Jan. 1989	n/a	+	−	−	n/a	+	−	+[3]
1 Feb. 1989	n/a	+	−	−	n/a	+	−	−
13 Mar. 1989	n/a	+	+	−	n/a	+	+	−
21 Apr. 1989	n/a	+	−	−	n/a	−	−	−

[1] n/a means that a category is inapplicable either because *N. ampliatum* was left undifferentiated, or because observations for the 'major' and 'minor' demes were kept separate.

[2] The results represent a rapid survey of a single 24 × 50 mm cover-slip used to harvest mixed epipelon from a mud sample after a few (generally *c.* 5) days. The data are not quantitative or exhaustive but indicate that sexualizable cells are often present and provide context for the claim that demes do not interbreed.

[3] On these occasions, rare pairs of *iridis* were observed but no vegetative cells were noted.

that remains within the gametangium that produced it and an active gamete, which migrates in from the other gametangium. Given the nongenetic effect of gametangium size on initial cell size that has been documented by various authors (see above), we expected that the slight dependence of initial cell size on gametangium size would be apportionable more or less equally to both gametangia, because the active and passive gametes produced by each gametangium are indistinguishable in size, shape, and structure. Instead, we found that only the 'mother' gametangium seemed to have any effect. It is possible that this is a genetically controlled characteristic of development in *Neidium.* Alternatively, the cause may be physical. The gametangia of *Neidium* do not dehisce fully (plasmogamy occurs via narrow copulation apertures), and the newly formed zygotes mature within the 'maternal' gametangium, during which they become ellipsoidal and surround themselves by a heavily silicified, bipartite wall; then bipolar expansion begins (Figs 31–33, 35, 38–40; Mann 1984a). The size of the mature zygote is thus partially dependent on the size of the maternal gametangium and so, unless auxospores are able to 'measure' absolute size, which is made very unlikely by Davidovich's (1994, 1998) observations of other raphid diatoms, there will be a knock-on effect, albeit slight, on the initial cell. Further progress in understanding how the relationship between initial cell size and the size of the parental cell is controlled – and in understanding why this relationship varies within and among different species – will be helped if analysis is extended beyond linear dimensions, to cover parameters such as the volumes of the gametangia, gametes, zygotes, and mature auxospores and the numbers and morphologies of the properizonial or perizonial elements.

Mating and mating system

Our data show that there are mechanisms preventing or restricting inbreeding in *N. ampliatum.* The 'minor' deme is heterothallic, and intraclonal auxosporulation occurred only in a mixed culture of compatible mating types where sexual activity was intense; it never occurred in monoclonal cultures. Unpaired 'singlet' gametangia are unable to produce auxospores, in contrast to the uniparental auxosporulation in, e.g. *Achnanthes* cf. *subsessilis* Kützing (Sabbe *et al.* 2004b). In the 'major' deme too, complementary mating types are present, but two clones also showed sexual reproduction in monoclonal culture. In BLA9, this was vigorous and produced initial cells more or less equal in size to those produced by interclonal pairs; the inbred initial cells grew and divided normally.

Heterothally has now been detected in several raphid diatom genera, representing widely divergent morphological

Table 2. *Neidium ampliatum.* Numbers of pairs and larger groupings of gametangia in seminatural populations from Blackford Pond.

Deme	Observation date	Pairs	Triplets	Quadruplets	Quintuplets	Sextuplets
'major'[1]	19 Jan. 1996	284	1	2	0	1
'major'	25 Jan. 1996	107	5	2	0	0
'major'	26 Jan. 1996	797	14	7	1	0
'major'	26 Jan. 1996	820	6	5	0	0
'major'	29 Jan. 1996	935	4	1	0	0
'major'	16 Feb. 1996	184	3	0	0	0
'major'	19 Feb. 1996	168	0	0	0	0
'minor'[1]	19 Jan. 1996	47	0	0	0	0
'minor'	25 Jan. 1996	32	0	0	0	0
'minor'	26 Jan. 1996	243	4	1	0	0
'minor'	26 Jan. 1996	207	2	0	0	0
'minor'	29 Jan. 1996	334	2	0	0	0
'minor'	16 Feb. 1996	63	1	0	0	0
'minor'	19 Feb. 1996	101	0	0	0	0

[1] Also two singlets.

types, e.g. *Eunotia, Neidium, Haslea* Simonsen, *Sellaphora, Seminavis, Amphora* Ehrenberg *ex* Kützing, *Nitzschia* Hassall and *Pseudo-nitzschia* H. Peragallo *in* H. Peragallo & Peragallo. In *Sellaphora* (Mann *et al.* 1999), *Nitzschia longissima* (Brébisson *ex* Kützing) Grunow (Chepurnov *in* Roshchin 1994), and *Pseudo-nitzschia* (Davidovich & Bates 1998; Chepurnov *et al.* 2004), the gametangia are differentiated, one producing an active gamete or gametes, the other passive gamete(s). Elsewhere among heterothallic diatoms, however, the gametangia cannot be distinguished from each other through any morphological or behavioural difference in the gametes they produce. *Eunotia* cf. *bilunaris* (Ehrenberg) Mills (Mann *et al.* 2003), *Haslea subagnita* (Proshkina-Lavrenko) Makarova & Karayeva (Chepurnov 1993), *Seminavis* cf. *robusta* Danielidis & D.G. Mann (Chepurnov *et al.* 2002), and *Amphora* cf. *proteus* Gregory (Sabbe *et al.* 2004a) are all isogamous, while in *Neidium* and *Nitzschia lanceolata* W. Smith (Roshchin 1990, 1994), heterothally is combined with *trans* behavioural anisogamy, i.e. each gametangium produces one active and one passive gamete. Clearly, gamete behaviour and mating system do not always evolve in tandem.

The incomplete heterothallism of *N. ampliatum* is paralleled in other species. *Nitzschia lanceolata,* though predominantly heterothallic, can also reproduce in monoclonal cultures (Roshchin 1990, 1994), and in *A. longipes* (see Introduction) some clones are unisexual and show only the slightest capacity for intraclonal reproduction, whereas other clones exhibit frequent intraclonal sex, although repeated inbreeding rapidly leads to a loss of vitality (Chepurnov & Mann 1999).

Currently, it is difficult to detect evolutionary trends in pennate diatoms with respect to heterothally vs homothally. Most araphid pennate diatoms that have been studied have proved to be heterothallic, with a clear differentiation of the gametangia into 'male' (producing active gametes) and 'female' (producing passive gametes) (Chepurnov & Mann 2004; Chepurnov *et al.* 2004). This suggests that the ancestral raphid diatoms were also heterothallic, because molecular data have confirmed what had already been suggested from morphological studies, that the raphid diatoms evolved from a lineage of araphid pennates (Kooistra *et al.* 2003; Medlin & Kaczmarska 2004). However, the presence of homothally in such divergent lineages as *Eunotia, Nitzschia,* and *Gomphonema,* and the presence of homothallic and heterothallic demes within the same species complex (e.g. Mann *et al.* 2004; Chepurnov *et al.* 2004), suggests that mating systems have been particularly labile during the evolution of the raphid diatoms.

The restriction of the formation of triplets (or quintuplets) to low levels in *N. ampliatum* must reflect the presence of a very effective mechanism that usually prevents extra compatible cells from joining an existing pair or leads to disengagement of superfluous cells. Presumably bonding between compatible cells is followed quickly by biochemical alteration to remove or deactivate surface molecules involved in cell–cell recognition. Our observations of sexual reproduction in culture, where almost all cells can be paired within 2 days of mixing compatible clones (Fig. 51), and the presence of large clusters of gametangia in seminatural populations (Figs 27–29) are consistent with an earlier suggestion (Mann 1984a) that long-distance signalling between compatible cells is achieved through a diffusible attractant. Alternatively, cells may find mates by following the trails that it has been shown (e.g. Edgar & Pickett-Heaps 1984; Higgins *et al.* 2003) are deposited from the raphe during movement. Some video footage of vegetative cells of *Achnanthes* Bory by Pickett-Heaps & Pickett-Heaps (2003) suggest strongly that cells do indeed trail each other. Mating through random encounters between compatible cells is inadequate to explain groups like those shown in Figs 27–29, 32. The formation of mixed clusters of 'minor' and 'major' pairs (Fig. 29) suggests that the attractant, whether diffusible or a trail component, is similar in both demes.

In heterothallic *Sellaphora,* where clones can be classified as 'male' or 'female' according to whether the gametangia produce active or passive gametes, Mann *et al.* (1999) reported preferential polyandry (a significant excess of male–female–male triplets) and suggested that this causes the observed larger mean size of male gametangia in this species (unfortunately, it is not possible to distinguish the two mating types of *Neidium* visually, and so the frequency and average cell size of the mating types cannot yet be estimated in natural populations). However, they did not offer an explanation of how polyandry is brought about. If, as suggested above, sexualized raphid diatoms locate each other through a pheromone, it is unlikely that each mating type would both produce and detect the same pheromone (Dusenbery 2000, p. 9). Probably, therefore, only one of the mating types provides chem-

Table 3. *Neidium ampliatum* clones and voucher samples held at the Royal Botanic Garden Edinburgh (E).

Clone	Deme	Source	Voucher	Isolation date	Cell length on 5 Sep. 1998 (μm)[1]
BS32	minor	Blackford Pond	E3218	7 Feb. 1997	n/a[2]
BM26	major	Blackford Pond	E3131	7 Feb. 1997	n/a[2]
BS59	major	Blackford Pond	E3225	7 Feb. 1997	n/a[2]
BS53	major	Blackford Pond	E3222, E3223	7 Feb. 1997	n/a[2]
4	minor	Blackford Pond	E3198	2 Mar. 1998	40.30 ± 2.47
11	minor	Blackford Pond	E3141, E3199	2 Mar. 1998	41.40 ± 1.96
44	minor	Figgate Loch	E3142, E3200	2 Mar. 1998	34.35 ± 2.37
BLA2	minor	Blackford Pond	E3228	22 Apr. 1998	40.75 ± 1.99
BLA7	major	Blackford Pond	E3231	22 Apr. 1998	74.25 ± 0.98
BLA8	major	Blackford Pond	E3140, E3232	22 Apr. 1998	86.60 ± 2.46
BLA9	major	Blackford Pond	E3233	22 Apr. 1998	73.90 ± 1.71
BLA10	major	Blackford Pond	—	22 Apr. 1998	ND[3]
BLA11	major	Blackford Pond	—	22 Apr. 1998	ND[3]
BLA12	minor	Blackford Pond	—	22 Apr. 1998	ND[3]
BLA13	minor	Blackford Pond	E3234	22 Apr. 1998	50.20 ± 1.32

[1] Mean ± *s* (*N* = 10). measurements were made shortly before mating experiments.
[2] n/a, not applicable.
[3] ND, no data; clones were small enough to be within the sexual size range.

ical clues as to its location, while the other responds to this information through directed movement. Preferential polyandry in *Sellaphora* suggests that it is the female that produces attractant, although female cells continue to move (presumably nondirectionally) after sexualization, so that some polygynous triplets do occur. Time lapse observations or cinemicrophotography are needed to test these ideas.

The preference of 'minor' cells for cells of the same size is surprising. Analyses of *Sellaphora* (Mann *et al.* 1999, 2004) revealed no size selectivity in the strictly heterothallic *S. capitata,* but a tendency of cells to pair with cells of similar size in *Sellaphora blackfordensis* D.G. Mann & S. Droop, which we now know (unpublished observations) to contain some facultatively homothallic clones like *N. ampliatum* 'major', clones BLA7 and BLA9. Therefore, after discovering heterothally in *N. ampliatum,* we decided to reanalyse our earlier data on sexualized 'minor' and 'major' cells in seminatural populations. Because 'minor' clones did not inbreed in monoclonal culture, we expected to find no evidence of size selectivity in 'minor' cells in nature. On the other hand, because of the occurrence of intraclonal auxosporulation in BLA7 and BLA9, we suspected there might be a tendency for 'major' cells to mate with cells of the same size in seminatural populations, because recently divided sibling cells might remain close enough to each other after separating for them to be likely to mate with each other, rather than with other compatible cells. Our results showed instead that 'major' cells did not tend to mate with cells of the same size, whereas 'minor' cells did. The 'minor' deme thus shows the opposite type of behaviour to centric diatoms, in which compatible clones are likely to differ in size because the sex of each clone often changes during the life cycle as the cells reduce in size, being female at first (in larger cells just below the sexual size threshold), then hermaphrodite, then male in the smallest cells (summarized by Drebes 1977). (It should be noted that we kept clones 4, 11, and 44 for a long period and tested mating between them in April and September 1998, with the same result on both occasions, despite size reduction.)

It seems unlikely that *Neidium* cells discriminate between potential partners on the basis of size, choosing those most similar to themselves, because (1) there are so many exceptions, where paired cells differ considerably in size (the smallest gametangium measured among the 24 September 1984 'minor' gametangia was 34 μm long and this was paired with a 56 μm cell) and (2) there is no obvious mechanism by which a cell could measure the size of another cell. We suggest instead that selectivity comes about (among cells that are below the sexual size threshold) because smaller cells are more easily and more rapidly induced to become sexual than large cells; this is suggested by experimental studies in *Seminavis* (Chepurnov *et al.* 2002) and by the size spectra of sexualized and nonsexualized cells in seminatural populations, both in *Neidium* (Fig. 47) and *Sellaphora* (Mann *et al.* 1999, figs 12–14). This would explain our observation that the gametangia were on average smaller (in the 24 September 1984 data set) in pairs where auxosporulation was already complete, as opposed to those that were at earlier stages of auxosporulation. Possibly sexualization is autocatalytic, so that larger cells are only induced to pair when many other, mostly smaller, cells have already become sexual and are secreting pheromones.

We did not observe any polyploid auxospores in *Neidium,* in contrast to several other diatoms that have been studied, e.g. *Craticula* Grunow, *Achnanthes, Dickieia* Berkeley *ex* Kützing, or *Seminavis* D.G. Mann *in* Round, R.M. Crawford & D.G. Mann (Mann & Stickle 1991; Roshchin 1994; Mann 1994b; Chepurnov *et al.* 2002). This may be significant. The species in which polyploid auxospores occur frequently seem to be those in which plasmogamy takes place isogamously within a relatively undifferentiated capsule of mucilage. In *Neidium* and *Sellaphora,* by contrast, the gametes move through well-defined copulation canals or apertures and are always contained within the gametangial thecae, although these split slightly apart on the side where the gametangia touch (Mann 1984a, 1989c). Here, then, the process of plasmogamy is physically constrained, making it unlikely that multiple fusion will occur among the gametes from two gametangia and making it almost impossible for there to be multiple fusion among gametes in a triplet or larger group of sexualized cells.

Table 4. *Neidium ampliatum:* results of crosses.[1]

BS32	minor	–																				
BM26	major	–	–																			
BS53	major	–	++	–																		
BS59	major	–	–	++	–																	
4	minor	NT	NT	NT	NT	–																
11	minor	NT	NT	NT	NT	++	–															
44	minor	NT	NT	NT	NT	–	++	–														
BLA2	minor	NT	NT	NT	NT	–	++	–	–													
BLA7	major	NT	NT	NT	NT	–	–	–	–	+												
BLA8	major	NT	NT	NT	NT	–	–	–	–	–	–											
BLA9	major	NT	NT	NT	NT	–	–	–	–	?	++	++										
BLA11	major	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	–									
BLA12	minor	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	–								
BLA13	minor	NT	NT	NT	NT	–	++	–	–	–	–	–	NT	NT	–							
		BS32	**BM26**	**BS53**	**BS59**	**4**	**11**	**44**	**BLA2**	**BLA7**	**BLA8**	**BLA9**	**BLA11**	**BLA12**	**BLA13**							

[1] +, some sexual reproduction; ++, vigorous sexual reproduction; –, sexual reproduction absent; NT, cross not tried.

Reproductive isolation

Although the formation of mixed clusters of 'minor' and 'major' pairs in seminatural populations implies that any chemoattractants are similar in the two demes, bonding of gametangia is almost always between compatible cells of the same deme. We never observed any interdeme mating in mixed cultures, and the only three 'minor' × 'major' pairs ever found in seminatural populations did not produce offspring. There are therefore strong barriers to mating between the 'minor' and 'major' demes, and this amplifies and confirms earlier claims (Mann 1989b, 1999) that these two demes are separate biological species. However, the fact that the three abortive mispairings occurred at all suggests that there could perhaps be very rare genetic exchange between the two demes, though this clearly does not compromise the integrity of the demes, in terms of cell size, morphology, and normal mate preferences. No interaction was seen between either of the *ampliatum* demes and the *iridis* deme.

Demonstration of compatibility between clone 44, from Figgate Loch, with clone 11, from Blackford Pond, is a small step toward extending use of the biological species concept in *Neidium* from sympatric populations to allopatric populations. Unfortunately, *Neidium* clones reduce in size inexorably in culture and are not very tolerant of infrequent subculturing (unlike most demes and species in the *S. pupula* complex), so that clones have a limited life of a few months to a couple of years, depending on how far they are through the life cycle when they are isolated. Hence, any new study in the experimental taxonomy of *Neidium* should be short-term and intensive.

Two other strands of evidence suggest that the demes studied here may be worthy of species status. First, it appears that the demes are not restricted to the few ponds studied in detail here: we base this conclusion on the morphological similarity between valves of different provenances, shown by our illustrations (e.g. Figs 13–16), figures published by Krammer & Lange-Bertalot (1986), and studies of various eutrophic British lakes. Second, the demes persist sympatrically from year to year, without intergradation. Clearly, the case for recognizing the demes as separate species would be strengthened by more extensive mating data, involving isolates from more distant locations, and by molecular genetic studies, e.g. sequencing of genes that evolve suitably rapidly. Such studies are in progress. Intriguingly, however, there appears to have been a change in the morphology of the 'major' deme, from a linear–lanceolate outline in the 1980s to a more linear, sharply angled morphology (retained in culture) in the 1990s. Unfortunately, no material of the 1980s morph is available for molecular genetic analysis, and the 'major' deme was often rare during intensive sampling of Blackford Pond in the late 1980s, so that herbarium material does not record details of the transition between the linear–lanceolate and linear morphologies. Two explanations for the change can be put forward: (1) the morphologies represent the same interbreeding population and the morphological change represents a fluctuating or directional change in the phenotypic and/or genotypic composition of the population or (2) the linear–lanceolate and linear morphologies are different demes (partially or fully isolated reproductively), one replacing the other as the dominant deme post-1990. These possibilities are being investigated further.

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