Why didn't Lund see sex in *Asterionella*? A discussion of the diatom life cycle in nature

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Abstract: Size reduction has been studied in a natural population of *Nitzschia sigmoidea*. Several size classes are present at all times, giving a multimodal size distribution. The spacing of the modes, together with the rate at which the cells of each class decrease in size, indicates that the classes are year classes, representing annual periods of auxosporulation. The classes can be affected differentially by selection. Previous studies of the diatom life cycle in nature are reinterpreted on the basis of the *N. sigmoidea* evidence, with the conclusion that the cycle can last from 2 to 20, or perhaps 40 years. There is evidence that intermediate size classes can increase as a proportion of the total population when small-celled classes auxosporulate; a mechanism by which this could be brought about is discussed.

INTRODUCTION

My title may seem impertinent since Festschriften usually commemorate achievements, not omissions or errors. It is not meant to be. Asked about many other phycologists the question would be meaningless, but asked about John Lund it becomes an intriguing problem. *Asterionella* Hass. appears in some cases to undergo size reduction (Lozeron 1902, Wesenberg-Lund 1908: see later), but after years of investigations by limnologists worldwide, including of course Lund, with his classic studies of *A. formosa* Hass. (1949, 1950), we still do not know how, or indeed if, auxospore formation takes place. And this is not because *Asterionella* has been studied only after the customary boiling in acid. Millions of cells must have been examined and counted, either alive or fixed to preserve cell contents and colony form, yet there is still no confirmed report of auxospores in the genus.

The question can be broadened to other phycologists and other diatoms. Few people ever see sexual reproduction in diatoms, unless they work with and manipulate cultures. Geitler is an exception, many of his accounts of auxospore formation (listed 1932, 1973, 1984) being derived from collections of natural material; it is noticeable, however, that his studies are biased towards attached diatoms. In any case, the proportion of diatoms whose sexual proclivities are known is very small. Good information is available for less than 200 species, whereas the genus *Navicula* Bory alone currently contains nearly 2000 (Mann 1986b). This lack of data contrasts strikingly with the situation in some other groups of algae, in which sexual stages are routinely used in taxonomy (e.g. see Lund 1962). Several explanations are possible.

The infrequency of sexual reproduction among diatom species may be real, most diatoms being asexual. But even where auxosporulation (which almost always involves sexuality) has not been demonstrated directly, the variation in shape and size of the vegetative cells suggests that it does occur (e.g. Geitler 1932, Lund 1945). Perhaps, then, phycologists are unobservant or indolent. In my experience it is easy to overlook occasional paired gametangia and auxospores in populations of pennate diatoms, especially if one has no clear idea of how a particular species is likely to look and behave during auxospore formation. But if auxosporulation were frequent in *Asterionella*, at least in the plankton, it would surely have been noticed and studied by now. So maybe *Asterionella* forms auxospores not in the open water but in the littoral, or perhaps the very act of auxosporulation causes loss from the water column. This is indeed possible, since if the method of auxospore formation in *Asterionella* were similar to that of other araphid pennate diatoms that have been investigated (see Geitler 1973, Mann 1982), the stellate colonies would probably fall apart or be disrupted during gamete formation and fusion, which would certainly alter the pattern of sedimentation (Reynolds 1984). But this would not explain why auxospore formation is detected so rarely in benthic taxa.

Finally, it could be that the life cycle of many diatoms is so long in nature, compared to the period spent in auxospore formation and development, that the chance of observing sex is minuscule. Lewis (1984), indeed, has suggested that the primary significance of size reduction in diatoms is to act as a timer for sex, preventing it from occurring too frequently. Lewis's argument is based on the costs of sex in actively growing unicells (because of the interruption of synthesis: Lewis 1983), and the likelihood that "mechanisms could easily evolve by which it [size reduction] would be circumvented". I am unconvinced that size reduction is "easily" avoided, given the Bauplan of the diatom, the inextensibility of silica, and a requirement for a wall capable of maintaining its integrity and resisting the turgor of the cell it encloses, despite fluctuations in the physical and chemical characteristics of the environment. Nevertheless, there *are* costs associated with sex, which *may* impose selection for a long life cycle, and so for this reason, and to answer the question in my title, it is necessary to know how long the life cycle is. I have therefore studied size reduction in a large epipelic diatom, *Nitzschia sigmoidea* (Nitzsch) W. Smith, a species known to reproduce sexually (Geitler 1949, Mann 1986a). The results are used to help reinterpret previous studies of the diatom life cycle in nature.

MATERIAL AND METHODS

Nitzschia sigmoidea was studied from the silty sediments of Blackford Pond and Figgate Loch in Edinburgh. Samples were collected and prepared as described by Mann (1984, 1986a). 24×50 mm coverslips were removed from the tissue-covered mud after incubation for 1-2 days and heated in a muffle furnace to 550°C for 30 min to remove carbon. This left cleaned

preparations that could be mounted in Naphrax for light microscopy. The harvesting method selects for live diatoms, so that the size spectra are not biased by old dead frustules. Preservation was generally good after the furnace treatment; the few twisted or broken frustules were omitted from the analysis.

All sampling was done from the same part of the pond, and each time a sample of 1 litre of mud slurry was taken from an area of c. 25 m² by repeated filling of a glass tube. Sampling was performed by the same method, using the same glass tubing, on every occasion, and the slurry put into the same size of box to settle before the supernatant was removed and tissue and coverslips added; this method gives consistent results even on a semiquantitative basis (Round 1953). No attempt was made to restrict sampling to a well-defined, smaller area, since the process of sampling inevitably disturbs the sediment. The consistency of the results from one sampling to the next (Figs 2-6) implies that heterogeneities in the distribution of different sizes of N. sigmoidea in the pond sediments are not a significant problem.

Measurements of length, from pole to pole of the girdle, were made using the drawing attachment of a Reichert Polyvar photomicroscope (see also Mann 1988), and were accurate to c. 1 μm. Whenever possible 500 cells were measured for a particular sample and the distribution of lengths plotted as a histogram (e.g. Fig. 2). Unfortunately, N. sigmoidea was sometimes very rare, especially in summer, and then far fewer cells could be measured, even after examination of three or more 24×50 mm coverslips. Since it is essential that we be able to follow the fate of particular size classes, these less satisfactory sets of data have had to be presented; this has been done by the use of ranked plots (Figs 1, 5, 8), like those of Bailey-Watts and Kirika (1981) and Bailey-Watts (1986), who discuss their derivation and properties. Briefly, a normal distribution of length will produce a straight line relationship between rankit value and length, the line's slope reflecting the variance of the population. Skewed, leptokurtic or platykurtic (more peaked or less-peaked than a normal distribution) distributions will produce various types of curve, while distinctly bi- or multimodal distributions, such as those found here, lead to stepped curves. In my plots each 'riser' represents a size class, the 'treads' representing the transitions from one class to the next. It will be noted that the risers have approximately equal slope, suggesting that the different size classes are alike in their variance.

In Fig. 10 I have replotted Nipkow's (1927) data on size change in Tabellaria fenestrata (Lyngb.) Kütz., to facilitate explanation and discussion. The material analysed by Nipkow was a core from the Zürichsee, consisting of easily datable, annually laminated sediments. Nipkow measured 100 valves of Tabellaria at one or more horizons for each year between 1896 and 1923, except 1907, and plotted the results as a series of frequency polygons. I have extracted the data from these graphs and displayed them in histograms. A few of Nipkow's curves have been omitted where two or more were available for a particular year, in order that the time intervals between the histograms should as far as possible be equal. The graphs omitted cover the following periods: summer 1897 (the two 1897 plots in Fig. 10 are for spring and autumn;

the omitted summer curve is very similar to the autumn one), spring 1901 (very similar to the autumn 1901 distribution plotted), autumn 1909 (fairly similar to the spring 1910 plot included in Fig. 10), autumn 1910 (which already shows the trend towards smaller size evident in 1911), and spring 1923 (intermediate between the autumn 1922 and 1923 distributions plotted in Fig. 10).

RESULTS

1983 and 1984 (Figs. 1, 2)

In the first two samples, taken early in 1983 and in August of the same year, most of the cells are below $300\,\mu$m long. There are no cells that could be considered to be the immediate descendants of auxospores, although as will become clear, this could be because the samples were small. By the winter of 1983-4 the picture has changed. Cells have appeared larger than any others found during the study period; the longest, $424\,\mu$m long, was recorded on 20.1.84.

The ranked plots and histogram for winter 1983-4 exhibit several important features. The lengths are clearly not normally distributed; instead, the distributions are multimodal. Most of the cells are small, below $300\,\mu$m long, and within this group there are at least two subpopulations, one giving a mode at $230\,\mu$m and the other, rather larger-celled, giving a mode at $270\,\mu$m. This is easily seen in the 20.1.84 histogram (Fig. 2) but the bimodality is also detectable in the 12.1.84 collection (Fig. 1) as a marked step in the left-hand portion of the curve. The data for January 1984 also suggest a much smaller and flatter peak at around $300-320\,\mu$m (Figs 1,2). As well as the small cells, there are very large cells which, on 20.1.84 have a mode at a little over $400\,\mu$m (Fig. 2); there is a small peak below this, at $360\,\mu$m, which may be significant in view of the fairly large number of cells measured (331) and the distributions encountered later, between 1985 and 1987.

By September 1984 the smallest cells, below $250\,\mu$m, have become much less abundant and the larger cells appear to have decreased in size, with a diffuse group at $350-390\,\mu$m and a cluster averaging *c*. $330\,\mu$m (Fig. 1).

1985-March 1986 (Figs. 2-4)

A good set of data is available for the early part of 1985, when *N. sigmoidea* was more abundant than at any other time during the study. At least 500 valves were measured in each sample from February to April. As in 1983-4 it is obvious that the lengths are not normally distributed. A series of modes can be picked out, some more obvious than others. On 19.3.85, for instance, there appear to be modes at 370, 315, 285 and $255\,\mu$m. These can be detected, with slight changes of position, in most of the other graphs. In addition there appears to be a minor peak at $340\,\mu$m. Very small cells, perhaps constituting another cluster, are present on 26.2.85 and 5.3.85, the smallest cell recorded so far ($204\,\mu$m long) being found in the 26.2.85 sample. For
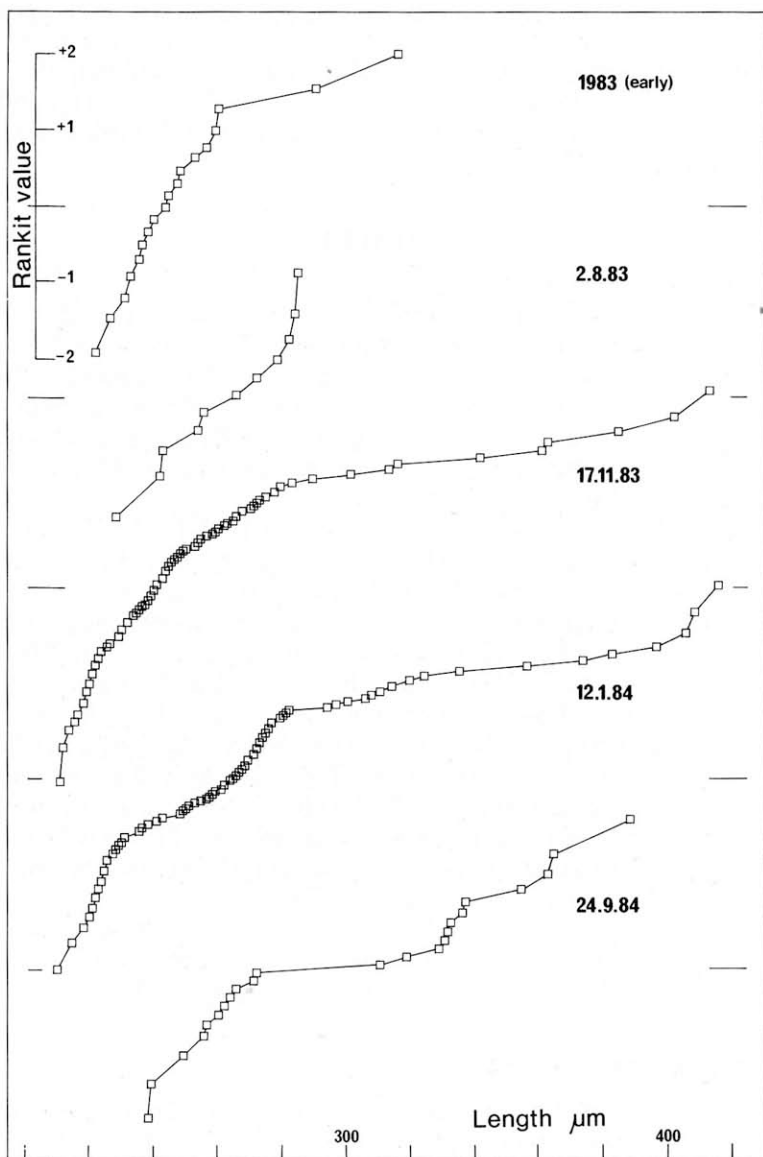
Fig. 1. Size variation in *Nitzschia sigmoidea* in Blackford Pond, 1983-4. Ranked arrays of length measurements plotted against ranked normal deviates (rankits). The x-axis for each graph, at rankit value 0, is indicated by the short horizontal lines at left and right. The pronounced steps in the 12.1.84 and 24.9.84 curves indicate that several size classes are present. The numbers of measurements were as follows: early 1983, 24; 2.8.83, 11; 17.11.83, 120; 12.1.84, 104; 24.9.84, 24.
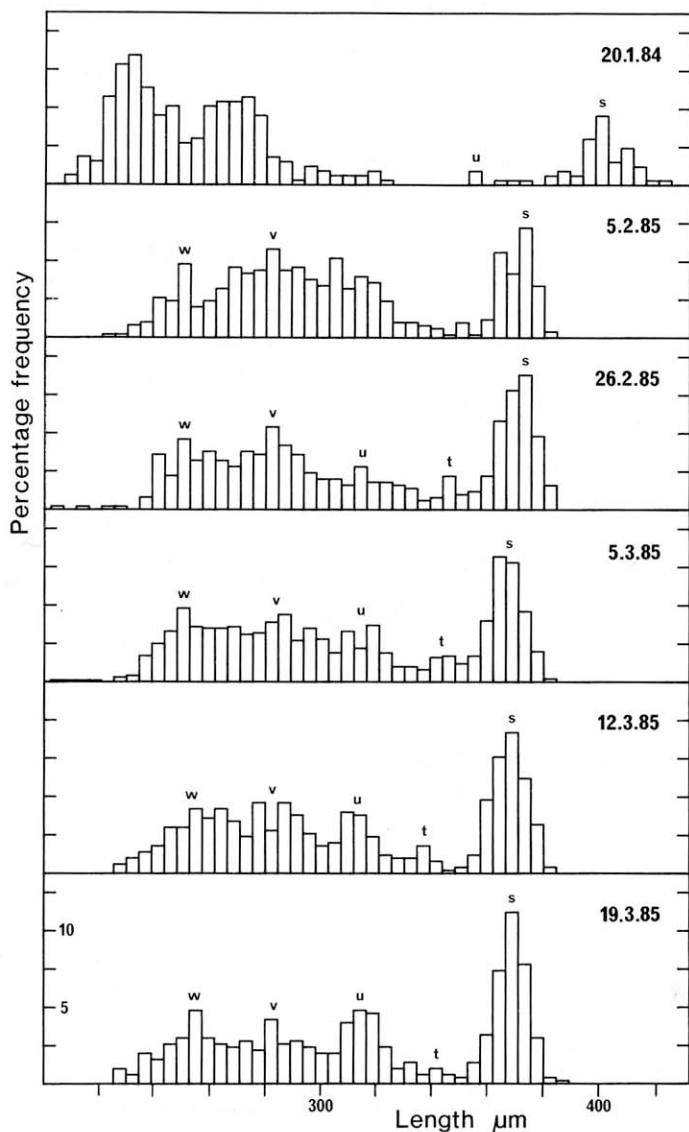
Fig. 2. Size variation in *N. sigmoidea*, January 1984 – March 1985, showing the presence of several size classes with modes (*s* to *w*) 25-30 μm apart. In the 26.2.85 and 5.3.85 graphs there is evidence of very small cells. All based on 500 measurements, except 20.1.84 (331) and 5.3.85 (1000).

convenience I will refer to the main peaks as s-w, as indicated on Figs. 2 et seq.

Besides reinforcing the conclusion that the length distribution of N. sigmoidea is multimodal, the early 1985 data show a shift in average cell size (Figs 2, 3). Peaks s-w remain, reflecting the underlying structure of the population, but superimposed on this is a general selection for larger size. Peak s grows until it constitutes more than 90% of the total, and it is the dominance of this peak in the two succeeding years that has made it possible to interpret the length distributions of N. sigmoidea in Blackford Pond.

The November 1985 and early 1986 frequency distributions are sufficiently similar to that for 30.4.85 for it to be possible to identify the s and u peaks, even though the whole distribution has shifted towards smaller cell sizes. By January or February 1986 the s mode is at c. 350 μm and the u mode at around 295 μm. Hence, between February 1985 (Fig. 2) and February 1986 (Fig. 4), the s mode has moved from 375 to 350 μm and the u mode by a roughly equivalent amount. Hence it is likely that the cluster of large cells present in January 1984 also represents the s peak, since they have a mode of just over 400 μm, 25 μm above the modal length for s a year later; the small peak at 340 μm in January 1984 (Fig. 2) could thus represent the u subpopulation.

The implication of this is that peaks s-w, each 25-30 μm above the next, represent year classes, resulting from discrete periods of auxosporulation occurring a year apart. The year classes are not represented equally within the population, and the early 1985 series shows that they can be affected differentially by selection.

March 1986 – January 1987 (Figs 4-7)

Subsequent observations confirm the conclusions already reached. N. sigmoidea was not very abundant during the latter part of 1986 and only in November was it possible to measure even as many as 300 cells. Nevertheless, it is clear from the histograms (Figs 4, 6) and the ranked data (Fig. 5) that during the whole period peak s is dominant, with a few cells present representing u. On 15.5.86 (Fig. 5) cells 260-285 μm long occurred, perhaps representing the v peak (Fig. 2). Between January 1986 and January 1987 the s mode declines from c. 350 μm to 320-325 μm, supporting the conclusions drawn from the 1985-6 data that the s-w peaks represent year classes.

The striking feature of this period is the way in which the s peak retains its dominance and characteristics, and the u peak its subsidiary rôle, while the whole distribution shifts to the left, to smaller sizes. This is obvious whether the results are presented as histograms (Figs 4, 6) or in ranked plots (Fig. 5). The variance of the s peak, as expressed by the spread of the histogram or the slope of the rankit plot, does not increase to any extent; the s peak is very similar on 26.11.85 (Fig. 3) and 26.1.87 (Fig. 7).

January-November 1987 (Figs 6-9)

In 1987 there is further size reduction of the existing year classes and also evidence of differential selection. In January the s peak is still overwhelmingly
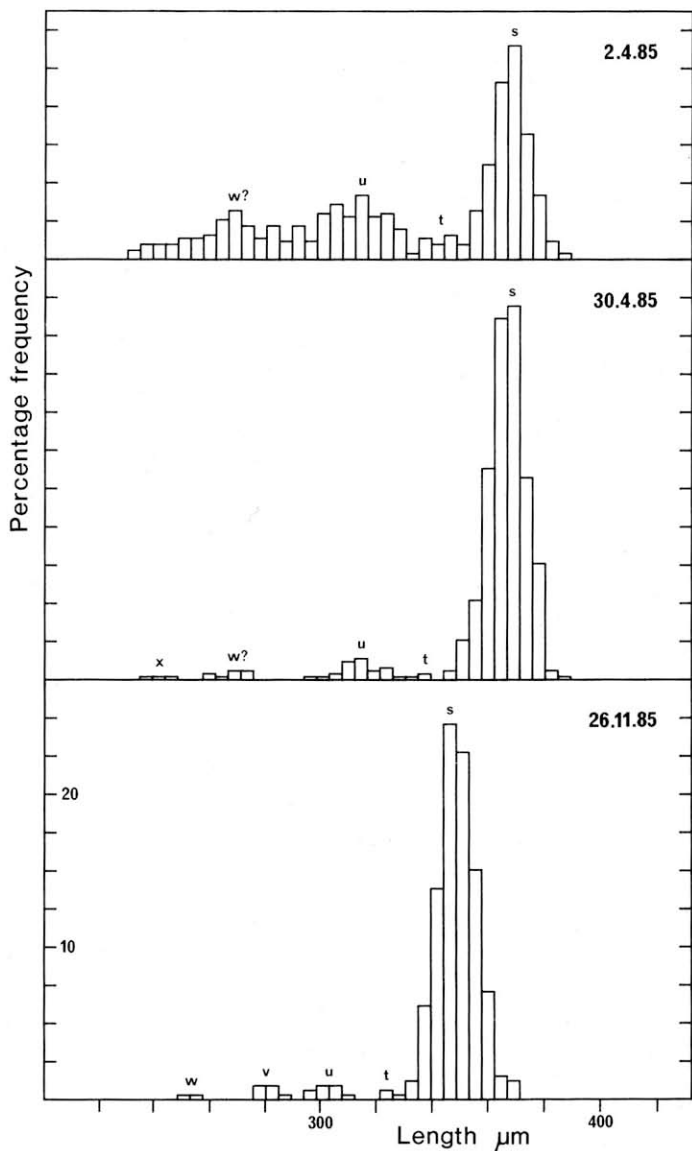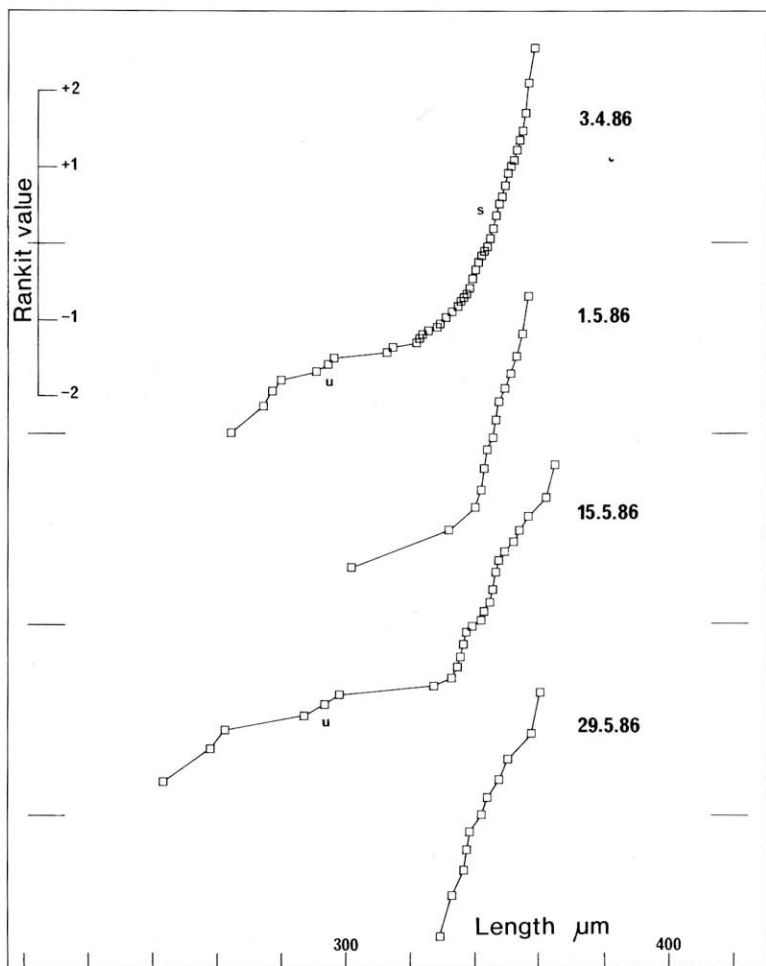
Fig. 3. Size variation in *N. sigmoidea*, April 1985 – November 1985. Classes *s, t* and *u* are still distinguishable, as are several classes of smaller cells, although the relationship of the latter to the classes present from January to April (Fig. 2) is unclear. There is still a marked tendency for the modes to be 25-30 μm apart; note the shift of all classes to the left by 26.11.85. Histograms based on 500 (2.4.85 and 30.4.85) or 325 (26.11.85) measurements.
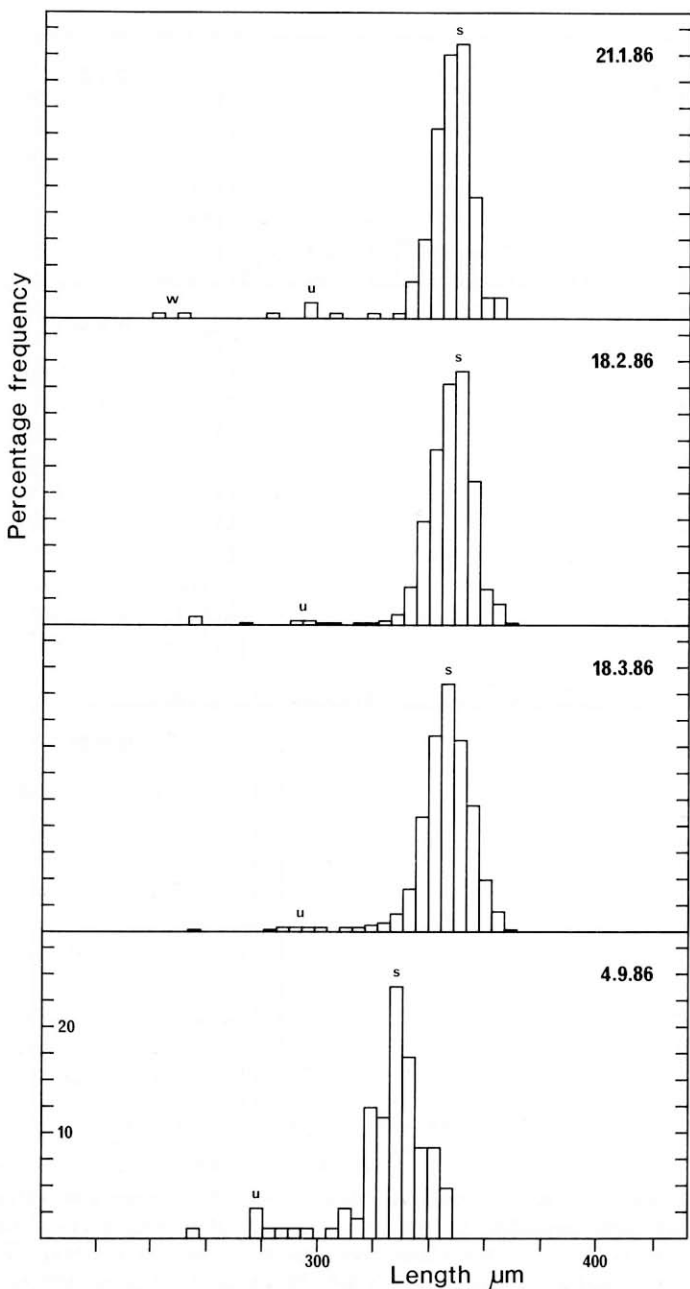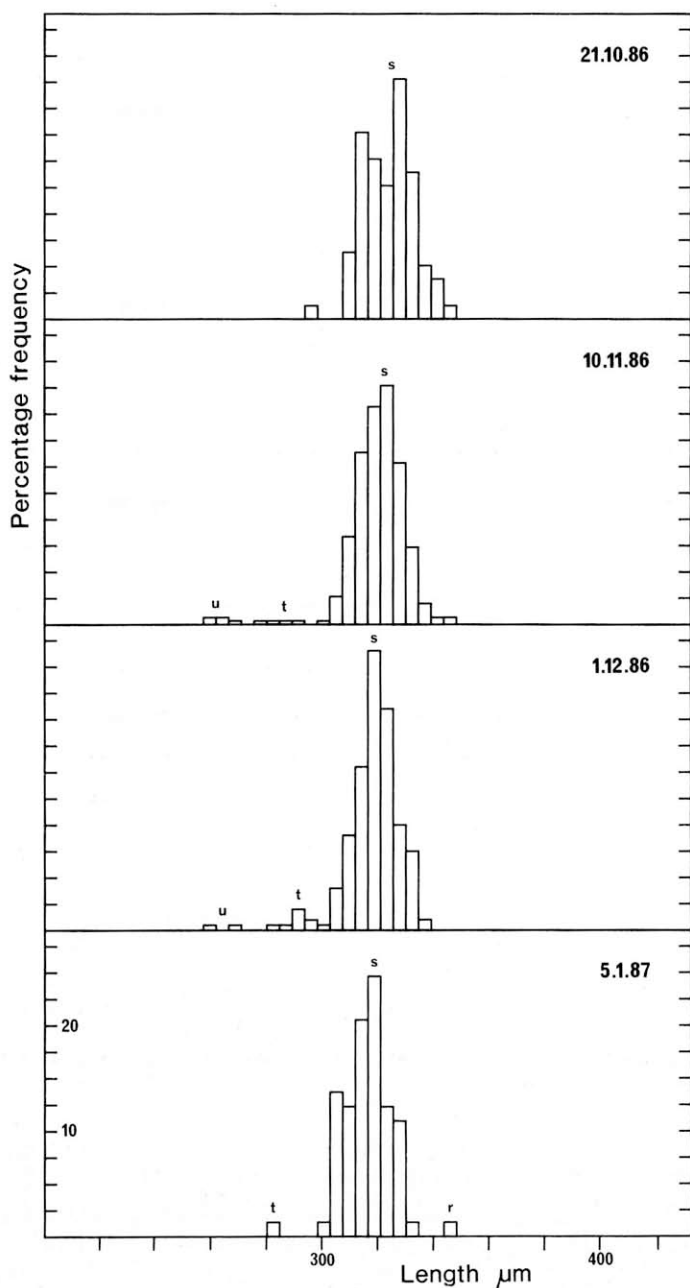
Fig. 4. Size variation in *N. sigmoidea*, January – September 1986 (see also Fig. 5). Note the dominance of class *s* and the persistence of class *u*, and the occasional appearance of smaller classes. Note also the shift to the left in all classes between 18.3.86 and 4.9.86. Histograms based on 200 (21.1.86), 500 (18.2.86), 470 (18.3.86) and 105 (4.9.86) measurements.

Fig. 5. Size variation in *N. sigmoidea*, April – May 1986. The ranked plots again reveal dominance by class *s*, and persistence of smaller-celled classes (e.g. *u*) in low numbers. Curves based on 106 (3.4.86), 16 (1.5.86), 32 (15.5.86) and 11 (29.5.86) measurements.

dominant, constituting over 90% of the total *N. sigmoidea* complement, as it has done since April 1985. During the next six months, however, the *s* peak collapses. The underlying structure of the *N. sigmoidea* population apparently persists but there is an increase in the proportion of smaller cells. This is shown quite clearly in the histograms (Fig. 7) but also in the ranked data (Fig. 8). The 23.2.87 measurements have been plotted in two ways to aid interpretation. In the ranked plot the two main peaks *s* and *u* are obvious as steep 'risers' separated by flatter 'treads'; peak *t* is visible in the histogram (Fig. 7) but is less so in the ranked plot, where it can be detected only as a

Fig. 6. Size variation in *N. sigmoidea*, October 1986 – January 1987. Continued dominance of class *s* and persistence of classes *t* and *u*. Histograms based on 79 (21.10.86), 300 (10.11.86), 200 (1.12.86) and 73 (5.1.87) measurements.
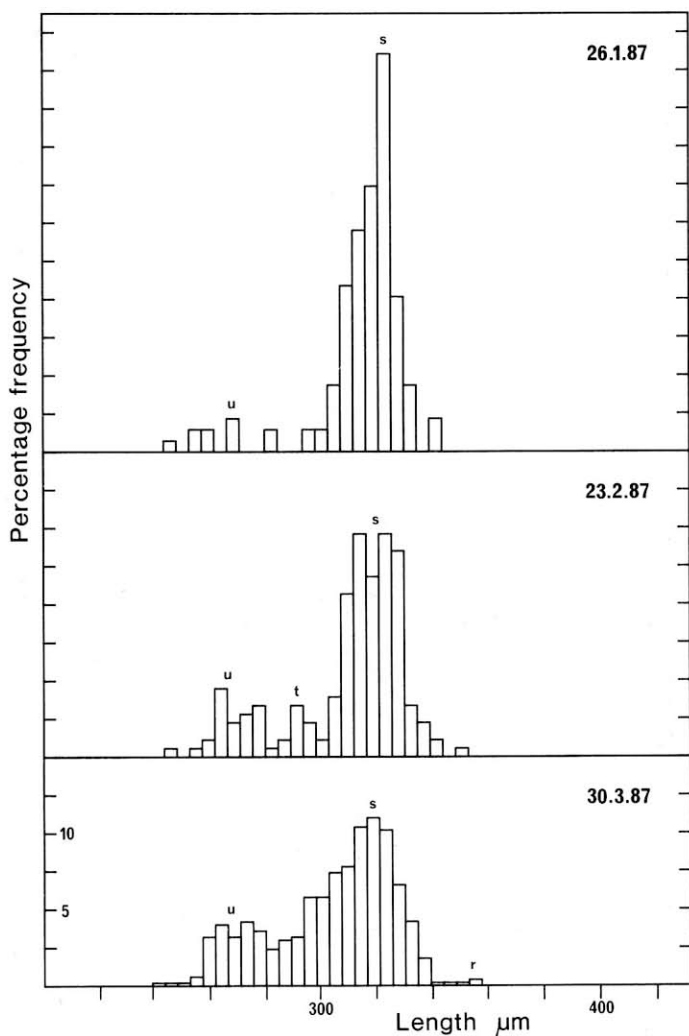
Fig. 7. Size variation in *N. sigmoidea*, January – March 1987. Collapse of the *s* peak and growth of the *u* peak with selection for small size. Histograms based on 138 (26.1.87), 178 (23.2.87) and 500 (30.3.87) measurements.

minor inflection in the curve. The decline of the *s* peak is well advanced by 30.3.87 (Fig. 7) and the general trend towards smaller cell size can be seen to continue for two months after that (Fig. 8, where the change manifests itself as a shift of the curve to the left: compare 23.2.87 with 4.5.87). Curiously, however, it is during this period that a new small size class appears to the right of the *s* peak, at 340-350 μm (Figs 7-9), apparently representing a more recent phase of auxosporulation than the one giving rise to *s*. When this peak (*r*) becomes detectable, it must already be several years old, judging by the fact that the cells are much smaller than the large cells present in 1983-4.
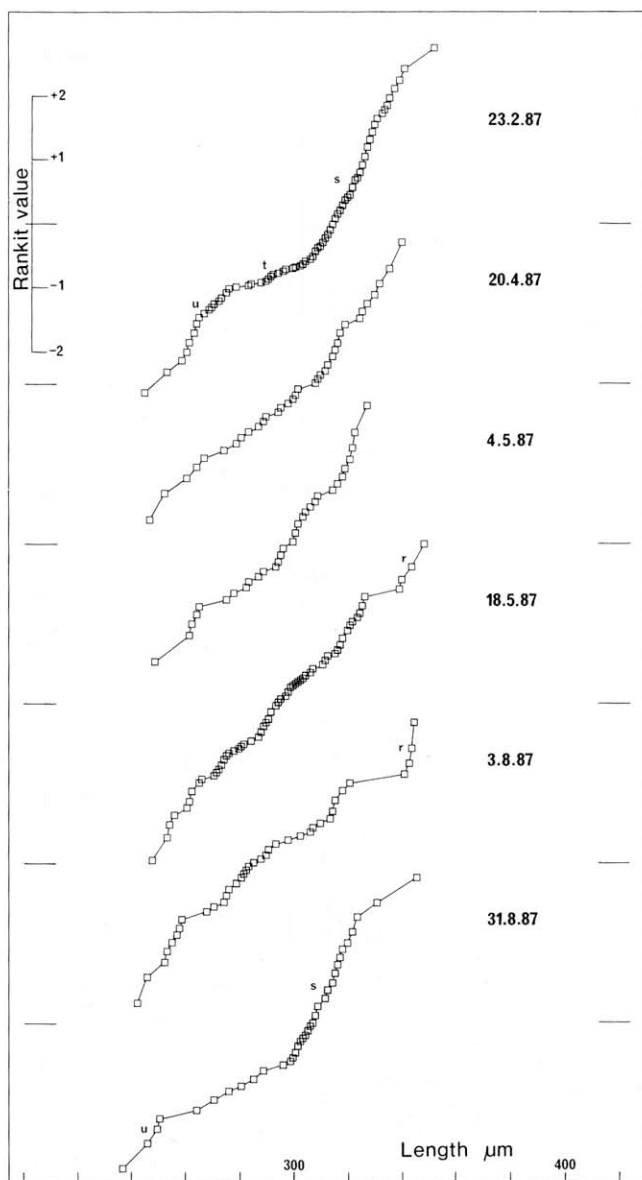
Fig. 8. Size variation in *N. sigmoidea*, February – August 1987 (see also Figs. 7 and 9). In the 23.2.87 curve class *s* is dominant, forming a long 'riser' to the right. By 4.5.87 and 18.5.87 there has been a pronounced shift of the whole population to smaller sizes. This is still noticeable in the 3.8.87 curve, but by 31.8.87 the *s* peak has again become predominant, the 31.8.87 curve resembling the 23.2.87 curve, but displaced downwards by *c.* 10 μm. Curves based on 178 (23.2.87), 40 (20.4.87), 36 (4.5.87), 93 (18.5.87), 43 (3.8.87) and 53 (31.8.87) measurements.

By 1.6.87 the *s* mode has shifted to *c.* 295 μm, a drop of over 20 μm since the beginning of the year. A shoulder is present at 260-270 μm, which may
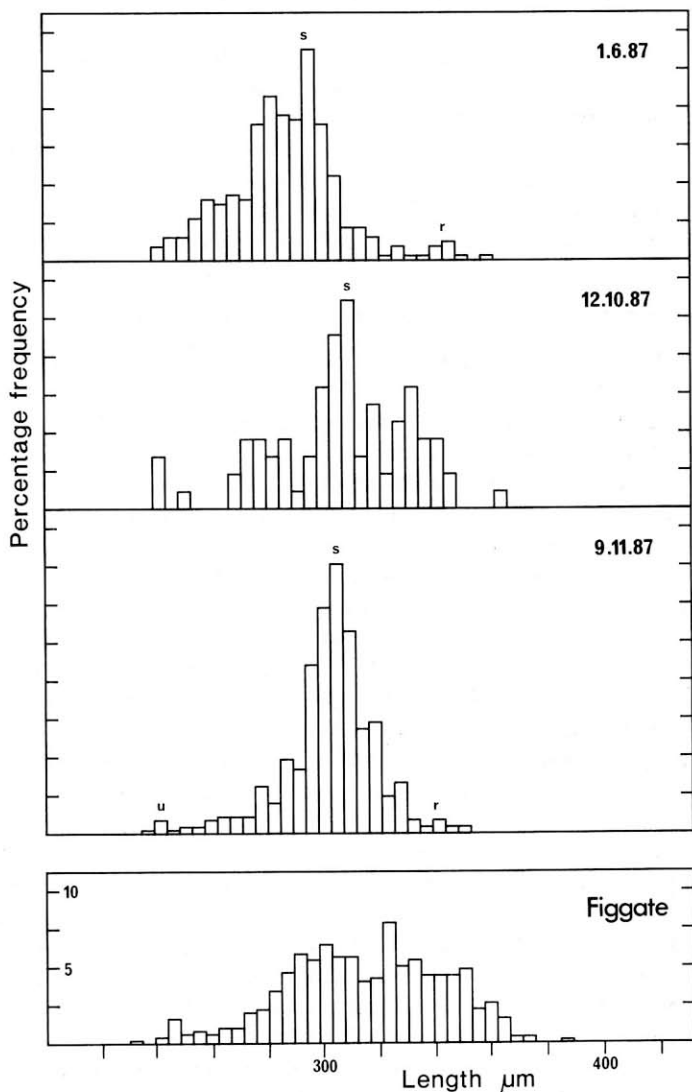
Fig. 9. Size variation in *N. sigmoidea* in Blackford Pond, June – November 1987, and in Figgate Loch, on 26.1.87. Note the shift back of the *s* mode, following reversal of size-selection. Curves based on 325 (1.6.87), 88 (12.10.87), 454 (9.11.87) and 500 (Figgate) measurements.

represent the *u* class (Fig. 9). The 1.6.87 distribution appears to be a markedly skewed derivative of the distributions present two or three months earlier (compare it with the 30.3.87 histogram on Fig. 7). It is noteworthy that the

lower limit of the distribution scarcely changes in spite of the decrease in average cell size, presumably because there are virtually no small cells left; peaks *v-x* (Fig. 2) have effectively disappeared and no longer respond to selection.

After this the selection for small size is relaxed and indeed reversed. During August, for example, the *s* peak shifts back (Fig. 8), so that by 31.8.87 the distribution resembles that present on 23.2.87, except that it is displaced downwards by $10 \mu m$ or so. Further recovery then occurs and in November 1987 there is again one dominant peak, now at $305 \mu m$, roughly $20 \mu m$ lower than the *s* mode for November 1986 (Fig. 6). The net decline in 1987 is therefore rather less than in either of the two preceding years.

Summary and discussion of the *N. sigmoidea* results

1. The *N. sigmoidea* complement of Blackford Pond is multimodal, divided into a number of size classes. Each undergoes size reduction, the decrease in modal length per year being consistent with the idea that the size classes are also year classes, representing annual periods of auxosporulation. The size classes are not equally abundant and there is overlap between them; in other ponds, e.g. Figgate Loch (Fig. 9), this makes interpretation of the size distribution very difficult.

Some other diatoms have also been shown to have multimodal size distributions, e.g. *Fragilaria crotonensis* Kitt. (Schröter and Vögler 1901) and *Eunotia pectinalis* (Müll.) Rabenh. (Steinman and Ladewski 1987), although most reports are of uni- or bimodal distributions (e.g. Wesenberg-Lund 1908, Nipkow 1927, Bellinger 1977, Round 1982); a good survey of the earlier literature is given by Hutchinson (1967). The multimodal curves for *Fragilaria* in the Zürichsee between 1896 and 1901 were interpreted by Schröter and Vögler (1901) as indicating that several varieties of the species were present; Nipkow (1927) later showed that they probably form parts of a single size reduction sequence. Steinman and Ladewski's (1987) interesting data on *Eunotia pectinalis* show that at any one time there are several size classes, but the interpretation is unclear. The authors are wrong in saying that auxospores have never been found in *E. pectinalis*: they were reported by Thwaites and W. Smith in the nineteenth century (see Geitler 1932), and by Geitler (1958b) for the var. *polyplastidica* Geitler. This case needs further investigation, to establish the significance of the size classes (no figures are given for the numbers of cells measured), and whether auxospore formation takes place in the particular races of *E. pectinalis* present.

2. The year classes can be affected differentially by selection. Sometimes small-celled classes are selected for (e.g. early 1987), sometimes larger-celled classes (e.g. early 1985). Selection for larger size could be achieved by the mechanism I describe in detail later, in relation to Nipkow's long-term studies of *Tabellaria*. In this, intermediate, non-auxosporing size classes are favoured and increase as a proportion of the total population when smaller-celled classes undergo size restitution. It may be significant that sexuality was induced

in Blackford Pond populations of *N. sigmoidea* in January 1985 (by transfer of populations to higher temperatures and light intensities in the laboratory: Mann 1986a), just before the growth of the *s* peak in nature, whereas since then similar treatments have failed to promote sexual reproduction.

The selection for small size in early 1987 occurred when the *N. sigmoidea* complement was being parasitised by the oomycete *Aphanomycopsis* Scherffel, which did not affect other species present (including, for instance, all those mentioned in Mann 1988) and which has already been noted to attack *N. sigmoidea* by Scherffel (1925) and Friedmann (1952). This parasite can attack along the whole length of its host (Friedmann, Fig. 5, and my own observations) and so large cells, having a larger surface area and presumably therefore a greater chance of encountering zoospores, are more likely to become infected than small ones. In contrast, the principal parasite of *Navicula capitata* Ehrenb. in Blackford Pond, a *Chytridium* Braun species (this may well be the *Navicula-Chytridium* system described by Sparrow 1933), usually attacks only via the poles of the diatom, so that the effective density of the host is probably controlled by the numbers, but not by the sizes of its cells.

3. The appearance and growth of a peak of large cells does not necessarily mean that auxosporulation is occurring, and it certainly does not mean that the new peak must represent newly formed initial cells and their immediate progeny. The growth of the *s* peak in 1985 could easily have been interpreted as evidence of auxosporulation, the smaller size classes giving rise directly to the *s* class, had it not been known that a peak of even larger cells was already present in winter 1983-4.

4. Failure to record a particular year class does not necessarily mean that it is absent. The appearance of the larger-celled *r* peak in 1987 suggests that auxosporulation took place in the year after the *s* class was produced, perhaps therefore in 1984, but at first this year class remained so rare that it was not detected in a sample of 1000 cells on 5.3.85.

5. The mode of each size class declines by between 20 and 30 μm per year. Preliminary measurements of *N. sigmoidea* frustules indicate that the epivalve is usually *c*. 1 μm longer than the hypovalve. If size reduction in *N. sigmoidea* takes place according to the McDonald-Pfitzer scheme (e.g. see Hutchinson 1967), the mean and mode for each size class should be equal and decline at the rate of $\frac{1}{2}\Delta l$ per generation, where Δl is the mean difference in length between epivalve and hypovalve; this is because at each division one of the two daughter cells is the same size as the parent. The implication is that *N. sigmoidea* divides roughly once per week in nature, a not unreasonable rate for a large diatom. The MacDonald-Pfitzer scheme requires, however, that as the mean and mode decrease by $\frac{1}{2}\Delta l$ per generation, the variance increases by $\frac{1}{4}\Delta l$. This does not occur either in *N. sigmoidea* or in the planktonic diatoms studied by Nipkow (1927) and Wesenberg-Lund (1908). An alternative scheme for size reduction was proposed by Müller (1884), following careful study of the chain-forming centric diatom *Ellerbeckia* Crawford ('*Melosira arenaria*'). His observations, which I can confirm, showed that one of

the daughter cells produced at each division, the smaller one, takes twice as long before it divides again as its larger sibling. Size reduction by this mechanism involves less increase in variance per generation (or per doubling of the population, if the smaller sibling is considered to miss a generation) but even so, as Hutchinson (1967) has pointed out, any scheme of division should involve some increase in variance. There appears, then, to be simultaneous selection against both large and small size. Hutchinson suggested that in planktonic species there might be selection against large size because of greater sinking speeds or lower rates of nutrient uptake per unit volume. The first clearly cannot apply to epipelic species like *N. sigmoidea*, while the second would require that larger size classes always decrease relative to small ones, which certainly did not occur in *N. sigmoidea* between January 1984 and January 1987.

It remains a mystery why each size class remains so 'tight'. One possibility is that in nature, if not in laboratory cultures, thecae and hence the cells that bear them have a limited life, set perhaps by the accumulation of epiphytic bacteria or even algae (*N. sigmoidea* often bears numerous cells of *Amphora pediculus* (Kütz.) Grun. and *Synedra parasitica* (W. Smith) Hust.), or by solution or abrasion of the frustule, etc. This, coupled with Müller's division scheme, would produce a much slower increase in variance during size reduction.

6. The smallest size class of *N. sigmoidea* observed in Blackford Pond was the 200-220 μm class present on 26.2.85 and 5.3.85. The largest cells were 400-420 μm long, on 12.1.84. At a rate of decline of 25-30 μm per year we can estimate that the life cycle of *N. sigmoidea* in Blackford Pond often takes at least six years to complete. This is probably an underestimate. Initial cells have not been detected at any time in this investigation and Geitler (1949) reported a range of 133-*c*. 460 μm, between the smallest gametangium and large cells apparently recently derived from auxospores. A range of 330 μm, coupled with rates of size reduction of 25-30 μm per year for each mode implies a life cycle lasting over a decade. It might be argued that this is a faulty calculation since many cells of a particular year class will reach sizes when they are potentially sexual (see Drebes 1977) long before the *modal* value has dropped to the same extent. This is undoubtedly true, but the fact that the variance of each size class increases only slowly, for whatever reason, indicates that my estimate for the life cycle is not unrealistic. It could also be said that many cells, indeed whole size classes, may become sexual long before they become as small as the smallest recorded (204 μm in this study, 133 μm in Geitler's). This is also true and my observations show that cells of *N. sigmoidea* can be induced to become sexual while still over 300 μm long (the gametangia in Mann 1986a, Fig. 18, measure 288 and 312 μm). Nevertheless, large numbers of cells well below 300 μm long were present in 1983 and 1984, indicating that small size is a necessary, but not a sufficient condition for auxosporulation in nature, and that induction of *mass* auxosporulation in a potentially sexual class does not occur every year.

7. In Blackford Pond *N. sigmoidea* is abundant only in the winter and

spring. From May to October it is usually rare, the 24×50 mm coverslips used to harvest the epipelon often bearing fewer than 20 cells; during winter the number can be 1-2 orders of magnitude higher. But although *N. sigmoidea* may be rare in summer, this does not imply that physico-chemical conditions are unsuitable for growth. Between 18.3.86 and 4.9.86 (Fig. 4) the *s* mode shifted by 20 μm, apparently as a result of vegetative division – there is little evidence that the reduction was brought about by selection like that occurring in early 1987, since the *u* class does not increase appreciably. The 20 μm shift is at least as much as one would expect to occur in six months, given an annual reduction of 25-30 μm, and this must imply that *N. sigmoidea* continues to grow and divide during the summer. The drop in abundance must be brought about primarily by an increase in the loss processes affecting the population (grazing, parasitism, burial) rather than by a decrease in the true growth rate, since this would retard size reduction unless Δl is very much greater in slowly growing cells.

8. The size spectrum of *N. sigmoidea* changes from year to year. This means of course that the response of the total *N. sigmoidea* population to the same size-dependent selection will also vary from year to year. If benthic invertebrates or parasitic fungi are 'fussy' about the size of the cells they ingest or attack, their effects on *N. sigmoidea* will depend on the structure of the particular population they encounter.

Previous studies reinterpreted

1. Tabellaria (Fig. 10)

In his classic study of the varved sediments of the Zürichsee and Baldeggersee, Nipkow (1927) used *Tabellaria fenestrata* as a model to help explain size changes in a variety of centric and pennate planktonic diatoms (*Aulacoseira [= Melosira] islandica* var. *helvetica* (O. Müll.) Simonsen, *Cyclotella comta* (Ehrenb.) Kütz., *Stephanodiscus hantzschii* Grun., *Asterionella gracillima* (Hantzsch) Heib., *Fragilaria crotonensis*, and *Synedra delicatissima* W. Smith). I have replotted most of Nipkow's *Tabellaria* results for the Zürichsee (Fig. 10) and will reinterpret them in the light of the *Nitzschia* data.

Nipkow considered the Zürichsee *Tabellaria* results to be 'die klarste der ganzen Serie'. He noted that the size distribution was usually unimodal, and that the mode declined progressively until it reached 40 μm or less, when it was generally replaced quite rapidly by another peak of rather larger cells: this replacement he referred to as rejuvenation (Verjüngung). Nipkow considered that the appearance of the larger cells inevitably entailed the demise of the smaller ones, because the larger cells were derived directly from them. By analogy with observations on *Cyclotella bodanica* Eulenst. by Bachman (1904) and his own studies of *Aulacoseira islandica* and *Stephanodiscus hantzschii*, Nipkow interpreted the replacement of one peak by another in *Tabellaria* as evidence of auxospore formation: 'eine andere Art der Zellvergrösserung wurde bis jetzt an Planktondiatomeen des Süsswassers nicht beobachtet, auch ist ein direktes Wachstum der alten Zelle mitsamt
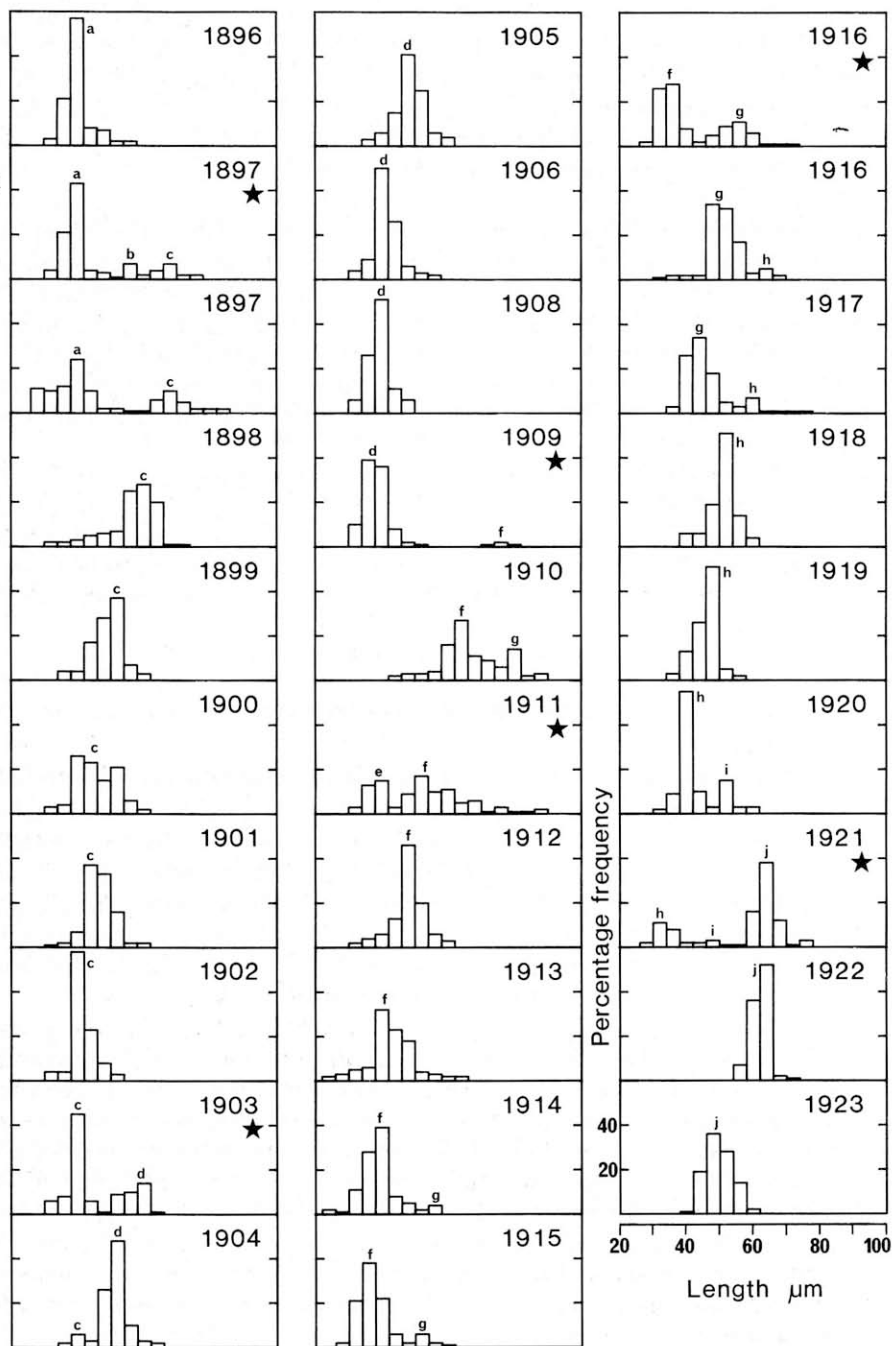
der starren Kieselschalen nicht möglich . . . Es dürfte demnach für alle Planktondiatomeen noch den häutigen Kenntnissen einzig die Auxosporenbildung . . . als Grössenregeneration in Frage kommen'. *Tabellaria* was therefore supposed to have undergone auxosporulation in 1897, 1903-4, 1909, 1911, 1915-16, and 1920-1 (Fig. 10). In each case Nipkow claimed *total* rejuvenation, since the old peak seemed to disappear completely, being replaced by a new peak of larger cells.

There are several problems with this interpretation. The first is that although old peaks of *Tabellaria* usually disappeared when their modes reached c. 40μm, the new peaks that replaced them had very different modal lengths (Fig. 10). The principal new peak in 1897 had a mode of 70μm (Fig. 10, *c*), while those of 1903 (*d*), 1910 (*g*), 1916 (*h*) and 1921 (*j*) had modes of 60, 80, 65 and 65μm respectively. The largest *Tabellaria* cells, however, were over 85μm long, recorded in 1910 and 1911. To accept that the 1903 and 1909-10 rejuvenations both present auxosporulation, we must suppose that gametangia ranging from 30-45μm produce auxospores of anything between 50 and 85μm long, the *mean* lengths of the auxospores also varying greatly from time to time. This seems unlikely in view of Geitler's (1932) dictum, well supported by experimental work and observations of natural populations (and see too Geitler 1958a, b, 1963, 1968, 1970, 1972, 1982), that each species has well-defined and narrow limits for the lengths of its gametangia and auxospores: "Zur Auxosporenbildung und sexuellen Fortpflanzung sind nur Zellen einer bestimmten Grössenklasse fähig. Ebenso ist den Auxosporen eine bestimmte Grösse eigentümlich. Die Entwicklungscyclus jeder Art wird somit durch für sie charakteristisch 'Kardinalpunkte' bestimmt' (Geitler 1932). The largest *Tabellaria* cells occurred in 1897 and 1910-11, when there are appreciable numbers of cells between 75 and 85μm: these appear to me to be the only cells it is fairly safe to assume represent the immediate descendants of auxospores. If so, the new peaks appearing in 1903 and 1914-16 do not represent auxospores. It remains true that whenever a peak reaches 40μm it soon disappears, and this is probably because of auxospore formation, but if the new peaks do not represent newly expanded cells, where do they come from?

Fig. 10. Size variation in *Tabellaria fenestrata* in the Zürichsee from 1897 to 1923 (data from Nipkow 19 / , replotted). Nipkow considered that rejuvenation of the population, via auxosporulation, took place in 1897, 1903, 1909, 1911, 1916 and 1921 (stars). The labelling of the peaks (*a-j*) represents my interpretation of Nipkow's results (see text); I have assumed that size reduction is an orderly process and traced the fate of particular classes from year to year. The bimodal distribution of 1900 is difficult to interpret and has been assigned to one class, *c*, on the basis of Nipkow's results for 1901, when he twice found a unimodal curve (only one of which is plotted), with no hint of a lower mode corresponding to that apparently present in 1900. All Nipkow's curves were based on 100 measurements. From 1896 to 1906 the curves were derived from sediments laid down in autumn, except the first curve given from 1897, which came from vernal sediments; 1908-10 are from vernal sediments; the remaining curves, 1911-23, came from autumnal sediments, except for the first 1916 curve, which was vernal.

An answer is suggested by the *N. sigmoidea* data. From mid-1985 to early 1987 the *N. sigmoidea* complement of Blackford Pond was dominated by a single size class *s*, but other classes (*t-w*) were present before the *s* class became dominant, and some at least of these persisted, though very much reduced, during the period of *s* dominance. Thus, when selection for smaller cell size occurred in spring 1987, the *u* peak 'reappeared'. Nipkow's *Tabellaria* populations were obviously dominated for most of the time by one size class, but others may have been present in small numbers, too small to be detected in a sample of 100 valves. Hints that this was so are given by the early 1897 curve, where there appears to be an intermediate peak *b*, between the 'gametangia' (*a*) and the 'initial cells' (*c*); in 1911 there are two peaks of medium to small cells and also a long tail of larger cells; and in 1920-1 it seems clear that at least three size classes are present. So, when a small-celled peak disappears, it *may* be replaced *as dominant* by a newly 'rejuvenated' line (as may be occurring in 1909), but it may instead be replaced *as dominant* by an intermediate size class, previously present in such low numbers as to be undetectable in a small sample. If so, however, a mechanism must exist by which an intermediate size class tends to become dominant during the auxosporulation of another, smaller-celled class. A simple model can be used to explain the problem. Assume that in one year there are two size classes A and B. A consists of small-celled individuals and constitutes 99% of the total population; B consists of medium-sized individuals and makes up the remaining 1%. The mechanism we require must explain how in a later year, as A disappears, giving rise by auxosporulation to the large-celled class C, the intermediate class B becomes dominant, coming to make up 99% or more of the population. The following may be elements of such a mechanism:

1. Paasche (1973) has shown in several species that growth rates (measured in divisions per day) are similar over most of the size reduction cycle. However, the smallest cells of *Ditylum brightwellii* (T. West) Grun. and *Licmophora hyalina* (Kütz.) Grun. had reduced growth rates and Geitler (1932) found evidence of structural abnormalities and reduced division rates in the smallest cells of a variety of pennate diatoms. Hence part of the decline in the proportion of small cells (class A in my example) could be due to reduced 'vitality' as the cells approach their lower size limit; there would therefore be a smaller proportion of cells to act as gametangia and thus give rise to the large C cells, and class B would begin to grow as a proportion of the total even before auxosporulation took place. Furthermore, if auxosporulation were incomplete in the A class, any cells not induced to form auxospores would probably soon disappear from the population. Nipkow's (1927) data show that small size in natural populations of planktonic species is often correlated with low abundance, which could indicate a reduction in true growth rate in the predominant size class (but see later).

2. Since auxospore formation in diatoms never involves the production of more than two zygotes per pair of gametangia, class C can only replace class A in my example *and* retain A's dominance if class B cells do not divide during the whole time that A cells are engaged in pairing, meiosis, fertilization,

auxospore expansion, and initial cell formation (itself involving two acytoki-netic mitoses!). In the diatoms I have studied, including *Navicula pupula* Kütz., *N. cuspidata* (Kütz.) Kütz., *Caloneis ventricosa* (Ehrenb.) Meister, *Achnanthes lanceolata* (Bréb. ex Kütz.) Grun., *Neidium affine* (Ehrenb.) Pfitzer, *Stauroneis phoenicenteron* (Nitzsch) Ehrenb., several forms in the *Amphora ovalis* (Kütz.) Kütz. aggregate, and *Cymatopleura solea* (Bréb. and Godey) W. Smith, the whole process of size restitution lasts several days (*Achnanthes lanceolata*) to over a week (*Caloneis ventricosa, Navicula cuspidata*), in conditions allowing frequent vegetative division (around $1.\text{day}^{-1}$ in several of the above) in unsexualized cells of the same species. Hence for class C to replace A without any relative increase in B it would have to be assumed that the rate of vegetative division at the time was minimal. Yet in most diatoms that have been studied it has been shown that there is no great difference between conditions promoting rapid vegetative growth and those inducing sexuality (e.g. see Drebes 1977). Thus, where a population consists of several size classes, auxosporulation by one class should be accompanied by an increase in the proportions of the non-auxosporulating size classes (of B in my example). In the case of a diatom taking a week to complete size restitution, a vegetative growth rate of 1 division per day would allow the non-auxosporulating cells to increase from 1% to over 50% of the total. And this assumes that auxospore formation is totally efficient, bringing about no mortality in excess of what would have affected the cells if they had remained vegetative; for instance, there must be no wastage of gametes, no disadvantage in the loss of motility or colonial organisation during auxospore formation, etc. Otherwise, the relative increase of the intermediate size classes will be even greater. This effect is a simple consequence of the interruption of synthesis, which Lewis (1983) has analysed in relation to the costs of sexual reproduction.

Let us examine the *Tabellaria* data (Fig. 10) with this mechanism in mind. In 1897 peak *a* begins to be replaced by *c*, which has established its dominance by 1898. As this occurs medium-sized cells (*b*) are also present, but in this case they do not remain a significant part of the population. From 1897 to 1902 the *c* class, which may have been derived from *a* by auxosporulation, undergoes size reduction, rapidly at first and then more slowly as the mode approaches $40\,\mu\text{m}$. This may reflect a decline in the intrinsic growth rate with decreasing cell size (I have already noted Nipkow's finding that a species was less abundant when the predominant size class was small-celled), or a decreasing Δl. In 1903 the mode of *c* reaches $40\,\mu\text{m}$ and *c* is then joined by *d*, with a mode at $60\,\mu\text{m}$. This peak becomes dominant after a year. Its cells are too small to be recently derived from auxospores and I suggest that it becomes dominant because of the interruption of synthesis in *c* cells as they undergo size restitution; the *d* class arose perhaps 2 or 3 years before it appears in Nipkow's graphs, perhaps through auxosporulation of the few medium-sized cells (*b*) present in 1897.

From 1905 to 1908 peak *d* shifts to the left. In 1909 a new peak *f* develops at $75\,\mu\text{m}$, which may reflect auxospore formation that year, although the cells

are smaller than the largest cells present in 1910 and 1911. The progress of peak *c* in 1897-9 suggests a possible interpretation of the 1909-12 series, which I have included in Fig. 10. There seems to be a group of cells larger than the *f* cells, resulting from a later phase of auxosporulation. This *g* peak becomes undetectable in 1912 but reappears later. In 1911 small cells occur (*e*), which may be the survivors of peak *d* but could well represent a hitherto undetected size class; the shape of the 1911 curve suggests that selection for small size occurred, as in *N. sigmoidea* during spring 1987. Very small cells are present in 1913 and 1914, which are probably derived from the *e* class. As the *f* peak approaches $40\mu m$, it is joined by another peak, *g*, which first reappears in 1913 but does not become dominant until the end of 1916. The slow decline of the *f* peak may mean that auxosporulation of its cells was divided between 1915 and 1916, the first phase producing the cells afterwards detectable as peak *h*, the second those appearing in 1920 as peak *i*. Neither the replacement of *f* by *g*, nor that of *g* by *h*, can reasonably be interpreted as representing direct restitution via auxospores. In each case there is an enhancement of a previously existing, intermediate size class.

In 1921, *h* and *i* are replaced by *j*. Again it is unlikely that *j* is a newly produced size class, since in 1921 its mode is already at $65\mu m$. Judging by the initial rates of size reduction in classes *c* and *f*, and assuming that the largest cells observed by Nipkow, in 1910 and 1911, represent the immediate descendants of auxospores, we can estimate that the *j* class arose in 1918 or 1919, in which case it may have arisen by auxosporulation of class *g*, the interruption of synthesis causing the shift in dominance to *h*. Finally, in 1922 and 1923 *h* and *i* disappeared, presumably giving rise to an undetectable class of large cells, while the single remaining peak *j* shifts to the left as its cells continue to reduce in size.

If this interpretation is correct, Nipkow's estimate that the life cycle lasts 2-6 years must be revised upwards to 7-8 years or more – approximately as long as in *N. sigmoidea*. Furthermore, the total rejuvenation Nipkow claimed to occur must be seen as an artefact, apparently produced by the mechanism I have outlined, coupled with the relatively small sample size Nipkow used (100 measurements). Auxospore formation probably takes place in most years but peaks of very large post-auxospore cells will appear only when the gametangial size class or classes are overwhelmingly dominant, as perhaps in 1908-10. Both Nipkow's data and mine imply that auxospore formation is limited to a few weeks each year – otherwise discrete size classes would not arise.

2. Other species

Are all diatom life cycles as long as in *Tabellaria fenestrata* and *Nitzschia sigmoidea*? Probably not. Mizuno & Okuda's (1985) studies of *Cocconeis scutellum* Ehrenb. indicate a two year cycle for this species in nature, although that authors seem to imply an annual cycle in their discussion. Mizuno and Okuda show convincingly that auxospore formation takes place in winter and

that in culture, gametangia measure 15-21 μm and initial cells 40-49 μm. Between November and February in nature a class of 15-25 μm cells was replaced as dominant by a class of larger cells; however, these did not measure 40-49 μm but were much smaller, between 25 and 37 μm, and the rate at which they subsequently decreased in size implies that they could not possibly have been derived from a 40-49 μm class within the time available since that year's auxosporulation. On the other hand, the rate at which the 25-37 μm cells reduced in size is entirely consistent with their having been formed the year before, giving a 2-year cycle for this species. This then, would be a case exactly like my hypothetical A-B-C type, the 'B' class of *C. scutellum* being favoured in winter because its cells continue to divide (albeit infrequently?) as the 'A' cells auxosporulate.

The other study known to me that suggests a short life cycle is that of Round (1982), which deals with a *Stephanodiscus* Ehrenb. population. Here there is no question that the largest forms, *c.* 60 μm in diameter, are the immediate descendants of auxospores, since initial cells were recorded among them. As in Mizuno and Okuda's studies, sampling was frequent, so that we can be confident that we are following the progress of particular size classes. The mode of Round's large-celled class declines from 60 to *c.* 40 μm in a year, which, providing size reduction does not slow down, implies an upper limit of 3 years or so for the life cycle! The predominant size class at the beginning of Round's series has a mode of around 30 μm; since at that time no smaller cells were sufficiently abundant to form a detectable peak, it must be assumed that it was the 30 μm class that provided the gametangia for sexual reproduction. In this case the life cycle must usually last 1-2 years. Auxospore formation was restricted to a short period, as shown by the fact that initial cells were common in the population for only a month or so. By no means all of the smaller cells took part in auxosporulation and their class was still detectable 5-6 months later, with little reduction in the sizes of the cells. Some cells apparently survive into their third year without undergoing size restitution, since very small 10 μm diameter cells were present in one sample. Round's data also show evidence of selective effects like those in *N. sigmoidea*, the two major *Stephanodiscus* size classes alternating as dominants in December and January 1973. During the year of investigation there was a marked discrepancy between the rates of size reduction in winter and summer, which Round left unexplained. It is worth noting that there are at least three ways, not mutually exclusive, in which this difference could be brought about: a change in Δl, a change in the true growth rate, or a change in the nature or intensity of size-selection (by grazers, through differential loss during sedimentation, and so on).

There are also cycles much longer than those of *T. fenestrata* and *N. sigmoidea*. The best documented is that of *Aulacoseira islandica* var. *helvetica*, studied by Nipkow (1927). Between 1901 and early 1904 there was a single dominant size class in the Zürichsee, which began with its mode at 6 μm (cell diameter) and shifted to 4 μm in the next three years. In 1905 auxosporulation occurred, shown by the appearance of hemispherical initial valves within the

population. Following this, from 1907 to 1919, there was again only a single dominant size class, whose mode moved down slowly and continously from $10\,\mu m$ in 1907 to $6\,\mu m$ in 1919. In 1920 large cells again appeared and two peaks persisted alongside each other from then to the end of the series of observations in 1923; by then the smaller cells still had a modal size of $6\,\mu m$ and still consituted the dominant size class. We can therefore say that many cells have a life cycle of at least 19 years (from the first appearance of large cells in 1904 to the end of the sequence in 1923). Careful examination of Nipkow's results reveals, however, that the life cycle could easily be much longer than this, perhaps twice as long, since the dominant peak present from 1905 to 1923 does not appear to have been derived from the auxospores undoubtedly formed in 1904-5. These auxospores had diameters of $16\text{-}22\,\mu m$, whereas the peak establishing itself as dominant by the end of 1905 (a peak already detectable alongside a $16\,\mu m$ peak in 1904 and early 1905) had a mode of around $10\,\mu m$. For the major peak present from 1905 to 1923 to have been derived from the auxospores formed in 1904-5, the cells would have had to have decreased in size more in the first few months after auxosporulation (16-22 down to $10\,\mu m$) than in the subsequent 18 years (10 down to $6\,\mu m$)! Nipkow explained this difficulty by reference to the enormous amount of *A. islandica* (a 5 mm thick layer) present in the sediments laid down in winter 1905-6, perhaps reflecting a very high true growth rate and hence rapid size reduction. Several points need to be made. Firstly, large numbers of cells in the sediments do not necessarily imply a greater rate of production. Large cells are likely to sink faster and so will have a shorter residence time in the water column. They are therefore less liable to wash-out loss (Reynolds 1984), and so a greater proportion of them may accumulate in the sediments. Secondly, high growth rates will greatly accentuate the tendency for intermediate, non-participating size classes to be favoured during auxosporulation. Thirdly, *A. islandica* was also abundant in 1921-3 (Nipkow 1927, tab. XI), when large cells were present as well as small ones; there was no sign of rapid size reduction in either class. The simplest explanation of Nipkow's data is that they indicate a life cycle of *c.* 40 years for *A. islandica* in the Zürichsee, the subpopulation dominant between 1905 and 1923 having arisen well before the beginning of Nipkow's sequence in 1897. The correlation between high abundance and recent auxosporulation was interpreted by Nipkow as indicating increased vitality of newly enlarged cells. There is no evidence of this kind of effect from Paasche's (1973) or Findlay's (1972) studies, and the results are probably better interpreted as showing that mass auxosporulation is favoured by high population densities, when the frequency of encounters between gametes (if released) or gametangia will be greatest.

The length of the *Aulacoseira* life cycle, long even in Nipkow's interpretation, should not perhaps be too surprising, since Lund's (1954) observations on *A. italica* subsp. *subarctica* O. Müll. suggest that winter phytoplankters divide far less often per year than species growing at other seasons, such as *Asterionella* or *Tabellaria*: Lund suggested a 100-fold increase for *Aulacoseira italica*, as against a 100,000-fold increase for *Asterionella formosa*.

Among the other species studied by Nipkow (1927), *Fragilaria crotonensis* appears to have a life cycle of 8-10 years in the Zürichsee, judging by the behaviour of a single dominant size class between 1898 and 1907; from 1907 to 1923, and between 1938 and 1953 (Nipkow 1953) the picture is far less clear. In the Baldeggersee an 8-year cycle was completed between 1912 and 1920 (Nipkow 1927). I cannot interpret Nipkow's (1927) *Stephanodiscus hantzschii* data. His curves for *Synedra delicatissima* both in the Zürichsee and Baldeggersee, strongly suggest the presence of several size classes at all times, only one of them usually being abundant. The abrupt shifts in mean length recorded for the species at various times between 1909 and 1919 in both lakes are difficult to interpret in any way other than that they represent shifts in the balance between two or more principal size classes, as in Round's (1982) *Stephanodiscus* data. Each of the classes seems to undergo slow size reduction and a long life cycle, well in excess of 10 years, is possible.

3. Asterionella

Some populations of *A. formosa* seem to maintain a constant size, e.g. 'var. *hypolimnetica*' in the Lunzer Untersee (Ruttner 1937). In other populations of *A. formosa* (Ruttner 1937) and also in *A. gracillima* there appears to be a size reduction cycle; Nipkow's (1927) long Zürichsee series is unfortunately too complex to admit interpretation at present. Wesenberg-Lund (1908) gave data on *A. gracillima* for seven Danish lakes, including a series for Furesø lasting from August 1900 to October 1906. Over the whole period a single peak was dominant, whose mode declined from 72 to 57 μm, a rate of approximately 2.5 μm per year. The largest cells of all were found in 1902 and measured nearly 100 μm in length; the smallest (found in 1906) measured 45 μm, although other Danish lakes had cells as small as 38 μm. If all these belong to the same race, with the same cardinal points, it could take 20 years for the life cycle to be completed. Lozeron (1902) also recorded a reduction of just over 2 μm per year in *A. gracillima*, both for a unimodal population in the Ober-Zürichsee, and for each of the modes in a complex multimodal population in the Zürichsee itself. Furthermore, the total range of length recorded for the Zürichsee (36-103 μm) was almost identical to that in the Danish lakes, supporting the idea of a very long life cycle for the species. This assumes, of course, that in these lakes *A. gracillima* is a single entity, not split into several races, each with its own size range.

CONCLUSION

If diatom life cycles last 2-40 years and sexual reproduction is nearly synchronous within a population or size class, lasting only a few weeks at the most, phycologists, even Lund, can be excused for having failed to observe it in the majority of species. Only in populations that are very dense and can be sampled without disturbing the spatial relationships between cells is it going to be at all easy to detect auxosporulation directly in nature, and then

only with frequent sampling through the year. This is surely why Geitler's tables (1932, 1973) listing the occurrence of different modes of auxospore formation among pennate diatoms, are dominated by records of epiphytic and epilithic taxa, and why we must rely on von Stosch's excellent studies of cultures (see Drebes 1977) for knowledge of the sexual processes of centric diatoms. I have not answered my question; perhaps Lund will.

ACKNOWLEDGEMENTS

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