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REGULAR PAPER

What constitutes *Gomphonema parvulum*? Long-term culture studies show that some varieties of *G. parvulum* belong with other *Gomphonema* species

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Background and aims – *Gomphonema parvulum* sensu lato contains a number of morphologically distinguishable varieties and forms that also show variation in their distribution and ecological ranges. As part of a study to investigate the ecophysiology of this taxon in relation to its use as a bio-indicator, clones from a range of sites in the UK were established and maintained in culture, monitoring their growth and morphology over extended periods of time.
Methods – Clonal cultures of strains of *G. parvulum* were isolated and maintained in liquid medium under standard conditions, harvesting old cultures after each sub-culturing event. Wall morphology was monitored by light microscopy of permanent preparations, and a number of morphometric measurements obtained.
Results – Four clones identified as belonging to two different varieties of *G. parvulum*, underwent autogamous sexual reproduction in culture, producing auxospores and initial cells, and then continuing to reproduce vegetatively. The two varieties gave rise to morphologically different cells that were identified as different *Gomphonema* species, i.e. *G. gracile* and *G. hebridense*. Continued culturing resulted in cell size reduction and changes in morphology towards that of the original isolates.
Conclusions – Cells identified as part of *G. parvulum* sensu lato represent one end of the morphological spectra of taxa that also exhibit morphologies that would be assigned to other *Gomphonema* species. The morphological range of these taxa has previously been inadequately documented and species definitions must be re-visited. Any nomenclatural revision must follow priority rules, but it will be necessary to check type material of all the relevant species before making any nomenclatural changes. The ecological tolerances of the taxa involved should also be re-evaluated; it is improbable that cells at the opposite ends of the morphological spectra have different ecological responses.

Key words – Bacillariophyta, culture studies, diatom size spectra, *Gomphonema*, species boundaries.

INTRODUCTION

Over the last few decades, studies of reproductive behaviour and the use of molecular tools to explore phylogenetic relationships and/or for taxon discrimination, have contributed to the proliferation of new diatom species descriptions (Mann 1984, 1988, 1989, Medlin et al. 1991, Mann et al. 2004, Sarno et al. 2005, Zingone et al. 2005, Lundholm et al. 2012). And even when only structural evidence is presented, it is clear that the morphological species concept for diatoms has narrowed considerably over the last 40 years (cf. Krammer & Lange-Bertalot 1986, 1991, and Jüttner et al. 2013). Yet, it is also usually assumed that diatom populations will show a gradual decrease in size as they reproduce, a phenomenon that can lead to proportional changes in their wall morphology (Hustedt 1955, Geitler 1958, 1985, Hohn 1959, Cox 1986, Round et al. 1990). While it is generally accepted that the restoration of maximum cell size is usually effected via sexual reproduction and auxosporulation (Mann 2011, Kaczmarska et al. 2013), there has been little comparative work on the morphology and identity of the extremes of the size range in most diatoms. Previous examples have usually been the result of serendipitous events in culture (Cox 1985, Trobajo et al. 2006, Poulíčková 2008, Poulíčková & Mann 2006, 2008) or based on observations of field material (Geitler 1932, 1952, 1958, 1960, 1970, 1985, Hohn 1959), although Veselá et al. (2009) used cultures to show that some

morphometric features were consistently informative over the entire size range of two *Navicula* Bory species.

More recently, two studies (Kermarrec et al. 2013, Abarca et al. 2014) have addressed the phylogenetics and phylogeography of *G. parvulum* clones from dispersed localities, using multi-gene and morphological approaches. While both found geographic patterns within their data, they also recognised the problems in distinguishing and identifying populations on morphological grounds alone.

Although many diatoms can be grown in culture, the gradual reduction in size often results in clones dying out because conditions are unfavourable or they are obligately allogamous (out-breeders), unable to reproduce sexually in the absence of another compatible strain and thereby restore their maximum cell size. Without being able to follow strains through their entire life cycle it is also difficult to be sure that the full size range has been observed. However, if clones are autogamous (self-fertile), they may undergo sexual reproduction and auxosporulation in clonal culture enabling both ends of the size range to be compared (Cox 1985, Trobajo et al. 2006). As part of a wider ranging study of *Gomphonema parvulum* (Kütz.) Kütz. sensu lato, clones of diatoms that would be assigned to this taxon were isolated and grown in culture over extended periods of time, monitoring their morphology at intervals (Rose 2008). Four clones in this study were observed to undergo sexual reproduction and auxosporulation followed by further vegetative growth, enabling the full size range to be investigated. This paper documents our findings and discusses the taxonomic implications.

MATERIALS AND METHODS

Clones were established by isolating putative *G. parvulum* cells from mixed cultures established from field collections (table 1) growing in Woods Hole MBL medium (Nichols 1973) for 5–7 days. Under an inverted microscope, cells were micropipetted through several drops of sterile medium to wash off and minimise contaminants (bacteria, fungi and other algae) before being transferred into petri dishes with fresh MBL (pH 7.2) to grow on for 3–5 weeks in culture cabinets at 20°C, under a 16hr light: 8 hr dark regime, with an average light intensity of 15cd m^{-2} sec^{-1}. Clonal cultures were established by isolating single cells from the initial cultures in the same way. Clones were sub-cultured every 4–6 weeks.

After each sub-culturing, the old cultures were harvested, digested in cold 60% nitric acid for 48 hrs, and washed five times in distilled water with centrifugation to remove the acid. A small portion of the cleaned diatoms was dried onto a coverslip, air-dried for 24hrs and mounted in Naphrax or Zrax to produce permanent slides for light microscopy. Slides were examined under a Zeiss Axioplan and photographed using a digital camera. Identifications were based on Krammer & Lange-Bertalot (1986, 1991).

RESULTS

Four clones identified as part of *G. parvulum* sensu lato were established in culture under the above standard conditions and monitored regularly over periods of between 10 and 14 months. At the start of the observation period cells initially identified as *G. parvulum* var. *parvulum* f. *saprophilum* Lange-Bert. & E.Reichardt (Krammer & Lange-Bertalot 1991: Taf. 77, figs 5–9) (clones 40/01, 41/01, 59/01) were 16–23 µm long and 5–7 µm wide (fig. 1A–M). Cells in a clone (17/02) identified as *G. parvulum* var. *exilissimum* Grunow (Krammer & Lange-Bertalot 1986: Taf. 154, fig. 24; 1991: Taf. 76, figs 14–18) were 26–27 µm long and about 6 µm wide (fig. 1N–S). Valves in all clones were markedly heteropolar, with narrow, acutely rounded foot poles with an apical pore field, and broader, more obtuse head poles. Stria densities were 12–14 in 10 µm for the first three clones and 15–16 in 10 µm in the last clone. Approximately one month after the start of observations, the first three clones (40/01, 41/01, 59/01) were observed to undergo auxosporulation, producing cells that were up to 60 µm long and 10.5 µm wide (59 × 9.5 µm, 60 × 10.5 µm, 58.5 × 9.5 µm, respectively) (table 2), and would be identified as *Gomphonema gracile* Ehrenb. (20–100 µm long, 4–11 µm wide, 9–17 striae in 10 µm) (figs 2 & 3). Valves were more lanceolate and almost isopolar in outline, although with an apical pore field at only one end. Stria densities were a little lower than in the parent clones, 10–11 in 10 µm. The fourth clone (17/02) (fig. 4A–E) auxosporulated after about two months in culture, producing cells that were up to 54 µm long and up to 8 µm wide (table 2), and that would be identified as *Gomphonema hebridense* Gregory (fig. 4F–K), 30–60 µm long, 4–8 µm wide with about 14 striae in 10 µm. Again, valves were almost isopolar but with more rounded apices than in the first three clones.

After auxosporulation, the production of initial cells and normal vegetative cells, the average size of the clones decreased as they continued to reproduce mitotically (fig. 5), and there was a gradual shift in morphology back towards the initial appearance. Valves became more heteropolar as

Table 1 – Sources of cultures of *G. parvulum* used in this study.

Clone ID	Collected	Locality (UK grid ref.)	Habitat	Original identification
40/01	25 Sep. 2001	Kings Mere Pond, Putney (TQ 232732)	fine gravel in shallow pond	*G. parvulum* var. *parvulum* f. *saprophilum*
41/01	25 Sep. 2001	Ham Gate, Richmond Park (TQ 188717)	sandy sediment in shallow pond	*G. parvulum* var. *parvulum* f. *saprophilum*
59/01	28 Sep. 2001	Llyn Idwal, North Wales (SH 646597)	on rock in lake	*G. parvulum* var. *parvulum* f. *saprophilum*
17/02	17 Apr. 2002	Parys Mountain, Anglesey (SH 437900)	fine sediment in pond	*G. parvulum* var. *exilissimum*

Table 2 – Comparison of sizes of parental cells and first vegetative cells after auxosporulation (mean values in parentheses).

Clone	Parental cells		First vegetative cells	
40/01 Kings Mere	16–19 (17.1)	6–8 (7.0)	56–59 (57.4)	7–10 (9.3)
41/01 Ham Gate	12–21 (15.6)	6–10 (7.0)	47–50.9 (49.2)	5.6–8.4 (7.6)
59/01 Llyn Idwal	14–17.5 (16.0)	6–7 (6.5)	46–58.5 (52.2)	4.5–9.5 (8.5)
17/02 Parys Mountain	14.5–18 (16.0)	5–7 (6.2)	48–53 (51.1)	7– 8.5 (7.6)

size decreased; foot poles remained narrow, while head poles became more obtuse (figs 6 & 7).

DISCUSSION

The results of the long term studies of these clones of *G. parvulum* demonstrate clearly that cells identified as part of this species complex can, after autogamous auxosporulation, give rise to cells that would be placed in other *Gomphonema* species. This is in striking contrast to much recent work that has led to many traditional diatom species being split into several, more narrowly defined, species (Mann 1984, 1988, 1989, Medlin et al. 1991, Mann et al. 2004, Sarno et al. 2005, Zingone et al. 2005, Lundholm et al. 2012, Jüttner et al. 2013). Yet it is clear from other studies on *G. parvulum*, that these diatoms present problems of specimen discrimination and identification, showing both inter- and intra-strain variation, and while some could be matched to named entities, others could not be identified (Kermarrec et al. 2013, Abarca et al. 2014).

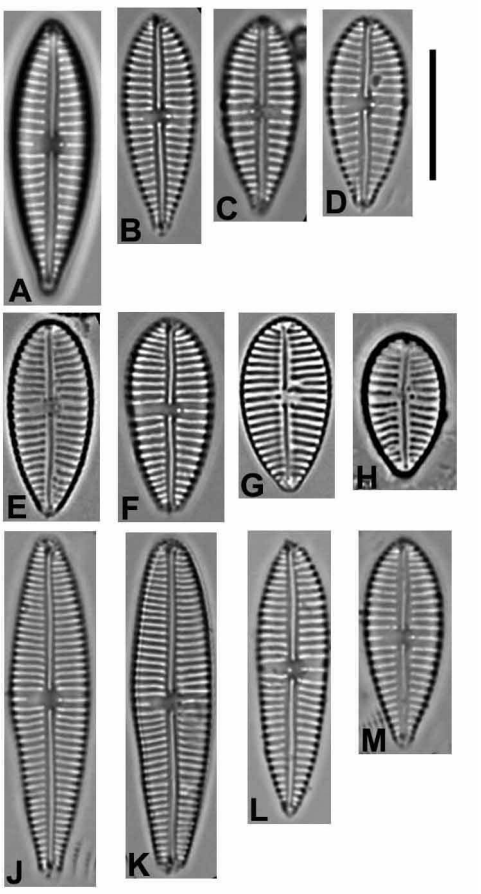


Figure 1 – Light micrographs of smallest cells from clones 40/01, 41/01 and 59/01. A–D, 40/01; E–H, 41/01; J–M, 59/01. Scale bar represents 10 µm.

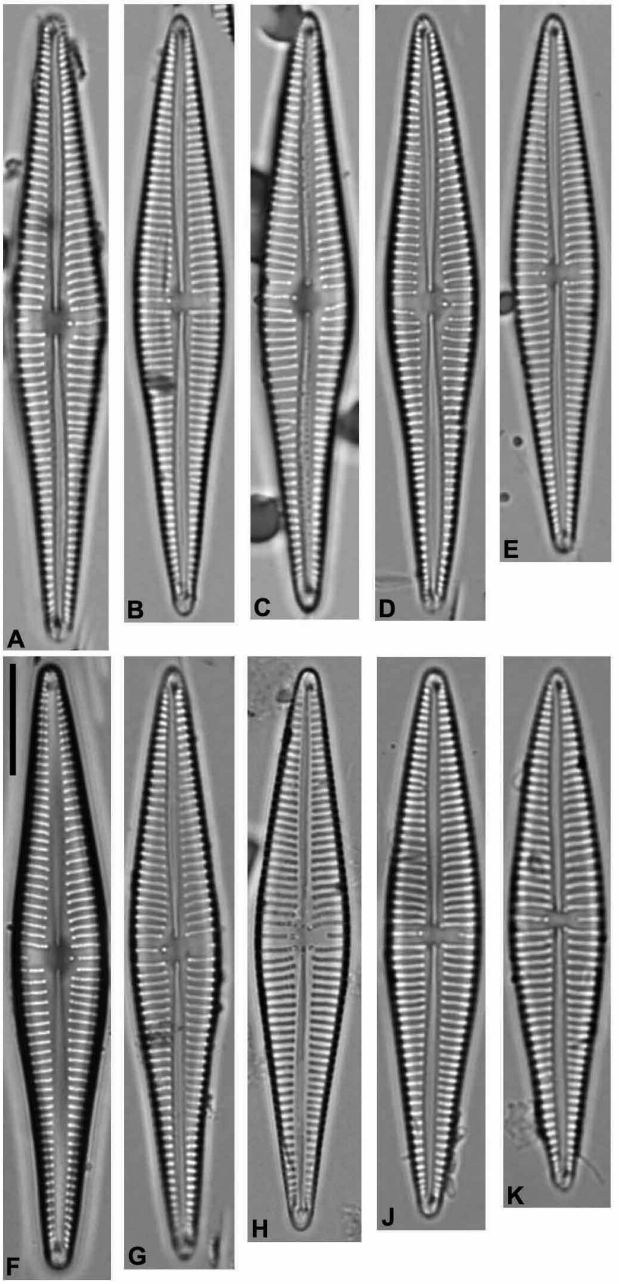


Figure 2 – Light micrographs of largest cells from clones 40/01 and 41/01. A–E, 40/01; F–J, 41/01. Scale bar represents 10 µm.

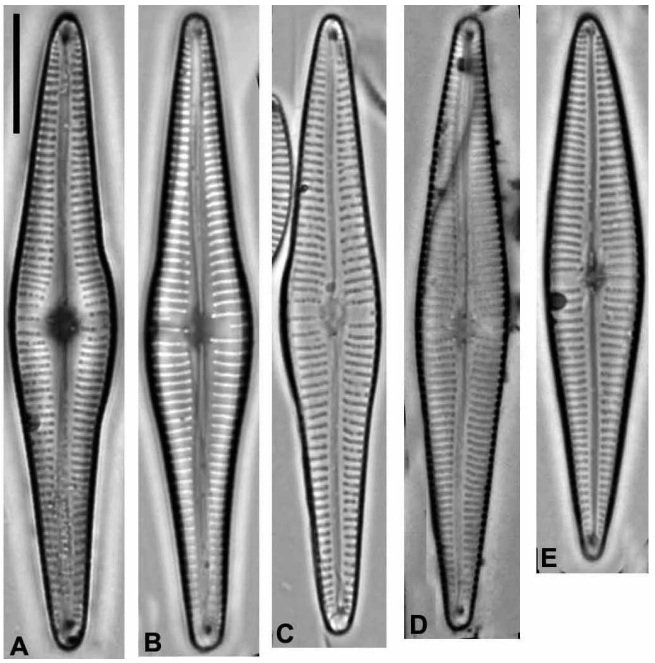
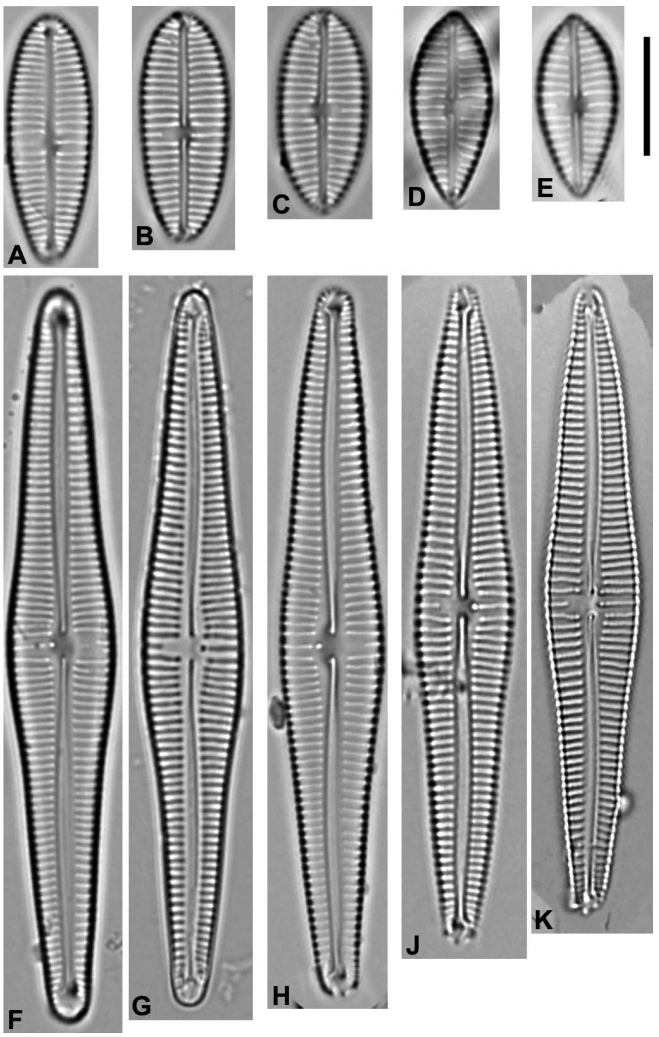


Figure 3 – Light micrographs of largest cells from clone 59/01 (A–E). Scale bar represents 10 µm.



◄ **Figure 4** – Light micrographs of smallest (A–F) and largest (G–L) cells from clone 17/02. Scale bar represents 10 µm.

While Kermarrec et al. (2013) reported the occurrence of auxosporulation in some of their cultures, they did not comment on the morphology of its products. Similarly, Abarca et al. (2014) only illustrated and described small cells; no links to other species were suggested. It may be that their cultures did not undergo auxosporulation; the authors do not indicate how long their clones were in culture.

Observing material from the Savannah River, Hohn (1959) recorded the size differences between mother cells and auxospores of two varieties of *G. parvulum*. *Gomphonema parvulum* var. *parvulum* had mother cells that were 14.6–19.2 µm long and auxospores that were 31.1–35.8 µm long, while *G. parvulum* var. *micropus* (Kütz.) P.T.Cleve mother cells were 21.8–27.5 µm long and their auxospores 45.2–54.5 µm long, a similar size change to that in our fourth clone (17/02). Geitler (1958) noted that two strains of *G. parvulum*, tentatively identified as var. *micropus*, had mother cells that were 20–24 µm and 13–17.5 µm long, that gave rise to initial cells that were 40–44 µm and 36–40 µm long respectively, while a third strain (resembling *G. parvulum* var. *exilissimum*) had even smaller mother cells (10–12.5 µm long) and initial cells that were 30.5–33 µm long. Hohn (1959) also noted that auxospores (and presumably initial cells) were only slightly asymmetrical about the transverse axis, whereas the vegetative cells were distinctly asymmetrical (i.e. heteropolar); Geitler's illustrations (1958) show the same phenomenon.

Although Geitler (1958) and Hohn (1959) demonstrated that *G. parvulum* cells could exceed the typically cited sizes, the range given by Krammer & Lange-Bertalot (1986) (10–36 µm long, 4–8 µm wide) effectively encompasses what would be expected for this complex. Abarca et al. (2014) summarized *G. parvulum* as a diatom that is 25 µm long and 6 µm wide. On the other hand, despite giving a maximum length for initial cells of 36–40 µm, Krammer & Lange-Bertalot (1986) did suggest a potential link between *G. parvulum* and the *auritum* form of *G. gracile*. However, to our knowledge, no link between *G. parvulum* and *G. hebridense* has been mooted; but the distribution of the latter seems to be poorly known (Krammer & Lange-Bertalot 1986) and it may therefore be unfamiliar to many.

As noted by Hohn (1959), the degree of valve heteropolarity is greater in smaller vegetative cells than at the upper end of the size range. This highlights differences in the constraints on valve shape with size reduction in *Gomphonema* compared to some other genera. Whereas while length:breadth ratios change with size reduction, isopolar and bilaterally symmetrical diatoms valves remain symmetrical (cf. Cox 1986). Dorsiventrality may become more marked in genera such as *Cymbella* Agardh, *Epithemia* Kütz. and *Eunotia* Ehrenb. (among others), but nevertheless these remain isopolar (cf. Geitler 1958, Geitler & Mack 1953, Geitler 1985, Steinman & Sheath 1984, Steinman & Ladewski 1987). However, in heteropolar diatoms, while foot pole shape is maintained, head poles can show greater variation and there is a striking shift in shape with size reduction (Mann 1982, Williams 1985). Presumably this is

linked to the presence, in most heteropolar diatoms, of some kind of apical pore field at the foot pole, through which polysaccharide can be excreted to attach the cell to a substratum. If this function is to be maintained, the apical pore field must be consistently developed in each new valve, whereas the absence of an apical pore field at the head pole means there is no functional constraint on head pole shape.

Our results also raise some questions about the inferred ecological tolerances of *G. parvulum* v. *G. gracile* and *G. hebridense*. Whereas *G. parvulum* had been suggested as indicative of organic pollution (Kelly & Whitton 1995), it has also been noted that parts of that species complex occur in oligotrophic waters (Krammer & Lange-Bertalot 1986, Jüttner et al. 2013, Abarca et al. 2014). On the other hand, *G. gracile* and *G. hebridense* are usually considered more typical of less eutrophic and lower conductivity waters (Krammer & Lange-Bertalot 1986). However, inferred tolerances for these taxa differ across European indicator systems. Thus, according to the IPS (the "Indice de Polluosensibilité") (Coste in

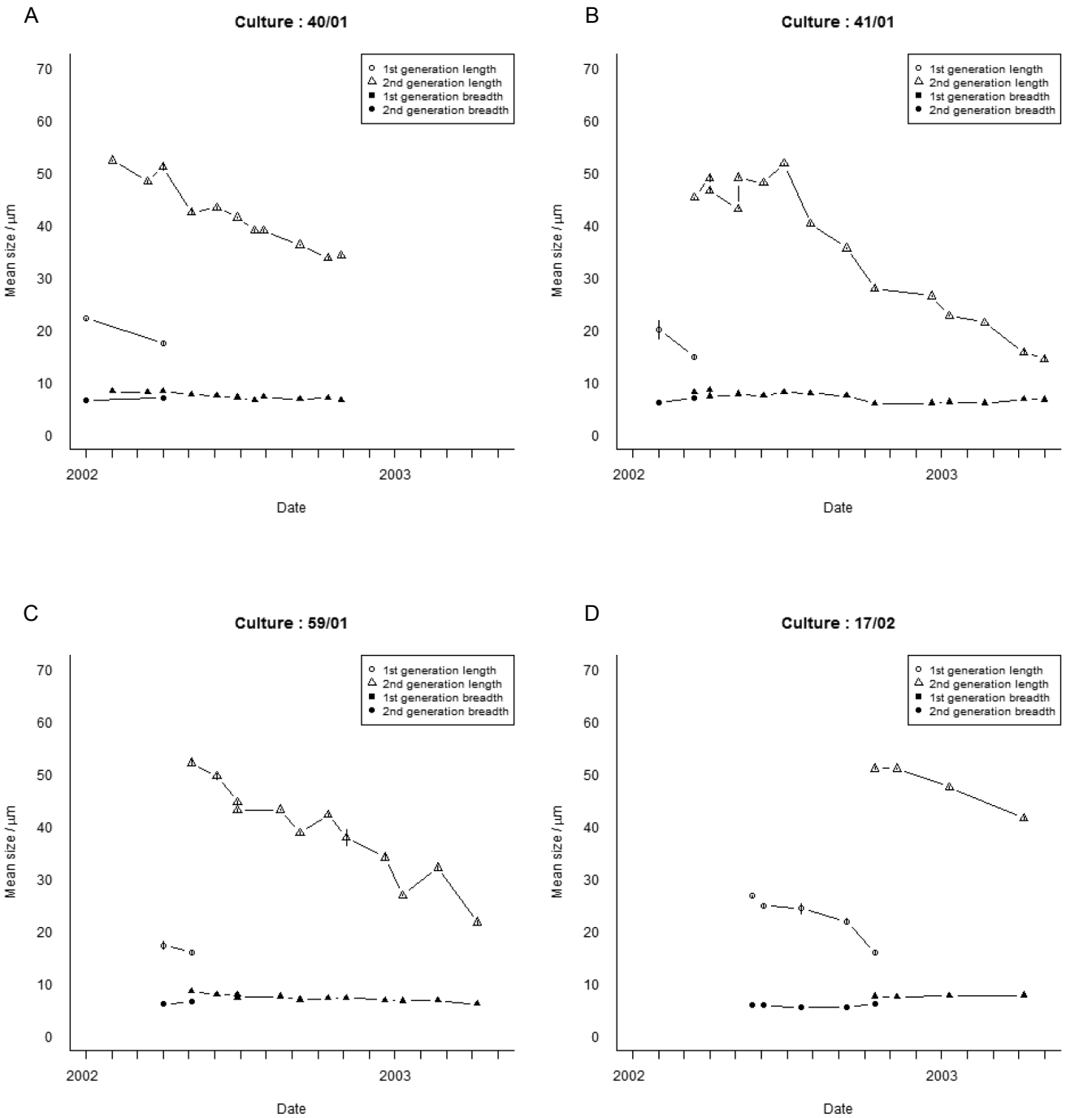


Figure 5 – Graphs of average length-breadth changes in clones over time. A, 40/01; B, 41/01; C, 59/01; D, 17/02.

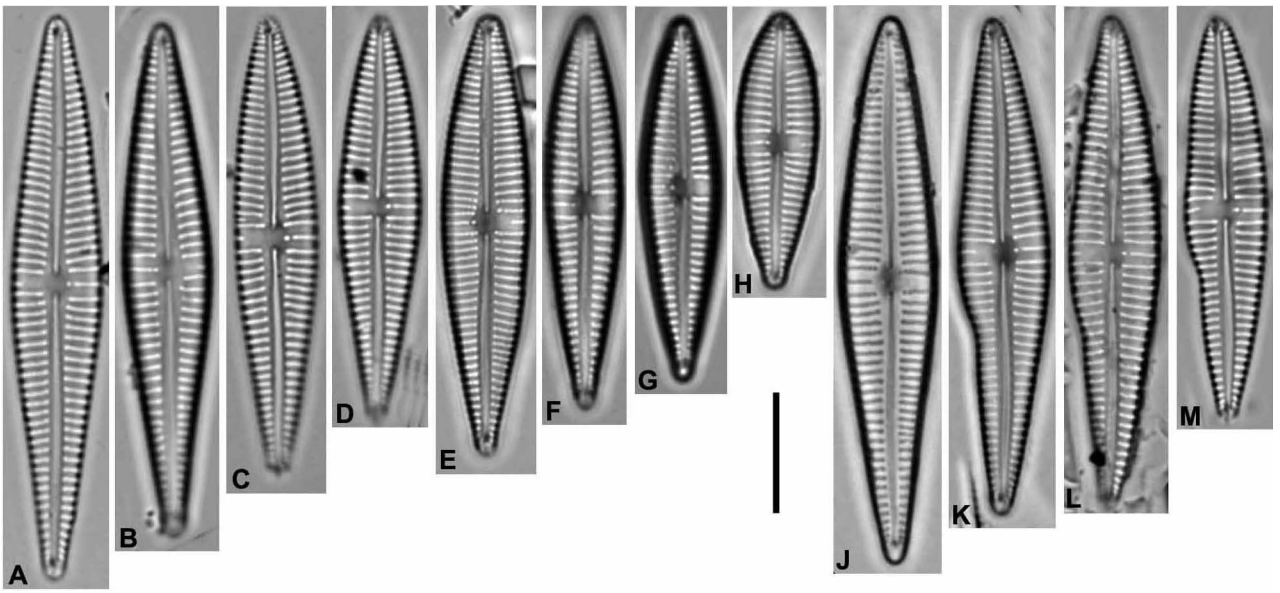


Figure 6 – Light micrographs of intermediate sized cells from the different clones. A–D, 40/01; E–H, 41/01; J–M, 59/01. Scale bar represents 10 µm.

CEMAGREF 1982) *G. gracile* is considered very sensitive to pollution, whereas according to the Trophic index in Austria it is considered tolerant of moderate to heavy organic pollution (Rott et al. 1999). While these indices may have been developed in different areas for different purposes, this still seems a marked disparity. If part of *G. parvulum* sensu lato is conspecific with *G. gracile*, and part is conspecific with *G. hebridense*, the ecological tolerances of all specimens within those strains would be expected to be comparable.

Demonstration that the morphological range of these taxa exceeds the classical definition of *G. parvulum*, and that one is linked to *G. gracile*, the other to *G. hebridense*, means that all these species definitions need emending, and nomenclatural changes will be necessary. However, this can only be finalised after close examination of all relevant type material. How these relate to strains that have been investigated using molecular techniques remains uncertain. Abarca et al. (2014) designated some of their morphodemes as *G. saprophilum* (Lange-Bert. & E.Reichardt) Abarca et al. (= *G. parvulum* var. *parvulum* f. *saprophilum*), but based on Krammer & Lange-Bertalot (1991: Taf. 76, figs 8–13), not their subsequent figures (Krammer & Lange-Bertalot 1991: Taf. 77, figs 5–9) to which our first three clones (40/01, 41/01, 59/01) were matched. Therefore it is uncertain whether or not our first three clones belong to *G. saprophilum* sensu Abarca et al. There may be similar uncertainty about the identity of our fourth clone when compared to type material of *G. exilissimum* (Grunow) Lange-Bert. & E.Reichardt (Jüttner et al. 2013).

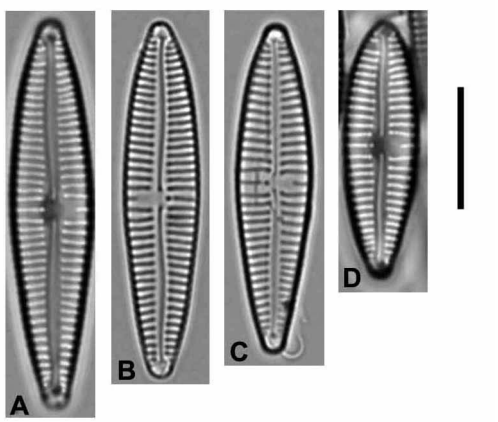


Figure 7 – Light micrographs of intermediate sized cells from 17/02. Scale bar represents 10 µm.

As has happened for other diatoms, shifts in the morphological concept of a species can occur over time, since most individuals inevitably and necessarily use published images to identify taxa rather than checking back to the original type specimens. Those published images may also represent only part of the morphological spectrum, further skewing the concept. Studies of type material can also reveal that names are now applied to completely different taxa, requiring either that the morphological concept associated with a name be changed (e.g. Ross & Håkansson 2000), a new species be described [as Abarca et al. (2014) have done for *G. saprophilum*] or a case be made for the conservation of the name (Cox & Ross 2004). However, our results revealing the striking differences between mother and first vegetative cells in these clones indicate that taxonomic revision of the *G. parvulum* complex will require more than simple examination of type specimens. Full documentation of the life cycle and any associated morphological variation will also be required. Molecular studies could help link different stages in the life cycle, while both the distribution and ecology of parts of this complex will require careful investigation if reliable ecological inferences are to be made from their occurrence.

The traditional focus on rather simple morphometric evaluations of cleaned diatom valves from variously collected, geographically dispersed, samples to define and

identify diatom species assumes more or less faithful valve replication with each cell division and minimal morphological change with size reduction. Yet it is clear that allometric size reduction can modify the relationships between different valve features (Theriot & Stoermer 1981, Theriot 1988, Beszteri et al. 2005), and simple generalisations about shape change with size reduction cannot be made (Cox 2014 and references therein). There is also increasing evidence that valve morphology in a range of diatoms can vary within a strain (Kistenich et al. 2014) and be modified by changing environmental conditions (Balzano et al. 2011, Trobajo et al. 2011, Erga et al. in press). The suggestion that ratios of maximal to minimal measurements can indicate whether or not specimens are likely to form part of a 'good' species or of a species complex (Krammer 2002), while perhaps logically attractive, is not supported by empirical evidence. Thus, average maximal:minimal length ratios for our clones are between 3.2 and 3.5, clearly above Krammer's 2.5 ratio for a 'clean' taxon, whereas similar ratios for breadths are lower than his maximum ratio of 1.5, i.e. 1.1–1.3.

In conclusion, it is increasingly important that we obtain empirical data on the size spectra of individual strains, that we move beyond simple measurements and stria counts to discriminate between taxa [cf the use of morphometrics by Poulícková et al (2010)], and that the effects of environment in generating different phenotypes from single genotypes are documented (Cox 2014).

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