Cryptogamie, Algol., 2002, 23 (3): 343-355

Morphological details of the life history of *Alexandrium minutum* (Dinophyceae)

Ian PROBERT [a†], Jane LEWIS [a] & Evelyne ERARD-LE DENN [b]*

[a] School of Biosciences, University of Westminster, 115 New Cavendish Street, London W1M 8JS, U.K.

[†] current adress: LBBM, Université de Caen, 14032 Caen, France

[b] IFREMER Centre de Brest, 29280 Plouzané, France

(Received 26 March 2001, accepted 13 March 2002)

Abstract — Different life stages and the processes of asexual division and sexual fusion of the toxic dinoflagellate *Alexandrium minutum* are reported. Asexual division is oblique, with the two identically sized daughter cells sharing the parent theca and synthesizing the remaining plates. As in many dinoflagellate species, gametes are indistinguishable from vegetative cells prior to mating. During gamete fusion, which is initiated by flagellar attachment, a wide range of relative gamete orientations were observed. The longitudinal flagella of resultant motile planozygotes are not necessarily situated adjacent to each other, and planozygotes have thus perhaps not been recognised in previous studies which used this characteristic for identification. There are similarities between the life histories of *A. minutum* and the closely related species *A. tamarense*. Dinoflagellates exhibit various modes of reproduction and the details of life histories which may cause confusion are highlighted.

dinoflagellate / *Alexandrium minutum* / life-cycle / marine microalgae / reproduction / morphology

Résumé — Détails morphologiques du cycle de vie *d'Alexandrium minutum* (Dinophyceae). Les différentes étapes et les processus de la division asexuée et de la fusion sexuée du dinoflagellé toxique *Alexandrium minutum* sont décrits. La division asexuée est oblique, avec deux cellules sœurs identiques partageant la thèque de la cellule-mère et synthétisant les plaques restantes. Comme pour d'autres espèces de dinoflagellés, les gamètes ne sont pas identifiables parmi les cellules végétatives avant qu'elles ne commencent à fusionner. Pendant leur fusion, initiée par l'attachement des flagelles, l'orientation respective des gamètes est très variable. Les flagelles longitudinaux du planozygote ne sont pas obligatoirement situés l'un à côté de l'autre. Les planozygotes n'ont peut-être pas été reconnus dans les études précédentes qui ont utilisé cette caractéristique pour les identifier. Il existe des similarités entre les cycles de vie d'*A. minutum* et de l'espèce très voisine *A. tamarense*. Il y a cependant plusieurs styles de reproduction chez les dinoflagellés, et les détails de ces cycles de vie qui peuvent sembler confus sont soulignés dans cette étude.

***Alexandrium minutum* / cycle de vie / dinoflagellé / microalgues marines / morphologie / reproduction**

* Correspondence and reprints: IanProbert@hotmail.com

INTRODUCTION

Since *Alexandrium minutum* Halim was first described from Alexandria harbour, Egypt (Halim, 1960), it has been reported from coastal waters in various locations in the northern hemisphere (reviewed by Nehring, 1994), as well as from Australasian waters (Bolch *et al.*, 1991; Chang *et al.*, 1995). Associations with paralytic shellfish toxin (PST) contamination have been demonstrated in Europe and Australasia (e.g. Franco *et al.*, 1994; MacKenzie & Berkett, 1997), and the production of PSTs by laboratory cultures of A. minutum has been confirmed using various methods (Oshima *et al.*, 1989; Flynn *et al.*, 1994; Franco *et al.*, 1994; Chang *et al.*, 1996).

The *A. minutum* vegetative cell and hypnozygote cyst have been described from several locations (Balech, 1995; Erard-Le Denn, 1991; Bolch *et al.*, 1991), but the morphological details of other life stages (gametes and planozygotes), and of the processes of asexual and sexual reproduction have not been reported. While increase in population size and the formation of zygote stages are indicative of the action of the two reproductive cycles, few detailed morphological descriptions of complete dinoflagellate reproductive cycles exist, with confusion arising from the fact that dinoflagellates exhibit various modes of asexual and sexual reproduction. Aspects of the life cycle of *A. tamarense* have been described (Anderson & Lindquist, 1985; Fritz *et al.*, 1989), but while similarities between the life histories of these two closely related species are likely to exist, there are presently not enough detailed reports to determine whether patterns can be elucidated within dinoflagellate genera.

MATERIALS AND METHODS

The stock *A. minutum* culture (AM89BM) maintained at the IFREMER laboratory in Brest was initiated from a single vegetative cell isolated from a sample collected during a bloom in the Morlaix estuary, Brittany, in 1989. Batch cultures were maintained at temperatures of 16°C and 20°C, with overhead illumination of 100 $\mu Em^{-2}s^{-1}$ in a 14:10 hour light/dark (L:D) cycle. Cultures were grown in modified K medium (Keller *et al.*, 1987), containing a range of N and P nutrient concentrations (between K and K/40).

Cells undergoing reproduction (two cells 'attached' in any manner) were selected under a binocular microscope and micropipetted into separate wells in a multi-well plate containing medium of the same nutrient concentration as the parent culture, and maintained under identical conditions to the parent culture. The cells were monitored twice daily over a period of several days in order to determine whether they subsequently divided or fused.

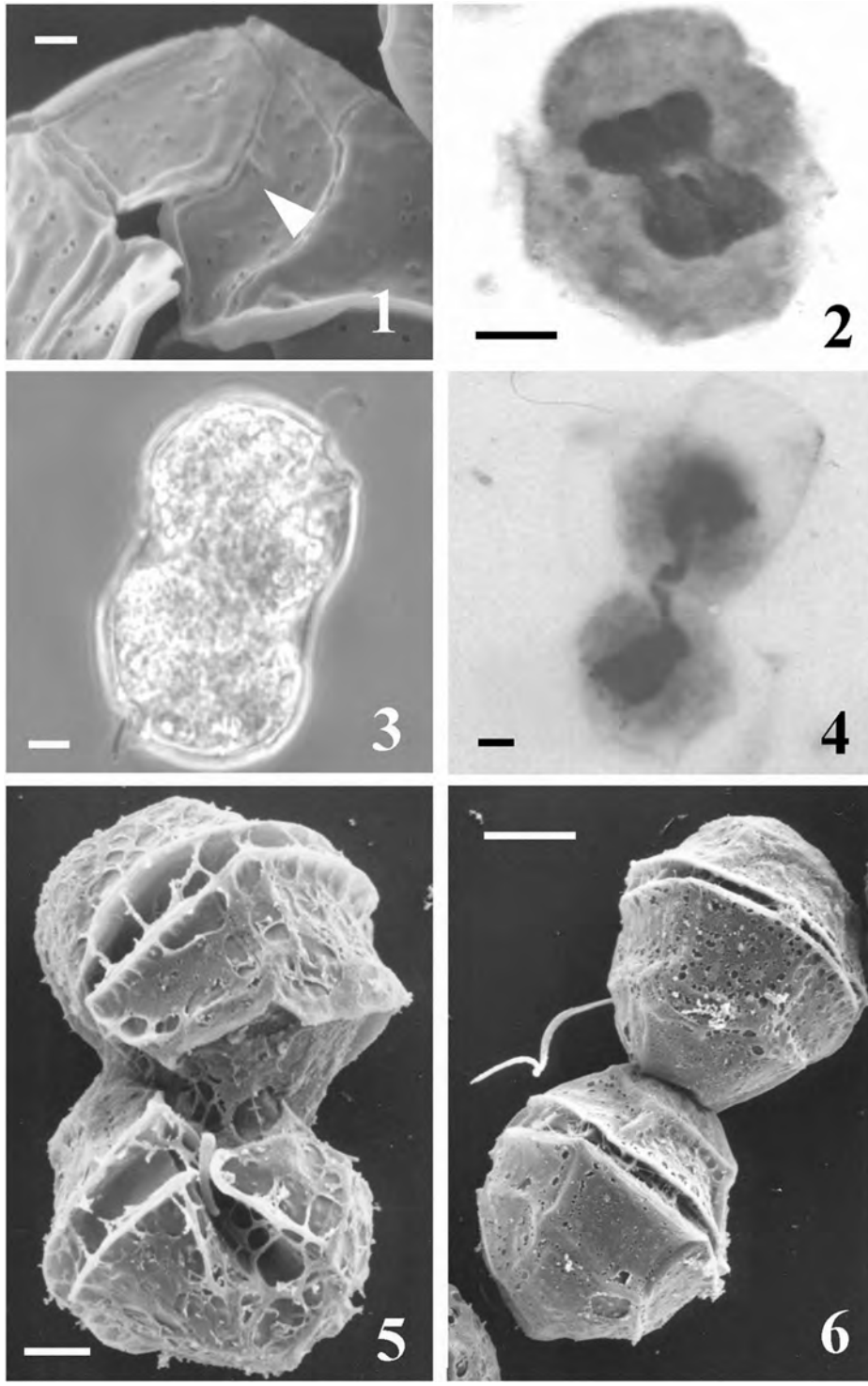
Live dividing/fusing cells were video recorded through an Olympus BH2 microscope and the images studied under slow motion to record morphology and behaviour of cells. A number of methods for slowing the swimming speed of cells to facilitate observation were tried (e.g. nickel sulphate, polyethylene oxide, agar, gelatine), but all methods caused cells to rapidly shed their flagella, and often to lyse. The dimensions of recorded images of cells were measured using Global Lab image analysis software. Unfixed cells and cells fixed in osmium tetroxide (2 % in filtered seawater) were photographed under bright field and phase contrast illu-

mination using an Olympus C-35AD camera through the Olympus BH2 microscope. The protargol silver staining technique of Montagnes & Lynn (1987) was employed to follow nuclear changes and the positioning of basal bodies through the reproductive cycles. Cells were prepared for the scanning electron microscope (SEM) following the methodology of Takayama (1985), a process developed for the preparation of fragile specimens. The preparations were gold/palladium sputter coated (Polaron E5100 Series II 'Cool' Sputter Coater), and viewed and photographed through an Hitachi S-2500 SEM.

RESULTS

The vegetative cells of strain AM89BM are small (typically between 17 and 26 μm long, absolute range 14-30 μm), more or less oval in ventral view, and are generally slightly longer than wide. The shape and arrangement of thecal plates confirmed this strain as *A. minutum*, although the ventral pore in the 1' plate, which Balech (1989) states is always present in this species, was consistently absent (Fig. 1). Live cells are pigmented a light brown-orange colour. The nucleus of the cell in the vegetative state is typically crescent shaped and situated in the central, hypothecal region of the cell.

In the earliest stage of asexual division the crescent shaped nucleus becomes ovoid and migrates to the centre of the cell. The mitotic division of the nucleus is initiated before any external signs of cellular division are apparent. The flagellar basal bodies replicate at this early stage in such a way that each new cell will subsequently possess one old and one new basal body. Nuclear division is oblique, with one newly forming nucleus migrating towards the top corner of the cell and the other towards the opposite bottom corner (Fig. 2). Throughout mid- and into late division, the dividing cells are enveloped by one outer cell membrane (Fig. 3). It is unclear whether, and exactly at which point, this membrane is either cleaved and shared between daughter cells, or shed and the two new cells form separate outer membranes. The flagella have replicated by this mid-division stage, such that each new cell possesses both transverse and longitutinal flagella. The nuclei move progressively apart, but typically remain attached by a thin projection of nuclear material until late in the division process (Fig. 4). The thecal plates are retained as the cell divides diagonally (Fig. 5). As division proceeds the two cells move apart and new thecal plates are synthesised. The basal bodies move further apart as, by late division, the cells appear 'stacked' (Fig. 6), with the hypothecal tip of one cell attached to the epithecal tip of the other in the region of the apical pore. The nuclei finally detach and the last thecal plates are synthesised. The two new cells are always identical in size. The total time for the division process up to this late 'stacked' stage is less than 1 hour, but the cells may remain in this last stage for considerably longer. Throughout the division process the direction of swimming is straight, whereas during early and mid-division swimming velocity is relatively slow, towards late division the 'stacked' cells swim much faster, with speeds comparable to single vegetative cells. 'Phased' cell division was observed in *A. minutum* cultures. Hourly observations of the percentage of a culture population in division (counts made through the light and dark cycles of a culture in the exponential growth phase) indicate a trend of increased division rate in the dark phase, with a peak (7.4 %) 2 hours before the onset of the light phase. Cells isolated at any stage of the division process described were always observed to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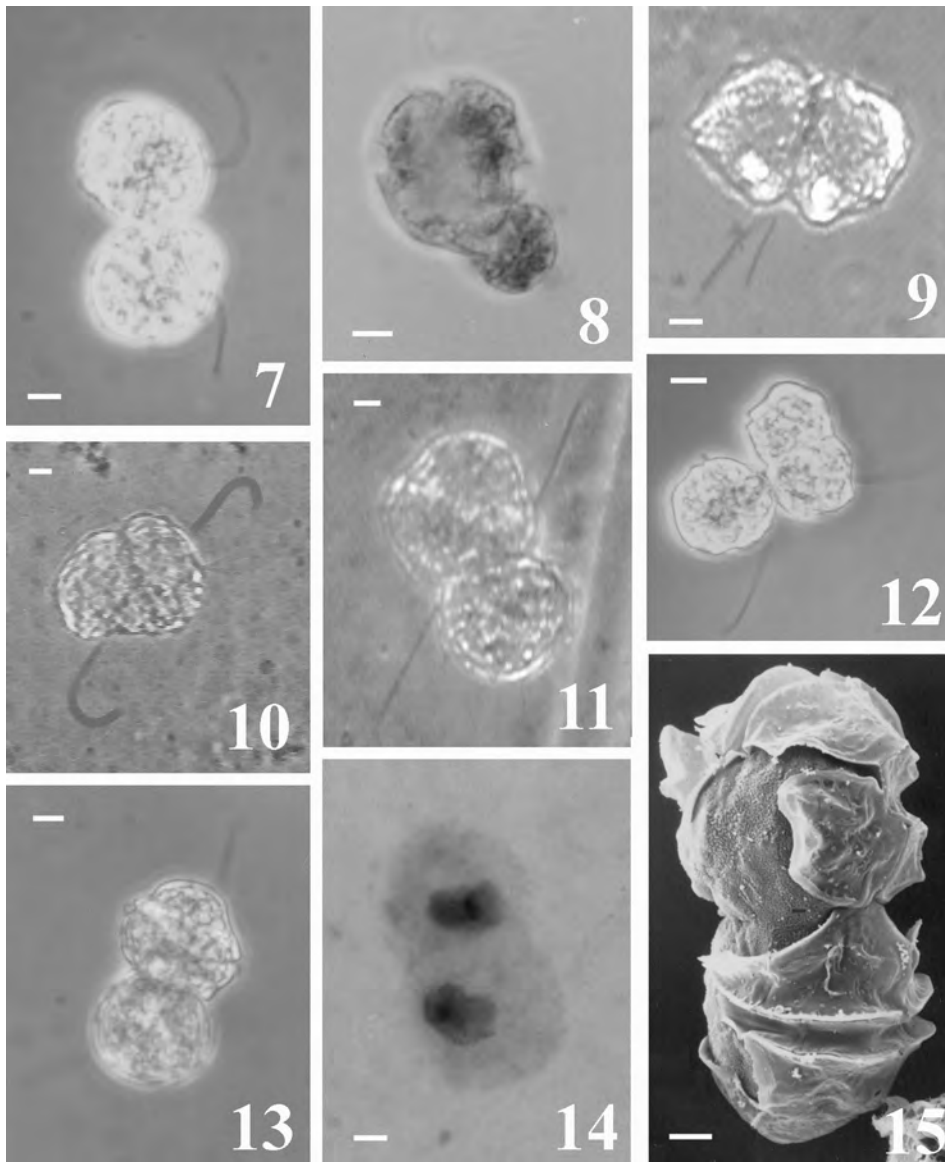
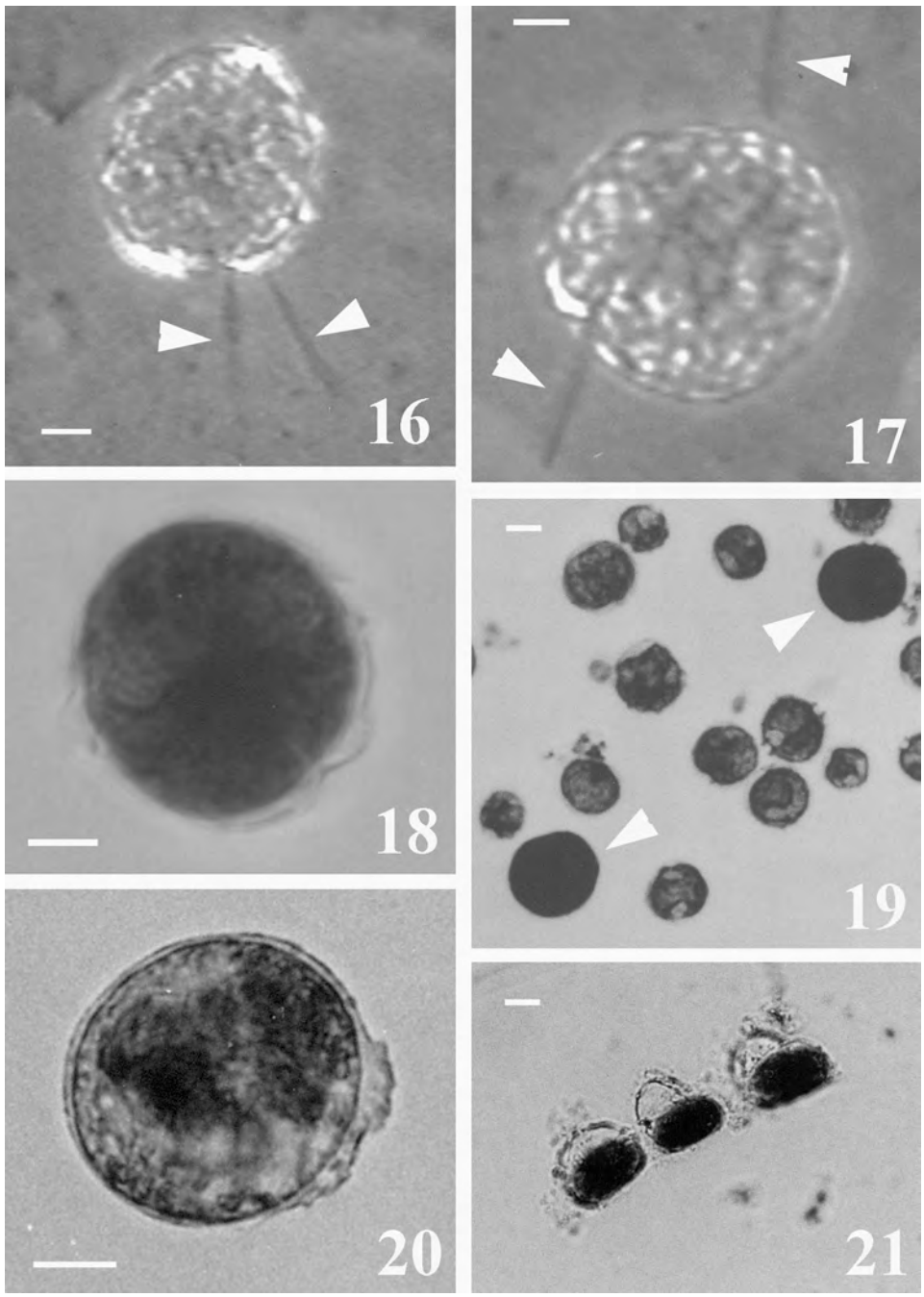
subsequently separate to two cells, confirming these observations as asexual reproduction. The average size of newly formed cells was 20.04 μm, s.d. 1.23 μm (n = 320). The average size of cells immediately prior to division was 25.16 μm, s.d. 1.44 μm (n = 140).

Mating gametes generally constituted a low proportion of culture populations. Mating may be between cells of similar size (Fig. 7), or of differing sizes (Fig. 8). In live cultures, two cells were occasionally observed in close proximity to each other swimming in tight circles, apparently attached by means of the longitudinal flagella of one of the cells. During the initial stages of gamete attachment the cell nuclei remain crescent shaped, and there are no obvious morphological differences from vegetative cells. The initial contact between cells is always between the hypocone of one cell and the sulcal region of the hypocone of the other. There does not appear to be, however, a set pattern for the orientation of the two cells with respect to each other. The cells may be joined cingulum:cingulum (Fig. 9), cingulum:antapical tip (Figs 10, 13), through to antapical tip:antapical tip (Fig. 11). The relative orientation of cingula ranges from approximately parallel (Fig. 9) to perpendicular (Figs 7, 13), and may be skewed to an oblique angle, and, notably, cells may be orientated inversely (Fig. 11). Occasionally three cells were observed in the process of fusion (Fig. 12 shows two dividing cells attached to another cell). Initial cellular fusion is between the hypocones of the two gametes. During this phase the nuclei become more or less ovoid, but remain apart while plasmogamy continues (Fig. 14). Some thecal plates at the point of contact between the gametes are shed as fusion progresses (Fig. 15). The relative orientation of gametes at the initial point of contact evidently affects aspects of subsequent fusion. A certain degree of reorientation of some cell configurations occurs early in fusion, allowing the hypocones to come into greater contact. Hypocone fusion is often observed to be almost complete before the epicones begin to fuse, and one of the transverse flagella is lost during this stage. After fusion of the epicones, gamete fusion is concluded when the nuclei fuse and the basal bodies come into close proximity, resulting in the formation of a motile planozygote. Throughout the process of gamete fusion the velocity of swimming is slow, the direction being dependent on relative orientation of gametes. The average size of gametes was 23.43 μm, s.d. 4.08 μm (n = 240), the size range being similar to that of vegetative cells. Some of the live pairs of cells isolated in very early fusion were observed to separate, presumably as the flagellar attachment was affected by agitation during isolation, but all cells in which cellular fusion had commenced were observed to remain paired (although in some cases fusion did not progress after isolation), confirming these observations as gamete fusion. Successful gamete mating of isolated pairs in multi-well plates sometimes took as long as seven days, but the average duration of fusion from early gamete attachment to the formation of a motile planozygote (based on twice daily external morphological observations) was two days (n = 214). The longitudinal flagella of the motile planozygote may or may not be situated adjacent to each other as has typically been reported for dinoflagellate planozygotes (Figs 16, 17). During this phase the cell swims very

◀ Figs 1-6. *Alexandrium minutum* vegetative cell and asexual division. Fig.1. 1' plate of vegetative cell lacking ventral pore (arrowhead) (SEM). Fig. 2. Early division (LM Protargol stained specimen). Fig. 3. Early division: cells surrounded by membrane (LM phase contrast). Fig. 4. Mid/late division: nuclei attached by thin projection (LM Protargol stained specimen). Fig. 5. Mid division: cells share plates diagonally (SEM). Fig. 6. Late division: 'stacked' cells (SEM). Scale bars: Fig 1 = 1 μm, Figs 2-6 = 5 μm.



Figs 7-15. Gamete fusion. Fig. 7. Isogamous mating (LM phase contrast). Fig. 8. Anisogamous mating (LM). Fig. 9. Hypocones fusing, cingula approximately parallel (still frame from video recording). Fig. 10. Perpendicular orientation (still video frame). Fig. 11. Inverse orientation (still video frame). Fig. 12. Three cells attached (LM). Fig. 13. Perpendicular orientation (still video frame). Fig. 14. Nuclei remain apart during fusion (LM Protargol stained specimen). Fig. 15. Some plates lost as fusion commences (SEM). Scale bars: Figs 7-11, 13-15 = 5 μm, Fig 12 = 10 μm.



Figs 16-21. Panozygotes and hypnozygote cysts. Fig. 16. Motile planozygote with adjacent longitudinal flagella (still video frame). Fig. 17. Motile planozygote with longitudinal flagella situated on opposite sides of cell (stillvideo frame). Fig. 18. Late non-motile planozygote (LM). Fig. 19. Larger, darker planozygotes (arrows) among vegetative cells (LM). Fig. 20. Hypnozygote cyst, top view (LM). Fig. 21. Hypnozygote cysts, side view (LM). Scale bars: Figs 16-18, 20 = 10 µm, Figs 19, 21 = 15 µm.

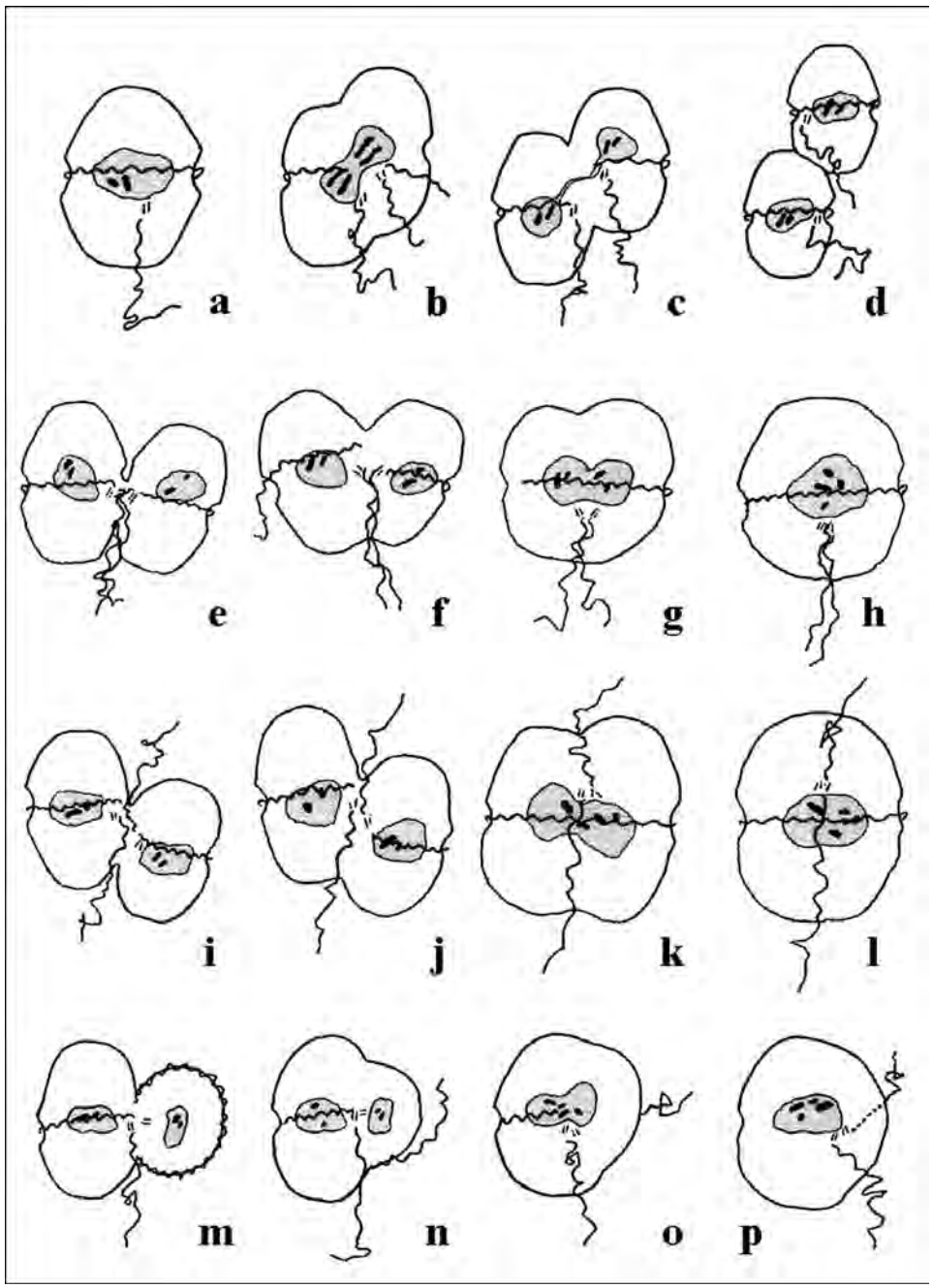


Fig. 22.

slowly and is often found towards the bottom of the culture vessel. The planozygote may remain motile for several days, becoming darker, before losing flagella (Figs 18, 19). Average planozygote size was 31.18 μm, s.d. 3.28 μm (n = 325).

The transformation of the non-motile planozygote to the hypnozygote resting cyst stage was never observed in this culture strain. Hypnozygote cysts were observed in sediments from the Abers of northern Brittany from which this species was isolated. The clear, mucoid cysts are roughly oval in shape when viewed from above (Fig. 20), and reniform when viewed from the side (Fig. 21). The cyst has a distinctive thick cell wall which surrounds the cytoplasm, which contains numerous storage granules and, in mature specimens, a single orange-red accumulation body. Prior to excystment the accumulation body becomes less obvious, and Brownian motion can clearly be seen in the granular cytoplasm. Hypnozygote size ranged from 25-33 μm, with an average value of 29 μm, s.d. 1.5 μm (n = 45).

The processes of cell division and gamete mating and fusion are diagrammatically illustrated in Fig. 22.

DISCUSSION

Asexual division

Walker (1984) states that in dividing pairs the cingula are usually parallel to each other. In late division, however, cingula are often reported to be orientated perpendicularly (e.g. *Scrippsiella* sp., Gao *et al.*, 1989; *Gyrodinium uncatenum*, Coats *et al.*, 1984). In *A. minutum*, division is oblique and the cingula are parallel throughout. Division in the related species *A. tamarense* (Anderson & Lindquist, 1985; Destombe & Cembella, 1990) also follows this pattern. The two newly forming cells share the thecal plates from the parent cell obliquely, and each cell synthesises the remaining plates during division (desmoschisis). The two new cells are always identical in size; there is no evidence of the budding division which results in cells of unequal size observed in some dinoflagellate species (e.g. *Gymnodinium* cf. *nagasakiense*, Partensky & Vaulot, 1989). It is unclear whether there is any advantage for the newly formed cells in remaining 'stacked' after division is apparently completed. When such cells are manipulated with a micropipette they typically separate, indicating that plate synthesis, and hence division, is indeed complete. As noted, cells in this configuration swim rapidly, although it is not known whether the swimming velocity is faster relative to that of the individual cells, a factor which could convey some advantage with respect to migration to optimal growth environments.

The sexual cycle

As in the asexual phase, the sexual cycle differs in details among dinoflagellate species. While certain trends are becoming evident, it must be noted that sexual reproduction has, as yet, only been observed and documented in a fraction of the known dinoflagellate species. *A. minutum* gametes remain thecate and vary in size over a range similar to that of vegetative cells. As in many other species, gametes appear morphologically and morphogenetically indistinguishable from vegetative cells. Anderson & Lindquist (1985) report that gametes of *A. tamarense*

are morphologically indistinct from vegetative cells, and note that it is not known whether gametes are formed by division of vegetative cells or whether vegetative cells are capable of sexuality directly. Fritz *et al.* (1989), in contrast, reported that *A. tamarense* gametes are somewhat smaller and less heavily pigmented than vegetative cells. *A. minutum* fusing gametes may be isogamous or anisogamous. In some strains of *Alexandrium* assignable to *A. tamarense* or *A. excavatum*, Destombe & Cembella (1990) report that both isogamy and anisogamy have been observed. The majority of dinoflagellate species studied, however, exhibit either one mode or the other.

From this study there is evidence that the initial contact between *A. minutum* gametes is through flagellar attachment as described for *Scrippsiella* sp. by Gao *et al.* (1989). In this process the transverse flagellum of one gamete migrates out of the girdle and grasps the longitudinal flagellum of the other before returning to the girdle. The two gametes subsequently move together, perhaps by helical movement of the transverse flagellum of the first gamete being pulled back into the girdle over the longitudinal flagellum of the second. This process is rapid, and hence will seldom be observed; in the present study two cells were occasionally observed swimming in tight circles in close proximity to each other, clearly linked by a flagellum. Gao *et al.* (1989) did not observe inverse orientation of *Scrippsiella* sp. gametes, and hence in this species there is presumably some mechanism by which the gametes are able to determine relative orientation. This mechanism must be related to the initial contact between the gametes, the flagellar attachment process described above. Gao *et al.* (1989) suggest that flagellar hairs may play an important role in the flagellar attachment process by providing better grip between the flagella, and that there may be macromolecules at the flagellar surface which act as cell recognition factors. There is perhaps some structural or biochemical difference between the flagella of the two species, which would account for the fact that flagellar attachment in this strain of *A. minutum* is not specific with respect to relative cell orientation.

Gao *et al.* (1989) summarised reports of sexual reproduction in dinoflagellate species, and concluded that they can be broadly divided into two groups. In group 1 gamete fusion starts in the sulcal region, and in the early stages the cingula are approximately parallel. In group 2 the gametes are thought to be oriented at 90° to one another and fuse such that the anterior apex of one is attached to the sulcal region of the other. Walker (1984) suggested that fusing pairs can generally be distinguished from dividing pairs since the cingula are perpendicular during fusion (i.e. group 2), but Gao *et al.* (1989) conclude that the observations on species in group 2 may need re-examination in order to determine whether cell division has been misconstrued as gamete fusion, or whether an alternate method of gamete mating and fusion exists in these species. Mating gametes of *A. minutum* do not fall directly into either of the categories outlined by Gao *et al.* (1989). Initial fusion is always between the hypocones (i.e. posterior) of each cell, and hence this species cannot be classed in group 2. The situation with respect to *A. tamarense* is confused; Fritz *et al.* (1989) report that fusion is similarly initiated between hypocones, but Destombe & Cembella (1990) state that in *A. excavatum* (= *A. tamarense*) fusing cells are characterised by contact between the epicones. The relative orientation of *A. minutum* cingula in early fusion varies from approximately parallel, as in group 1, to perpendicular. The relative position of flagellar bases in early fusion is consequently not necessarily close, another characteristic of group 1. In *A. tamarense*, Fritz *et al.* (1989) report that while fusion is typically initiated at the hypothecal region of each cell with one slightly higher than the other (i.e. approximately parallel), other orientations were observed. For the same species, Anderson &

Lindquist (1985) report that the cingula of fusing cells are typically at oblique angles to each other, an observation they used to distinguish gametes from dividing cells, in which cingula were observed to be parallel. In *Gymnodinium catenatum*, Blackburn *et al.* (1989) report that gametes can either be joined equatorially or with the girdles more or less perpendicular to each other. In *A. minutum* there was not a dominant early fusion orientation, however by late fusion the cingula are most often oriented parallel and the basal bodies are close. *A. minutum* exhibits some of the characteristics of group 1 species, but is evidently not a typical example. Gao *et al.* (1989) emphasised the interspecific differences in relative orientation of cingula in fusing gametes, but it is evident from comparison of different reports on certain species, and from the present study, that a range of cingula orientations may occur within a single species. This variability confuses attempts at classification of modes of fusion, and may affect estimates of abundance of dividing and fusing cells based on cingular orientation which have been employed in previous studies.

In *A. tamarense*, the fusion process involves not the fusion of thecae, but instead the fusion of gamete pellicular layers, which subsequently play a major role in the development of ensuing stages (Fritz *et al.*, 1989). As observed in *A. minutum* in this study, some *A. tamarense* thecal plates are lost during the fusion process, although others may remain to surround the gametes until late in cytoplasmic fusion (Fritz *et al.*, 1989).

The duration of gamete fusion, at least several hours, is comparable with that reported for most dinoflagellate species. The swimming behaviour of *A. minutum* gametes is quite distinct from that of dividing cells. Throughout division cells swim straight and relatively fast, and are distributed throughout the culture. In contrast, fusing gametes swim slowly, often in tight circles, and are generally observed towards the bottom of the culture vessel. Similar behaviour is observed for many dinoflagellate species, including *A. tamarense* (Anderson & Lindquist, 1985; Fritz *et al.*, 1989).

The observation in this study that *A. minutum* planozygotes may not exhibit the typical flagellar orientation is unique, and may be particularly significant. The fact that the two planozygote trailing flagella are located alongside one another is widely accepted. In view of the fact that planozygotes were not observed to mature into hypnozygotes in the present study, the observation of alternative flagellar orientations may be representative of immature planozygotes or be an artefact caused by culture conditions, but it is also possible that alternative orientations may not always have been sought or recognised as planozygotes in some previous studies.

Comparison of the results of this study with reports on observations of related *Alexandrium* species indicate that many similarities exist, particularly between *A. minutum* and *A. tamarense*. These two species are closely related, and if such similarities are observed between members of other dinoflagellate species groups, it may in the future be possible to reliably predict morphological and other details of the reproductive cycle of a given species by comparisons with reports on closely related species. At present insufficient numbers of detailed dinoflagellate life-cycle studies have been reported to allow such comparisons to be drawn, and it should be noted that significant differences do exist between the reproductive modes of this species group and other *Alexandrium* species.

Acknowledgements. We gratefully acknowlege the technical assistance of the staff at the EM Unit of the Natural History Museum, London. This study was supported by a NERC (CASE) studentship (GT4/94/427/A) with additional financial assistance from IFREMER.

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