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Life cycle of *Peridinium bipes* f. occulatum (Dinophyceae) isolated from Lake Kizaki.

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ABSTRACT

Encystment and excystment in the life cycle of a dinoflagellate *Peridinium bipes* f. occulatum (Lindem.) Lef., which was isolated from Lake Kizaki, were investigated by use of a clonal population. Sexual reproduction was induced in *P. bipes* inoculating exponentially growing cells into N-free Carefoot's medium. The sexual life history of *P. bipes* resembles that reported for *P. willei* and *P. cinctum* in many respects: isogamous and homothallic gamete; the gametes resemble small, naked, vegetative cells; lateral fusion with a long living planozygotic stage in which the zygotes enlarge and become warty; hypnozygote with 3 walls (exsospore, mesospore and endospore); hypnozygote characterized by one or more large red oil droplets. However, the difference of *P. bipes* from *P. cinctum* and *P. willei* is that the post-zygotic cell of *P. bipes* divided into one cell with a red oil droplet and one without it.

From the encystment experiment of *P. bipes*, very low encystment was observed below 10°C. The temperature range which permitted encystment was 15 to 25°C. Although cyst formation was observed even in continuous dark, the frequency of encystment increased with the light intensity.

Under continuous dark conditions, no excystment was observed at any temperature from 5 to 25°C. The cysts illuminated at 105 $\mu E/m^2/S$ excysted at the frequency of 81 % after 13 days incubation at 15°C, and lower light intensities led to decreases in germination frequency. Excystment frequency of this species depended on light intensity. The experiments described here demonstrate that light is a critical factor in the germination of *P. bipes* cysts, affecting excystment frequency.

Key words: Cyst; Encystment; Excystment; Life cycle; *Peridinium bipes*

INTRODUCTION

Water bloom of so-called freshwater red tides has been recently observed in many

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reservoirs in western Japan (Ito, 1979). In most cases freshwater red tides are caused by the dinoflagellate *Peridinium*, which is a well-known typical phytoplankter of red tide in reservoirs, lakes and coastal regions (Nakamoto, 1975 ; Pollinger & Berman, 1975 ; Watanabe & Shiraishi, 1983 ; Hata, 1983 ; Kim, 1987 ; Kawabata & Ohta, 1989). Dense blooms of this organism clog filtration systems and give an unpleasant taste and smell to the drinking water (Pollinger & Berman, 1975). Mass mortality of fish has also been linked to these blooms (Hashimoto et al., 1968). Freshwater red tides of dinoflagellates have been reported in many countries. Most species of dinoflagellate have life cycles which are affected by several environmental factors such as temperature, nutrients, light and dissolved gases. The most common culture manipulation that induces encystment is nutrient depletion, particularly nitrogen depletion. *Peridinium* has a cyst stage in its life cycle (Eren, 1969 ; Pollinger & Serruya, 1976 ; Pfiester & Skvarla, 1979 ; Sako et al., 1984, 1987). Water temperature is often emphasized as the major environmental factor regulating germination of dinoflagellate cysts (Wall & Dale, 1968 ; Anderson & Morel, 1979 ; Fukuyo et al., 1982 ; Endo & Nagata, 1984), while light conditions have been found to exert little or no effect on excystment of some species of *Peridinium* (Endo & Nagata, 1984 ; Sako et al., 1985). Several published reports on other dinoflagellates (Binder & Anderson, 1986 ; Anderson et al., 1987) demonstrate that light does affect the excystment. This difference may be due to differences in experimental method or species differences, since the exposure to a small amount of light before the start of experiments could be significant, and the light requirement for excystment may be different among species (Binder & Anderson, 1986 ; Anderson et al., 1987).

This paper reports the previously unknown life cycle of *Peridinium bipes* f. occulatum (Lindem.) Lef.

MATERIALS AND METHODS

In Lake Kizaki, dense bloom of the dinoflagellate, *Peridinium bipes*, has been occurring regularly every year since 1986 (Kida et al., 1989). A vegetative cell of *P. bipes* was isolated from Lake Kizaki. This clonal culture was utilized in all the following experiments. Stock cultures were maintained in Carefoot's medium (Carefoot, 1968) at ca. 15°C. An incident light source of ca. $105\mu E/m^2/S$ was provided by cool white fluorescent lamps set on 12 : 12 h L/D cycle. No bubbling or shaking of culture was employed. Sterilized Pasteur pipettes were used for inoculation, transfer and isolation. The sexual life cycle was followed by isolating each individual pair of fusing gametes in 0.1ml of media poured into Corning multiple well plates (96 wells). The basic medium with nitrogen omitted was used to induce sexual reproduction. The zygotes formed were checked daily with an inverted microscope.

Encystment experiments

Quantitative observation of the encystment was made with the clonal culture of the *P. bipes*. When the population density of cells reached ca. 1×10^4 cells/ml about one month after the inoculation to the Carefoot's medium, the cells were transferred to fresh Carefoot's medium without nitrogen at a final concentration of ca. 500 cells/ml. At short intervals the total number of cysts were counted. The standard conditions for the encystment experiment were N-free Carefoot's medium, incubation temperature at 15°C and illumination of $105\mu E/m^2/S$ and 12 : 12 h L/D cycle. All experiments were performed in triplicate. Light and temperature conditions during the encystment experiments were modified according to experimental design. A frequency of cyst formation (Katoda et al., 1984) was calculated using the following formula :

$$\text{Frequency of cyst formation (\%)} = 2Nc/(Nv+2Nc)\times100,$$

where Nc and Nv are the numbers of cysts and vegetative cells in one milliliter, respectively.

Excystment experiments

The cysts for the excystment experiment were provided from the encystment experiment. Dark-cold (5°C) treated and sufficiently matured cysts were employed. During the dark-cold treatment, the cysts were tested to determine the duration of mandatory dormancy. After the dark-cold treatment the cysts were washed with fresh Carefoot's medium, then tested for excystment using Corning multiple well plates (96 wells). Each cyst was put into a well (volume : 0.37ml) of the plate containing 0.1ml of Carefoot's medium. The plates were incubated under various conditions of temperature, light and nutrients for 12 days. The standard condition for the excystment experiment was 15°C, $105\mu E/m^2/S$ and 12 : 12 h L/D cycle in the Carefoot's medium. More than one hundred cysts were scored under an inverted microscope for the determination of the excystment percentage. An overview of the life cycle of *P. bipes* was obtained with an inverted differential interference microscope (Nikon DIAPHOT-TMD, Type 114).

Samples for scanning electron microscope were fixed with 2% O_sO_4 for 2 h or with 3% glutaraldehyde for 1 h. These samples were washed twice with distilled water or filtered lake water and dehydrated in a graded ethanol series (25, 50, 75, 90, 95 and 100%) for 10-15 min each. Samples were critical point dried with liquid CO_2, mounted on stubs and coated with a thin gold film. The specimens were observed with scanning electron microscope (JEOL Ltd., JSM-25S).

RESULTS

Morphological observations of life cycle

Figure 1 shows the scanning electron micrographs of *P. bipes*. This species is characterized by its tabulation. Additional features are a distinctive apical pore complex and well developed girdle and sulcus (Huber-Pestalozzi, 1968). The species of

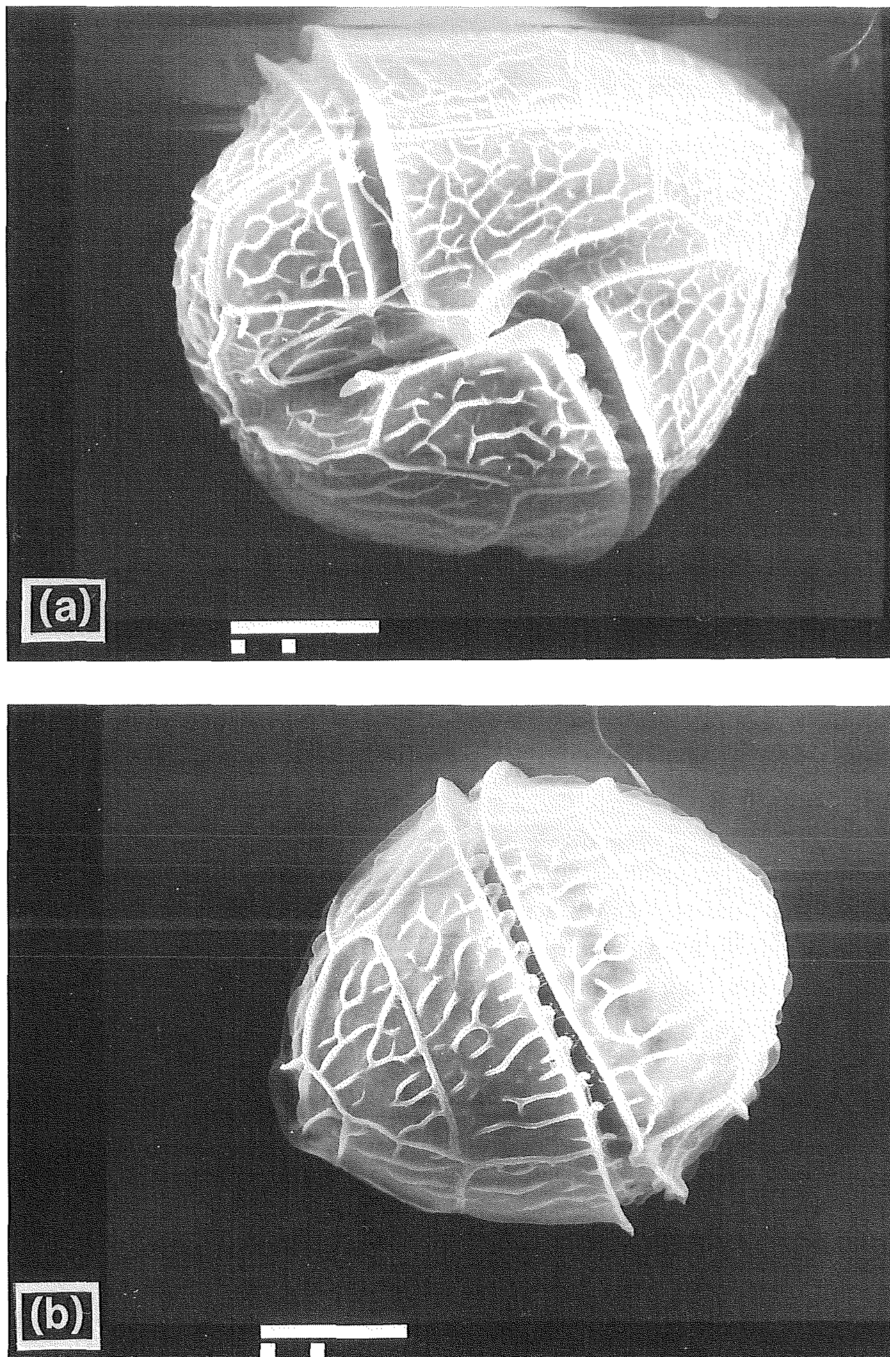

Fig. 1. Scanning electron micrographs of *P. bipes*.
(a) Ventral view. (b) Dorsal view. Scale bars=10μm.

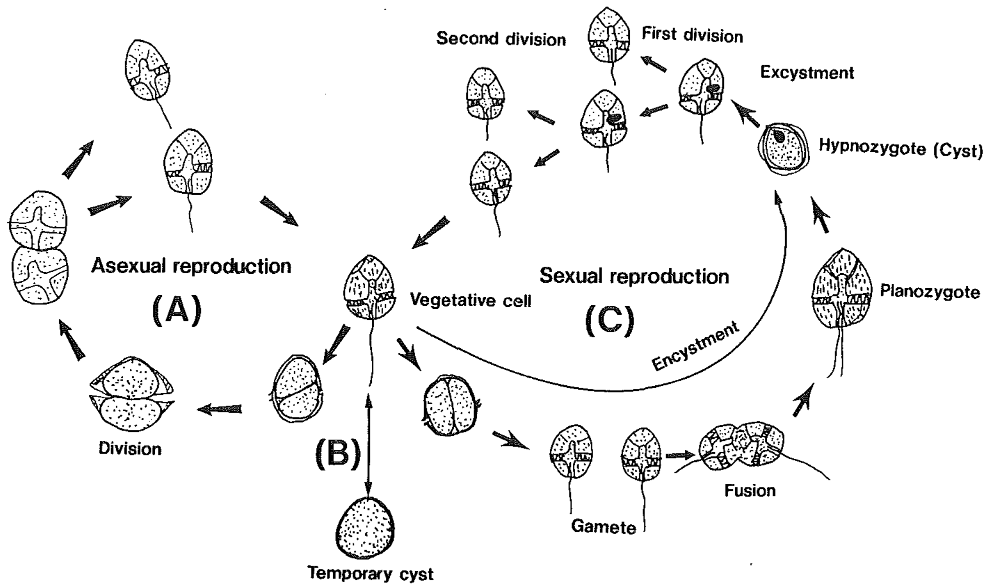

Fig. 2. Proposed scheme for life cycle of *P. bipes*.

Peridinium was identified as *Peridinium bipes* f. occulatum (Lindem.) Lef. The complete life cycle of *P. bipes* as detailed from light microscopic studies is depicted in Fig. 2. Loop A depicts asexual cell division, the most common process of cell reproduction of dinoflagellates. Loop B depicts temporary cyst formation : short term cysts formed when vegetative cells encountered adverse environmental conditions. Loop C is the sexual cycle.

Sexual reproduction was induced in *P. bipes* inoculating exponentially growing cells into N-free Carefoot's medium. Under these conditions, gamete formed within 3 days on a 12 : 12 h L/D cycle. In the asexual reproduction, exponentially growing phase cells divided horizontally into two young vegetative cells (Fig. 3) but in the sexual division, gamete forming cells divided longitudinally into young gamete cells (Fig. 4) not involving the parent theca (Pfiester, 1975). Gametes turned before being released, so the plane of cell division appears to be horizontal. Gametes, approximately 30μm in diameter, were released by the breakdown of the parent theca. Small, light colored cells, measuring 35$\times$38μm and similar in appearance to young vegetative cells, acted as gametes. Initially gametes are naked (i. e., they lack a theca), but a theca begins forming immediately after release.

Sometimes *P. bipes* formed a round dark, thin-walled asexual cyst, a temporary cyst, when inoculated in N-free medium (Fig. 5). That some cells formed round, sexual cysts rather than gametes may be explained by the genetic differences within an clonal, unialgal population. Five vegetative clones each produced zygotes when

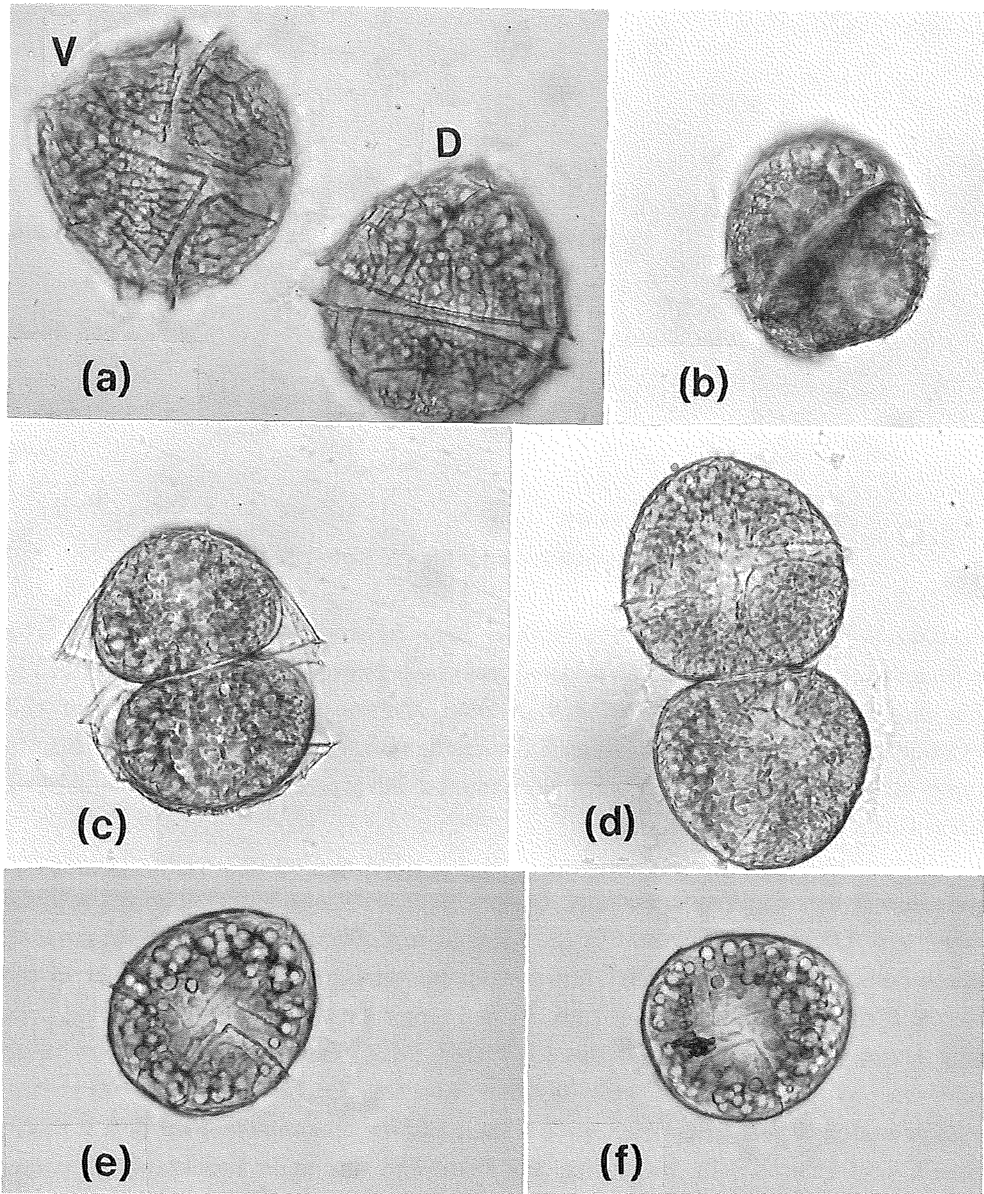

Fig. 3. Asexual reproduction of *P. bipes* in Carefoot's medium. (a)-(f) Successive stages of cell division. ×400. (a) Light micrograph of vegetative cell (V: Ventral view. D: Dorsal view).

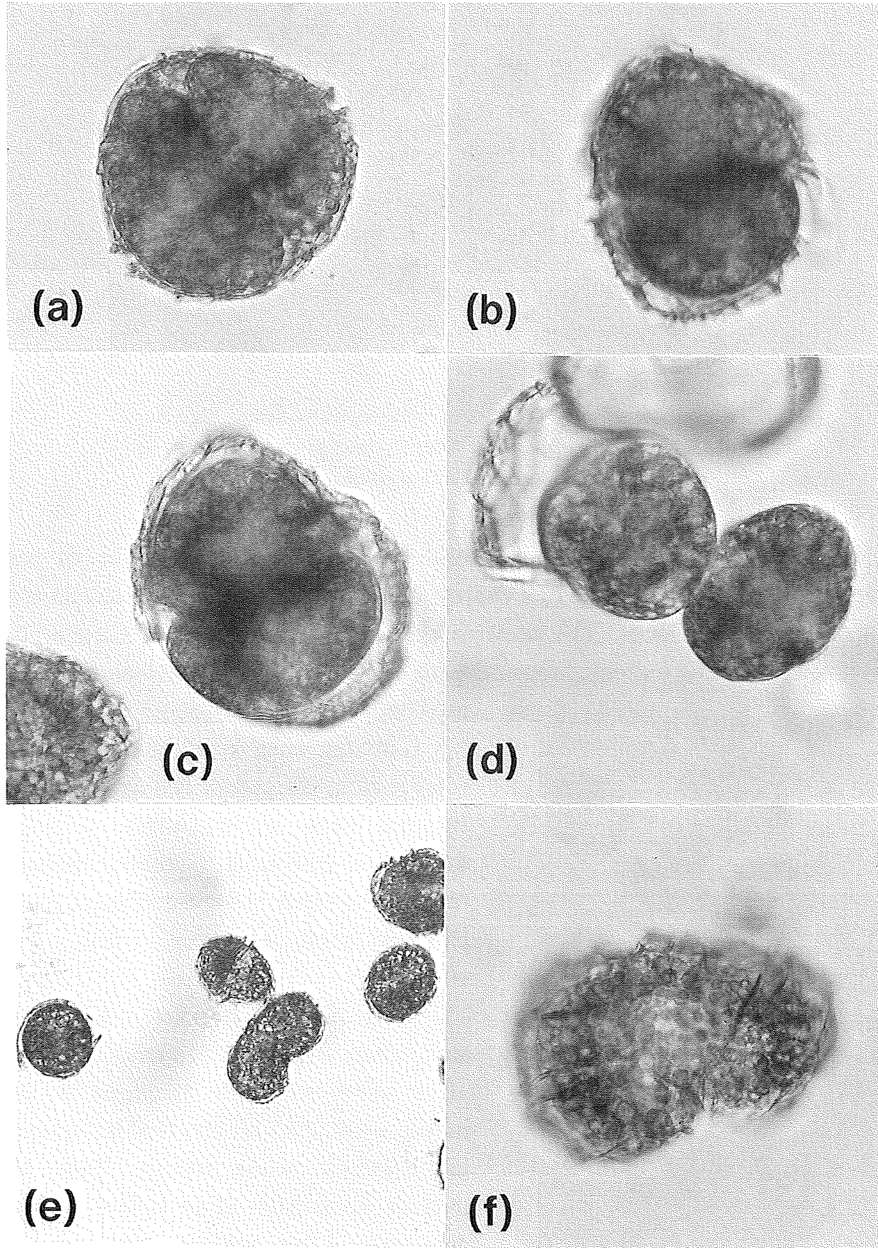

Fig. 4. Gamete formation (a-d) and fusing gametes (e-f) in the sexual reproduction of *P. bipes* in N-free Carefoot's medium. ×400, (e) ×200

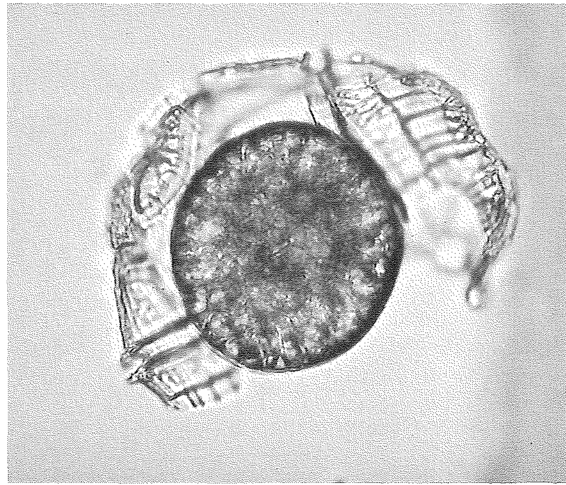

Fig. 5. Light micrograph of temporary cyst (asexual cyst) in nitrogen depletion Carefoot's medium. ×400.

inoculated into an N-free medium. Interclonal crosses were made in all possible combinations from 5 clones. Sexual reproduction was more prevalent in crosses than within the respective clones. The gametes were thus isogamous and homothallic, with some clones exhibiting a greater formation of thin-walled cysts than zygotes in N-free medium.

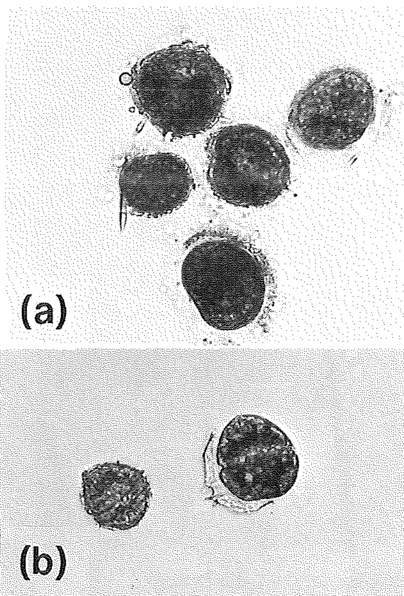

Fig. 7. Light micrographs of planozygote (a)-(b) which reached maximum size and appeared almost black. ×200.

Gametes that fuse immediately often release from the parent cell or remain motile for 1-2 days, during which period they develop a cell wall. Gametes fusing with theca have been observed (Fig. 4), but their thecate gametes seemed not to fuse completely.

Many pairs of gametes, settling next to each other, were observed in aliquots from N-free cultures. Pairs of gametes became motile without apparent stimulation and then swam about while fusing. They always became nonmotile again before fusion was complete. After settling, the membranes gradually disappeared at the point of contact. Flagella on opposite sides of the fusing cells remained active until fusion was almost complete. The gamete and the vegetative

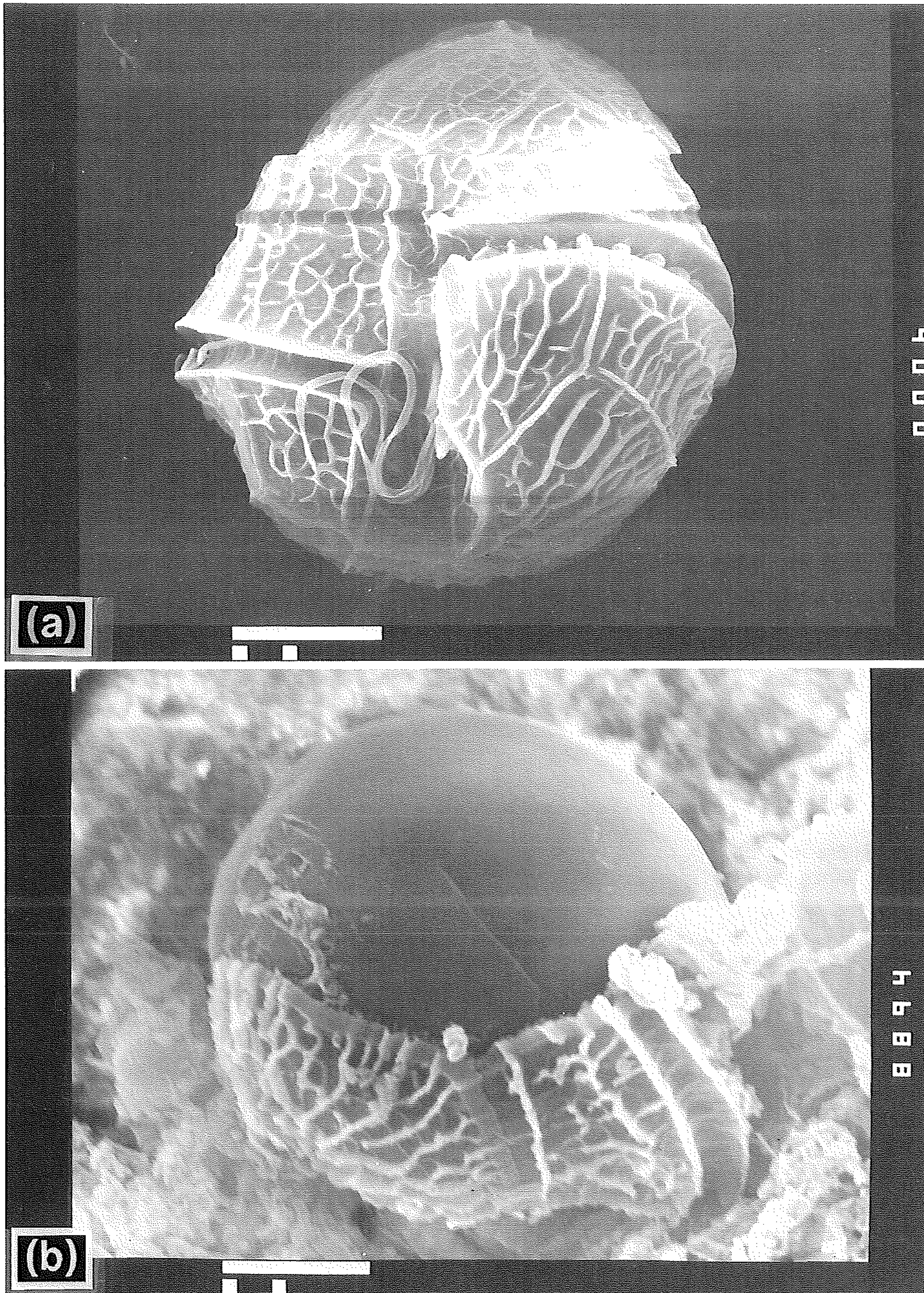

Fig. 6. Scanning electron micrographs of planozygote.
(a) Thecal tabulation similar to vegetative cell, which has two trailing flagella.
(b) Nonmotile planozygote. Scale bar $=10\mu$m.

cells were very delicate and easily damaged by mechanical transfer. Many pairs of gametes would not complete fusion once isolated from a culture plate onto a microscope slide. Thus the same pair of fusing gametes was not photographed throughout the fusion process (Fig. 6). Sexual fusion required ca. 1 hour from gametic contact to completion. Once gametes fused completely, the zygote remained motionless for ca. 1 hour, during which time it came to resemble a vegetative cell. The zygote was at first spherical but within 1 hour resembled a *P. bipes* vegetative cell ranging in size from 50-55μm to 55-60μm. Zygotes secreted thecae within ca. 1 day of completion of

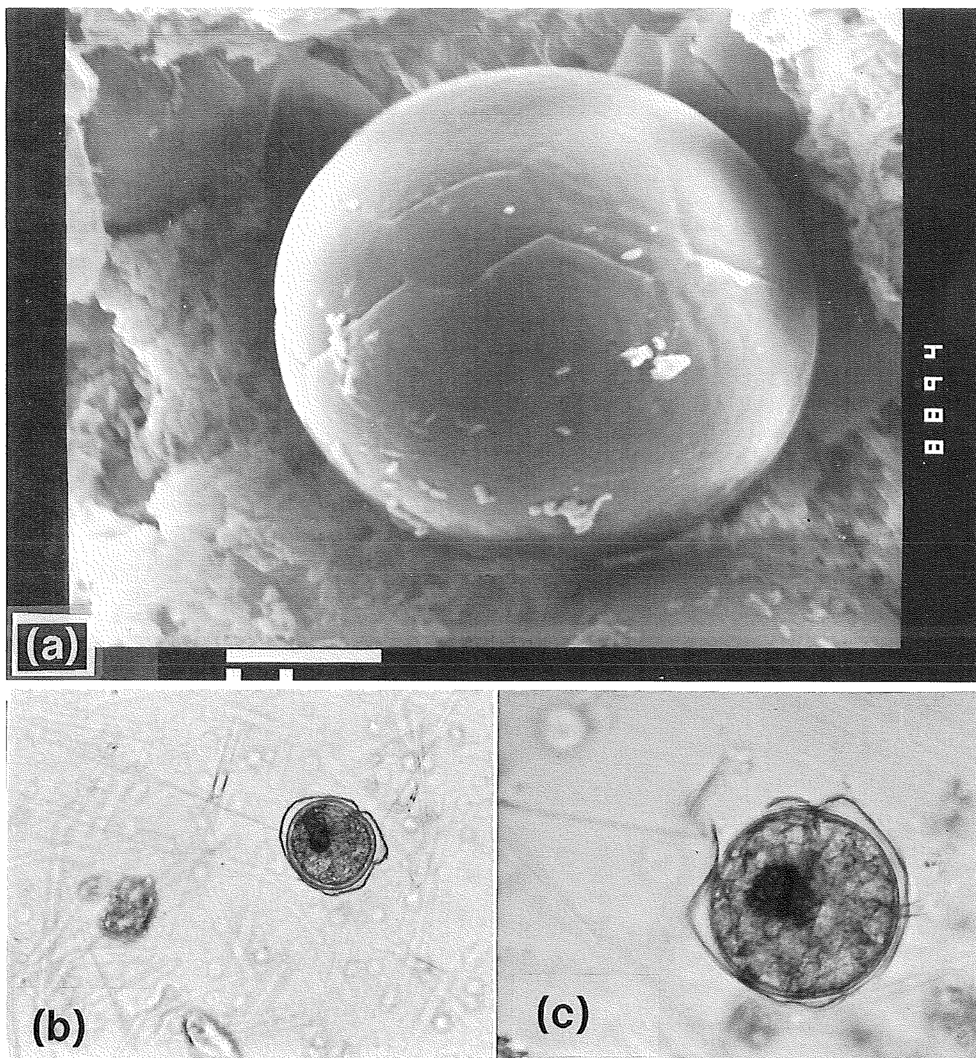

Fig. 8. Scanning electron micrograph of planozygote. (a) Light micrographs of hypnozygote. Scale bar=10μm. (b) Three distinct walls of *P. bipes* cyst and red pigment, ×200. (c) ×400.

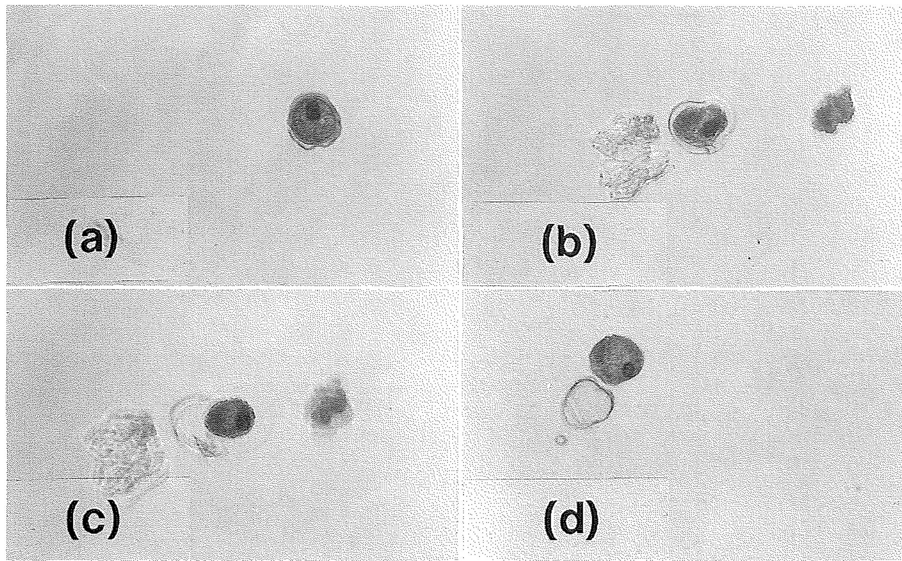

Fig. 9. Light micrographs of the excystment process (a-d), (post-zygotic cell). ×200.

fusion. They remained motile for about half a month, darkening and enlarging, with the thecae becoming warty. Sometime later, a wall is laid down internal to the thecal plates. At this stage the planozygote, which has two trailing flagella (Fig. 6), reached a maximum size of $65\times70\mu$m within 2 weeks, at which time it appeared to be almost black (Fig. 7). At the same time that the zygote became nonmotile, the theca thickened and pigments distributed throughout the cell concentrated toward the center to form 1 or 2 red, bean-shaped spots. According to Graham (1951), the red bean-shaped spots are oil droplets. The protoplast was bleached and contracted. Once the maximum size was reached, a third wall developed.

Zygotes, the treated cysts at 5°C and in the continuous dark, germinated between 15 and 16 weeks after gametic fusion, to produce one post-zygotic cell which retained the large red pigment (Fig. 9). The post-zygotic cell divided within 24 h into one cell with a red body and one without ; within several days these daughter cells subdivided into 2 ordinary young vegetative cells (Fig. 10). At this time, the young vegetative cells left a red body in the theca of the parent cell.

Encystment experiments

Encystment was induced in *P. bipes* by transferring exponentially growing cells into N-free Carefoot's medium. Encystment occurred in N-free Carefoot's medium over one month period. Figure 3 shows the effect of temperature on the encystment of *P. bipes* in N-free Carefoot's medium at $105\mu E/m^2/S$. The optimum temperature for encystment of *P. bipes* was 20°C and the incubation temperature wcich was required

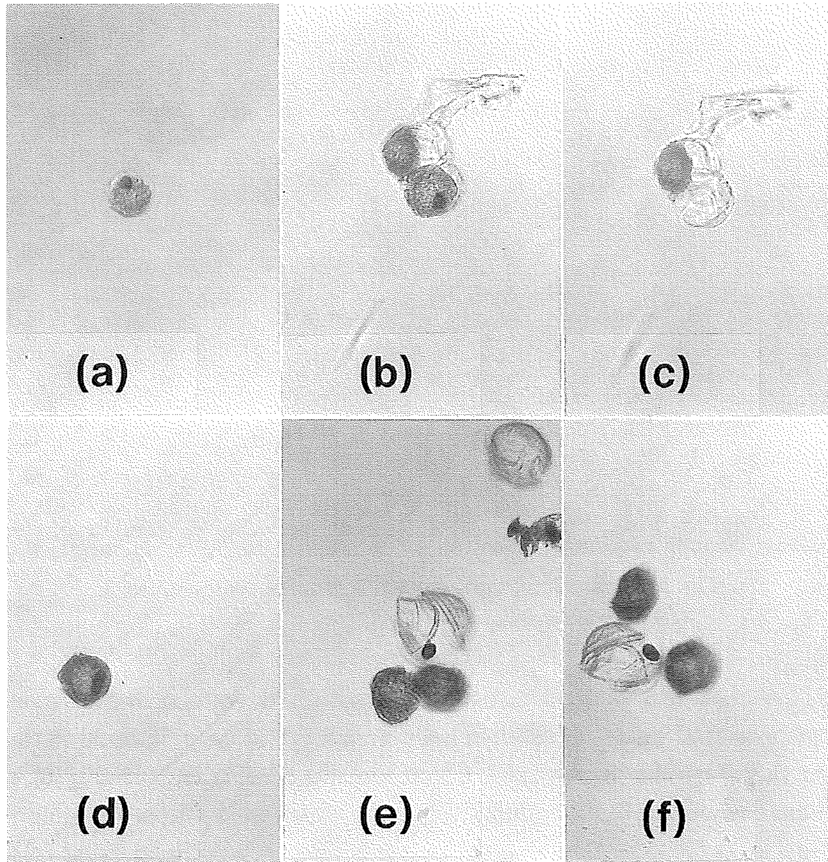

Fig. 10. Light micrographs of the first and second division after excystment (a-f). ×200.

for an encystment frequency of over 35% ranged widely from 15 to 25°C. Very low encystment was observed at 5 and 10°C during 30 days incubation. Most of the cells retained in the form of vegetative cells.

The dependence of encystment on light intensity in N-free Carefoot's medium at 15°C is shown in Figure 4. Although the cyst formation was observed even in the dark condition, the frequency of encystment increased with the light intensity (12 to 34%). The highest frequency obtained here was 34% under the light intensity of $105 \mu E/m^2/S$.

Excystment experiments

No excystment was observed in the experiment in spite of optimum conditions for germination during the 15 weeks in which the cysts incubated in the cold room regulated at about 5°C and shut out from light. After 16 weeks the cysts started to excyst more than 80%. The mandatory dormancy period of *P. bipes* was 16 weeks

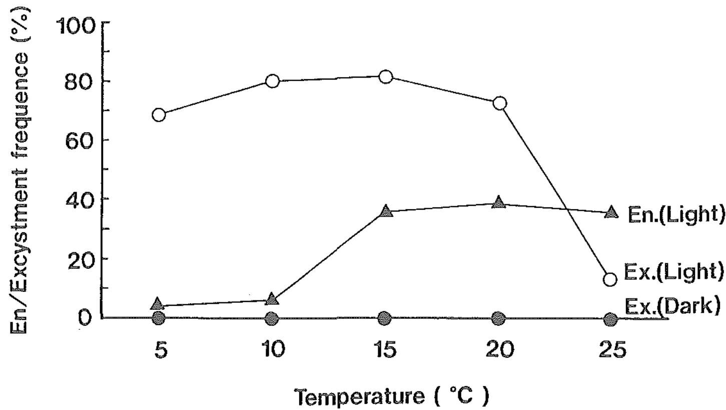

Fig. 11. Effect of temperature on the encystment and excystment frequency of *P. bipes*. The cells of encystment were transferred to N-free Carefoot's medium and incubated in 30 days at 105μE/m²/S (12 : 12 h L/D). Cysts were incubated in Carefoot's medium for 12 days at 105μE/m²/S (12 : 12 h L/D) and continuous dark condition.

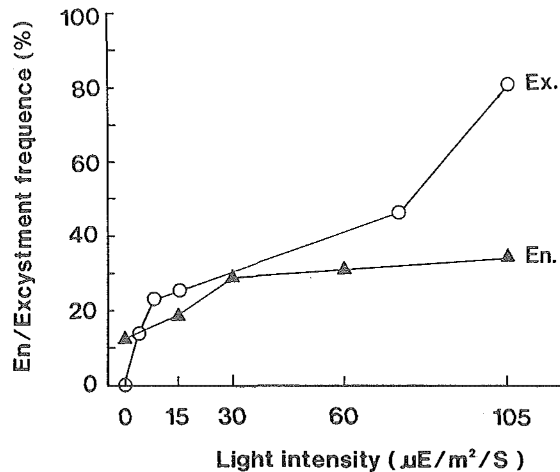

Fig. 12. Effect of light on the encystment and excystment frequency of *P. bipes*. The cells of encystment were transferred to N-free Carefoot's medium and incubated in 30 days at 15°C, (12 : 12 h L/D). Cysts were incubated in Carefoot's medium for 13 days at 15°C, (12 : 12 h L/D).

under dark-cold condition. Once the cysts were mature, the resting state was maintained (quiescence ; see Pfiester and Anderson, 1987) until external conditions became suitable for excystment. The matured cysts which were incubated at 5, 10, 15,

20 and 25°C under 105μE/m^2/S illumination in Carefoot's medium began to excyst after 4 days incubation. After 12 days the cyst excysted at the maximum frequency of 81% at 15°C. The frequency of excystment after 12 days incubation between 5 and 25°C is summarized in Figure 11. The optimum temperature for excystment of *P. bipes* was 10 to 15°C and the incubation temperature which was required for the excystment frequency of more than 50% ranged widely from 5 to 20°C. A very low excystment frequency was obtained at 25°C.

Under continuous dark condition, no excystment was observed at any temperature from 5 to 25°C (Fig. 11). The cysts illuminated at 105μE/m^2/S (12 : 12 h L/D cycle) excysted with a frequency of 81% for 13 days incubation at 15°C, and lower light intensities led to decreased germination frequency (Fig. 12). Which component on Carefoot's medium is effective in excystment of *P. bipes* was examined at 15°C in 105μE/m^2/S. After 12 days incubation in N-free, P-free and N- and P-free media, the cysts excysted. The frequencies were almost the same as for excystment in the complete Carefoot's medium. Therefore, the excystment of *P. bipes* was independent of nutritional conditions, such as nitrogen and phosphorus.

DISCUSSION

Sexual reproduction

Gamete formation began within 3 days after inoculation and continued for ca. 1 month. The cell density of the population increased during this time as some cells continued asexual division under conditions stimulating sexual fusion or encystment in others. According to Fogg (1959), only those cells in which the nucleic acid content had increased prior to transfer to N-deficient medium can divide in N starved populations. This would explain the extended gamete formation. The majority of gametes formed by *P. bipes* were poorer in plastids as well as pigments than the vegetative cells. The same true for gametes of *Amphidinium carteri* Hurlburt (Stosch, 1972) and *Gymnodinium pseudopalustre* Schiller reported that apart from their smaller size and lighter color, the gametes differed little from vegetative cells (Stosch, 1973).

The hyponzygote of *P. bipes* was similar in appearance to the descriptions given for the hypnozygote of *P. cinctum* (Pfiester, 1975) and *P. willei* (Pfiester, 1976). Three distinct walls of *P. bipes* cyst, the exospore, the mesospore and the endospore, were observed (Fig. 8). The exospore was thick, the mesospore very thin, and the endospore thick. They have been described for hypnozygotes of *Woloszynskia apiculata* Stosch, (Stosch, 1973), *Gymnodinium pseudopalustre* (Stosch, 1973), *P. cinctum* (Pfiester, 1975) and *P. willei* (Pfiester, 1976). According to Pfiester (1975 & 1976), *P. cinctum* and *P. willei* the post-zygotic cell divided within 24 h into 2 daughter cells, each with a prominent red oil droplet, and within several days these daughter cells subdivided into

ordinary vegetative cells. Eren (1969) collected the cysts of *P. cinctum* during the bloom in Lake Kinneret and showed that during the first division after excystment two cells each with one red body were formed and then the second division produced one cell with a red body and one without it. Sako et al. (1985) observed that all vegetative cells of *P. cunningtonii* had one red body after the second division.

The sexual life history of *P. bipes* resembles that reported for *P. willei* (Pfiester, 1976) and *P. cinctum* (Pfiester, 1975) in many respects: isogamous and homothallic gamete; the gametes resemble small, naked, vegetative cells; lateral fusion with a long living planozygotic stage in which the zygotes enlarge and become warty; hypnozygote with 3 walls (exospore, mesospore and endospore); hypnozygote characterized by one or more large red oil droplets. However, the difference of *P. bipes* from *P. cinctum* and *P. willei* is that the post-zygotic cell of *P. bipes* divided into one cell with a red oil droplet and one without it.

Encystment

Environmental factors such as temperature, nutrient depletion, light intensity and dissolved gases have been suggested as possible causes of encystment in dinoflagellates (Stosch, 1969; Anderson & Wall, 1978: Pfiester & Anderson, 1987). The most common culture manipulation that induces encystment in dinoflagellates is nutrient starvation, especially nitrogen depletion (Andeson & Wall, 1978; Pfiester & Anderson, 1987). Nitrogen deficiency is known to induce encystment in *P. cinctum, P. gatunense* and *P. willei* (Pfiester, 1975; 1976; 1977). Encystment was induced in *P. bipes* by transferring exponentially growing cells into N-free Carefoot's medium. Cyst formation occurred in N-free medium over a 1 month period. Temperature alone has never been shown to be a factor that directly induces encystment, but it can alter the encystment process once initiated by nutrient depletion. From the experiment with *P. bipes*, very low encystment was observed below 10°C (Fig. 11), and the range of temperature which permitted encystment was 15 to 25°C. Although cyst formation was observed even in continuous dark, the frequency increased with the light intensity (Fig. 12).

Excystment

Water temprature is often cited as the major environmental factor regulating germination of the cyst of dinoflagellates (Wall & Dale, 1968; Anderson & Morel, 1979; Fukuyo et al. 1982; Endo & Nagata, 1984; Binder & Anderson, 1987). The optimum temperature for the excystment of *P. bipes* was 10 to 15°C under $105\mu E/m^2/S$, and the incubation temperature required for the germination frequency of more than 50% ranged widely from 5 to 20°C.

Light conditions have beeo found to exert little or no effect on excystment of some species of *Peridinium* (Endo & Nagata, 1984; Sako et al., 1985). Several published reports on other dinoflagellate (Binder & Anderson, 1986; Anderson et al., 1987) demonstrated that light does affect the excystment. This difference may be due to

differences in experimental method or species differences, since the exposure to a small amount of light before the start of experiments could be significant and the light requirement for excystment may be different among species (Binder & Anderson, 1986 ; Anderson et al., 1987). Under continuous dark conditions, no excystment was observed at any temperature from 5 to 25°C (Fig. 11). The cysts illuminated at 105μE/m^2/S excysted at the frequency of 81% after 13 days incubation at 15°C, and lower light intensities led to decreases in germination frequency (Fig. 12). The excystment frequency of this species depended on light intensity. The experiments described here demonstrate that light is a critical factor in the germination of *P. bipes* cysts, affecting excystment frequency.

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