The cyst-motile stage relationship of three *Protoperidinium* species from south-east coast of Iran

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Abstract

Resting cyst and motile thecate cell stages of three heterotrophic *Protoperidinium oblongum*, *Protoperidinium* sp and *Protoperidinium claudicans* were assessed. Cysts were isolated from sediment collected from southeast coast of Iran. Individual live cysts were incubated under optimal conditions for germination. Results showed that *Protoperidinium oblongum* cysts were pentagonal smooth walled and three cysts of this species were identified from Iranian sediment. The germinated cell of *P. oblongum* was colourless and elongated pentagonal in shape. *P. claudican* theca cell has a pointed antapical horn with a four sided apical plate. *Protoperidinium* sp. had dark brown pentagonal cyst. Its germinated cell differed from other *Protoperidinium* species. Two divergent distally antapical horns formed 90˚ between the horn and Posterio-lateral of main body edge, thus, comprising a unique species in the *Protoperidinium* genus. This kind of antapical horn has not been previously reported among *Protoperidinium* spp.

Keywords: Phytoplankton, *Protoperidinium*, Taxonomy, Cyst, Germinated cell, Sediment, Chabahar bay, Iran

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Introduction

There is an increased understanding of phytoplankton knowledge in Iran, especially after the Persian Gulf *Cochlodinium* bloom that caused massive mortality of aquatic organisms. Correct identification of species is vital in all biological studies, and this is not possible unless all stages and formations of a species life cycle are assessed and identified.

Dinoflagellates are important group of phytoplankton, of which some of them produce resting cysts during their life cycle. Cysts are dormant stages with reduced metabolism that can survive in very low or high temperature, anoxic conditions and darkness (Anderson et al., 1987; Bravo and Anderson, 1994). These cysts are physiologically and morphologically different from vegetative or motile stages (Dale, 1983; Binder and Anderson, 1990). Dinoflagellate can also produce temporary cysts that are not a dormant stage. This stage is relatively a short period between benthic and planktonic stages. Dinoflagellates produce temporary cysts under adverse conditions such as ageing culture nutrient deficiency, temperature fluctuation and bacterial attack; these temporary cysts can help population loss reduction (Anderson and Wall, 1978; Nagasaki et al., 2000).

During the last two decades, planktologists were more concerned in distributing of cysts because of their potential to form a seed bank in sediment, representing key stages for geographic distribution and successionally spread of dinoflagellate species in space and time (Fritz and Timer, 1985; Bolch et al., 1999; Bolch and Reynolds, 2002; Morquecho and Lechung-Deveze, 2003). Morphology of both resting cysts and motile cells have proved useful in helping to clarify phylogentic relationships and the taxonomy of some groups (Faust, 2006; Matsuka et al., 2006; Attaran-Fariman and Bolch, 2007; Attaran-Fariman et al., 2007). The genus *Protoperidinium* Bergh is a large genus with approximately 151 species (Bisby et al., 2007) and a dominant heterotrophic group in recent dinoflagellate population (Matsuka et al., 2006; Yamaguchi and Horiguchi, 2008). The genus may be very abundant during dinoflagellate bloom and less abundant during diatoms bloom (Jeong and Latz, 1994; Ganjian et al, 2010). *Protoperidinium* spp has a major role in red tide dynamic by grazing on some former dinoflagellate blooms (Jacobson and Anderson, 1986; Hansen, 1991).

Protoperidinium species have been known to produce cysts with varied morphology from simple spheres to complex pentagonal shapes with process and horn. The common feature for identifying cyst is the shape of archeoplyle which involves intercalary paraplate with or without an adjacent paraplate (Matsuka et al., 2006). Morphology of some *Protoperididium* cyst species is similar and difficult to identify (Faust, 2002; Matsuka et al., 2006; Gribble et al., 2009). However, some show an anterior intercalary containing one or two paraplates which can be used in species identifications. Classification of *Protoperidinium* is based on cyst morphology, cell size, shape, horn, spine and also plate tabulation. Vegetative cells of this genus have cellulose thecal plates;

shape, size, and the number of intercalary and cingular plates with the tabulation pattern and also the shape of the first apical plate are significant features for taxonomy of the genus (Harland, 1982; Faust, 2002). There is no descriptive information on *Protoperidinium* recent cyst and their germinated vegetative cell species in Iran. In this paper we describe the vegetative stages produced by the cyst germination of three *Protoperidinium* species from recent sediments of south east coast of Iran.

Materials and methods

Sediment samples were collected by Ekman grab in 2004 from Chabahar bay on the southeast coast of Iran (Fig. 1) at a water depth of 3m. Water temperature at the sampling site was 25˚C. Surface sediment from the grab sample were kept in the dark at 20˚C for later study.

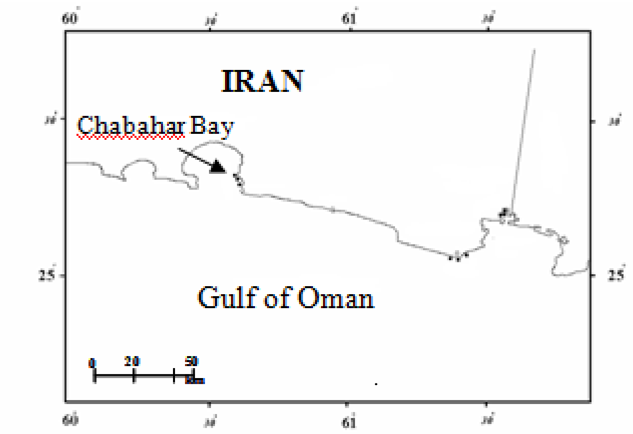


Figure 1: Geographical position of the study area

Approximately 2-4g of wet sediment were mixed with 0.2µm filtered seawater and sonicated for 2min using a Microson ultrasonic cell disruptor. Samples were sieved at 125µm and retained at 20µm sieve (Bolch and Hallegraeff, 1990). Live resting cysts were concentrated by centrifuging (Bolch et al., 1999).

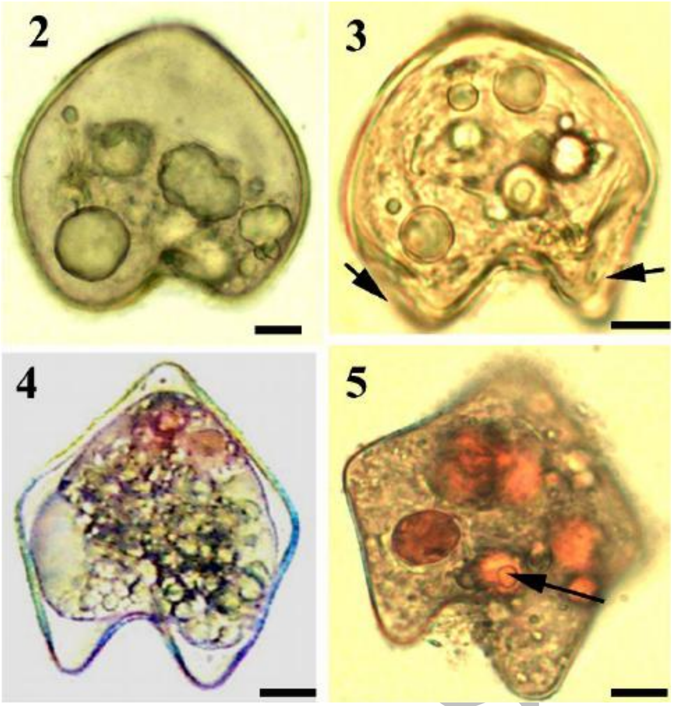
Individual cysts of *Protoperidinum* then were isolated by micropipette under a Leica stereomicroscope and washed twice in f2 growth media containing trace metal, vitamin mix, nitrate and phosphate stock solution (Guillard and Ryther, 1962) and placed in 55mm polystyrene Petri dishes containing 10ml of medium. The samples were then incubated at 26˚C ± 0.5˚C, under cool white fluorescent light (70-90µmol photon $m^{-1}s^{-1}$) with a 12h light: 12h dark cycle. The remaining mixed sediment was also incubated in the same condition. The plates were checked daily for germination.

After excystment, the cysts and excysted motile cells were photographed with an Olympus BH-2 light microscope equipped with Leica DC300F digital imaging system. The plates' pattern of germinated thecate cells were also examined by fluorescent microscopy using a broadband UV filter (Olympus filter set no. 488) after staining with fluorescence brightener M2R (Fritz and Treimer, 1985).Individual cysts were collected on Nuclepore filter (1 µm pore size), air dried, sputter coated with gold (Blazers, SCP004, Germany) and examined under Philips Quanta 600 scanning electron microscope (SEM).

Results

The cysts were observed under light microscope and *Protoperidinium* spp was dominant group among cyst flora of Chabahar bay with 51% relative abundance (other cyst flora of Iran's south east coast will be published elsewhere). Results showed different cyst types belonging to *Protoperidinium oblongum* (Aurivillius) Parke and Dodge species. The synonym of this species is *Peridinium divergens* Ehrenberg var. oblonga Aurivillius and its Paleontological name is *Votadinium carvum* Reid. Morphological analysis of the species demonstrated that the resting cysts of *P. oblongum* are pentagonal and dorso-ventrally compressed. Cysts were varied in size from 53-59µm (n=50) in width and 50-63µm (n=50) in length. These smooth-walled cysts possess a pale brown outer wall (Fig. 2). *P. oblongum* has straight or slightly concave sides with a symmetrical and triangular epicysts. Cysts showed variety in cyst content and colour. Three cyst types were found in Iranian sediments. The first type had rounded and roughly rounded apical and antapical horns respectively, with short antapical horns. In the second type, the epicyst hadconvex sides, but hypocyst hadstraight sides and ed two clear lobes (Fig. 3). In the third type, the cysts were rhomboidal in outline with straight or somewhat concave sides with a triangular epicysts. The hypocyst also had straight sides pointed into two rounded distinct horns. The paracingulum is weakly defined (Figs 4, 9). In some cysts the protoplasmic membrane is clear (Figs 4, 6, 8); this appears in the cyst prior to excystment (Wall and Dale, 1968). Cysts sometimes contain numerous pink oil globules (Fig. 7).

Cysts may show convex sides on the epicysts (Fig. 8). The archeopyle was not observed as all specimens were intact. The Result of the germination experiment for this species revealed that two specimens (Fig. 4) were successfully germinated to produce colourless and elongate *Protoperidinium oblongum* cells (Figs. 10, 11). The theca has two roughly parallel elongate and conical antapical horns.The sulcus is set asymmetrically in the hypotheca; the right antapical horn is slightly displaced laterally from the sulcal furrow (Fig. 11). Another resting cyst that has been examined in this study was a dark brown, pentagonal, dorso-ventrally compressed cyst of *Protoperidinium* sp. with a smooth wall. A cyst is 70µm in length and 65µm in width.



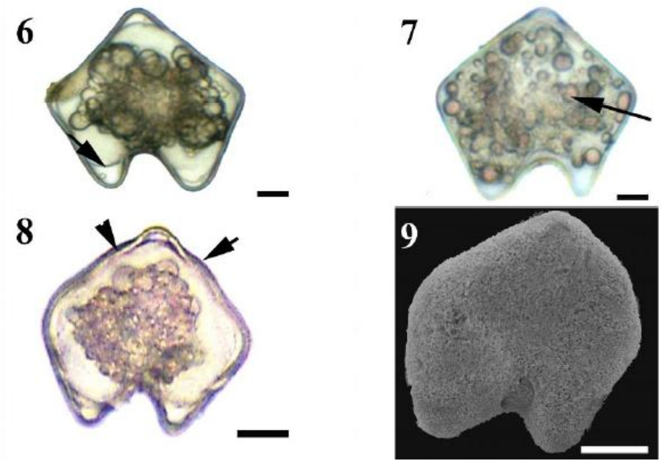
Figures 2-5: LM. *Protoperidinium oblongum.* All scale bars=10µm

Figure 2: Cyst type 1 showing two short antapical lobes.

Figure 3: Cyst type 2 showing round epitheca and straight sides in hypocyst (arrows).

Figure 4: Cyst type 3 showing conical epitheca with straight sides.

Figure 5: Cyst showing large orange oil droplets (arrow).



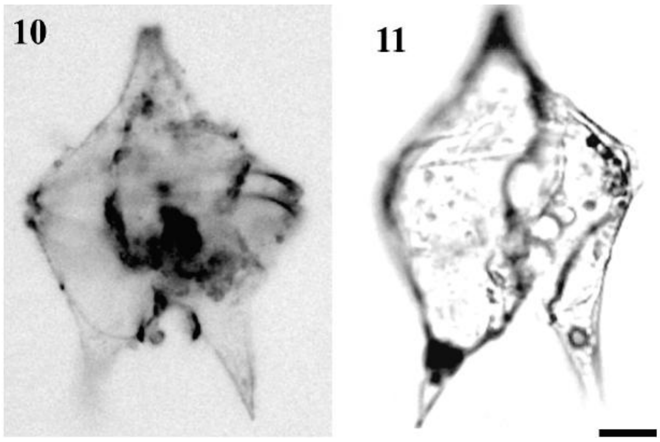
Figures 6-9: *Protoperidinium oblongum.* All scale bars=10µm

Figure 6: L.M. Cyst showing clear protoplasmic membrane (arrow)

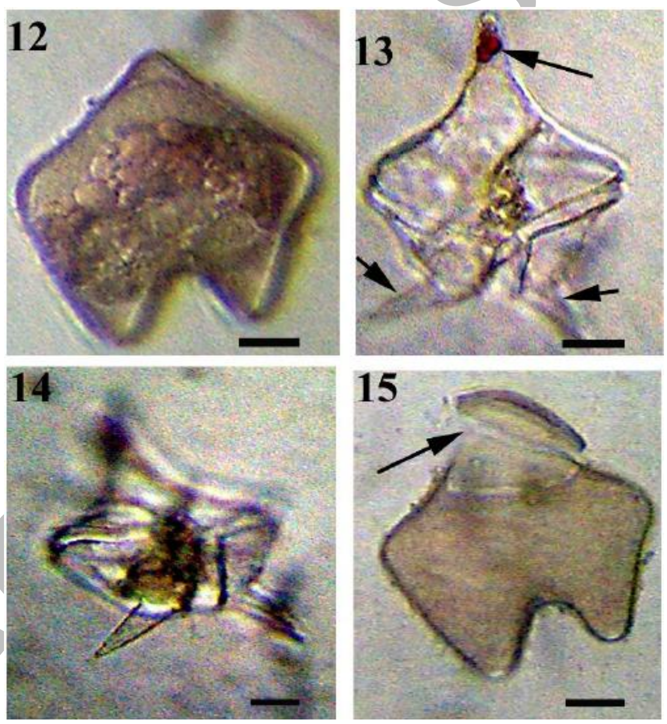
Figure 7: L.M. Cyst showing lots of pink oil droplets

Figure 8: L.M. Cyst type 3 with slightly convex epicyst (arrow)

Figure 9: SEM of cyst type 3



Figures 10-11: L.M. of *Protoperidinium oblongum*. All scale bars=10µm. Ventral view of the germinated cells showing an elongated pentagonal body

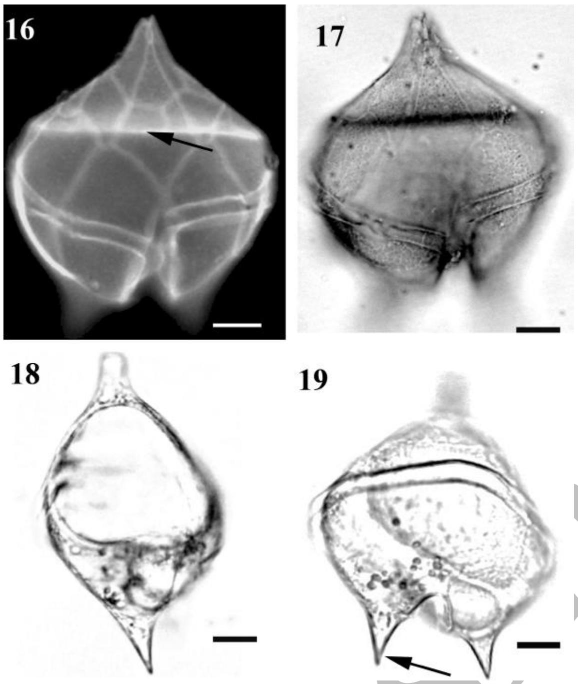


Figures 12-15: LM. *Protoperidinium* sp. All scale bars=10µm

Figure 12: Live cyst with contents.

Figures 13-14: Motile cell germinated from the cyst in Fig. 12. withe diverging antapical horns (arrows). Note red spot (arrow).

Figure 15: Empty cyst showing archeopyle with attached operculum. Note the broad hump with a flattened top on ventral side of the archeopyle (arrows)



Figures 16-19: Protoperidinium *claudicans.* All scale bars=10μm
Figure 16: F.M. Ventral view of cell exhibiting the four sided first apical plate (arrow).
Figure 17: LM. Ventral view of same cell showing first apical plate.
Figure 18: Lateral view of theca cell of *P. claudicans* isolated from mix-incubated sediment.
Figure 19: LM. Posterio-dorsal view of vegetative cell of P. *claudicans* showing pointed antapical horn (arrow)

The cyst has a red accumulation body and globular contents (Fig. 12). The epicyst has a large triangular archeopyle that may reflect the loss of intercalary and apical paraplates. Results of the germination experiment for *Protoperidinium* sp. cyst detect that the cyst was successfully germinated. The erminated cell produced a colourless peridinioid cell with a convex conical epitheca extending into a conical apical horn (Figs 13, 14). The hypotheca is characterised by two conical antapical horns that diverge distally forming an angle of 120˚ between them and approximately 90˚ between the horns and the posterio-lateral body edge of the main body. The archeopyle exhibits a distinct flattened hump on its anterior side and the operculum may remain attached to this part (Fig. 15). The hypocyst is straight-sided and forms two antapical lobes. The third species in this study that has been examined belongs to *Protoperidinium* claudicans (Paulsen) Balech. Its paleontological name is *Votadinium spinosum*. The motile cell of *P. claudicans* was germinated in mix-incubated sediment and the cell was isolated from mixed samples. Morphological analysis of vegetative cells illustrated that cells have pentagonal bodies. Motile cells are broadly rounded along the girdle. The concave conical epitheca is tapering to an apical horn (Fig. 17). The cell has a four-sided first apical plate (Figs. 16, 17). The hypothecae is pointed, with the two diverging conical antapical horns standing (Figs 18, 19). The left antapical horn is shorter than the right one.

Discussion

The morphological feature of cysts and those cells germinated from cysts were alike for non-pigmented heterotrophic armoured dinoflagellate. In the dinoflagellate group, cyst morphology, especially the archeopyle shape has provided valuable insight into taxonomy of the species (Dale, 1983). On the first consideration, before excystment in the filed sample, cyst of *Protoperidinium oblongum* could not be distinguished from cysts of *Protoperidinium* sp. Both species have pentagonal without any ornamented cysts (Compare Figs. 6, 12). However, after germination the empty cyst of *Protoperidinium* sp. has a distinct saphopylic archeopyle with a flattened bump on the anterior edge. Many closely related cyst types have been described as *P. oblongum* (Dale 1986; Baldwin1987; Macminn, 1991; Nehring, 1994; Okolodkov, 2008). Dodge (1985) documented a *P. oblongum* cyst with subtrapezoidal archeopyle with rounded angels that positioned subapically in the dorsal surface. Joyce (2005) demonstrated that an empty cyst of *P. oblongum* has broad and large intercalary archeopyles. Sonneman and Hill (1997) illustrated cordate cysts with rounded apices and a hexagonal archeopyle. The cyst of *Protoperidinium oblongum* from Iranian sediments is different from those cysts described by Bolch and Hallegraeff (1990) and Sonneman and Hill (1997) from temperate area due to its lack of paracingular grooves. They referred that the groove is unusual for this cyst type. Iranian cyst is more similar to *P. oblongum* from South Africa , Orkney (Joyce, 2005) and also Russian Pacific coast (Orlova et al., 2004). Beside the archeopyle shape, the size of *P. oblongum* cyst is smaller than *Protopeidinium* sp. cyst of *P. oblongum* from SE Australian coastal area has 50-79µm length and 56-70µm width (Sonneman and Hill, 1997). Okolodkov (2008) documented 70.0µm Length and 62.5µm width for this cyst from Gulf of Mexico. *P. oblongum* from Iranian sediment has 53-59µm width and 50-63µm length which is more comparable to those described by Morquecho and Lechuga-Deveze (2003) and Joyce (2005) with respect to its size. On the basis of the motile cell morphology, cell size of *P. oblongum* from Iran is not resembled to that illustrated from Australian coastal regions (Bolch and Hallegraeff, 1990; Sonneman and Hill, 1997). In the study of Sonneman and Hill (1997) cell length is 91-94µm and also 55-62µm length has been reported from Tasmanian coastal region (Bolch and Hallegraeff, 1990). As results showed cell length from Iranian coastal region is 71µm. *P. oblongum* cell appearance from Iran is similar to *Protoperidinium claudican* from Australia and *P. divergens* from Gulf of Mexico (Okolodkov, 2008). Some authors documented the *P. oblongum* as a neritic variety of *Protoperidinium oceanicum* Balech (Halim, 1967; Balech, 1988). Some others have documented the *P. oblongum* as different species from *Protoperidinium oceanicum* because of its smaller size and less elongated body (Dodge 1982; Evagelopoulos, 2002). Okolodkov (2008) illustrated 100-130µm length for *P. oceanicum* which is bigger than *P. Oblongum*-cell length which has been reported so far. However, incubation experiments that have established cyst/theca relationship of the *Protoperidinium oblongum* can explain any morphological variation of the species and confirm the taxonomy of Iranian species. In fact, there is no any report on producing resting cysts by *P. oceanicum* and cyst of *Protoperidinium claudican* is differed from *P. oblongum* by its ornamentation and shape. *Protoperidinium claudican* cysts are heart-shaped, 52-57µm long, light brown and ornamented with numerous short pointed spines (Bolch and Hallegraeff, 1990; Morquecho and Lechuga-Deveze, 2003; Orlova et al., 2004; Joyce, 2005). But, this spiny empty cyst that released the motile cell of the *P. claudican* in our mixed-incubated sediment was not observed.

Protoperidinium sp. possesses an unusual morphology compared to those of other typical *Protoperidinium* species including *P. oblongum* and *Protoperidinim claudican*. It is characterised by distally divergent antapical horns that form an angle of 120º between the two horns. This extraordinary antapical horn morphology is quite unique in the *Protoperidinium* genus. Species of genus *Protoperidinium* has an almost parallel or a little divergent antapical plate that forms a V shape antapical ridge (Fensome et al., 1993; Sonneman and Hill, 1997; Matsouka et al., 2006; Okolodkov, 2008). *Protoperidinium divergens* Ehrenberg (Balech, 1998) and *Protoperidinium divaricatum* Meuneir (Dodge, 1985) have been documented to have little divergent antapical horns (Evagelopoulos, 2002; Okolodkov, 2008), which are rounded in outer margin of the cell. So far, no theca cell has been reported to have an antapical horn with such a high degree of distally deviation. This type of antapical plate has not been previously described in the genus *Protoperidinium* and strongly is a new species that has a different shape of archeopyle and motile cell. But only one cyst was germinated and its thecal plate pattern was not observed very clear with light microscopy, therefore the species remains unknown. In this study, we examined morphology of cysts and motile cultured cells of only three *Protoperidinium* species out of 200 described *Protoperidinium* species. Form the taxonomic point of view, among the three *Protoperidinium* species that have been examined *P. oblongum* and *Protoperidinium* sp. were most interesting. Many more *Protoperidinium* species from our study area must be investigated in future analysis which will help to determine any morphological differences for this important group of dinoflagellate.

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