

Karlodinium veneficum* in India: effect of fixatives on morphology and allelopathy in relation to *Skeletonema costatum

R. K. Naik, R. R. Chitari and A. C. Anil*

National Institute of Oceanography (CSIR), Dona Paula, Goa 403 004, India

Dinoflagellates form an important component of marine and freshwater phytoplankton. They are a remarkably diverse and complex group with various modes of nutrition and ability to produce toxins, and are major contributors to harmful algal blooms. In the present work, we identify the presence of *Karlodinium veneficum* in India. Its detection in the coastal waters of India was possible because of live sample analysis. This species has both toxic and nontoxic strains and due to its bloom-forming capability, it is known to influence the coexisting phytoplankton community structure. In this communication we provide a description of this species, possible methods for its identification in preserved samples and effect of its extracellular and intracellular extract on the growth of *Skeletonema costatum*, an important and abundant diatom in the phytoplankton community in the Indian waters.

Keywords: Allelopathy, cell extract, harmful algal blooms, *Karlodinium veneficum*, phytoplankton, *Skeletonema costatum*.

In 1950, near Plymouth Sound, UK, a non-thecate dinoflagellate was isolated by Parke and later described and named as *Gymnodinium veneficum* by Ballantine¹. Further studies on the same strain using light and electron microscopy and partial LSU rDNA, found matching features with *Karlodinium micrum*. Thus Bergholtz *et al.*² suggested a change of name for both *G. veneficum* and *K. micrum* to *Karlodinium veneficum*.

K. veneficum (as *K. micrum*) is a widespread species, reported from various bioregions, Australia³, North America⁴, southern Africa⁵ and Europe⁶. Bergholtz *et al.*² also described its wide distribution around different water bodies. This species often gets overlooked due to its small size (<20 µm)⁷. Artifacts due to fixation could be another reason, since previous studies showed that artifacts induced by fixation are a potentially significant factor of bias to phytoplankton sample analysis^{8,9}. The majority of earlier studies conducted in the Indian waters are preservative-based. Identification of species belonging to *Karlodinium* often requires live samples². In this study, in the course of routine monitoring programme involving collection of phytoplankton samples with the

intention of raising cultures, we have isolated and cultured *K. veneficum*. This is the first report of this species from waters around the subcontinent of India.

K. veneficum is considered as a common member of the coastal phytoplankton, generally found at relatively low cell abundance, but capable of forming blooms under appropriate conditions. Such blooms have resulted in fish mortality^{4,7,10,11}. Karlotoxins produced by certain strains of *K. veneficum* and its mixotrophic capabilities are likely to be important factors contributing to the formation and continuation of blooms^{12,13}. But, in some of the cases, by releasing secondary metabolites such as allelopathic chemicals, some toxic algae can also negatively influence co-occurring phytoplankton¹⁴. Such production of toxins or release of organic compounds is considered a part of the defence mechanisms or competition strategies among phytoplankton^{15,16}.

In light of the above, we conducted two experiments on *K. veneficum* isolated from the Indian waters. The first experiment analysed the effect of four different fixatives on characteristic morphological features of *K. veneficum*. The second experiment analysed the allelopathic effect of *K. veneficum* on *Skeletonema costatum*, a common diatom in coastal, marine habitats throughout the tropical and temperate regions^{17,18}.

Dinoflagellates were isolated from sea-water samples collected from the vicinity of Dona Paula Bay along the central west coast of India (15°27'N, 73°48'E). The isolated dinoflagellates were maintained in f/2 medium¹⁹ without silicate at 22 ± 2°C using a 12 h : 12 h light : dark cycle.

The culture was examined using light microscopy (LM) and scanning electron microscopy (SEM). For SEM, the samples were fixed in 1% osmium tetroxide, placed on a nucleopore polycarbonate filter, and dehydrated in ethanol series consisting of 25%, 50%, 75%, 95% and 100% ethanol. Samples were kept for 15 min in each ethanol concentration except for 100%, where it was kept for 30 min. The samples were then critical-point dried. The filters were mounted on a stub, sputter-coated with gold and examined with a JEOL JSM-6700F field emission scanning microscope (at 5.0 kV). Samples were also stained with 4'-6-diamidino-2-phenylindole (DAPI), kept in dark for 30 min and then observed under epifluorescence microscopy to determine the arrangement of the nucleus and chloroplasts. Light and epifluorescence photomicrographs were taken using Olympus BX51 microscope equipped with Olympus DP71 camera (12.5 megapixels) and ImagePro-plus software.

An experiment was designed to study the effect of fixatives on the morphology of *K. veneficum*. The fixatives used were buffered formalin, glutaraldehyde, Lugol's iodine and osmium tetroxide. After fixation, the samples were kept in a cool, dark place for a maximum period of 30 days. Samples from all fixatives were observed at four different time intervals (days 1, 7, 15 and 30), under a

*For correspondence. (e-mail: acanil@nio.org)

light microscope using 100 \times , 200 \times , 400 \times and 1000 \times magnification. Observations at each magnification were captured using an Olympus DP71 digital camera. All the images were tiled together using ImagePro-plus software for easy comparison.

Culture filtrate (CF) and cell extracts (CE) were obtained during the exponential phase of *K. veneficum* culture (cell density 2.7×10^4 cells ml $^{-1}$). The CF was obtained by gravity filtration of *K. veneficum* culture (250 ml) through GF/F (glass fibre) filters. To obtain the CE, 250 ml of the culture was centrifuged for 15 min at 5000 rpm followed by sonication with a 40T Titanium needle probe (max power 50 W) for 2 min with 30 s interval (three cycles). After sonication, the contents were filtered through GF/F filter to obtain the CE.

S. costatum culture was inoculated in f/2 medium at a concentration of 100 cells ml $^{-1}$. Three sets of culture flasks (150 ml) in triplicate, containing 100 ml *S. costatum* culture were used. One replicate was treated with CF (7 ml), the second with CE (7 ml) and the control was levelled by adding same amount of plain f/2-silicate medium (7 ml), which was used for *K. veneficum* culture. Samples (2 ml) for cell count were taken from the control as well as from CF and CE-treated flasks every alternate day, starting from the second day of the experiment. Cell counting was carried out using a haemocytometer. Counting was possible only up to 12th day and thereafter the counting was stopped in order to avoid error due to clumping of *Skeletonema* cells.

The allelopathic effect (AE) was calculated using the equation¹⁶: $AE = [(Ctn-Ftn)/Ctn] \times 100$, wherein the difference between the cell numbers in the controls (Ctn) and in the filtrate treatments (Ftn) for the same sampling, was normalized by the cell numbers in the control, and expressed as percentage. It represents the percentage of decrease or increase of cells in the filtrate relative to the control.

The log-transformed *S. costatum* abundance data [in controls and treatments (CF/CE)] was subjected to two-way ANOVA to assess the variation across treatments and days.

The isolated dinoflagellate was identified as *K. veneficum* (Ballantine) J. Larsen. Identification of the species was carried out based on the characteristic features described in Bergholtz *et al.*² using LM and SEM.

In the case of LM, the cell outline is oval; the epicone and hypocone are of about equal size (Figure 1a). The cell dimensions ranged from 9 to 12 μ m length, and 6 to 9 μ m width. This range is smaller compared to that given by Bergholtz *et al.*². Each cell has four large chloroplasts, two each in the epicone and hypocone (Figure 1a and b). The nucleus is large, round and situated centrally in the cell (Figure 1c).

In the case of SEM the apical groove and ventral pore are clearly noticeable just above the sulcal extension (Figure 1d). The apical groove is deep and approximately 0.4 μ m wide (Figure 1e and f). The ventral pore is elongated, approximately 1.7 μ m in length and 0.2 μ m in

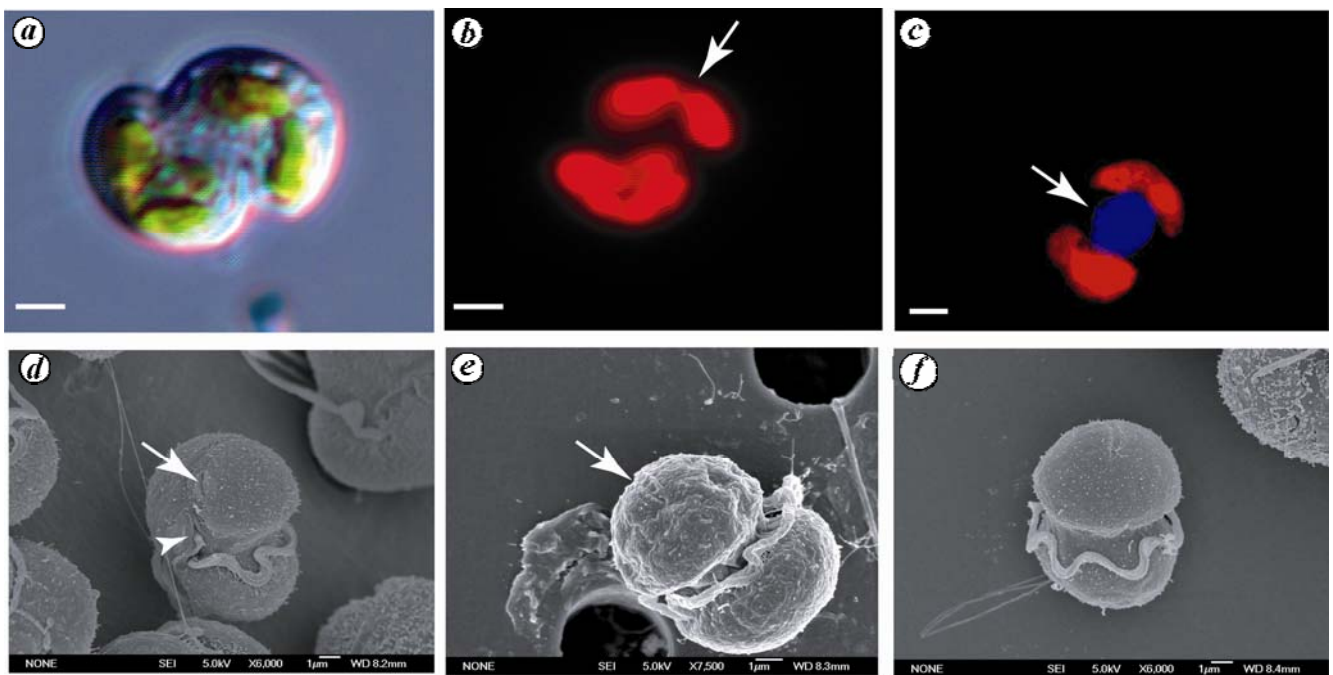


Figure 1. Light, epifluorescence and scanning electron photomicrographs of *Karlodinium veneficum*. *a*, Cell showing four large chloroplasts, two each in the epicone and hypocone. Scale bar = 2 μ m. *b*, Depression of apical groove as observed in epicone (arrow). Scale bar = 2 μ m. *c*, Cell stained with DAPI showing the position of the nucleus (arrow). Scale bar = 2.5 μ m. *d*, Ventral pore (arrow) and sulcal intrusion on the ventral side of the epicone (arrowhead). Scale bar = 1 μ m. *e, f*, Dorsal view of the cell showing apical groove (arrow) and transverse flagella. Scale bar = 1 μ m.

width and situated on the left side of the apical groove (Figure 1f). The outline of the epicone and the hypocone is round (Figure 1d-f). The transverse and longitudinal flagella can be seen clearly (Figure 1d).

We carried out an evaluation of the effect of fixatives on the morphology of *K. veneficum*. It is generally difficult to identify *K. veneficum* up to species level using light microscope alone. Some minute characteristic features like apical groove, apical pore and sulcal intrusion are important for its identification², but cannot be seen clearly under a LM.

Observation of *K. veneficum* under 100X and 200X magnification provides a pinhead appearance, and it is quite possible that it will be overlooked during routine microscopic analysis. Observation under 400X magnification can provide clues to identifiable features at the genus level (*Karlodinium/Gymnodinium*) due to a slight, dumbbell-shaped appearance. Under 1000X magnification, our observation indicated that it is possible to identify the genus and by carefully observing the chloroplast structure and arrangement, species-level features could also be visualized. But this observation is possible only if the analysis is done within one week of fixation. With increasing number of days in fixatives, the morphology changes, leading to identification errors. The results obtained indicate that till seven days, all the fixatives provided similar results. However, observations on days 15 and 30 indicated that the species in buffered formalin and glutaraldehyde had artifacts, such as no clear demarcation between the epicone and hypocone, and chloroplast disruption. However, in Lugol's iodine and osmium tetroxide, the demarcation between epicone and hypocone was still visible and artifacts were less obvious (Figure 2).

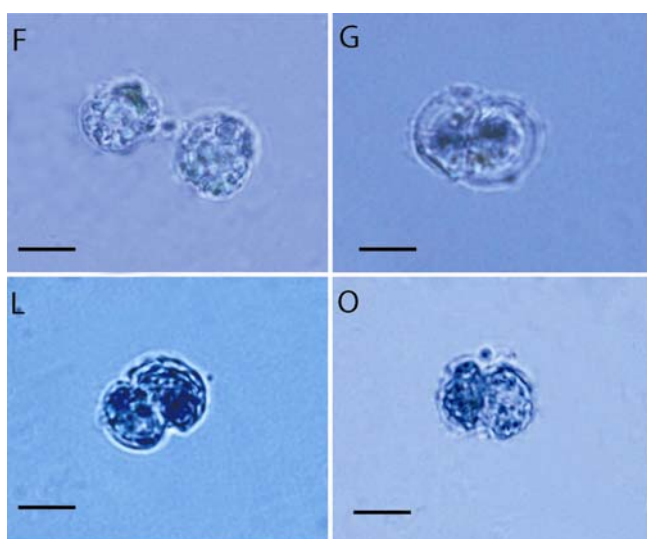


Figure 2. Light photomicrograph of *K. veneficum* taken at 1000X magnification on day-30 showing the effect of fixatives. F, Formalin; G, Glutaraldehyde; L, Lugol's iodine and O, Osmium tetroxide. Scale bar = 5 μ M.

In the case of allelopathic evaluation, initially till day-4, the CF was not inhibitory to *S. costatum*. In fact, the cell count of *S. costatum* in CF-treated flasks (in triplicates) was higher till day-4 compared to the control count. However, from day-6 onwards, an inhibitory effect was observed, which lasted till day-10 (Figure 3a). The highest AE/percentage of inhibition (33.9) was observed on day-8 (Figure 3b).

Statistical analysis indicated that *S. costatum* abundance in the CF treatment did not vary significantly from that in the control (two-way ANOVA, $P =$ not significant); it varied significantly only across days (two-way ANOVA, $P = 0.00002$).

CE inhibited the growth of *S. costatum* throughout the observation period (Figure 3a). Maximum AE (48.9%) was observed on day-10 (Figure 3b). However, the inhibitory effect lowered from day-10 to day-12 (Figure 3b).

Statistical analysis indicated that *S. costatum* abundance in the CE treatment varied significantly from that in the control (two-way ANOVA, $P = 0.00338$) and also across days (two-way ANOVA, $P = 0.00004$).

Identification of *K. veneficum* from coastal waters of India adds one more geographic location in the information about its global distribution. Non-reporting of this species from the region can possibly be related to preservative-induced artifacts. Each method of preservation

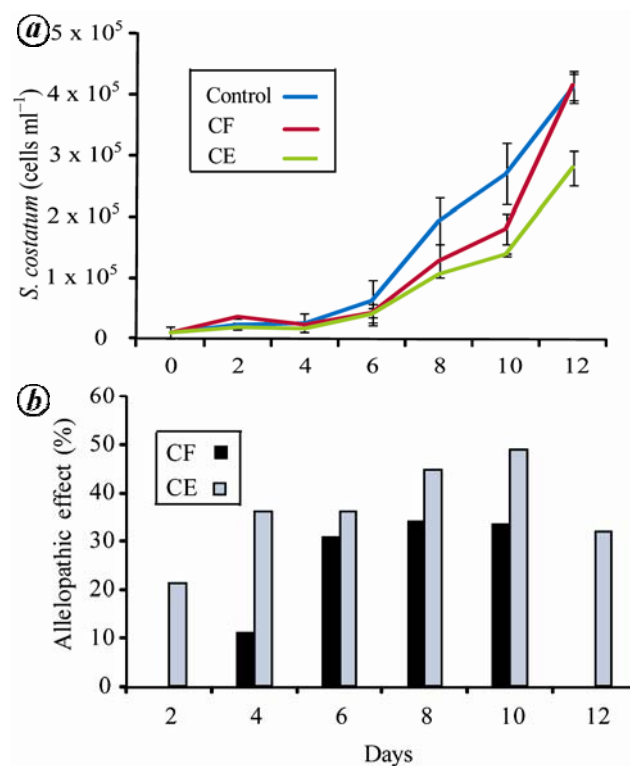


Figure 3. Allelopathic effect of *K. veneficum* on the growth of the diatom *Skeletonema costatum*. **a**, Growth curve of *S. costatum* in control, with culture filtrate (CF) and culture extract (CE). **b**, Allelopathic effect (%) in CF and CE-treated *S. costatum*.

has its own merits and demerits in preservation of taxa⁸. For example, the most important advantage of the Lugol's iodine method is that flagellates do not lose their flagella. Ingredients for the preparation of Lugol's iodine are relatively easy to obtain and this stock solution keeps well for many years. The demerits of this method are that samples fixed in Lugol's iodine need monitoring during storage as iodine oxidizes with time. The formaldehyde method maintains coccolithophorids, diatoms and thecate dinoflagellates in identifiable condition, whereas the demerit of this method is that formaldehyde fixation bleaches the cell content and thus, it becomes difficult to distinguish between pigmented and non-pigmented cells⁸. In view of this, the method of preservation depends on the objectives of the work and the targeted taxa²⁰. The goal of our first experiment was to verify the temporal changes in morphological characteristic features of *K. veneficum* in different fixatives and to determine the suitable fixative for taxonomic identification of this species. The results obtained indicate that Lugol's iodine and osmium tetroxide provide better results. Nevertheless, the danger of using osmium tetroxide outside a fume-hood, its expense and the possibility of the sample staining black limits its value²¹. Lugol's iodine, considered to be a widely used fixative⁹, was recommended earlier for preserving ciliates and flagellates⁸. *K. veneficum* fixed in Lugol's iodine provides better images depicting the characteristic outline of the epicone and hypocone. However, excess of Lugol's iodine can stain the chloroplast and render one of the identifying features of *K. veneficum* (chloroplast structure, two each in epicone and hypocone) non-recognizable. However, this problem can be solved by using sodium thiosulphate for cleaning the iodine stain⁸.

Apart from the cell outline, structure of the epicone and hypocone and chloroplasts, which are visible using LM, there are several additional features which are necessary to identify *K. veneficum* up to species level. These features are the presence and position of the ventral pore and structure of the apical groove and sulcal intrusion, and cannot be discerned in preserved samples using LM. In such cases, the use of electron microscopy facilitates identification up to species level, and therefore must be used in conjunction with LM whenever possible.

The detection of *K. veneficum* from the study area may not be surprising, since the species is known as a commonly found dinoflagellate in coastal waters around the world. But what makes its detection interesting are the characteristic features of the west coast of India, where the study area is located. This area is influenced by the southwest monsoon and enhanced nutrient loading from land²² that is known to trigger primary productivity²³. The southwest monsoon also has a role in changing the dinoflagellate community structure²⁴. The appropriate biological and ecological mechanisms of this species to attain a large biomass in nature are not clear¹². But the general understanding or the occurrence concepts of

dinoflagellate blooms are linked with eutrophication or altered nutrient ratios²⁵. Therefore, the presence of *K. veneficum* in the coastal waters of India (west coast) is of ecological interest. The Indian southwest monsoon may provide appropriate conditions for *K. veneficum* to bloom. The bloom-forming capability of *K. veneficum*, its toxin-producing potential and the consequences for the ecosystem have been reported for other water bodies in earlier studies^{4,7,10}. These studies indicate its adverse effect on fish and copepods.

It has been reported that karlotoxin produced by *K. veneficum* confers not only self-protection from grazers, but also to co-occurring species as well¹². Sheng *et al.*²⁶ showed that karlotoxins are a vital instrument in the predation process. In the present study, we examined the possibility of extracellular compounds released by *K. veneficum* as CF and intracellular compounds as CE. The results point out that the intracellular compounds can be more inhibitory to the co-existing diatom, *S. costatum* compared to the extracellular compounds. The extracellular compounds appear to be relatively short-lived. This could be one of the reasons for the substantial increase in *S. costatum* growth (compared to the control) on the 12th day in the presence of CF. This aspect needs to be explored further.

The relatively high allelopathic effect of CE indicates the important role of contents of the organism. Adolf *et al.*¹² and Waggett *et al.*¹³ have pointed out that, under grazing pressure, *K. veneficum* secretes anti-grazing metabolites. In view of this, it will be important to understand the differences between grazing-induced defensive mechanisms and population sustenance interactions with the co-existing phytoplankton. To elucidate this aspect, further studies on prey-predator interactions and competition with other phytoplankton through micro- or mesocosm experiments will be a step forward.

Experiments carried out on the effect of fixatives point out that it is possible to recognize morphological features of *K. veneficum* for as long as 30 days, when Lugol's iodine is used as a fixative. The allelopathic effect of *K. veneficum* on the growth of the diatom *S. costatum* observed in the study, suggests its potential ecological implications. However, several factors influencing the allelopathic effect of *K. veneficum*, e.g. cell concentration (dose-dependence), its relation with nutrient concentration, and the role of predation-induced defensive metabolites need to be elucidated in future experiments.

1. Ballantine, D., Two new marine species of *Gymnodinium* isolated from the Plymouth area. *J. Mar. Biol. Assoc. UK*, 1956, **35**, 467–474.
2. Bergholtz, T., Moestrup, N. D. Ø. and Fernandez-Tejedor, M., On the identity of *Karlotinium veneficum* and description of *Karlotinium armiger* sp. nov. (Dinophyceae), based on light and electron microscopy, nuclear-encoded LSU rDNA, and pigment composition. *J. Phycol.*, 2005, **42**, 170–193.

3. Hallegraeff, G. M., *Aquaculturists, Guide to Harmful Australian Microalgae*, Print Center, Hobart, Australia, 2002, p. 136.
4. Deeds, J. R., Terlizzi, D. E., Adolf, J. E., Stoecker, D. K. and Place, A. R., Toxic activity from cultures of *Karlodinium micrum* (= *Gyrodinium galatheanum*) (Dinophyceae) – a dinoflagellate associated with fish mortalities in an estuarine aquaculture facility. *Harmful Algae*, 2002, **1**, 169–1896.
5. Tengs, T., Bowers, H. A., Ziman, A. P., Stoecker, D. K. and Oldach, D. W., Genetic polymorphism in *Gymnodinium galatheanum* chloroplast DNA sequences and development of a molecular detection assay. *Mol. Ecol.*, 2001, **10**, 515–523.
6. Bjornland, T. and Tangen, K., Pigmentation and morphology of a marine *Gyrodinium* (Dinophyceae) with a major carotenoid different from peridinin and fucoxanthin. *J. Phycol.*, 1979, **15**, 457–463.
7. de Salas, M. F., Bolch, C. J. S. and Hallegraeff, G. M., *Karlodinium australe* sp. nov. (Gymnodiniales, Dinophyceae), a new potentially ichthyotoxic unarmoured dinoflagellate from lagoonal habitats of south-eastern Australia. *Phycologia*, 2005, **44**, 640–650.
8. Throndsen, J., Preservation and storage. In *Phytoplankton Manual* (ed. Sournia, A.), UNESCO, Paris, 1978, pp. 69–74.
9. Zarauz, L. and Irigoien, X., Effects of Lugol's fixation on the size structure of natural nano-microplankton samples, analyzed by means of an automatic counting method. *J. Plankton Res.*, 2008, **30**, 1297–1303.
10. Abbott, B. C. and Ballantine, D., 1957. The toxin of *Gymnodinium veneficum* Ballantine. *J. Mar. Biol. Assoc. UK*, 2008, **36**, 169–189.
11. Adolf, J. E., Bachvaroff, T. R. and Place, A. R., Can cryptophyte abundance trigger toxic *Karlodinium veneficum* blooms in eutrophic estuaries? *Harmful Algae*, 2008, **8**, 119–128.
12. Adolf, J. E., Bachvaroff, T. R., Krupatkina, D. N. and Place, A. R., Karlotoxin mediates grazing of *Oxyrrhis marina* on *Karlodinium veneficum* strains. *Harmful Algae*, 2007, **6**, 400–412.
13. Waggett, R. J., Tester, P. A. and Place, A. R., Anti-grazing properties of the toxic dinoflagellate *Karlodinium veneficum* during predator-prey interactions with the copepod *Acartia tonsa*. *Mar. Ecol. Prog. Ser.*, 2008, **366**, 31–42.
14. Legrand, C., Rengefors, K., Fistarol, G. O. and Granéli, E., Allelopathy in phytoplankton – biochemical, ecological and evolutionary aspects. *Phycologia*, 2003, **42**, 406–419.
15. Smetacek, V., A watery arms race. *Nature*, 2001, **411**, 745.
16. Fistarol, G. O., Legrand, C., Selander, E., Hummert, C., Stolte, W. and Graneli, E., Allelopathy in *Alexandrium* spp.: effect on a natural plankton community and on algal monocultures. *Aquat. Microb. Ecol.*, 2004, **35**, 45–56.
17. Hasle, G. R., Morphology and taxonomy of *Skeletonema costatum* (Bacillariophyceae). *Norw. J. Bot.*, 1973, **20**, 109–137.
18. Huang, C. J., Wang, C., Dong, Q. X. and Lin, X. P., Population dynamics of *Skeletonema costatum* in Zhelin Bay of Eastern Guangdong. *Acta Ecol. Sin.*, 2007, **27**, 142–151.
19. Guillard, R. R. L. and Ryther, J. H., Studies on marine planktonic diatoms I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.*, 1962, **8**, 229–239.
20. Gifford, D. J. and Caron, D. A., Sampling, preservation, enumeration and biomass of protozooplankton. In *ICES Zooplankton Methodology Manual* (eds Harris, R. P. et al.), Academic Press, London, 2000, pp. 193–221.
21. Taylor, F. J. R., Some specific preparations. In *Phytoplankton Manual* (ed. Sournia, A.), UNESCO, Paris, 1978, pp. 143–147.
22. Naqvi, S. W. A. et al., Coastal versus open-ocean denitrification in the Arabian Sea. *Biogeosciences*, 2006, **3**, 621–633.
23. de Sousa, S. N., Kumar, M. D., Sardesai, S., Sarma, V. V. S. S. and Shirodkar, P. V., Seasonal variability in oxygen and nutrients in the central and eastern Arabian Sea. *Curr. Sci.*, 1996, **71**, 847–851.
24. D'Costa, P. M., Anil, A. C., Patil, J. S., Hegde, S., D'Silva, M. S. and Chourasia, M., Dinoflagellates in a mesotrophic, tropical environment influenced by monsoon. *Estuarine Coastal Shelf Sci.*, 2008, **77**, 77–90.
25. Global ecology and oceanography of harmful algal blooms, harmful algal blooms in eutrophic systems. In *GEOHAB* (ed. Glibert, P.), IOC and SCOR, Paris and Baltimore, 2006, p. 74.
26. Sheng, J., Malkiel, E., Katz, J., Adolf, J. E. and Place, A. R., A dinoflagellate exploits toxin to immobilize prey prior to ingestion. *Proc. Natl. Acad. Sci. USA*, 2010, **107**, 2082–2087.

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European *Cryptothallus mirabilis* v. Malmb. matured in India – its morphological and LM & SEM palynological studies, with comments on utility for modern and fossil studies

Asha Gupta

Birbal Sahni Institute of Palaeobotany, 53 University Road, Lucknow 226 007, India

The present communication provides an account of the Scottish *Cryptothallus mirabilis* – a unique, non-chlorophyllous thalloid bryophyte which grows hidden underneath the ground layer. The studied plants were immature during collection, but have successfully matured in India under the artificial conditions. Plants show unbranched-branched fleshy thalli and enlarged calyptra which protects the sporophyte. Palynological studies has shown that spores remain adherent in their tetrad after maturity, crytopolar type, showing irregularly reticulate sculpturing at exposed surface (i.e. distal), which under SEM exhibits double ornamentation. This taxon is widely distributed but the extent of distribution is not certain due to subterranean habit. The present study will be useful in both modern and fossil studies.

Keywords: *Cryptothallus mirabilis*, fossil studies, morphological-palynological studies, Scotland India.

e-mail: ashagupta_2000@yahoo.co.uk