

## *Chapter 5*

*Effect of preservation on the morphology of  
Karlodinium veneficum, a non-thecate, potentially  
harmful dinoflagellate and allelopathy in relation  
to Skeletonema costatum*

### **5.1. Introduction**

In 1950, near Plymouth Sound UK, a non-thecate dinoflagellate was isolated by Parke and later described and named as *Gymnodinium veneficum* by Ballantine (1956). Further studies on the same strain, using light and electron microscopy and partial LSU rDNA, found matching features with *Karlodinium micrum* and thus Bergholtz et al. (2005) 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, Australia (Hallegraeff 2002), North America (Deeds et al. 2002), Southern Africa (Tengs et al. 2001) and Europe (Bjornland and Tangen 1979). Bergholtz et al. (2005) also described its wide distribution around different water bodies. This species often gets overlooked due to its small size (de Salas et al. 2005) (< 20 µm). Artifacts due to fixation could be another reason, since previous studies showed artifacts induced by fixation are a potentially significant factor of bias to phytoplankton sample analysis (Thronsen 1978, Booth 1987, Stoecker et al. 1994, Mender-Deuer et al. 2001, Zarauz and Irigoien 2008). The majority of earlier studies conducted in Indian waters are preservative-based. Identification of species belonging to *Karlodinium* often requires live samples (Bergholtz et al. 2005). 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 (Abbott and Ballantine 1957, Deeds et al. 2002, Fensin 2004, de Salas et al. 2005, Place 2005, Adolf et al. 2008). 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 (Adolf et al. 2007, Waggett et al. 2008). But, in some of the cases, by releasing secondary metabolites such as allelopathic chemicals, some toxic algae can also negatively influence co-occurring phytoplankton (Legrand et al. 2003). Such production of toxins or release of organic compounds is considered as part of the defense mechanisms or competition strategies among phytoplankton (Smetacek 2001, Tillmann and John 2002, Tillmann 2003, Fistarol et al. 2004, Tillmann 2004).

In light of the above, two experiments on *K. veneficum* isolated from Indian waters were conducted. The first experiment analyzed the effect of four different fixatives on characteristic morphological features of *K. veneficum*. The second experiment analyzed the allelopathic effect of *K. veneficum* on *Skeletonema costatum*, a common diatom in coastal, marine habitats throughout the tropical and temperate regions (Hasle 1973, Castillo et al. 1995, Chen et al. 2007, Haung et al. 2007).

## **5.2. Materials and methods**

### *5.2.1. Dinoflagellate culture*

Dinoflagellates were isolated from seawater 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 medium (Guillard and Ryther 1962) without silicate at 22±2°C using a 12h:12h light:dark cycle.

### *5.2.2. Morphological characterization*

The culture was examined using light and Scanning Electron Microscopy (SEM). For SEM, 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 5.0kV). Samples were also stained with 4'-6-diamidino-2-phenylindole (DAPI), kept under 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.

### 5.2.3. Effect of preservatives/fixatives

An experiment was designed to study the effect of fixatives on morphology of *K. veneficum*. The preservatives/fixatives used were buffered formalin, glutaraldehyde, Lugol's iodine and osmium tetroxide. After fixation, samples were kept in a cool, dark place for a maximum period of 30 days. Samples were observed at 4 different time intervals (days 1, 7, 15 and 30), under a light microscope using 100X, 200X, 400X and 1000X magnification. Observations at each magnification were captured using a DP71 Olympus digital camera. All the images were tiled together using ImagePro-plus software for easy comparison.

### 5.2.4. Allelopathy experimental set-up

Culture filtrate (CF) and cell extracts (CE) were obtained during the exponential phase of *K. veneficum* culture (cell density  $2.7 \times 10^4$  cells mL<sup>-1</sup>). The CF was obtained by gravity filtration of *K. veneficum* culture (250 mL) through GF/F (glass fiber) filters. For getting the CE, 250 mL of 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 sec intervals (3 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<sup>-1</sup>. Three sets of culture flasks (150 mL) in triplicate, containing 100 mL of *S. costatum* culture were used. One replicate was treated with CF (7 mL), the second

with CE (7 mL) and the control was leveled 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 control as well as from CF and CE-treated flasks every alternate day, starting from the 2<sup>nd</sup> day of the experiment. Cell counting was carried out using a haemocytometer. Counting was possible only up to 12<sup>th</sup> day and thereafter the counting was stopped in order to avoid error due to clumping of *Skeletonema* cells.

#### 5.2.5. Data analyses

The allelopathic effect was calculated using the equation,  $AE = [(Ctn-Ftn)/Ctn] \times 100$  (Fistarol et al. 2004) wherein, the difference between the cell numbers in the controls (Ctn) and in the filtrate treatments (Ftn) for the same sampling occasion, 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.

### **5.3. Results**

#### *5.3.1. Morphological characterization*

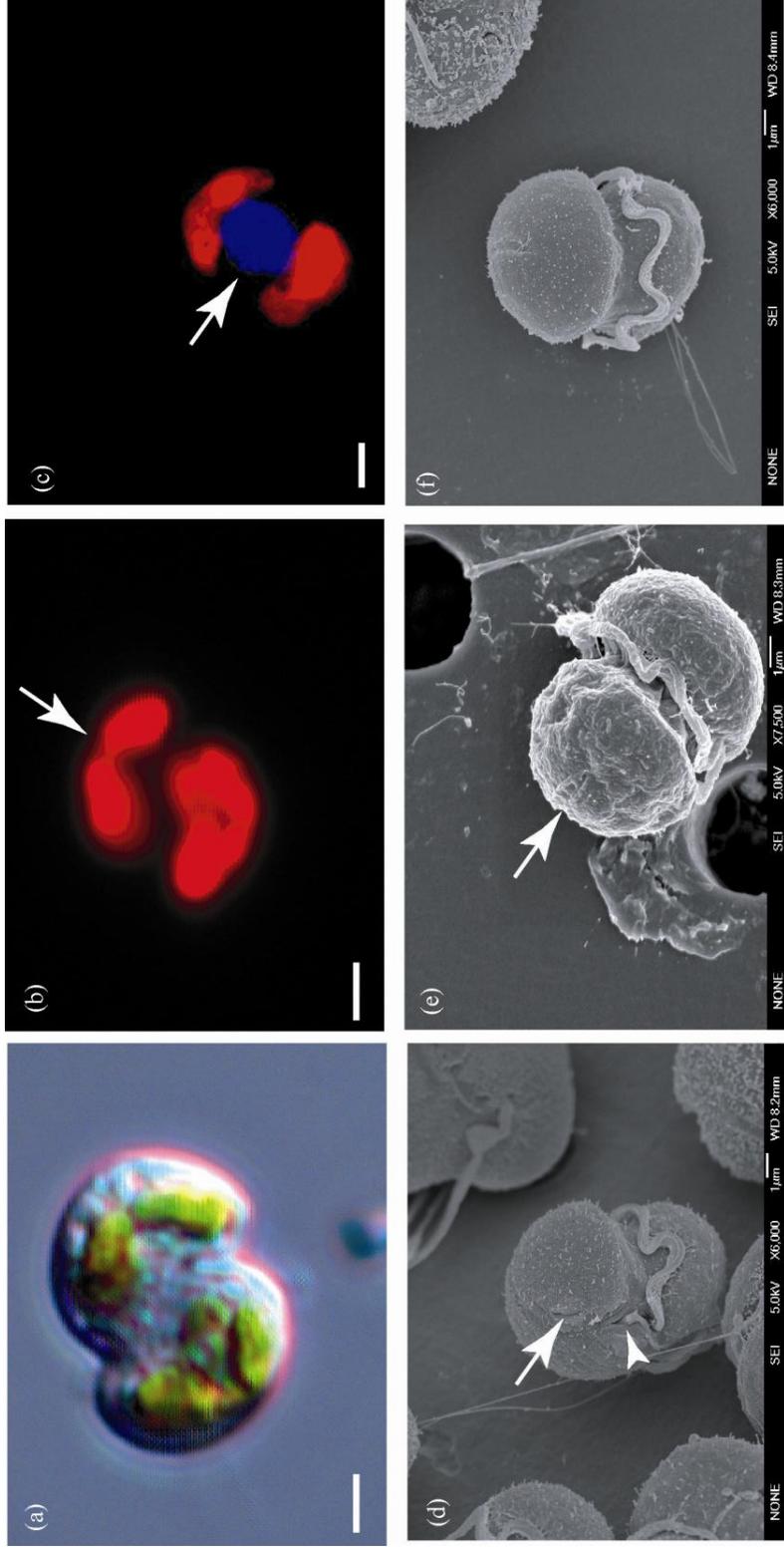
The isolated dinoflagellate was identified as *K. veneficum* (Ballantine) J. Larsen. The identification of the species was carried out based on the characteristic features described in Bergholtz et al. (2005), using light and scanning electron microscopy.

#### *5.3.2. Light microscopy (LM)*

The cell outline is oval, epicone and hypocone are of about equal size (Fig. 5.1a). The cell dimensions ranged between 9-12  $\mu\text{m}$  length and 6-9  $\mu\text{m}$  width. This range is smaller compared to the range given by Bergholtz et al. (2005). Each cell has two to four large chloroplasts, 1-2 in each epicone and hypocone (Fig. 5.1a,b). The nucleus is large, round and situated centrally in the cell (Fig. 5.1c).

#### *5.3.3. Scanning electron microscopy (SEM)*

The apical groove and ventral pore are clearly noticeable just above the sulcal extension (Fig. 5.1d). The apical groove is deep and approximately 0.4  $\mu\text{m}$  wide (Fig. 5.1e,f). The ventral pore is elongated, approximately 1.7  $\mu\text{m}$  in length and 0.2  $\mu\text{m}$  in width and situated on the left side of the apical groove (Fig. 5.1f). The outline of the epicone as well as hypocone is round (Fig. 5.1d-f). The transverse (Fig. 5.1d-f) and longitudinal flagella can be seen very clearly (Fig. 5.1d).

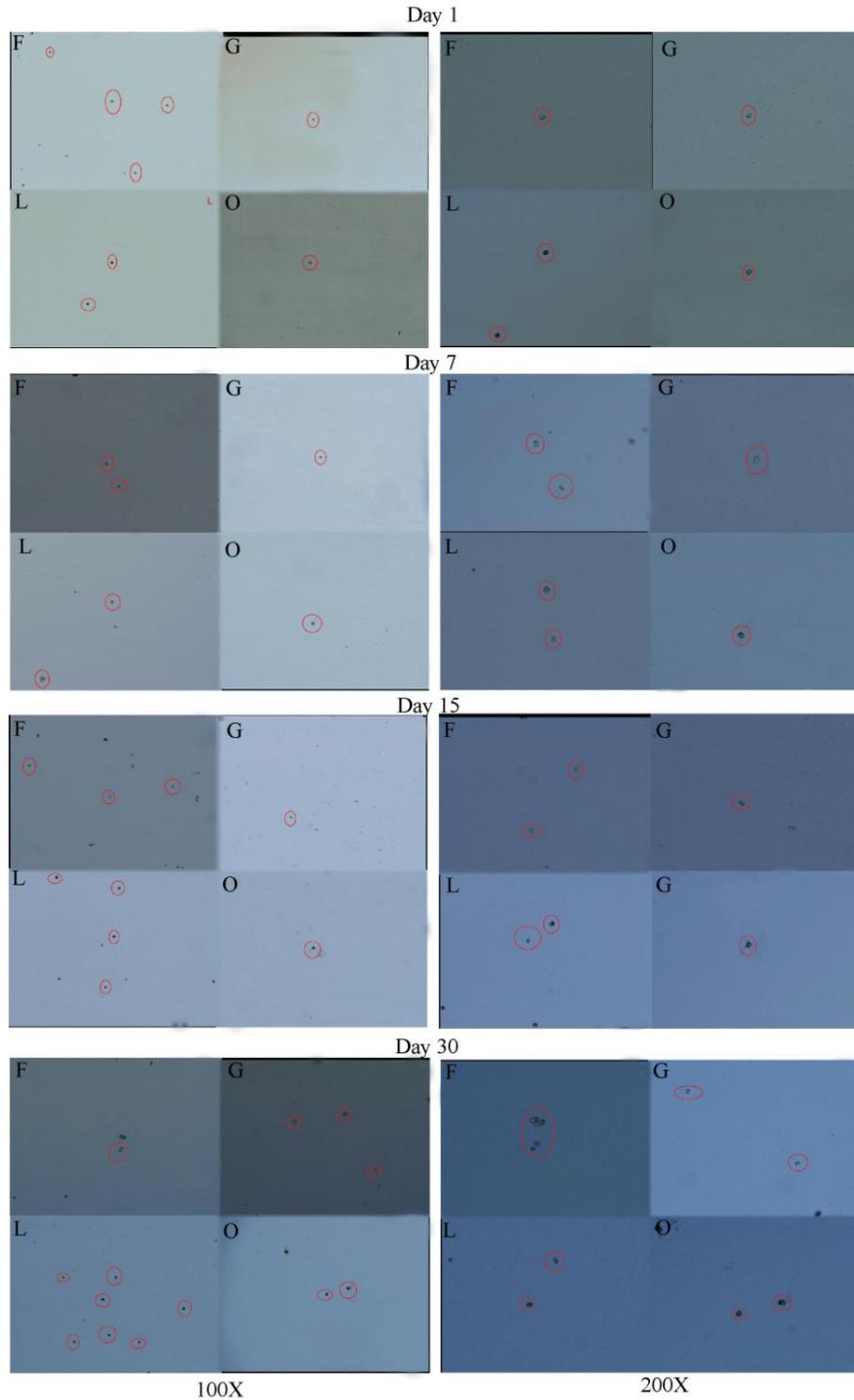


**Fig. 5.1.** Light, epifluorescence and scanning electron photomicrographs of *Karlodinium veneficum*. (a) Cell showing four large chloroplasts, two each in epicone and hypocone. Scale bar = 2  $\mu$ M. (b) Depression of apical groove as observed in epicone (arrow). Scale bar = 2  $\mu$ M. (c) Cell stained in DAPI showing the position of nucleus (arrow). Scale bar = 2.5  $\mu$ M. (d) Ventral pore (arrow) and sulcal intrusion on ventral side of the epicone (arrowhead). Scale bar = 1  $\mu$ M. (e,f) Dorsal view of cell showing apical groove (arrow) and transverse flagella. Scale bar = 1  $\mu$ M.

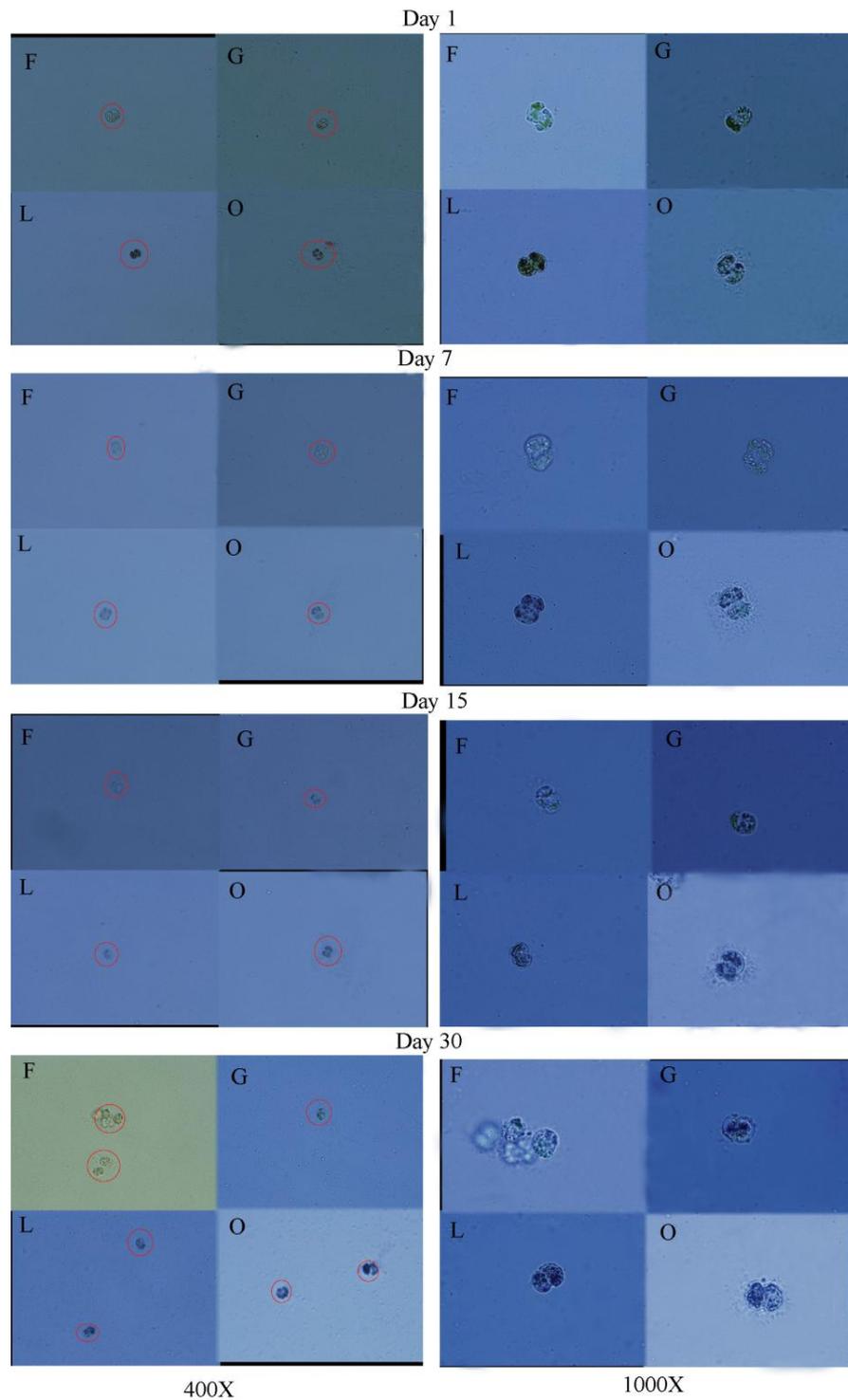
#### 5.3.4. *Effect of preservatives/fixatives*

These observations suggest the probable identification problems during light microscopy and also about the suitability of preservatives/fixatives. However, it is difficult to identify *K. veneficum* up to species level using a light microscope alone. Some minute characteristic features like apical groove, apical pore and sulcal intrusion are important for its identification (Bergholtz et al. 2005) but cannot be seen clearly under a light microscope.

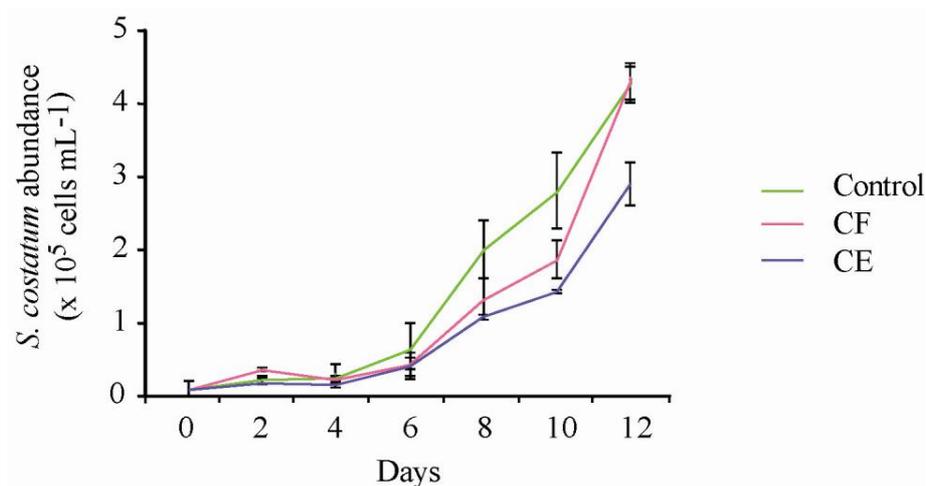
On observing under 100X and 200 X magnification, *K. veneficum* appears like a pinhead structure (Fig. 5.2) and it is quite possible that it will be overlooked during routine microscopic analysis. The observation under 400X magnification can provide identifiable features at genus level (*Karlodinium/Gymnodinium*) due to a slight, dumbbell-shaped appearance (Fig. 5.3). Under 1000X magnification (Fig. 5.3), it was 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 analysis is done within one week of fixation. With increasing number of days in preservatives/fixatives, the morphology changes, leading to identification errors. The results obtained indicate that till 7 days, all the preservatives/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 epicone and hypocone, and chloroplast disruption. However, in Lugol's iodine and osmium tetroxide, the demarcation between epicone and hypocone were still visible and artifacts were less obvious (Fig. 5.3).



**Fig. 5.2.** Light photomicrographs of preserved *K. veneficum* (100X and 200X magnification) taken at different time intervals (days 1,7,15 and 30). F-Formalin, G-Glutaraldehyde, L-Lugol's iodine and O-Osmium tetraoxide.



**Fig. 5.3.** Light photomicrographs of preserved *K. veneficum* (400X and 1000X magnification) taken at different time interval (days 1,7,15 and 30). F-Formalin, G-Glutaraldehyde, L-Lugol's iodine and O-Osmium tetroxide.



**Fig. 5.4.** Growth curve of *S. costatum* in control, with Culture Filtrate (CF) and Cell Extract (CE).

#### 5.3.5. Allelopathic effect of Culture Filtrate (CF)

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 as compared to the control count. However, from day 6 onwards, an inhibitory effect was observed, which lasted till day 10 (Fig. 5.4). The highest AE/percentage of inhibition (33.9%) was observed on day 8 (Fig. 5.5).

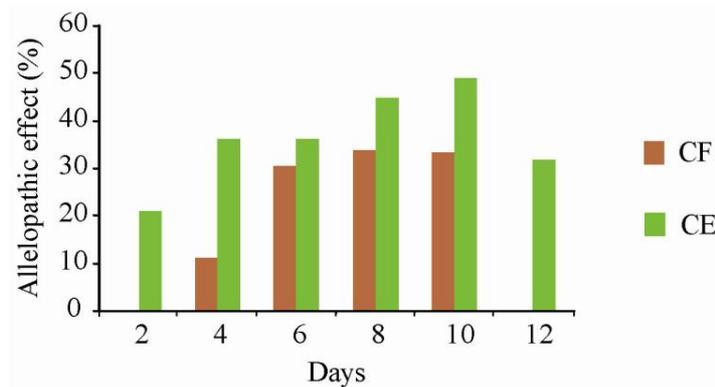
Statistical analysis indicated that *S. costatum* abundance in the CF treatment did not vary significantly from that in the control. Significant variation was observed only across days (two-way ANOVA, Table 5.1).

**Table 5.1.** Two-way ANOVA to evaluate the variation in abundance of *S. costatum* in the Culture Filtrate (CF) and Cell Extract (CE) treatments.

Factor	df	SS	MS	p-value
<b>Culture Filtrate</b>				
Treatments	1	0.0108	0.0108	0.3000
Days	6	4.0933	0.6822	<b>1.75E-05</b>
Within sub-group error	6	0.0502	0.0084	
Total	13	4.1543		
<b>Cell Extract</b>				
Treatments	1	0.1409	0.1049	<b>0.0033</b>
Days	6	3.9916	0.6653	<b>3.59E-06</b>
Within sub-group error	6	0.0287	0.0048	
Total	13	4.1251		

#### 5.3.6. Allelopathic effect of Cell Extract (CE)

Effect of CE inhibited the growth of *S.costatum* throughout the observation period (Fig. 5.4). Maximum AE (48.9%) was observed on day 10 (Fig. 5.5). However, the inhibitory effect lowered from day 10 to day 12 (Fig. 5.5).



**Fig. 5.5.** Allelopathic effect of *K. veneficum* in Culture Filtrate (CF) and Cell Extract (CE)-treated *S. costatum*.

Statistical analysis indicated that *S. costatum* abundance in the CE treatment varied significantly from that in the control and also across days (two-way ANOVA, Table 5.1).

#### **5.4. Discussion**

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 has its own merits and demerits in preservation of taxa (Thronsdén 1978). For example, the most important advantage of the Lugol's iodine method is that flagellates do not lose their flagella. Ingredients for 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 cells (Thronsdén 1978). In view of this, the method of preservation depends on the objectives of the work and the targeted taxa (Gifford and Caron 2000). The goal of the first experiment was to verify the temporal changes in morphological characteristic features of *K. veneficum* in different preservatives/fixatives and to determine the suitable choice for taxonomic identification of this species. The results obtained indicated 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 sample staining black limits its value (Taylor 1978). Lugol's iodine, considered to be a widely used fixative (Menden-Deuer et al. 2001, Zarauz and Irigoien 2008), was recommended earlier for preserving ciliates and flagellates (Thronsen 1978, Leakey et al. 1994, Karayanni et al. 2004). *K. veneficum* fixed in Lugol's iodine, provide 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, 2 each in epicone and hypocone) non-recognizable. However, this problem can be solved by using sodium thiosulphate for cleaning the iodine stain (Thronsen 1978).

Apart from the cell outline, structure of the epicone and hypocone and chloroplasts, which are visible using light microscopy, 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 light microscopy. In such cases, the use of electron microscopy facilitates identification upto species-level (Hasle 1978), and therefore must be used in conjunction with light microscopy 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 make its detection interesting are the characteristic features of the west coast of India where the study area is located. The west coast of India is

influenced by the South West Monsoon (SWM) coupled with upwelling that results in high nutrient conditions (de Souza et al. 1996). This, in turn, triggers high primary production (Raghukumar and Anil 2003). The SWM also has a role in changing the dinoflagellate community structure (D'Costa et al. 2008). The appropriate biological and ecological mechanisms of this species to attain a large biomass in nature are not clear (Adolf et al. 2007). But the general understanding or the occurrence concepts of dinoflagellate blooms are linked with eutrophication or altered nutrient ratios (GEOHAB 2006 and references therein). Therefore, the presence of *K. veneficum* in the coastal waters of India (west coast) is of ecological interest. The Indian SWM conditions 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 (Abbott and Ballantine 1957, Deeds et al. 2002, Fensin 2004, Place 2005, de Salas et al. 2005, Adolf et al. 2008); 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 well (Adolf et al. 2007). Sheng et al. (2010) showed that karlotoxins are a vital instrument in the predation process. In the present study, the possibility of extracellular compounds released by *K. veneficum* as CF and intracellular compounds as CE, was considered. 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 12<sup>th</sup> day in 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. (2007) and Waggett et al. (2008) 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 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.

Summarizing the results, the experiments carried out on the effect of fixatives, point out that it is possible to recognize morphological features of *K. veneficum* as long as 30 days, when Lugols 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*, for e.g., cell concentration (dose-dependence), its relation with nutrient concentrations, and the role of predation-induced defensive metabolites need to be elucidated in future experiments.

## *Chapter 6*

### *Summary*