

Genotoxicity of cadmium in marine diatom *Chaetoceros tenuissimus* using the alkaline Comet assay

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Abstract Genotoxic effects of Cadmium on phytoplankton *Chaetoceros tenuissimus* have been evaluated using DNA damage by comet assay. Cadmium concentrations ranging from 2.4 to 10 mg/L were used to evaluate the effects. Results showed that as the concentration of Cd increased growth of the diatom decreased. Alkaline single-cell gel electrophoresis (Comet assay) method, which is highly sensitive in detection of DNA damage in eukaryotic cells, was used to observe genomic changes in marine diatom cells. DNA damage was measured as percent number of comets and normal cells. 65% cells were found to be damaged at 10 mg/L concentration of Cd as compared to 23% in 2.4 mg/L and only 5% in controls. More than 50% apoptic cells were observed on 8th day at 10mg/L and 12th day at 7.5 mg/L concentrations. At lower Cd concentrations (4.5 mg/L and below) the damage was below 30% till the last day. This suggested that higher Cd levels have early damaging effects on cell nuclear material and that % injury increases with advancement of exposure period. One advantage of use of *C. tenuissimus* is the ease with which it can be cultured in a defined medium. *Chaetoceros tenuissimus* diatom can be used as an in vivo model for ecogenotoxicity assessment using the Comet assay.

Keywords: Growth, *Chaetoceros tenuissimus*, Comet assay, Single-cell gel electrophoresis, DNA damage

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Introduction

Coastal waters in India are subject to serious threat of heavy metal (Cd, Pb and Hg) pollution arising from the industrial and domestic effluents from major cities such as Mumbai, Chennai, Kolkata, as also the industrial towns situated along the coast. Govindasamy and Azariah (1999) while studying the metal concentrations along Coromandal coast, India, indicated that high Cd concentration near Mahabalipuram was due to the discharge of bulk quantities of industrial and sewage discharges of at least 16 major and minor industries, directly into the sea. Similarly Krishnamurti and Nair (1999a, b) observed alarmingly high bioaccumulation of metals in fishes, prawns and crabs in Thana-Bassein Creeks in Mumbai, which receives large industrial and domestic

effluents. Senthilnathan and Balasubramanian (1999) while studying metal distribution along Pondicherry harbour, observed a relationship between metal concentration in water and plankton growth and suggested that plankton plays an active role in removal of metal from ambient seawater and also acts as pollution indicator.

Since phytoplankton are the primary organisms subjected to pollution stress, the effects of metal contamination on these organisms gain importance. Besides interfering in metabolic enzyme systems, Cd, complexes with amino acids, peptides and proteins, there by affecting the conformation of polyriboadenylic acid, as well as physical properties of DNA (Conway, 1978). Some of the reports on the effects of Cd on bacteria (Volesky and Holan, 1995), cyanobacteria (Inthorn et al., 1996), algae (Holan et al., 1993) and marine phytoplankton (Torres et al., 1998) have shown its bioaccumulation potential and toxicity. However little is known about the genotoxicity of Cd especially in phytoplankton. Since Cd is a hazardous environmental pollutant, widespread along the Indian coastal waters, the need was felt to study the influence of this metal on the growth and genomic DNA of phytoplankton.

Genotoxicity assessment using single cell gel electrophoresis or Comet assay in the recent years is gaining importance. Comet assay is a valuable technique, which allows detection of DNA damage and repair at single cell level and provides a unique opportunity to investigate intercellular differences. Aoyama et al. (2003) studied the effect of benzo[a]pyrene, mitomycin C and actinomycin D on the DNA of *Euglena gracilis* cells by comet assay; and Wilson et al. (1998) studied the detection of DNA damage in the cells of a marine invertebrate *Mytilus edulis* using the comet assay

In the present investigation Comet assay was used to evaluate the DNA damaging potential of Cd in laboratory cultures of phytoplankton species *Chaetoceros tenuissimus*, isolated from the coastal waters.

Material and Methods

Chaetoceros tenuissimus was isolated from the coastal waters of Kandla harbor, Gujarat and grown into unialgal cultures in laboratory at NIO, Goa. They were further treated with antibiotics to maintain as axenic cultures during the experimental period. Batch cultures were maintained in 50ml volume in 100ml Erlenmeyer flasks using filtered autoclaved seawater enriched with F/2 medium (Guillard and Ryther, 1962). The initial cell density was 2×10^4 cells per liter. The subcultures were kept in a culture chamber with fluorescent light (40 W) on a 14:10; light:dark cycle at 25°C.

Preparation of test cultures

Test concentrations of Cd were decided based on preliminary toxicity test. For the pilot experiment, Cadmium ($\text{CdCl}_2 \cdot \text{H}_2\text{O}$) concentrations of 1.15, 1.55, 2.1, and 2.8 mg/L were tested on the growth of *C. tenuissimus*. Since growth in these concentrations was almost similar as in control, higher concentrations of 2.4, 4.2, 7.5 and 10mg/L of cadmium were taken for the final experiments. For each series of concentrations controls were maintained in which Cd was not added. A subculture in the exponential growth phase was added as inoculum for the test cultures (1 ml for each flask) maintaining equal initial cell density. Growth was assessed on alternate days by Chlorophyll *a* analysis by removing 0.5 ml aliquots of cultures over a period of 20 days depending on the longevity of the experiments. Chl *a* was measured by acetone extract method using fluorometric techniques. All the experiments were run in triplicates.

Alkaline single cell gel electrophoresis / Comet assay

The Comet assay with *C. tenuissimus* was performed by the modified method of Singh et al. (1988) and Aoyama et al. (2003). 1ml of culture was harvested on alternate days from each flask. After centrifugation, at 2000 rpm for 5 min, cell pellets were resuspended in 500µl of 0.5% Low Melting Agarose (LMA GeneI) and 100µl aliquots were poured onto frosted microscopic slides, which were previously coated with 200µl of 0.5% Normal Melting Agarose (NMA GeneI) dissolved in 1X TAE buffer. The agarose was allowed to

set for 10 to 15 minutes on an aluminium foil at 4⁰C before addition of a final layer of 100 µl LMA carrying the treated or untreated cell suspensions. After the LMA has solidified, the slides were placed in lysing solution (300mM NaOH, 30mM Na₂EDTA and 0.01% SDS, pH10) in a Coplin jar for 5 min at 4⁰C, followed by a rinsing in two changes in distilled water to remove excess salts.

Alkaline unwinding was carried out at 15⁰ C in a gel electrophoresis chamber (GeneI) containing chilled alkaline solution (300mM NaOH, 1mM Na₂EDTA) for 20 minutes and electrophoresis was performed in the same chilled buffer to prevent overheating during electrophoresis for 20 minutes at 20 volts and 300 milliamps. The slides were then neutralized by dropwise coating of slides with neutralizing buffer (400mM Tris pH 7.4) for about 5 minutes in a drain tray. The slides were drained for 2 more times and stained with 50 µl ethidium bromide (10 µg/ml of distilled water) for 5 mins and dipped in chilled distilled water to remove excess stain. A coverslip was placed over the slides and examined in an epifluorescence microscope under 10X eye lens and 40X objective and number of normal nuclei and number of comets were counted, converted into percentages and tabulated.

Analysis of data

Growth for test cultures was measured by analyzing Chl a (mg/ml) on every alternate day and two- way analysis of variance (ANOVA) was applied to find the variations among test concentrations. Observed percent comets nuclei were analysed individually using Two-way ANOVA, to find the variations among treatments, by assuming the variation within each treatment as equal. Student-Newman-Keuls test was used to test the difference between treatment samples by comparing Least Significant Difference (LSD) (Winner, 1971).

Results

Figure 1 depicts the effect of Cd on the growth of *C. tenuissimus*. Data were statistically analysed by ANOVA and Student 't' test was done ('t' table value at 0.01). Statistical

analysis revealed that growth in control and 2.4 mg/L Cd concentration was almost similar ($p>0.01$) with slightly more growth in 2.4 mg/L concentration than in control from 10th day onwards. Gradual increase in growth of *C. tenuissimus* in control cultures reached maximum on 10th day followed a stationary phase up to 16th day and declined after 18th day. While in Cd concentration of 2.4 mg/L growth enhanced the control cultures after 10th day to attained a peak on 16th day and thereafter a decline phase from 18th day onwards. Further increase in Cd concentrations (4.2, 7.5 & 10 mg/L) decreased the growth. Of these concentrations growth in 4.2 mg/L followed the pattern of 2.4 mg/L, however, difference was insignificant (LSD, $p>0.01$). Growth in 7.5 and 10 mg/L was significantly inhibited as compared to control cultures and 2.4 mg/L (LSD, $p<0.01$).

From the Fig. 1, it is also clear that, in all the concentrations including control, growth showed, exponential increase form 2nd day onwards. However maximum growth was attended on different days in different concentrations. In Cd concentrations of 4.2 mg/L growth attended the maximum on 16th day and declined on 18th day. While growth pattern of cultures in 7.5 and 10 mg/L was similar from initial day to the end of the experiment.

Figure 2 depicts the DNA damage in *C. tenuissimus* exposed to different Cd concentrations observed as percent nuclei with comets. Among the different Cd concentrations, statistical tests showed significantly higher DNA damage ($p<0.01$) in 10 mg/L (65% damage) and lowest in 2.4 mg/L (23% damage), with control remained below 5%. Difference in DNA damage at control, 2.4 mg/L and 4.2 mg/L was insignificant ($p>0.01$). More than 50% apoptic cells were observed on 8th day at 10 mg/L and 12th day at 7.5 mg/L concentrations. At lower Cd concentrations (4.5 mg/L and below) the damage was below 30% till the last day. Fig 2, also reveals that, from 2nd day onwards, on 12th and 14th day at Cd concentrations below 4.2 mg/L and on 14th and 16th day at higher levels the declining damage could be noticed.

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Discussion

It was observed, from the two way analysis of variance (ANOVA) for growth and comet cells that, both the concentration and days were highly significant at $p \leq 0.01$ and for comet cells, days and concentration together was also observed to be significant ($P \leq 0.01$).

From Fig (1) and statistical analysis it is clear that, there was a significant effect on growth of *C. tenuissimus* at Cd concentrations of above 2.4 mg/L. There were no significant differences between control cultures without Cd and cultures with 2.4 mg/L ($P > 0.01$). This Cd concentration was not inhibitory to *C. tenuissimus* as the growth in 2.4 mg/L enhanced the control cultures after 10th day to attain a peak on 16th day and thereafter a decline phase from 18th day onwards. While in control cultures peak on 10th day followed a stationary phase up to 16th day and declined after 18th day.

Cultures in 4.2 mg/L also followed the growth peak up to 16th day and declined from 18th day onwards as seen in 2.4 mg/L Cd levels. However as a whole growth at 4.2 mg/L concentration was not significantly different from 2.4 mg/L, which is shown by LSD values ($p > 0.01$). At higher concentrations i.e. 7.5 and 10 mg/L growth was drastically reduced as compared to lower concentrations. This suggests that higher concentrations of 7.5 mg/L and above have inhibitory effects on the growth of *C. tenuissimus*. Rebhun and Ben-Amotz (1984) while working with marine microalga *Chlorella stigmatophora* observed similar trend and reported that Cd concentrations above 5 mg/L had inhibitory effects on growth. The inhibitory effects above 7.5 mg/L concentrations were further confirmed by the LSD values ($P < 0.01$). Similar patterns have been obtained by Torres et al. (1998) while studying the removal of Cd ions by the marine diatom *Phaeodactylum tricorutum*; and Garnham et al. (1992) while studying uptake and intracellular location of cobalt, manganese and zinc in the estuarine green alga *Chlorella salina*.

Since the changes in pigment concentrations may not necessarily indicate the changes at cellular components, the comet assay procedure was standardized to understand the

effect on nuclear materials of phytoplankton cells exposed to Cd. Fig 2 depicts the % DNA damage in *C. tenuissimus* over 20 days experiment. It can be seen that among the different Cd concentrations, higher DNA damage was detected in 10 mg/L (65% damage) and lowest in 2.4 mg/L (23% damage), with control remained below 5%. Statistical tests (LSD) showed significant difference ($P < 0.01$) comet cells at control, 2.4 and 4.2 mg/L with 7.5 mg/L Cd concentrations. However this relation was insignificant within control, 2.4 and 4.2 mg/L and between 7.5 and 10 mg/L Cd levels. More than 50% apoptic cells were observed on 8th day at 10 mg/L and 12th day at 7.5 mg/L concentrations. At lower Cd concentrations (4.5 mg/L and below) the damage was below 30% till the last day. This indicates that higher Cd levels have early damaging effects on cell nuclear material and that % injury increase with advancement of exposure period.

A close look at Fig 2 indicates that although DNA damage showed exponential increase at all concentration, from 2nd day onwards, on 12th and 14th day at Cd concentrations below 4.2 mg/L and on 14th and 16th day at higher levels the declining damage could be noticed. Similar trend was evident in chlorophyll growth curve also (Fig 1). This could be indicative of the repair mechanisms which is inherent in the organism's cell as indicated by Hook and Lee, (2004) upon studying the different stages of the embryos of grass shrimp (*Palaemonetes pugio*) exposed to various concentrations of benzo[a]pyrene (BP), Cr(VI) and hydrogen peroxide, DNA repair studies showed that oxidative lesions were repaired more rapidly in stage 7 than in stage 4. Declining damage could also be due to the induction of phytochelatins, which could bind the excess metal ions, thereby reducing the Cd toxicity (Morelli and Pratesi, 1997).

However from 18th day onwards DNA damage increased with consequent fall in growth curve, which could be due to inadequacy in tolerance capacity of the phytochelatins to arrest the metal ions. Pruski and Dixon, (2002) observed decrease in post-treatment DNA repair in gill cells of *Mytilus* that had been pre-treated with Cd, in vivo or in vitro. Decreased growth of *C. tenuissimus* may be due to combined effect of increased DNA damage (as observed by Wilson et al., 1998), while studying effect of different

concentration of NDMA and H₂O₂ on *Mytilus*) and competition among cells for the nutrients and space.

It is concluded from this study that DNA damage in *C. tenuissimus* induced by Cadmium can be evaluated by comet assay. Based on the comparative study of growth pattern and % DNA damage in different Cd concentrations this organism seems to be sensitive to the heavy metals. It is noted that higher the concentrations of Cd higher the damage and lower the growth of *C. tenuissimus*. One advantage of use of *C. tenuissimus* is the ease with which it can be cultured in a defined medium. Thus *Chaetoceros tenuissimus* diatom can be used as an *in vivo* model for ecogenotoxicity assessment using the Comet assay.

Acknowledgement The authors are grateful to Dr. S. R. Shetye, Director, NIO for encouragement. Thanks are also due to Dr. S. G. Dalal for his help in statistical analysis. This work was carried out with funding (GAP 0323) from the Department of Ocean Development, New Delhi. This is NIO contribution No.4119.

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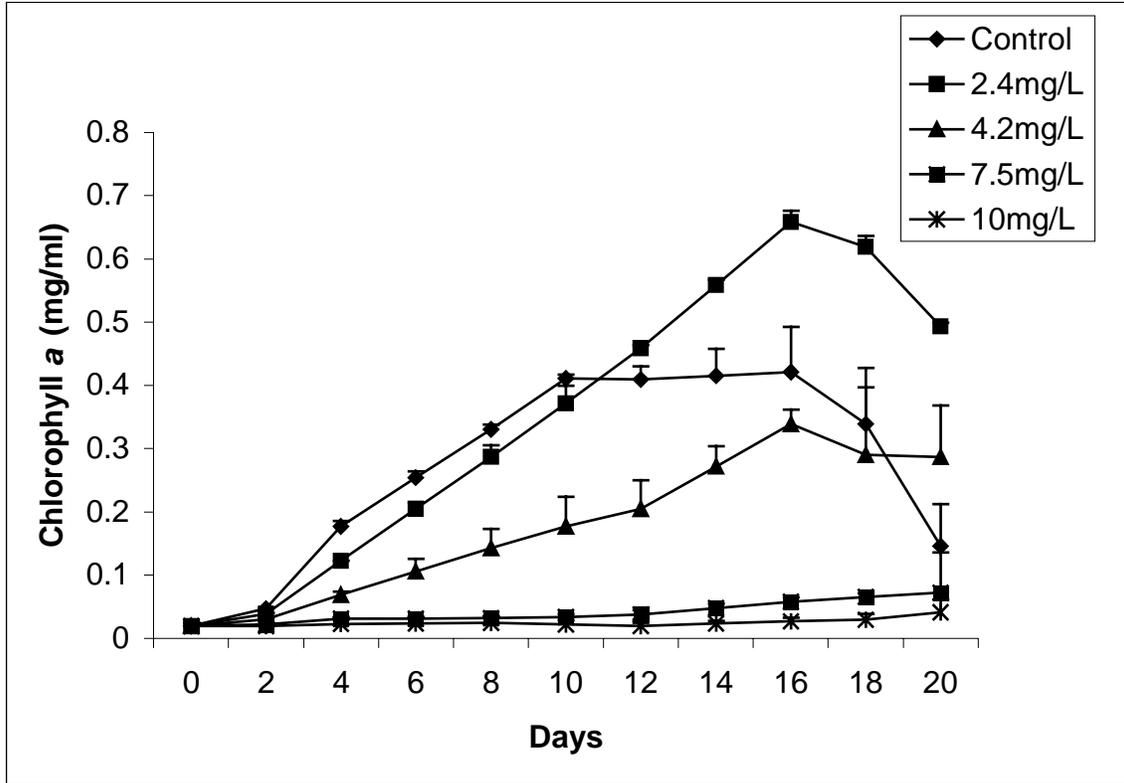


Fig 1: Effect of Cd on the growth of *Chaetoceros tenuissimus*
 (data are expressed as mean \pm SD, n=3)

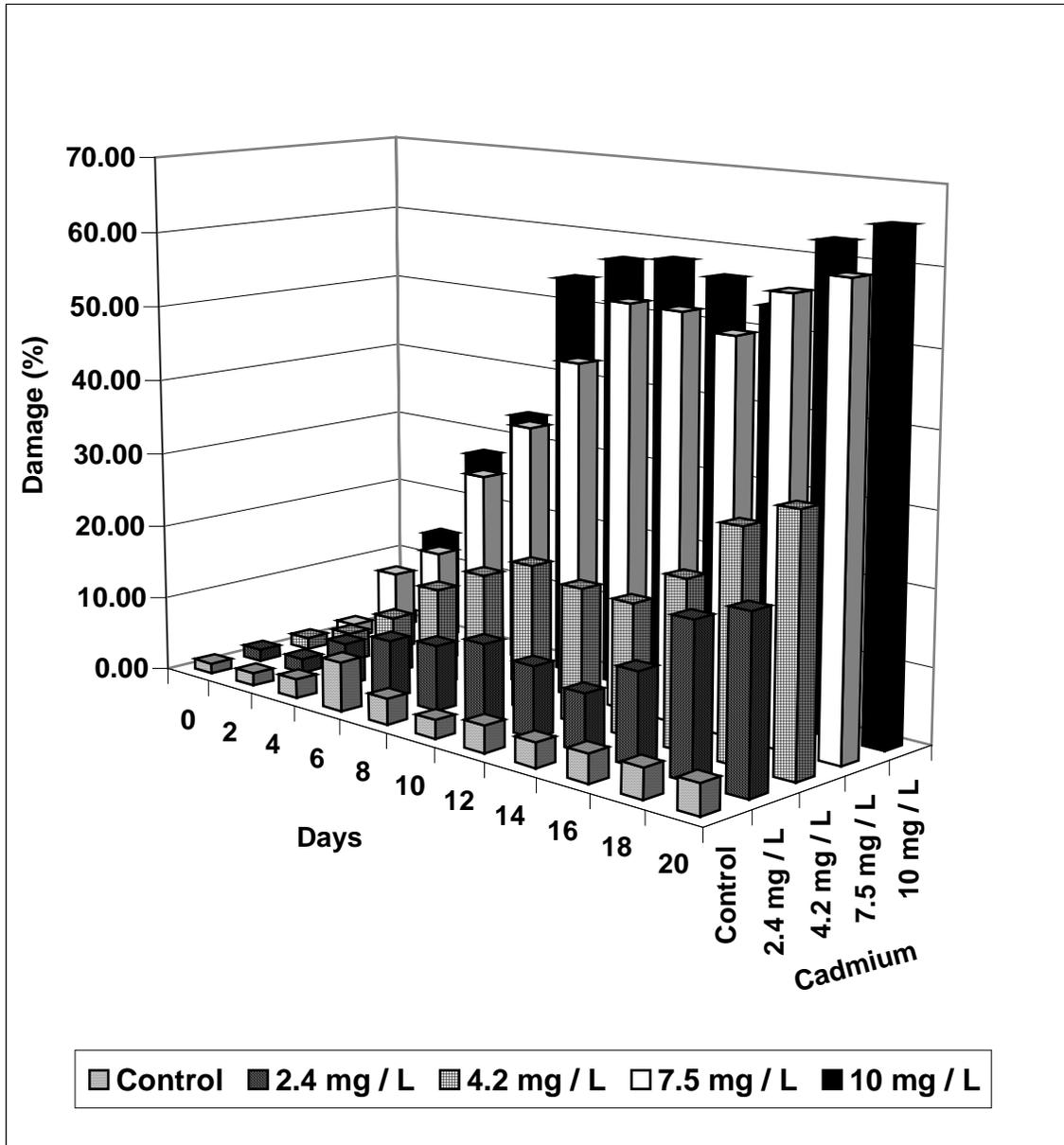


Fig 2. DNA damage as percent nuclei with comets in *Chaetoceros tenuissimus* exposed to Cd.