

Genotoxicity of cadmium chloride in the marine gastropod *Nerita chamaeleon* using comet assay and alkaline unwinding assay

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Abstract

This paper presents an evaluation of the genotoxic effects of cadmium chloride (CdCl₂) on marine gastropod, *Nerita chamaeleon* following the technique of comet assay and the DNA alkaline unwinding assay (DAUA). In this study the extent of DNA damage in gill cells of *N. chamaeleon* were measured after *in vivo* exposure to four different concentrations (10; 25; 50 and 75 µg/L) of CdCl₂. *In vitro* exposure of H₂O₂ (1; 10; 25 and 50 µM) of the gill cells showed a significant increase in the % tail DNA, Olive tail moment (OTM) and tail length (TL). Significant changes in % tail DNA by CdCl₂ exposure were observed in all exposed groups of snails with respect to those in control. Exposure to 75 µg/L of CdCl₂ produced significant decrease in DNA integrity as measured by DAUA at all duration with respect to control. *In vivo* exposure to different concentrations of CdCl₂ (10; 25; 50 and 75 µg/L) to *N. chamaeleon* showed considerable increase in DNA damage as observed by both alkaline comet assay and the DAUA. The extent of DNA damage in marine gastropods determined by the application of alkaline comet assay and DAUA clearly indicated the genotoxic responses of marine gastropod, *N. chamaeleon* to a wide range of cadmium concentration in the marine environment.

Keywords: genotoxicity; comet assay; alkaline unwinding assay; gastropods; DNA integrity; cadmium chloride

1. Introduction

Cadmium is one of the most toxic compounds that pose serious threat to the health of the marine ecosystem, the mechanisms of its toxicity are still not clearly understood (Baršienė et al., 2013; Michel Cornet, 2007). It is a systemic poison affecting many cellular functions (Cao et al., 2007; Waalkes, 2003; Abdulla and Chmielnicka, 1989). The toxicity of cadmium was assessed in different species of marine organisms by several scientists all over the world (Jose et al., 2011; Sébastien et al., 2010; Sokolova et al., 2005). It has been classified as a human carcinogen by IARC (1993). Cadmium exposure induces intracellular damage through several mechanisms. In cultured cells, cadmium produces direct and indirect genotoxic effects such as DNA strand breaks, DNA-protein cross links, oxidative DNA damage and chromosomal aberrations (Hwua and Yang, 1998; Misra et al., 1998; Dally and Hartwig, 1997). Recent works have demonstrated the interest of using the comet assay for assessing the genotoxicity of cadmium in aquatic ecosystems (Ahmed et al., 2010; Chang et al., 2009; Emmanouil et al., 2007; Hook and Lee, 2004 and Faverney et al., 2001).

In view of the ongoing problems of contamination of the marine environment, the use of molecular biomarkers such as DNA integrity and DNA strand breaks measured at the molecular level are of immense significance for biomonitoring of pollution (Osterauer et al., 2011; Fernando et al., 2005; Carajaville et al., 2000).

The DNA alkaline unwinding assay (DAUA) was used to detect DNA damage caused by complex environmental contamination in aquatic test organisms (Bechmann et al., 2010; Oliveira et al., 2010; Pisanelli et al., 2009; Ahmad et al., 2008; Sarkar et al., 2008). This assay enables the assessment of primary DNA damage in tissue from exposed aquatic test organisms. In DAUA, whole cells or crude DNA extracts are subjected to alkaline assay conditions to allow controlled 'unwinding' of double-stranded DNA into single-stranded DNA, beginning at each strand break (Erixon and Ahnstrom, 1979). The number of DNA strand breaks in the original sample is inversely proportional to the fraction (F) of DNA remaining double stranded after the assay (Rydberg et al., 1975). A simple method for quantifying strand breaks uses a dye (Hoechst dye 33258) that fluoresces preferentially in the presence of double-stranded DNA and was developed for use with single cells. The measurement of DNA damage in control samples exposed with known concentration of toxicants following the technique of DAUA is useful for screening potential genotoxicants and for investigating relationships between DNA processes such as damage, synthesis and repair (Nacci et al., 1992).

Comet assay also called as single cell gel electrophoresis assay, is a simple, sensitive, rapid and versatile tool to access DNA damage and repair. In the last few years it has become one of the most sophisticated and reliable tool to detect genotoxicity in cells exposed to chemical or physical agents.

Since its inception, comet assay has been used in several animals from fungi (*Saccharomyces cerevisiae*, Banerjee et al., 2008), algae (*Cryptophyta*, Sastre et al., 2001), plants (*Vicia faba*, Menke et al., 2000), mussels (*Limnoperna fortunei*, Villela et al., 2007; *Dreissena polymorpha*, Riva et al., 2007), polychaetes (*Nereis virens*, Boeck and Volders, 1997), fruit flies (*Drosophila melanogaster*, Siddique et al., 2005), amphibians (*Rana tigrina*, Ralph and Petras, 1997) and birds (*Ciconia ciconia*, *Milvus migrans*, Baos et al., 2006). Few studies have reported on the occurrence of DNA damage in gastropods (Hubert et al., 2012; Osterauer et al., 2011; Grazeffe et al., 2008; Hagger et al., 2006; Regoli et al., 2006; Benton et al., 2002).

The choice of marine gastropods as a model organism for environmental biomonitoring of genotoxic pollutants took into account that invertebrates represent more than 90% of aquatic species. The phylum mollusca have the highest number of animal species after arthropods and 80% of molluscs species are represented by gastropods (Strong et al., 2007). Moreover gastropods are easy to breed, need little space, can reproduce throughout the year under controlled conditions and have a short life-span. Gill cells were selected for this study since they were easy to obtain, and in nature they come into contact with relatively large volumes of seawater compared with the rest of the animal, thus conferring them with the potential for being a suitable target tissue for mutagen exposure (Dixon and Clarke, 1998; Venier and Canavo, 1996).

2. Materials and Methods

2.1. Chemicals

CdCl₂ (analytical grade, purity ≥98%), calf thymus DNA, ethidium bromide, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), gluaiacol glycerol ether, hydrogen peroxide, trypan blue and sephadex G-50 were purchased from Sigma Aldrich Pvt. Ltd. (Mumbai, India); Frosted slides and cover slips were supplied by Yogeshwari Agencies (Himedia, Goa, India); DMSO was obtained from Scitech Scientific (Qualigens, Goa, India); Tris buffer and triton X-100 were obtained from Sadhale Enterprise (Merck, Goa, India).

2.2. Sampling site and Gastropods

The marine gastropods were collected from the site at Palolem (Fig. 1a,b) which is a 1.5 km falcate shaped beach, located on the west coast of India about 37 kms away from the city of Margao and 2 kms west of Chaudi in the Canacona taluka of Goa, India. The sampling site was chosen because of its clean environment. The beach lies on the southern coastline of Goa amidst outstanding natural beauty. There are no known industries close to the site.

Marine gastropods (*Nerita chamaeleon*) were collected during low tide from intertidal rocks scattered along the Palolem beach, Goa, India. Organisms were identified by the experts from Zoological Survey of India; Kolkata, India using the certified reference samples (Subba Rao, et al., 1992). A typical picture of the marine gastropod, *Nerita chamaeleon* is shown in Fig.2. The samples were thoroughly washed and stored with aeration in sea water at room temperature for 48h to acclimatize them to laboratory condition. The organisms were exposed for 5 days to various concentrations of CdCl₂ (10; 25; 50; 100 µg/L and control) in aerated seawater. Seawater was changed on daily basis and replaced with fresh seawater with respective concentrations of CdCl₂. The CdCl₂ stock solution was dissolved in ultra pure water (100 mg/L) and stored at 4°C.

2.3. Alkaline unwinding assay

DNA integrity in *N. chamaeleon* was determined by following the technique of partial alkaline unwinding assay (Everaarts and Sarkar, 1996; Rao et al., 1996; Everaarts et al., 1995). Immediately after collection, the snails were sacrificed by breaking the shells with the help of a hammer and the tissue removed from the shell was kept on ice on a petri dish during the sample processing for preservation of the activity of DNA. The tissues were placed in the bottom of a dounce homogeniser with 1 ml of 1N NH₄OH/0.2% TritonX-100 per 200-400 mg of tissue. After homogenization, 1 ml of triple distilled water was added per 200 mg of tissue. The DNA purification was accomplished by extraction with CIP chloroform/isoamyl alcohol/phenol (24/1/25, v/v). The samples were shaken to fully denature all proteins and centrifuged at 19,000xg at 4°C for 60 min to separate the different phases. The aqueous phase, pipetted out of the centrifuge tube was purified by passing through the Sephadex column (G-50) to isolate pure DNA. The purified DNA sample collected in eppendorf tube was stored in a refrigerator at 4°C until further processing.

DNA strand-breaks were determined according to alkaline unwinding procedure in which three parameters were measured: the amount of double-stranded (ds), single stranded (ss) and the fraction of double-stranded (au) DNA after alkaline unwinding. After isolation of DNA all determinations were made on Modulus Multimode Microplate Reader.

The DNA integrity is expressed in terms of F-values which are in fact, the ratio of the expression, (au-ss)/ (ds-ss). The calculation is the following:

$$F = (F_{au} - F_{ss}) / (F_{ds} - F_{ss})$$

With F_{au} : fluorescence of the partially unwinded DNA

F_{ds} : fluorescence of double stranded DNA

F_{ss} : fluorescence of single stranded DNA.

In order to validate the technique the same procedure was followed with a standard DNA sample from calf thymus and the F-value was determined (Everaarts and Sarkar, 1996).

2.4. Preparation of Single Cell Suspension

The collected gastropods shells were then broken and the gills were carefully transferred into a tube containing 1 ml of cold extrusion buffer (71.2 mM NaCl, 5 mM EGTA, 50.4 mM guaiacol glycerol ether, pH 7.5). The tissues were slightly chopped and the suspension was then left for 2-3 minutes on ice. The supernatant were then centrifuged at 5000 rpm for 3 minutes. The pellet was washed thrice with Phosphate Buffer Saline (PBS, 1.2 M NaCl, 0.027 M KCl, 11.5 mM K₂HPO₄, 0.08 M Na₂HPO₄, pH 7.3) and suspended in 150 µl of PBS. The cellular suspension was kept on ice to minimize endogenous damage occurring during slide preparation. The trypan blue exclusion test was used for viability assessment to ensure the presence of optimum number of cells to perform the assay (Absolom, 1986). 50 µl of diluted cell suspension was mixed with 50 µl of 0.4% Trypan blue dye solution. The cells were counted using a hemocytometer under Leica microscope (Leica Microsystems, Germany). Gill cells with more than 75% viability were used in the assays.

2.5. *In vitro* exposure of gill cells to hydrogen peroxide

To validate the comet assay protocol, freshly dissociated gill cells were treated with hydrogen peroxide (1; 10; 25 and 50 µM) in PBS for 30 min. The control cells were incubated in PBS without hydrogen peroxide. Three replicates per condition were performed.

2.6. The Comet Assay

Comet assay was carried out following the methods described by Singh et al., (1988) with minor modifications. First layer was made using 1% normal melting agarose (10 mg/ml in ultra pure water) on pre-cleaned and methanol treated dry slides. 100 µl of 0.55% low melting agarose (LMA, 5.5 mg/ml in PBS) mixed with 20 µl of diluted cells was used to make the second layer. After the agarose layer hardens, 100 µl of 0.5 % LMA (50 mg per 10ml Tris Buffer) was poured as third and protecting layer. It was then kept on freshly prepared lysis buffer (2.5 M NaCl, 0.1 M di- sodium EDTA, 0.01 M Tris Buffer, 0.2 M NaOH, pH 10.0) at 4°C in dark for 1 hour.

After lysis, slides were subjected to unwinding in the electrophoretic buffer (300 mM NaOH, 1 mM EDTA, pH 13.0) for 15 minutes. Electrophoresis was conducted in the same buffer at 20V (cc. 1V/cm) and 300 mA. Current and voltage was kept constant throughout the electrophoresis by adjusting the buffer level. Slides were neutralized using neutralizing buffer (0.4 M Tris, pH 7.5) drop wise for four

times at an interval of 5 minutes each. Slides were kept in cold 100 % methanol overnight for dehydration. Staining was performed using ethidium bromide (20 µg/ml).

2.7. Comet Capture and Image analysis

The presence of comets was examined in cells at 40X magnification using an image analysis system (Kinetic Imaging, Liverpool, UK) attached to a fluorescent microscope (DM100 Leica, Leica Microsystems, Germany) equipped with appropriate filters (Excitation wavelength of 515-560 nm and emission wavelength of 590 nm). All slides were coded and the whole slide was randomly scanned. Fifty cells per slide were analyzed with 2 slides per incubation (total 100 cells). All experiments were carried out in triplicate to take into account possible variations between different cell preparations. Results are expressed in terms of the percentage of DNA migrated from the comet head to the tail region (tail % DNA) (Anderson et al., 1994), tail length (TL), Olive tail moment (OTM).

2.8. Measurement of physico-chemical parameters and cadmium concentration

The physico-chemical parameters such as temperature, pH, salinity, dissolved oxygen (DO) biochemical oxygen demand (BOD) and nutrients (nitrate, nitrite and phosphate) were determined following the standard procedure (Grasshoff, et al., 1983). The concentration of cadmium in the tissues of a marine gastropod, *Cronia contracta* from the same sampling station (Palolem) was found to be in the range, 0.1 ± 0.03 – 0.2 ± 0.03 µg/g (dry weight) (Sarkar et al., 2008). Moreover the concentration of cadmium in water from the Goa coastal region was measured to be 0.24 µg/l (KrishnaKumari et al., 2006).

2.9. Statistical analysis

Statistical analysis was performed using XLSTAT (Version 2012.5.02, Halseon Solutions Private Limited, Bangalore, India). The Kolmogorov–Smirnov test was performed to verify whether the results follow a normal distribution. Mutagenic and genotoxic results from the control and exposed animals were statistically analyzed using analysis of variance (ANOVA) and Dunnett's multiple comparisons of means. Three levels were considered significant: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3. Results

3.1. *In vitro* exposure of gill cells to hydrogen peroxide

Fig. 3 shows a significant increase of the % tail DNA, OTM and TL after exposure to H₂O₂ (1; 10; 25 and 50 µM) of the gill cells isolated from *N. chamaeleon*. H₂O₂ at 50 µM induced the highest level of tail DNA, a 2.5-fold increase, compared with the negative control ($p < 0.001$). Four fold increase in OTM

was observed at 50 μM compared with negative control ($p < 0.001$). H_2O_2 induced a concentration dependent increase of the tail DNA, OTM and tail length.

3.2. *In vivo* exposure of gastropods to CdCl_2

In vivo exposure of gastropods did not induce any mortality at any of the concentrations tested. The lowest observed effect concentration (LOEC) and induction factors (IFs) are given in table 1. IFs are calculated by dividing the respective DNA damage at the concentrations by the DNA damage at the control.

3.2.1. Alkaline unwinding assay

No significant changes in DNA integrity was observed in the control throughout the duration of the experiments. The results showed that the gastropods exposed to 10 $\mu\text{g/L}$ CdCl_2 did not produce any significant changes in the integrity at any duration with respect to that of control. After one day of exposure a slight decrease in DNA integrity was observed at all concentrations of CdCl_2 with respect to that of the control (Fig. 4). DNA integrity decreased with the increase in CdCl_2 concentration from 10 - 75 $\mu\text{g/L}$. For the first two days of the experiment, there was no significant change in DNA integrity (F-values) due to 50 and 75 $\mu\text{g/L}$ CdCl_2 exposure. After 2 days of exposure, a significant decrease in the DNA integrity was observed in the group of gastropods treated with 25 and 75 $\mu\text{g/L}$ CdCl_2 ($p < 0.05$ and $p < 0.01$). DNA integrity decreased significantly ($p < 0.01$ and $p < 0.001$) from the third day onwards when exposed to 25, 50 and 75 $\mu\text{g/L}$ CdCl_2 . Interestingly, exposure to 75 $\mu\text{g/L}$ of CdCl_2 produced significant decrease in DNA integrity with respect to that of control at all duration. The most significant observation is that the F value was reduced by 50% and 20% with respect to that of control after 3 days and 5 days of exposure with 75 $\mu\text{g/L}$ CdCl_2 respectively.

3.2.2. Comet Assay

During the course of the experiment a control with equal number of gastropods from Palolem without the exposure to any external contaminants was maintained. The water quality parameters of seawater was analyzed for Temperature, pH, conductivity, dissolved oxygen, biochemical oxygen demand, phosphate, nitrate, nitrite (table-2). The cadmium concentration was also measured in the tissues of the samples of gastropods. Compared with the control samples, significant changes in % tail DNA ($p < 0.001$) was observed in the gastropods due to CdCl_2 exposure (Fig. 5). After 3 days of exposure of the gastropods with different concentration of CdCl_2 (10, 25, 50 $\mu\text{g/L}$) no significant changes were observed among the gastropods of different groups. % tail DNA was found to increase by almost three fold (48.47 ± 2.33) when treated with 50 $\mu\text{g/L}$ of CdCl_2 with respect to that of the control (15.65 ± 1.03) on day3. After 4 days of exposure with 50 $\mu\text{g/L}$ of CdCl_2 the % tail DNA increased by almost four fold

(53.54±2.34). Interestingly, no significant differences were observed in the % tail DNA for the groups of gastropods exposed to 25 and 50 µg/L CdCl₂.

As far as OTM is concerned, there was no significant change after one day of exposure to cadmium at all concentrations tested (Fig. 5). However, a significant increase ($p < 0.01$) in OTM values was observed in gastropods exposed to 25 µg/L, 50 µg/L and 75 µg/L of cadmium after 2 days of exposure with respect to that of the control gastropods ($p < 0.01$). Interestingly, as the exposure time increases, significant increase ($p < 0.001$) in OTM values was observed after 3, 4 and 5 days at 10 µg/L CdCl₂ exposures. At 25 µg/L CdCl₂ exposure there was significant increase in OTM values from 6.66±2.16 on day 1 to 9.36±1.03 on day 5. However, there was slight decrease in the OTM values from day 2 to day 3 of exposure with 25 µg/L and 75 µg/L of CdCl₂ respectively. It has been observed that there was no significant difference between the exposure with 25 µg/L and 50 µg/L of CdCl₂ at all duration.

An increase in Tail Length of DNA was observed in marine gastropods exposed to CdCl₂ in comparison to those in control. However after one day of exposure with different concentrations of CdCl₂ there was no significant change in the tail length. Significant changes in TL of DNA were observed in marine gastropods exposed to 75 µg/L CdCl₂ in comparison to those in control at all durations. Interestingly, after 3 days of exposure to different concentrations of CdCl₂ except at 10 µg/L all the groups of gastropods showed significant increase in TL of their DNA compared to those in control. However, there was slight decrease in TL of DNA in gastropods exposed to 75 µg/L of CdCl₂ during the period from day 2 to day 3. After 4 days of exposure of all the groups of gastropods to different concentrations CdCl₂ except at 25 µg/L showed significant increase in TL in comparison to those in control ($p < 0.05$, $p < 0.01$). However, decrease in TL of DNA was observed from day 4 to day 5 due to exposure to 10 µg/L and 75 µg/L of CdCl₂ respectively. Decrease in TL of DNA was also observed in gastropods exposed to 25 µg/L CdCl₂ from day 4 to day 5. It has been observed that TL of DNA increased significantly due to exposure of marine gastropods to 10 µg/L CdCl₂ for a period of 5 days (41.01±2.57) as compared to those in control (26.02±1.82).

4. Discussion

The usefulness of gastropods as sentinel organism in metal biomonitoring studies are widely recognized (Itziou and Dimitriadis 2011; Sarkar et al., 2008, 2006 and Downs et al., 2001). Gill cells are used as an attractive cellular model in ecotoxicology; as the gills are constantly exposed to dissolved contaminants and are capable of metabolizing carcinogens and mutagens into reactive products (Mitchelmore et al., 1998; Wilson et al., 1998). An assessment of the integrity of DNA is highly important when determining genotoxic pollution related stress in living organisms. So in the present

study gill cells were used to investigate the damage occurring in the DNA due to cadmium contamination. The measurement of integrity of DNA was carried out following the technique of partial alkaline unwinding assay and comet assay.

Using the partial alkaline unwinding assay we have demonstrated a concentration dependent response relationship between the level of DNA strand breaks and environmentally relevant concentrations of CdCl₂. The DNA damage occurred in this study could be due to various reasons such as DNA single strand breaks, DNA double strand breaks, DNA adduct formations, DNA–DNA and DNA–protein cross-links (Mitchelmore et al., 1998) resulting from the interaction of heavy metal or their metabolites with DNA (Fairbairn et al., 1995). Chandra and Khuda-bukhsh (2004) showed the induction of genotoxic effects of cadmium chloride (CdCl₂) singly and also in combination with azadirachtin (Aza) on a fish, *Oreochromis mossambicus*. Ahmed and his coworkers (Ahmed et al, 2010) measured the induction of DNA damage by CdCl₂ in freshwater climbing perch *Anabas testudineus* (Bloch) using the alkaline single cell gel electrophoresis (comet assay). They determined the extent of DNA damage in gill, kidney and liver of the organisms as the percentage of DNA in comet tails and comet heads in the tissue of the fish specimens exposed to 0.1, 1.0, 2.0 mg L⁻¹ concentrations of CdCl₂. It has been observed from their investigation that the DNA damage was found to be concentration dependent, with the highest DNA damage at 2 mg L⁻¹ concentration, followed by 1.0 and 0.1 mg L⁻¹.

The results of the DAUA suggested that CdCl₂ induces DNA damage in *N. chamaeleon*. DAUA have shown significant differences in DNA integrities among the four groups of marine gastropods exposed to four different concentrations (10; 25; 50 and 75 µg/L) of CdCl₂ irrespective of the duration of exposure. Moreover, it has been observed that among the different groups of marine gastropods exposed to different concentrations of CdCl₂ for different duration have clearly shown concentration dependent responses with respect to the loss of DNA integrity whereas there were significant differences in those among the groups due to exposure to the lowest concentration (10 µg/L) and the highest concentration (75 µg/L) of CdCl₂ for all the durations. Vincent-Hubert et al., (2012) determined the DNA strand breaks in both embryonic cells and on adult gill cells of freshwater mud-snail (*Potamopyrgus antipodarum*) using comet assay. They also investigated the stability of DNA strand breaks in adult reproducing snails and neonates exposed to cadmium (Cd) and bisphenol A for 8 days. They reported that Cd was genotoxic for both embryos and neonates during the exposure time and also after 7 days of depuration, suggesting that Cd could inhibit DNA repair enzymes. Vincent-Hubert et al., (2011) assessed the genotoxic potential of environmentally relevant concentrations of Cd on the zebra mussel, an important freshwater sentinel organism. They measured the oxidative DNA damage and the co-genotoxicity of Cd in combination with B[a]P. They determined the DNA damage in haemocytes and

gill cells of zebra mussels exposed for 11 days to a constant concentration of Cd (10 µg/L), B[a]P (10 µg/L) or the two combined chemicals (10 µg/L + 1 µg/L). They observed that bioaccumulation of cadmium in the soft tissues of mussels exposed to CdCl₂ or CdCl₂ + B[a]P increased in a time-dependent manner indicating that both exposures were effective. Omar et al (2012) evaluated the genotoxic effects of toxic metals such as Cu²⁺, Zn²⁺, Pb²⁺, Fe²⁺ and Mn²⁺ in cultured and wild Nile tilapia (*Oreochromis niloticus*) and mullet, (*Mugil cephalus*) collected from the contaminated aquatic habitats in comparison with fish from a non-polluted reference site. They indicated that high concentrations of heavy metals have potential genotoxic effects and is possibly related to agricultural and domestic activities. *In vivo* and *in vitro* exposures of freshwater fish (*Prochilodus lineatus*) to lead (Pb) showed significant genotoxicity as confirmed by comet assay (Monteiro et al., 2011). Mai et al., (2012) studied the genotoxicity of two dissolved metals copper and cadmium (Cu and Cd) and two pesticides (metolachlor and irgarol) occurring in Arcachon Bay (SW France) in Pacific oyster (*Crassostrea gigas*) larvae by measuring the DNA strand breaks using comet assay. They indicated that after 24 h exposure, the DNA strand breaks were found to be highly significant with 0.1 µg L⁻¹ for Cu, 10 µg L⁻¹ for Cd. Frenzilli, et al (2009) reported that comet assay is a very useful technique for evaluation of the impact of genotoxins in aquatic environment. *In vivo* and *in situ* studies were carried out in various marine and freshwater sentinel species using comet assay for biomonitoring of pollution.

Chemical analyses of trace metals in mussel tissues were integrated with a multi-biomarker approach for the early detection of biological responses at several cellular targets. Gorbi et al., 2008 analyzed the trace metals in mussel tissues and confirmed the variations in antioxidant and lysosomal stability as sensitive early warning signals for biological disturbances of both natural and anthropogenic origin. Recent studies on the integrity of DNA in marine snail (*Planaxis sulcatus*) clearly indicated the impact of pollution at Goa coast (Sarker et al., 2006). High level (3.8 µg/g) of Cd was reported in gastropod, *Cronia contracta* in Goa region leading to loss of DNA integrity by 73.5% (Sarkar et al., 2008). Stronkhorst et al., (2003) reported a decrease in DNA integrity measured using DAUA in Sea star *Asterias rubens* at dumping sites contaminated with heavy metals (Cd and Hg) in North Sea. Everaarts and Sarkar (1996) showed the impact of highly persistent pollutants on the integrity of DNA in pyloric caeca of the sea star (*Asterias ruben*) and classified the different regions of the North Sea in terms of loss of DNA integrity due to pollution. Bolognesi et al., (1999) observed a statistical increase of DNA damage induced by Cd in mussel species *Mytilus galloprovincialis* exposed in aquarium for 5 days.

Recent studies have reported the use of comet assay in gastropods (Ali et al., 2012; Itziou et al., 2011; Mohamed, 2011; Rank, 2009). In this study three parameters namely % tail DNA, OTM and TL were used in comet assay for the measurement of DNA damage. We observed that exposure to cadmium

induces concentration dependent increase in DNA strand breaks in gill cells of gastropod measured using all the three parameters. The tail DNA content values increased over time with their highest values recorded on day5 for all exposed group. Similar trend was observed for OTM and TL, the highest value was observed on day4 for 10 µg/L and 50 µg/L. However the highest value for 75 µg/L was observed on day5 for OTM and TL. For 25 µg/L, the highest value for OTM and TL was observed on day2 and 3 respectively. However the OTM value for 25 µg/L was almost same for day2 and 3.

Cadmium have shown to be genotoxic to mussels (Wilson et al., 1998), fish (Chandra and Khuda-Bukhsh, 2004), crustaceans (Hook and Lee, 2004). An increase in DNA strand breaks is reported on snail *Potamopyrgus antipodarum* exposed to 10 µg/L of cadmium (Hubert et al., 2012). Hubert et al., (2011) reported increase in bioaccumulation of cadmium in the soft tissues of mussels exposed to CdCl₂ in a time-dependent manner indicating that cadmium exposures produces damage in the DNA. They found that cadmium (10 µg/L) is genotoxic after 3 days of exposure in gill cells in zebra mussel *Dreissena polymorpha*. Juhel et al., (2007) reported DNA strand breaks in haemocytes of zebra mussels exposed to a very high cadmium concentration (>733 µg/L) after 7 day of exposure. For marine mussels, cadmium was reported to induce DNA damage in gill cells after a 10 day exposure to 200 µg/L (Emmanouil et al., 2007).

Genotoxicity is considered one of the most important toxic endpoints in chemical toxicity testing and risk assessment (Hayasi et al., 2005); however, little is known about the genotoxicity of cadmium especially towards gastropod *N. chamaeleon*. The results of both the comet assay and alkaline unwinding assay presented here have demonstrated clear concentration and time dependent responses to genotoxicant (Cadmium) exposure in the *N. chamaeleon*. Overall, the assays provide a set of convenient, highly sensitive, monitoring tools of environmental exposure to genotoxicants and both the comet assay and the integrity of DNA (F value) of alkaline unwinding assay explains its suitability as a biomarkers in gastropods. This investigation points out the need for regular biomonitoring of pollution due to genotoxicants like cadmium in the marine ecosystems. The results provide valuable information in regard to the genotoxic potential of cadmium to marine gastropods like *Nerita chamaeleon*. The results from this investigation will act as a base line data for evaluation of the state of pollution by genotoxic pollutants like cadmium along the marine coastal region using marine gastropods like *Nerita chamaeleon* as the sentinel organisms.

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Figure captions:



Fig. 1a. The sampling site at Palolem beach

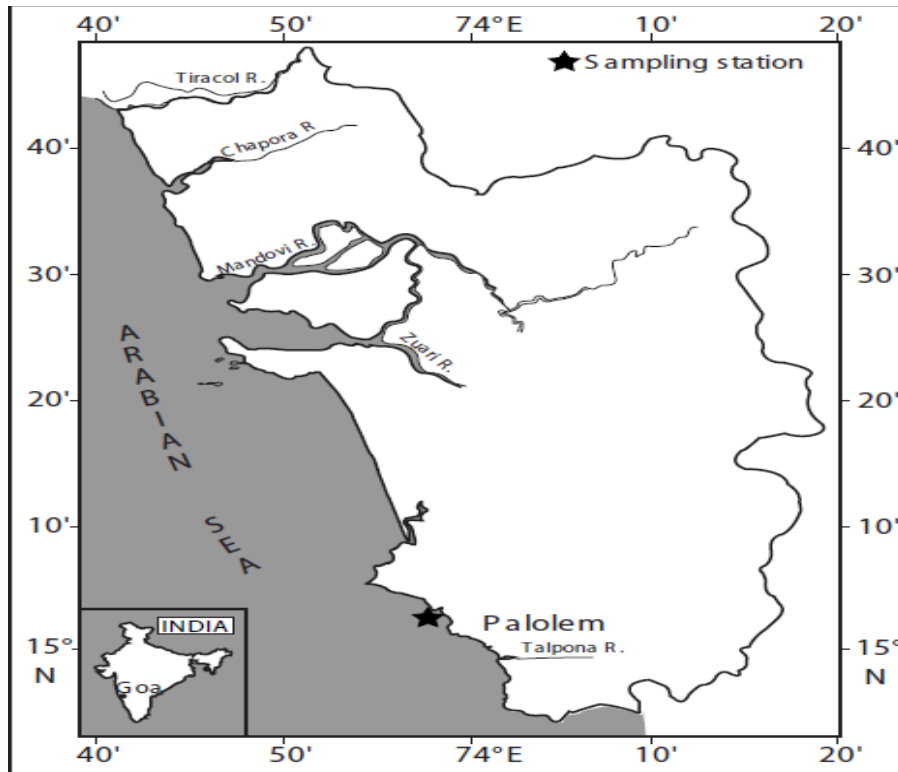


Fig. 1b. Location of the sampling site, Palolem, Goa, India



Fig. 2. A typical picture of the marine gastropod, *Nerita chamaeleon* at the sampling site (Palolem, Goa, India)

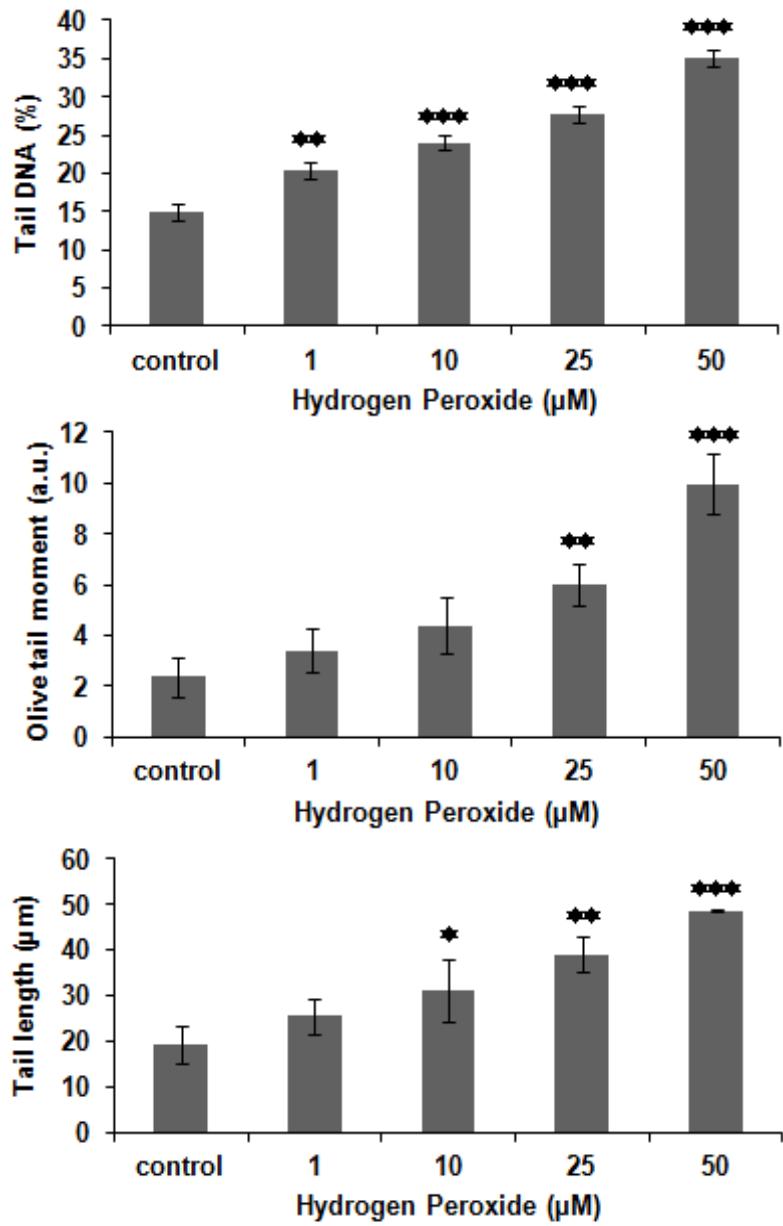


Fig. 3. DNA strand breaks (tail DNA %, OTM and TL) in gill cells of *Nerita chamaeleon* exposed to hydrogen peroxide. Isolated gill cells were exposed at 4°C for 30 min in the dark to hydrogen peroxide (n=3 replicates, 300 nuclei). Comet parameters were reported as mean ± standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from the control (ANOVA, Dunnett's test).

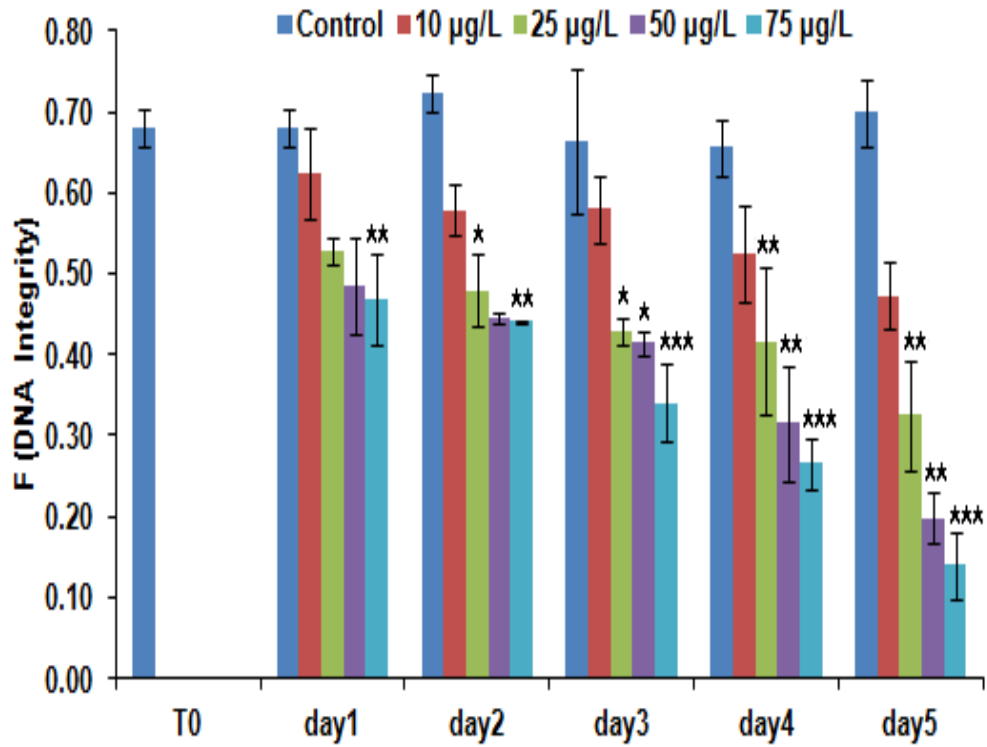


Fig. 4. DNA Integrity (F value) in gills of *Nerita chamaeleon* exposed to CdCl₂. Snails were exposed for 5 days to CdCl₂. T0 refers to time zero or zero day of exposure. Data expressed are means of triplicate values of DNA integrity. Results were reported as mean ± standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from the control (ANOVA, Dunnett's test).

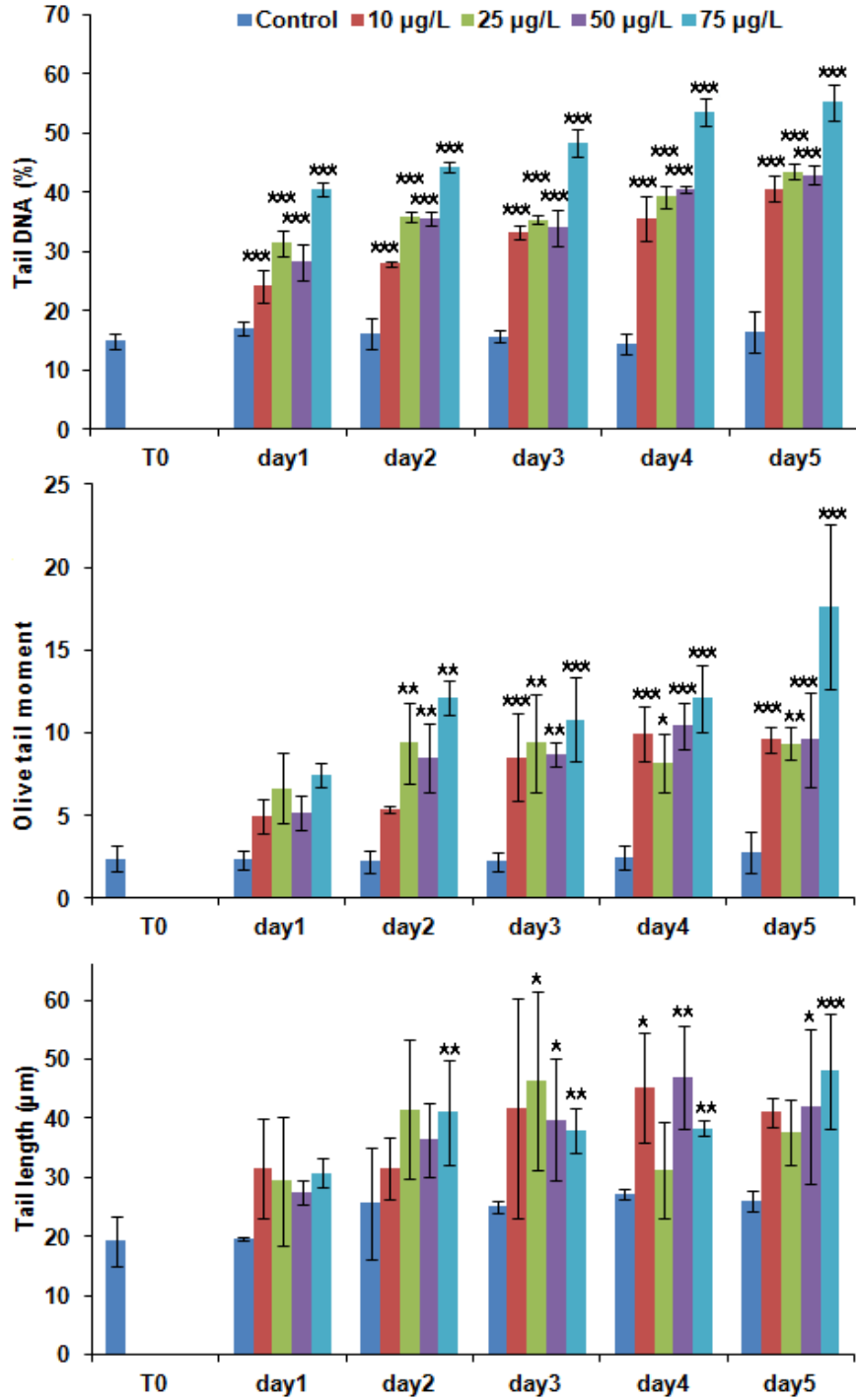


Fig. 5. Induction of DNA damage in terms of (a) tail DNA %, (b) OTM and (c) TL in *Nerita chamaeleon* gill cells following exposure to different concentrations of cadmium chloride *in vivo*. T0 refers to time zero or zero day of exposure. Comet parameters were reported as mean \pm standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from the control (ANOVA, Dunnett's test).

Tables

Table 1: Lowest Observed Effect Concentration (LOEC) and Induction factors (IFs) in gill cells of *Nerita chamaeleon* exposed *in vivo* to CdCl₂

	Lowest Observed Effect Concentration (LOEC) in µg/ml			Induction Factors (IF)											
	Tail DNA (%)	Olive tail moment	Tail length	Tail DNA (%)				Olive tail moment				Tail length			
				10 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml
Day1	10	-	-	1.41	1.83	1.65	2.37	2.16	2.88	2.24	3.21	1.61	1.50	1.41	1.57
Day2	10	25	75	1.73	2.23	2.21	2.75	2.45	4.28	3.86	5.52	1.23	1.62	1.42	1.60
Day3	10	10	25	2.13	2.26	2.17	3.10	3.85	4.24	3.94	4.89	1.67	1.86	1.59	1.52
Day4	10	10	10	2.46	2.71	2.80	3.70	4.02	3.31	4.21	4.89	1.66	1.14	1.72	1.40
Day5	10	10	50	2.46	2.64	2.60	3.35	3.44	3.35	3.44	6.30	1.58	1.45	1.61	1.84

Table 2: Water quality parameters at sampling site, Palolem, India

Temperature	pH	Turbidity (NTU)	Conductivity (mS/cm)	DO (mg/L)	BOD (mg/L)	P0 ₄ (µM/L)	N0 ₃ (µM/L)	N0 ₂ (µM/L)
28	7.858	1.65	48.5	1.468	3.726	0.107	0.386	0.463