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Carbon Nanoparticle Toxicity to marine algae *Navicula longa* and *Isochrysis galbana.*

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Present study reports cytotoxicity of Carbon Nanoparticles to *Navicula* and *Isochrysis*. Cytotoxicity was determined by morphological changes, cell density count, Acid Phosphatase and Lactate dehydrogenase assays. Carbon nanoparticles (CNPs) promote concentration as well as exposure dependent toxicity to Navicula & Isochrysis, which exhibited cell death, necrotic changes such as cell shrinkage and pycnosis of nucleus. Lactate Dehydrogenase (LDH) & Acid Phosphatase (ACP) activities elevated significantly, in both algae, in a dose and exposure dependant manner. In Navicula and Isochrysis, LDH activity was elevated by 42.3% and 60.7%, and 44.5% and 90.3%, on exposure to 0.05 mg/mL and 1 mg/mL CNPs for 10 days respectively. Similarly, ACP activity in both Navicula and Isochrysis was elevated by 34.6% and 55.4%, and 11.7% and 18.5%, on exposure to 0.05 mg/mL and 1 mg/mL of CNPs for 10 days respectively, as compared to the controls. The occurrence of tube like structures under the influence of CNPs in algae was also observed.

[Keywords: Carbon, Nanoparticle, Algae, Toxicity, Cell necrosis, Tube like structures]

Introduction

Advances in nanotechnology have led to the development and production of many new nanomaterials with unique characteristics for industrial and biomedical uses. The size (<100 nm) of these new Nanoparticles (NPs) with their high surface area and unusual surface chemistry and reactivity, promote threat to the environment, in particular to flora and fauna [1].

NPs vary in chemistry, structure, size and shape as well as behavior and ability to interact with the biological materials. They have significantly large surface to size and mass ratio with high potency electrons at the surface[2]. Therefore, they have greater applications as well as hazardous properties. Though the researchers have expressed concern for the potential hazards of NPs to human health, [3,4,5] the impact of NPs on the environment is yet relatively less known[6,7,1].

The effect and mode of action of nanoparticles (NPs) on the environment and within living systems is difficult to predict.[8] It is known that CNPs have toxic effects on human and rodent tissues in vivo as well as in vitro[9-16]. CNPs are reported to promote cytotoxicity to human hepatocytes (Hep G 32 cell lines) in a concentration dependent manner as evidenced by proportionate LDH leakage[17]. Oberdorster *et al*.[18] and Tempelton *et al*.[19] have reported toxic effects of CNPs on marine and estuarine invertebrates. It is known that dry CNPs adhere extensively to body surface and wings, disturb natural grooming mechanisms leading to impairment of locomotion and death of adult Drosophila melanogaster[20]. Canesi *et al*.[21], reported inflamatory responses and decrease in mitochondrial mass and membrane potential in blue mussel.

Studies on *Fucus serratus* (seaweed) have revealed effects of CNPs on sperm concentration, fertilization, body axis alignment, germination and rhizoid elongation. CNPs at 100 mg/mL promoted large agglomerates that removed sperm from suspension and reduced fertilization success, while 50 mg/mL reduced correct alignment of polar axis. The clear evidence for the uptake of CNPs in zygote was not obtained in EM imaging. Instead zygotes were found covered by agglomerates of CNPs that may have shaded the incident light that is crucial for alignment of the polar body axis[22].

Presence of Carbon Nanoparticles (CNPs) in an array of modern day products, such as sports equipments, automobiles, computers, household, kitchen appliances, ink, dyes, paints, photocopier toners, car tyres, lubricants, pharmaceuticals, carbon

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rods etc, elevate chances of their predominant release into freshwater, estuarine and marine environments[23] which may affect the fate of other pollutants[24] and may cause necrosis of algal cells and other floral components. It may also invade into the marine animal cells and tissues thereby causing inflammation and necrotic death. Further, if these nanoparticles accumulate in the body of animals which are consumed by humans they may enter the human body systems causing health hazards. As the CNPs are likely to reach the aquatic bodies they are likely to influence the natural inhabitants of these bodies.

Generally diatoms are producers within the food chain and microalgae are useful as feed in aquacultures especially for larval and juvenile forms. In shell fisheries Chaetoceros, Pavlora, Thalassiosira are used as feed [25,26]. Similarly Isochrysis sp. is used as feed in aqua industry, rearing juveniles and filter feeders[27]. As apparently there is scanty information on CNPs toxicity to microalgae in particular and diatoms at large that form integral part of marine food web and aqua feed, the present study was undertaken to investigate the effects of CNPs on these forms.

Materials and Methods

CNPs {nanospheres, size 10-45 nm, surface area = 30-50 m2/g, purity 99%, carbon (3N), having no metal content with a zeta potential of -15mV} were obtained from P. Sen of JNU, New Delhi. F2 Medium composed of $NaNO_3$ (75 mg/L), NaH_2PO_4 H_2O(5mg/L), Na_2SiO_3 $9H_2O$(30mg/L), trace metal solution{$FeCl_3$ $6H_2O$(0.44 mg/L), Na_2EDTA $2H_2O$ (0.16 mg/L), $CuSO_4$ $5H_2O$ (1.37 µg/L), Na_2MoO_4 $2H_2O$(0.88 µg/L), ZnSO4 $7H_2O$ (3.08 µg/L), $CoCl_2$ $6H_2O$(1.4 µg/L), $MnCl_2$ $4H_2O$ (25.2 µg/L)}, vitamin solution{thiamine HCl (vit.B_1) 33.2 µg/L, biotin (vit. H) 16.6 µg/L, cyanocobalamin (vit. B_{12}) 166 µg/L},[28,29] Microalgae (Isochrysis) & Diatom (Navicula) were obtained from National Institute of Oceanography, Dona Paula, Goa, India.

Sub-culturing of Algae

Algae were maintained in separate conical flasks and were allowed to grow in F2 culture medium under the artificial incandescent light. The algal samples were sub cultured under horizontal laminar flow hood using 100 mL of F2 medium and 0.5 mL of algal sample (10^6 cells/mL) in a conical flask. The algal cultures were kept for 8 days under observation for the cell growth and proliferation. Subsequently, algae were subcultured again in the same manner after every 8-10 days.

Exposure of algae to CNP

After the algal cells attained a good proliferation and growth rate, they were used for the experiment. The pilot experiment was carried out to decide the doses, to see whether they had any effect on the cells. It was observed that the cells exposed to less than 0.05 mg/mL showed non toxic effects, as they did not produce any detectable alterations in cell morphology. Two concentrations *viz*., 0.05 mg/mL and 1 mg/mL of CNPs were used for the toxicity study. The normal algal cultures (not exposed to CNPs) were treated as controls, and those exposed to CNPs were treated as experimentals. Control, experimental I (exposed to 0.05 mg/ mL) and experimental II (exposed to 1 mg/ mL) sets were prepared in triplicates. After exposure of algae to CNPs for the desired period (10 days) and concentrations, the morphological alternations of algal cells and their population density was determined using Olympus inverted microscope and hemocytometer. The photographs were taken with Nikon digital camera (model coolpix 4500) at 20X × 10X magnification with a resolution of 300dpi. These results were compared with controls.

LDH and Acid Phosphatase Assay

The control and experimental algae were collected along with the media and cold centrifuged at 1000 rpm, the supernatant thus collected was labelled (A) and kept in the refrigerator at 4^0 C until used. Then to the sediment 2 % of triton 100X was added and the mixture was vortexed for 5 minutes followed by cold centrifugation at 1000 rpm. The supernatant was labeled (B) and kept in the refrigerator at 4^0 C until used. Both the supernatants (A) & (B) obtained for control, experimental I and experimental II were used for the Lactate Dehydrogenase (LDH) and Acid Phosphatase (ACP) assay. The assays were performed after 24 hrs, 5 days and 10 days of exposure to CNPs. The Percentage release of the cells was determined using the following formula.

$$\% \text{ Release of LDH/ACP} = \frac{\text{Release of LDH / ACP (supernatant)}}{\text{Total LDH / ACP}} \times 100$$

Statistical Analysis

The various groups were statistically compared using t-test (Microsoft Office Excel 2003) and the differences were considered significant at $p \leq 0.05$, and highly significant at $p \leq 0.001$.

Results

Population density – Navicula & Isochrysis

The population density of controls (*Navicula & Isochrysis*) elevated progressively up to five days and subsequently remained unchanged upto ten days. But on exposure to 0.05 and 0.1 mg/mL concentration of CNP, their population density declined gradually and significantly ($p \leq 0.05$) depending upon the concentration of CNP and exposure period (Fig. 1 & 2).

Percentage release of Acid Phosphatase & LDH by Navicula and Isochrysis

The percentage of ACP & LDH release by controls remained constant at all the exposure periods indicating that the microalgal viability was unaffected (Fig. 3, 4, 5 & 6). The augmentation of ACP and LDH release (%) by algae exposed to CNPs was dependent on concentration and exposure periods. This indicates CNP mediated cell necrosis (Fig. 3, 4, 5 & 6).

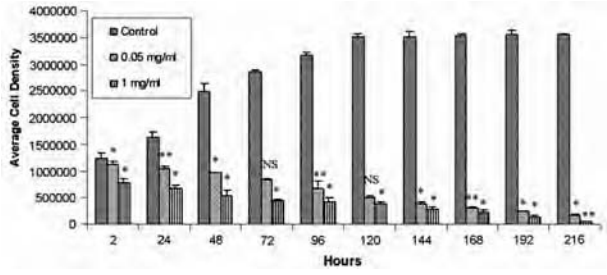

Fig 1—Effect of Carbon Nanoparticle on *Navicula* cell density against Control (*-$p \leq 0.05$, **-$p \leq 0.001$, NS- Non significant).

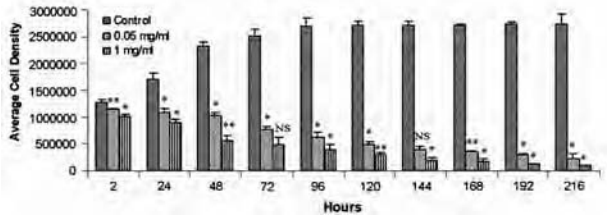

Fig 2—Effect of Carbon Nanoparticle on *Isochrysis* cell density against Control (*-$p \leq 0.05$, **-$p \leq 0.001$, NS- Non significant).

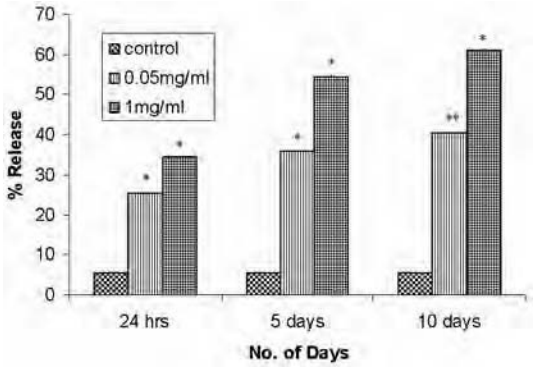

Fig 3—Effect of 0.05 mg/mL and 1 mg/mL dose on Navicula by Acid Phosphatase percent release against Control (*-$p \leq 0.05$, **-$p \leq 0.001$).

Morphological changes in Navicula & Isochrysis.

The controls (*Navicula and Isochrysis*) appeared elongated, slightly spindle shaped with a distinct

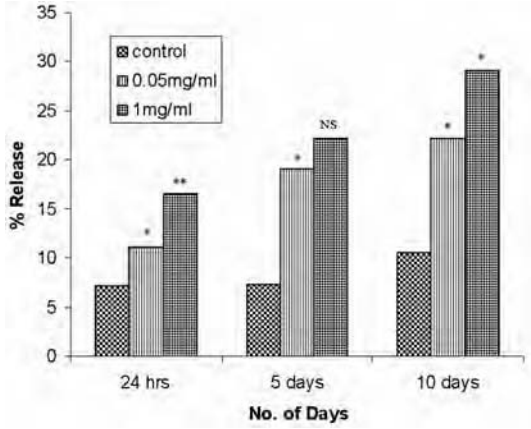

Fig 4—Effect of 0.05 mg/mL and 1 mg/mL dose on *Isochrysis* by Acid Phosphatase percent release against Control (*-$p \leq 0.05$, **-$p \leq 0.001$, NS- Non significant).

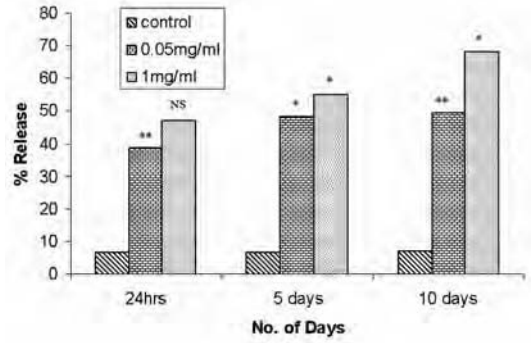

Fig 5—Effect of 0.05 mg/mL and 1 mg/mL dose on *Navicula* by LDH percent release against Control (*-$p \leq 0.05$, **-$p \leq 0.001$, NS- Non significant).

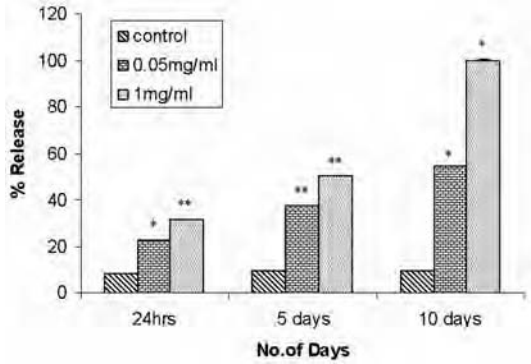

Fig 6—Effect of 0.05 mg/mL and 1 mg/mL dose on *Isochrysis* by LDH percent release against Control (*-$p \leq 0.05$, **-$p \leq 0.001$).

nuclei (Fig. 7-a1, 8-b1). On exposure to CNPs (1 mg/mL) the cells lost their spindle shape and became irregular with pycnotic nuclei (Fig. 7-a2, 8-b2). As the exposure period progressed the cytoplasm of the cells became sparser with cell shrinkage (Fig. 7-a3, 8-b3) at the end of 48 hrs and then subsequently cell necrosis progressed with accumulation of debris and occurrence of Tube like structures (TLS) (Fig. 7-a4, 8-b4). Further, after 5 to 10 days, along with the progression of cell necrosis & lysis, the formation of TLS was substantially prominent (Fig. 7-a5, 8-b5, 7-a6 & 8-b6). The necrosis induced by CNP at 0.5 mg/mL concentration was relatively less.

Discussion

Many researchers have demonstrated that CNP toxicity to tissues depends upon dose/ concentration and exposure period. [9-10-11-12-13-14-15] Earlier studies have reported toxicity of CNPs and CNTs to rodents, human cells, large mouth bass [30] but less is known of CNP toxicity to marine algae. Here we report CNP toxicity to marine algae such as *Navicula* and *Isochrysis*. We have observed that the toxicity of CNP depends upon the dose/ concentration and exposure period. The loss of cell integrity especially, pycnosis of nucleus indicates cell necrosis. The decline in population density therefore could apparently be due to progression of necrosis leading to cell death.

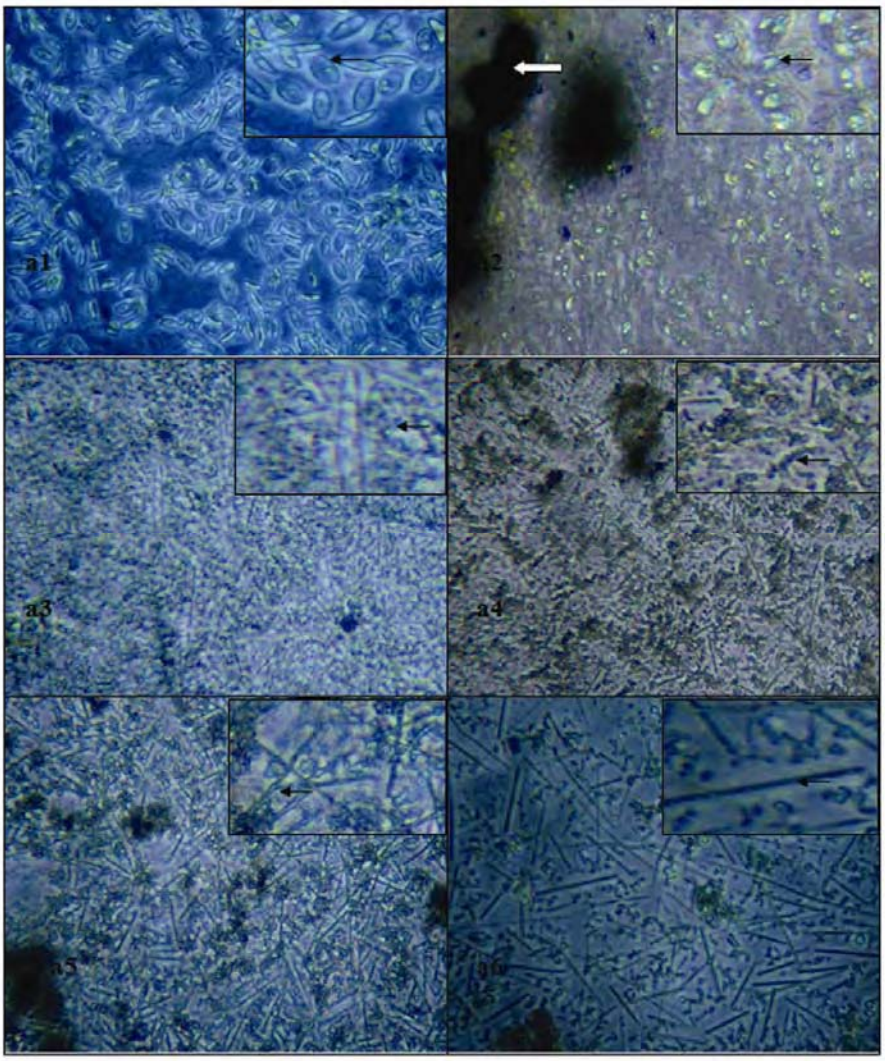

Fig 7—a1- *Navicula* under control conditions (← shows elongated spindle shaped cell). Exposure to 1 mg/mL CNP; a2- 24 hrs (← shows irregular cells with pycnotic nucleus, shows accumulation of CNPs), a3- 48 hrs (← shows cells shrinkage), a4- 72 hrs (← shows accumulation of debris), a5- 5days (← shows TLSs) and a6- 10 days (← shows prominent TLSs).

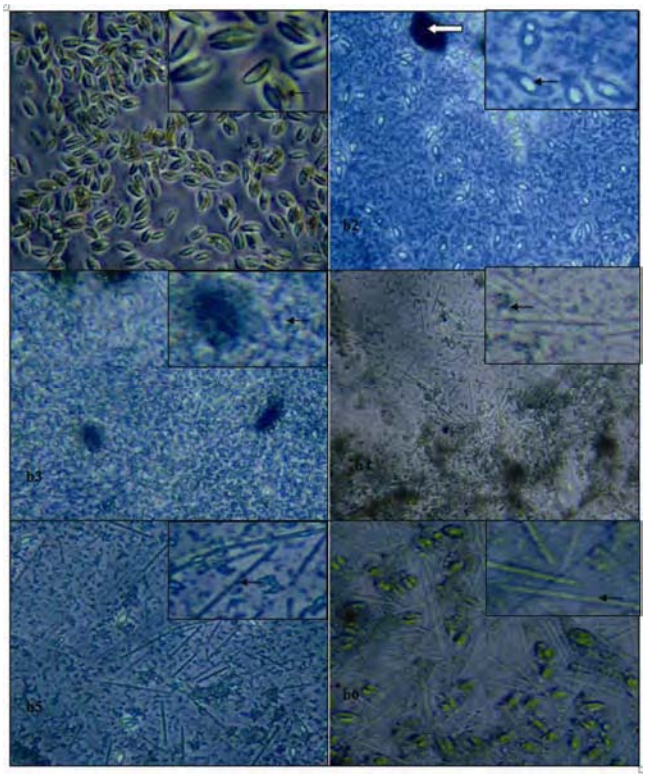

Fig 8—b1 - *Isochrysis* under control conditions (← shows elongated spindle shaped cell). Exposure to 1mg/mL CNPs; b2- 24 hrs (← shows irregular cells with pycnotic nucleus, shows accumulation of CNPs), b3- 48 hrs (← shows cells shrinkage), b4- 72 hrs (← shows accumulation of debris), b5- 5days (← shows TLSs) and b6- 10 days (← shows prominent TLSs).

ACP is a lysosomal enzyme, which is involved in cell necrosis. The relationship between enhanced ACP activity and progressive necrosis is well established. [31] The exposure period and CNP concentration dependent ACP release (%) substantiates the induction of cell necrosis leading to cell death. Normally, ACP is not released in the cell culture medium but is released to the exterior environment only when cells are injured. Similarly, LDH is a cytoplasmic enzyme and gets released into the milieu exteriors/ external environment when cells are injured. The CNT (3-50 μg mL^{-1}) mediated LDH release in human hepatocytes (Hep G 32 cell lines) is reported by Anreddy *et al*[1]. The CNPs and exposure dependent release of LDH indicates the concentration and exposure period dependent damage inflicted by CNP to the cultured algae. CNPs are known to pass through the protein lipid bilayers of cell membrane and accumulate in the cells[30]. Thus, when they enter the cell they may promote formation of Reactive Oxygen Species (ROS) as observed in human cells leading to oxidation of lipids resulting in cell deaths [30]. The present work shows the necrotic cell damage promoted by ACP, a lysosomal enzyme.

The present study shows the occurrence of TLS under the influence of CNPs in algae. The exact mechanism of its formation and its chemical nature are not known, but CNPs can pass through the membranes and on damaging cells through activation of lysosomal ACP, promote cell necrosis resulting in cell death. Subsequently CNPs might have come out of dead cells along with the cytoplasmic exudates, and might have invaded other intact cells leading to decline in the cell density. The TLS formed looks similar to Carbon nanotubes (CNT). The diameter of TLS formed in our study ranges from 0.5 to 1 μM and length ranges from 1 to few microns. However, the CNTs synthesized in laboratories vary in diameter from 10 to 50 nM and length varies from 1 to few microns[32]. Since both vary in their diameter it is very difficult to say that the TLS formed are carbon nanotubes. However, it needs further investigation to understand the mechanism of TLS formation by the algae or algal debris.

The increased industrial usage and applications of CNPs could increase their chances of release in Aquatic ecosystem and therefore could be toxic to flora and fauna. Our study shows that carbon

nanoparticles cause cell death of the microalgae and diatoms which are the primary producers of the marine environment, thus proving its potential to disrupt the ecosystem.

Conclusion

The morphological changes that occur on exposure to CNP and release of ACP as well as LDH indicate the onset of necrosis of algae. CNP is able to induce toxicity to algae by inducing cell necrosis. The carbon nanoparticle doses less than sub lethal level have no consequential significant effect as compared to the sub lethal doses. One needs to find out the mechanism of activation of lysosomal enzymes as well as lysosomes. The death of microalgae and diatoms in a natural environment would reduce their population density and disrupt the food chain of which they are integral part.

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