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Acute Toxic and Cytogenetic Effects of Carbon Nanotubes on Aquatic Organisms and Bacteria

A. A. Gusev[a], I. A. Fedorova[b], A. G. Tkachev[c], A. Yu. Godymchuk[d], D. V. Kuznetsov[e], and I. A. Polyakova[a]

[a] *Derzhavin State University, Medical Institute, ul. Sovetskaya 93, Tambov, 392000 Russia*
[b] *National Research Center Chernyshevskii State University, ul. Astrakhanskaya 83, Saratov, 410012 Russia*
[c] *NanoTekhTsentr, ul. Sovetskaya 51, Tambov, 392000 Russia*
[d] *Tomsk Polytechnic University, pr. Lenina 30, Tomsk, 634050 Russia*
[e] *National University of Science and Technology "MISiS," podr. 217, Leninskii pr. 4, Moscow, 119049 Russia*
e-mail: nanosecurity@mail.ru

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Abstract—This paper summarizes a comprehensive study concerning the acute toxicity of a commercial carbon nanomaterial consisting mostly of carbon nanotubes to larvae of Chironomidae, crustaceans *Ceriodaphnia affinis*, algae *Scenedesmus quadricauda*, and bacteria *Escherichia coli*. It is shown that the nucleolar organizer region (NOR) index of polytene chromosomes in the salivary gland cells of midge larvae depends on the duration of concentration and exposure. This fact is indicative of the switching on of cell adaptation pathways in response to a xenobiotic stressor to restore cell homeostasis. The investigated nanomaterial is labeled as a Class III environmentally hazardous material (moderately hazardous). Safe concentrations of the carbon nanomaterial in aquatic media are less than 2 mg/l. It is concluded that larvae of Chironomidae are the most resistant to the material of all test species, whereas *Scenedesmus quadricauda* and *Escherichia coli* are the most sensitive.

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INTRODUCTION

Since the early 1990s, the development, manufacture, and introduction of carbon nanostructured materials have been the most rapidly growing branch of nanotechnologies [1]. In particular, carbon nanotubes (CNTs) are promising for obtaining ultrastrong threads and composite materials, transistors and fuel cells, capsules for the storage of metals and gases, high-selective molecular sieves, display devices, and light-emitting diodes [2]. They are also used in targeting pharmaceuticals and the production of biomaterials and biosensors [3]. Therefore, the safety of this class of nanomaterials in regards to labor and health have come to the fore [4] and investigating the toxic action of carbon nanomaterials (CNMs) is among the most urgent tasks of toxicology and environmental toxicology [5].

The number of publications on nanoparticle toxicology has been exponentially growing within the last six years [6, 7]. Because CNMs are produced in greater amounts than any other nanomaterial, numerous studies are focused on their toxic action, migration, and entry into the environment.

Many authors demonstrate the high stability of nanotubes in the environment. For example, CNTs can form stable aerosols in disposal by incineration [8]. They are also resistant to degradation in soil [9]. These are prerequisites for their migration to the hydrosphere followed by absorption or adhesion by living organisms.

After entry to the hydrosphere with sewage or polluted air, engineered nanoparticles first enter aquatic organisms: zoo- and phytoplankton, fishes, benthic invertebrates, etc. These organisms are the bases of food webs, which makes them the most probable site for the primary accumulation of all fine abiogenic pollutants, including nanoparticles [10].

Single- or multiwalled CNTs can enter the alimentary tract of aquatic invertebrates and accumulate there [11]. They cause disorders in hydrosphere inhabitants [12] and delay embryonic development [13]. Fullerene C60 adhesion to crustacean bodies (parasites *Acartia tonsa*) has been reported to disturb their behavior and physiology [14].

The factors conjectured to determine the toxicity of CNTs include heavy-metal penetration [15], the mechanical breakdown of physical barriers in cells and cell membranes (membrane stress) [16, 17], the catalytic and inhibitory action of nanomaterials on biochemical processes [17], and free radical formation [9, 18, 19].

Little information on the cytogenetic action of CNMs is available. An analysis of the mitotic index (MI), chromosomal aberrations, micronuclei, and DNA

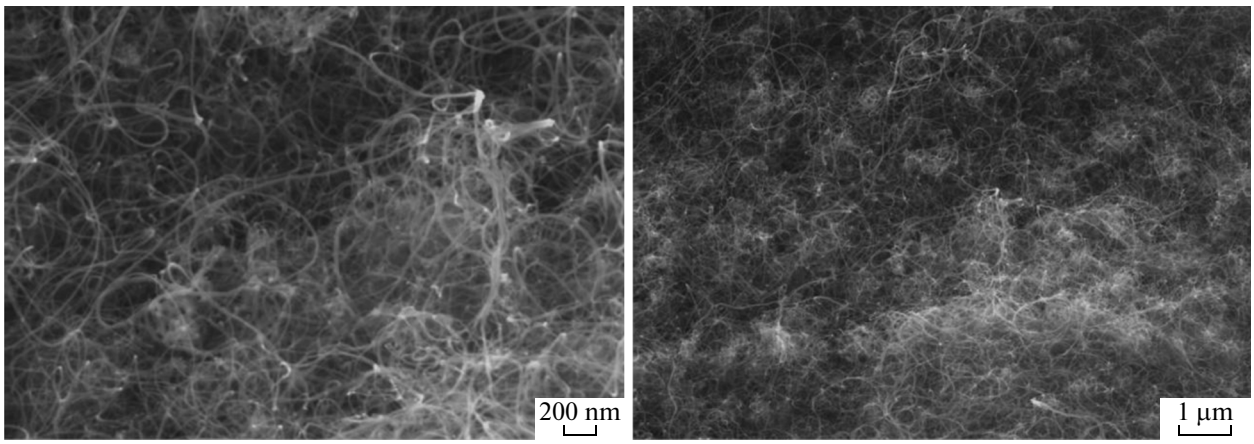

Fig. 1. Morphology of the carbon nanomaterial under study.

damage in bone marrow cells reveals an increase in the number of structural chromosomal aberrations, micronucleus frequency, and the rate of DNA injuries with decreasing MIs in mice exposed to the acute action of CNMs in comparison with the control group [20]. The MI decrease is supposedly caused by synthesis repression at the interphase stage of the cell cycle. The authors of [20] associate the cytogenetic effects with both the primary effect of CNMs on the nuclear machinery and the indirect action on cells mediated by inflammatory reactions and oxidative stress.

Carbon nanotubes display much greater genotoxicity than other nanoparticles [21, 22, 23] and induce a severe oxidative stress. It is presumed that the fibrous structure of CNTs facilitates their penetration to cell nuclei through nuclear pores, where they disrupt the double helix of DNA [24].

Polytene chromosomes of salivary glands of chironomids (Chironomidae, Diptera) are a unique model for analyzing the cytotoxic hazard of nanomaterials. These chromosomes are constantly transcriptionally active [25, 26].

Changes in the functional activity of interphase chromosomes in eukaryotic cells are a measure of the molecular toxicity of xenobiotics [27]. The current view of puffing as a sufficient condition of RNA synthesis allows transcriptional activity to be estimated from the size of active regions visualized by common ethyl orcein staining. Glow may be absent even with [^{3}H] uridine labeling, whereas transcription is quite intense [28]. This phenomenon is related to the absence of any incorporation of exogenous [^{3}H] uridine to nucleoside triphosphates owing to large amounts of NTPs accumulated hitherto [25, 28].

Thus, the toxic action of nanomaterials on aquatic organisms and bacteria depends largely on the various structural features of the materials rather than on their chemical nature. For this reason, an estimation of the hazard of nanomaterials by analogy with formerly examined samples can yield erroneous results and cause adverse consequences for the environment and human health in the course of the manufacture, storage, use, and disposal of nanomaterials. Therefore, an ecotoxicological assessment of each of the industrially produced nanomaterials should be performed.

A proper choice of test species belonging to groups most representative of the ecological diversity of potential victims of nanomaterials entering the environment is essential. In our opinion, such species should include freshwater filter feeders (larvae of chironomids and ceriodaphnias), unicellular green algae as a component of phytoplankton, and *Escherichia coli* bacteria (the commonest subject in testing the toxicity of polyelectrolyte microcapsules with zinc oxide nanoparticles) [29].

This study is dedicated to estimating the toxicity of a CNM on the base of multiwalled CNTs to the following test subjects: *Chironomus riparius* Meigen, 1804; *Ñeriodaphnia affinis* Lilljeborg, 1900; *Scenedesmus quadricauda* (Turpin) Brebisson, 1835; and *Escherichia coli*, 1885, M-17.

MATERIALS AND METHODS

Experiments were performed with the CNM Taunit. It looks like a dark powder and consists of multiwalled nanotubes (≤95–98% w/w), cones, fullerenes, graphite, and amorphous carbon. The nanotubes are 11 to 28 nm in diameter and 5 to 10 μm in length. Carbon layers in the nanotubes are spaced about 0.25 nm apart. In the common state, Taunit forms 1 to 1000-μm agglomerates of entangled carbon nanotube bundles (Fig. 1). The material is synthesized by catalytic hydrocarbon pyrolysis with chemical vapor deposition. It is commercially manufactured at NanoTekhTsentr, Tambov, Russia.

Taunit is a promising material for the aircraft, atomic power, space, and pharmaceutical industries; medicine; and the production of supercomputers, video equipment, flat screens, video displays, and

multipurpose filters. Doping with Taunit improves the quality of lubricants, structural composite materials, and construction materials. Taunit granules can serve as supports for catalysts or pharmaceuticals, sorbents, and sources for field electron emission [30].

Prior to experiments, Taunit was dispersed in distilled water by sonication at 300 W and 23.740 kHz for 5 min. Distilled water for control experiments was treated in the same way.

We used common ecotoxicological methods for toxicity evaluation from changes in the death and birth rates of ceriodaphnias, unicellular green algae, and transgenic *Escherichia coli* bacteria [31, 32, 33]. The protocol based on the crustacean (*Ceriodaphnia affinis* Lilljeborg, 1900) death rate [34] is recommended for nanomaterial safety tests.

Growth media for biotests were prepared by conventional methods. Stable CNM sols of required concentrations were obtained by sonication.

Bioluminescence was measured with a Biotox 10 luminometer.

The class of environmental hazard determined for the subject most sensitive to the toxicant tested was taken to be final. Hazard classes were determined from the dilution factors of water extracts not affecting test subjects (Table 1).

The method based on ceriodaphnia death involves a comparison of the death rates in a sample with CNM and growth water (control).

Toxicity was considered acute lethal if it caused death of no less than 50% of ceriodaphnias within 48 h in comparison to the control, where the death rate did not exceed 10%. A 30-mL vessel was half-filled with liquid to be tested, and one ceriodaphnia was placed there. The animals were taken from a culture synchronized for no more than 2 days. Ten vessels were used for each concentration tested. Temperature was maintained within 22–24°C, and illumination was 900–1000 lx. The oxygen content at the end of the experiment was no less than 4 mg/l, and pH was 7.0–7.3. Ceriodaphnias were fed with algal suspension made prior to the experiment according to [31] and then daily. Death rates were determined in experimental and control groups hourly until the end of the first day of the experiment and then twice a day until the end of the experiment. Unmoving individuals were considered dead if they did not move within 15 s after the vessels were rocked. Median lethal ($LC_{50\text{-}48}$) and safe ($LC_{10\text{-}48}$) concentrations were calculated by probit analysis.

The toxicity test with the *Scenedesmus quadricauda* (Turpin) Brebisson, 1835, green alga involves a record of the growth rate (population decrease) of algal cells exposed to toxicants with reference to the culture in control samples without the toxicant tested. The acute toxic or inhibiting (IDF_{50-72}) dilution factor was determined in water extracts as that reducing the algal cell population by 50% or more after 72 h of exposure.

Table 1. Hazard classes of chemicals according to [35]

Hazard class	Dilution factor of the water solution of a substance tested at which its adverse action is not recorded
I	>10000
II	10000 to 1001
III	1000 to 101
IV	<100
V	1

The safe dilution factor (not causing acute toxicity) reduced the cell population by no more than 20% within 72 h with reference to the control.

A suppression of algae in experiments was judged from the reduction of cell populations in comparison with the control after 72 h of the test.

The test was performed with an algologically pure culture of *Scenedesmus quadricauda* in the log phase (3–5 days after inoculation). Tested samples (100 mL) were placed in 250-mL Erlenmeyer flasks. The same volumes of distilled water pH 7.0–7.5 were used as the control. All tests were done in duplicate. Reagent solution (0.1 mL) was added to each flask under sterile conditions in a burner flame according to [32]. The mixtures were stirred. Equal volumes of algal suspension were added so that the cell density in each flask was within 25000–35000 cells/mL. The flasks were stirred again. Algal cells were counted in a hemocytometer for all tested and control samples. The count was done twice for each flask. The mixtures were stirred again, closed with cotton plugs, and kept in a luminostat in the temperature range from +22 to +25°C and constant illuminance 3000–10000 lx. Cells were suspended by shaking once or twice a day to improve aeration and mitigate pH variation caused by carbon dioxide release by the algae.

Cells were counted in a hemocytometer in test and control flasks after 72 h of incubation. Algal cell density in control flasks was found to increase no less than tenfold, and the pH shift at the end of the test did not exceed 1.5.

The mean cell population was determined for each dilution factor from two independent measurements in the acute toxic action test according to the equation

$$\bar{X} = \frac{\sum X_i}{n},$$

where $\bar{X}$ is the mean value of the parameter tested (fluorescence or cell population), X_i is the parameter value in the ith replication, and n is the number of replications.

Table 2. Degrees of toxicity of a solution tested

Deviation from the control, %	Toxicity degree
below 20	nontoxic
50 or more	acute toxicity

Table 3. Cumulative effects of CNM Taunit in the acute experiment with *Ch. riparius* larvae

Cumulation degree	Cumulation index range
	TL_{50}, h
1. Hypercumulation	>48
2. Strong	>24–48
3. Moderate	12–24
4. Weak	<12

Relative changes (%) of algal cell populations were determined for each dilution factor with reference to the control (I):

$$I = \frac{\bar{X}_C - \bar{X}_T}{\bar{X}_C},$$

where $\bar{X}_C$ is the mean parameter value in the control experiment and $\bar{X}_T$ is the value in the test.

The toxicity grades are defined in Table 2.

Stimulation (action opposite to suppression) up to 30% with reference to the control was interpreted as a nontoxic action.

The IDF_{50-72} and IDF_{20-72} values were calculated by probit analysis.

The test of acute toxicity to Ecolum biosensor bacteria (luminescent transgenic *Escherichia coli*, 1885, M-17) is based on the measurement of luminescence in the bacteria exposed to toxicants [33]. The acute toxic action of a sample on Ecolum was determined from bioluminescence quenching after 30-min exposure. Samples were diluted with distilled water at 20°C, pH within 7.0–7.2. A volume of 0.9 mL of a tested dilution or water (control) was added per 0.1 mL of the bacterial suspension in a luminometer cell. The following ambient conditions were maintained during the exposure: air temperature 20°C and relative air humidity not exceeding 80%. Median effective toxic concentrations (ELC_{50}) and harmless concentrations (ELC_{20}) were determined from Biotox 10 records.

Another test object for analyzing cytogenetic effects was polytene chromosomes of summer larvae of the *Chironomus riparius* bipteran of the fourth instar, seventh maturation phase. Being highly sensitive to toxicants, polytene chromosomes were taken as models for the detection of cytogenetic effects and larvae were test subjects for the characterization of effects at the organismal and subcellular levels. The age and phase of larvae were determined according to the table by Ilyinskaya and Iordan [36].

Prior to the tests, larvae were kept at the laboratory for 24 h to let individuals injured during transportation die. The tests were conducted in 250-mL vessels in settled and filtered tap water (pH 7) without any substrate at room temperature under stagnant conditions. The animals were not fed, because the exposure time corresponded to an acute time span [37].

We tested 10 and 5% water sols of the nanomaterial at exposure times 24, 48, 72, and 96 h in two replications. Ten larvae (and ten salivary gland cells from each larva) were used in each experimental variant. A total of 240 larvae and 2400 cells were used: 40 in control experiments and 200 in experiments with the nanomaterial. The median lethal concentration LC_{50} was found by probit analysis [38]. The toxicity in acute experiments was additionally assessed from the cumulative effect based on the median lethal time LT_{50}. This parameter was calculated by the same method as the medial lethal concentration.

Changes in the function of polytene chromosomes were assessed mainly from the response of the nucleolar organizer region (NOR), controlling cellular homeostasis. Larvae were fixed in a 3 : 1 mixture of 96% ethanol and glacial acetic acid. Polytene chromo-

Table 4. Assessment of the influence of the carbon nanomaterial on test subjects

Test subject	Concentration of the CNT sol %. Water dilution factors are parenthesized.					Hazard class
	100 (1×)	10 (10×)	1 (100×)	0.1 (1000×)	0.01 (10000×)	
Ceriodaphnias	0	0	1	1	1	IV
Scenedesmus quadricauda	0	0	0	1	1	III
Ecolum biosensor	0	0	0	1	1	III
Chironomus riparius larvae	0	1	1	1	1	IV

Note: (0) death of the organisms; (1) survival of the organisms.

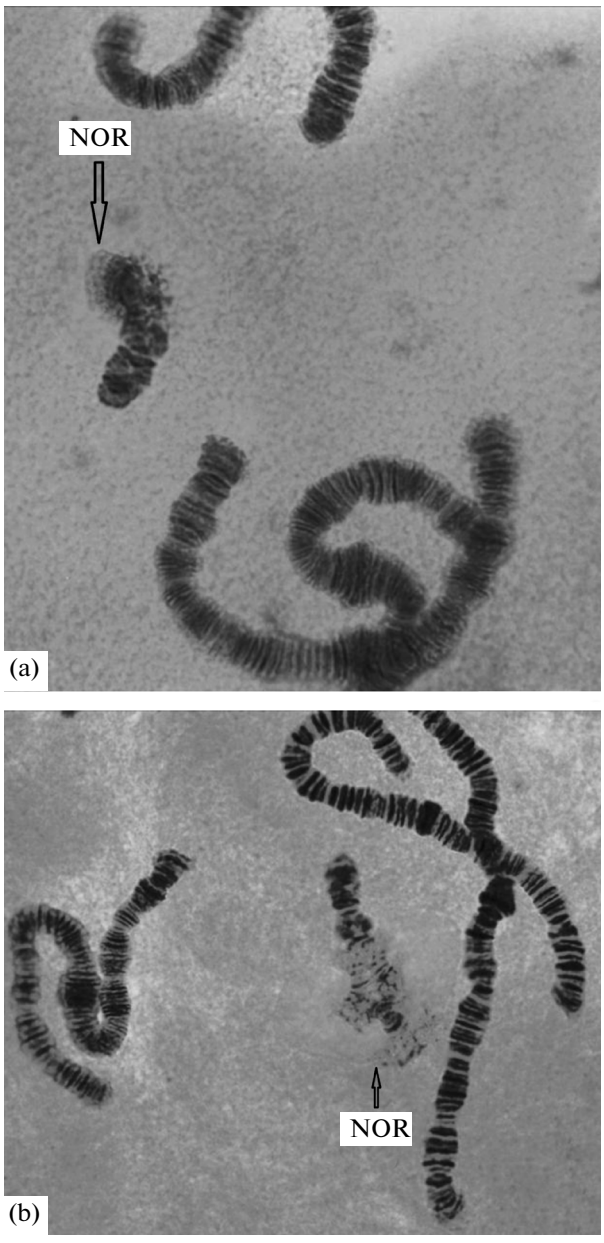

Fig. 2. (a) Activity of chromosome IV NOR of *Chironomus riparius* in the control (24 h). (b) Increase in NOR activity after 24-h exposure to Taunit extract, concentration 50 g/L.

some slides were prepared by the ethyl orcein technique [39] and microscopically examined at the 10 × 40 magnification.

The chromosome set of *Ch. riparius* ($2n = 8$) has four chromosome pairs: I (AB), II (CD), III (EF), and IV (G)). The NOR is located on arm G of chromosome IV. Changes in the function of polytene chromosomes were assessed from the NOR index (the ratio between its largest diameter and the width of the intact region of chromosome IV [40]). The measurement was done with Autodesk software.

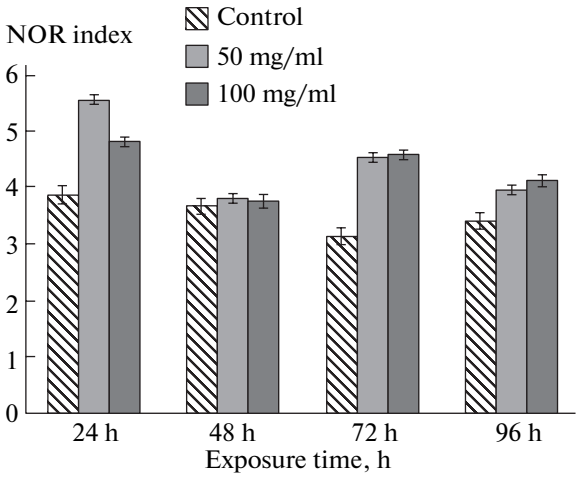

Fig. 3. NOR index change in response to the action of the water extract of Taunit.

A statistical evaluation was conducted with Excel and Statistica 6. The significance of differences was determined by a one-way analysis of variance at $P < 0.05$.

RESULTS AND DISCUSSION

Biotests with ceriodaphnias showed the toxic action of the water extract of Taunit, undiluted and diluted tenfold, on the test subject. No toxic action was detected at 100-fold dilution (1%). Because the safe dilution factor is below 100, the test assigns the material to class IV environmentally hazardous, or marginally hazardous, materials.

Biotests with *Scenedesmus quadricauda* revealed a toxic action at 100% and 1% concentrations. No toxic action was detected in 0.1% extract. The safe dilution factor is within 101–1000. Thus, the material belongs to class III, moderately hazardous substances.

The water extract of CNM Taunit exerted toxic action on the Ecolum biosensor at concentrations of 100, 10, 1, and 0.5%, but not at 0.1% (1000-fold dilution). The safe dilution is within 101–1000 times; hence, the material belongs to class III (moderately hazardous).

Biotests of Taunit with chironomid larvae at the organismal and subcellular levels showed that the toxic effect increased with concentration and exposure time.

Chironomid larvae proved to be highly resistant to the toxic action of Taunit. The corresponding median lethal concentration (LC_{50-96}) was 86 g/L; LC_{10}, 52 g/L; and LT_{50}, 60 h. Thus, the solution that was tested showed hypercumulation, and it can be assigned to class IV (Table 3).

The guide criterion for evaluating the cytogenetic action of CNMs was chosen to be the change in NOR activity in polytene chromosomes of chironomids, because this region functions in 99% of cells [41], sup-

Table 5. Median lethal and safe CNM concentrations for the test species studied

Test subject	Median lethal concentration of the CNM, g/l	Safe CNM concentration, g/l
Ceriodaphnias	0.5	0.1
Scenedesmus quadricauda	2.15	0.002
Ecolum biosensor	0.13	0.074
Chironomus riparius larvae	86	52

ports homeostasis in the cell, and serves as a sensor of many stress factors. Its dysfunction causes apoptosis [42]. It is known that the response of NOR to stress factors is specific and it can serve as a cell-activity indicator at the cellular and subcellular levels [42].

It is apparent from Fig. 2 that the activity of NOR in water extracts of Taunit increases significantly at all concentrations and exposure times (except 48 h). The maximum index was recorded after 24-h exposure to the water extract concentration 50 mg/mL. This effect points to the triggering of short-term cell hypercompensation pathways during the primary response to the stress factor aimed at the restoration of somatic cell homeostasis. The subcellular response (NOR activity change) is more sensitive and more informative than the organismal response, death of test organisms (Fig. 3).

The experimental results are summarized in Table 4.

Thus, our complex biotests indicate that CNM Taunit belongs to Class III (moderately hazardous substances). This class is the same as that of soot, a widespread natural nanostructured material [43]. The relatively low toxicity of multiwalled carbon nanotubes is in agreement with other studies [44, 45].

The toxic action of the nanomaterial may be mediated by cell membrane injury, oxidative stress [46], or mechanical action of nanomaterial agglomerates on respiratory organs of ceriodaphnias [29]. The toxic action of chironomid larvae may be associated with disorders in their alimentary and respiratory organs.

Median lethal and safe concentrations were determined for the test subjects used (Table 5). Chironomid larvae proved to be the least sensitive to Taunit, whereas bacteria and algae were the most sensitive. The safe concentration of Taunit in water for the most sensitive subject, the *Scenedesmus quadricauda* alga, was 2 mg/L.

CONCLUSIONS

The acute toxic action of the commercial carbon nanotube material Taunit on chironomid and ceriodaphnia larvae, unicellular green algae, and bacteria has been assessed. The material belongs to class III (moderately hazardous substance). Chironomid larvae are most resistant to Taunit, whereas the microalgae and bacteria are the most sensitive. Safe Taunit concentrations in aquatic media are below 2 mg/L.

The significant increase in the NOR activity in polytene chromosomes of salivary glands of chironomids exposed to water extracts of the carbon nanomaterial points to a switching on of short-term cell hypercompensation to mitigate the stressing action of the toxicant and restore homeostasis in somatic cells. The subcellular response is more sensitive and informative than the organismal one.

The results of this study can be used in the development of safety precautions in the manufacture, storage, transportation, use, and disposal of products containing carbon nanomaterials.

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