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The toxic influence and biodegradation of carbon nanofibers in freshwater invertebrates of the families Gammaridae, Ephemerellidae, and Chironomidae



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ABSTRACT

Carbon nanofibers (CNFs) are widely used in consumer products today. In this study, we assessed the effects of CNFs on the digestive system of three freshwater invertebrate species (Gammaridae, Ephemerellidae, and Chironomidae). The aquatic insects *Diamesa* sp., *Drunella cryptomeria*, and *Gammarus suifunensis* were incubated with the CNFs at the concentration of 100 mg/L during the 7-days period. Histological examination of the whole specimens and the longitudinal sections revealed no toxic effects of CNFs. However, a noticeable change in the structure of the CNFs accumulated in the intestines of the aquatic insects was found by Raman spectroscopy. The registered decrease in the relative proportion of amorphous carbon included in the CNF sample was found in the intestines of *Diamesa* sp. and *D. cryptomeria*. The registered effect can indicate a biodegradation of amorphous carbon in the digestive tract of these two insect species. In contrast, the decrease of highly structured carbons and the decrease of G-bonds intensity were registered in the digestive tract of *G. suifunensis*. This observation demonstrates the partial biodegradation of CNFs in the digestive tract of *G. suifunensis*.

1. Introduction

The growing industry of nanotechnology results in design and development of new types of nanomaterials with different properties and applications [1–3]. In the past few decades, carbon nanomaterials have

become one of the most widely used type of nanomaterials because of their light weight, high strength, and high conductivity [4]. Among these carbon nanomaterials, carbon nanofibers (CNFs) attracted great research interest due to their promising properties and novel applications in electronics, medicine, energy storage, and catalysis [5,6]. A

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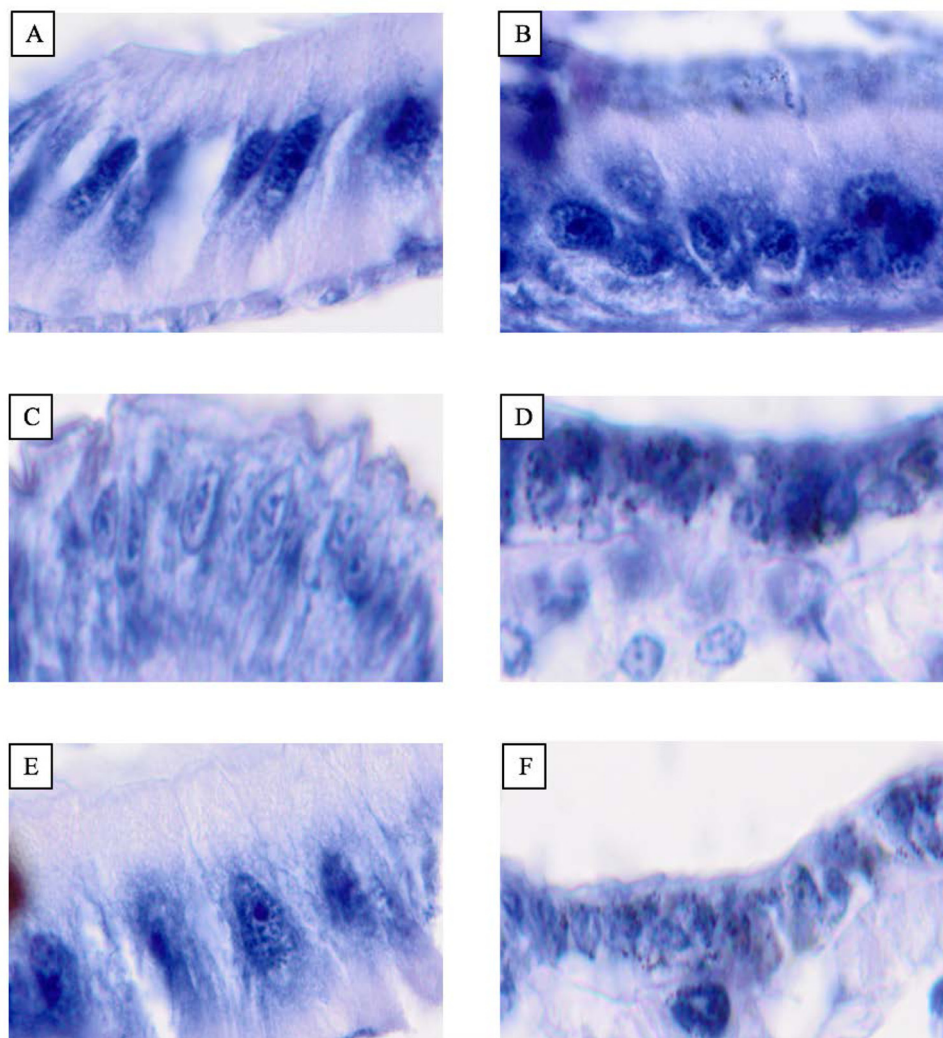


Fig. 1. The intestinal epithelium of the insect specimens used in the bioassay (Hematoxylin-Eosin 10×40). (A) CON group of *G. suifunensis*, (B) EXP group of *G. suifunensis*, (C) CON group of *Diamesa* sp., (D) EXP group of *Diamesa* sp., (E) CON group of *D. cryptomeria*, (F) EXP group of *D. cryptomeria*.

CNFs are carbon cylindrical nanostructures that are formed from stacked layers of graphene in the form of cones, cups, or plates [7]. It should be noted that with an increasing annual production of CNFs, the risks of their harmful influence to the environment and human health is rising. Many research works have been devoted to studying of environmental toxicity of different nanomaterials including CNF [8–12]. However, the toxic properties of CNF are still not fully understood.

Previously, the ecotoxic influence of CNFs has been studied on algae [13,14], bivalves [15,16], and fish models [17]. It was reported that the other carbon nanomaterials, such as fullerenes and carbon nanotubes demonstrate a significantly higher level of toxicity to aquatic organisms compared to CNFs [18]. In particular, fullerene C_{60} induced both necrotic and apoptotic cell death in zebrafish embryos [19]. Consequently, there is a lack of studies aimed to reveal the environmental behavior and the safety of less toxic carbon nanomaterials such as CNFs.

At the same time, it was reported that carbon nanomaterials can penetrate the environment and accumulate in tissues of organisms, in bottom sediments, and in various parts of food chains [20–22]. The other area that required more research is the toxic influence of carbon nanomaterials on benthic aquatic invertebrates, inhabiting the bottom layers of reservoirs. Freshwater plants and animals are continuously exposed to anthropogenic toxicants, which leads to their death and destruction of the biocenosis [23–26]. Macroinvertebrates play a key

role in the trophic chain of freshwater ecosystems, and they are excellent indicators of water quality, especially larvae of amphibiotic insects due to the high sensitivity to any type of pollution [27–29]. Moreover, macroinvertebrates play a pivotal role in the transformation of substances and energy in water bodies, they create an environment for the aquatic inhabitants and participate in the enhancement of water quality [30,31]. However, there are few studies of carbon nanomaterial toxicity in freshwater invertebrates [32–34], and, to the best of authors' knowledge, still there is no study of CNFs influence on freshwater invertebrates.

This study aims to investigate the toxic influence of CNFs on the typical representative species of freshwater insects (*Gammarus suifunensis*, *Drunella cryptomeria*, and *Diamesa* sp.) and to assess the possible biodegradation of the nanomaterials in the digestive tract of the insects.

2. Material and methods

2.1. Synthesis and characterization of nanomaterials

Carbon nanofibers were synthesized and characterized in the Borekov Institute of Catalysis (Novosibirsk, Russia) [35]. The sample of CNFs used for toxicity bioassays was synthesized by catalytic decomposition of the propane-butane mixture on a 90 % NiO and 10 % Al_2O_3 catalyst at the temperature of 800 °C. The obtained CNFs sample

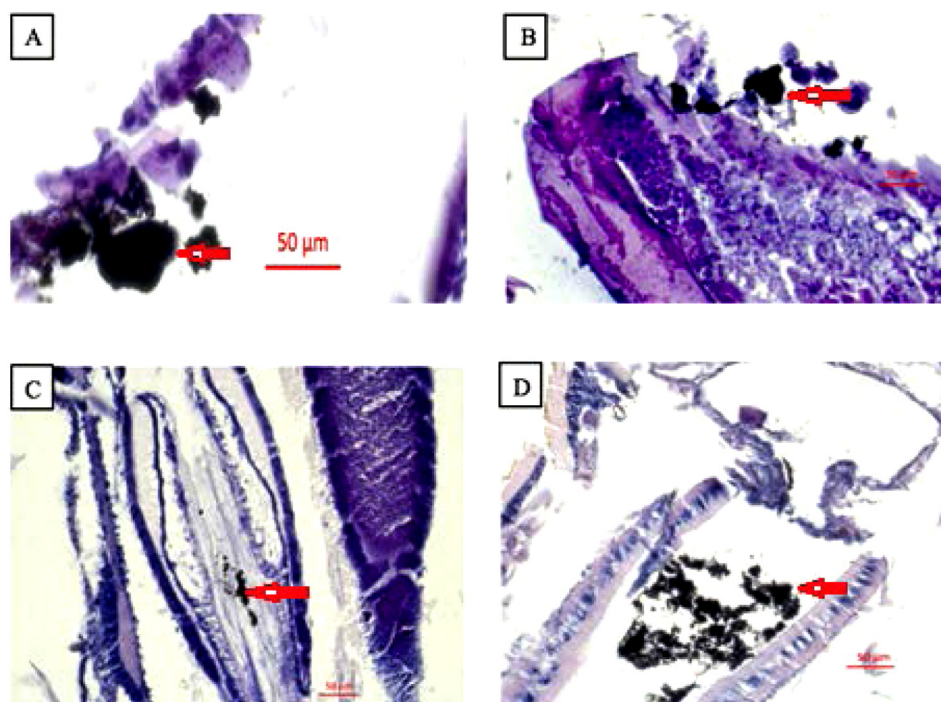


Fig. 2. The conglomerates of carbon nanofibers in EXP groups of the insects (Hematoxylin-Eosin 10×20). (A) intestinal lumen of *G. suifunensis*, (B) tarsus of *G. suifunensis*, (C) intestinal lumen of *Diamesa* sp., (D) intestinal lumen of *D. cryptomeria*.

had an average diameter of 90–120 nm and contained small amounts of metal impurities (Ni, 3.6 % and Al_2O_3 , 0.4 %). The structural features of CNFs were additionally assessed by Morphologi G3-ID dispersive Raman microscope equipped with a 785 nm diode laser (Malvern Instruments Ltd, UK). The resulting laser power at the sample was 4 mW in a low power mode.

2.2. Insects sampling

Three invertebrate species, namely *Gammarus suifunensis* Martynov, 1925 (Amphipoda, Gammaridae), *Drunella cryptomeria* Imanishi, 1937 (Ephemeroptera, Ephemerellidae), and *Diamesa* sp. (Diptera, Chironomidae) were chosen and collected for the experiment. All the chosen species belong to the functional feeding group of collectors-gatherers which collect fine particulate organic matter from the stream bottom. The invertebrates were identified by taxonomic specialists from the Laboratory of Freshwater Hydrobiology of the FSC Biological Diversity (Vladivostok, Russia).

The samples of zoobenthos were collected from the Chernaya River (third order stream) located in Vladivostok, Russia (43.216442°N, 132.043871°E) in January 2017. The water temperature during the sampling procedure was 3.5 °C. The invertebrates were gathered by kick sampling method using a deep net. The captured samples were transferred into the basin, and the remnants of the soil were rinsed away using the water from the Chernaya River. The samples were placed alive into glass containers and transferred to the laboratory. The benthic organisms were sorted and identified the same day and kept in Petri dishes placed in a refrigerator.

2.3. Bioassay and analytical procedures

For the toxicity bioassay, we have used 20 individuals of each insect species (60 in total). The chosen insects of each species were divided into two groups of 10 insects, namely control (CON) and experimental (EXP) groups. Each group was incubated separately in a 250 mL cell tissue flask (Greiner Bio-One GmbH, Germany) with air access. All the groups were incubated in the water taken from the original habitat of

the insects (Chernaya River, Vladivostok) at the temperature of 4 °C in a refrigerator without light for 7 days. Before the experiment, the used insects had an acclimatization period of 72 h.

The CNFs were added to the same river water to obtain the working suspensions with a concentration of 1000 mg/L. Before the bioassay, the working suspension was sonicated with ultrasonic homogenizer Bandelin Sonopuls GM 3100 (Bandelin Electronic GmbH & Co. KG, Germany) with a power intensity of 100 W for 10 min. After that, the suspension of CNFs was added to the EXP groups to obtain the final concentration of 100 mg/L.

Histological examination was performed for the whole insects and for the longitudinal sections. Histological preparations were prepared from insect tissues by the passing of the specimens through the alcohols of gradually increasing concentrations, followed by pouring in paraffin blocks. Histologic preparations were stained with hematoxylin-eosin. The insect samples were imaged using an optical microscope Axio Imager Z2 (Carl Zeiss, Germany) with a magnification of $\times 200$ and $\times 600$. Specialized software ZEN 2 (Carl Zeiss, Germany) was used for the output of the results. Some sections of the intestine were fixed but not stained to obtain Raman spectra of insect intestinal contents.

2.4. Statistical analysis

Statistical analysis was performed using the software package STATISTICA 10 (StatSoft, Inc., USA). The differences between the CON and EXP groups were analyzed using one-way ANOVA test. A value of $p \leq 0.05$ was considered statistically significant. All the experimental data were presented as mean \pm standard deviation.

3. Results

Morphological analysis of the tissues of the respiratory, circulatory, nervous, excretory, and digestive systems of *G. suifunensis* revealed no visible pathological changes in the CON group. On the histological slides, the intestinal walls were lined by a single layer of cylindrical epithelium (Fig. 1A). In the lumen of the intestine, there were discovered heterogeneous contents of different colors, shapes, and sizes

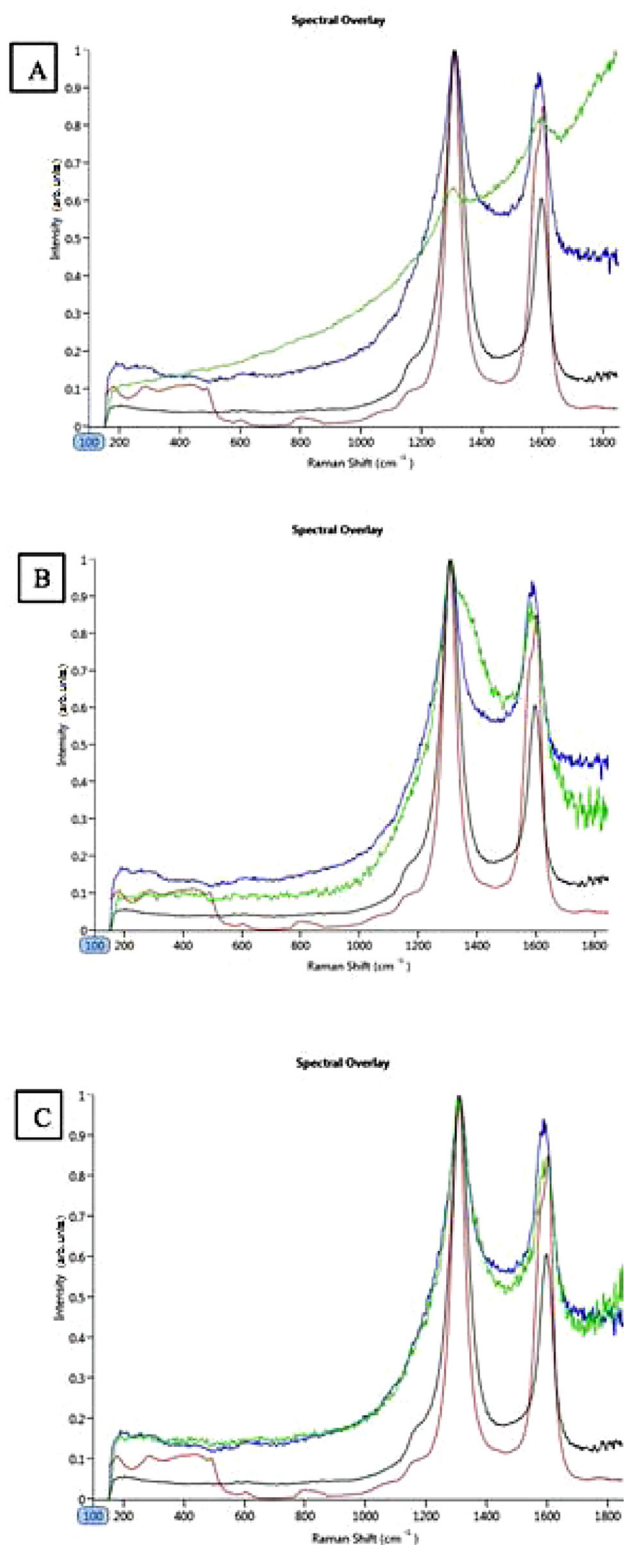


Fig. 3. The Raman spectrums of the aggregates from the intestine of the EXP groups of insects (A) *G. suifunensis*, (B) *Diamesa* sp., (C) *D. cryptomeria*.

The tissue of the EXP group of *G. suifunensis* also had no visible injuries and had a histological structure similar to the CON group. Signs of inflammation, dystrophy, and necrosis in the tissues of the respiratory, circulatory, nervous, excretory, and digestive systems were not found. The intestinal wall was lined by a single layer of cylindrical epithelium as in the CON group. In the apical part of the epithelial cells, there were

discovered many inclusions (presumable conglomerates of nanofibers) (Fig. 1B).

Morphological examination of tissues of respiratory, circulatory, nervous, excretory, and digestive systems of *Diamesa* sp. had no visible damage in the CON group. The intestinal walls were lined by a single layer of cylindrical epithelium (Fig. 1C). Heterogeneous contents of different colors, shapes, and sizes were found in the lumen of the intestine. In the EXP group, the morphological study of the tissues of *Diamesa* sp. also revealed no visible differences compared to the CON group. Signs of inflammation, dystrophy, and necrosis in the tissues of the respiratory, circulatory, nervous, excretory, and digestive systems were not found. The intestinal wall was lined by a single layer of cylindrical epithelium. Many inclusions (presumable conglomerates of CNFs) were observed in the apical part of epithelial cells (Fig. 1D). Morphological analysis of the tissues of the larvae of *D. cryptomeria* in the CON group revealed no visible damage. The intestinal wall was lined by a single layer of cylindrical epithelium (Fig. 1E). In the EXP group, *D. cryptomeria* tissue also had no damage and had a structure similar to the CON group. The intestinal wall was lined by a single layer of epithelium. Many inclusions (presumably conglomerates of CNFs) were observed in the apical part of epithelial cells (Fig. 1F).

The black conglomerates (Fig. 2A) found in the lumen of the intestine of the EXP group of *G. suifunensis* were investigated using Raman spectrometry (Fig. 3A). The obtained results confirmed that these inclusions were the aggregates of CNFs. In addition, we found the change in the ratio of D (1305 cm^{-1}) and G (1595 cm^{-1}) lines in the Raman spectra and the decrease of the G-bond signal intensity. The observed conglomerates were covered by a large amount of mucus. Moreover, the similar individual conglomerates were found on the surface of the insects (Fig. 2B). The conglomerates from the surface of the insects showed no significant change in the ratio of D and G bonds of the Raman spectra.

The black conglomerates discovered in the intestinal wall of the EXP group of *Diamesa* sp. (Fig. 2C) were also investigated by Raman spectroscopy (Fig. 3B). The results confirmed that these agglomerates were CNFs. It should be noted that the intensity of the D and G bonds of Raman spectrums had no significant difference compared to the spectrums of initial CNFs but the peak corresponding to D bonds was remarkably broadened. Thus, the Raman spectrums indicated the appearance of multiple defects in the CNFs.

The Raman spectroscopy (Fig. 3C) of the black conglomerates from the intestinal lumen of the EXP group of *D. cryptomeria* (Fig. 2D) also shown no significant difference in the intensity of the signals related to G and D bonds.

The morphometry of the intestinal epithelium shown no statistically significant differences in the EXP groups of *G. suifunensis*, *Diamesa* sp., and *D. cryptomeria* as shown in Figs. 4–6, respectively.

Single cells loaded with dark inclusions were found in the intestinal walls of the EXP group of *D. cryptomeria* as shown in Fig. 7.

The Raman spectrometry investigation of the intestinal contents in the CON groups of insects shown the presence of the following minerals: potassium, calcium, iron, magnesium, zinc, and selenium. It is known that freshwater insects provide minerals and several vitamins for organisms who eat them. Vitamin and mineral profiles of insects significantly depend on the composition of the insect diet, especially benthic aquatic invertebrates inhabiting the bottom layers of reservoirs, which swallow mineral grains [28]. In the CON groups of the specimens used in the experiment, we compared the spectra of the intestinal contents with various minerals as shown in Fig. 8 and Table 1.

4. Discussion

In the current study, we examined the possible effects of 7-days exposure of CNFs on the digestive system of three freshwater invertebrate species. The digestive system of invertebrates was chosen as an object of the investigation because of its high sensitivity to the

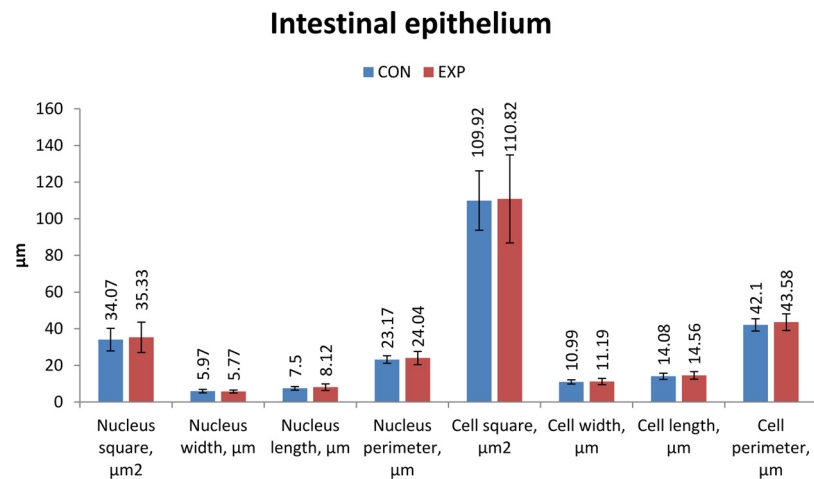


Fig. 4. Morphometric parameters of the intestinal epithelium in the EXP group in *G. sulfunensis*.

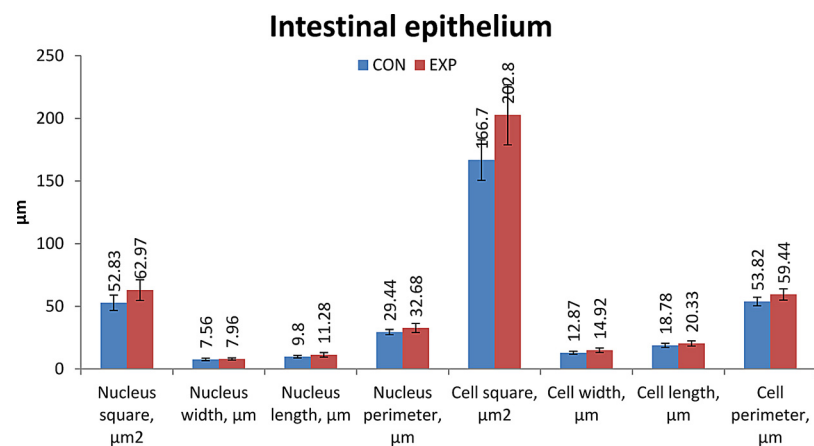


Fig. 5. Morphometric parameters of the intestinal epithelium in the EXP group in *Diamesa* sp.

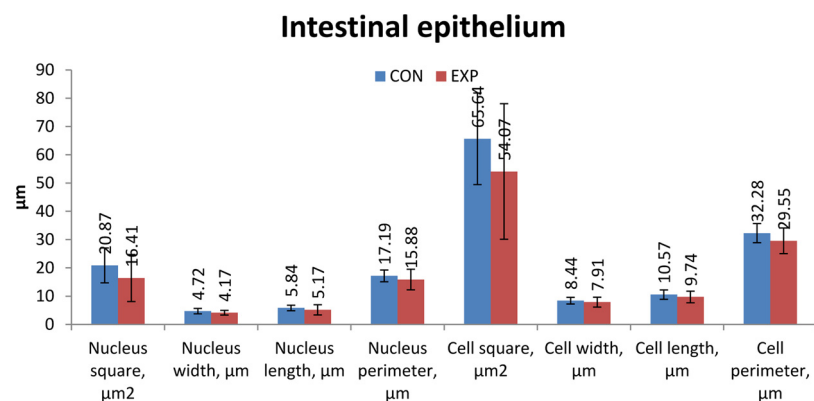


Fig. 6. Morphometric parameters of the intestinal epithelium in the EXP group in *D. cryptomeria*.

influence of toxic particulate matter [36,37]. The results of histological examination (Fig. 2) and Raman spectroscopy analyses (Fig. 3) confirmed the intense absorption of conglomerated CNFs by the intestine of the insects. The inclusions were located mostly in the apical part of the epithelial cells of all used insect species (Fig. 2). However, single cells loaded with CNFs were found in the intestinal walls of the EXP groups of all used insect species (Fig. 7). According to these data, we can infer that the inclusions observed in this case might be the macrophage cells of the local immune system of the insects with absorbed nanofibers [38].

The presence of inclusions in the cells of the lining of intestines

(Fig. 2) and in the cells of the immune system (Fig. 7) suggests that carbon nanoparticles can enter the body of freshwater insects and can have a negative effect on them. In study the authors examined the effect of nanoparticles on two species of lepidopterans (*Spodopteralitura* F. and *Achaea janata* L.) the accumulation of nanoparticles in cellular organelles was observed in the intestinal cells of the insects [39]. The results of our study did not show any pronounced toxic effect of CNFs on the benthic insects. The used insects feed on organic and mineral micro-particles of bottom sediments in the natural environment, as it was confirmed by Raman spectroscopy of aggregates from the intestine of the CON groups of insects (Fig. 8). A possible explanation for the

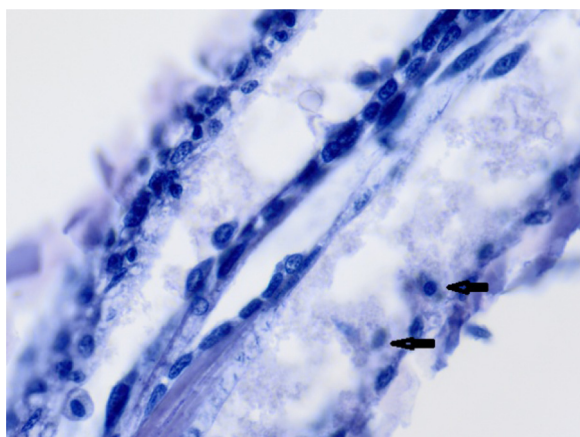


Fig. 7. Single cells loaded with dark inclusions in the intestinal wall of the EXP group of *D. cryptomeria* (Hematoxylin-Eosin 10 × 20).

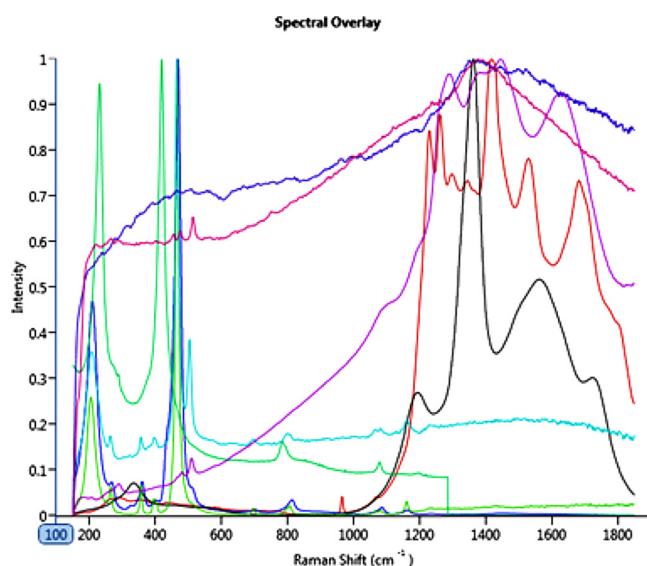


Fig. 8. Raman spectrum of aggregates from the intestine in CON groups.

Table 1

The degree of correspondence between the spectra of minerals and aggregates from the intestine in CON groups.

Mineral	The degree of spectrum identity to the minerals from the intestine in CON groups
Quartz	0.132
Phosphohedyphane	0.059
Flintstone	0.143
Cornelian	0.225
Pumice-pecutan-trachyte	0.542
Granite	0.098
Pumice	0.444
Amazonite	0.215

tolerance of the sediment feeding insects to the CNFs may be concluded in the presence of an innate protection mechanism which is able to isolate the harmful substances by mucus covering and prevent the inflammation of tissues and reduce the toxic influence of the toxicants [40].

Another important discovered finding was that the agglomerates of CNFs in the digestive system of *Diamesa* sp. and *D. cryptomeria* had a slight decrease in the amount of amorphous carbon and an increase in the intensity of the G bond signal of Raman spectra in relation to the D bond intensity (Fig. 3B, C). Thus, amorphous carbon was partially

biodegraded in the digestive system of these two insect species. The particles remaining in the intestine of the insects were highly structured CNFs, according to the Raman spectroscopy data (Fig. 3B, C). This result may be explained by the fact that the enzymes produced at the gastrointestinal tract of these insects are capable of oxidizing amorphous carbon [41]. The biodegradation of carbon nanomaterials in living organisms has been previously shown in several studies [42–44]. The influence of carbon nanomaterials on *Daphnia magna* shown a high absorption of nanomaterials in the organism of crustaceans within several hours but a slow rate of nanomaterial elimination and biodegradation [45], which induce the possible risk for the higher levels of the food chain.

In contrast to *Diamesa* sp. and *D. cryptomeria*, Raman spectrometry of the agglomerates found in the intestine of *G. suifunensis* reveals an increase in the signal intensity of the D bonds in relation to G bonds (Fig. 3A). The observed correlation between the intensity of the registered signals demonstrated a significant increase in the number of defect and unstructured nanofibers. This finding suggests that the CNFs were partially biodegraded in the digestive tract of *G. suifunensis*. However, it is still unclear whether the biodegradation of CNFs in the digestive tract of *G. suifunensis* was carried out by the internal enzymatic system of *G. suifunensis* or was produced by the microorganisms located in the digestive tract of the insects.

The results of our study provide evidence of the fact that novel synthetic nanomaterials can be biodegraded by aquatic organisms to a certain extent. However, the mechanisms of such a biodegradation process are still poorly understood. The design of this study limited by the absence of real-life exposure simulation, where long-term, low-dose exposure to chemical mixtures is the case [46]. Further study could assess the long-term effects of CNFs and implement the assessment of genotoxicity, metabolomic changes, and other crucial responses of the organisms to xenobiotic exposure [47–50]. Also, further studies should evaluate the mechanisms of the toxic influence of carbon nanomaterials on different trophic levels, on various freshwater and marine animals, and humans.

5. Conclusions

Our study shown that freshwater invertebrate species represents a sufficient model to assess the negative effect of CNFs on the aquatic environment. This study has identified the absence of pronounced toxic influence of CNFs on freshwater insects *G. suifunensis*, *Drunella cryptomeria*, and *Diamesa* sp. at the concentration of 100 mg/L during the 7 days. However, Raman spectroscopy analysis revealed the biodegradation of amorphous carbon in the intestines of *Diamesa* sp. and *D. cryptomeria* and partial biodegradation of CNFs in the digestive tract of *G. suifunensis*. The findings of this study have significant implications for further filling the lack of understanding of the mechanisms of accumulation and biodegradation of nanostructures in different species of aquatic organisms.

CRedit authorship contribution statement

Vladimir Chaika: Investigation, Methodology, Visualization, Writing - original draft. **Konstantin Pikula:** Investigation, Writing - review & editing. **Tatyana Vshivkova:** Data curation, Resources, Validation. **Alexander Zakharenko:** Formal analysis, Writing - original draft. **Galina Reva:** Visualization. **Konstantin Drozdov:** Resources. **Alexander I. Vardavas:** Writing - original draft, Writing - review & editing. **Polychronis D. Stivaktakis:** Writing - original draft, Writing - review & editing. **Taxiarchis K. Nikolouzakis:** Writing - original draft, Writing - review & editing. **Antonios K. Stratidakis:** Writing - original draft, Writing - review & editing. **Manolis N. Kokkinakis:** Writing - original draft, Writing - review & editing. **Alexandra Kalogeraki:** Writing - original draft, Writing - review & editing. **Tatyana Burykina:** Software. **Dimosthenis A. Sarigiannis:** Writing - original draft,

Writing - review & editing. **Aleksei Kholodov:** Writing - original draft. **Kirill Golokhvast:** Conceptualization, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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