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Effects of carbon and silicon nanotubes and carbon nanofibers on marine microalgae *Heterosigma akashiwo*

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ABSTRACT

The effect of carbon and silicon nanotubes (CNTs and SiNTs) and carbon nanofibers (CNFs) to microscopic marine algae *Heterosigma akashiwo* was studied, using algal growth inhibition for 3 days (acute effect) and 7 days (chronic effect) as toxicity endpoints. The criterion of the toxic effect was the statistically significant reduction of the number of algal cells in the exposed samples compared to the control. Samples did not demonstrate toxic effects at doses 1 mg/l and 10 mg/l. CNTs and SiNTs samples at 100 mg/l exhibited both acute and chronic toxic effects. We assume that the main cause of cell death in these samples was related to the mechanical damage of cell integrity. CNFs at concentrations of 100 mg/l did not inhibit algal growth, but cells with irregular shapes were observed, which were not observed after exposure to CNTs and SiNTs. Nickel impurities present in CNFs samples are presumably the main cause of observed cell deformations.

1. Introduction

Rapid increase in applications of carbon nanotubes (CNTs) and carbon nanofibers (CNFs) raises questions on their potential adverse environmental effects (Piperigkou et al., 2016). The global CNTs market is expected to increase from 2.26 Billion USD and more than 2000 t in 2015–5.64 Billion USD by 2020, at a compound annual growth rate of 20.1%. Silicon nanotubes (SiNTs), have also become more popular. Nowadays, nanotubes of different nature are also proposed as the drug adsorbents and drug delivery systems, however, several toxicity problems have been associated with their use (Bostan et al., 2016; Sayapina et al., 2016). Nowadays all kinds of synthesized carbon-based nanoparticles are well described concerning their physical parameters (Liu et al., 2014). However, there is still a lack of toxicity data required for risk assessment and modeling (Sarigiannis et al., 2016; Guseva Canu et al., 2016; Neagu et al., 2017).

In order to understand the mechanisms of action of the biological adverse effects, a detailed physico-chemical characterization of nanoparticles (NPs) is required to be performed in parallel to the bioassays. However, NPs used in testing biological activity of (NPs) often do not characterized physically well (Juganson et al., 2015).

In continuation of our studies on the toxic effects of CNTs on mammals (Golokhvast et al., 2013), we were interested in the environmental adverse effects of well characterized nanoparticles, in terms of their physicochemical properties, as there are only a few studies concerning ecotoxicological data of NPs (Kahru and Ivask, 2013).

Approximately 60–80% of the world's plastic materials and different composites, and almost 10% of their the annual production ends up into the oceans, where degradation of such objects can take several hundred years (Avio et al., 2017). Most of composites contain several nanoparticles, used for the improvement of their properties. With regard to the NPs life-cycle in the ecosystem, they are released to the environment via various waste streams and most of them sooner or later reach the ocean. There are a few nanotoxicity studies on marine species such as bivalves, which filter a significant amount of water (Anisimova et al., 2017; Anisimova et al., 2015; Balmuri et al., 2017). Toxicological studies on marine microalgae are also of great importance, because they are widely distributed and constitute the base of the food chain in the

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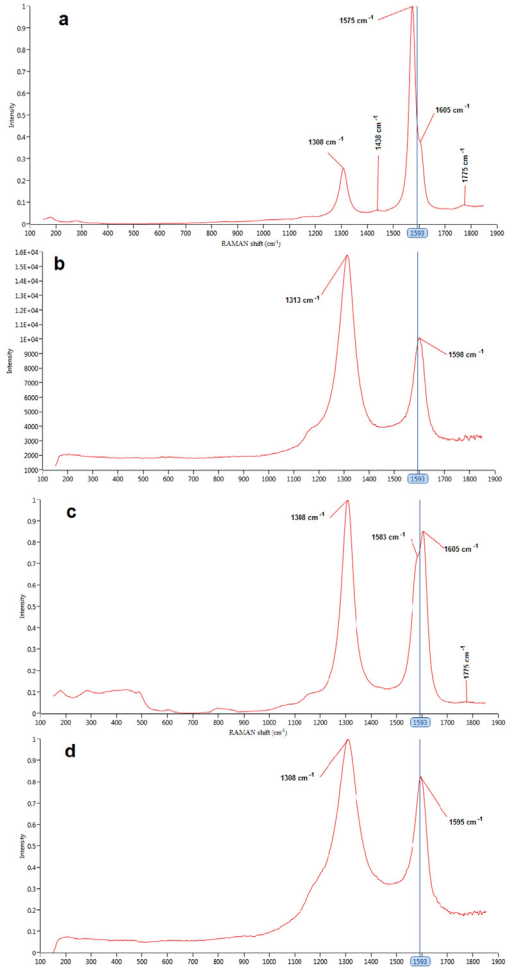

Fig. 1. RAMAN spectra of carbon nanotubes (CNTs) and carbon nano fibers (CNFs). a - **CNTs CE2**; b - **CNTs SC3**; c - **CNFs 500**; d - **CNFs 800**.

ocean. To the best of our knowledge, there is a small number of studies on the toxicity of MWCNT for marine microalgae in the recent literature (Wei et al., 2010; Kowk et al., 2010). It has also been reported that for high concentration levels, growth inhibition was highly correlated with the shading effect of CNTs (Schwab et al., 2011). The inhibitory effect of cotton-derived cellulose nanofibers on growth of freshwater filamentous eukaryotic green microalgae *Klebsormidium flaccidum* was recently showed (Munk et al., 2015). In the current work, we studied the toxicity of carbon and silicon nanotubes (CNTs and SiNTs) and carbon nanofibers (CNFs) to unicelular marine microalgae *Heterosigma akashiwo* isolated from Peter the Great Gulf of Japan sea.

2. Materials and methods

2.1. Materials

Carbon nanotubes and nanofibers used in this research were synthesized in Boreskov Institute of Catalysis (Novosibirsk, Russia) and their toxic effects were previously studied on rats (Golokhvast et al., 2013). Un-annealed **CNTs SC3** with d ≥ 18–20 nm, specific surface area based on BET (BrunauerEmmett-Teller) results, 130 m^2/g, contained small amounts of impurities: Fe – 0.6%, Co – 0.3%, Al – 0.9%. Annealed at 2600 °C **CNTs CE2** 2600 °C had the following parameters: d ≥ 18–20 nm, specific surface area 150 m^2/g, contained small amounts of impurities: Fe – 0.0014%, Ca – 0.0036%, Si – 0.0098%. The impurities content in MWNTs was determined using X-ray fluorescent analysis XRF (ARL-Advant'x analyzerwith Rh anode of the X-ray tube) (Kuznetsov et al., 2010).

CNFs 500 and **CNFs 800** were obtained by catalytic decomposition of propane-butane mixture on a 90% NiO + 10% Al_2O_3 catalyst at 500 and 800 °C, respectively. CNFs contained small amounts of impurities, Al_2O_3 – 0.4%, Ni – trace for **CNFs 500** and Ni – 3.6%, Al_2O_3 – 0.4% for **CNFs 800**. Specific surface area (based on BET results), 90–100 m^2/g, length are 5–50 μm for both samples.

Silicon nanotubes **SiNTs** and **SiNTs INC-2** were kindly provided by the Department of Chemistry, Inha University Republic of Korea (Han and Park, 2010). Specific surface area (based on BET results) 395 m^2/g, d ≥ 40–45 nm.

2.2. Observation methods

Raman spectroscopy was used to characterize samples of CNTs. Raman spectra were collected 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 at a low power mode.

The microalgae were imaged using confocal microscopy by optical microscope Axio Imager A2 (Carl Zeiss, Germany) with a magnification of 200 × and 600 × .

Algal cell analysis and counting of the propidium iodide stained cells were conducted by CytoFLEX flow cytometer (Beckman Coulter, USA) with the excitation light of 405 nm, 488 nm and 638 (Suman et al., 2015). Aliquot volumes for the analysis were 0.1 ml per sample.

2.3. Algal growth inhibition assay

All experiments to determine CNTs and CNFs toxicity with the use of a microalgae model were conducted in accordance to the guidance OECD No.201 (OECD, 2006) with minor modifications. The method is based on the determination of the difference between the intensity of algal growth in control medium and the test sample. The criterion of the toxic effect is the statistically significant reduction of the number of algal cells in the samples compared to the control for the 72 h bioassay (conditionally "acute toxicity") and for 7 days ("chronic toxicity"). The toxicity tests were performed in 24-well cell culture plates. Each substance, the medium and the algal inocula were mixed to obtain an initial algal concentration of 10,000 cells/ml in 2.0 ml of bioassay volume. Final exposure concentrations were 1, 10 and 100 mg/l. At least four replicates per concentration were used.

Microscopic alga *H. akashiwo* (class *Raphidophyceae*) were used as a test strain. The medium was prepared using filtered and sterilized seawater with a salinity of 32‰ followed by the addition of sterile solution of F medium (Guillard and Ryther, 1962). Millipore filter 0.22 μm were used for filtration. To 1 L of seawater were added following components: 75 mg of $NaNO_3$, 5 mg of $NaH_2PO_4{\cdot}H_2O$, 30 mg of $NaSiO_3{\cdot}9H_2O$; 100 mg of thiamine-HCl, 0.5 mg of biotin and 0.5 mg of vitamin B12, trace elements stock solution 1 ml. Cultures were incubated at 20 ± 2 °C with 70 μmol m^{-2} s^{-1} light provided from fluorescent lamps and a 12:12 h light-dark cycle; the flasks were shaken once a day. All cells were illuminated from the top and were equidistant from the light sources. The method of flow cytometry with propidium iodide staining was used to count the number of algal cells (Suman et al., 2015).

Statistical analyzes were performed using STATISTICA 10 software (StatSoft, Inc., USA). One-way ANOVA test were used for analysis. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

Samples of CNTs were additionally studied by Raman spectroscopy. All the spectra exhibited two typical bands of carbon material: D-band (1100–1400 cm^{-1}) and G-band (1575–1600 cm^{-1}). Sample **CNTs CE2**

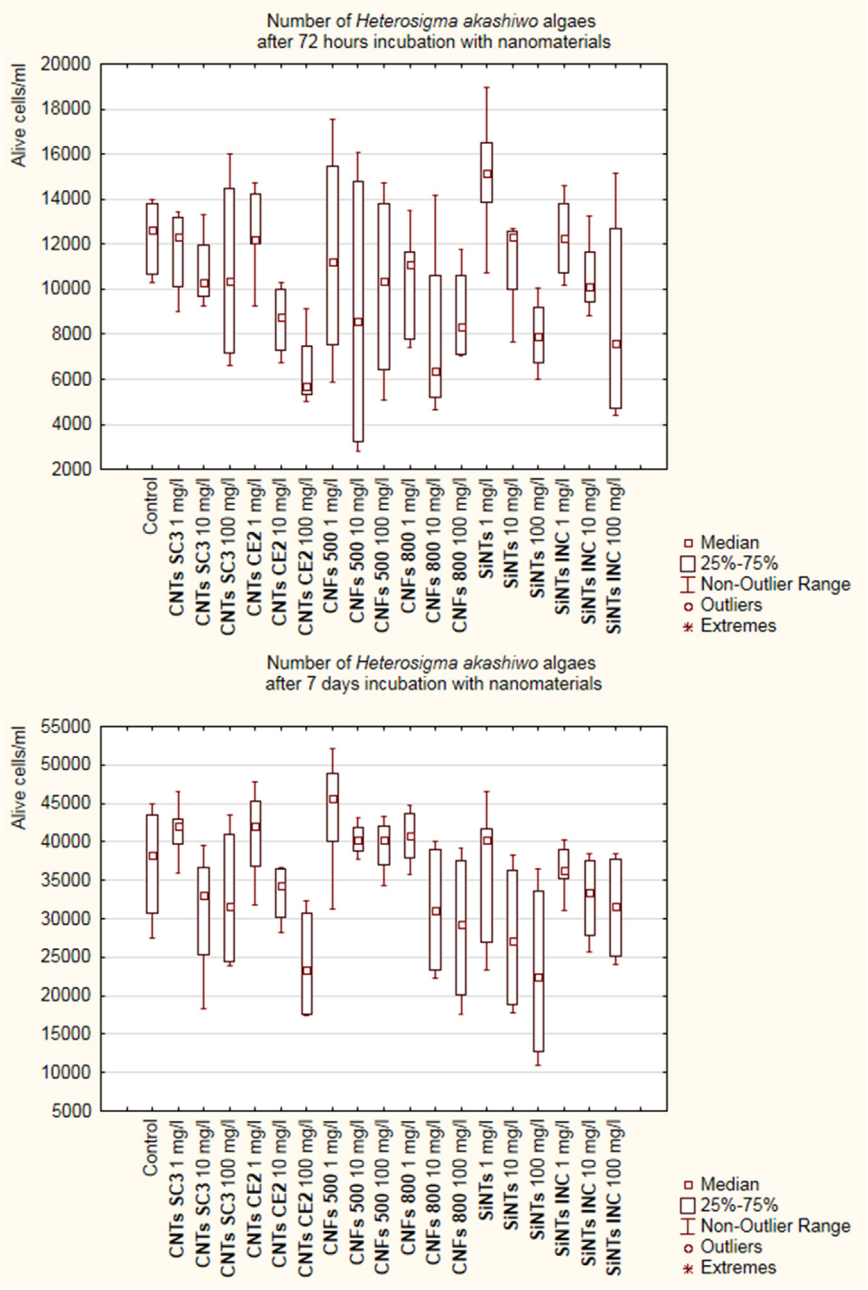

Fig. 2. Survival of the marine microalgae *H. akashiwo* in 72 h and 7 days upon exposure to different concentrations of silicon nanotubes (SiNTs), carbon nanotubes (CNTs) and carbon nano fibers (CNFs) at 20 °C and 12:12 h light-dark cycle.

had more regular structure and G-band provided a good representation of the sp^2 bonded carbon that is present in planar sheet configurations which include the sp^2 bonded carbon of carbon nanotubes (Fig. 1a). Moreover, a big difference in signal intensity indicates the multilayered ordered structure. Sample **CNTs SC3** is characterized with less ordered structure (Fig. 1b). D-band (1308 cm^{-1}) can be assigned to disorder or defect, which originates from edge configurations in graphene where the planar sheet configuration is disrupted. It is detected on the edge of the open end of a carbon nanotube, where defects are located. Sometimes, some sp^2 bonded amorphous carbon contributing to this band can also be observed. Line at 1605 cm^{-1} corresponding to D′-band is also indicative of sp^2 bonded carbon that represents surface defect modes.

A small difference in signal intensity between D-band (1308 cm^{-1}) and G-band (1583 cm^{-1}) indicates the amount of layers in the structure between 2 and 4.

$$\omega_G = 1581.6 + 11/(1 + n1.6)$$

Raman spectrum of carbon nanotubes has a peak at 1575 cm^{-1}, which corresponds to a number of single graphite nanocrystals. There is also a peak of 1605 cm^{-1}, corresponding to multilayered carbon nanotubes (MWCNTs) (Fig. 1a). A large number of peaks in the region of the radial breathing modes (RBM) in the low-frequency region of the spectrum (peaks at 178 cm^{-1}, 278 cm^{-1}, 357 cm^{-1}, 464 cm^{-1}) indicate a large number of nanotubes in a sample having a double layer (Fig. 1b).

Results of algal growth inhibition test revealed that acute toxicity of all samples were dose dependant (Fig. 2). Sample of **CNTs CE2** exhibited greater inhibitory activity on algal growth than **CNTs SC3**. The sample with a large amount of Ni impurities **CNFs 800** exhibited greater inhibitory activity than **CNFs 500**. The most toxic sample with acute and chronic toxicity was **CNTs CE2**. The sample of **CNFs 500**

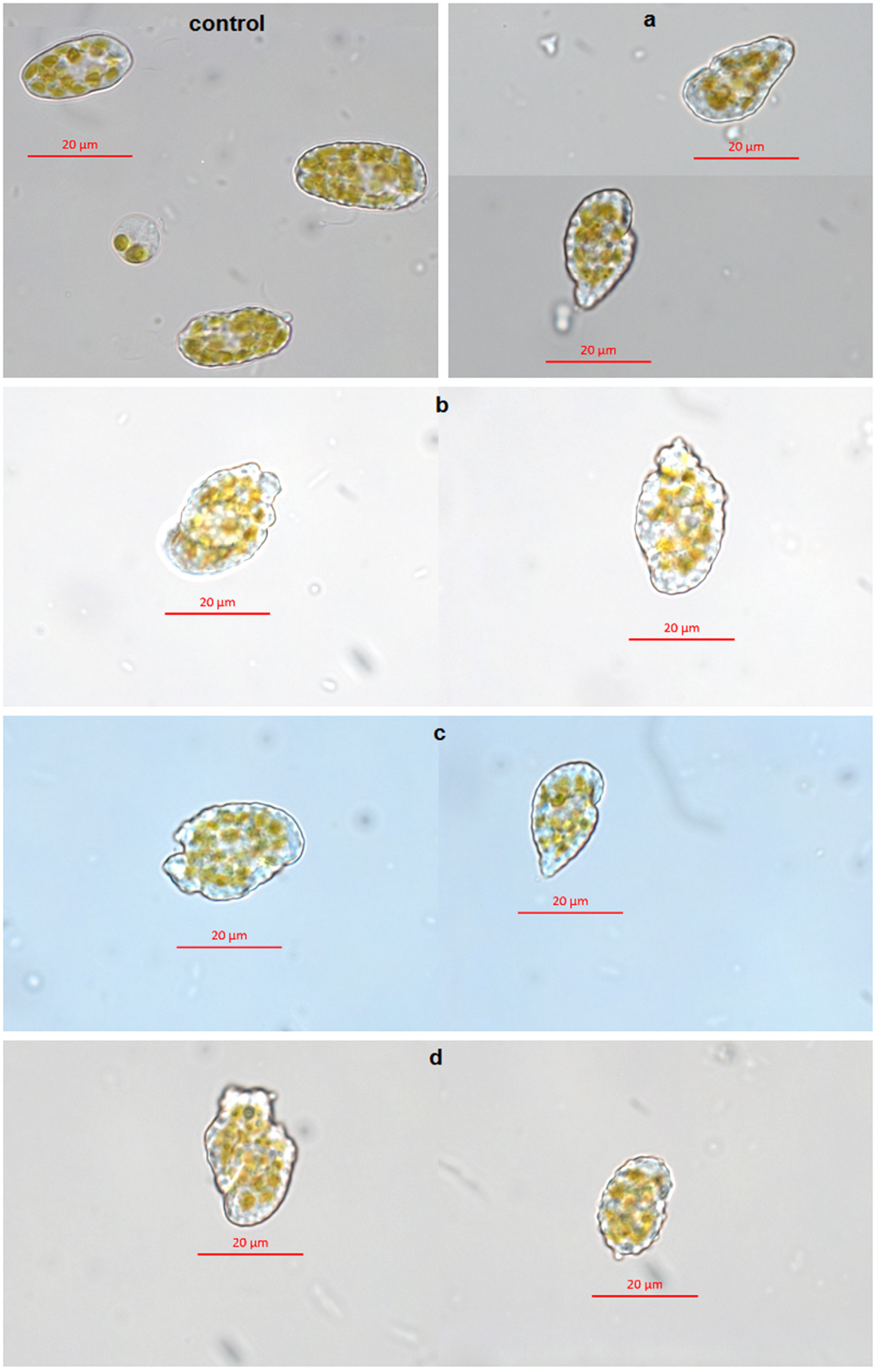

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Fig. 3. Confocal microscopy of marine microalgae with two samples of carbon nanotubes CNTs and two samples of carbon nano fibers CNFs samples after 7 days of experiment at exposure 100 mg/l. **a - CNTs CE2; b - CNTs SC3; c - CNFs 500; d - CNFs 800.**

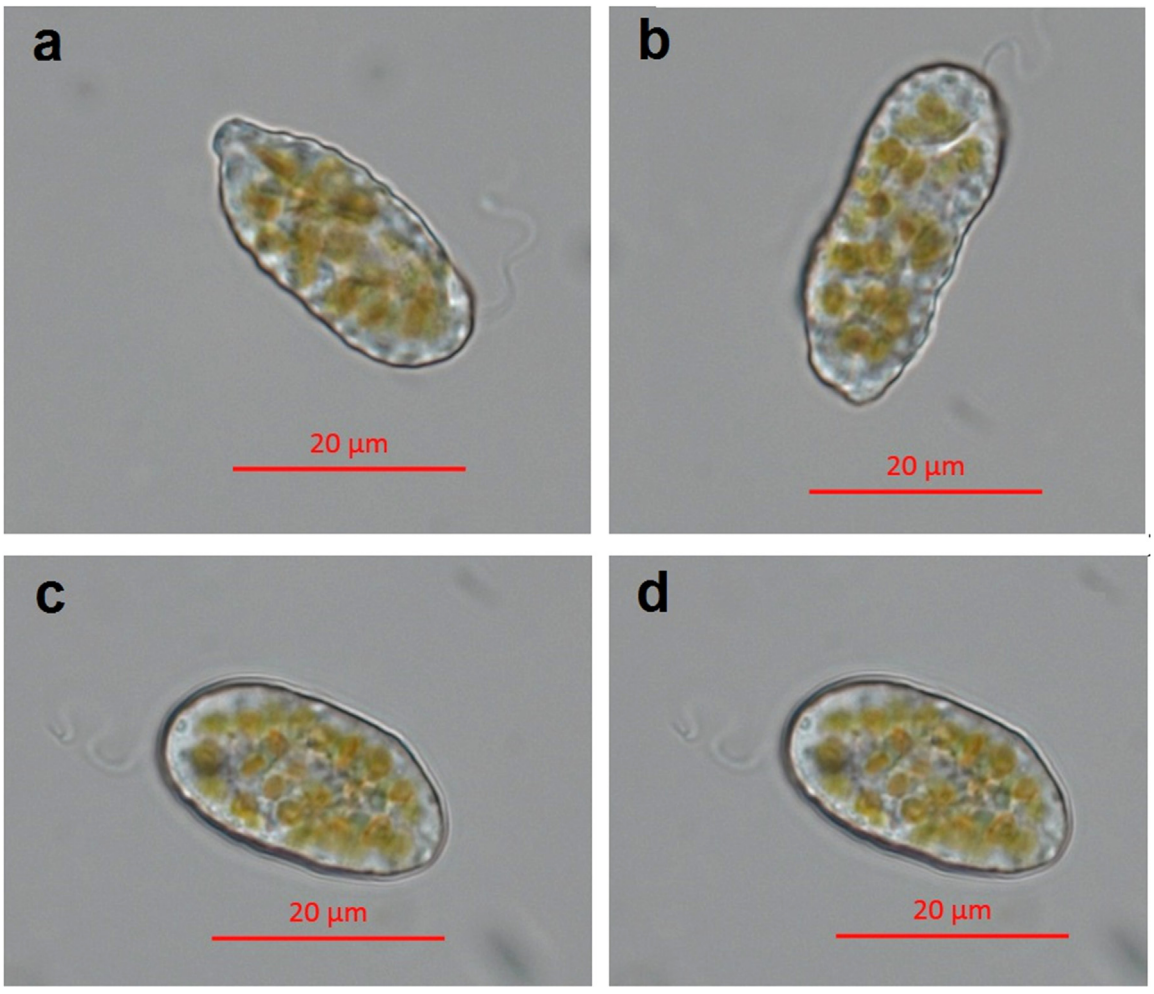

Fig. 4. Confocal microscopy of marine microalgae with two samples of silicon nanotubes **SiNTs** and **SiNTs INC-2** samples after 7 days of experiment at exposure 100 mg/l. **a, b - SiNTs; c, d - SiNTs INC-2.**

exhibited almost no toxicity even in concentration 100 mg/l. Moreover, microalgae exposed to this sample grew better than the control for the 7 days experiment.

Observation with the optical confocal microscopy revealed that the number of cells with irregular shape in **CNTs CE2** samples was minimal (Fig. 3a), while there were more malformed cells in **CNTs SC3** samples (Fig. 3b) with predominate D-band in the structure (see Fig. 1b). The experiments with all CNFs samples revealed a large number of cells with irregular shape (Fig. 3c,d).

Similarly to **CNTs CE2**, all samples of **SiNTs** showed high toxicity on microalgae and almost absence of cells with abnormal shape (Fig. 4).

Micro structure of CNTs and SiNTs were studied with transmission and scanning electron microscopy (TEM and SEM) (Han and Park, 2010; Kuznetsov et al., 2010). Silicon nanotubes have much more branched microstructure, and ratios of length to diameter were smaller (Fig. 5). Both samples of CNTs have a length hundreds of times greater than their diameter. CNTs form balls in the size of several tens of micrometers (Fig. 6).

4. Discussion

The range of exposure concentrations for our studies was chosen in accordance to previously published data. Nanoparticles exposure concentration levels for fresh water algae ranged from 0.1 to 60 mg/l (Aruoja et al., 2015, 2009; Suman et al., 2015); fresh water green algae were exposed to annealed multi-walled carbon nanotubes (MWCNTs) at levels ranging from 5 to 30 mg/l (Figarol et al., 2015; Zhang et al., 2015) and to graphene-family materials at levels ranging from 37 to 180 mg/l (Zhao et al., 2017); marine algae were exposed to MWCNTs at levels ranging from 0.1 to 10 mg/l (Wei et al., 2010). We choose a little higher concentrations of MWCNTs for *H. akashiwo* than the levels used for marine seaweed *Dunaliella tertiolecta*, because the latter is able to release reactive oxygen species (hydrogen peroxide and superoxide) (Marshall et al., 2005), which significantly increase its resistance to pollution and stress (Twiner et al., 2001; Dring, 2005).

Microscopic alga *H. akashiwo* (class *Raphidophyceae*) were chosen because it is important to analyze the toxicity of carbon nanomaterials

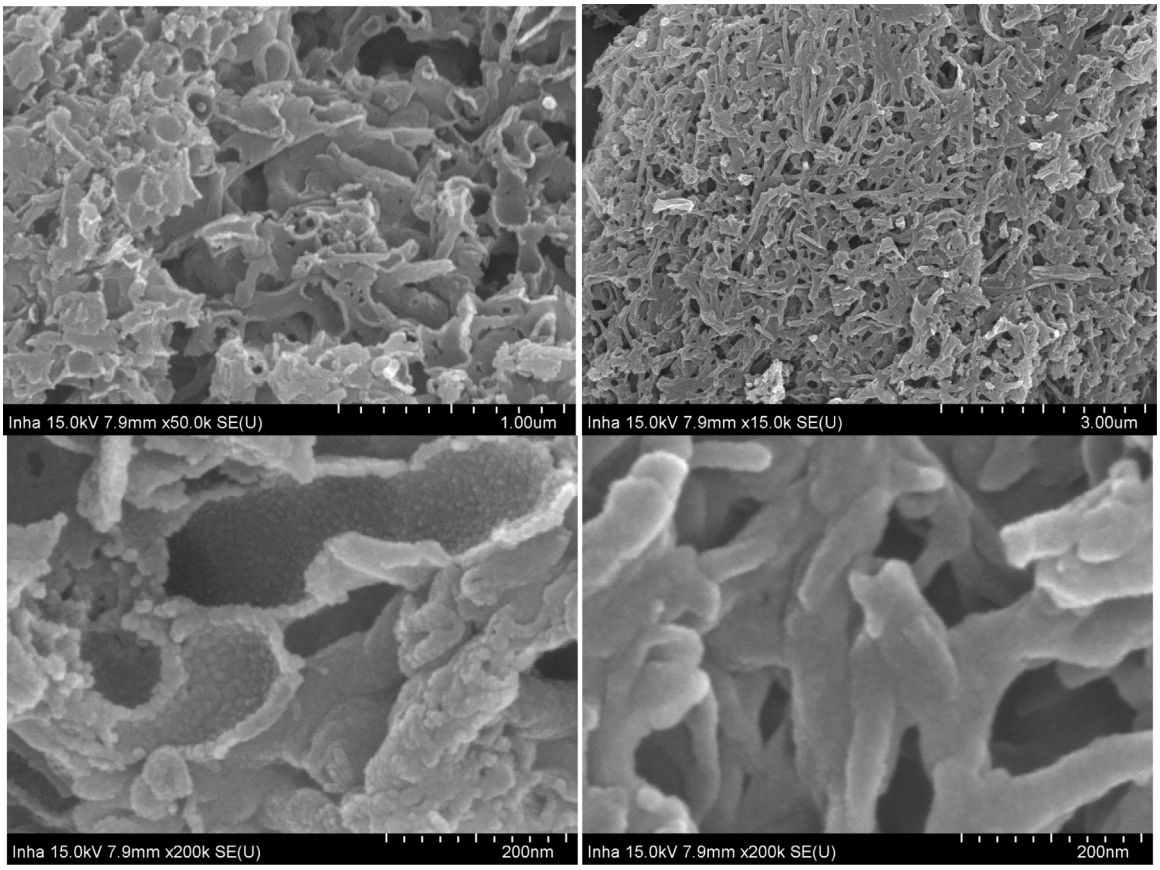

Fig. 5. TEM images of carbon extraction replica of silicon nanotubes SiNTs.

with respect to Far East of Russia local marine microalgae, moreover this species have thin cell wall, which can render it rather susceptible to chemical pollution.

It is known that growth inhibition was highly correlated with the shading of CNTs and the agglomeration of algal cells (Schwab et al., 2011, 2014). We did not notice a direct relationship between shading and growth inhibition, but in the case of nanofibers, we observed how they agglomerated with them and formed large clusters.

Results of algal growth inhibition test revealed a good correlation with the number of G-band that represents the amount of well-formed nanotubes in the samples (Fig. 1a, Fig. 2). Probably, a large number of well-formed nanotubes affect the viability of microalgae population. Sample of **CNTs CE2** exhibited greater inhibitory activity on algal growth than **CNTs SC3**. With regard to CNFs samples, sample with a large amount of Ni impurities **CNFs 800** exhibited greater inhibitory activity than **CNFs 500**. Probably this difference is due to the fact that in CNTs samples most of the metal impurities are inside the tubes and are inaccessible to algae. It is known that metals like Ti, Ni, Cu, Zn, Cr and rare earth oxide particles inhibit algal growth and induce direct and indirect toxic effects (Joonas et al., 2017; Miazek et al., 2015). Importance of metal impurities for toxic effects of carbon nanotubes were shown previously (Vitkina et al., 2016). Moreover it was shown previously that Ni leads to the disturbance of the microalgae cell shape (Osman et al., 2004). The CNFs samples on the contrary, have a small amount of graphene-type bonds, but all impurities are on the surface and are easily accessible. Probably in the case of CNFs, the inhibitory effect is caused by impurities only. Understanding of mechanistic interactions of nanoparticles with extracellular matrix is very important for nanotoxicology (Engin et al., 2017).

Silicon nanotubes revealed high acute toxicity, even though it has been reported that silicon oxide itself is not toxic to microalgae (Ji et al., 2011). Absence of algae with an irregular shape in the experiment with SiNTs means that physical damage remains the main assumption of the cause of death. It is likely that the toxicity of silicon nanotubes is directly related to their structure and the fact that they cause physical damage the integrity of the cells (Fig. 5). Both samples of CNTs have a length hundreds of times greater than the diameter and the probability of causing physical damage is not as great as in the case of silicon nanotubes (Fig. 6).

We assume that greater toxicity of **SiNTs** in comparison to CNTs is due to the fact that they are smaller and more hydrophilic. Both of these factors increase their penetration and travel speed in the solution. In the case of **SiNTs**, the sample does not contain a large amount of impurities, so there was no reason for the detection of altered shapes of cells. Finally, this research has revealed that carbon and silicon nanotubes at concentrations of 100 mg/l exhibited both acute and chronic toxicity. It can be concluded from the obtained results that the main cause of cell death among these samples was mechanical damage that disrupted the integrity of cells.

At the same time, carbon nanofibers at 100 mg/l showed almost no toxicity, although a significant number of cells with irregular shapes was observed. It must be noted that such irregularly shaped cells were

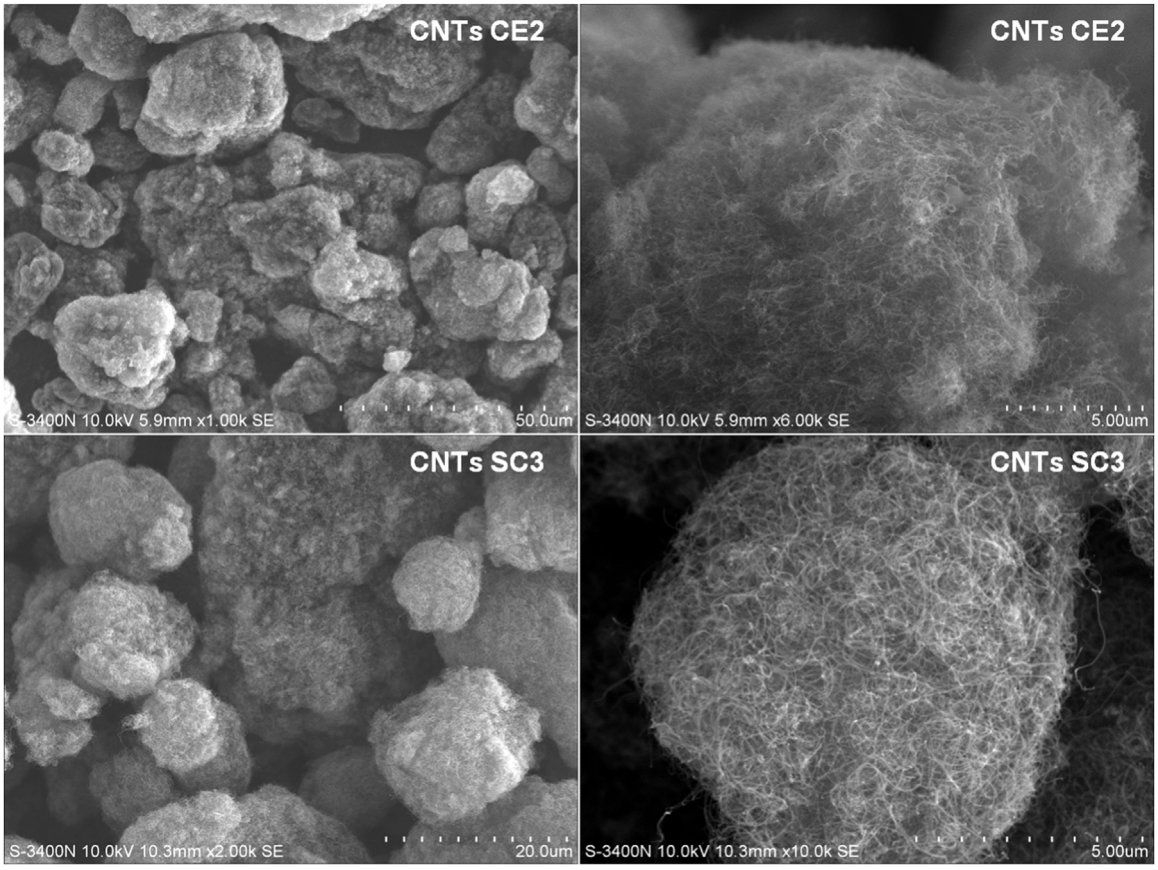

Fig. 6. SEM images of carbon nanotubes CNTs.

not observed in the case of nanotubes with well defined structure. This observation allows us to conclude that the appearance of these irregular cells was probably associated with the presence of small amounts of impurities in the CNFs samples as algae are very sensitive to heavy metals (Aruoja et al., 2009, 2015; Joonas et al., 2017).

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