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Carbon nanotubes enhanced the lead toxicity on the freshwater fish

D S T Martinez¹, O L Alves¹ and E Barbieri²

¹Solid State Chemistry Laboratory (LQES), Institute of Chemistry, University of Campinas – UNICAMP, P. O. Box 6154, 13083-970, Campinas-SP, Brazil.

²Instituto de Pesca, APTA – SAA/SP, P.O. Box 61, 11990-000, Cananéia-SP, Brazil.

E-mail: diegostefani.br@gmail.com, oalves@iqm.unicamp.br,
edisionbarbieri@yahoo.com.br

Abstract. Carbon nanotubes are promising nanostructures for many applications in materials industry and biotechnology. However, it is mandatory to evaluate their toxicity and environmental implications. We evaluated nitric acid treated multiwalled carbon nanotubes (HNO₃-MWCNT) toxicity in Nile tilapia (*Oreochromis niloticus*) and also the lead (Pb) toxicity modulation after the nanotube interaction. Industrial grade multiwalled carbon nanotubes [Ctube 100, CNT Co. Ltd] were treated with 9M HNO₃ for 12h at 150°C to generate oxygenated groups on the nanotube surface, to improve water dispersion and heavy metal interaction. The HNO₃-treated multiwalled carbon nanotubes were physico-chemically characterized by several techniques [e.g. TEM, FE-SEM, TGA, ζ-potential and Raman spectroscopy]. HNO₃-MWCNT did not show toxicity on Nile tilapia when the concentration ranged from 0.1 to 3.0 mg/L, and the maximum exposure time was 96h. After 24, 48, 72 and 96h the LC50 values of Pb were 1.65, 1.32, 1.10 and 0.99 mg/L, respectively. To evaluate the Pb-nanotube interaction influence on the ecotoxicity, we submitted the Nile tilapia to different concentrations of Pb mixed with a non-toxic concentration of HNO₃-MWCNT (1.0 mg/L). After 24, 48, 72, 96 h the LC50 values of Pb plus nanotubes were: 0.32, 0.25, 0.20, 0.18 mg/L, respectively. These values showed a synergistic effect after Pb-nanotube interaction since Pb toxicity increased over five times. X-ray energy dispersive spectroscopy (EDS) was used to confirm lead adsorption on the carbon nanotube oxidized surface. The exposure of Nile tilapia to Pb plus HNO₃-MWCNT caused both oxygen consumption and ammonium excretion decrease, when compared to the control. Finally, our results show that carbon nanotubes interact with classical pollutants drawing attention to the environmental implications.

1. Introduction

Safe development of nanotechnology depends on the ability to control both health and environmental risks [1].

Carbon nanotubes (CNTs) are among the most promising materials for nanotechnology and their applications demand chemical treatments (e.g. purification and oxidation) of the raw samples. Nitric acid treatment is one of the most used methods for achieving this goal [2]. The surface of CNTs is oxidized during this process, which generates oxygen functional groups such as –OH, –C=O, and –

COOH [3]. Environmental applications have explored these oxygenated surface groups on nanotubes and their large surface area to remove and monitor polar and non polar pollutants in the environment (e.g. PHAs, dioxins, pesticides and heavy metals) [4].

Lead (Pb) is a cumulative and insidious environmental pollutant that induces a broad range of physiological, biochemical and behavioral dysfunctions in living organisms [5]. With the increasing aquatic environment contamination, the potential interaction of Pb with nanomaterials needs to be considered. The CNTs industry is growing very fast and industrial production has already reached several tons a year. Therefore, the investigation of the interaction of classical pollutants with nanotubes is an important issue and requires more attention [6].

Freshwater fish is an important source of protein and lipids to human beings and animals. Nile tilapia (*Oreochromis niloticus*) is not only a very nutritious food, but also an important indicator of environmental contamination and water quality [7,8,9].

We evaluated the nitric acid treated multiwalled carbon nanotubes (HNO₃-MWCNT) ecotoxicity on the Nile tilapia as well as lead (Pb) toxicity modulation after nanotube interaction. This work aims to contribute to the development of a safe nanocarbon-based technology.

2. Experimental

Commercially available chemical vapour deposition (CVD) grown multiwall carbon nanotubes (MWCNT) [*Ctube 100*, CNT Co. Ltd., Incheon – South Korea] were used. PbCl₂ (analytical grade) was obtained from Reagen Ltd and used without further purification.

2.1.1. *Nitric acid treatment of multiwalled carbon nanotubes.* The raw MWCNT (1.0 g) was refluxed in HNO₃ 9.0 mol/L (200 mL) for 12 h at 150 °C. After cooling to room temperature, they were filtered through a 0.2 µm PTFE membrane and washed with deionized water until neutral pH of the filtrate was reached. The HNO₃-MWCNT sample was dried in a vacuum system for 24 h.

2.1.2. *Characterization of HNO₃-MWCNT sample.* HNO₃-treated multiwalled carbon nanotubes were physico-chemically characterized by using the following techniques: a) Transmission electronic microscopy [TEM – Zeiss], b) Field emission scanning electronic microscopy [FEG-SEM – FEI], c) thermogravimetric analysis [TGA, SDTQ600 TA Instruments], d) ζ-potential [Malvern Instruments], e) Raman spectroscopy [WiTech].

2.1.3. *HNO₃-MWCNT stock-solution preparation.* 100 mg of HNO₃-MWCNT were dispersed in 200 mL of deionized water and sonicated for 60 min in an ultrasound bath [Cole-parmer 8891].

2.1.4. *Acute ecotoxicity experiments.* The acute toxicity of HNO₃-MWCNT and Pb was evaluated in Nile tilapia (*Oreochromis niloticus*) juveniles cultivated in the laboratory of Fisheries Institute (Cananéia, Sao Paulo state, Brazil). They were exposed to different concentrations of these chemicals for a period of up to 96 h. Groups of 15 individuals were exposed to each of the following concentrations of HNO₃-MWCNT: 0.1, 0.5, 1.0, 2.0, 3.0 and Pb: 0.00, 0.04, 0.4, 0.8, 1.12 and 2.12 mg/L, respectively. After this procedure, we submitted the juveniles to concentrations of 0.04, 0.4, 0.8, 1.12, and 2.12 mg/L Pb mixed with a non-toxic concentration of HNO₃-MWCNT (1.0 mg/L). Prior to this, nanotubes were maintained in contact with Pb for 1 h. Dead fishes were removed from the tanks and counted at 24, 48, 72, and 96 h of exposure. The lethal concentration (LC50 with 95% confidence limits) was calculated by Probit analysis. These experiments were performed in triplicates using glass tanks of 50 L.

2.1.5. *Oxygen consumption and Ammonia excretion measurements.* The respirometer was made in our laboratory, with an acrylic tube and PVC covers. Ten fish were subjected, individually, to oxygen

consumption and ammonia excretion measurements in the presence of HNO₃-MWCNT (1.0 mg/L), Pb (1.0 mg/L) and the mixture of nanotubes and Pb (1:1 ratio). Before beginning the experiments, the animals were maintained in the respirometer with continuous water circulation for at least 90 min to attenuate handling stress. The difference between the oxygen and ammonia concentrations determined at the beginning and the end of the confinement was used to calculate consumption and excretion after three hours. Dissolved oxygen was determined according to Winkler Method (Winkler, 1888). Determination of ammonium-nitrogen in the freshwater was based on the phenolhypochlorite method (Solarzano, 1969). Nile tilapia juveniles oxygen-specific consumption and ammonium excretion average were analyzed by using the Tukey's multiple comparisons test (P<0.05).

3. Results and Discussion

Due to the economical and ecological importance of freshwater fish and the problems related to water pollution, we evaluated the effects of oxidized multiwalled carbon nanotubes and Pb interaction on Nile tilapia juveniles. Industrial grade multiwalled carbon nanotubes were oxidized with nitric acid, aiming to generate oxygenated groups on the nanotube surface as well as to improve their water dispersion. Fig. 1 shows the nanotubes transmission electronic microscopy (TEM) and scanning electronic microscopy (FEG-SEM) representative images, after the oxidation process with nitric acid (9.0 mol/L) at 150 °C for 12 hours under conventional reflux system. Table 1 shows a summary of the main physico-chemical properties of HNO₃-MWCNT.

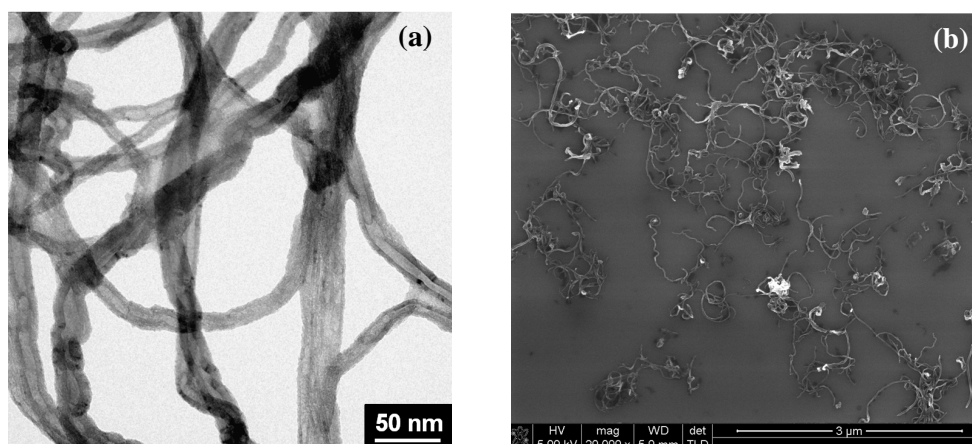


Fig. 1. (a) Transmission electronic microscopy [TEM] and (b) scanning electronic microscopy [FEG-SEM] of acid treated multiwalled carbon nanotubes (HNO₃-MWCNT).

Table 1. Summary of the physico-chemical properties of HNO₃-treated MWCNT used in this work.

Physico-chemical properties	HNO ₃ -MWCNT	Technique used
Diameter (nm)	10 ~ 40	TEM
Length (μm)	< 5	FEG-SEM
Surface area (m ² /g)	264	BET method
Surface charge (mV)	-27	Zeta-potential
Oxidation temperature (°C)	587	TGA
Iron oxide metallic residue (%)	< 2.0	Analytical microbalance
Defects (I _D /I _G ratio)	1.02	Raman

The effects of HNO₃-MWCNT sample (from 0.1 until 3.0 mg/L) were evaluated according to the acute toxicity assay for Nile tilapia (24, 48, 72 and 96 h). No deaths were observed (data not shown). However, the higher the concentration of metal the fish were exposed to, the higher the observed mortality. After being exposed to Pb, death was first observed at a concentration of 1.12 mg Pb/L in the first 24 h. Mortality rate of 100% was observed after a 24h exposure at concentrations of 2.12 mg Pb/L and after 72 h at a concentration of 1.12 mg Pb/L. The acute toxicity of Pb to Nile tilapia exposed to different concentrations of this metal, expressed as the medium lethal concentration (LC50), were 1.64, 1.32, 1.10 and 0.98 mg/L for 24, 48, 72, and 96h exposure, respectively. Furthermore, we exposed the Nile tilapia to different concentrations of Pb mixed with a non-toxic concentration of HNO₃-MWCNT (1.0 mg/L) to evaluate the influence of Pb-nanotube interaction on the ecotoxicity. After 24, 48, 72, 96 h the LC50 values of Pb plus nanotubes were: 0.32, 0.25, 0.20, 0.18 mg/L, respectively. It was observed an increase over five times of Pb toxicity on the freshwater fish, which demonstrates that there is a synergistic effect after Pb-nanotube interaction (Fig. 2).

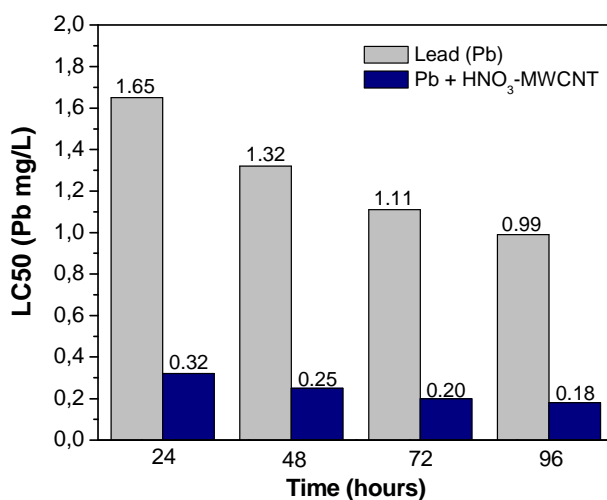


Fig. 2. Modulation of the LC50 toxicity value of lead (Pb) in the freshwater fish, Nile tilapia (*Oreochromis niloticus*) after the interaction with nitric acid treated multiwalled carbon nanotubes (HNO₃-MWCNT). 1.0 mg/L of nanotubes were used during this experiment, a non lethal dose for freshwater fish.

Xu et al. 2011 [10] investigated acute toxicity and synergism of four antifouling biocides (e.g. Irgarol 1051, dichlofluanid, tolylfluanid and Sea-Nine 211) and five heavy metals (e.g. Ni, Pb, Zn, Cd and Cu) using sea urchin embryos (*Glyptocidaris crenularis*). This study reported synergistic effects in almost all 25 mixtures. We also found a synergism effect when we used a mixture of Pb and oxidized multiwalled carbon nanotubes for Nile tilapia.

In order to get further insights into the adsorption of lead on the nanotubes, we performed analysis of HNO₃-MWCNT after incubation in freshwater containing 1.0 mg/L of Pb by using X-ray energy dispersive spectroscopy (EDS) (Fig. 3). We can observe that after the interaction with freshwater containing Pb the nanotubes presented this element on their composition as well as Mg and Ca. Similar results have been reported in the literature [11]. The proposed mechanism to this chemical interaction is the electrostatic force between the lead ions (e.g. Pb²⁺, positively charged) and the oxygenated groups on the nanotube surface (e.g. -COOH, negatively charged) [12]. However, colloidal chemistry aspects (e.g. adsorption/desorption and aggregation/agglomeration) on the toxicity assessment of this mixture (Pb + nanotubes) should be studied in more detail.

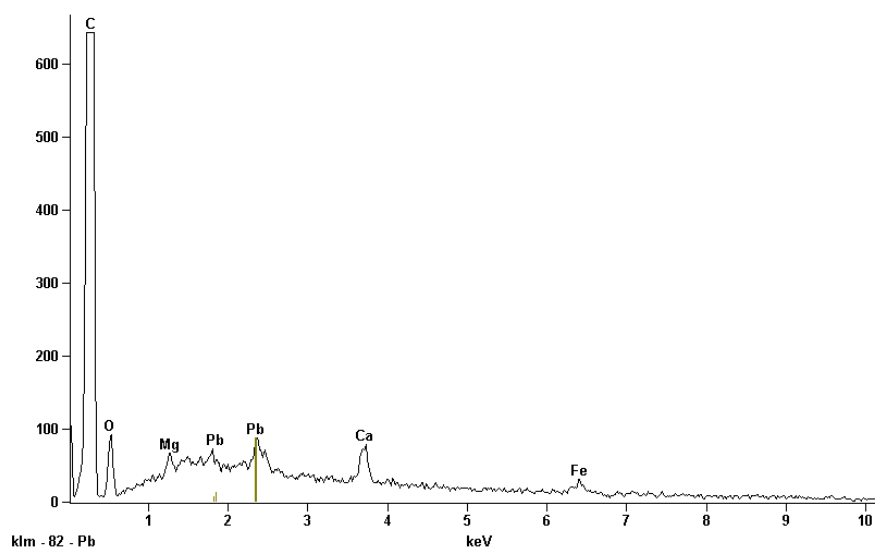


Fig. 3. X-ray energy dispersive spectroscopy (EDS) of HNO₃-MWCNT after incubation in freshwater containing of 1 mg/L of Pb.

Oxygen consumption and ammonia excretion measurements provide a good indicator of animal stress and water quality [13]. We evaluated the effects of Pb, HNO₃-MWCNT and mixture (Pb + HNO₃-MWCNT) at a non lethal concentration (1.0 mg/L) on these physiological parameters after three hours of exposure.

The specific oxygen consumption decreased in the presence of Pb, carbon nanotubes and the mixture of Pb-nanotubes treatments. The average of specific oxygen consumption of fish exposed to Pb, HNO₃-MWCNT and mixture (Pb + HNO₃-MWCNT) at a fixed concentration (1.0 mg/L) were 0.32, 0.52, 0.05 mL oxygen/g/min, respectively. These values represent a metabolic level decrease of 74%, 58% and 95% when compared to the control (Fig. 4).

Using the Tukey ($p < 0.05$) statistical test, it was verified that the averages of the specific ammonia excretion in the groups treated with nanotubes and Pb were not different when compared to the control (Fig. 5). However, the ammonia excretion decreased considering the mixture (Pb + HNO₃-MWCNT). Thus, we found a clear synergistic effect when the mixture was evaluated. Our future work will focus on both the acute effects of these nanotubes on Nile tilapia at other biological levels such as histological and biochemical, and chronic effects on metabolism, molting and growth rates, which are also very important for the toxicological effects.

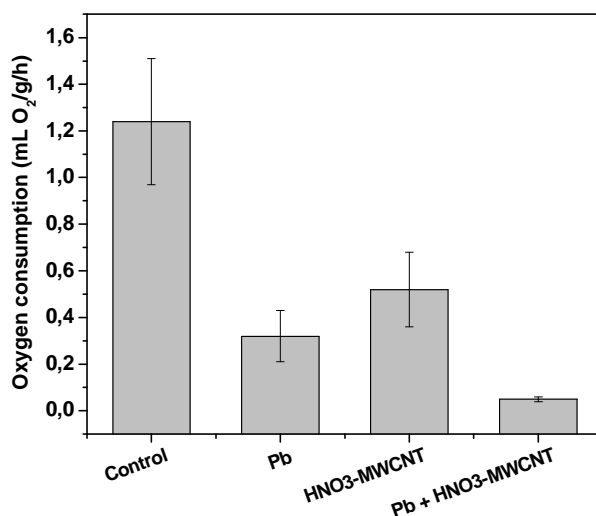


Fig. 4. Average of oxygen consumption in the freshwater fish, Nile tilapia (*Oreochromis niloticus*) after exposure to nitric acid treated multiwalled carbon nanotubes (HNO₃-MWCNT), Pb and mixture (Pb + HNO₃-MWCNT).

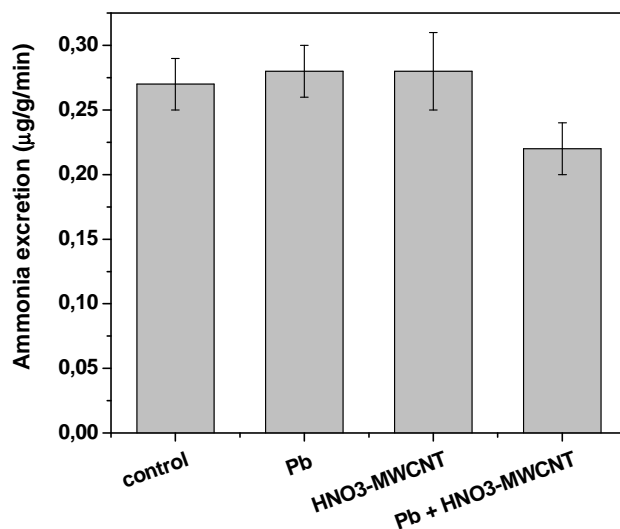


Fig. 5. Average of ammonia excretion in the freshwater fish, Nile tilapia (*Oreochromis niloticus*) after the exposure with nitric acid treated multiwalled carbon nanotubes (HNO₃-MWCNT), Pb and mixture (Pb + HNO₃-MWCNT).

4. Conclusion

The oxidized multiwall carbon nanotubes (HNO₃-MWCNT) enhanced the lead acute toxicity on the freshwater fish Nile tilapia (*Oreochromis niloticus*). An increase over five times on the LC₅₀ value of lead after the nanotube interaction was observed. The carbon nanotubes studied did not show acute toxicity on the freshwater fish up to 3.0 mg/L. Furthermore, the nanotubes decreased the oxygen consumption and ammonia excretion after lead interaction. Since the industrial production of carbon nanotubes has already reached tons per year, this work draw attention for the implications of carbon nanomaterials released in the aquatic environment and their interaction with classical pollutants.

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