Chinese Journal of Oceanology and Limnology
Vol. 31 No. 2, P. 306-311, 2013
http://dx.doi.org/10.1007/s00343-013-2131-1

Toxicity of single-walled carbon nanotubes on green microalga *Chromochloris zofingiensis**

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Received Jun. 18, 2012; accepted in principle Jul. 31, 2012; accepted for publication Nov. 15, 2012

Abstract Nanoparticles, or particles in size of 1–100 nm, are extensively used in the world in different applications. For instance, single-walled carbon nanotubes (SWCNTs) are commonly used in consumer products, such as biosensors, drug and vaccine delivery transporters, and novel biomaterials. Although nanoparticles do not cause safety concerns to consumers who use nanoparticle-containing products, these small particles are potentially harmful for workers who produce them in factories or in cases of discharge to aquatic ecosystems. SWCNTs do not have a natural analogue, so the effects on health of their disposal remain largely unknown. In this study, we evaluated the effects of SWCNTs on a population of the green microalga *Chromochloris zofingiensis* and the profile and production of pigments and fatty acids. The alga was incubated with SWCNTs for 6 days in 0 (control), 40, 80, 160, or 320 mg/L concentrations. SWCNTs showed both positive and negative effects on the growth of *C. zofingiensis*, with a biomass enhancement at low levels (40–160 mg/L) but inhibition at high levels (320 mg/L). By contrast, a decreased accumulation of fatty acids and pigments of *C. zofingiensis* was observed over the range of the tested concentrations. These results indicate that the markers on the inhibitive toxicity of SWCNTs are increasingly sensitive in the following order: biomass and fatty acids < primary carotenoids < chlorophylls < secondary carotenoids. *C. zofingiensis* is a suitable microalga for evaluating the ecotoxicological hazards of SWCNTs, especially in terms of pigmentation response.

Keyword: carbon nanotubes; pigmentation; cell growth; *Chromochloris zofingiensis*

1 INTRODUCTION

The use of nanoparticles, or particles in size of 1–100 nm, has been increasing worldwide. Nanotechnology is an innovation that has great potential to enhance the quality of a wide variety of industrial products and services (Alvarez et al., 2009). Nanomaterials exhibit unique size- and structure-dependent thermal, optical, magnetic, electronic, chemical, and mechanical properties (Salvetat et al., 1999). Therefore, these materials possess potential radar, optic, electronic, and material science applications. Single-walled carbon nanotubes (SWCNTs) are one of the most widely used nanomaterials in consumer products, such as biosensors, drug and vaccine delivery transporters, and novel biomaterials (Lekas, 2005). The annual worldwide production of SWCNTs is estimated to be over 1 000 tons (Lekas, 2005).

SWCNTs do not have a natural analogue; therefore, the effects on health of their disposal remain largely unknown (Klaine et al., 2008; Rogers et al., 2010). With increasing interest in industrial production and commercial goods, the raw materials of SWCNTs will inevitably contaminate workplaces, either accidentally through spillage or deliberately through discharge. Although nanoparticles do not cause safety concerns to consumers who purchase nanoparticle-containing products, these small particles are potentially harmful for workers who produce them in factories or in cases of discharged to aquatic ecosystems. Nanoparticles are said to behave like

* Supported by the National Natural Science Foundation of China (No. 50904051), the Science and Technology Planning Project of Yantai, China (No. 2010247), and the Open Fund of Shandong Oriental Ocean Sci-Tech Co., Ltd. (No. 200803)
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asbestos fibers, which cause mesothelioma. In addition, nanotubes can cross membrane barriers, and if raw materials reach the organs, they can induce harmful effects, such as inflammatory and fibrotic reactions (Warheit et al., 2004).

Organic matter can enhance the mobility of SWCNTs in water. Therefore, released raw materials of SWCNTs can be easily transported to large aquatic environments (Hyung et al., 2007; Wang et al., 2008). In aquatic ecosystems, microalgae serve as food for organisms at high trophic levels and as an oxygen source for respiration (Lebeau et al., 2006). Microalgae are fundamental constituents of food chains in almost all aquatic ecosystems. Therefore, any sensitivity to toxic SWCNTs is likely to be important to aquatic ecosystem dynamics. *Chromochloris zofingiensis* has been used as a model microalga in numerous studies, such as those that focus on pigments (Bar et al., 1995; Ip et al., 2004; Ip and Chen, 2005). Although the relevance of *C. zofingiensis* to aquatic ecosystems may be somewhat limited, it is a commonly used microalga in evaluating the ecotoxicological hazards of SWCNTs, especially in terms of pigmentation response. *C. zofingiensis* was tested in this study for the toxic effects of SWCNTs. The population of *C. zofingiensis* and profile and production of pigments and fatty acids were used to assess the toxic effects of SWCNTs to obtain an improved understanding of the potential toxicity risks of SWCNTs in aquatic environments.

2 MATERIAL AND METHOD

2.1 Microalga and growth conditions

Green microalgae *Chromochloris zofingiensis* (ATCC 30412) were maintained on an agar slant containing Kuhl's medium (Kuhl and Lorenzen, 1964) at 4°C. Cells from slants were inoculated with liquid Kuhl's medium, and the alga was grown in flasks at 26±1°C with continual fluorescence light of 150 μmol/(m^2·s) at the flask surface and orbital shaking at 150 r/min. The cells in the linear growth phase were used as inoculum. An inoculum of 10% (by volume, average cell concentration of 0.2 g/L) was inoculated into each 250-mL Erlenmeyer flask containing 100 mL Kuhl's medium supplemented with 30 g/L of glucose as a carbon source. All media were adjusted to pH 6.5 and then autoclaved at 121°C for 20 min. The algal cells were incubated with a range of SWCNT concentrations (40–320 mg/L). A culture without any SWCNTs was used as a control. Kinetics of cell dry biomass, fatty acid, and pigment profiles in these treatments were monitored for 6 d.

2.2 Determination of algal dry biomass

For cell dry biomass (DW, g/L) determinations, 5-mL aliquots of the cell culture were filtered through a pre-dried Whatman GF/C paper and washed three times. The filters containing cells were dried for 24 h until a constant weight.

2.3 Pigments analysis

Total pigments were extracted with acetone following the method by Ip et al. (2004). Twenty microliters per extract was separated by HPLC on a Waters Spherisorb® 5 μm ODS2 4.6 mm×250 mm analytical column according to the method described by Baroli et al. (2003). The entire process was performed in darkness.

2.4 Fatty acids analysis

Fatty acid methyl esters (FAMEs) were prepared by acid transesterification (Christie, 2003). Briefly, lyophilized cells were incubated with a solvent mixture of toluene and 1% sulfuric acid in methanol (1:2, v/v) overnight at 50°C to produce FAMEs, which were then extracted with hexane. The FAMEs were analyzed by using an Agilent 7890 capillary gas chromatography equipped with a flame ionization detector (FID) and a DB-23 capillary column (30 m×0.25 mm×0.25 μm). Nitrogen was used as a carrier gas. The temperature programming consisted of 5 min at 140°C and a subsequent increase to 240°C at 2.5°C/min. The injector was kept at 250°C with an injection volume of 1 μL under split mode (5:1). The FID temperature was set at 260°C. FAMEs were identified by chromatographic comparison with analytical standards (Sigma). The quantities of the individual FAMEs were estimated from the peak areas on the chromatogram using nonadecanoic acid (Sigma, St. Louis, MO, USA) as the internal standard.

2.5 Calculation of degree of fatty acids unsaturation

The degree of fatty acids unsaturation (∇/mol) was calculated according to Chen and Johns (1991) as follows:

∇/mol=[1.0(% monoene)+2.0(% diene)+3.0(% triene)]/100.

Table 1 Fatty acid profile and quantification of *C. zofingiensis* incubated with SWCNTs (0 mg/L to 320 mg/L) after 6 days

Fatty acid compositions	Fatty acid content (mg/g)				
	SWCNTs (0 mg/L)	SWCNTs (40 mg/L)	SWCNTs(80 mg/L)	SWCNTs (160 mg/L)	SWCNTs (320 mg/L)
Myristate (C14:0)	0.48 ± 0.02	0.40 ± 0.02*	0.34 ± 0.01**	0.32 ± 0.01**	0.32 ± 0.01**
Palmitate (C16:0)	43.42 ± 1.10	34.30 ± 0.90**	34.76 ± 1.00**	34.06 ± 1.00**	32.89 ± 1.30**
Palmitoleate (C16:1)	3.55 ± 0.10	2.83 ± 0.10**	2.78 ± 0.10**	2.92 ± 0.10*	4.27 ± 0.20*
Hexadecadienoic acid (C16:2)	9.89 ± 0.30	8.01 ± 0.30*	8.27 ± 0.20*	8.24 ± 0.30*	5.66 ± 0.20**
Hexadecatrienoic acid (C16:3)	3.15 ± 0.10	2.33 ± 0.10**	2.20 ± 0.10**	2.04 ± 0.10**	0.80 ± 0.04**
Stearate (C18:0)	10.66 ± 0.50	8.43 ± 0.30*	7.46 ± 0.20**	7.02 ± 0.20**	5.13 ± 0.20**
Oleate (C18:1)	113.75 ± 2.80	97.80 ± 2.40*	103.13 ± 2.60*	107.69 ± 3.00	141.04 ± 3.10**
Linoleate (C18:2)	38.99 ± 1.00	27.06 ± 0.90**	26.64 ± 0.90**	23.71 ± 0.60**	15.72 ± 0.40**
Linolenate (C18:3)	20.78 ± 0.50	16.77 ± 0.50**	15.96 ± 0.40**	15.30 ± 0.40**	11.00 ± 0.20**
Total fatty acids	244.68 ± 8.60	197.92 ± 7.10*	201.53 ± 5.90*	201.30 ± 6.00*	216.83 ± 7.20*
Degree of fatty acids unsaturation (∇/mol)	1.17 ± 0.04	1.15 ± 0.04	1.14 ± 0.03	1.13 ± 0.04	1.03 ± 0.03*

Data are mean values ± SD of three independent measurements. *: Significant and **: highly significant for the same fatty acid compared with the control group at $P<0.001$.

2.6 Statistical analysis

All treatments were performed in triplicate. Means and standard deviations were calculated for each treatment. One-way analysis of variance (ANOVA) was used for statistical analysis, and all tests were considered statistically significant at $P<0.05$ or highly significant at $P<0.001$.

3 RESULT

3.1 Cell growth

Figure 1 shows the kinetics of cell dry biomass in *C. zofingiensis* incubated with a range of SWCNT concentrations (0–320 mg/L). In the control culture without supplemented SWCNTs, the cell dry biomass increased from 0.20 g/L to a maximum of 10.10 g/L on Day 4. Low levels of SWCNTs (40–160 mg/L) improved the production of cell dry biomass to different extents. With reference to the control group (100%), for instance, the culture exposed to 80 mg/L of SWCNTs increased biomass to 119% (12.50 g/L). However, a high-level SWCNTs (320 mg/L) resulted in cell growth inhibition, which was manifested by a lower production of dry biomass and a longer time to reach the maximum compared with that of the control group.

3.2 Fatty acid profile and quantification

Table 1 shows the variations in fatty acid profile and quantification of *C. zofingiensis* incubated with a range of SWCNT concentrations (0 mg/L to 320 mg/L). *C. zofingiensis* showed similar qualitative but different quantitative compositions of fatty acids in different SWCNT treatments. The fatty acids were C14:0, C16:0, C16:1, C16:2, C16:3, C18:0, C18:1, C18:2, and C18:3 in all treatments. The SWCNT concentrations increased as the contents of C16:1 and C18:1 increased, whereas other types of fatty acid decreased. In the 320 mg/L SWCNT-incubated culture, for instance, the compositional changes were as follows: C14:0 decreased from 0.48 mg/g to

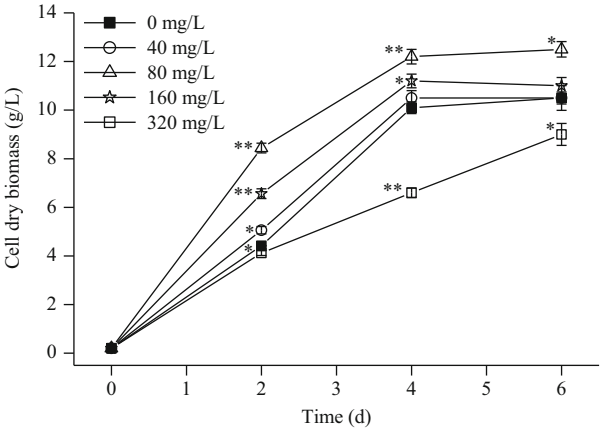

Fig.1 Kinetics of cell dry biomass in *C. zofingiensis* incubated with a range of SWCNT concentrations (0 mg/L to 320 mg/L)

Data are mean values ± SD of three independent measurements. The comparison was performed over one time period. * indicates significant and ** indicates highly significant compared to control group at $P<0.001$.

Table 2 Pigment profile and quantification of *C. zofingiensis* incubated with SWCNTs (0 mg/L to 320 mg/L) after 6 days

Pigment compositions	Pigment content (mg/g)				
	SWCNTs (0 mg/L)	SWCNTs (40 mg/L)	SWCNTs (80 mg/L)	SWCNTs (160 mg/L)	SWCNTs (320 mg/L)
Chlorophylls	1.66 ± 0.08	1.45 ± 0.07*	1.53 ± 0.05	1.32 ± 0.06*	1.10 ± 0.05**
Chlorophyll *a*	1.22 ± 0.03	1.10 ± 0.03*	1.21 ± 0.03	1.03 ± 0.02**	0.80 ± 0.02**
Chlorophyll *b*	0.43 ± 0.01	0.35 ± 0.01**	0.32 ± 0.01**	0.29 ± 0.00**	0.30 ± 0.01**
Primary carotenoids	0.32 ± 0.01	0.26 ± 0.01*	0.29 ± 0.01*	0.23 ± 0.01**	0.24 ± 0.01**
β-Carotene	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.01	0.04 ± 0.00
Lutein	0.19 ± 0.00	0.15 ± 0.01*	0.15 ± 0.00**	0.13 ± 0.00**	0.14 ± 0.01**
Zeaxanthin	0.07 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
Secondary carotenoids	1.60 ± 0.04	1.59 ± 0.05	1.56 ± 0.05	1.45 ± 0.04*	0.52 ± 0.02**
Astaxanthin	1.03 ± 0.03	0.97 ± 0.02*	0.91 ± 0.02*	0.85 ± 0.03*	0.17 ± 0.01**
Adonixanthin	0.48 ± 0.01	0.48 ± 0.01	0.53 ± 0.02*	0.49 ± 0.02	0.33 ± 0.01**
Canthaxanthin	0.09 ± 0.00	0.13 ± 0.01*	0.13 ± 0.00**	0.12 ± 0.01*	0.02 ± 0.00**

Data are mean values ± SD of three independent measurements. *: Significant and **: highly significant of the same fatty acid compared with the control group at $P<0.001$.

Table 3 Accumulation of cell dry biomass, fatty acid and pigment in *C. zofingiensis* exposed to 320 mg/L of SWCNTs after 6 days

	Without SWCNTs	With SWCNTs (320 mg/L)
Cell dry biomass (g/L)	10.50 ± 0.51	9.00 ± 0.45
	100%	86%
Total fatty acids (mg/g)	244.68 ± 8.60	216.83 ± 7.20
	100%	89%
Primary carotenoids (mg/g)	0.32 ± 0.01	0.24 ± 0.01
	100%	75%
chlorophylls (mg/g)	1.66 ± 0.08	1.10 ± 0.05
	100%	66%
Secondary carotenoids (mg/g)	1.60 ± 0.04	0.52 ± 0.02
	100%	33%

Data are mean values ± SD of three independent measurements.

0.32 mg/g; C16:0 from 43.42 mg/g to 32.89 mg/g; C18:0 from 10.66 mg/g to 5.13 mg/g; C18:2 from 38.99 mg/g to 15.72 mg/g; and C18:3 from 20.78 mg/g to 11.00 mg/g. Finally, the contents of total fatty acids in the SWCNT treatments were less compared with that in the control group without supplemented SWCNTs. However, except for the 320 mg/L SWCNT-incubated culture, the degree of fatty acids unsaturation (1.13–1.17 ∇/mol) did not significantly vary among experimental groups.

3.3 Pigment profile and quantification

Table 2 shows both qualitative and quantitative compositions of chlorophylls and primary and secondary carotenoids in SWCNT-incubated *C. zofingiensis*. Secondary carotenoid and chlorophyll levels decreased gradually at low SWCNT concentrations from 160 mg/L downward, but these levels decreased steeply at a high concentration of 320 mg/L. For example, secondary carotenoid levels decreased from 1.59 mg/g at 40 mg/L of SWCNTs to 1.45 mg/g at 160 mg/L of SWCNTs, but secondary carotenoid levels declined to 0.52 mg/g at 320 mg/L of SWCNTs. Primary carotenoid levels decreased gradually over the entire concentration range of SWCNTs tested.

Chlorophyll comprises chlorophylls *a* and *b*. Chlorophyll *a* is more sensitive to the inhibitive effects of SWCNTs than chlorophyll *b*. With reference to the control group (100%), the culture supplemented with 320 mg/L of SWCNTs decreased chlorophyll *a* to 66% and chlorophyll *b* to 70%. Secondary carotenoids were astaxanthin, adonixanthin, and canthaxanthin, of which astaxanthin is the most sensitive pigment to the negative effects of SWCNTs. With reference to the control group (100%), the culture supplemented with 320 mg/L of SWCNTs decreased astaxanthin to 17%, canthaxanthin to 22%, and adonixanthin to 69%.

Collectively, unlike the two-sided effects of SWCNTs on cell growth, SWCNTs over the concentration range tested in this study showed no significant positive effects. Instead, they exhibited more or less negative effects on the accumulation of both fatty acids and pigments in *C. zofingiensis*. SWCNTs demonstrated strong inhibitive toxicity at a

high level of 320 mg/L. Table 3 indicates that in reference to the control group (100%), the culture supplemented with 320 mg/L of SWCNTs decreased cell dry biomass to 86%, total fatty acids to 89%, primary carotenoids to 75%, chlorophylls to 66%, and secondary carotenoids to 33%.

4 DISCUSSION

The toxicity of pollutants in higher plants and microalgae commonly acts in a concentration-dependent manner. However, microalgae are known to grow better in the presence of additives than when no additives are used. For example, microalgae that grow with bacteria as a co-culture or co-immobilization yield higher microalgal populations (Gonzalez and Bashan, 2000; de-Bashan et al., 2005). In this study, the SWCNTs showed both positive and negative effects on *C. zofingiensis* growth. SWCNTs at low levels (40–160 mg/L) could improve the production of cell dry biomass, whereas SWCNTs at high levels (320 mg/L) resulted in biomass inhibition. Similar two-sided (positive and negative) effects of other types of carbon nanotubes (CNTs) on cell growth have also been observed in higher plants. For example, Cañas et al. (2008) reported that the application of CNTs enhanced root elongation in onion and cucumber and inhibited root elongation in tomato. Khodakovskaya et al. (2009) observed that if the CNT concentration range was limited from 10–40 mg/L, no discernible toxic effects could be found on the root elongation or root development of tomato seedlings. The CNT concentration ranges that yield positive or negative effects vary across organisms in different species. Therefore, cell growth does not seem to be a definitive parameter to assess the side effects of pollutants, including nanomaterials, in microalgae.

Unlike the two-sided effects of SWCNTs on cell growth, SWCNTs over the concentration range tested in this study showed no significant positive effects. Instead, they demonstrated negative effects on the accumulation of both fatty acid and pigment in *C. zofingiensis*. The markers on the inhibitive toxicity of SWCNTs are increasingly sensitive in the following order: biomass and fatty acids < primary carotenoids < chlorophylls < secondary carotenoids (Table 3). Therefore, pigmentation, especially the accumulation of secondary carotenoids, is more sensitive than cell growth in assessing the side effects of SWCNTs on *C. zofingiensis*.

Studies on bacteria and mammalian cells suggest that oxidative stress might be a direct cause of cell inactivation during exposure to metal nanomaterials, including unrefined CNTs with residual metals that may lead to oxidative stress (Geslin et al., 2001; Ding et al., 2005; Manna et al., 2005; Sayes et al., 2006). However, these studies focused on the presence of residual metals within CNTs, which may lead to oxidative stress (Geslin et al., 2001). Recent research suggests that rather than oxidative stress, the physical interaction of carbon-based nanomaterials with cells is the primary mechanism for cell death (Ali et al., 2004; Tang et al., 2007). Not only oxidative stress but also associated bacteria generally improve carotenoid production in microalgae (de-Bashan et al., 2002; Ip and Chen, 2005). The toxicity of SWCNTs unexpectedly lowered secondary carotenoid synthesis in the microalga examined. Therefore, we conclude that pure CNTs, including SWCNTs, may exert a toxic effect by killing cells through different cytotoxic mechanisms associated with nanomaterials and CNTs with residual metals.

5 CONCLUSION

We tested *C. zofingiensis* to evaluate the toxic effects of SWCNTs. Our results suggest that exposure to SWCNTs at levels of 40–320 mg/L could affect the accumulation of biomass, fatty acid, and pigment in *C. zofingiensis*. The dry biomass exhibited a two-sided (positive and negative) response to SWCNTs, which was concentration-dependent. By contrast, fatty acid and pigment exhibited only a negative response to SWCNTs. We found secondary carotenoids to be the most sensitive parameter for the inhibitive toxicity of SWCNTs.

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