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ARTICLE

EVALUATION METHODS FOR EFFECTS OF NANOMATERIALS ON DIATOM GROWTH

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ABSTRACT

Background: *We proposed two evaluation methods to show the effects of nanomaterials on diatom cell growth. Firstly, a "diatom chip" using an adhesive diatom, Navicula sp., was prepared on a functionalized glass surface. Further, after culturing the diatom chip with sodium dodecyl sulfate (SDS) or carbon nanotubes (CNTs), a significant decrease in diatom cell division was observed. Secondly, a floating diatom, Melosira nummuloides, was used for assessing the cell growth. In this diatom, the increase in the number of cells was not counted; instead, cell growth was calculated by estimating the area covered by the cells in a Petri dish. By using this method, inhibition of cell division by single-walled nanotube (SWNT) suspension was observed. Thus, in the presence of a SWNT aqueous suspension, higher inhibition of cell division was observed than that in a SWNT ethanol suspension. Our results showed that both adhesive and floating diatoms could be used to evaluate the effects of nanomaterials on cell growth.*

KEY WORDS
diatom, carbon nanotube, toxicity, biochip

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INTRODUCTION

Carbon nanotubes (CNTs) are one of promising nanomaterials because of their sophisticated and robust nanostructures [1-3]. However, because the morphology of CNTs resembles that of asbestos, the safety of CNT use has become a subject of debate [4-10]. To evaluate the safety of CNTs, until now, dosage experiments using animals and animal cells have been intensively conducted [11-14]. Recent studies have shown methods for reducing the toxicity of CNTs [15]. On the other hand, such evaluation experiments on microorganisms, especially algae, have been initiated recently [16-23]. Green algae, in particular, have been frequently used to evaluate the effects of CNTs [24-26]. For example, Schwab et al. reported that the growths of *C. vulgaris* and *P. subcapitata* were inhibited in the presence of 1.8 mg and 20 mg CNT/L in well-dispersed suspensions with 50% half maximal effective concentrations (EC50) [25].
A diatom is one of the major photosynthetic planktons [27-29]. Diatom cells are sensitive to water quality, therefore water assessment methods using diatoms have been established [30-31]. However, we found only one study investigating the toxic effects of CNTs on diatoms [32][34][35]. This study used several types of diatom cells to evaluate the toxicity of double-walled carbon nanotubes (DWNTs).

In this paper, we studied the effects of CNTs on diatom growth. Two marine diatoms, *Navicula* sp. and *Melosira nummuloides*, were chosen because of their rapid growth rates. Several types of CNTs such as dispersed single-walled carbon nanotubes (SWNTs) and powder of multi-walled carbon nanotubes (MWNTs) were used.

MATERIALS AND METHODS

Materials: MWNT was purchased from Tokyo Chemical Industry Co. Ltd. (C2149, Tokyo, Japan). SWNT suspension was purchased from Meijo Nano Carbon Co. (FH-P, Aichi, Japan). Sodium dodecyl sulfate (SDS) and other chemicals used were of analytical grade.

Diatom cells: Isolated *Navicula* sp. cells were cultured from seawater using Daigo IMK culture medium (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) and sodium metasilicate (final concentration, 1 mM) in a Petri dish [33]. Isolated *M. nummuloides* cells were cultured from seawater using f/2 culture medium (G9903; Sigma-Aldrich Inc., St. Louis, MO).

Observations: In the case of *Navicula* sp., the previously reported two-dimensional culture method was employed [33]. A glass slide surface was functionalized using 3-aminopropyltriethoxysilane (APS). A drop of 100-µL diatom cell suspension was pipeted onto the functionalized glass surface. After a 1-h incubation period at 18 °C, the glass slide was rinsed using the culture medium to remove unadsorbed cells. A Petri dish was then filled with 35 mL of the culture medium. Finally, the SDS, or powder or suspension SWNT was added to the Petri dish. Sample culture was performed at 18 °C under fluorescent light. To count the number of cells, the samples were regularly observed by inverse optical microscopy. All experiments using the 3 cultures were replicated using 3 separate Petri dishes. In the case of *M. nummuloides* cells, 100 µL of cell suspension was added to a Petri dish containing 35 mL of the culture medium. Then, powder or suspension SWNT was added to the Petri dish. Sample culture was performed at 18 °C under fluorescent light. The sample surface was regularly observed using an optical microscopy, and the amount of the diatom cells was estimated using ImageJ analysis and reported as the coating ratio. All experiments using the 3 cultures were replicated using 3 separate Petri disheses.

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RESULTS

[Fig. 1] shows scanning electron microscopy (SEM) images of the frustules of *Navicula* sp. and M. *nummuloides*. Each species was isolated and prepared by passage culture prior to the dosage experiments.

[Fig. 2] shows the schematic view of the sample preparation for *Navicula* sp., which is an adhesive diatom. Thus, we could attach *Navicula* cells onto the functionalized glass surface, after which, the increase in cell numbers was observed in the presence and absence of CNT. For this, 100-μL cell suspension was placed as a drop onto the surface of a glass slide functionalized using APS. Before the previously shown step, the cell suspension was gently shaken for 30 min to obtain uniform distribution of the cells. After 1-h incubation, the sample was rinsed with water. By rinsing, only a few cells remained on the glass surface, thus, it could be referred to as a "diatom chip."

To evaluate the effects of SDS and CNT on diatom cell growth, 3 pieces of the prepared diatom chips were placed in a Petri dish containing 35 mL of culture medium. Concentrations of either SDS or CNT were added to the dish [Fig 3a]. [Fig. 3b] shows a typical image of diatom cells on the glass surface as observed using an optical microscope. The majority of diatom cells grew two-dimensionally, which enabled precise counting of mature cells.

[Fig. 4] shows the effects of SDS on *Navicula* sp. growth. SDS was selected as a test compound, because of it being a typical surfactant that is widely used in laboratories and at home. The data clearly showed that diatom cell growth was inhibited in the presence of SDS. Further, 0.01% SDS was particularly effective at inhibiting diatom growth, which was observed when the cells on the glass surface became white. Typically, living and dead cells are identified based on their colors, namely, brown and white, respectively.
There were several advantages to the toxicity evaluation method in this study. The diatoms used in the experiment, for instance, matured rapidly and the results were quickly obtained over the course of 1 week. Furthermore, because culturing was easy and costless, averaged data of many samples could be obtained. Figure 5 shows the effects of powder MWNT on *Navicula* sp. growth. When 1 mg of MWNT was added to the sample, increase in cell numbers was clearly inhibited. However, the inhibition effect remained constant even after increasing the amount of powder MWNT to 10 mg. It is possible that the inhibition effect of the CNT powder was limited because the powder was insoluble in the culture medium. Most of the MWNT was precipitated in the Petri dish.

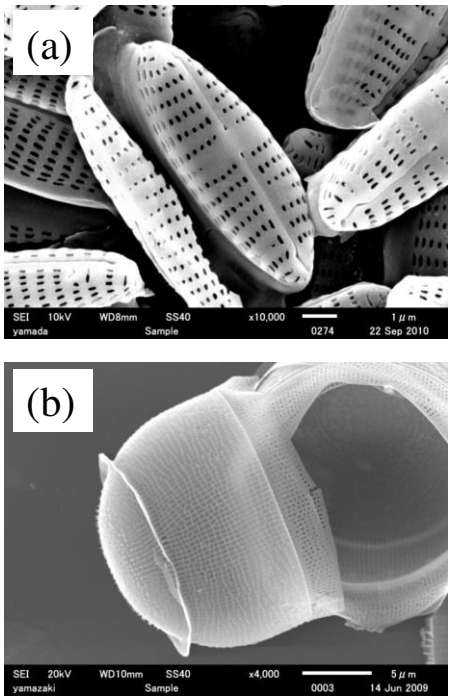

Fig. 1: Scanning electron microscopy (SEM) images of diatom frustules. (a) *Navicula* sp.; scale bar, 1 μm. (b) *Melosira nummuloides*; scale bar, 5 μm.

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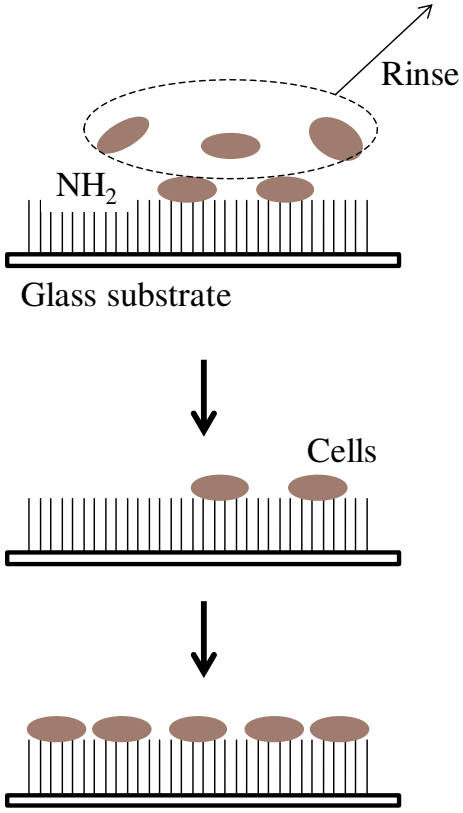

Fig. 2: Schematic view of the preparation procedure of a "diatom chip." Cell suspension was placed as drop onto the surface of a functionalized glass slide. After a 1-h incubation period, unadsorbed cells were removed by rinsing. Thus, diatom cells were cultured on the functionalized glass surface.

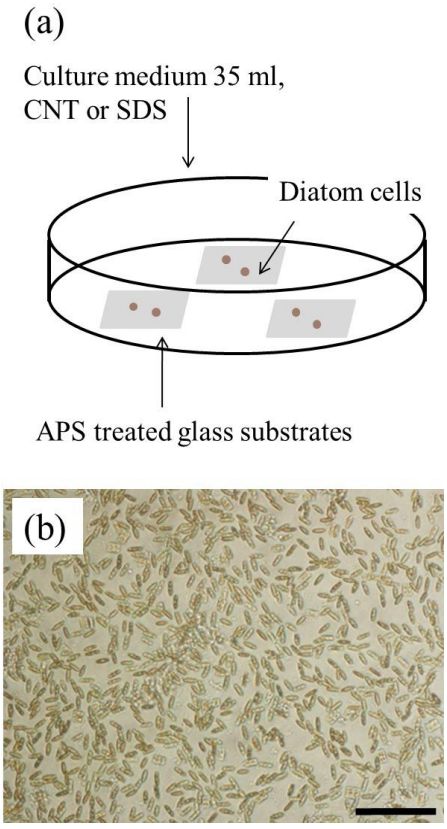

Fig. 3: Evaluation procedure using a "diatom chip" (*Navicula* sp.). (a) Three chips were placed in a Petri dish and increase in the cell numbers was observed using an optical microscope. (b) A typical image of diatom cells cultured on a glass surface; the scale bar is 1 mm.

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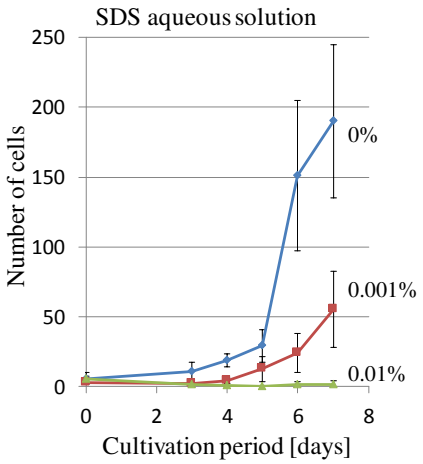

Fig. 4: Effects of sodium dodecyl sulfate (SDS) aqueous solution on *Navicula* sp. growth. Diamond, square, and triangle markers represent 0%, 0.001%, and 0.01% SDS concentrations, respectively.

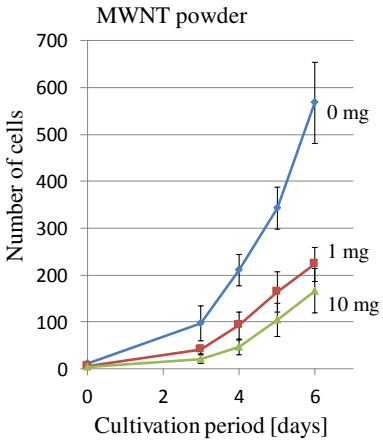

Fig. 5: Effects of multi-walled carbon nanotube (MWNT) powder on *Navicula* sp. growth. Diamond, square, and triangle markers represent 0 mg, 1 mg, and 10 mg of MWNT, respectively.

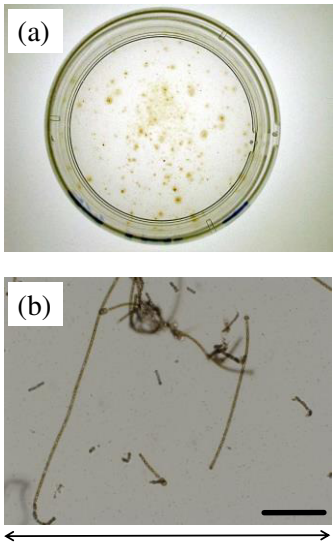

Fig. 6: *Melosira nummuloides* cells cultured in a Petri dish. (a) A photograph of a Petri dish during the cultivation. Brown objects in the dish show the diatom cells. Diameter of the Petri dish was 90 mm. (b) A typical image of diatom cells cultured in the Petri dish; the scale bar is 1 mm.

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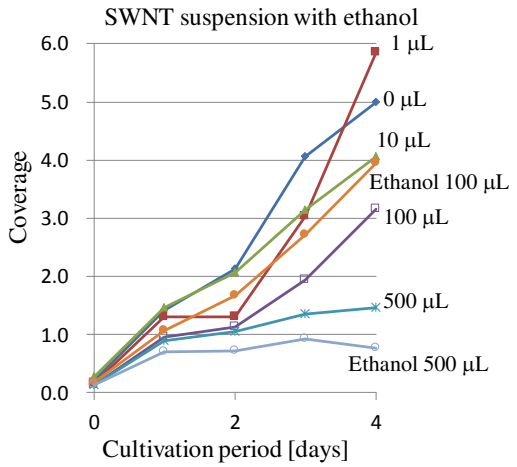

Fig. 7: Effects of single-walled nanotube (SWNT)-ethanol suspension (FH-P) on *Melosira nummuloides* growth. Diamond, square, triangle, white square, and star represent the addition of 0, 1, 10, 100, and 500 µL of the suspension, respectively. Circle and white circle, respectively, represent the addition of 100 and 500 µL of ethanol without SWNT.

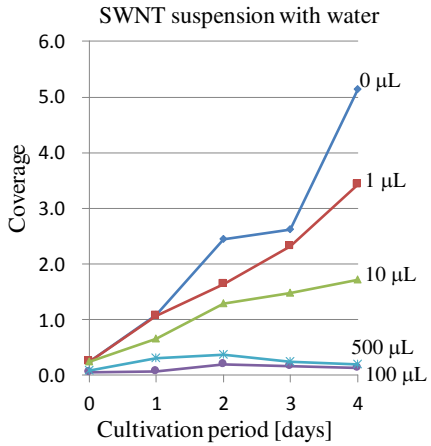

Fig. 8: Effects of single-walled nanotube (SWNT)-aqueous suspension (FH-P) on *Melosira nummuloides* growth. Diamond, square, triangle, circle, and star represent the addition of 0, 1, 10, 100, and 500 µL of the suspension, respectively.

In the current study, we showed a method, which is different from those previously shown, for evaluating the effects of SWNT suspension on diatom cell growth. We used *M. nummuloides* that associate similar to fibers and do not adhere to solid surfaces. Therefore, the glass substrate method used in the case of *Navicula* sp. would be ineffective in this diatom. Instead, we used a 100-µL cell suspension of *M. nummuloides* and cell growth was monitored by continuously observing the surface of the culture Petri dish using an optical microscope. The photographs were binalized using ImageJ software and cell coverage on the Petri dish surface was calculated. [Fig. 6(b)] shows a typical photograph of *M. nummuloides* cells in a Petri dish.

The effects of SWNT were evaluated using the second method with *M. nummuloides* cells. [Fig. 7 and 8], respectively, show the diatom cell growths after adding SWNT suspension using ethanol and water. Although inhibition of diatom growth was observed in both the cases, the effect was stronger in SWNT suspension using water than that using ethanol. The addition of more than 100-µL SWNT water suspension completely inhibited cell growth. In contrast, diatom cell growth was not completely inhibited even after the addition of 500-µL SWNT ethanol suspension. This difference in results might be because of precipitation of SWNT when dispersed using ethanol after adding to the culture medium, whereas the aqueous suspension of SWNT might have been retained in the dispersed state in the culture medium.

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CONCLUSION

We proposed two methods for evaluating the toxicity of nanomaterials (e.g. CNT) using two types of diatom cells. In all instances, the effects of SDS and CNT on cell division were clearly observed. An aqueous suspension of SWNT significantly inhibited cell growth. We believe the methodology used in this study will be recognized as a convenient and effective technique for evaluating the safety of nanomaterials.

CONFLICT OF INTEREST
There is no conflict of interest.

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FINANCIAL DISCLOSURE
None

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