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Multi-walled carbon nanotubes, natural organic matter, and the benthic diatom *Nitzschia palea*: "A sticky story"

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Abstract

Different effects of multi-walled carbon nanotubes (MWCNTs) on the freshwater diatom *Nitzschia palea* were examined. MWCNTs used in this study (MWCNT) were dispersed either by sonication without (MWCNT$_{sonicated}$) or with a realistic concentration (10 mg L^{-1}) of Natural Organic Matter (MWCNT$_{+NOM}$). A pocket-size device was designed to distinguish shading effect (using MWCNT suspensions as external filters) from total exposure effect of MWCNT$_{sonicated}$ and MWCNT$_{+NOM}$ on benthic algae. This study demonstrates that cell division was strongly inhibited after a 48 h exposure to MWCNT$_{+NOM}$ compared to MWCNT$_{sonicated}$. This device did not yield a quantifiable contribution of shading to growth inhibition of MWCNT$_{sonicated}$ and below 10 mg L^{-1} of MWCNT$_{+NOM}$. In all cases, neither lethal effects nor drops in photosynthetic quantum yield were observed. After a 6-d exposure, a complete growth recovery was observed for all conditions except at the highest concentration of MWCNT$_{+NOM}$. Different microscopic approaches using carbohydrates markers revealed the strong affinity between MWCNT and extracellular polymeric substances (EPS) produced by *N. palea*. These seem to constitute a defensive mechanism against MWCNT.

Introduction

Although observed for the first time in 1952, it was only during the 1990s, with the first controlled synthesis of carbon nanotubes (CNTs) that their widespread use became possible (Monthioux & Kuznetsov, 2006). Owing to their nanoscale and their physico-chemical properties (mechanical, electrical, thermal, and optical), they are used increasingly in many fields (Ajayan & Zhou, 2001). Currently most CNTs produced are multi-walled carbon nanotubes (MWCNTs). Annual production is more than 3 Kt/year (Keller et al., 2013). Currently, it is already possible to find MWCNTs in a variety of everyday products such as plastic additives, batteries, or some sporting goods (Endo et al., 2008).

For several years now, increased production and use of MWCNTs, as well as wastes that will result have been raising the question of their potential environmental impact, especially on aquatic ecosystems. Indeed, their location downstream of terrestrial ecosystems favors the concentration of all kinds of pollution, especially in the case of non-biodegradable compounds such as CNTs (Kümmerer et al., 2011). Thus, organisms inhabiting aquatic ecosystems might be particularly exposed to MWCNTs.

Several studies have investigated the toxicity of MWCNTs on various aquatic organisms. These have provided conflicting results while emphasizing both toxic and non-toxic effects. Acute toxicity, chronic toxicity, cytotoxicity, or genotoxicity of MWCNTs are usually explained by cell membrane disruption or breakage and oxidative stress (Gusev et al., 2012; Hsieh et al., 2013; Kang et al., 2008; Kwok et al., 2010; Mouchet et al., 2007, 2010; Nel, 2006; Singh et al., 2009; Wei et al., 2010; Von Moos & Slaveykova, 2013). Other studies have also demonstrated that in the case of non-purified CNTs, metal catalyst residues could significantly influence the observed toxicity (Ge et al., 2012; Matorin et al., 2010; Mwangi et al., 2012; Shvedova et al., 2012). However, other papers have reported inhibiting effects of MWCNTs on green algae growth and clearly demonstrated the negligible influence of catalyst residue (Long et al., 2012; Schwab et al., 2011). Long et al. (2012) have also observed the presence of MWCNTs in the cytoplasm of *Chlorella* sp. Finally, decreases in photosynthetic yield and inhibition or delay of cell growth were reported to be a combinatory effect of MWCNTs on oxidative stress, agglomeration, physical interactions, and shading (Long et al., 2012; Matorin et al., 2010; Schwab et al., 2011; Wei et al., 2010). Many authors have also investigated the effect on biota of dispersed-CNTs by non-covalent functionalization using synthetic organic compounds such as carboxymethyl cellulose, sodium dodecyl sulfate, sodium dodecylbenzene sulfonate, sodium cholate, Triton X-15, Triton X-100, polyvinylpirrolidone, and tetrahydro-furan

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(Gao et al., 2012; Vaisman et al., 2006; Petersen et al., 2011). Unfortunately, due to the inherent toxicity of some of these products, it is difficult to clearly separate their effect from those of CNTs (Dong et al., 2008). Although widely used by industry to provide well-dispersed CNTs, none of these above compounds are encountered in aquatic environments at sufficient concentrations to play a role in CNT dispersion. Assessment of CNT toxicity in the presence of non-toxic natural organic compounds such as gum Arabic (Bourdiol et al., 2013; Mouchet et al., 2011; Youn et al., 2012) or natural organic matter (NOM) (Schwab et al., 2011) demonstrated that these compounds could interfere with the toxicity of CNTs. NOM, being widely present in aquatic media, is probably the best choice to investigate the influence of the dispersion on CNTs toxicity in natural environment.

To date, few studies have focused on the effects of nanoparticles on algae (Von Moos & Slaveykova, 2013). Nevertheless, their abundance in the environment, their primordial place in the carbon cycle as well as their intrinsic properties such as photosynthesis and the presence of cell walls, make them of particular interest for the assessment of contaminant effects (Debenest et al., 2010). Among algae, diatoms are responsible for more than 25% of the global primary productivity (Scala & Bowler, 2001). Diatoms can be planktonic or benthic. They represent the main component of many freshwater photo-autotrophic biofilms during autumn and spring. In addition, they produce a cell wall composed of amorphous silica, similar to glass, called frustule. The frustule is known to provide great protection against environmental physical stresses (Hamm et al., 2003). Another feature of benthic diatoms is the production of extracellular polymeric substances (EPS) mainly composed of polysaccharides and proteins. These allowing them to adhere, move, and stabilize supports they colonize (Stal, 2003). EPS production also confers strong resistance to many organisms against biocides (Flemming & Wingender, 2001) and metallic ions released by nanoparticles (Miao et al., 2009).

Currently, studies using NOM as dispersant focus on an optimal dispersion of CNTs. However, none has addressed the effect of MWCNTs in the presence of a realistic concentration of NOM in order to simulate the behavior and effects of CNTs in natural conditions. The effect of CNTs on benthic freshwater diatoms has also never been investigated despite their key role at the base of many aquatic food chains.

In this study, a common freshwater diatom, *Nitzschia palea* (Kützing) W. Smith (*N. palea*) was chosen to assess the effects of MWCNTs in the presence of NOM. The first part of this work focuses on quantitative effects (growth inhibition, photosynthetic yield decrease, and viability) using a device inspired by previous studies (Aruoja et al., 2009; Hund-Rinke & Simon, 2006). It was designed to separate shading (using external light filters) and total exposure effects of nanoparticles on benthic photosynthetic organisms. In the second part, the affinity and the potential input of MWCNTs into the cells were investigated and characterized using various microscopic approaches while taking into account two major characteristics of diatoms known to protect them from environmental stress: EPS and the silica frustule.

Methods

Carbon nanotubes and natural organic matter

MWCNTs were provided by the ARKEMA Company under the reference Graphistength® C100 (MWCNT; note that in this paper, MWCNTs is the general term for MWCNTs and MWCNT refers to those used in this study).

They were synthesized by CCVD using a fluidized bed process. According to the supplier and Bourdiol et al. (2013),

MWCNT have 5–15 walls with an outer diameter ranging from a few nanometers to 20 nm and a specific surface area of $270 \, m^2 \, g^{-1}$. Their length ranges from 0.1 to 10 μm. Their initial mean agglomerate size ranges between 200 and 500 μm in deionized water. The carbon content of dried MWCNT is ca. 95 wt%. MWCNT were provided suspended in deionized water (100 mg in 20 mL). Suwannee river Natural Organic Matter (NOM; Cat no. 1R101N) was purchased from the International Humic Substances Society (IHSS, St. Paul, MN). Characterization of MWCNT suspensions will be described in a later section.

Diatom strain and cultures

The axenic strain of *N. palea* (Ref. CPCC-160) purchased from the Canadian Phycological Culture Center (CPCC) was grown in a CHU no. 10 basic medium (CHU10; $6.4 < pH < 6.6$) modified by J. Acreman using ethylenediamine tetraacetic acid ferric sodium salt (EDTA–Na–Fe) as an iron source (for more detail see: http://uwaterloo.ca/canadian-phycological-culture-centre/cultures/culture-media/chu-10). All bioassays were performed in a growth room at $20 \pm 1 \, °C$ on a rotary shaker at 90 rpm during a light/dark period of 16 h/8 h provided by high pressure sodium lamps (VIALOX® NAV® (SON) SUPER 4Y®, 600W, OSRAM GmbH) with an illumination of 5500 lux. CHU10 was always replaced by fresh medium 72 h before the experiments and prior to preparing inoculum. All manipulations during the experiments were carried out under a class II laminar flow hood to avoid biotic contamination.

MWCNT suspensions

Deionized water was removed (95%) by pipetting from provided suspensions before re-suspending MWCNT in CHU10 thus obtaining MWCNT stock suspension of $100 \, mg \, L^{-1}$. Stock suspension was homogenized by ultrasonication for 1 h using a BRANSON digital sonifier S-250D with a 1/8 in Tapered Microtip (200 W; amplitude: 35% 5 s/2 s). Then, four dilutions were carried out ($0.167 \, mg \, L^{-1}$, $1.67 \, mg \, L^{-1}$, $16.7 \, mg \, L^{-1}$, and $83.5 \, mg \, L^{-1}$) for the algal tests, and four ($0.1 \, mg \, L^{-1}$, $1 \, mg \, L^{-1}$, $10 \, mg \, L^{-1}$, and $50 \, mg \, L^{-1}$) for analyses (metal dosages in solution, optical densities, and microscopic observations). MWCNT suspensions were homogenized again as described above, but only for 20 min after autoclaving (20 min, 121 °C, 1 bar) prior to the beginning of the experiments. Prior to the second homogenization, MWCNT suspensions dispersed with NOM were carried out in the same way, adding $16.7 \, mg \, L^{-1}$ of NOM for algal tests and $10 \, mg \, L^{-1}$ for the other tests. This concentration was not chosen to allow optimal dispersion of MWCNT but to be representative of the average NOM abundance in the river Save near Toulouse, France (Oeurng et al., 2011). Two kinds of MWCNT suspensions were obtained after sonication: (i) without NOM (MWCNT$_{sonicated}$) and (ii) with NOM (MWCNT$_{+NOM}$) for all concentrations (e.g. MWCNT$_{50mg-sonicated}$ and MWCNT$_{50mg+NOM}$ are suspensions of MWCNT at concentration of $50 \, mg \, L^{-1}$ without or with $10 \, mg \, L^{-1}$ of NOM, respectively).

Characterization of MWCNT suspensions

MWCNT$_{sonicated}$ and MWCNT$_{+NOM}$ were characterized by transmission electron microscopy (TEM, JEOL JEM-1400, 120 kV, JEOL, Tokyo, Japan) and by field effect gun scanning electron microscopy (SEM, JEOL JSM-6700F, 5 kV, JEOL, Tokyo, Japan). Samples of each $10 \, mg \, L^{-1}$ suspension were first sonicated for 5 min. A droplet was dried over a perforated carbon copper grid before TEM observation and on aluminum holders before SEM observation.

The stability of MWCNT$_{sonicated}$ and MWCNT$_{+NOM}$ was assessed by optical density at the chosen wavelength of $\lambda = 439$ nm corresponding to the maximum absorbance of *N. palea* photosynthetic pigments. For that purpose, 90 mL of each suspension was divided into three test tubes. About 1 mL at half height of the water column was sampled and analyzed at regular intervals (1, 24, 48, and 72 h).

Effect of MWCNT on the CHU10 composition

Investigation of MWCNT effects on CHU10 was done by analyzing the elemental composition of triplicated MWCNT$_{50mg-sonicated}$ and MWCNT$_{50mg+NOM}$. Each suspension was incubated 48 h in identical conditions to the algal tests (see above). Samples were then centrifuged 30 min at 20 675g (Sigma Laborzentrifugen 3-18, Osterode, Germany). Supernatants were filtered on a Minisart high flow polyethersulfone membrane (0.1 μm; SARTORIUS-STEDIM Biotech). Major and trace elements were measured by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent-7500ce, Agilent Technologies, Palo Alto, CA). pH was controlled at the beginning of the tests in each MWCNT suspension and directly in wells at the end of the experiment.

Investigation of MWCNT toxicity and shading effect on *N. palea*

The experimental device used for algal growth tests was designed to assess total exposure effect and shading effect of MWCNT$_{sonicated}$ and MWCNT$_{+NOM}$ on benthic algae. Moreover, it also allows *in situ* observations of MWCNT behavior on a photosynthetic biofilm.

Each device consists of two stacked 12 well plates (COSTAR®-3513, Corning Incorporated, Corning, NY) with a black opaque film stuck on covers around the wells. The stack of two plates, after being surrounded by Parafilm® to avoid evaporation, was placed in an open-topped opaque box to allow light to enter by the wells only (Figure 1). One device was used per condition.

Before the beginning of the exposures, lower plates of each device were inoculated with 1 mL of algal inoculums (2.5×10^5 cells mL^{-1}) and the uppers ones with 1 mL of CHU10. The devices were then placed, without shaking, in a growth room for 24 h to allow a homogenous sedimentation and adhesion of algae. Then, 1.5 mL of MWCNT suspensions (see above) were added to each well of the lower plates, to obtain a final volume of 2.5 mL per well and MWCNT concentrations of 0.1, 1, 10, and 50 mg L^{-1} per plate. Shading effect tests were performed by adding 1.5 mL of MWCNT$_{sonicated}$ or MWCNT$_{+NOM}$, but only to the upper plates. All remaining wells were filled with 1.5 mL of CHU10 to obtain a final volume of 2.5 mL per well. The same procedure was followed for MWCNT$_{+NOM}$ tests except NOM was added to CHU10. Control devices were prepared with only CHU10 or CHU10 complemented with NOM (CHU10+NOM). The duration of incubation was 24, 48, 72, and 144 h.

Each time, the content of three wells per plate were scraped, homogenized, and fixed with 3.6% of formaldehyde. Algal concentrations were determined using a Malassez cell counter to perform two counts per well. A one-way analysis of variance (ANOVA) followed by Tukey HSD tests were implemented using the statistical open source software ''R'' (SSR; R Development Core Team 2012, Bio-RAD, Charlottesville, VA) to determine significant differences between conditions of the growth test. Effect concentrations of 50% (EC$_{50}$) values were estimated with the Excel® Macro: REGTOX 7.0.3 (Copyright© 2001, Eric Vindimian, Boston, MA) using the Hill model. The 95% confidence intervals for the EC$_{50}$ values were calculated by
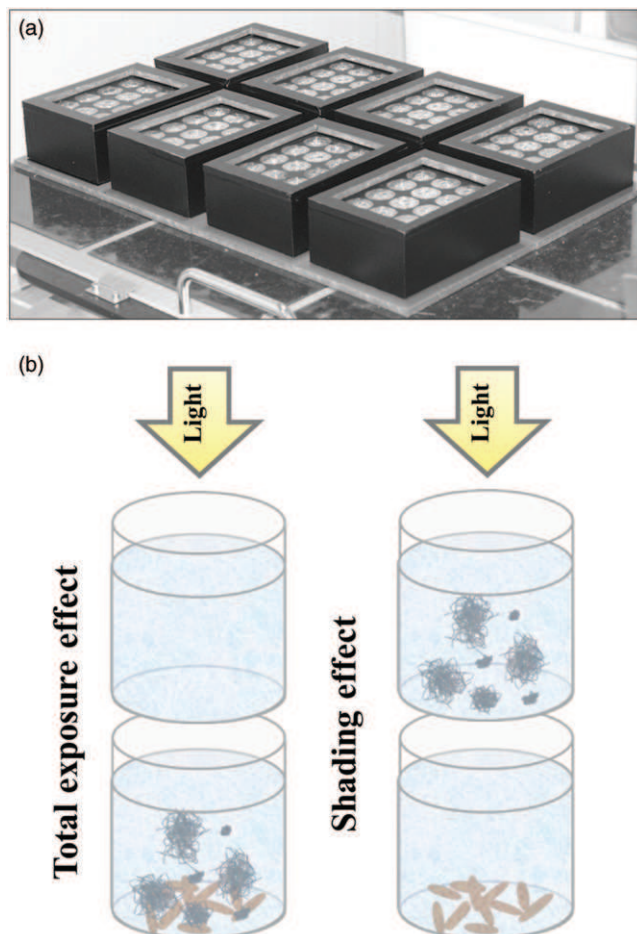

Figure 1. Experimental devices composed of open-top opaque boxes covering two stacked 12 wells plates with opacified inter-wells (a). Schematic representation of the device used for toxicity test (b). Bottom wells contain benthic diatoms exposed to (left) or shaded by (right) multi-walled carbon nanotube suspensions without (MWCNT$_{sonicated}$) or with the presence of natural organic matter (MWCNT$_{+NOM}$). Top boxes contain only culture medium (CHU10) in the case of exposure tests and MWCNT$_{sonicated}$ or MWCNT$_{+NOM}$ in the case of shading tests.

bootstrap simulations ($n = 500$). Growth rates (r) were calculated from the following equation ($n_0 = 1.22 \times 10^5$ cell mL^{-1} = number of cells mL^{-1} at the beginning of exposures and n_x = number of cells mL^{-1} after x hours of exposure to MWCNT ($x = 48$ or 144 h)

$$r = \frac{n_x - n_0}{n_0}$$

Algal mortality was controlled after 48 h of exposure to MWCNT suspensions using Sytox green® (Molecular Probes, Inc., Eugene, OR). Living samples were incubated 10 min in Sytox green® (100 nM) and then observed using a fluorescence microscope (BX-41, Olympus, Center Valley, PA) equipped with an HG lamp (U-LH100HG, Olympus, Center Valley, PA) using a 470–490 nm/520 nm excitation/emission filter and a 500-nm dichromatic filter (U-MNB2, Olympus, Center Valley, PA). The nucleus of dead (or injured) cells fluoresces in green while the nucleus of intact cells is not stained. Photosynthetic active radiations (PAR) received by *N. palea* were measured after 48 h of exposure, with the sensor placed between the two plates, using a light-meter (Li-250A light meter equipped with Li-COR Quantum sensor; Li-COR Biosciences, San Diego, CA). Interference between Sytox green® and MWCNT was assessed following the works of Horst et al. (2013). It consisted to labeling diatom just before or right after the addition of MWCNT. No significant

interference was revealed at chosen concentration (data not shown).

Influence of MWCNT and NOM on photosynthetic activity and chloroplasts

The effect of MWCNT on photosynthetic activity was investigated by pulse amplitude modulated fluorometry (PAM) using a Phyto-PAM (Heinz Walz GmbH, Effeltrich, Germany). The photosystem II quantum yield (PSII) was investigated to establish the ratio of emitted photons to photons absorbed by chlorophyll after an illumination pulse. In the case of total inhibition, quantum yield is close to 0 and rises with increased photosystem II activity (Phyto-PAM user guide). Measurements were done 48 h after the beginning of the exposure for each MWCNT suspension and concentration and after removing background signals from the corresponding test conditions. Any interference of NOM was revealed after illumination pulse. Each condition was triplicated. The Kruskal–Wallis analysis of variance (non-parametric data) was implemented using SSR to determine significant differences between conditions. Kendall tests were subsequently implemented to determine correlations between MWCNT concentration and the presence of NOM on PSII of *N. palea*.

Integrity of chloroplasts was also qualitatively controlled with a fluorescence microscope using a 330–385 nm/420 nm excitation/emission filter and a 400-nm dichromatic filter (U-MWU2, Olympus, Center Valley, PA) thus allowing auto-fluorescence observation of chloroplasts.

Nature of MWCNT affinity for a biofilm of *N. palea*

Investigation of the interaction between MWCNT and *N. palea* at the cellular level was provided by both light microscopy and SEM. For this purpose, diatoms were grown on coverslips in devices used for growth tests and exposed 48 h to $MWCNT_{10mg - sonicated}$ or $MWCNT_{10mg + NOM}$. Samples were then fixed and stained with Alcian blue (Sigma-Aldrich, Paris, France) directly in the wells, following Erlandsen's (2004) protocol with some modifications. Fixation consisted in a first 24 h-incubation in a solution of 0.1% Alcian blue in acetic acid (0.5 M), paraformaldehyde (2%), and glutaraldehyde (2%) and buffered using sodium cacodylate (0.15 M). Samples were then gently rinsed in cacodylate buffer (0.15 M) and then observed *in situ* at a maximum well depth using an inverted microscope (IX51, Olympus, Center Valley, PA, × 400). Samples exposed to $MWCNT_{10mg + NOM}$ were subsequently 2 h-post fixed in a solution of cacodylate buffer containing potassium ferro-cyanide (1.5%) and OsO_4 (1%). They were rinsed again and dehydrated in an ascending ethanol gradient [50, 70, 80, and 95%] each for 10 min and twice for 15 min in [100%] before critical point drying with N_2. Coverslips were then placed on SEM mounts and platinum coated before observation (JEOL JSM-6700F, 3 kV, detection mode: Secondary Electron Imaging).

SEM observations of frustules were performed after a chemical treatment following the normalized AFNOR protocol (NF EN 13946) digested cellular content and EPS. This was achieved by boiling hydrogen peroxide (H_2O_2, Sigma-Aldrich® 30%) for 10 min and boiling hydrochloric acid (HCl, 35%) for 5 min. Samples were then rinsed, dried on SEM mount and platinum coated before being observed as stated above with voltage switched to 5 kV.

Results

Characterization of MWCNT suspensions

Absorbance spectrometry showed that after 24 h, the measured water column absorbance of all $MWCNT_{sonicated}$ was close to zero which corresponds to complete settling of the MWCNT out of suspension. However, $MWCNT_{+NOM}$ exhibited stable absorbance, up to $10 mg L^{-1}$ of MWCNT and similar to those of the beginning of the test (data not shown). Figure 2 shows examples of collected TEM images of MWCNT in three different dispersion states depending on the MWCNT/NOM ratio (*w/w*). Without NOM, MWCNT were grouped into pellets ranging from 0.1 to 1 μm in diameter which were almost interconnected by individualized nanotubes forming clusters of several tens of micrometers (Figure 2a). In the presence of five times more MWCNT than NOM (w/w = 5/1), MWCNT form fewer pellets than previously observed and exhibited more or less individualized clusters of tubes (Figure 2b). Finally, with a ratio MWCNT/NOM of 1or less, all MWCNT tend to be individualized in the suspension (Figure 2c).

Higher magnification TEM observations of MWCNT without NOM presented in Figure 2(d) show the presence of catalyst residue, always encapsulated in carbon shells or directly embedded into the tubes. ICP-MS analysis revealed only significant increased concentrations of molybdenum (Mo) in both 48 h $MWCNT_{50mg - sonicated}$ and $MWCNT_{50mg + NOM}$. Thus, Mo concentrations were $20.77 μg L^{-1}$ (SD = 3.85) for $MWCNT_{50mg}$ and $22.42 μg L^{-1}$ (SD = 0.15) for $MWCNT_{50mg + NOM}$, namely around two times higher than in CHU10. The pH of the culture media measured at the beginning (6.5 ± 1) and at the end of each experiment (7.1 ± 1) was never significantly modified by the presence of either NOM or MWCNT.

The effect of MWCNT suspensions on photosynthetic yield, chloroplasts and viability

Significant positive correlation between the presence of NOM in the MWCNT suspensions and PSII was observed ($\tau = 0.62$; $Z = 5.77$; $p < 0.001$), highlighting a positive effect of NOM on photosynthetic activity. However, no significant correlation was found between MWCNT concentration and PSII ($\tau = 0.18$; $Z = 1.075$; $p = 0.06$). Moreover, no significant difference in PSII was observed between control and treated diatoms regardless of MWCNT concentration. Thus, diatoms exposed to $MWCNT_{sonicated}$ exhibited an average PSII of 0.58 ± 0.03 ($\pm = 95\%$ confidence interval) whereas those exposed to $MWCNT_{+NOM}$ exhibited an average PSII of 0.63 ± 0.03. Observations of chloroplasts have not revealed any abnormality whatever the test conditions. Viability tests after 48 h of exposure have not revealed any significant mortality induced by $MWCNT_{sonicated}$ or $MWCNT_{+NOM}$. Thus, the test have revealed an average mortality of $7.2 \pm 1\%$ ($\pm = 95\%$ confidence interval) under all tested conditions.

MWCNT and NOM effects on *N. palea* growth

Figure 3(a) shows the growth kinetics of *N. palea* in controls with or without NOM. A positive effect of NOM ($p < 0.01$) was observed on *N. palea* growth from 48h ($+21 \pm 9\%$) extending to 144 h ($+8 \pm 3\%$) but was significant only during the exponential growth phase. EC_{50} and growth rates were determined after 48 h of exposure to MWCNT corresponding to the end of the exponential phase. Diatoms exposed to $MWCNT_{sonicated}$ exhibited an estimated 48 h EC_{50} of $118 mg L^{-1}$ (95% confidence interval: 76; 201). Diatoms exposed to $MWCNT_{+NOM}$ exhibited a 48 h EC_{50} of $2.83 mg L^{-1}$ (95% confidence interval: 0.84–8.82). Figure 3(b and c) shows growth inhibition after both 48 h of direct exposure to $MWCNT_{sonicated}$ and shading. Significant growth inhibition of *N. palea* was only observed for direct exposure to $MWCNT_{50mg - sonicated}$ ($23.4 \pm 7\%$) (Figure 3b). Direct exposure to $MWCNT_{+NOM}$ (Figure 3c) caused a dose–response growth inhibition from $0.1 mg L^{-1}$ to $50 mg L^{-1}$ with
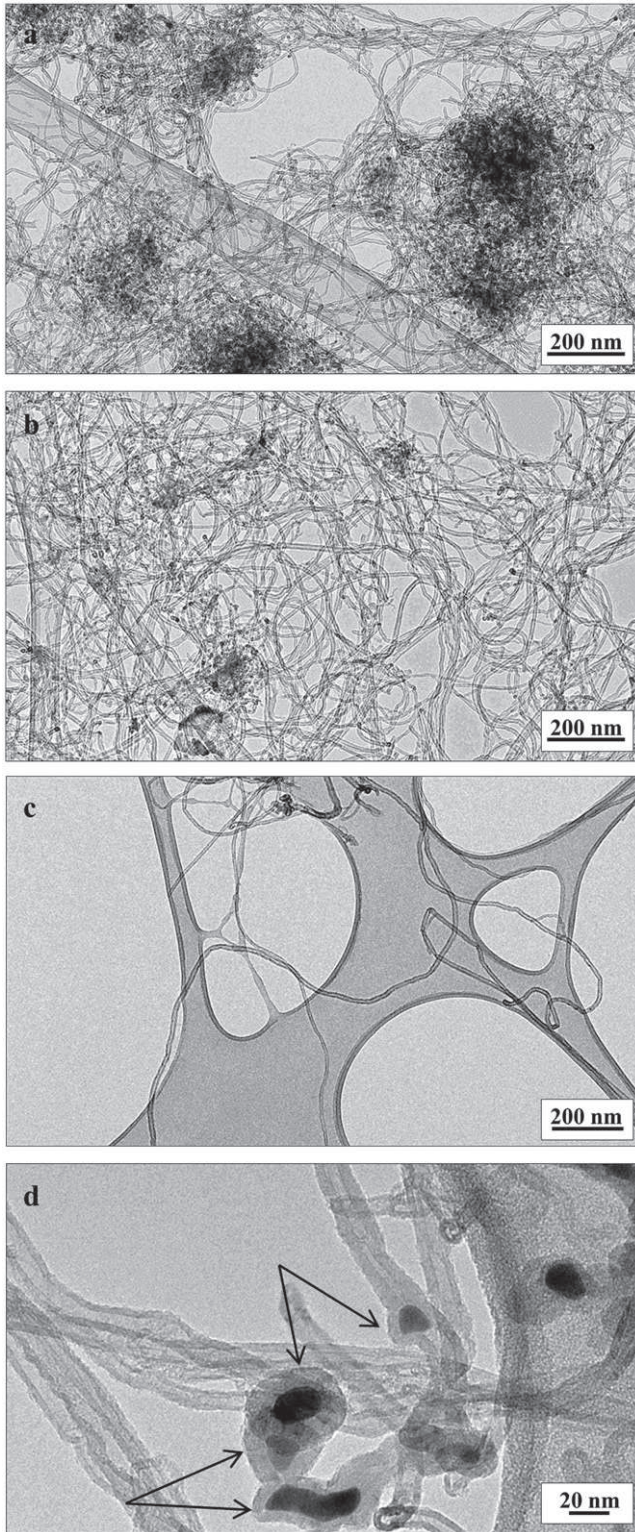
Figure 2. Transmission electron microscopy (TEM) images of multi-walled carbon nanotubes (MWCNT) dried suspensions with various concentrations of natural organic matter (NOM). (a) Without natural organic matter (MWCNT_sonicated). (b) With NOM (MWCNT_+NOM; ratio 1/1). (c) MWCNT_+NOM (ratio 5/1). (d) Magnified view of (a). Arrows indicate metallic residues encapsulated in carbon shells or directly embedded in MWCNT.

inhibition values of $28.9 \pm 12\%$, $52.4 \pm 3\%$, $71.2 \pm 7\%$, and $81.4 \pm 2\%$ and a calculated 48 h EC_{50} of $2.83 \, mg \, L^{-1}$. Moreover, MWCNT_+NOM caused growth inhibition by shading from $10 \, mg \, L^{-1}$ ($67 \pm 12\%$) to $50 \, mg \, L^{-1}$ ($67.1 \pm 8\%$) although shading

effect at lower concentrations cannot be ruled out. The decrease of PAR caused by shading depends on the MWCNT concentrations. This is shown in the caption for Figure 3. Figure 3(d and e) shows growth rates after 144 h of exposure. All conditions exhibited growth rates similar to controls, except for cultures exposed to MWCNT_{50mg + NOM} which exhibited an inhibition of $34 \pm 11\%$.

MWCNT adherence to the algal biofilm

The interaction of MWCNT with the biofilm of diatoms was investigated using stereo microscopy, light microscopy, and SEM. Examples of collected stereo-microscopy images show an overview of the MWCNT pellet size and structure in MWCNT_sonicated in the presence or absence of *N. palea* (Figure 4). In the absence of *N. palea*, MWCNT_sonicated formed only free sparse large pellets of several mm in diameter. In the presence of *N. palea*, MWCNT form many scattered pellets agglomerated onto the biofilm despite the presence or not of NOM. The staining of EPS using Alcian blue showed an agglomeration of the MWCNT onto these compounds (Figure 5a–c). Collected images also showed that this agglomeration was highly impacted by the NOM driven dispersion of MWCNT. Without NOM, MWCNT presented large clusters, mostly agglomerated on areas with a high quantity of diatoms and EPS (Figure 5b). NOM-dispersed MWCNT clusters were smaller and a large quantity of MWCNTs was stock to the EPS network (Figure 5c).

Figure 5(d–g) shows examples of collected SEM images of *N. palea* in control culture and in cultures exposed for 48 h to MWCNT_{10mg + NOM}. SEM allows higher magnified observations of the EPS network produced by diatoms and their interaction with MWCNT. These figures show that MWCNT present a high affinity for the EPS and are mostly included-in or stocked to the EPS matrix. However, few are found on the surface of cells. Finally SEM observations highlighted a $\leq 10 \, nm$ porous nanostructure inside the pores of frustules (Figure 5i). The size of these ''nanopores'' is smaller than that of most MWCNT (Figure 5h).

Discussion

MWCNT suspensions, dispersion, and catalyst metal release

The importance of CNT dispersion on the observed effects on organisms has often been described (Dong et al., 2008; Kwok et al., 2010; Schwab et al., 2011; Gao et al., 2012). Ultra-sonication is a widely employed technique to disperse CNTs in solvents (Hilding et al., 2003). In CHU10, ultra-sonication was not efficient in maintaining MWCNT in suspension. According to Hyung et al. (2007), the addition of NOM ($10 \, mg \, L^{-1}$) during sonication allowed a stable suspension of MWCNT from $0.1 \, mg \, L^{-1}$ to $10 \, mg \, L^{-1}$. However, $^-$MWCNT_{50mg + NOM} sedimented rapidly despite the observed reduction of pellet size (Figure 2). These results emphasize three distinct states of dispersion of MWCNT: (i) agglomerated (MWCNT_{0.1mg – sonicated}; MWCNT_{1mg – sonicated}; MWCNT_{10mg – sonicated}; MWCNT_{50mg – sonicated}) (Figure 2a), (ii) partially dispersed but unstable in the suspension (MWCNT_{50mg + NOM}) (Figure 2b), and (iii) dispersed and stable (MWCNT_{0.1mg + NOM}; MWCNT_{1mg + NOM}; MWCNT_{10mg + NOM}) (Figure 2c).

NOM affinity for CNTs results in a non-covalent adsorption due to different mechanisms: electrostatic, hydrophobic, $\pi-\pi$, and hydrogen bonding (Yang & Xing, 2009). It might be attributed to the size of hydrophilic groups, with high molecular weights and long polymeric chains (Vaisman et al., 2006) which compose humic and fulvic acids. This coating/interaction induces
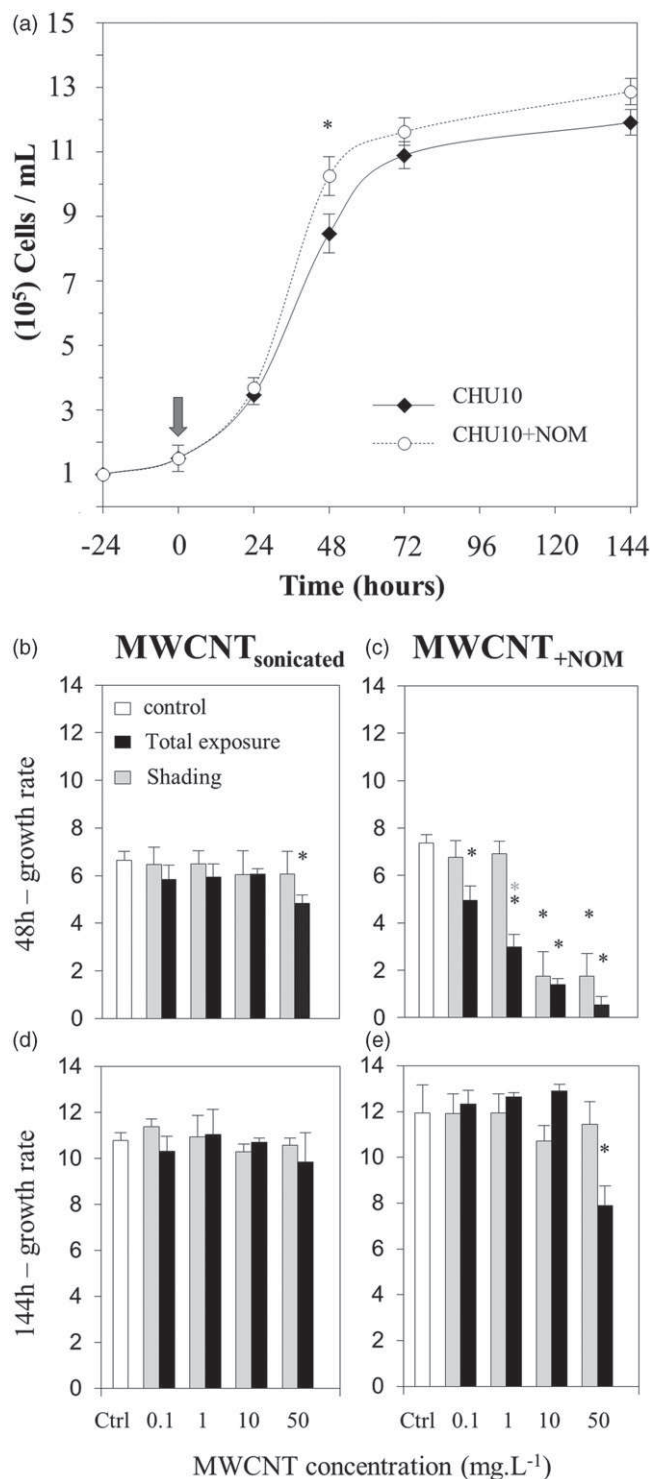
Figure 3. (a) Growth kinetics in control culture of *N. palea* growth in CHU no. 10 medium (CHU10; solid line) and in CHU10 plus $10 \, \text{mg L}^{-1}$ of natural organic matter (CHU10 + NOM; dashed line). Arrow indicates the time of NOM addition. (*) Indicates a significant difference ($p < 0.01$). (b) *Nitzschia palea* growth rates (r) after 48 h of exposure to multi-walled carbon nanotubes (MWCNT) sonicated (MWCNT$_{\text{sonicated}}$) and (c) to MWCNT$_{\text{sonicated}}$ with $10 \, \text{mg L}^{-1}$ of natural organic matter (MWCNT$_{+\text{NOM}}$). (d) and (e) are the same conditions but after 144 h of exposure. Error bars are standard error. (*) Indicates significant difference versus controls. (*) Indicates a significant difference between total exposure effect and shading effect ($p < 0.01$). $r = (n_x - n_0)/n_0$ (n_x = number of cells mL^{-1} after 48 or 144 h of exposure; $n_0 = 1.22 \times 10^5 \, \text{cell mL}^{-1}$ = number of cells mL^{-1} at the beginning of exposures). Photo synthetically active radiations (PAR) values (µmole $\text{s}^{-1} \text{m}^{-2}$ µA) after 48 h are 22.9 ± 0.8, 24 ± 3.2, 24.4 ± 1.7, 23.3 ± 4.8, $11.7 \pm 2.1^*$ for MWCNT$_{\text{sonicated}}$ and 23.8 ± 1.5, 24.2 ± 2, 23.8 ± 3.4, $15.1 \pm 1.4^*$, $9.6 \pm 1.6^*$ for MWCNT$_{+\text{NOM}}$ values correspond, respectively to: control $0.1 \, \text{mg L}^{-1}$, $1 \, \text{mg L}^{-1}$, $10 \, \text{mg L}^{-1}$, and $50 \, \text{mg L}^{-1}$. (*) Indicates significant difference versus controls.

electrostatic or steric repulsions that counterbalance van der Waals attractions and changes surface energy. This creates a thermodynamically stable dispersion (Hilding et al., 2003). These results demonstrated that organic matter can strongly modify the behavior of MWCNT in aqueous media. These also show that dispersion is modulated by the NOM/MWCNT ratio. In our experimental conditions, MWCNT forms dispersed and stable suspensions as long as MWCNT/NOM ratio is less than or equal

Figure 4. Examples of collected stereo-microscopy images of well bottoms after 48 h of diatoms exposed to $50 \, \mathrm{mg \, L^{-1}}$ of MWCNT without natural organic matter ($\mathrm{MWCNT_{50mg - sonicated}}$). (a) Overview of $\mathrm{MWCNT_{50mg - sonicated}}$ without diatoms. (b) Same suspension in the presence of diatoms. (c) and (d) higher magnification of (a) and (b), respectively.
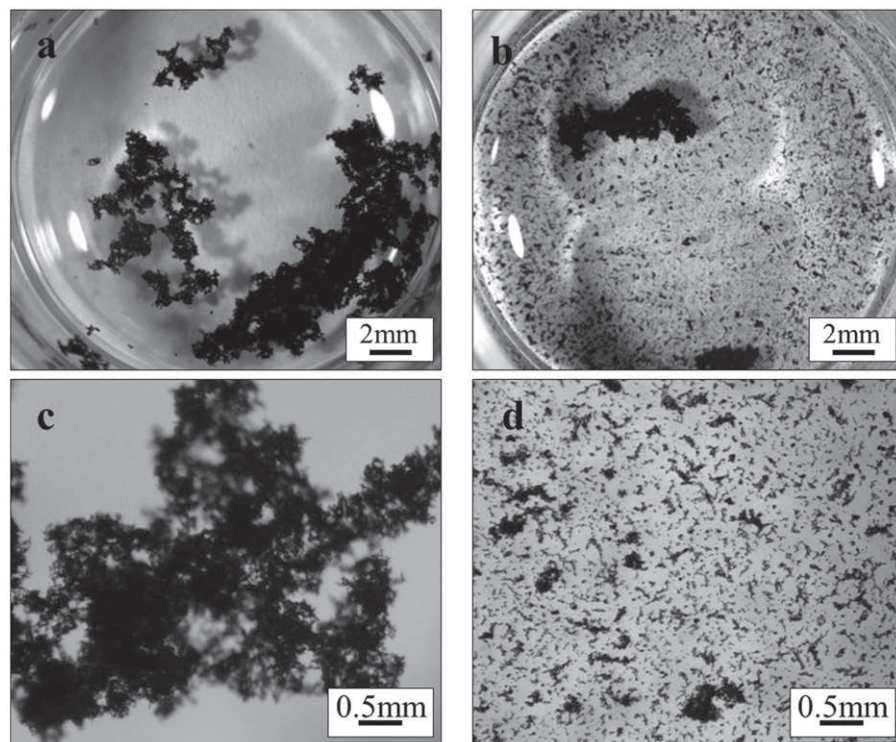
to 1. This is the case for all experimental conditions except for $\mathrm{MWCNT_{50mg + NOM}}$.

The presence of metallic ions and nanoparticles in MWCNT suspensions was shown by TEM and quantified in solution by ICP-MS. In agreement with Pumera (2007), TEM only revealed carbon-encapsulated metallic nanoparticles that were physically and chemically protected from the medium (Figure 2d). However, ICP-MS analysis revealed the presence of significant free Mo concentration about two times more than in CHU10. Investigations of structural defects by TEM and SEM did not provide any evidence of MWCNT alteration caused by ultrasonication, although TEM is probably not the best suited tool to elucidate this question. Both Bourdiol et al. (2013) working on the same batch of MWCNT and the works of Chowdhury & Cui (2011) came (using Raman, TEM and Dynamic light scattering) to the same conclusions.

Investigation of MWCNT toxic effects

Oxidative stress induced by the release of nanoparticles or metal ions is often involved in toxic effects such as alteration of cellular membranes, mortality, and decreases in PSII (Von Moos & Slaveykova, 2013). ICP-MS analysis revealed only the presence of Mo both in $\mathrm{MWCNT_{50mg - sonicated}}$ and $\mathrm{MWCNT_{50mg + NOM}}$. Mo concentrations were about 750 times less ($\sim 20 \, \mathrm{\mu g \, L^{-1}}$) than those leading to acute toxicity ($> 15 \, \mathrm{mg \, L^{-1}}$) for some unicellular algae (Dooren de Jong, 1965; Sakaguchi et al., 1981). Furthermore, Mo concentrations were similar for these two conditions while strong differences in terms of growth inhibition were observed (Figure 3b and c). No significant decrease in *N. palea* PSII was measured after 48 h of exposure whether for diatoms exposed to MWCNT or to $\mathrm{MWCNT_{+NOM}}$ when compared with control. These data show that MWCNT had no effect on the relative efficiency of energy conversion of the photosynthetic centers of *N. palea*. According to several studies (Kang et al., 2008; Wei et al., 2010; Wild & Jones, 2009), viability, plasma membranes, and chloroplasts were also unaltered whatever the MWCNT dispersion conditions. All these results suggest the absence of disorders caused by oxidative stress on exposed diatoms. In line with previous works on photosynthetic organisms (Ilyash et al., 2007; Kulikova et al., 2005), a positive effect of NOM on PSII was emphasized. This increase in PSII may explain the positive effect of NOM on *N. palea* growth (Figure 3a). Finally, the lack of effect on PSII and chloroplasts, together with the absence of acute mortality or plasma membrane disruption, hint at as an inhibitory effect of MWCNT on *N. palea* rather than a toxic effect. Moreover, growth inhibition of *N. palea* was not related to the presence of metal ions in MWCNT suspensions.

MWCNT effect on growth: total exposure effect versus shading effect

In this study, total exposure and shading effects on the growth of *N. palea* were investigated simultaneously. Without NOM, only exposure to $\mathrm{MWCNT_{50mg - sonicated}}$ led to growth inhibition after 48 h (Figure 3b) with an $\mathrm{EC_{50}}$ of $\sim 118 \, \mathrm{mg \, L^{-1}}$. However, exposure to $\mathrm{MWCNT_{+NOM}}$ led to a dose–response growth inhibition from $\mathrm{MWCNT_{0.1mg + NOM}}$ (Figure 3c). Thus NOM decreased the $\mathrm{EC_{50}}$ by around a factor forty ($2.83 \, \mathrm{mg \, L^{-1}}$). Addition of NOM ($10 \, \mathrm{mg \, L^{-1}}$) has strongly increased growth inhibition caused by MWCNT despite a positive effect of NOM observed in control culture (Figure 3a). According to Kwok et al. (2010), shading tests have shown that MWCNT without NOM agglomerate quickly (Figures 2a and 4a–c). They were also not effective in causing growth inhibition by shading in the range of chosen concentrations (Figure 3b). A recent study estimated between 20% and 40% the contribution of shading to the observed toxicity of MWCNTs ($10 \, \mathrm{mg \, L^{-1}}$) on *Chlorella* sp. after 96 h of exposure (Long et al., 2012). Thus, our results suggest that *N. palea* growth is less affected by the shading of $\mathrm{MWCNT_{sonicated}}$ than the planktonic green algae *Chlorella* sp. According to Schwab et al. (2011), shading of $\mathrm{MWCNT_{+NOM}}$ caused a strong growth inhibition from $10 \, \mathrm{mg \, L^{-1}}$ ($\sim 67\%$) due to dense and homogeneous clouding. Nevertheless, evaluation of shading using external filters as they were used in the present study, is prone to underestimate the part of shading plays in growth inhibition
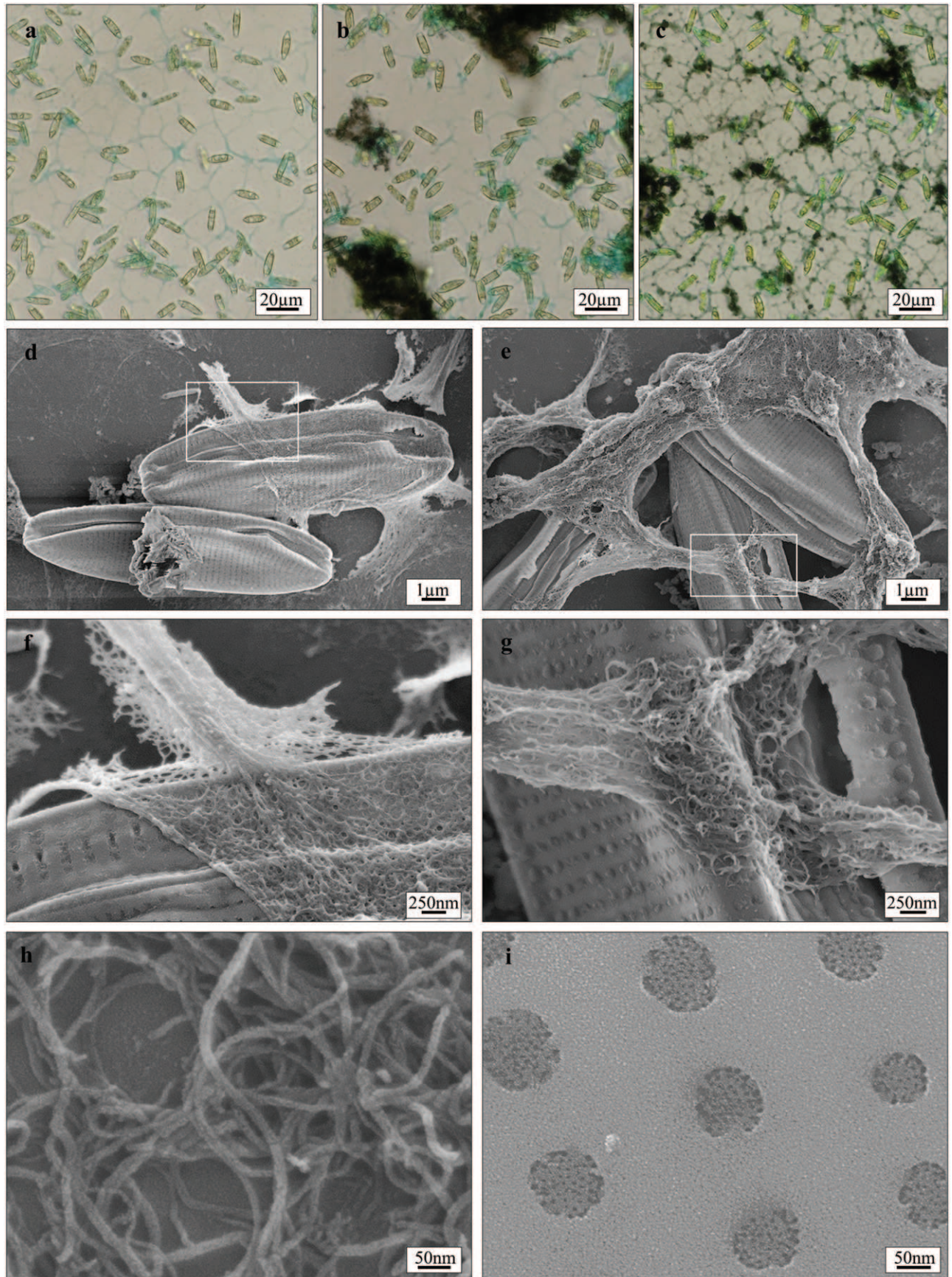
Figure 5. Biofilm of *N. palea* in devices after staining extracellular polymeric substances (EPS) using Alcian blue. (a) Control culture. (b) Culture exposed to suspension of multi-walled carbon nanotubes at $10\,mg\,L^{-1}$ (MWCNT$_{10mg-sonicated}$). (c) Culture exposed to suspension of $10\,mg\,L^{-1}$ MWCNT with a natural organic matter (MWCNT$_{10mg+NOM}$). EPS are stained in blue. MWCNT are in black. Scanning electron microscopy (SEM) images of *N. palea* (d) in control cultures and (e) in MWCNT$_{10mg+NOM}$. (f) and (g) Higher magnification of previous images focused on an EPS structure without or with MWCNT$_{10mg+NOM}$, respectively. (h) SEM images of MWCNT. (i) Detail of the nanostructure observed in pores of frustule.

(Cleuvers et al., 2002; OECD, 2002). Indeed, shading will be slightly higher for diatoms in contact with MWCNT (especially in the case of MWCNT$_{sonicated}$) as compared with diatoms in externally shaded cultures. This makes it difficult to properly estimate the true part of inhibition by shading for exposed diatoms.

Stereo microscopy showed that MWCNT bonded strongly to the biofilm (Figure 4) whatever their state of dispersion and the presence or absence of NOM. The presence of biofilm, therefore, reduced their dispersion. Agglomeration of MWCNT and more broadly of nanomaterials onto cells is often involved in the growth inhibition or toxicity observed on algae (Aruoja et al., 2009; Long et al., 2012; Schwab et al., 2011). Agglomerates observed on the biofilm may, therefore, explain growth inhibition. At the end of the experiment, a full recovery of growth was observed for all conditions except MWCNT$_{50mg + NOM}$. Thus, the inhibitor effect caused both by lack of light and contact appears to be reversible. This suggests that (i) shading at tested concentrations only delayed growth of *N. palea* and (ii) *N. palea* became less sensitive to MWCNT over time, as already observed on green algae (Wang et al., 2008; Youn et al., 2012).

It has been hypothesized that CNT self-agglomeration and macromolecules production, such as EPS by algae, both helped to neutralize the reactive surface sites of nanoparticles (Wei et al., 2010), reducing CNT toxicity. Thus, agglomeration that is partly responsible for growth inhibition early during the exposure could lead to detoxifying MWCNT. Furthermore, the mobility of *N. palea* gives it the opportunity to leave contaminated areas of the biofilm and to better reach MWCNT-free areas, making the evaluation of shading over time even more unpredictable.

Biofilm-MWCNT interaction: role of EPS and frustules

Focusing on EPS, light microscopy revealed the EPS network formed by *N. palea*, resulting from excretion involved in their shifting on and adherence to the substrate (Figure 5a). Strong agglomeration of MWCNT on EPS (Figure 5b and c) was also observed. In the absence of NOM, MWCNT form large agglomerates in high EPS concentration areas. MWCNT dispersed by NOM homogeneously covered the EPS network.

SEM observations confirmed and specified the interaction. The EPS network is fully covered by MWCNT and highly disrupted in the case of MWCNT$_{10mg + NOM}$ (Figure 5d–g). This affinity may result from different kinds of interactions depending on the presence or absence of NOM. EPS could form many hydrogen and sulfate bonds, bivalent bridging or covalent peptide bonds with NOM coating MWCNTs because of their numerous uronic acids, sulfonated sugars, and/or ketal-linked pyruvate groups (Stal, 2003). Finally, NOM adsorbed to MWCNT could, like Arabic gum, act as adhesion promoters, leading to the formation of highly adhesive interfaces between CNTs and the EPS matrix (Bandyopadhyaya et al., 2002; Gennes, 1990). These data show the efficiency of EPS in binding MWCNT and their potential role in diatom protection. More generally, our results according to the literature show the widespread role of EPS in efficiently protecting organisms against (nano) particles, whether natural (Brouwer et al., 2005; Staats et al., 1999) or manufactured (Luongo & Zhang, 2010; Jachak et al., 2012; Wei et al., 2010). Environmental conditions are known to strongly influence the production of EPS (Stal, 2003). An energetic trade-off could be achieved by *N. palea*, allocating more energy to their protection (sequestration of MWCNT as well as fleeing from high density MWCNT areas) and less to cell division. This could explain both the strong growth inhibition during early stages of exposure and the growth recovery observed at the end of the experiment. Studies are currently in progress in order to better

quantify the contribution of this mechanism to the growth inhibition caused by MWCNT.

Frustules are at the interface between the cell membrane and the extracellular environment. They represent the area of exchange between intracellular and external medium and confer strong protection to diatoms against mechanical stresses (Hamm et al., 2003). SEM shows that the frustule was not altered by MWCNT. Contrary to what was observed by other authors, SEM did not reveal any direct affinity of MWCNT for the outer surface of the diatom cells (Figure 5e–g). Furthermore, magnified observation revealed ''nanopores'' ($\sim$10 nm or less), forming nano-metric filters (Figure 5h and i). These filters enable both nutrient uptake and exclusion of particles, bacteria, and viruses (Losic et al., 2006). In the present case, it might completely prevent the entry of MWCNT into the cells.

This study reveals two important features of *N. palea* and numerous benthic diatoms which efficiently protect them against MWCNT and possibly explain the absence of toxicity: (i) the affinity of MWCNT for EPS rather than diatoms cell wall and (ii) the nano-porous structure of the frustule pores.

Conclusion

NOM strongly increases MWCNT-induced growth inhibition on the diatoms *N. palea* compared with raw MWCNT material, especially at short exposure times. Only growth inhibition was observed and seems partially explained by the shading effect that MWCNT caused when dispersed by NOM. However, the affinity of MWCNT for the biofilm and the mobility of *N. palea* were not taken into account in shading effect assessment, and a misestimating of the shading effect cannot be ruled out in the present study.

Microscopic observations provided an explanation for the commonly observed adherence of MWCNTs to organisms. Our results suggest that EPS provide considerable protection against MWCNTs, by reducing water column opacity and contact opportunities. EPS excretion also confers mobility to *N. palea*, an opportunity for escape from MWCNTs contaminated area. The intrinsic-to-diatom frustule, also appears as an efficient barrier preventing MWCNTs cellular uptake, thereby limiting toxic effects. Thus, our study highlights two protective means that benthic diatoms naturally possess against MWCNTs and probably numerous other non-soluble nanoparticles too. For a better assessment of MWCNTs effect, determining to what extent an overproduction of EPS induced by MWCNTs may be involved in the observed growth inhibition could be the next step.

From an ecological point of view, CNTs adhesion to biofilm could greatly increase the persistence of these nanoparticles in aquatic environments. The predominant role of aquatic biofilms for many primary consumers could also potentiate the transfer of CNTs along the aquatic food chains.

Acknowledgements

Declaration of interest

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