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Direct and indirect toxic effects of cotton-derived cellulose nanofibres on filamentous green algae

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ABSTRACT

Recently, cellulose nanofibers (CNFs) have attracted considerable attention as natural, abundant polymers with excellent mechanical properties and biodegradability. CNFs provide a new materials platform for the sustainable production of high-performance nano-enable products for various applications. Given the increasing rates of CNF production, the potential for their release to the environment and the subsequent impact on ecosystem is becoming an increasing concern that needs to be addressed. Here, we used the *Klebsormidium flaccidum* as a bioindicator organism of terrestrial and freshwater habitats pollution using a battery of biomarkers. Our results show that cotton CNFs inhibit the proliferation of algae and induce morphological changes in them. The two main toxicity mechanisms induced by cotton CNFs are: (i) a direct contact of CNFs with the cell wall and cellular membrane and (ii) an indirect effect through the generation of reactive oxygen species (ROS).

1. Introduction

Cellulose is a structural component of the cell walls of many plants. This polymer is one of the most important natural polymers, an almost inexhaustible raw material and a key source of sustainable materials on an industrial scale (Klemm et al., 2011). Cellulose nanofibers (CNFs, also known as whiskers or nanocrystals) are materials composed ofnanosizedcellulosefibrils with a high aspect ratio (length-to-width ratio). Their typical average diameter are 2–20 nm nanometers and their length vary between 10^{-6} and 10^{-3} cm, depending on the cellulose source (Zhou et al., 2011). Moreover, they are biodegradable (Kolakovic et al., 2011) and can be obtained from a range of renewable biosources, such as cotton, wood, hemp, tunicin, straw, algae and microorganisms (Klemm et al., 2011).

The annual global market for CNF applications is estimated to be 35 million metric tonnes (Cowie et al., 2014). CNFs have technological applications in several sectors including the automotive and aerospace industries as well as packaging, paint, water purification, cosmetics (Shatkin et al., 2014), drug delivery (Kolakovic et al., 2012) and tissue engineering (Pooyan et al., 2013). In particular, CNFs enable the manufacturing of new environmentally friendly materials. This purely natural product may replace synthetic petroleum-based fibers commonly used to reinforce composite materials (Bulota et al., 2013). Thus, CNFs represent an important niche for more sustainable product design and development (Shatkin et al., 2014).

Despite their great potential applications, little is known about the effects of CNFs on the environment and on human health. CNF has similar morphology to carbon nanotubes (CNT) such as

Abbreviations: ATP, adenosine triphosphate; BB, Bold's basal medium; CNF, cellulose nanofibers; CNT, carbon nanotube; EPS, extracellular polymeric substance; Fv/Fm, variable over maximal fluorescence; NP, nanoparticle; PAM, pulsed amplitude modulation; PPF, photosynthetic photon flux; PSII, photosystem II; ROS, reactive oxygen species; SEM, scanning electron microscopy; SOD, superoxide dismutase; TEM, transmission electron microscopy

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nanometer diameter and nano or micrometer length (Eichhorn et al., 2010). The toxicity of CNTs has been attributed mainly due to their fibershaped (Donaldson et al., 2006). Thus, cotton CNF may potentially have toxicity dependent on its morphology, which reinforces the need for studies on the toxicity of this nanomaterial.

In this study, we investigated the interaction of cotton CNFs with *Klebsormidium flaccidum* as a biological model in terrestrial and freshwater environments. *K. flaccidum* is a green alga that belongs to the Charophyceae family and comprises multicellular and non-branching filaments without differentiated or specialized cells (Hori et al., 2014). Yet, these algae are well known for their wide distribution and for being the dominant microorganisms in many biofilms (Barberousse et al., 2006). The use of primary producers as biological indicators is important because they are situated at the base of the food chain and any change in the dynamics of their communities can affect higher trophic levels of the ecosystem. Thus, the aim of the current study was to evaluate the toxicity of cotton CNFs on filamentous green algae *K. flaccidum*. We examined the effect of cotton CNF on cell proliferation, morphology and metabolic activity. The direct and indirect toxic effects of the cotton CNFs on *K. flaccidum* were elucidated.

2. Methods

2.1. Reagents

Unless stated otherwise, all chemicals were from Sigma Chemicals (St Louis, MO, USA).

2.2. Synthesis of cotton CNFs

Commercial cotton fibers were purchased from the local market. The fibers were finely chopped in a knife mill, passed through a 10-mesh sieve, dewaxed with 1:1 (v/v) ethanol:cyclohexane for 12 h in a Soxhlet apparatus, and then vigorously washed with tap water. The dewaxed samples were dried for 12 h at 100 °C in an air-circulating oven. Approximately 5 g of fibers were dispersed in 100 ml of 6.5 M sulfuric acid at 45 °C and stirred vigorously for 75 min. After that, 500 ml of cold distilled water was added to stop the reaction. The sulfuric acid was partially removed from the resulting suspension by centrifugation at 8000 × *g* for 15 min. The non-reactive sulfate groups were removed by centrifugation followed by dialysis. Then, the fibers were re-suspended and dialyzed against tap water with a tubing cellulose membrane (76 mm, D9402-Sigma) until the pH reached 6–7. The resulting suspension was sonicated (Branson 450 sonifier, Branson Ultrasonics, Danbury, USA) for 5 min (in an ice bath) and stored in a refrigerator.

2.3. Characterization of cotton CNFs by transmission electron microscopy (TEM) and X-ray diffraction (XRD)

An aliquot of the cotton-CNF suspension was diluted and sonicated for 5 min. A drop of this resultant diluted suspension was deposited on a carbon grid (400 meshes) and the grid was stained with a 1.5% solution of uranyl acetate and dried at room temperature. TEM images were obtained using a Hitachi H-700 transmission electron microscope with an acceleration voltage of 75 kV and equipped with a Hamamatsu camera. The dimensions (length and diameter) were calculated with the ImageJ software (National Institutes of Health, Bethesda, Maryland).

XRD patterns were recorded using an X'Pert PRO (PANalytical) diffractometer with Co Kα radiation. The diffractometer was calibrated using a standard Si sample. The samples were placed on a Si holder (absence of Si peaks).

2.4. Microalgal culture

K. flaccidum, a benthic filamentous eukaryotic green alga, was isolated from a sample of a black soiling developing on a building near Paris, France (Barberousse et al., 2006). The *K. flaccidum* cells were grown in 250 ml Erlenmeyer flasks in a sterile Bold's basal medium (BB medium) buffered with 3.5 mM phosphate buffer at a controlled temperature of (20.0 ± 0.5) °C and a luminosity of 50–80 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) under ambient CO_2 conditions. The pH of the medium was adjusted to 7.0 using a 1 M NaOH solution.

2.5. Toxicological assessment

Stock suspensions containing 1000 µg ml^{-1} cotton CNFs were obtained by sonicating 10 mg aliquots of CNFs in 10 ml of BB medium for 10 min at 200 W (VWR, USA). Sonication was applied to break micrometric aggregates and stabilize the suspensions. Aliquots of these suspensions were then added to the batch cultures (exponential growth organisms prepared three days before starting the test at a concentration of 5.0×10^5 cells ml^{-1}) to obtain the final cotton CNF concentrations corresponding to 1, 50 and 100 µg ml^{-1}.

The toxic response was evaluated by means of cell counting at 24, 48, 72 and 96 h after cotton CNF spiking as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures.

2.6. Viability assay

The viability of *K. flaccidum* cells were screened by staining the cells with trypan blue (HiMedia, India) following the principle of dye exclusion (Rocco et al., 2015). Cell counting was performed by bright field microscopy using a Cellometer Auto X4 instrument (Nexcelom, USA), which simultaneously calculates the percentage of cell viability (this is achieved through live/dead tests conducted using the trypan blue dye that selectively colors dead cells blue).

2.7. Microscopic observation

The effects of the different treatments on the test organisms (100 µg ml^{-1} after 48 h of exposure) were studied by optical microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Optical microscopy studies were performed using a Zeiss Primo Star microscope equipped with an AxioCam IcC1 cam and an AxioVision LE software. The production of mucilage by the cultures was assessed by India ink staining. For the electron microscopy studies, the control and treated samples were fixed with a mixture containing 2.5% glutaraldehyde and 1.0% picric acid in a BB medium (0.1 M, pH 7.4). Dehydration was then achieved in a series of ethanol baths (50–100%). For the TEM measurements, post-fixation using osmium tetroxide (OsO_4) before the dehydration was performed, and after this, the samples were processed by flat embedding in a Spurr resin. Ultrathin sections were obtained using a Reicherd-Young Ultracut microtome (Leica). Sections were contrasted with a 4% aqueous uranyl acetate solution and Reynold's lead citrate before visualization. For the SEM measurements, the samples were dried with a BAL-TEC CDP 030 supercritical point dryer after ethanol baths.

SEM images were obtained using a Zeiss Supra 40 microscope equipped with an in-lens detector. A very low excitation voltage (2.5 kV) and a small working distance (3 mm) were used.

TEM imaging was performed with a Tecnai 12 operating at 80 kV equipped with a 1 k × 1 k Keen View camera.