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Ecotoxic effects of paclitaxel-loaded nanotherapeutics on freshwater algae, *Raphidocelis subcapitata* and *Chlamydomonas reinhardtii*

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The contamination of water bodies and water pollution with pharmaceuticals are global issues receiving increasing attention, stemming from population growth and the resultant rises in pharmaceutical consumption, disposal, and excretion. However, little is known about how emerging classes of pharmaceuticals, in particular nanopharmaceuticals, influence water bodies and organisms living in them. In this work, we investigate the interactions of paclitaxel-loaded nanomedicine with freshwater algae *Raphidocelis subcapitata* and *Chlamydomonas reinhardtii*. For a given paclitaxel concentration, the nanomedicine form of paclitaxel led to a higher localization of paclitaxel on/in algal cell surfaces and inhibited algal growth more than molecular (free) paclitaxel. In addition, while the molecular paclitaxel at the solubility limit in water could not significantly hinder algal growth to reach an IC_{50} level, the nanomedicine form had a 120 h IC_{50} value of 1.1 ± 0.1 μg paclitaxel ml^{-1} for *C. reinhardtii* and a 72 h IC_{50} value of 1.6 ± 0.1 μg paclitaxel ml^{-1} for *R. subcapitata*. In the case of paclitaxel-loaded nanomedicine, concentrations above 16.2 μg paclitaxel mL^{-1} for *R. subcapitata* and above 5.4 μg paclitaxel mL^{-1} for *C. reinhardtii* resulted in an algaecidal effect, *i.e.* algal necrosis and complete stoppage of algal growth. The presence of paclitaxel-loaded nanomedicine also hindered the photosynthetic activity while free-paclitaxel caused no significant effect on it. These findings indicate that nanopharmaceuticals can cause ecotoxic effects on freshwater algae, which is otherwise not possible with traditional pharmaceuticals, owing to their ability to solubilize water-insoluble drug molecules in them.

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Environmental significance

With the increasing consumption and production of nanomedicines, the occurrence and fate of nanomedicines in the environment, and the potential consequences on human health have been increasingly recognized as issues warranting consideration. Nanomedicine is an emerging environmental concern because recent *in vivo* studies have indicated that some of the administered nanomedicines can be excreted from the body intact through the kidney or the liver/bile duct. After excretion, the nanomedicines can reach the sewer system and may eventually find their way into groundwater, reservoirs, and river systems, thereby entering into the food chains of living organisms. In this study, we investigated the influence of paclitaxel-loaded nanomedicine on fresh water algae because algal communities have many characteristics as biological indicators of spatial and temporal environmental variations. The key finding is that nanomedicines can cause enhanced ecotoxic effects, such as growth and photosynthesis inhibition on freshwater algae, due to their ability to solubilize water-insoluble therapeutics at concentrations above the solubility limit of such therapeutics in water.

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Introduction

Nanotherapeutics are defined as nanoscale or nanostructured materials used for medical diagnosis and treatment.[1] The main motivation behind using nanotherapeutics is their size-specific unique medical and physiological properties. For instance, the bioavailability of therapeutics increases with decreasing size and increasing surface area to volume ratio of drug particles, which is especially beneficial for water-

insoluble therapeutics.[2] In addition, nanopharmaceuticals in the range of 10 nm to 200 nm tend to passively target disease sites through the enhanced permeability and retention effect.[3,4] By relying on such intriguing properties, a number of nanotherapeutics have successfully been developed from the laboratory to the clinic to the market; according to a recent study, there were 22 nanomedicines approved in Europe by the European Medicines Agency (EMA) and between 71 and 87 approved by the US-FDA in 2015.[5] Another study identified 158 start-ups and small/medium enterprises focusing on the development of nanotherapeutics.[1] The global market value of nanotherapeutics was estimated to reach about $7 billion in 2004,[1] between $17 and $73 billion in 2011,[6,7] and between $178 and $528 billion in 2019.[7,8] The increased production and consumption of nanotherapeutics have brought along concerns regarding the potential consequences of their occurrence and distribution on environmental health.

The above-mentioned concerns have intensified in light of recent studies indicating that some of the administered nanotherapeutics can be excreted from the human body *via* the excretory or hepatobiliary systems.[9–11] The hepatobiliary system generally provides partial or full metabolization of the nanomedicines which is followed by fecal or biliary excretion.[12,13] On the other hand, the kidney and other parts of the excretory system rapidly remove them, in particular the small ones (*i.e.* <50 nm), from the vascular compartment in a relatively unaltered form.[12] Upon excretion, the nanotherapeutics are destined to reach the sewer system and then go through a waste treatment facility. However, the elimination of nanoparticles from wastewater is a challenge and standard wastewater treatment plants (WWTP) do not completely capture some nanomaterials.[14,15] While it is currently unknown how nanotherapeutics interact with waste treatment plants, the occurrence and detection of conventional therapeutic agents in water bodies[16–18] suggest that nanotherapeutics may also find their way to rivers, lakes, and oceans through the discharge of liquid effluent from wastewater treatment plants. In addition, sewage systems may leak or become defective, and these leaks can eventually infiltrate into subsoil and reach underground streams.[19–21] Overall, owing to these reasons, the ecotoxicity of nanotherapeutics is an emerging concern from an environmental science perspective.

Algal communities have many characteristics as biological indicators of spatial and temporal environmental variations because of their position at the base of aquatic foodwebs.[22] Furthermore, algae play a role in the purification of polluted water.[23,24] Algae respond promptly to exposure to various types of pollutants and provide warning signals related to the deterioration of ecological conditions, making them useful as model organisms for assessing the toxicity of pollutants.[25–30] There is extensive literature on the interactions of pharmaceuticals and nanomaterials with algae and the subsequent outcomes of these interactions on survival and growth of algae.[31–38] Mechanisms by which nanomaterials cause ecotoxicity on algae include modifications of membranes and other cell structures, local nutrient depletion and shading induced by physical restraints (clogging effects), solubilization of toxic compounds, and/or production of reactive oxygen species.[39–58] Regarding pharmaceuticals, the main modes of pharmaceutical toxicity for algae are specific and nonspecific inhibition of photosynthesis and reactive toxicity.[59–65] The severity of these ecotoxic effects depend on the chemical nature of pharmaceuticals. For instance, cytostatics, which are used for cancer therapy, tend to inhibit algal growth at doses much below those that analgesics and antibiotics can.[37,38] Despite the existence of numerous ecotoxicity studies of nanoparticles and pharmaceuticals with algae, few have focused on nanopharmaceuticals. In addition, the very nature of nanopharmaceuticals responsible for increased bioavailability, the ability to solubilize hydrophobic molecules, higher payload capacity, excellent stability in aqueous media, and prolonged blood circulation times further motivates ecotoxicity studies with nanotherapeutics. This is because the above-mentioned properties can cause nanotherapeutics to persist for extended periods of time in the environment, and thus have a larger impact on uncontrolled releases and accidental spills.

In this work, we study the interactions of paclitaxel-loaded nanomedicine with *Raphidocelis subcapitata* and *Chlamydomonas reinhardtii*. Here, the focus is on paclitaxel since it is a commonly used antineoplastic agent for the treatment of breast, ovarian, lung, pancreatic, and other types of cancer and is the active ingredient of three different nanomedicines on the market.[66] While it is unknown how the nanomedicine form of paclitaxel is metabolized in humans, the three main metabolites of free (molecular) paclitaxel detected in human bile and the human liver are 6α-hydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel and 6α-3′-*p*-dihydroxy paclitaxel.[67,68] Walle *et al.*[69] reported that the total cumulative urinary excretion of paclitaxel (Taxol) and its metabolites accounted for 14.3 ± 1.4% (mean ± SE) of the dose in human patients. Fecal excretion accounted for 71.1 ± 8.2% of the dose, leading to a total excretion of 85.4 ± 7.9% of the dose. Martin *et al.*[70] reported 0% removal rate for free (non-nanomedicine form) paclitaxel in WWTP sites near Seville, Spain where WWTP processes involve pretreatment and primary (settling) and secondary (activated sludge) treatments. *R. subcapitata* and *C. reinhardtii* are well-established algae models for ecotoxicity studies.[52,71–73] To systematically study how the nanomedicine adsorbs and absorbs on/in algae and how such processes influence algal proliferation, a comprehensive set of experimental techniques were used, including transmission electron microscopy, dynamic light scattering, spectrofluorometry, and optical and laser confocal microscopy.

Experimental

Materials

Paclitaxel ($C_{47}H_{51}NO_{14}$, 99%) was purchased from Selleck Chemicals (Houston, TX); poly(ethylene oxide)-*block*-poly(ε-

caprolactone) (PEO-*b*-PCL, 5k-b-6k, Mw/Mn = 1.3) was purchased from Polymer Source Inc. (Dorval, Quebec, Canada); and tetrahydrofuran (THF, 99+%) and poly-L-lysine were procured from Sigma-Aldrich Co. (St. Louis, MO). Oregon Green® 488 Conjugate (Oregon Green® 488 Taxol, Flutax-2) was purchased from Thermo Fisher Scientific (Waltham, MA). All chemicals were used as received.

Preparation of paclitaxel-loaded nanotherapeutics

The paclitaxel-loaded nanomedicine was prepared as described elsewhere.[74–76] Briefly, paclitaxel (0.002 g) and PEO-*b*-PCL (0.03 g) were dissolved in 1 ml THF, which was then intensely mixed with 9 ml Milli-Q water using a probe sonicator at 1200 W for 5 min (SJIA-2000W, Ningbo Haishu Sklon Electronics Instruments Co., Zhejiang, China). Then, the dispersion of paclitaxel-loaded particles was dialyzed in algal growth medium using a standard regenerated cellulose membrane (molecular weight cut-off 12 000–14 000 Da, Spectrum Laboratories, Inc., Rancho Dominguez, CA) to remove THF from the dispersion. To facilitate the removal of THF, the OECD medium was replaced with fresh OECD medium every 30 min until no THF was detected by olfactory analysis.

Characterization of paclitaxel-loaded nanotherapeutics

The particle size distribution of paclitaxel nanoparticles after the dialysis was measured using dynamic light scattering (DLS) with a Zetasizer ZS90 particle size and zeta potential analyzer (Malvern Instruments, Ltd., Westborough, MA). The measurements were carried out at a scattering angle of 90° at 25 °C. The morphology of the nanotherapeutics was characterized using a JEOL JEM-2010 transmission electron microscope (JEOL USA, Inc., Peabody, MA). In these measurements, the nanomedicine dispersion was added dropwise onto a copper grid (400 mesh) with a carbon film (CF400-Cu, Electron Microscopy Sciences, Hatfield, PA). Then, the sample was fully dried under ambient conditions prior to TEM analysis. Observations were done at 200 kV accelerating voltage, $<2.5 \times 10^{-5}$ Pa pressure, and 25 °C.

The release rates of paclitaxel from the nanomedicine were examined *via* a dialysis-based assay.[77,78] Briefly, 10 ml of the nanomedicine dispersion was placed in a standard regenerated cellulose dialysis membrane (MWCO: 12 000–14 000, Spectrum Laboratories Inc., California, USA) and dialyzed against 200 mL water or algae medium. The aliquots from the reservoir were collected at various times and analysed with a UV/vis spectrometer (UV-1800, Shimadzu Corporation, Kyoto, Japan).

Algal growth and exposure experiments

R. subcapitata and *C. reinhardtii* algae cells were obtained from Carolina Biological Supply Company (Burlington, NC). The algae were grown in algal growth medium described in the guidelines by the Organisation for Economic Cooperation and Development (OECD TG 201 medium).[79] The resultant algal cultures were counted using a hemocytometer and then diluted into 200 mL of fresh OECD medium to yield a concentration of 10^5 cells per L. Then, the algae cultures were exposed to free paclitaxel at the solubility limit in water (0.2 μg mL^{-1})[80] or to the paclitaxel-loaded nanomedicine at varying concentrations where the net paclitaxel concentration was 0.2, 0.6, 1.8, 5.4, or 16.2 μg ml^{-1} in the suspension. The algal culture with no treatment was the control group. Each of these conditions was performed in triplicate. The point of exposure was taken as time zero. The number of algae after the treatments was determined by taking 3 mL aliquots from the treated solutions and measuring their fluorescence levels at an excitation wavelength of 350 nm using a spectrofluorometer (PTI QuantaMaster, The Fluorescence Solutions Company, Edison, NJ) at day 0, 1, 2, and 3 for *R. subcapitata* and at day 0, 1, 2, 3, and 5 for *C. reinhardtii*. This analysis was carried out by preparing a calibration curve using cell counts at time zero, T_0, and converting the rest of the fluorescence values to cell numbers. The inhibitory concentration of paclitaxel-based nanomedicine leading to a 50% reduction in algal growth rate compared to the controls (*i.e.* IC_{50}) was calculated using a linear interpolation.[81]

Characterization of algae and nanoparticulate uptake by algae

The shape and dimensions of algae were characterized using optical microscopy (Zeiss LSM 780 NLO, Carl Zeiss Microscopy GmbH, Pleasanton, CA). To enable fluorescence tracking of the therapeutic agent needed in uptake studies, paclitaxel/Oregon Green® 488 conjugate rather than just paclitaxel was used for the preparation of paclitaxel-loaded nanomedicine. The exposure studies were conducted using paclitaxel/Oregon Green® 488 conjugate and the nanomedicine containing paclitaxel/Oregon Green® 488 conjugate. Here, Oregon Green® 488 was particularly selected to ensure that the fluorescence emission of the tracked particles peaks at a wavelength sufficiently away from that of algal chlorophylls. After the preparation of fluorescent-tagged materials, 1.0 ml of an aliquot from each algae stock solution at a concentration of 10^5 cells per L was mixed with 1.0 ml of fluorescent-tagged free paclitaxel (0.2 μg ml^{-1}) or paclitaxel-based nanotherapeutics with a net paclitaxel concentration of 0.2 μg mL^{-1} and incubated for 2 h. Before confocal microscopy imaging, the exposed algal cultures were washed once with OECD medium and immobilized on glass cover slides that were coated with poly-L-lysine solution. The images were obtained using a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems Inc., Buffalo Grove, IL) at an excitation wavelength of 488 nm.

Photosynthetic activity measurements

The effect of nanoparticles on the photosynthetic activity of both *R. subcapitata* and *C. reinhardtii* was measured by fast repetition rate fluorometry (FRRF) using a FASTtracka instrument (Chelsea Technologies Group Ltd.). FRRF was used with an acquisition sequence of 100 saturation flashes of 1 μs

duration and 10 ms sleep time between acquisitions, after a 30 min dark adaptation period of the cultures. The operating efficiency of photosystem II, F_v/F_m ($(F_m - F_0)/F_m$, dimensionless), was obtained for four different treatments realized in triplicate, including control (without any drug), free drug (0.2 μg ml^{-1}), and the lowest (0.2 μg ml^{-1}) and the highest (18 μg ml^{-1}) nanomedicine concentrations.

Statistical analysis

Data and statistical analyses were carried out using ORIGIN® v8 software (OriginLab Corp., Northampton, MA). One-way analysis of variance (ANOVA) was used to test the differences between treatments and Tukey's Honestly Significant Differences (HSD) test was performed to separate means differing at $p < 0.05$. Error bars in the figures are the standard deviations.

Results and discussion

Characterization of paclitaxel-based nanomedicine and microorganisms

The nanoparticle size is known to affect the efficacy and pathway of cellular uptake and blood circulation of nanoparticles. Commercial nanomedicines for intravenous administration are often prepared in a way that results in a particle size in the range of 50–300 nm to ensure prolonged blood circulation and passive targeting.[78,82,83] The average intensity-weighted hydrodynamic size of the prepared paclitaxel-based nanomedicine was found to be 84 ± 4 nm with a polydispersity index of 0.19 *via* DLS (Fig. 1a). Since the size of the prepared nanomedicine lies within the range of optimum size for intravenous drug delivery applications, the prepared nanomedicine represents a suitable model from a size perspective.

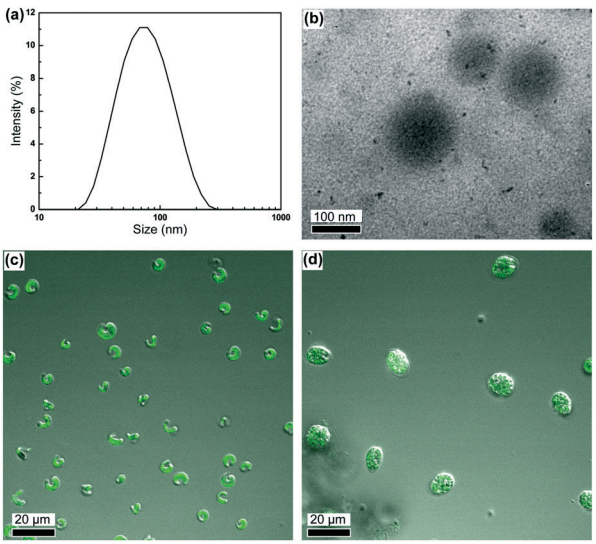

Fig. 1 (a) Size distribution for paclitaxel NPs obtained *via* DLS analysis, (b) transmission electron micrograph of paclitaxel NPs, and microscopy images of (c) *R. subcapitata* and (d) *C. reinhardtii* algae cells.

The shape of nanoparticles is another important parameter influencing their cellular uptake.[84] For instance, Chithrani *et al.*[85,86] reported that spherical particles of similar size were taken up 500% more than rod-shaped particles, which was attributed to the greater membrane-wrapping time required for the elongated particles. Hence, to better interpret the algal uptake data, we characterized the morphology of the prepared nanomedicine using TEM (Fig. 1b), which revealed that the particles are spherical in shape as most commercial nanotherapeutics.

Fig. 1c and d show the size and shape of *R. subcapitata* and *C. reinhardtii* cells, respectively, before any treatments. It was observed that while *R. subcapitata* cells have a curved and twisted appearance, with an arc length of 9.3 ± 1.8 μm and a width of 1.9 ± 0.4 μm, *C. reinhardtii* cells were mostly spherical with a diameter of 9.8 ± 1.2 μm. In summary, these microorganisms are approximately two orders of magnitude larger in size than the nanomedicine (*i.e.*, about six orders of magnitude larger in volume).

Fig. 2 shows the release kinetics of paclitaxel from the nanocarrier as a function of time in water and algae medium. The rate of release did not depend on the concentration of paclitaxel and if the dispersing medium is water or algae medium. For all cases, the release profile reached a plateau at around 22–25 h. The steady-state cumulative release percentage was slightly higher in algae medium and at lower concentrations.

Effect of free-paclitaxel and paclitaxel-based nanomedicine on algal growth

To compare the effect of paclitaxel in solution and nanoparticulate formulation on algae growth, *R. subcapitata* and *C. reinhardtii* cells were exposed to free paclitaxel at the solubility limit in water or at varying concentrations of paclitaxel-based nanomedicine for up to 5 days, and the resultant algal growth was spectrofluorometrically determined as a function of time (Fig. 3). In the case of *R. subcapitata*, these studies

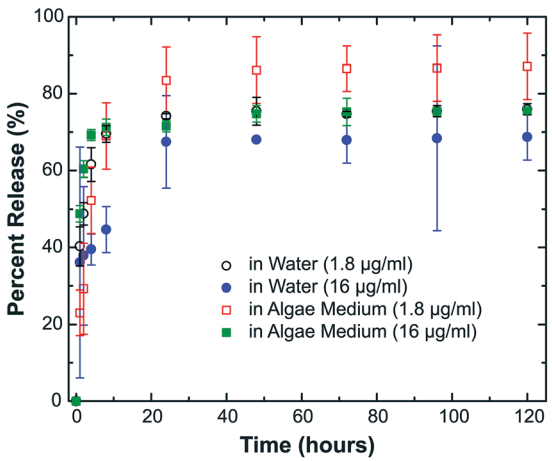

Fig. 2 Release kinetics of paclitaxel-loaded nanomedicine in water and algae medium at two different concentrations.

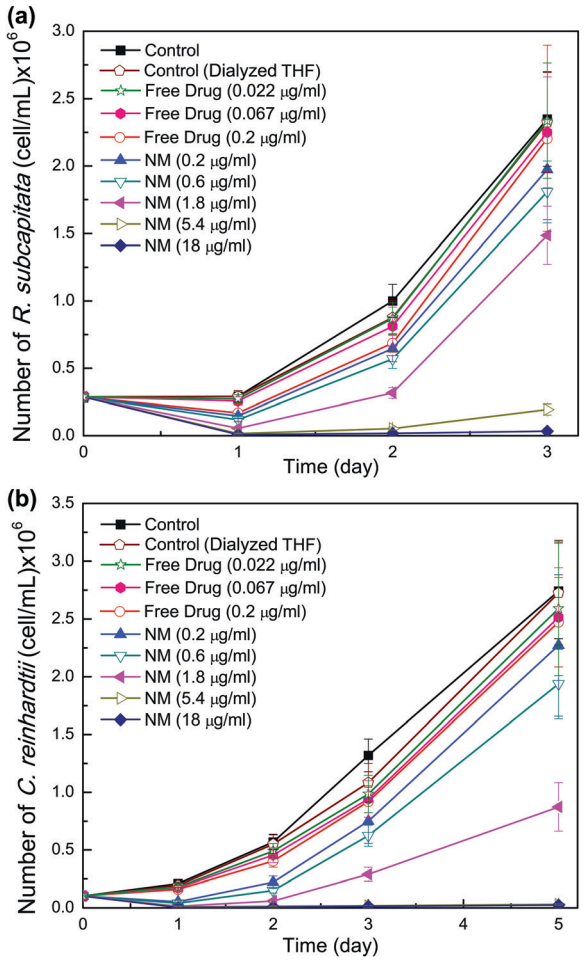

Fig. 3 Effect of free (molecular) paclitaxel and paclitaxel-based nanomedicine on the growth of (a) *R. subcapitata* and (b) *C. reinhardtii* algae cells. The concentrations of nanomedicine (NM) are given in terms of the nominal paclitaxel concentration.

revealed the following (Fig. 3a): first, both the free and nanoparticulate forms of paclitaxel reduced the algal population in the short term (up to day 1). Second, for a given paclitaxel concentration (0.2 μg ml^{-1}), the nanoparticulate form of paclitaxel hindered the algal growth more than the free form, indicating an enhanced algal toxicity of the nanoparticulate form. Third, the algal growth slowed down with increasing nanomedicine concentration in the range of a net paclitaxel concentration of 0.2 to 5.4 μg ml^{-1} and completely ceased at a nanomedicine concentration corresponding to 16.2 μg paclitaxel ml^{-1}. Fourth, in the case of paclitaxel nanomedicine, the IC_{50} value was 1.6 ± 0.1 μg paclitaxel ml^{-1} for 72 h. On the other hand, free-paclitaxel could not lead to growth inhibition to the IC_{50} level due to the poor water solubility of paclitaxel in water, 0.2 μg ml^{-1}. The highest dose that is not statistically significantly different from the control response was 0.022–0.067 μg ml^{-1}, which is the no observed effect concentration (NOEC) for free-paclitaxel. Fifth, empty nanoparticles (nanocarriers) gave rise to identical data with the control (not shown), which is because polymers used in the formation of the nanocarriers are proven non-toxic, biocompatible materials. TFH, which may remain in the medium after the dialysis stage, did not result in any statistical difference in algal growth at concentrations corresponding to the residual THF after dialysis.

To put the IC_{50} values into perspective, we compare this value with the IC_{50} values of other nanomaterials and chemicals in the literature. For instance, toxicity studies involving 10–20 nm CeO_2 nanoparticles and *R. subcapitata* indicated a 72 h IC_{50} value of 10.3 ± 1.7 μg ml^{-1}.[87] Franklin *et al.*[88] reported that *R. subcapitata* was sensitive to ZnO nanoparticles (30 nm), with a 72 h IC_{50} value of 68 μg Zn L^{-1}, mostly due to dissolved zinc. Aruoja *et al.*[89] found that the *R. subcapitata* EC_{50} values (72 h) of most nonpolar narcotic chemicals, including pentachloroethane, 2,4-dichlorotoluene, *m*-xylene, trichloroethene, and hexanol, were in the range of 2–200 μg ml^{-1}. Common antibacterial agents, such as triclosan, triclocarban, roxithromycin, and clarithromycin, were shown to inhibit *R. subcapitata* growth with a 72 h IC_{50} of 0.5 to 46 μg L^{-1}.[90]

Overall, while most nonpolar narcotic compounds and CeO_2 nanoparticles yield lower toxicity to *R. subcapitata* compared to the paclitaxel-based nanomedicine, ZnO nanoparticles and antibacterial agents, such as triclosan, triclocarban, roxithromycin, and clarithromycin, were significantly more toxic to *R. subcapitata* than the paclitaxel-based nanomedicine.

Similar trends were also observed for the case of *C. reinhardtii* with a few differences (Fig. 3b). First, molecular paclitaxel did not cause a decrease in algal population at any time point. Second, the paclitaxel-based nanomedicine inhibited *C. reinhardtii* growth to a greater extent in comparison with *R. subcapitata* growth. The IC_{50} value of paclitaxel-based nanomedicine was 1.1 ± 0.1 μg paclitaxel ml^{-1} for *C. reinhardtii* while it was 1.6 ± 0.1 μg paclitaxel ml^{-1} for *R. subcapitata*. Third, the algaecide effect of paclitaxel-based nanomedicine was observed at a lower concentration (5.4 μg paclitaxel ml^{-1}) instead of 16.2 μg paclitaxel ml^{-1}. Considering that paclitaxel is highly lipophilic, a higher fatty acid content of *C. reinhardtii*, *i.e.* ~9% of dry cell weight (DCW)[91] compared to ~7% fatty acid of DCW in *R. subcapitata*[92] may account for this difference. Furthermore, the ratio of unsaturated fatty acids to all fatty acids is larger for *R. subcapitata* (~75%)[92] than for *C. reinhardtii* (~65%).[91] *C. reinhardtii* cells are much more fragile than *P. subcapitata* cells because the *R. subcapitata cell* wall is composed of cellulose and other polysaccharides while the cell wall of *C. reinhardtii* does not contain cellulose and other polysaccharides.[93,94] This difference implies a more rigid and ordered membrane structure and, hence, a lower permeability of the cell membrane for *R. subcapitata*. Fourth, as in the case of *R. subcapitata*, the NOEC of free-paclitaxel for *C. reinhardtii* was 0.022–0.067 μg ml^{-1}.

Regarding the prior studies focusing on the effect of nanomaterials on *C. reinhardtii* growth, Chen *et al.*[95] reported that *C. reinhardtii* algae cells were significantly damaged by an

increased concentration of TiO_2 nanoparticles. The algae cells were exposed to nanoparticulate dispersions with 0.1, 1, 10, 20 and 100 μg ml^{-1} TiO_2 nanoparticles, and the growth of algae cells were shown to stop at an exposure concentration of 100 μg ml^{-1}. Perreault *et al.*[96] measured a 30 min and 24 h EC_{50} of 0.114 mg ml^{-1} and 0.083 mg ml^{-1} for the polyamidoamine-coated gold nanoparticle–*C. reinhardtii* system, respectively. CuO NPs were shown to cause growth inhibition on *C. reinhardtii* with a 72 h EC_{50} of 150.45 ± 1.17 μg ml^{-1}.[42] Hu and co-workers[97] found that 4 nm CdTe quantum dots (QDs) inhibited *C. reinhardtii* growth above a concentration of 1 μg ml^{-1} and these microorganisms were more sensitive to QDs than to TiO_2 nanoparticles.

Interactions of free paclitaxel and paclitaxel-based nanomedicine with algae cells

To gain mechanistic insights into the interactions of paclitaxel-based nanomedicine and algal *R. subcapitata* and *C. reinhardtii* cells, we carried out confocal microscopy studies with the aid of paclitaxel conjugated with a fluorophore, in the free (molecular) form as well as in the nanoparticulate form. As can be seen from Fig. 4a and b, for a given paclitaxel/fluorophore concentration, the ratio of *R. subcapitata* cells with fluorophores to all cells was higher in the case of exposure to paclitaxel-loaded nanomedicine: >79 ± 11% in comparison with 38 ± 5%. While the exposure to paclitaxel-loaded nanomedicine of *C. reinhardtii* led to a percent localization of 88.1 ± 10.3%, which is statistically not different from that for *R. subcapitata* (one-way ANOVA, $P > 0.05$), the exposure to free-paclitaxel gave rise to a higher localization for *C. reinhardtii* cells (66 ± 2%) compared to *P. subcapitata* cells (38 ± 5%) (Fig. 4 and 5). The reason behind the enhanced localization of paclitaxel in/on algae for the nanoparticulate form can be attributed to two phenomena. First, the adsorption of free-paclitaxel and paclitaxel-based nanomedicine on the algae surface is mainly governed by van der Waals interactions, which are well-known to be body-forces. This indicates a stronger attraction between larger objects *i.e.*, nanomedicine and algae cells compared to drug molecules and algae cells. Furthermore, due to the existence of hydrogen bonding groups on an algae cell[98] and since the stabilizing shell of the nanomedicine, PEO-*b*-PCL, contains a large number of hydroxyl and ether groups,[99] favorable hydrogen bonding interactions arise between the nanomedicine and the algae cells.

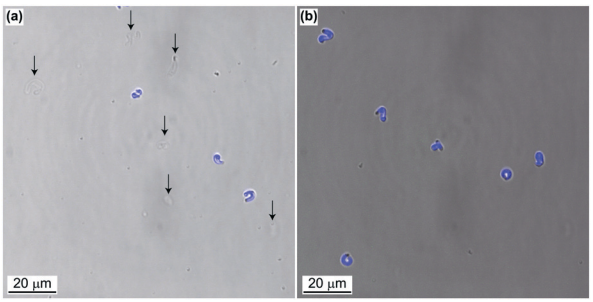

Fig. 4 Confocal microscopy images of *R. subcapitata* cells in the presence of (a) the free drug (paclitaxel) and (b) paclitaxel-based nanomedicine. Arrows indicate the empty cells (*i.e.* cells without drug localization).

The presence of paclitaxel (both forms) resulted in flocculation of *C. reinhardtii* cells but not *R. subcapitata* cells (Fig. 3 and 4), suggesting a stronger interaction between paclitaxel/paclitaxel-based nanomedicine and *C. reinhardtii*. To explain this discrepancy between *C. reinhardtii* and *R. subcapitata*, we measured their zeta potentials and found the zeta-potentials to be −21.9 ± 0.9 mV for *R. subcapitata* and −8.51 ± 0.2 mV for *C. reinhardtii*. These values indicate that double-layer electrostatic repulsion between *C. reinhardtii* cells are much weaker, *i.e.* more likely to agglomerate. Furthermore, the introduction of paclitaxel having hydroxyl, carbonyl, and amino groups[100] or poly(ethylene oxide)-*block*-poly(ε-caprolactone) having hydroxyl and ether groups[99] may link the algae cells and form "algal coacervates" through hydrogen bonding. It is also possible that aggregation could be a self-protection mechanism of algal cells, which relies on the minimization of their surface area through aggregation.[101] Similar to our findings, Perreault *et al.*[102] also found that when *C. reinhardtii* cultures were exposed to mannose-functionalized Au NPs, 90.5 ± 6.3% of algae cells were in an aggregated form, indicating nanoparticle-induced aggregation and clustering of algal cell cultures. Likewise, Behra and co-workers[43] reported that exposure to CeO_2 nanoparticulate aggregates resulted in flocculation of *C. reinhardtii* cells.

For both types of microorganisms, the cells exposed to paclitaxel or paclitaxel-loaded nanomedicine slightly shrank and deformed (Fig. 1, 3, and 4). The deformations were slightly more noticeable for the case of *C. reinhardtii* presumably owing to soft and flexible hydroxyproline-rich glycoprotein layers. In addition, lipophilic paclitaxel prefers to internalize in a lipid-rich environment as in the case of *C. reinhardtii* compared to *R. subcapitata*.[91,92] However, the degree of morphological alterations induced by paclitaxel-loaded nanomedicine is much less than that observed with hard inorganic nanoparticles such as alumina, silica, titania, and nickel oxide.[103–106] This difference may be ascribed to the dynamic nature of the nanomedicine in which there are continuous rearrangements of building blocks (*i.e.*, diblock copolymers) due to thermal energy and entropic factors, its ability to reassemble and disassemble in the presence of certain stimuli, and its soft and deformable nature.

Effect of free paclitaxel and paclitaxel-based nanomedicine on photosynthetic activity

The FRRF methodology is commonly used to evaluate primary productivity in aquatic systems. F_v/F_m values were compared for *R. subcapitata* and *C. reinhardtii* in Fig. 6. The highest (18 μg ml^{-1}) and the lowest (0.2 μg ml^{-1}) nanoparticle concentrations significantly decreased (student's *t*-test, *p*-value

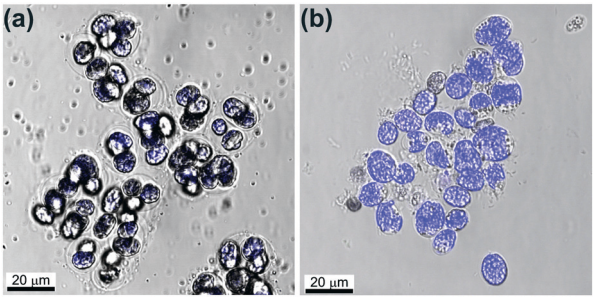

Fig. 5 Confocal microscopy images of *C. reinhardtii* cells in the presence of (a) free paclitaxel and (b) paclitaxel-based nanomedicine.

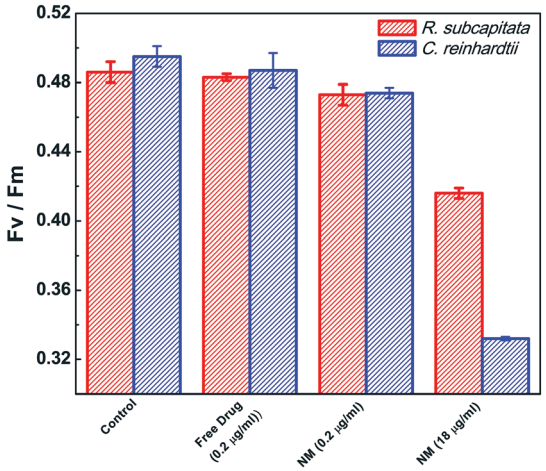

Fig. 6 F_v/F_m values for *R. subcapitata* and *C. reinhardtii* depending on the treatment.

< 0.05) the photosynthesis efficiency of both algae, while no significant difference was observed between the control and the free drug treatment. Therefore, it can be concluded that paclitaxel nanoparticles negatively influence photosynthesis efficiency compared to the free drug, and their negative effect can be elevated by increasing the nanoparticle concentration. Moreover, the photosynthesis efficiency of *C. reinhardtii* showed higher sensitivity to paclitaxel nanoparticle exposure since *C. reinhardtii* showed a significantly (Student's *t*-test, *p*-value < 0.05) lower F_v/F_m value than *R. subcapitata* at NM (18 μg ml^{-1}).

Conclusions

This work is concerned with the interactions of paclitaxel-loaded nanomedicine with *R. subcapitata* and *C. reinhardtii* algae cells as well as the potential consequences of these interactions on the dynamics of algal growth. The key findings are as follows: first, for a given drug concentration, paclitaxel-load nanomedicine inhibits algal growth more than molecular (free) paclitaxel. While the molecular paclitaxel at the solubility limit (*i.e.* maximum solubility in water, ~0.2 μg ml^{-1}) was not enough to hinder the algal growth to reach an IC_{50} level, paclitaxel-loaded nanomedicine had a 120 h IC_{50} value of 1.1 ± 0.1 μg paclitaxel ml^{-1} for *C. reinhardtii* and a 72 h IC_{50} value of 1.6 ± 0.1 μg paclitaxel ml^{-1} for *R. subcapitata*. This result indicates that due to its ability to solubilize water insoluble (lipophilic) drug molecules in it, the nanomedicine can cause ecotoxic effects on algae that are not otherwise possible. Second, the nanomedicine form of paclitaxel also demonstrates higher localization/internalization on algal cell surfaces suggesting favorable interactions between hydrogen bonding groups on algae cells and the stabilizing shell of the nanomedicine, poly(ethylene oxide)-*block*-poly(ε-caprolactone), which contains a large number of hydroxyl and ether groups. Third, an increasing exposure concentration of paclitaxel-loaded nanomedicine results in a decrease in the growth rate. In addition, concentrations above 16.2 μg paclitaxel ml^{-1} for *R. subcapitata* and above 5.4 μg paclitaxel ml^{-1} for *C. reinhardtii* lead to an algaecidal effect (*i.e.* inability to grow any algae). Fourth, the presence of paclitaxel-loaded nanomedicine also hindered the photosynthetic activity while free-paclitaxel caused no significant effect on it. Overall, the increasing production and consumption of nanomedicines is a valid ecological and environmental concern given that the nanomedicine form of paclitaxel, a commonly used drug for cancer treatments, can inhibit the growth of freshwater algae and even show an algaecidal effect at concentrations above several parts per million (ppm).

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