



# Investigating the Impact of Cerium Oxide Nanoparticles Upon the Ecologically Significant Marine Cyanobacterium *Prochlorococcus*

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Cerium oxide nanoparticles (nCeO<sub>2</sub>) are used at an ever-increasing rate, however, their impact within the aquatic environment remains uncertain. Here, we expose the ecologically significant marine cyanobacterium *Prochlorococcus* sp. MED4 to nCeO<sub>2</sub> at a wide range of concentrations (1 μg L<sup>-1</sup> to 100 mg L<sup>-1</sup>) under simulated natural and nutrient rich growth conditions. Flow cytometric analysis of cyanobacterial populations displays the potential of nCeO<sub>2</sub> (100 μg L<sup>-1</sup>) to significantly reduce *Prochlorococcus* cell density in the short-term (72 h) by up to 68.8% under environmentally relevant conditions. However, following longer exposure (240 h) cyanobacterial populations are observed to recover under simulated natural conditions. In contrast, cell-dense cultures grown under optimal conditions appear more sensitive to exposure during extended incubation, likely as a result of increased rate of encounter between cyanobacteria and nanoparticles at high cell densities. Exposure to supra-environmental nCeO<sub>2</sub> concentrations (i.e., 100 mg L<sup>-1</sup>) resulted in significant declines in cell density up to 95.7 and 82.7% in natural oligotrophic seawater and nutrient enriched media, respectively. Observed cell decline is associated with extensive aggregation behaviour of nCeO<sub>2</sub> upon entry into natural seawater, as observed by dynamic light scattering (DLS), and hetero-aggregation with cyanobacteria, confirmed by fluorescent microscopy. Hence, the reduction of planktonic cells is believed to result from physical removal due to co-aggregation and co-sedimentation with nCeO<sub>2</sub> rather than by a toxicological and cell death effect. The observed recovery of the cyanobacterial population under simulated natural conditions, and likely reduction in nCeO<sub>2</sub> bioavailability as nanoparticles aggregate and undergo sedimentation in saline media, means that the likely environmental risk of nCeO<sub>2</sub> in the marine environment appears low.

**Keywords:** *Prochlorococcus*, ecotoxicology, nanomaterials, cerium oxide, marine pollution, phytoplankton

## INTRODUCTION

Cerium oxide nanoparticles (nCeO<sub>2</sub>) represent an emerging contaminant with widespread use across a range of industries. Nano-sized cerium possesses unique properties compared to the bulk material and exhibit properties such as redox activity, scavenging of free radicals and inhibition of biofilm formation (Singh et al., 2020), leading to their use across a variety of applications including; biomedical applications (Das et al., 2013; Nyoka et al., 2020), drug delivery and therapeutics (Nadeem et al., 2020), glass production and incorporation into electronic components (Merrifield et al., 2013).

Annual production of nCeO<sub>2</sub> is estimated to be approximately 10,000 tonnes (European Commission, 2012), and their ever-increasing use is likely to result in greater release of engineered nCeO<sub>2</sub> into the environment (Collin et al., 2014). The use of nCeO<sub>2</sub> as an additive for diesel fuels is a major ecological concern, and is believed to be the main source of nCeO<sub>2</sub> particles into the natural environment (Johnson and Park, 2012). Overall, it is predicted up to 70 million metric tonnes of ceria could be released annually by road transport worldwide (Dale et al., 2017), with the highest environmental levels of nCeO<sub>2</sub> predicted to be present in water draining from road surfaces (Johnson and Park, 2012), where nanoparticles may then enter the aquatic environment. Additionally, aquatic transport responsible for 80% of global trade shipping (Hallquist et al., 2013) may represent an understudied source of nCeO<sub>2</sub> release. Boat engines have been recorded to utilise fuels containing nanosized additives including cerium (Somasundaram et al., 2017; Jiaqiang et al., 2018; Somasundaram et al., 2020). It is possible that nCeO<sub>2</sub> may enter the ocean in this manner if used in boat fuels to enhance efficiency (Somasundaram et al., 2020), and may represent a previously understudied source of nCeO<sub>2</sub> entry into the environment. Aside from transport, nCeO<sub>2</sub> may enter the natural environments *via* release into wastewater, use of wastewater treatment sludge as fertiliser, or as a consequence of industrial production of nCeO<sub>2</sub> or related products (Hu et al., 2006; Limbach et al., 2008).

Research carried out to date suggests that the environmental risk of nCeO<sub>2</sub> is low (Johnson and Park, 2012), however, limited evidence exists outlining the potential impact of nCeO<sub>2</sub> upon marine microorganisms. Upon entry into the environment, airborne and waterborne nCeO<sub>2</sub> may become widely dispersed (Merrifield et al., 2013). In some cases, nanoparticulate matter emitted from diesel engines has been traced internationally, for example nCeO<sub>2</sub> released in the United Kingdom has been identified in mainland Europe (Charron and Harrison, 2005). However, insufficient data exists to accurately predict the current levels of nCeO<sub>2</sub> in the natural environment (Johnson and Park, 2012; Neil et al., 2021), and great variation exists in such estimations. For example, Dale et al. (2017) predict a global release of 70 million metric tonnes of Ce-based pollution into the environment by road transport, a value considerably higher than the estimated 15.6–114.9 kg year<sup>-1</sup> predicted in the United Kingdom by Johnson and Park (2012). To address this uncertainty, increased efforts must be placed into improving methods by which we are able to accurately monitor

the release of engineered nanomaterials into the environment. Subsequently, experimental work can be carried out under environmentally relevant conditions, facilitating the effective assessment of the environmental risk of nanosized contaminants in natural systems.

The marine environment represents the sink for pollutants entered into aquatic systems. Here, microbial organisms which display high surface area-to-volume ratios are at significant risk from waterborne pollutants, particularly in coastal regions (Lucas et al., 2011). These microorganisms represent the base of the marine food chain and play key roles in the functioning of the entire marine ecosystem (Field et al., 1998; Flombaum et al., 2013). Therefore, understanding the impact of pollutants upon these key species is of great importance. Metal oxide nanomaterials including; titanium dioxide (Clement et al., 2013; Manzo et al., 2015; Xia et al., 2015; Deng et al., 2017), zinc oxide (Miao et al., 2010; Wong et al., 2010) and iron oxide (Demir et al., 2015) have previously been reported to exert adverse effects upon marine phytoplankton. Such effects include growth inhibition (Wong et al., 2010; Clement et al., 2013), reduced photosynthetic performance (Deng et al., 2017), induction of oxidative stress (Xia et al., 2015; Deng et al., 2017) and physical attachment (Demir et al., 2015; Manzo et al., 2015; Deng et al., 2017). However, limited evidence is available examining the impact of nCeO<sub>2</sub> upon marine microbial species. A significant reduction in growth of marine phytoplankton has been recorded in response to nCeO<sub>2</sub> exposure at concentrations in the range 10–40 mg L<sup>-1</sup> (Deng et al., 2017; Sendra et al., 2017), although findings vary. At concentrations of up to 5 mg L<sup>-1</sup> no adverse effects of nCeO<sub>2</sub> exposure upon the green algae *Chlorella autotrophica* and *Dunaliella salina* were observed (Sendra et al., 2018). Currently, the effect of nCeO<sub>2</sub> upon marine microbial species under environmental conditions remain uncertain and further research is required to reveal potential impacts.

In this study, we examine the impact of nCeO<sub>2</sub> exposure upon the ecologically significant marine cyanobacterium *Prochlorococcus sp.* MED4. This phototroph has previously been shown to display increased sensitivity to nano-pollutants (i.e., silver) (Dedman et al., 2020) and represents the most abundant phototroph in the world's oligotrophic oceans (Scanlan et al., 2009; Bagby and Chisholm, 2015). Cultures were incubated with nCeO<sub>2</sub> at both environmentally relevant and supra-environmental concentrations under simulated natural conditions (i.e., ambient cell densities grown in oligotrophic natural seawater) (Mella-Flores et al., 2011; Ma et al., 2017), or in cell-dense cultures grown in nutrient-rich media, for a period of 72–240 h. Alterations in cyanobacterial cell density were monitored by flow cytometry. An assessment of nCeO<sub>2</sub> behaviour in saline media was carried out by dynamic light scattering (DLS) and microscopic methods, revealing extensive aggregation behaviour, believed to drive the observed declines in cyanobacterial abundance recorded during exposures. Such research presents novel insight into the likely behaviour and impact of an emerging contaminant within the marine environment for which limited information exists, hence, facilitating the effective evaluation of their likely environmental risk and interaction with microbial species.

## MATERIALS AND METHODS

### Materials

Nanomaterials utilised for research were purchased from Sigma Aldrich [cerium oxide, <25 nm (Material No. 544841)]. Natural oligotrophic seawater (NSW) used to culture cyanobacteria and during experimental work was collected from Station L4, Plymouth (50°15.0'N; 4°13.0'W). Prior to use, NSW was autoclaved (120°C, 20 min) and filtered through a 0.22 μm polyethersulfone membrane (Corning®). For each experiment nCeO<sub>2</sub> stocks were added directly to NSW prepared as described and sonicated for 15–30 min using a Branson 1210 Sonicator operating at 40 kHz to ensure suspensions were well-mixed and minimise aggregation of nanoparticles. MilliQ ultrapure water used throughout laboratory work was 0.22 μm filter operated at 18.2 MΩ at 298 K. Sterile filter capped tissue culture flasks were used for all experimental work with cyanobacteria and glassware was acid-washed before use. Axenic *Prochlorococcus* sp. MED4 cultures (isolate source: Mediterranean Sea, South France) were grown on-site using Pro99 media (see **Supplementary Section “Pro99 Media”**; Moore et al., 2002).

### Characterisation of nCeO<sub>2</sub> and Dynamic Light Scattering Assessment of Behaviour in Natural Seawater

To determine primary particle size and examine the morphology of nCeO<sub>2</sub> utilised during study, transmission electron microscopy (TEM) was used. Here, images were taken using a JEOL 2100 TEM (200 kV, LaB<sub>6</sub> instrument; beam current ~115 mA), equipped with a Gatan Orius 11-megapixel camera. To prepare samples for TEM imaging, nCeO<sub>2</sub> stock was made up in MilliQ ultrapure water and 10 μL deposited into formvar-coated 300 mesh copper grids (EM Resolutions). Following image acquisition, the average particle diameter was determined by measuring 100 nanoparticles using Image J v.3.2 software. Visual observation of particle morphology was also carried out at this time. UV-visible spectra were collected using an Agilent Cary 60 UV-vis spectrophotometer of a sample of 100 mg L<sup>-1</sup> nCeO<sub>2</sub> in MilliQ ultrapure water. Dynamic light scattering (DLS) was used to evaluate the aggregation behaviour of nCeO<sub>2</sub> (1 and 100 mg L<sup>-1</sup>) when entered into NSW for a period of 240 h. Using a Malvern Zetasizer Nano ZS instrument, equipped with a 4 mW He-Ne 633 nm laser module, measurements of z-average size and polydispersity in MilliQ ultrapure water were first obtained. Following this, triplicate suspensions of nCeO<sub>2</sub> were made up in 20 mL NSW in 50 mL tissue culture flasks, maintained at ambient room temperature under shaking (orbital shaker, 100 rpm) to simulate natural water motion. At 0, 1, 2, 4, 24, 48, 72, 168, and 240 h, a 200 μL sub-sample was collected from the mid-point flasks, representing the suspended fraction, and average values for z-average size, polydispersity index (PDI) and mean count rate recorded based on 3 measurements consisting of 11 sampling runs each lasting 10 s. Zeta-potential of nCeO<sub>2</sub> (1 and 100 mg L<sup>-1</sup>) in MilliQ ultrapure water, and NSW diluted 1:1 in MilliQ ultrapure water, was additionally monitored at 0 h.

### Short-Term (72 h) Toxicity Testing at Environmentally Relevant Concentrations

*Prochlorococcus* sp. MED4 was exposed to nCeO<sub>2</sub> (0, 1, 10, and 100 μg L<sup>-1</sup>) for a period of 72 h under two test conditions: (i) ambient cell density (2.8 × 10<sup>4</sup> cells mL<sup>-1</sup>) grown in NSW and (ii) cell-dense cultures grown in nutrient-rich Pro99 media (8.9 × 10<sup>5</sup> cells mL<sup>-1</sup>). Seventy-two hours prior to experimentation, axenic culture was added to NSW or Pro99 media, respectively, and preadapted to experimental conditions: 23°C and 10 μmol photons m<sup>-2</sup> s<sup>-1</sup> light intensity (Lifelite™ full spectrum bulb) whilst shaking (100 rpm). To establish exposures, triplicate 30 mL samples were added to 50 mL filter-capped tissue culture flasks under sterile conditions and spiked with a defined volume of nCeO<sub>2</sub> stock to make up test concentrations. Flasks were subsequently maintained under the experimental conditions described and the cyanobacterial population monitored at 0, 24, 48, and 72 h using a Becton Dickinson Fortessa Flow Cytometer. Briefly, a 1 mL sample was collected from the mid-point of flasks and for NSW cultures and analysed directly, whilst those obtained from cultures grown in Pro99 media were first diluted 10-times in NSW. To calculate values for cell density, FACSDiva software was used to gate *Prochlorococcus* cells based on their natural autofluorescence and their abundance relative to standard polystyrene reference beads (2.2 μm high Intensity fluorescent Nile Red particles, Spherotech FH-2056-2) was recorded.

### Extended (240 h) Exposure

Following 72 h toxicity testing, incubations with nCeO<sub>2</sub> were repeated and extended to 240 h to examine longer-term impacts of exposure. Initial cell densities were 1.7 × 10<sup>5</sup> and 6.6 × 10<sup>5</sup> cells mL<sup>-1</sup> in NSW and Pro99 media, respectively. The nCeO<sub>2</sub> test concentrations utilised were also expanded to include both environmentally relevant and supra-environmental values (0, 1, 10, and 100 μg L<sup>-1</sup>; and 1, 10, and 100 mg L<sup>-1</sup>), hence allowing for inference of likely impacts in hot spots of pollution or should release of nCeO<sub>2</sub> into the marine environment increase. Exposures were established as described above and the cyanobacterial population monitored by flow cytometry at 0, 24, 48, 72, 168, and 240 h. A summary of test conditions examined is provided in **Supplementary Table 2**.

### Fluorescent Microscopic Analysis of Cyanobacterial-nCeO<sub>2</sub> Interactions

To investigate the presence of cyanobacteria within precipitated material observed during extended (240 h) toxicity testing (section “Extended (240 h) Exposure”), sub-samples of deposited material were collected from the base of culture flasks and stained using SYBR Gold nuclear acid stain (diluted to 1X, ThermoFisher) for > 15 min under darkness. After staining, material was imaged under brightfield and GFP fluorescence at 40x magnification using a Nikon Widefield Fluorescence Microscope. Images obtained from each channel were subsequently merged to produce a composite image and assess hetero-aggregation of cyanobacteria and nCeO<sub>2</sub>.

## Statistical Analysis

To examine the occurrence of statistically significant variations in *Prochlorococcus* MED4 populations exposed to nCeO<sub>2</sub> in short-term (section “Short-Term (72 h) Toxicity Testing at Environmentally Relevant Concentrations”) and extended toxicity tests (section “Extended (240 h) Exposure”), one-way analysis of variance ANOVA tests were carried out using IBM SPSS Statistics v27. Additionally, *post hoc* Dunnett’s *T*-tests were utilised to identify significant alterations in cell density in treated cultures compared to the untreated control at respective timepoints ( $p \leq 0.05$ ).

## RESULTS

### Characterisation of nCeO<sub>2</sub> and Aggregation Behaviour in Natural Seawater

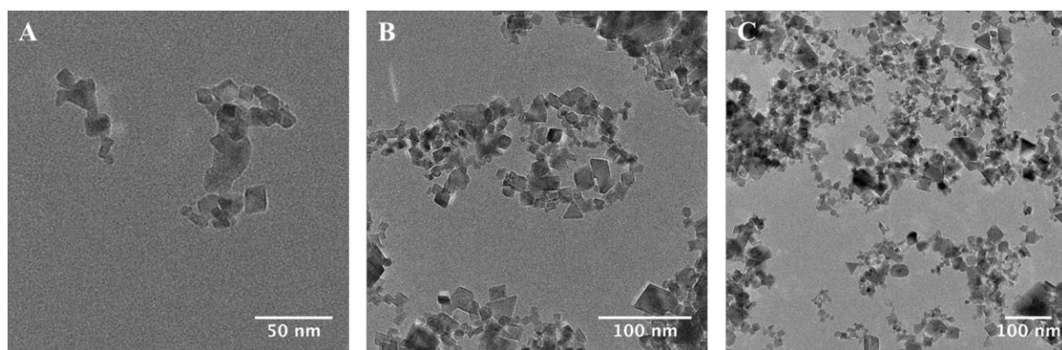
#### Primary Particle Size and Morphology

Analysis of TEM images revealed a primary particle size of  $20.6 \pm 12.1$  nm, close to that stated by the manufacturer (<25 nm). However, nanoparticles displayed a great variation in primary particle size. The size distribution of primary nCeO<sub>2</sub> particles measured during analysis is presented in **Supplementary Figure 1**. In terms of morphology, nCeO<sub>2</sub>

were generally cuboid in shape, although a range of forms could be observed, including diamond- and triangular-shaped particles (**Figures 1A,B**). As is common in TEM analysis, nCeO<sub>2</sub> readily formed aggregates, as a result of drying onto TEM grids (**Figure 1C**). UV-vis spectroscopy demonstrated an absorption band between 250 and 400 cm<sup>-1</sup> with a maximum absorbance at 317 nm (**Supplementary Figure 2**), attributed to the electronic structure of ceria and defects, including oxygen vacancies, and is in line with literature (Fronzi et al., 2009; Calvache-Munoz et al., 2017).

#### Behaviour of nCeO<sub>2</sub> in NSW

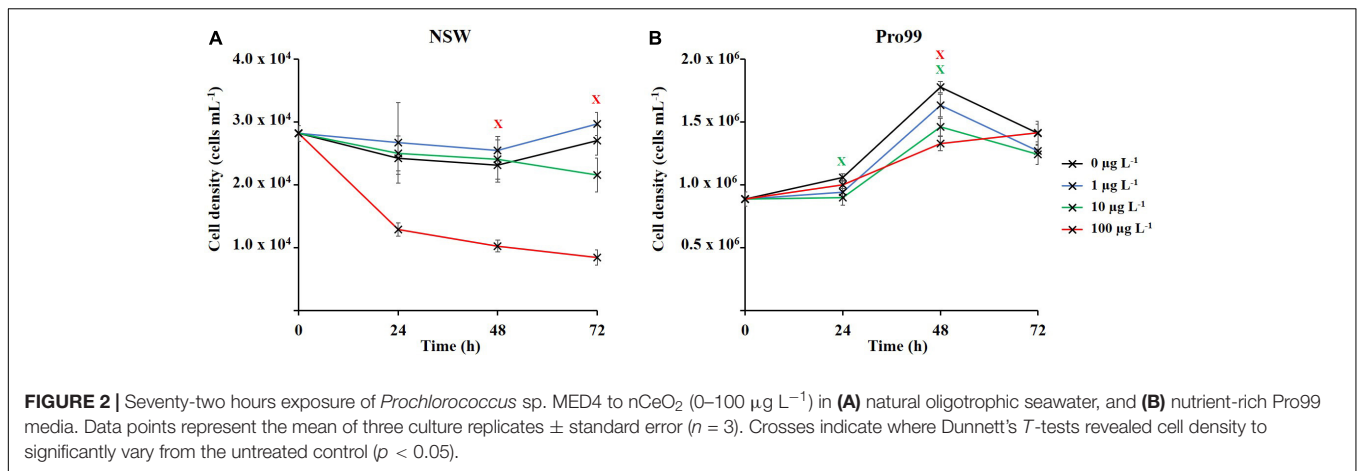
The aggregation behaviour of nCeO<sub>2</sub> ( $20.6 \pm 12.1$  nm, TEM) was examined by monitoring the z-average size of nanoparticles, as measured by DLS, after entry into NSW over a period from 0 to 240 h (**Table 1**). Analysis of nCeO<sub>2</sub> suspensions made up in MilliQ ultrapure water revealed respective z-average sizes of  $136 \pm 2$  nm and  $125 \pm 11$  nm for concentrations of 1 and 100 mg L<sup>-1</sup>. Upon entry into NSW (0 h), z-average size was recorded as  $1,293 \pm 141$  nm and  $1,196 \pm 140$  nm for 1 and 100 mg L<sup>-1</sup> samples, respectively, indicating the immediate aggregation of nCeO<sub>2</sub>. The aggregation of nCeO<sub>2</sub> within saline media with high ionic strength is expected and in accordance with previous research (Keller et al., 2010; Ottofuelling et al., 2011; Quik et al., 2014; Sendra et al., 2017). Measurements of zeta-potential were obtained in MilliQ ultrapure water and NSW at 0 h, revealing



**FIGURE 1** | Transmission electron microscope imaging of nCeO<sub>2</sub> (Sigma-Aldrich, <25 nm) used for experimental work (**A–C**).

**TABLE 1** | Summary of data obtained during DLS analysis of nCeO<sub>2</sub> ( $20.6 \pm 12.1$  nm) added to natural seawater for a period of 240 h at a concentration 1 and 100 mg L<sup>-1</sup> ( $n = 3$ ).

Time (h)	1 mg L <sup>-1</sup>		100 mg L <sup>-1</sup>	
	Z-average size (nm)	Polydispersity index	Z-average size (nm)	Polydispersity index
0	$1,293 \pm 141$	0.525	$1,196 \pm 140$	0.336
1	$2,576 \pm 1,933$	0.683	$3,601 \pm 1,794$	0.716
2	$2,382 \pm 1,924$	0.760	$3,097 \pm 2,784$	0.638
4	$1,862 \pm 489$	0.772	$2,875 \pm 1,757$	0.655
24	$1,767 \pm 187$	0.893	$3,122 \pm 2,622$	0.794
48	$2,338 \pm 1,428$	0.845	$3,414 \pm 2,027$	0.910
72	$1,016 \pm 298$	0.607	$1,549 \pm 449$	0.729
168	$1,390 \pm 971$	0.812	$1,724 \pm 992$	0.816
240	$1,218 \pm 914$	0.799	$1,638 \pm 359$	0.909



**FIGURE 2** | Seventy-two hours exposure of *Prochlorococcus* sp. MED4 to nCeO<sub>2</sub> (0–100 μg L<sup>-1</sup>) in (A) natural oligotrophic seawater, and (B) nutrient-rich Pro99 media. Data points represent the mean of three culture replicates ± standard error ( $n = 3$ ). Crosses indicate where Dunnett's  $T$ -tests revealed cell density to significantly vary from the untreated control ( $p < 0.05$ ).

respective surface charges of  $-8.6 \pm 0.5$  mV and  $-2.8 \pm 0.1$  mV in ultrapure water; and  $-10.6 \pm 1.5$  mV and  $1.4 \pm 0.3$  mV in NSW for 1 and 100 mg L<sup>-1</sup> nCeO<sub>2</sub> samples. Zeta-potential appeared to vary between 1 and 100 mg L<sup>-1</sup> samples, likely due to concentration-dependent effects which appear exacerbated in NSW where ionic strength is increased. Upon entry into NSW, zeta-potential values appeared to vary between the two concentrations, likely due to the extensive aggregation behaviour of nanoparticles upon entry into saline media, as demonstrated by DLS measurements (Table 1). The highly variable nature of the surface charge of nCeO<sub>2</sub> nanoparticles, and related metal oxides (e.g., TiO<sub>2</sub>), upon addition to various experimental media has previously been reported in the literature (Rodea-Palomares et al., 2010; Sendra et al., 2017).

During the first 2 h, nCeO<sub>2</sub> displayed an increase in z-average size at both concentrations, indicating a continuation of the aggregation process. Alongside increases in z-average size, the polydispersity index (PDI) also increased, rising from 0.525 to 0.760 in 1 mg L<sup>-1</sup> samples, and 0.336 to 0.638 in 100 mg L<sup>-1</sup> during the initial 2 h, further indicating the presence of aggregation of the particles. For both concentrations, a slight decrease in z-average size is recorded at the 4 h timepoint, however, in this case PDI continued to increase, implying continued aggregation (slight reductions in size observed may be due to precipitation of the largest aggregates, leaving smaller particle species in suspension). The highest z-average size of nCeO<sub>2</sub> were reached after 48 h entry into NSW at both concentrations, reaching average values of  $2,338 \pm 1,428$  nm and  $3,414 \pm 2,027$  nm for 1 and 100 mg L<sup>-1</sup>, respectively. Generally, following this the average particle size at both concentrations displayed a gradual decrease for the rest of the 240-h experiment, ultimately reaching sizes similar to those recorded at the start of the experiment. This gradual decrease likely represents the reduction in abundance of larger particles which precipitated out of the water column at a faster rate. PDI values displayed a less generalised trend and remained considerably higher than those recorded at 0 h, indicating the consistent presence of aggregates in suspension. Throughout the experiment, the deposition of nCeO<sub>2</sub> at the bottom of flasks was observed, clearly evident from 24 h onwards, indicating the precipitation of aggregated particles

(Supplementary Figure 3). Mean count rate was recorded to vary throughout the 240-h experiment (Supplementary Table 1), however, the reduction of average count rate from 225 kcps at 0 h to 108 kcps at 240 h observed in the 100 mg L<sup>-1</sup> treatment supports the visual observation of nCeO<sub>2</sub> sedimentation.

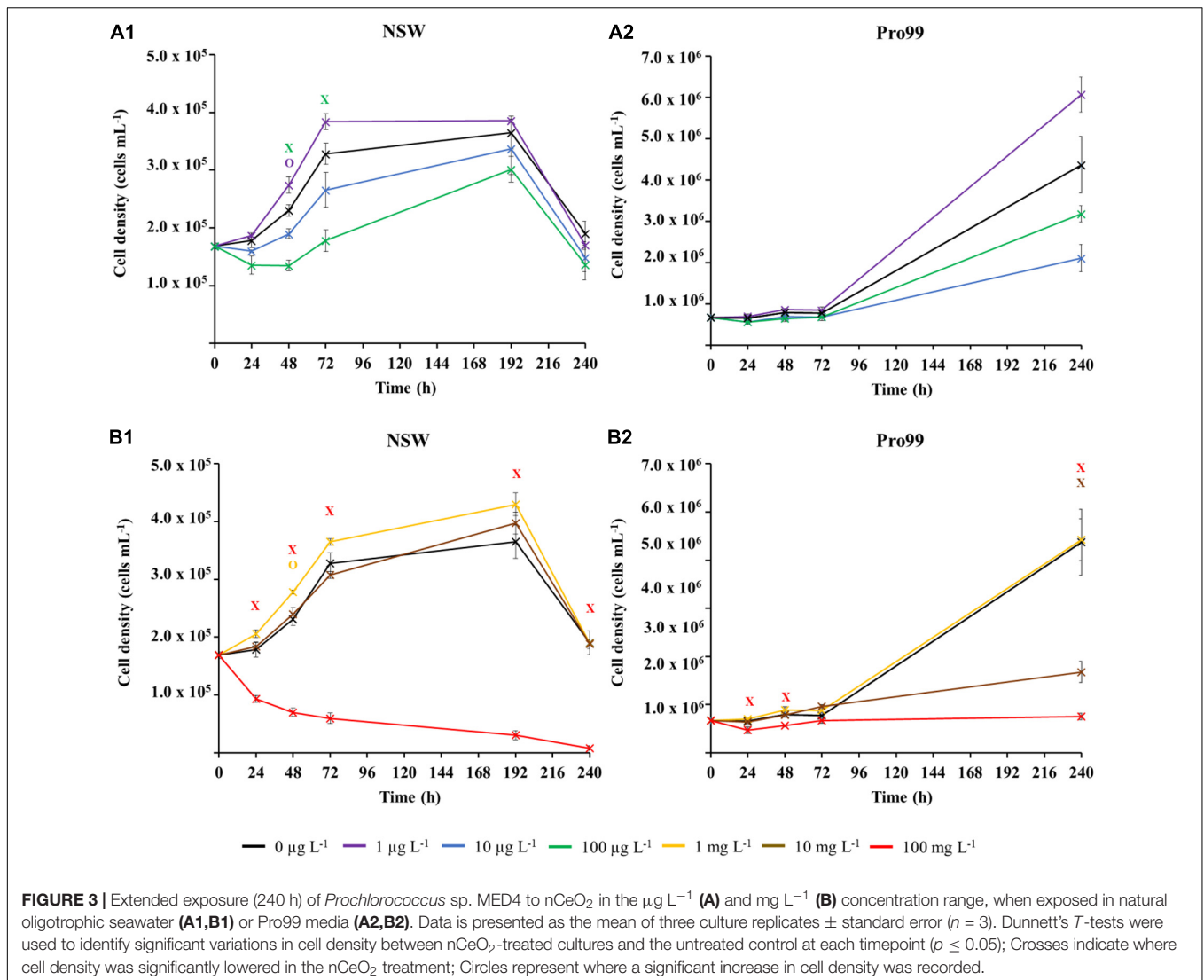
### Short-Term (72 h) Exposure of nCeO<sub>2</sub> Toward *Prochlorococcus*

Figure 2 displays the effects of nCeO<sub>2</sub> (20.6 ± 12.1 nm) upon 72 h growth of *Prochlorococcus* sp. MED4. At ambient cell densities grown in NSW (Figure 1A) ANOVA revealed significant alterations in cell density of *Prochlorococcus* between treatments at both the 48 and 72 h timepoints ( $p \leq 0.05$ ). However, only exposure to the highest concentration (100 μg L<sup>-1</sup>) had a significant negative effect upon growth of *Prochlorococcus*. After 48 h incubation, cell density was decreased on average 46.6% in this treatment compared to the untreated control, increasing to 68.8% by the end of the 72-h incubation (Dunnett's  $T$ -tests,  $p \leq 0.05$ ). At the final 72 h timepoint the average cell density of the 10 μg L<sup>-1</sup> treatment was also lower than that of the control, however, this result was not significant. Likewise, when grown in nutrient rich Pro99 media (Figure 1B), significant variations in cell density of various treatments were identified by ANOVA at the 24 and 48 h timepoints ( $p \leq 0.05$ ). However, no negative effect upon *Prochlorococcus* following 72 h incubation with nCeO<sub>2</sub> was recorded at any concentration. Significantly reduced growth was observed at the 24 h and 48 h timepoints in the 10 μg L<sup>-1</sup> treatment, where cell density was recorded on average 14.4 and 17.8% lower than the untreated control (Dunnett's  $T$ -tests,  $p \leq 0.05$ ). Additionally, at the 48 h timepoint a 25.2% decrease was also recorded in the 100 μg L<sup>-1</sup> treatment (Dunnett's  $T$ -tests,  $p \leq 0.05$ ).

### Alterations in Cell Density of *Prochlorococcus* in Extended Exposure (240 h)

#### Environmentally Relevant Concentrations (μg L<sup>-1</sup>)

Extended (240 h) exposure of *Prochlorococcus* to nCeO<sub>2</sub> is displayed in Figure 3. In accordance with short-term (72 h)



experiments, the lowest tested concentrations of 1 and 10 µg L<sup>-1</sup> had no effect on *Prochlorococcus* grown in NSW (Figure 3A1) throughout the 240-h experiment. As recorded in 72 h toxicity testing (section “Short-Term (72 h) Exposure of nCeO<sub>2</sub> Toward *Prochlorococcus*”), exposure to 100 µg L<sup>-1</sup> nCeO<sub>2</sub> within NSW caused a decline in average cell density at each timepoint during the initial 72 h of exposure. Here, significant declines in cell density were recorded at both the 48 and 72 h timepoints, representing declines of 41.4 and 45.7% compared to the untreated control (Dunnett's *T*-test, *p* ≤ 0.05). The extent of population decline in this treatment appears less severe as recorded in 72 h toxicity testing presented above (68.8%), likely due to the initial cell density of *Prochlorococcus* being relatively lower upon the establishment of the first experiment, indicating a possible impact of varying cell:nanoparticle ratios as recorded in previous research examining AgNP toxicity (Dedman et al., 2020). However, despite early declines, at later stages (i.e., 192 and 240 h), no significant variation was recorded between the cell density of *Prochlorococcus* in the 100 µg L<sup>-1</sup> treatment and

untreated cultures. Between 192 and 240 h, all cultures grown in NSW declined in cell density, likely due to nutrient depletion. In contrast, *Prochlorococcus* grown in the nutrient-enriched Pro99 media did not suffer any adverse effect of exposure to nCeO<sub>2</sub> concentrations in the µg L<sup>-1</sup> range during early stages of exposure (Figure 3A2). Due to the presence of nutrient-rich culture medium, no cell decline was expected to occur because of depleted nutrient availability as observed with cultures exposed in oligotrophic conditions. By the end of the 240-h incubation, both the 10 and 100 µg L<sup>-1</sup> treatments were observed to reduce average cell growth. Unexpectedly, the 10 µg L<sup>-1</sup> treatment had the greatest effect, reducing cell density on average by 27.3% compared to the untreated control. However, neither of these declines were recorded to be statistically significant. The lowest concentration of 1 µg L<sup>-1</sup> exerted no negative impact on *Prochlorococcus* throughout the 240-h exposure in Pro99 media but resulted in an increase in cell density (38.8%) than the control after 240 h. However, this increase was not statistically significant.

## Supra-Environmental Concentrations (mg L<sup>-1</sup>)

Concentrations in the mg L<sup>-1</sup> range were investigated to examine the impact of supra-environmental concentrations of nCeO<sub>2</sub> should nanoparticles accumulate in the environment or their release increase. ANOVA revealed significant variations in cell density between treatment at all but one timepoint ( $p \leq 0.05$ ). In NSW (**Figure 3B1**) 100 mg L<sup>-1</sup> nCeO<sub>2</sub> caused a significant decline in *Prochlorococcus* at every timepoint with a maximal response observed after 192 h where cell density declined by over one order of magnitude in comparison to the untreated control, representing an approximate 95.7% decline (Dunnett's *T*-test,  $p \leq 0.05$ ). Exposure to  $\leq 10$  mg L<sup>-1</sup> nCeO<sub>2</sub> in NSW did not appear to have any negative effect upon growth at any timepoint. As mentioned above, in NSW all cultures displayed a reduction in cell density at 240 h, this had no additional effect upon toxicity, only the highest concentration continued to exert an adverse effect. In cultures supplemented with Pro99 media (**Figure 3B2**), 100 mg L<sup>-1</sup> nCeO<sub>2</sub> also exerted a negative effect upon growth of *Prochlorococcus* observed at the 24, 48, and 240 h timepoints. In addition, the 10 mg L<sup>-1</sup> treatment also resulted in a significant decrease in cell density after 240 h. By the end of the 240-h experiment, cell density was reduced approximately 61.6 and 82.9% in the 10 and 100 mg L<sup>-1</sup> groups, respectively, in comparison with the untreated control (Dunnett's *T*-test,  $p \leq 0.05$ ). As expected, throughout these supra-concentration treatments, small aggregates were observed by flow cytometry and large aggregates were visible at the bottom of the culture flasks confirming nanoparticle aggregation and possible cyanobacteria-nanoparticle aggregation which deserved further investigation.

## Physical Cyanobacterial-nCeO<sub>2</sub> Interactions

nCeO<sub>2</sub> aggregates alone do not fluoresce and, hence, would not be picked up during flow cytometric analysis. Nevertheless, the occurrence of large fluorescent particles were observed only at mg L<sup>-1</sup> treatments and are believed to represent hetero-aggregates of *Prochlorococcus* cells and nCeO<sub>2</sub>. By gating these aggregates using FACSDiva software, it was possible to estimate their abundance relative to free *Prochlorococcus* cells (described above, section "Alterations in Cell Density of *Prochlorococcus* in Extended Exposure (240 h)") and reference beads. Here, stocks of NSW in the absence of cyanobacteria and nCeO<sub>2</sub>, as well as untreated cultures were utilised to control for the occurrence of such hetero-aggregated material. It should be noted that no such events were recorded during analysis of samples derived from cultures exposed to nCeO<sub>2</sub> in the  $\mu\text{g L}^{-1}$  range and therefore these are not described herein.

The estimated concentration of cyanobacterial-nCeO<sub>2</sub> aggregates in both NSW and Pro99 media, recorded at the 24, 48, 72, and 240 h timepoints is presented in **Figure 4**. The number of aggregates in suspension appeared considerably higher in cell-dense cultures grown in Pro99 media compared to those grown in NSW, reaching peak concentrations of  $2.8 \times 10^5$  and  $\sim 7.0 \times 10^4$  aggregates mL<sup>-1</sup>, respectively, at the 24 h timepoint. In NSW, aggregate density appeared to follow a dose-wise trend,

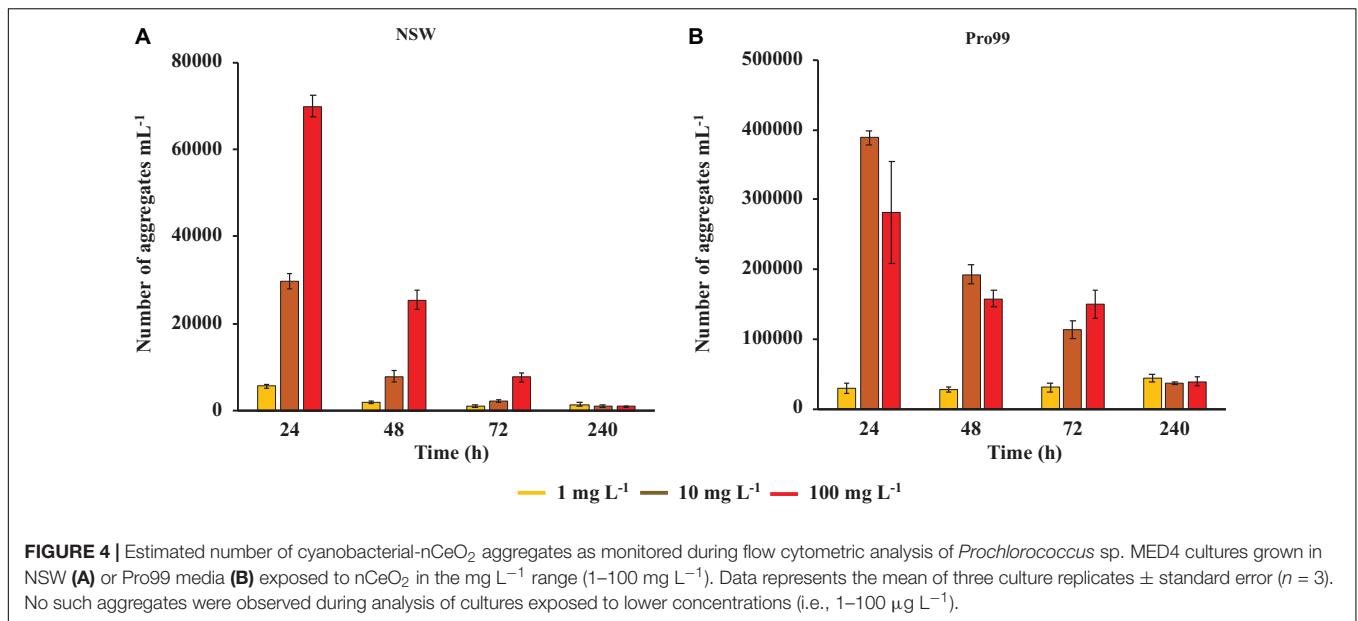
increasing with increased concentration, however, this was not the case at the 240 h timepoint, where it is likely that aggregated material had precipitated out of the media and was therefore not present in the suspended fraction monitored by flow cytometry. In Pro99 media, this dose-wise trend was not observed, and highest values were recorded in the 10 mg L<sup>-1</sup> treatment. Notably, aggregate density decreased rapidly throughout the course of the experiment, up to 73% decrease recorded between the 24 and 48 h timepoints. This decrease is likely the result of rapid aggregation and precipitation of large, aggregated material due to increased density and, interestingly, at the last time point i.e., 240 h the number of aggregates estimated at each concentration were in the same order of magnitude in both NSW and Pro99 media (**Figure 4**).

Fluorescent microscopy was utilised to confirm the presence of cyanobacteria within aggregated material. Sub-samples were collected from the bottom of culture flasks and imaged using brightfield and GFP fluorescent channels after staining with SYBR Gold nuclear stain (**Figure 5** and **Supplementary Figure 4**). Here, clear evidence of cyanobacterial-nCeO<sub>2</sub> hetero-aggregation was observed at concentrations of 10 and 100 mg L<sup>-1</sup> in both NSW (**Figures 5A,B**) and Pro99 media (**Figures 5C,D**). Observed hetero-aggregates reached sizes in the 10s-of-micron range but showed great variation in size and morphology. Some evidence of hetero-aggregation was observed within the 1 mg L<sup>-1</sup> treatment; however, this was not apparent in cultures exposed to lower concentrations (1–100  $\mu\text{g L}^{-1}$ ).

## DISCUSSION

Herein, the toxicity of the emerging nano-pollutant nCeO<sub>2</sub> toward the marine cyanobacterium *Prochlorococcus sp.* MED4 was examined under environmentally relevant (ambient cell density within oligotrophic NSW) and optimal growth (cell-dense cultures grown in nutrient-enriched Pro99 seawater) conditions during short-term and extended exposure. Investigation revealed a potential for nCeO<sub>2</sub> to exert significant declines in cyanobacterial cell density, dependent on nCeO<sub>2</sub> concentration, culture media and exposure length. Examination of nCeO<sub>2</sub> behaviour within NSW, and during incubation with cyanobacteria, displays the extensive aggregation of nCeO<sub>2</sub> under saline conditions and ability to form hetero-aggregates with microbial cells. The physical interaction between cyanobacteria and nCeO<sub>2</sub> is believed the key driver of significant alterations to cell density recorded during laboratory investigation.

It is widely acknowledged that the fate and behaviour of nanomaterials within the natural environment will determine their bioavailability and hence likely effect upon biota (Rodea-Palomares et al., 2010; Hazeem et al., 2016; Thiagarajan et al., 2019). Upon entry into the aquatic environment, nCeO<sub>2</sub> may be transported freely in suspension, aggregate to other nCeO<sub>2</sub> particles (homo-aggregation) or aggregate to other particulate matter (hetero-aggregation). To improve our understanding of nCeO<sub>2</sub> behaviour in the marine environment, the aggregation behaviour of nanoparticles in NSW was assessed using DLS for a period of 240 h as previously described (Petosa et al., 2010;



Afroz et al., 2014; Clavier et al., 2019). Concentrations of 1 and 100 mg L<sup>-1</sup> were selected for analysis, as limitations of DLS at lower concentrations meant that environmental concentrations (i.e., μg L<sup>-1</sup>) could not be accurately measured (Handy et al., 2012). Evidence of nCeO<sub>2</sub> aggregation was recorded immediately upon entry into saline media, reaching sizes of up to 1,293 ± 141 nm, considerably higher than the primary particle size determined by TEM (20.6 ± 12.1 nm). Average size of nCeO<sub>2</sub> remained high throughout the 240-h experiment, indicating that particles remained in an aggregated state, with a slight decrease recorded in later stages suggesting the possible sedimentation of larger aggregated material. The aggregation of particles causes a reduction in the surface-area-to-volume ratio of nanomaterials and is likely to influence the toxicity and reactivity of particles (Lowry et al., 2012). Following aggregation, particles may sink through the water column due to increased mass and sedimentation may occur. This process was evident through visual inspection of nCeO<sub>2</sub> suspensions and supported by mean count data at high concentrations (100 mg L<sup>-1</sup>).

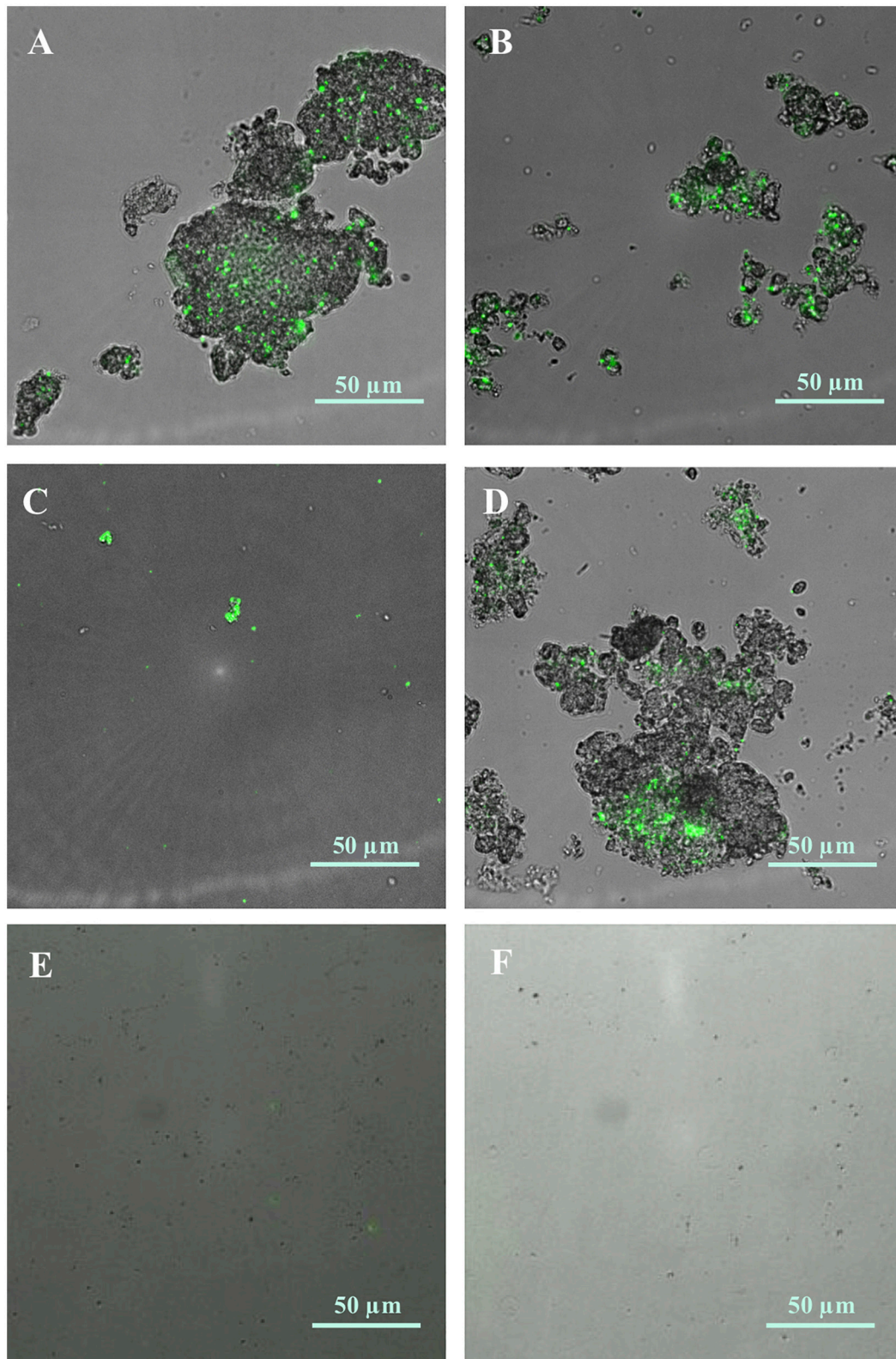
In accordance with previous research, it is clear that nCeO<sub>2</sub> is likely to aggregate upon entry into saline media with high ionic strength such as seawater (Keller et al., 2010; Ottofuelling et al., 2011; Quik et al., 2014; Sendra et al., 2017). In the natural environment a number of additional factors will also govern the fate and behaviour of engineered nanomaterials, including; particle specific physicochemical properties (Dale et al., 2017) and local water chemistry with pH, natural organic matter (NOM) and particulate matter all playing influential roles (Quik et al., 2010; Collin et al., 2014; Velzeboer et al., 2014; Van Koetsem et al., 2015). For example, in the presence of NOM, Quik et al. (2010) observed up to 88% of nCeO<sub>2</sub> added to deionised water to remain stable in suspension. Therefore, the presence of NOM in natural waters is likely to influence particle stability, although this will also largely depend on specific surface characteristics of nanoparticles. Hence, in our work we

utilised NSW to simulate natural waters that nCeO<sub>2</sub> may enter. Given nCeO<sub>2</sub> nanoparticles' propensity to aggregate and undergo sedimentation in saline media, they have the potential to interact with marine biota throughout the water column as they are transported toward deeper zones.

Flow cytometry was used to monitor alterations in populations of the marine cyanobacterium *Prochlorococcus*. It is known that dead cells of *Prochlorococcus* lose fluorescence rapidly (Christie-Oleza et al., 2017; Roth-Rosenberg et al., 2020), therefore flow cytometry can be used to monitor changes in the viable cyanobacterial population. During short-term (up to 72 h) exposure, presence of μg L<sup>-1</sup> nCeO<sub>2</sub> was recorded to significantly reduce the cell density of marine cyanobacterium *Prochlorococcus*, when grown under environmentally relevant conditions in NSW, causing up to a 68.8% decrease in cell density. However, no such effect was observed in cultures grown to higher cell densities in nutrient rich Pro99 media after 72 h exposure, highlighting the influence of specific exposure conditions. Upon extending incubations to 240 h; the trend observed in early stages of exposure (<72 h) continued. However, despite the significant declines recorded during the initial 72 h to 100 μg L<sup>-1</sup> nCeO<sub>2</sub>, *Prochlorococcus* cultures grown in NSW were observed to recover to cell densities comparable to that of the untreated control by the 192 h timepoint. Whilst in short-term exposure, cell-dense cultures appeared more resilient to nCeO<sub>2</sub>, in the long-term this trend was reversed as cultures grown in Pro99 media experienced declines in average cell density up to 27.3% in response to both 10 and 100 μg L<sup>-1</sup> treatments.

To further evaluate the impact of nCeO<sub>2</sub> should emissions into the environment rise or hotspots of pollution occur due to accumulation of nanoparticles, the response of *Prochlorococcus* toward supra-environmental concentrations was also assessed. In both NSW and Pro99 exposure to the highest concentration (100 mg L<sup>-1</sup>) exerted a negative impact upon *Prochlorococcus*, causing a maximum decline in cell density of 95.7%. As with





**FIGURE 5** | Hetero-aggregation of *Prochlorococcus* sp. MED4 (green) and nCeO<sub>2</sub> (10 and 100 mg L<sup>-1</sup>) formed in natural oligotrophic seawater [(A); 10 mg L<sup>-1</sup> and (B); 100 mg L<sup>-1</sup>] and Pro99 media [(C); 10 mg L<sup>-1</sup> and (D); 100 mg L<sup>-1</sup>]. Images were captured using fluorescent microscopy under brightfield and GFP fluorescent and subsequently were merged. No such aggregates were visible in *Prochlorococcus* culture (E) or natural oligotrophic seawater (F) in the absence of nanoparticles.

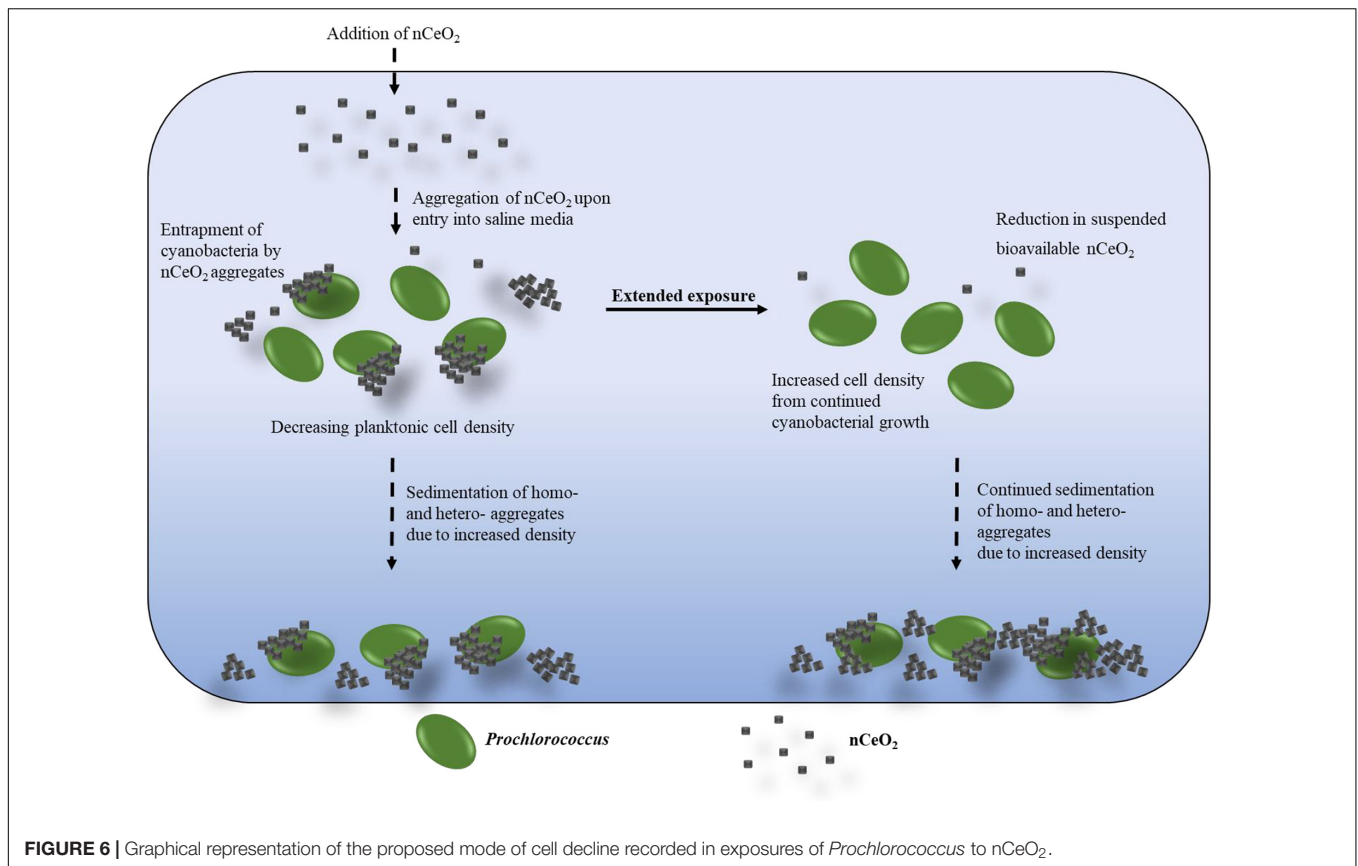
lower concentrations, differences in response between the two culture conditions could also be observed in mg L<sup>-1</sup> treatments. Whilst 10 mg L<sup>-1</sup> nCeO<sub>2</sub> exerted no effect on *Prochlorococcus* grown in NSW, cell-dense cultures experienced a 61.6% decrease in cell density by the end of the experiment. The enhanced cell decline observed in cell-dense cultures, may arise due to increased likelihood of encounter between nanoparticles and cyanobacteria. Previous research has also shown that cultures grown to higher cell densities suffer greater adverse effect to metal oxide nanomaterial exposure (Bessa da Silva et al., 2016). Interestingly, following 240 h exposure no effect was recorded in 1 and 10 mg L<sup>-1</sup> nCeO<sub>2</sub> treatments in NSW, or the 1 mg L<sup>-1</sup> treatment in Pro99 media. However, declines were observed at lower concentration at specific timepoints (10 and 100 μg L<sup>-1</sup>). We propose that the difference in effect caused by these treatments is likely due to the extensive aggregation of nCeO<sub>2</sub> upon entering saline media. It is possible that in μg L<sup>-1</sup> treatments, stability of nCeO<sub>2</sub> in suspension is increased following lowered homo-aggregation due to reduced rate of encounter between individual nCeO<sub>2</sub> particles, hence facilitating their interaction with planktonic cells. In contrast, at higher concentrations (1–10 mg L<sup>-1</sup>) homo-aggregation occurs at a higher rate causing nCeO<sub>2</sub> to aggregate and sink before interacting with cyanobacteria. At the highest concentration (100 mg L<sup>-1</sup>) where effects are most severe, and in the 10 mg L<sup>-1</sup> in cell-dense cultures where particle-cell contact is more likely, the aggregation process is extensive enough to significantly reduce *Prochlorococcus* numbers.

Adverse effects associated with nCeO<sub>2</sub> exposure have previously been recorded in studies with marine phytoplankton (Deng et al., 2017). The diatom *P. tricornutum* suffered adverse effects following exposure to 10–30 nm nanoparticles at concentrations >10 mg L<sup>-1</sup>, resulting in significant reductions in growth (Deng et al., 2017). However, interestingly, lower concentrations <5 mg L<sup>-1</sup> were recorded to stimulate growth of this diatom (Deng et al., 2017). Such enhanced growth following exposure to nCeO<sub>2</sub> has also been recorded in marine algal species (Sendra et al., 2018).

Due to the limited evidence available, the exact mechanisms by which nCeO<sub>2</sub> drives significant declines in phytoplankton abundance remain unclear. Based on work in freshwater, nCeO<sub>2</sub> is thought to exert oxidative stress upon microbial species via photocatalytic ROS production (Rogers et al., 2010). This suggests that the presence of natural light may be a key factor in determining toxicity of nCeO<sub>2</sub>, hence in our study full-spectrum bulbs were used to best simulate natural conditions. Oxidative stress and associated lipid peroxidation have been recorded in the marine diatom *P. tricornutum* in response to nCeO<sub>2</sub> (Deng et al., 2017). Although, whether such effects arise due to the nanoparticles' presence, or as a consequence of physical interactions with cells is not clear. In that work, concentrations of 10–40 mg L<sup>-1</sup> led to considerable increases in activity of superoxide dismutase, peroxidase and malondialdehyde, associated with oxidative stress and lipid peroxidation, respectively (Deng et al., 2017). It is possible ROS production and associated oxidative stress may have played a role in the significant declines of *Prochlorococcus* recorded during our

study. However, it has also been suggested that due to nCeO<sub>2</sub> being a redox catalyst, nanoparticles display the potential to both mitigate, as well as exert, oxidative stress (Arnold et al., 2013; Pulido-Reyes et al., 2015). For example, nCeO<sub>2</sub> has been recorded to display antioxidant activities toward secondary oxidative stress exerted upon phagocytic and human bronchial epithelial cell lines (Xia et al., 2008), and during incubation with phytoplankton (Sendra et al., 2017). It is possible any antioxidant properties may account for the enhancement of phytoplankton growth recorded in previous research in response to nCeO<sub>2</sub>, and lack of decline observed at concentrations of 1 and 10 mg L<sup>-1</sup> observed in our study. Although, such enhanced growth can also be attributed to hormetic effects (Mattson, 2008). The exact mechanisms by which nCeO<sub>2</sub> exerts and mitigates oxidative stress requires attention, particularly in seawater. Additionally, nCeO<sub>2</sub> exposure has been linked to a decrease in photosynthetic activity (Taylor et al., 2016; Deng et al., 2017), as well as reducing carbon fixation in freshwater phytoplankton (Taylor et al., 2016), suggesting phototoxic properties of nCeO<sub>2</sub>. Deng et al. (2017) recorded a decline in quantum yield of PSII by approximately 29% following 96 h exposure to nCeO<sub>2</sub> in the diatom *P. tricornutum*. However, such effects are only observed at relatively high concentrations (2.5–40 mg L<sup>-1</sup>) (Taylor et al., 2016). As such, it is unlikely photosynthetic processes will be adversely affected by nCeO<sub>2</sub> in the natural marine environment at current levels.

During exposures of *Prochlorococcus* to nCeO<sub>2</sub> the formation and precipitation of aggregates of cyanobacteria and nanoparticles (which aggregate readily in the high ionic strength of seawater, as demonstrated and described by DLS analysis) were visible in culture flasks. Whilst monitoring cyanobacterial populations by flow cytometry, it was possible to estimate the number of hetero-aggregates within suspension (**Figure 4**). Only freely suspended *Prochlorococcus* were used to calculate cell density presented in the work above (section “Alterations in Cell Density of *Prochlorococcus* in Extended Exposure (240 h)”) as those entrapped within hetero-aggregates would likely be removed from the water column by co-precipitation with nCeO<sub>2</sub>. The presence of cyanobacteria within precipitated aggregates of nCeO<sub>2</sub> was confirmed by carrying out fluorescent microscopy on precipitated material collected from the bottom of culture flasks that was subsequently stained with SYBR Gold (**Figure 5**). Here, *Prochlorococcus* was clearly visible within aggregates of nCeO<sub>2</sub> which reached sizes in the 10s-of-micron range. The hetero-aggregation between metal oxide nanomaterials and phytoplankton has previously been reported in the literature (Rodea-Palomares et al., 2010; Manzo et al., 2015; Xia et al., 2015; Wang et al., 2016; Morelli et al., 2018; Dedman et al., 2021), and has been observed with nCeO<sub>2</sub> during 96 h exposure (Deng et al., 2017). It is our belief that the process of entrapment and subsequent co-precipitation of cyanobacteria and nCeO<sub>2</sub> from the water column (**Figure 6**) is a key mechanism behind the temporary declines in *Prochlorococcus* presented herein (sections “Short-Term (72 h) Exposure of nCeO<sub>2</sub> Toward *Prochlorococcus*” and “Alterations in Cell Density of *Prochlorococcus* in Extended Exposure (240 h)”). However, given the various impacts of nCeO<sub>2</sub> upon phytoplankton physiology described above, further



testing such as proteomic analysis and the determination of ROS generation is required to confirm this.

As expected, the estimated number of hetero-aggregates was substantially higher in cell-dense cultures (**Figure 4B**) than those grown in NSW (**Figure 4A**), likely due to the increased rate of encounter between nanoparticles and cyanobacteria. The number of hetero-aggregates in suspension within culture flasks was recorded to decline rapidly, with up to 73% decrease between the 24 and 48 h timepoints, suggesting their rapid precipitation out of the water column along with attached cyanobacteria. Previous research using the filamentous cyanobacterium, *Anabaena* CPB4337, also observed physical interaction between nCeO<sub>2</sub> and cyanobacterial cells (Rodea-Palomares et al., 2010). Here, positively charged particles appeared to be attracted to the negatively charged cell walls of cyanobacteria (Rodea-Palomares et al., 2010). The researchers proposed that toxicity may be induced by the direct contact of nCeO<sub>2</sub> to the cell wall; causing mechanical damage to the cell membrane, disrupting the exchange of nutrients and metabolites and exerting oxidative stress (Rodea-Palomares et al., 2010). The same research group investigated the effects of nCeO<sub>2</sub> upon *P. subcapitata* in freshwater, observing damage to the cell membrane, and leakage of the cytoplasm (Rodea-Palomares et al., 2010). Once again, this was believed to result from direct contact between nanoparticles and cells. Similar findings upon *P. subcapitata* have been recorded in previous studies (Rogers et al., 2010), providing further evidence that direct contact induces the toxicity of nCeO<sub>2</sub>.

Hetero-aggregation between phytoplankton and metal oxide nanomaterials has also been proposed to cause shading effects which may reduce photosynthetic efficiency (Hu et al., 2018), possibly causing the declines in photosynthetic output previously described (Taylor et al., 2016; Deng et al., 2017). Thus, it is concluded by Rodea-Palomares et al. (2010) that the cytotoxicity of nCeO<sub>2</sub> requires direct contact of particles to the cell wall or membrane. During this process it is likely that physical damage to cells will be caused, such as that described above, although additional study is required to confirm this. The belief that physical effects are a key driver of cyanobacterial decline in our work, rather than oxidative stress, is supported by the fact that *Prochlorococcus* is highly susceptible to ROS and is sensitive to H<sub>2</sub>O<sub>2</sub> concentrations as low as 200 nM (Morris et al., 2011; Zinser, 2018). Hence, given that the viable free-living population recorded by flow cytometry is able to recover to ambient cell densities in extended exposure, this suggests that ROS-mediated stress is likely not a feature of our work.

Over time the concentration of suspended nCeO<sub>2</sub> in *Prochlorococcus* cultures will display a continual decline, hence reducing its bioavailability and allowing the surviving cyanobacterial population to recover, as observed in *Prochlorococcus* populations grown in NSW (**Figure 6**). It is important to note that in our work, uncoated nCeO<sub>2</sub> was utilised during experiments. The behaviour of these nanoparticles likely differs to those which are surface-modified, likely playing a key role in the effects recorded herein. It has been suggested

that surface characteristics influence differences in toxicity by altering NM bioavailability (Booth et al., 2015). For example, in contrast to the nanoparticles used in our work, Poly-(acrylic acid)-stabilised nCeO<sub>2</sub> appear well-dispersed in saline media, remaining biologically available to species occupying the water column, and hence enhancing toxicity toward these organisms (Booth et al., 2015). However, findings vary and appear case-specific. Dextran-coated nCeO<sub>2</sub> have been recorded to exert negligible toxicity upon *E. coli* (Shah et al., 2012), believed likely due to stabilisation of the oxidative state nanoparticles by the polymer coating, as well as a reduction in contact between particles and cells (Shah et al., 2012). Going forward it will be increasingly important to assess the surface characteristics of nCeO<sub>2</sub> entering the environment, such that greater insight into their likely fate and interaction with biota can be gained.

The occurrence of hetero-aggregation between nanoparticles and phytoplankton may increase the risk of bioaccumulation and transfer of particles to higher trophic levels by ingestion. Evidence of trophic transfer of nCeO<sub>2</sub> has previously been reported in a terrestrial plant model (Hawthorne et al., 2014). However, it must be noted that the widespread occurrence of hetero-aggregation between phytoplankton and nanoparticles recorded in laboratory investigation is likely driven at least partially by the relatively high concentrations examined and the closed-system nature of most experimental set-ups. As a result, such physical interactions between cells and nanoparticles are less likely in the natural environment at lower concentrations where nanoparticles appear more dilute and may alternatively interact with other particulate matter.

The recovery of *Prochlorococcus* recorded under environmentally relevant conditions in extended exposure and lack of adverse effect of concentrations <10 mg L<sup>-1</sup> in NSW suggests that at current, the likely environmental risk of nCeO<sub>2</sub> exposure toward marine microbial species under natural conditions is low. The recovery of microbial populations in long-term exposure has previously been recorded during investigation with metal oxide nanomaterials (Hazeem et al., 2016; Li et al., 2019; Mitchell et al., 2019), often attributed to the aggregation behaviour of metal oxide nanomaterials, leading to their reduced bioavailability over time (Hazeem et al., 2016; Thiagarajan et al., 2019), in accordance with DLS analysis and microscopic imaging described above. This evidence suggests that similarities between the impact of various metal oxide nanomaterials exist, allowing researchers to better understand their likely effect.

## CONCLUSION

In this study we provide new insight into the effect of nCeO<sub>2</sub> upon marine microbial species using the ecologically significant marine cyanobacterium *Prochlorococcus*. To date, limited research has been carried out to assess the potential impact of this emerging contaminant upon marine microbial organisms, and hence the works presented herein address this gap in knowledge. Additionally, information is provided on the possible ecotoxic effects of engineered NMs upon an ecologically significant marine cyanobacterium, for which little

ecotoxicological data exists. We have shown that despite evidence of short-term (<72 h) declines in cell density, nCeO<sub>2</sub> appears to exert little toxicity toward *Prochlorococcus* under natural conditions in the longer term, except at exceptionally high concentrations (i.e., 100 mg L<sup>-1</sup>). Cultures grown to higher cell densities in nutrient-rich media appear to suffer greater effects of exposure when incubations are extended to 240 h, believed due to the increased rate of encounter between cyanobacteria and nanoparticles. The occurrence of hetero-aggregation between nCeO<sub>2</sub> and *Prochlorococcus* is a key feature of exposure, in accordance with a number of studies examining the ecotoxicity of metal oxide NMs, (Rodea-Palomares et al., 2010; Manzo et al., 2015; Xia et al., 2015; Wang et al., 2016; Morelli et al., 2018) driven by aggregation behaviour of nCeO<sub>2</sub> in saline media confirmed by DLS. The processes of entrapment and subsequent co-precipitation of nanoparticles and cyanobacteria are believed to be important mechanisms of the declines in cell number recorded, although additional testing is required to confirm this. Over time, the concentration of suspended nCeO<sub>2</sub> is reduced, hence reducing the likelihood of interaction between nanoparticles and biota, allowing the remaining free-living cyanobacterial population to resume normal growth. However, it remains that the mechanism of cell decline observed in laboratory investigation is far less likely to occur at the low nCeO<sub>2</sub> concentrations expected in the natural environment, where dilution effects likely limit the extent of both homo-aggregation and hetero-aggregation with phytoplankton, where nanoparticles may also interact with natural colloids or NOM. As such, in accordance with previous research (Johnson and Park, 2012), it appears that the likely environmental risk of nCeO<sub>2</sub> toward marine microorganisms is low. One drawback from the works presented herein is the use of pristine research-grade nanomaterials. Such particles may not accurately represent those particles released into the environment. nCeO<sub>2</sub> is likely to undergo transformation upon its release into the environment, particularly nCeO<sub>2</sub> released from fuel *via* combustion (Dale et al., 2017). Echoing the beliefs of Dale et al. (2017), our future experimental work will focus upon investigating the environmental impact of nCeO<sub>2</sub> and other metal oxide nanomaterials using materials that best represent those released into the environment. It will be important to comprehensively characterise physicochemical properties as well as nanoparticle state in the environment. Here, synthetic approaches may be a useful tool to produce nanomaterials which accurately match environmental particles. Efforts must also be directed toward accurately predicting the likely environmental concentration of nCeO<sub>2</sub>, where marine transport may contribute substantially to the entry of nanoparticles into the ocean. In future studies, experiments conducted on whole communities may aid analysis of impacts upon ecological function and identify any taxonomic groups particularly susceptible to exposure.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

CD performed all experiments, data acquisition, data analysis, and wrote the manuscript. MR assisted with nanomaterial characterisation. G-LD and JC-O conceived the idea, assisted with data analysis and interpretation and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.668097/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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