NANOPLASTIC EXPOSURE CAUSES GROWTH DELAYS AND INCREASED MORTALITY TO CHLAMYDOMONAS.

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ABSTRACT

Chlamydomonas reinhardtii is a single-celled green algae that survives almost anywhere on Earth where moist soil or water are present, making it a foundation of countless food webs. Previous work by Ben Kossman, a past student in the Sanderfoot lab, found that *C. reinhardtii* grown in liquid cultures appeared to aggregate (clump together) when exposed to nanoplastics, behavior not observed in liquid cultures excluding nanoplastics. The same research also found potential evidence that cells grown in darkness produce less intense aggregation phenotypes or don't aggregate at all compared to those kept in a typical day and night cycle. This research project was focused upon advancing the understanding of how *C. reinhardtii* respond to nanoplastics at a culture and cellular level while under different light exposures with the presence or absence of a cell wall. *C. reinhardtii* cells were cultured in 24 and 96 well plates, treated with varying concentrations of nanoplastics, and left to grow in a day/night cycle or complete darkness. Differences in cell proliferation under various treatments was monitored using absorbance of cultures over time and characteristics of aggregates was monitored using confocal and fluorescence microscopy. Relative abundance of time suggested that increased nanoplastic exposure correlated with delayed cell culture proliferation. The viability assay performed provided reason to believe nanoplastic exposure resulted in increased cell mortality. Microscopy revealed that nanoplastics localized to the extracellular matrix of individual cells forming a hetero aggregate during exposure despite differences in light treatment and presence of a cell wall. All treatments ultimately resulted in an aggregative phenotype.

Keywords: Nanoplastic, algae, toxicology, cell biology, confocal microscopy, spectrophotometry.

INTRODUCTION

Transfer of energy from lower to higher trophic levels is an essential component of ecological food webs. The foundational organisms of most food webs are photosynthetic primary producers that provide energy that an entire food web may rely upon. Many photosynthetic primary producers are microscopic, such as *Chlamydomonas renhardtii. C. reinhardtii* is a single-celled green algae that survives almost anywhere on Earth where moist soil or water are present, making the genus a primary producer in many food webs. *C. reinhardtii* is also commonly used as a model organism for indicating pollution and environmental change (Míguez et al. 2020; Esperanza et al. 2015). Scientific interest in the widespread pollution of microplastics, (referred to as "nanoplastics" when they are less than 1 μm in size) and the possible effects they have upon unicellular organisms like *C. reinhardtii* has been growing in recent years (Long et al. 2017; Mattsson et al. 2018; Shi et al. 2024; Verdú et al. 2022; Yang et al. 2021). Large plastics and microplastics in aquatic environments are exposed to processes that lead to fragmentation, ultimately generating nanoplastics (Kung et al. 2023). Recent publications by (Déniel et al. 2020) and (Tamayo-Belda et al. 2023) found that *C. reinhardtii* exposure to nanoplastics led to damage and change in the biochemical composition of cells. Furthermore, (Shi et al. 2024) found that nanoplastics are currently present in bodies of water across Asia, Europe, Antarctica, and the Arctic Ocean at levels of 0.3–488 microgram per liter. Undergraduate research within the Sanderfoot lab at the University of Wisconsin La-Crosse has observed that *C. reinhardtii* cells tend to aggregate in the presence of nanoplastic particles when grown under normal conditions for photosynthetic organisms. Oddly, when cultures were grown in darkness under heterotrophic conditions cells in liquid media cultures did not exhibit

aggregative phenotypes visible to the naked eye. This research project aimed to advance the understanding of the effect polystyrene nanoplastics have upon *C. reinhardtii*, primarily focusing upon identifying differences in aggregative phenotypes and cell mortality when cultures were grown in various light conditions with and without the presence of an algal cell wall.

METHODS

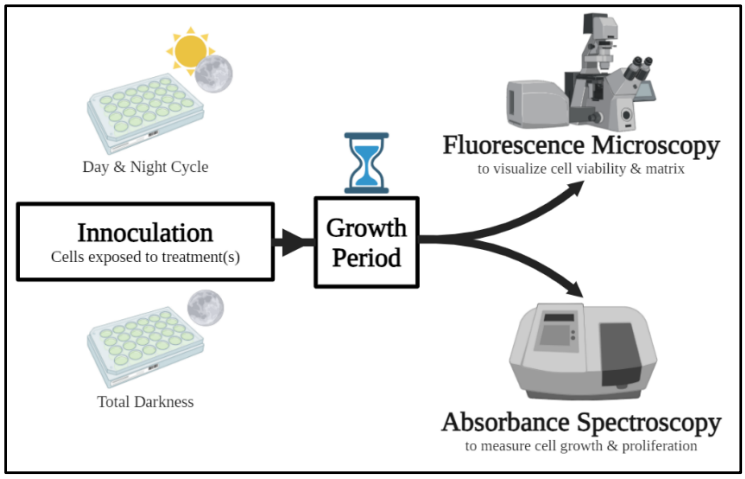

Figure 1. Visualization of methodology starting with the creation of treated *C. reinhardtii* cell cultures (left), permitting time for culture growth (center), and the methods used to monitor the influence of nanoplastic exposure upon treated cultures (right).

Cell Culture

Cell cultures destined for spectrophotometry were grown in standard flat-bottom 24 well plates, while cultures to be examined using confocal microscopy were grown in thin-bottom 96 well microplates (DOT Scientific 4ti-0223) to permit higher magnification of cultures in vitro. Thin-bottom 96 well plates used specifically in microscopy mimicked all variables of their 24 well plate counterparts. Two separate strains of *C. reinhardtii* were used; wildtype (WT) (CC-1690), and *cw15* cell-wall-less strain (CC-406). The cell-wall-less strain was included to examine the possible role the presence of an algal cell wall may have in response to nanoplastic exposure. All clear 24 well plate wells contained equal volumes of 1mL of TAP (Tris-acetate-phosphate), a liquid growth medium that includes acetate to be respired by *C. reinhardtii* cells for energy, permitting cell survival while in the dark (Kropat et al., 2011).

Nanoplastic treatment

Cells were treated with two separate concentrations of polystyrene nanoplastics (Sigma LB1), 50 µg/mL and 100 µg/mL. Preliminary research within the Sanderfoot Lab suggested that *C. reinhardtii* grown in a culture containing 50 µg/mL of nanoplastics displayed an aggregative phenotype visible to the naked eye only days after inoculation. The higher dosage of 100 µg/mL was included to gather insight into if increasing nanoplastic concentration results in increased aggregative phenotypic response, pr perhaps increased cell death. Previous published research by (Shi et al. 2024) suggests that these levels of nanoplastics occur within polluted aquatic environments. Control cultures received no nanoplastics.

Light treatment

Following the creation of two separate identical 24 well plates, one plate was selected to be kept in darkness and wrapped tightly in standard aluminum foil to prevent all light from reaching *C. reinhardtii* cultures within. The aluminum foil used also served a function to prevent evaporative loss of TAP media in cultures grown in darkness. Plates that were kept in darkness were only removed from foil for examination, and light exposure was limited to the fullest extent. The other 24 well plate received 14:10 (light:dark) day/night cycle with an intensity of 300 µmol/m^2•s of white LED light. Day/night cultures were wrapped tightly in parafilm to prevent evaporative loss of TAP media. 96 well plates received identical light treatments as their 24 well counterparts. All cultures were grown on an oscillating table at 200 rpm.

Monitoring cellular proliferation

*C*ell proliferation was monitored using an ID3 spectrophotometer (Molecular Devices) with a wavelength of 680 nm, exploiting chlorophyll's natural absorption capabilities to monitor the number of cells within cultures. It is worth mentioning that *C. reinhardtii* grown in darkness do continue to produce chlorophyll despite a lack of light (Choquet et al., 1992), ensuring that spectroscopy could still be used to accurately measure culture growth. Absorbance readings were taken periodically to generate growth curves representing relative absorbance over time. Absorbance readings from all cultures were normalized prior to statistical and graphical analysis to account for potential differences in initial cell count at time of inoculation. Absorbance readings were not recorded at evenly spaced intervals due to scheduling interferences and equipment availability.

Cell viability assay & visualizing nanoplastic localization

Fluorescein diacetate (FDA; Thermo Scientific, 191660050) stain was used to visualize cell viability. The FDA vital stain was dissolved in acetone (stock 5 mg/mL) and then diluted 1:100 in TAP immediately before a dilution of 1:10 into cell cultures, resulting in a final concentration of 12 µM in wells. Viability assays and nanoplastic visualization was done in 96 well plates to allow visualization of cultures without disruption of the extracellular matrix. Cultures stained with FDA were visualized after 5-10 minutes of darkness on a Stelaris 5 confocal microscope (Lecia) with an excitation at 498 nm and a detection ranging 520-550 nm. Chlorophyll was excited at a wavelength of 405 nm and detected at 660-700 nm. FDA stain can become internalized in both dead and alive cells, however, only living cells possess active enzymes used to cleave acetate groups off of FDA to activate its fluorescent capabilities. Cells that fluoresced both chlorophyll and FDA wavelengths were determined to be alive, while cells that only fluoresced residual chlorophyll and were unable to activate FDA were determined to be dead. Visualization of nanoplastic particle localization within the extracellular matrix was conducted by exposing cells to fluorescent tagged polystyrene nanoplastics (Sigma L9902) in place of typical polystyrene nanoplastics. The fluorescent tagged nanoplastics used were excited at 538 nm and were detected from 580-620 nm.

RESULTS

Cellular proliferation

Absorbance measurements monitoring cellular proliferation over time revealed a significant delay in growth between control and nanoplastic treated cultures across all treatments (Figures 2 & 3). Cultures that were not exposed to any nanoplastics consistently reached peak growth rates earlier than both 50 µg/mL and 100 µg/mL nanoplastic exposed cultures.

Cultures grown in a typical day/night cycle that were treated with 50 ug/mL and 100 ug/mL treatments generated significant differences in relative abundance, and our data suggests that in day/night cultures increased nanoplastic concentration negatively correlated with culture growth rate (Figure 2).

Darkness cultures also experienced delayed growth due to nanoplastic exposure like the previously mentioned day/night cycle cultures, however, they differ in that increasing nanoplastic concentration from 50 µg/mL to 100 µg/mL did not result in a significant change in delayed growth (Figure 3). Our results also suggested that the

presence of a cell wall has little to no effect in the response to nanoplastic exposure, with CC-1690 and CC-406 closely resembling one another. Control cultures grown in darkness saw a significant difference in relative abundance (Figure 3C).

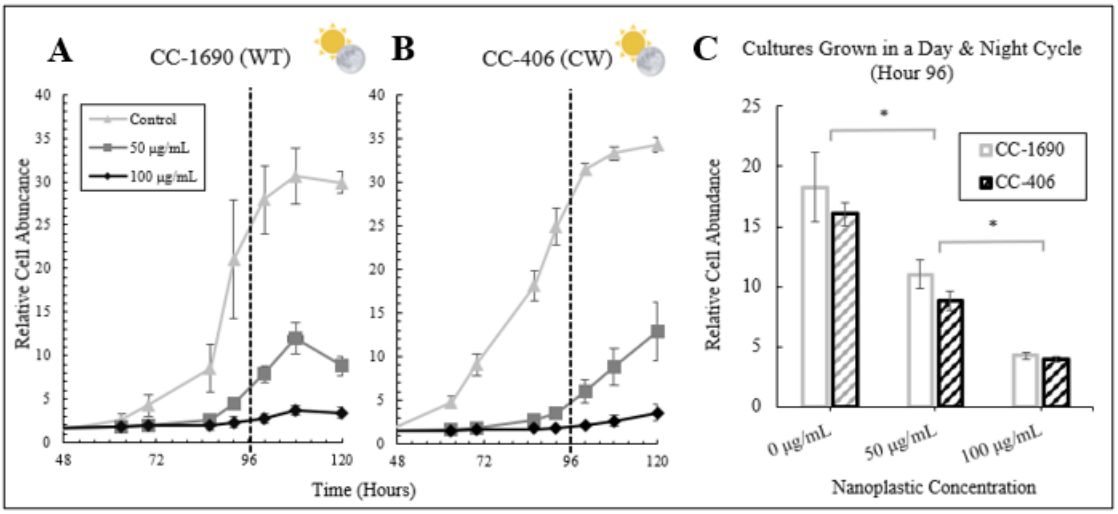

Figure 2. (A & B) Relative abundances of wildtype (CC–1690) and cell-wall-less (CC–406) strains of *C. reinhardtii* grown in a typical day/night cycle over time in hours (n = 4). **(C)** Relative abundances of CC-1690 and CC-406 grown in a day/night cycle at hour 96 while exposed to nanoplastic treatments (n = 8). (*) danotes significant difference, $p < 0.05$ T-test (two tail, two sample).

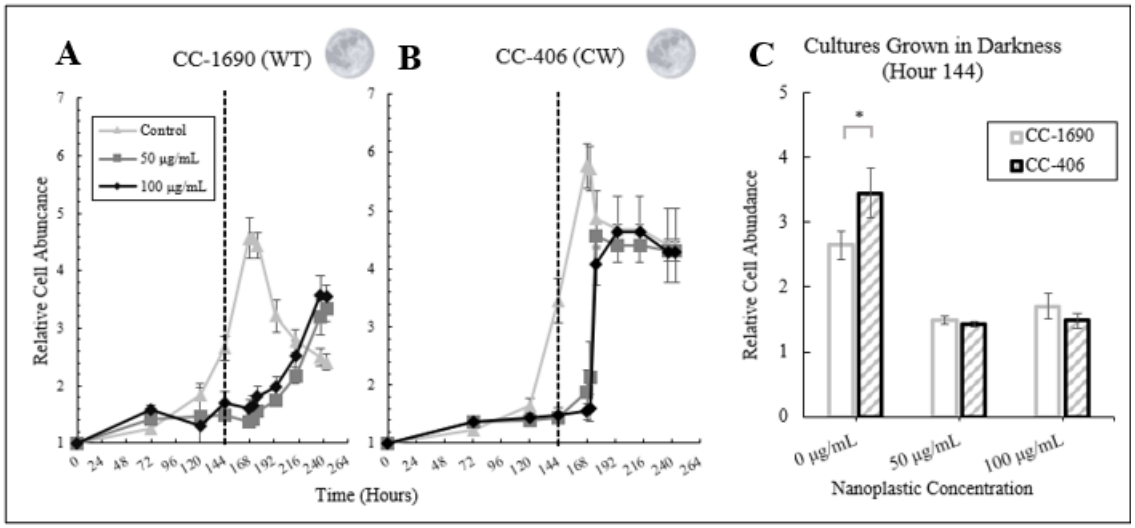

Figure 3. (A & B) Relative abundances of wildtype (CC–1690) and cell-wall-less (CC–406) strains of *C. reinhardtii* grown in darkness over time in hours (n = 4). **(C)** Relative abundances of CC-1690 and CC-406 grown in darkness at hour 144 while exposed to nanoplastic treatments (n = 8). (*) denotes significant difference, $p < 0.05$ T-test (two tail, two sample).

Visualization of Nanoplastic Localization

Use of fluorescent tagged nanoplastics to visualize polystyrene nanoplastic localization revealed that nanoplastics localized to the perimeter of cells (Figure 4). Hetero aggregation and nanoplastic localization around the perimeter of cells was observed occurring in as little as one day after nanoplastic exposure in both 50 µg/mL and 100 µg/mL treatments (not pictured). Staining and microscopy was performed at hours 120 for day/night cultures and 144 for darkness cultures because cells surrounded by fluorescent nanoplastics could be visualized alongside termed 'free cells' that appeared to be free of nanoplastic encapsulation. The timing of the microscopy photographs in (Figure 4) were not selected for the purpose of strict comparison of treated cultures to one another. The photographs were taken later in culture growth to reveal trends in nanoplastic localization between light treatments once there were observable free cells alongside those that had been encapsulated.

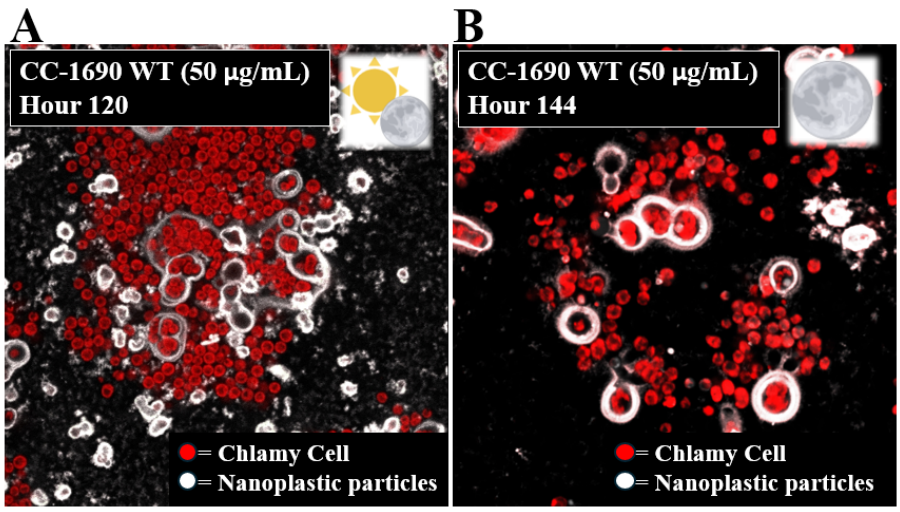

Figure 4. Wildtype *C. reinhardtii* (CC-1690) cultures treated 50µg/mL of fluorescent tagged polystyrene nanoplastics for grown in a day/night cycle (A) and darkness (B). Red represents chlorophyll autofluorescence (found in both live and dead cells). White indicates fluorescent tagged polystyrene nanoplastics that appear as clouds because they are too small to be resolved individually.

Cell Viability Assay

Exposure to nanoplastics was observed to increase cell mortality (Figure 5). Staining and exciting cultures using an FDA and chlorophyll viability assay provided observational evidence that the presence of nanoplastics positively correlated with an increased abundance of dead cells. Live cells fluoresced both the activated FDA stain (green) and chlorophyll (red), combined to make yellow/orange. Dead cells were unable to activate FDA by cleaving acetate groups and consequently only fluoresced residual chlorophyll (red), noted with white arrows.

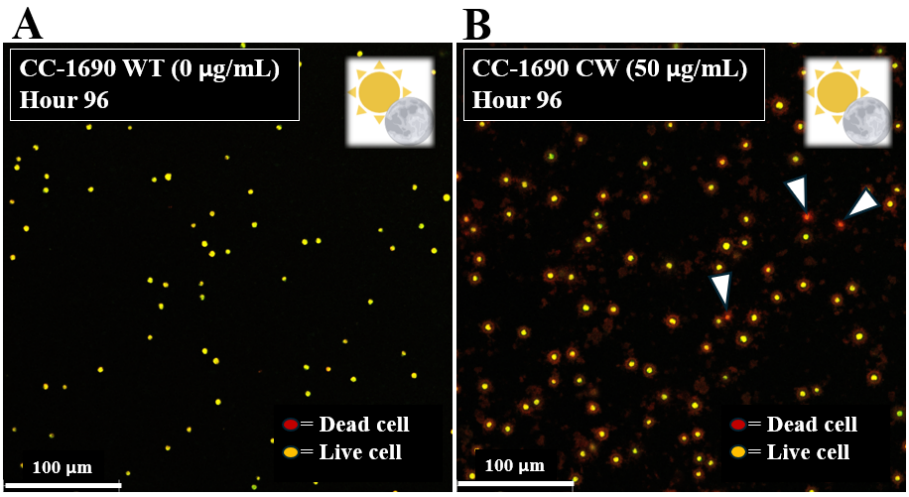

Figure 5. Wildtype *C. reinhardtii* (CC-1690) grown in a typical day/night cycle treated with polystyrene nanoplastic concentrations 0µg/mL **(A)** and 50µg/mL **(B)** stained with FDA at hour 96 after inoculation. Red indicates chlorophyll autofluorescence (found in both live and dead cells) while green indicates FDA stain only apparent in living cells. A combination of red and green results in the yellow/orange appearance of living cells. White arrows indicate dead cells.

DISCUSSION & CONCLUSION

Our results revealed a significant delay in growth between control and nanoplastic treated cultures across all variables. We expected a significant difference in relative abundance between dark and day/night treatments due to a lack of light for *C. reinhardtii* to perform photosynthesis while grown in darkness. The difference in delayed growth between cell variants grown in darkness remains unexplained, and differences in growth between the variants was not observed anywhere else within the experiment. Our evidence of delayed growth in a lab setting may suggest that *C. reinhardtii* could experience similar delays in polluted aquatic environments. The concentrations used in this experiment are well within the range of polluted aquatic environments, which can reach nanoplastic concentrations topping out around 400 µg/mL (Shi et al. 2024).

The direct cause of growth delays in *C. reinhardtii* remains unclear, however, existing publications involving different algae species and similar plastic treatments has previously investigated cell growth, viability, and aggregation. Research investigating the effect polystyrene nanoplastics have upon the growth of *Chlorella pyrenoidosa* attributed inhibited growth to the blockage of expression of aminoacyl tRNA synthetase and other related enzymes (Yang et al. 2021), however, the concentrations used were 10-fold higher than ours. Previous publications also documented polystyrene nanoplastic induced aggregation in the species *Chaetoceros neogracile* outlining hetero aggregation that resembles the hetero aggregation we observed in *C. reinhardtii* (Long et al. 2017).

The methods we used to qualify viability will need to be developed further to be able to quantify viability to be able to statistically reinforce that nanoplastic exposure is truly resulting in increased mortality. Potential causes of cell death may be associated with harmful chemicals that leach from nanoplastics that have previously been attributed to cellular damage and changes in biochemical makeup of cells (Déniel et al. 2020; Tamayo-Belda et al. 2023). Existing publications have also attributed very high nanoplastic concentrations to an inhibitory effect upon photosynthesis (Yang et al. 2021) that could also potentially cause cell death. I hypothesize that the ultimate causes of hetero aggregation, cell death, and growth delays related to nanoplastic exposure in *C. reinhardtii* could resemble the findings of the previously mentioned studies.

LIMITATIONS

The lack of additional confocal photographs of nanoplastic localization and viability to accompany the full range of treatments that were included in proliferation monitoring trials was a consequence of time. The entirety of this experiment was conducted within one academic semester; however, this leaves a framework for new incoming students to expand upon these findings.

The use of data closest to the inflection point of control cultures to produce bar graphs in (Figures 2C & 3C) was chosen arbitrarily to provide a potentially insightful comparison in a separate figure where a visual difference in growth would be more apparent. It may be worth investigating where inflection points occur to better quantify and compare delays in growth across nanoplastic and light treatments. Alternative methods to clearly visualize differences within cultures at a specific timepoint should also be considered. It is likely that the growth rate at hour 144 of cultures grown in darkness does not correspond well with the hour 96 growth rate of day/night cycle cultures. Gaining a better understanding of when cultures grown in darkness are nearing peak growth rates may provide higher quality insights to trends and allow better comparison of growth rates between light treatments.

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