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The combined effects of chemicals used in textile processing on plankton communities

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Honors Thesis

Department of Biology

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Under the Direction of

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and

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Introduction:

Chemical pollution in rivers and ponds has many repercussions for the organisms who live there including direct (e.g. mortality, behavioral abnormalities) and indirect (e.g. food reduction, increased predation, competition) effects (Fleeger et al. 2003). One major pathway for chemical entry into surface waters is through industrial discharge (de Zwart 2018). Because many chemicals are present in industrial effluent at the same time, they may interact with each other, resulting in potentially more toxic effects than each might have on its own (Fleeger et al. 2003, de Zwart 2018).

The textile industry discharges numerous chemicals simultaneously (Moore & Ausley 2004; EPD 2013) including flame retardants (e.g. tetrakis hydroxymethyl phosphonium chloride; THPC), curing agents (ammonia) and oxidizers (hydrogen peroxide) (WHO 2000; EPD 2013; Zope 2017). Individually, these chemicals are known to alter the abundance of microscopic algae (phytoplankton) and zooplankton grazers, such as *Daphnia magna*, that are important food resources for freshwater fish in river and lakes. THPC decreased the growth rate of the green microalga *Selenastrum capricornutum* at concentrations less than 0.8 mg L^{-1}, which is a concentration observed in surface waters (WHO 2000). In contrast, ammonium may increase biomass of microalgal species like *Chlorella* sp. because the inorganic nitrogen is used as a nutrient source (Li et al. 2008). Finally, hydrogen peroxide (H_2O_2) not only reduces algal abundance but is recommended as a treatment for cyanobacterial blooms at concentrations of 2.3 mg L^{-1} (Weenik et al 2015). However, its presence is short-lived in the environment; H_2O_2 no longer affected algal abundance after approximately 96 hours (Weenik et al 2015).

Zooplankton are also affected by these chemicals, though not necessarily in the same way as phytoplankton. Osier and Cohen (2020) found that THPC concentrations of 0.8 mg L^{-1}

decreased abundance of large freshwater cladocerans, like *Daphnia magna* in plankton communities. Bartolome (2005) reported that THPC decreased saltwater *Artemia salina* larvae phototactic response at 25 µg L^{-1} (>30 times lower than their calculated 24-hour LC50). At environmentally relevant concentrations (≤ 0.58 mg L^{-1}), ammonium decreased survival, development, and reproduction in three *Daphnia* species (Lyu et al 2013, Cao et al. 2014). Hydrogen peroxide decreased *D. magna* survival rates and reproductive abilities above concentrations of 1.25 mg L^{-1} (Meinertz 2007), which is within the range of concentrations (0.5-2.0 mg L^{-1}) found around a textile company located along the Ogeechee River (32°36'17.1"N 81°44'28.2"W) in Southeast Georgia (Tetra Tech EM Inc. 2011). While individual effects of each chemical occur at concentrations found in the environment, their effects could differ in mixture.

Chemicals in mixture may interact in different ways, including additive (toxicity is the sum of individual chemical effects), antagonistic (toxicity of a chemical is reduced in the presence of another), synergistic (chemicals' effects are equal to more than the sum of each chemical's effect alone), or potentiation (the addition of one chemical with no toxic effect increases the toxicity of another chemical) interactions. For example, Liu et al. (2015) found that the toxicity of commercial antimicrobial formulations made of PAA (Peracetic acid), acetic acid, hydrogen peroxide, stabilizers and water increased *D. magna* mortality due to the additive effects of PAA and hydrogen peroxide. However, it is not certain how mixtures with ammonium will affect chemical effects on aquatic organisms. Combining ammonium and THPC decreased copepods, such as *D. magna*, in a plankton community (in an additive fashion) while the individual chemicals did not (Osier and Cohen 2020). Ammonium combined with algal toxin (microcystin-LR) delayed *D. magna* development and reduced offspring output synergistically to

controls (Cao et al. 2014). However, Liang et al (2018) found that *Brachionus calyciflorus* post-reproductive period and posterolateral spine length were antagonistically affected by the chemical combination of ammonia and microcystin-LR. Furthermore, ammonium can indirectly affect zooplankton. The positive relationship between nitrogen sources (e.g. ammonium) and algae has the potential to maintain or increase the amount of food available to zooplankton grazers in the presence of contaminants (Duff et al 2017). Liu et al (2015) found that ammonium at concentrations of 0.5 mM have an antagonistic interaction with chromium toxicity on the chlorophyll *a* and *b* in *Chlorella vulgaris*. These studies indicate that mixtures of ammonium, THPC, and hydrogen peroxide likely have different and possibly unpredictable effects on aquatic organisms than each chemical individually, and therefore should be investigated.

In addition to the presence of multiple chemicals, changes in environmental conditions, such as temperature, can stress aquatic organisms. For example, *Daphnia pulex* exposed to increasingly higher temperatures (from 20°C to 25°C to 30°C), increased stress protein production (Becker et al. 2018). When aquatic organisms are under stress from increasing temperatures, they can become more susceptible to the effects of contaminants in their environment (Shamsollahi et al 2015). Increasingly negative effects of zinc on *D. magna* reproduction, growth, and survival occurred after the temperature increased from 20 to 24°C (Zhang et al. 2018). Thus, responses to chemical mixtures under a range of conditions must be studied to predict effects on planktonic species exposed to chemical combinations in the environment.

I hypothesized that the individual effects of THPC (T), ammonium (A), and H_2O_2 (H) on freshwater plankton communities differ from effects of the chemicals in mixture. Individually, each chemical was expected to adversely affect *D. magna* survival and increase stress response

(catalase production), while the mixtures should have additive effects (Table 1). The *Chlorella* sp. abundance was predicted to increase in the presence of the ammonium addition but decrease in the THPC and peroxide treatments. In THPC and peroxide mixtures with ammonium, antagonistic effects were expected on algal abundance due to the nutrient addition aiding cell growth that would increase with temperature, however the THPC+H_2O_2 mixture was predicted to have additive effects from interactions between the chemicals. A decrease in algal abundance was anticipated to negatively affect *D. magna* by decreasing the food supply. The THPC+ H_2O_2+NH_4 mixture was expected to have additive effects on both organisms with the THPC and H_2O_2 expected to negate the positive effects of nutrient addition from the ammonium.

Methods:

Study Organisms

The green microalga *Chlorella* sp. was purchased from Carolina Biological Supply (Catalog No.: S07465ND) and cultured in Alga-Gro medium for freshwater. These cultures were maintained at 22°C on a 16:8 light-dark cycle.

Daphnia magna were purchased from Carolina Biological Supply (Catalog No.: S07268ND) and cultured in tanks with sufficient *Chlorella* sp. to avoid food limitation, and kept on a 16:8 light-dark cycle (80 µmol m^{-2} s^{-1}) (Nyholm and Källqvist 1989; Teresa et al. 2019) at 18°C. The *D. magna* used in all experiments were less than one week old.

1. Pilot Study – Determination of Temperature and Chemical Concentration Treatments

A pilot study was conducted to determine appropriate temperatures, THPC, and H_2O_2 concentrations to be tested in mixture on *D. magna* and *Chlorella* sp. Experimental treatments

consisted of two peroxide concentrations (0 or 2.0 mg L^{-1} H_2O_2 (H)), two THPC concentrations (0 or 1.0 mg L^{-1} THPC (T)), and two temperatures (20°C or 30°C) in a fully-crossed design resulting in eight treatments with 4-fold replication (n=4). These treatments were selected based on values that occur in the environment (20-30°C; Dyar & Alhadeff 1997; Zeis 2013; Weenik et al. 2015; Osier & Cohen 2020; Cambronero 2018). Sampling occurred after 0, 24, 48, and 96 hours of exposure. The microcosms were 250 ml beakers containing 200 ml of treatment solution, five *D. magna* (to avoid stress effects from overcrowding; Burns 1995), and *Chlorella* sp. at a density of $2.25*10^4$ cells ml^{-1} to provide *D. magna* sufficient food for the duration of the experiment (Kelly & Cohen 2018) The microcosms were randomized by location in one of two environmental chambers (Thermo Scientific-Precision) maintained at either 20°C or 30°C with a 16:8 light-dark cycle and an average light intensity of 21.43±0.80 µmol m^{-2} s^{-1} (Teresa et al. 2019).

At each time point, the temperature in each microcosm was measured immediately upon removal from the chamber, followed by hand-held meter measurements of water quality: pH (Oakton phTestr10 MP-35634-10), dissolved oxygen (Cole Palmer Traceable® Dissolved Oxygen Pocket Tester UX-19601-31), and conductivity (Oakton ECTestr11 WD-35662-35). To determine abundance of *Chlorella* sp., a 1 ml sample from each beaker was taken after homogenization by shaking followed by measurement on a flow cytometer (BD Accuri, Becton-Dickinson, CA, USA). The flow cytometer was used to count the *Chlorella* sp. cells using lasers to characterize each cell based on the fluorescence emitted by the cell. The number of live *D. magna* were enumerated visually, and the number of dead *D. magna* were calculated by subtracting the number alive at the time of measurement from the initial number of *D. magna*.

Statistical Analysis:

Data were first tested to determine whether assumptions of parametric tests were met using Levene's test (homogeneity of variances) and the Shapiro-Wilk *W* test (normality). To determine the effects of THPC, peroxide and temperature alone or in mixture on *Chlorella* sp. cell density and *D. magna* mortality, data were analyzed using 3-way ANOVA followed by Tukey-Kramer post-hoc multiple comparisons [all analyses were performed using JMP®, Version 13 (SAS Institute Inc., Cary, NC, 1989-2019)].

2. Greenhouse Experiment

Experimental Design

A greenhouse experiment was conducted to determine how daily temperature fluctuation affects toxicity of textile chemicals individually and in mixture to *D. magna* and *Chlorella* sp. The experimental treatments consisted of two peroxide treatments (0 or 2.0 mg L^{-1} H_2O_2 (H); Hydrogen peroxide, Acros Organics, 35 wt% in H_2O_2, CAS: 7722-84-1), two THPC treatments (0 or 1.0 mg L^{-1} THPC (T); Tetrakis(hydroxymethyl)phosphonium chloride solution, Aldrich Chemistry, 80% in H_2O_2, CAS: 124-64-1), two ammonium treatments (0 or 0.5 mg L^{-1} NH_4 (A); Ammonium chloride, Fisher Scientific, CAS: 12125-02-9). These chemical treatments were tested in a fully crossed design yielding a total of 8 treatments X 4 time points X six-fold replication (N=192). Concentrations of each chemical were selected to reflect concentrations measured in the environment but known to have effects on plankton species abundance from the published literature (WHO 2000; Xiang et al. 2010; Cao et al. 2014; Meinertz et al. 2007). In addition, the concentrations of THPC and peroxide were determined based on results from the pilot study. The experimental duration was one week to allow for the possibility of observing

chronic effects on *D. magna*. Measurements of *D. magna* mortality, *D. magna* stress, *Chlorella* sp. cell density, *Chlorella* sp. pigment concentration, and water quality were taken from each treatment (n=6) initially and after 24 h, 72 h, and one week.

The hydrogen peroxide, THPC and ammonium were administered as a single dose at the beginning of the experiment to simulate a discharge event (Constable et al. 2003; Moore and Ausley 2004). This experiment was conducted in the summer to observe the effects of moderately high temperatures on *D. magna* mortality and stress and *Chlorella* sp. cell density and pigment (chlorophyll *a*) concentration. The microcosms (independent 250 ml beakers) contained 200 ml of treatment solution, 5 *D. magna*, and enough food for the duration of the experiment (*Chlorella* sp. at a density of $2.25*10^4$ cell ml^{-1}; Kelly & Cohen 2018) and were randomized by location in the Georgia Southern University Biology greenhouse (32.4216, -81.7910). Microcosms were subjected to diel variation in temperature (23-27.5°C) and covered with one layer of window screening reduce incident light by 50% and simulate light levels in shallow surface waters while avoiding algal photoinhibition (Kim et al. 2015).

Sampling and Processing

At each time point, measurements of water column pH, dissolved oxygen, conductivity, and temperature were taken first with handheld meters (Traceable Dissolved Oxygen Meter; Eutech EC/TDS/SALT Testr; Eutech pH Testr) to monitor water quality. Then, *D. magna* abundance and mortality were measured visually, and the living *D. magna* were collected and stored at -80°C for catalase analysis. *Chlorella* sp. abundance was determined last, because the microcosm had to be homogenized by vigorous shaking to resuspend the cells before sampling could occur.

To measure *Chlorella* sp. abundance as cell density, a 1 ml sample was collected at each time point, and the cells were counted using a flow cytometer (BD Accuri, Becton-Dickinson, CA, USA). To measure *Chlorella* sp. abundance as pigment concentration, a 100 ml sample was vacuum filtered through a Whatman GF/F glass fiber filter (nominal pore size 0.7 µm) to collect algal cells, followed by storage at -20°C until analysis. The pigments were extracted using 90% acetone for 24 hours at -20°C, and fluorescence was measured using a Trilogy fluorometer (Turner Designs Sunnyvale, CA) according to EPA method 445.0 (Arar & Collins 1997).

Stress in *D. magna* was measured using catalase assays according to methods of Laspoumaderes et al. (2017) using a standard Catalase Assay Kit (Cayman Chemical Item No. 707002). Because of the high *D. magna* mortality on Day 7, evidence of stress was measured after 3 days of exposure to treatments. Prior to running the assay, each sample received 100 µL of phosphate buffered saline followed by homogenizing using a pestle in 1 ml centrifuge tubes and centrifugation for 15 minutes at 10,000 x g at 4ºC. Each sample well in the assay plate received 100 µL of diluted assay buffer, 30 µL of methanol, and 20 µL of sample, and samples were analyzed in duplicate. Each of the samples were activated with 20 µL of diluted hydrogen peroxide, deactivated with 30 µL of potassium hydroxide, and given 10 µL of catalase potassium periodate to dye the solution before using a plate reader to determine the absorbances of the samples at 540 nm.

Because the catalase samples did not contain the same amount of *D. magna* tissue, a protein assay was required to standardize the stress protein measurements. The protein assay was performed using a Coomassie Plus (Bradford) protein assay kit (Thermo Scientific No. 23236) with an adjusted Albumin (BSA) standard curve (0-1000 µg BSA mL^{-1}) to fit the range of protein we observed. The protein samples were taken from the catalase assay after they had been

prepared but before they were run. In a microplate, 10 µL of the standards were distributed in duplicate into the appropriate wells, and 10 µL of sample were administered in duplicate to the remaining wells making sure to keep the samples cold to avoid sample breakdown. Each well received 300 µL of the Coomassie Plus reagent and allowed to incubate on a shaker table for 10 minutes. Then the samples' absorbances were recorded at 595 nm by a plate reader.

Statistical Analysis

The data were first tested for the assumptions of homogeneity of variances using Levene's test and normality using the Shapiro-Wilk *W* test. One-way ANOVA was used to test for effects of chemical treatment on *D. magna* mortality, *D. magna* stress, *Chlorella* sp. chlorophyll *a* concentration, and *Chlorella* sp. cell density. Only catalase concentration met the assumptions of parametric tests and was followed by Tukey-Kramer post-hoc multiple comparisons. Chemical effects on *D. magna* mortality, and both measures of *Chlorella* sp. abundance could not be transformed and were therefore analyzed using Kruskal-Wallis tests followed by nonparametric post hoc analyses. All analyses were conducted in JMP®, Version 13. (SAS Institute Inc., Cary, NC, 1989-2019).

Results:

1. Pilot Study – Determination of Temperature and Chemical Concentration Treatments

Initially, there were negative effects of hydrogen peroxide on *Chlorella* sp. cell density with a decrease in cell abundance of almost 20% without THPC addition (Table 2a; Fig 1a, b,). A trend of hydrogen peroxide addition decreasing the cell density in the 20°C was observed throughout the entire experiment (Figure 1).

Cell density was affected by the interaction between peroxide and temperature (Table 2b) and the interaction of THPC, peroxide, and temperature after 24 hours of exposure (Table 2b). After 24 hours, both chemicals (H_2O_2 and THPC), appeared to decrease the cell density by approximately half at 20°C, but they did not have the same effect at 30°C where hydrogen peroxide seemed to increase the cell density when THPC was not present and decreased cell density combined with THPC (Fig 1c, d). Only temperature affected *D. magna* mortality to increase ~20% in the no peroxide treatments and ~50% in the peroxide addition treatments after 24 hours, (Table 2b; Fig 2b).

After 48 hours of exposure, all three factors influenced cell density individually (Table 2c). Temperature increased *Chlorella* sp. abundance; all treatments at 30°C had cell densities >26,000 cells mL^{-1} higher than the treatments at 20°C (Fig 1). Also, THPC addition decreased all cell densities by >5,000 cells mL^{-1} in both temperature treatments (Fig 1e, f). In contrast, hydrogen peroxide decreased *Chlorella* sp. cell density (3,000-21,000 cells mL^{-1}) at 48 hours whether THPC was present or not (Table 2, Fig 1). After 48 hours, almost all the *D. magna* were dead in all the 30°C treatments (Table 2c, d, Fig 2d, f).

After 96 hours, there was a significant interaction between THPC and temperature (Table 2d) and hydrogen peroxide and temperature (Table 2d) on cell density. There was no longer any influence from the hydrogen peroxide. *Chlorella* density was still decreasing in the hydrogen peroxide treatment groups at 20°C (>5,000 cell mL^{-1} lower), but at 30°C the hydrogen peroxide treatment groups were benefiting the *Chlorella* sp. cell abundance without THPC (Fig 1g, h). In the 30°C treatment, the cell densities were similar in the presence of THPC regardless of the peroxide addition

Overall, 2 mg L^{-1} hydrogen peroxide decreased the *Chlorella* sp. cell density in the 20℃ treatments regardless of whether THPC was present. However, treatments with 1 mg L^{-1} THPC present generally had lower cell densities than those without in the 20℃ treatments. After the first 48 hours, part of the reason why the 30℃ treatments had significantly higher cell density than the 20℃ treatments due to the lack of grazing pressure from the *D. magna.* As a result of the pilot study, the hydrogen peroxide and THPC treatments were selected for subsequent experiments, however, the high temperature treatment for the laboratory experiment (Appendix 1) was decreased to 25°C to avoid high *D. magna* mortality rates due to heat stress (Folt et al 1999).

2. Greenhouse Experiment

A. Daphnia magna mortality and stress responses to textile chemicals

After one week, exposure to textile chemicals increased *D. magna* mortality ($X^2_7 = 40.2$, $p<0.0001$). In particular, the differences occurred in the presence of THPC; *D. magna* mortality in all treatments containing THPC reached 95-100%, while mortality in the control, and all other chemical treatments averaged 25% or less (Figure 3). It appears that this high mortality was unaffected by the presence of other chemicals in mixture, since mortality was equally high regardless of whether THPC was alone or mixed with one or both of the other chemicals.

Catalase (stress protein) concentration increased due to the chemical additions, however results from post hoc tests showed that the only treatment that was elevated relative to the others was the T+H+A mixture ($F_{7,23} = 22.2$, $p<0.0001$). Each of the chemicals alone had very similar effects as the control, while the mixtures with two chemicals (one of which was ammonium)

exhibited a nonsignificant pattern of increase; *D. magna* in these treatments had nearly twice the catalase activity (Figure 4).

B. Changes in Chlorella sp. abundance in response to textile chemicals

The addition of textile chemicals altered *Chlorella* sp. abundance, but the results differed depending on whether abundance was measured as cell density ($X^2_7 = 28.1$, p=0.0002) or chlorophyll a concentration ($X^2_7 = 35.2$, p<0.0001) (Figure 5). Increased cell density occurred only in the THPC treatment, although there were nonsignificant patterns of increase in the peroxide and ammonium treatments and the T+H treatment (76-300%), and patterns of decrease in the T+A and T+H+A treatments (43-51%) (Figure 5). Chlorophyll *a* concentrations were elevated by more than 190% in mixtures containing THPC and hydrogen alone treatment (Figure 5).

Another influential factor on *Chlorella* sp. abundance was the grazing pressure from *D. magna*. Total mortality of *D. magna* occurred in the THPC treatment (Figure 3). This lack of grazing pressure on *Chlorella* sp. in the THPC treatment helps to explain the increased cell density relative to the control. However, this pattern of increased *Chlorella* sp. cell abundance with complete *D. magna* mortality was not observed in the other THPC-mixture treatments (Figure 5), suggesting chemical toxicity to *Chlorella* sp. The T+H, T+A, and T+H+A treatment bars on Figure 5 had cell density values that were similar to the control and significantly higher chlorophyll *a* than the control and the THPC alone because of the toxicity of the chemical mixtures.

Throughout the experiment, the water quality measurements (Table 3) stayed consistent and within acceptable ranges for *D. magna* and *Chlorella* sp.

Discussion:

My hypothesis that the effects of THPC, ammonium, and hydrogen peroxide alone would differ from their effects in combination was generally supported. Stress in *D. magna* increased only when all three chemicals were present, and *Chlorella* sp. abundance as chlorophyll *a* differed from the control in all of the mixtures but one. However, *D. magna* mortality was sensitive to THPC. Although mortality was high in all of the mixtures containing THPC, the same loss of *D. magna* occurred in the presence of THPC alone.

It was predicted that all the chemicals would decrease *D. magna* mortality individually (Osier & Cohen 2020, Bartolome 2005, Lyu et al 2013, Cao et al 2014, Meinertz 2007), and this decrease would be additive in combination (Osier & Cohen 2020, Liu et al 2015, Cao et al 2014). However, THPC was the only individual chemical to decrease zooplankton abundance, and any mixtures with THPC had the same effect, suggesting that neither peroxide nor ammonium negatively affected *D. magna* at the concentrations tested. These results are consistent with the outcome of the pilot study where temperature was the only stressor that decreased *D. magna* mortality (Table 2 c, d). This may be because hydrogen peroxide breaks down only a few hours after it is added to water (Weenick 2015), and the ammonium is being taken up as nutrients by the *Chlorella* sp. throughout the week (Duff et al 2017). Therefore, the effect of the combination of ammonium and peroxide was not additive, but rather similar to the control.

Each chemical alone was predicted to increase the concentration of the stress protein catalase in *D. magna*, and the combination of the chemicals to cause additive increases in catalase concentration (Fan et al 2012). While the H+A and T+H mixtures appeared to have additively increases in catalase concentration over the individual chemicals the T+H+A treatment synergistically increased *D. magna* catalase concentration. This may be due to these

chemicals interacting and increasing each other's toxicity along with indirect effects on the *Daphnia*'s food source and food quality (Fleeger et al 2003). It is also likely catalase activity was affected by heat stress brought on by the greenhouse conditions, like those seen during the pilot study (Folt et al 1999).

Chlorella sp. abundance did not respond to chemical treatments as predicted. The addition of ammonium showed a pattern of increased *Chlorella* sp. abundance (Li et al 2008). However, the peroxide and THPC treatments did not decrease the abundance values like predicted (WHO 2000, Weenik et al 2015). Instead, the cell density values of the THPC only treatment were increased by 344% without *Daphnia* grazing pressure, while the cell densities of the peroxide and ammonium treatments were similar to the control. The mixtures did not have significantly lowered abundance values than the chemicals alone as predicted. Instead, the change in cell densities for the mixtures were similar to the control, and the change in chlorophyll a was increased by 190-339%. The abundance values were likely influenced by the toxicity and interactions of the chemicals in mixture. It has been shown that under stressful conditions, such as metal pollution (Franqueira et al 2000) that chlorophyll *a* in microalga can increase without a corresponding increase in cell density (Beale & Appleman 1971), and is consistent with what was observed in this study.

Reduced abundance of plankton species that form the base of aquatic food webs worldwide that could adversely affect energy transfer to higher trophic levels. Such bottom up effects occur in fisheries where primary producers affect the amount of fish produced for consumer use (Chassot et al 2007). This is why it is important to consider the chemicals and the concentrations of these chemicals that are being deposited into lakes and rivers because removal of trophic levels has been shown to have adverse effects to ecosystems (Duffy 2002). Removal

of a species or group from aquatic ecosystems via pollutants can cause indirect effects on the surrounding trophic levels due to changes of energy flow through the food web (Relyea and Hoverman 2006). The chemical mixture effects observed in this study affected organism abundance in ways that would not have been apparent if any of these chemicals had been tested by themselves. In the future, other environmental stressors like changes in dissolved oxygen or salinity should be observed alongside these chemicals to simulate more realistic environmental conditions. Future research should also expand to higher trophic levels to observe the chemical and energy transfer effects textile chemicals have on aquatic food webs.

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Figure Captions:

Figure 1. *Chlorella* sp. density at 20 ºC and high 30 ºC after 0 (a,b), 24 (c,d), 48 (e,f) and 96 (g,h) hours of exposure to treatments in the pilot study. Error bars are ± one standard error of the mean (SEM) and n=4.

Figure 2. Mortality of *Daphnia magna* in pilot study at 20 ºC and 30 ºC after 24 (a,b), (c,d), and 96 (e,f) hours of exposure to treatments no mortality occurred at time zero (not shown). Error bars are ± one SEM and n=4.

Figure 3. Mean *D. magna* percent mortality after seven days of chemical exposure in greenhouse experiment. Means with asterisks are significantly different from the control. Error bars are ± one SEM and n=6.

Figure 4. Mean catalase activity for surviving *D. magna* after three days of chemical exposure in greenhouse. Asterisk indicates significant difference from the control. Error bars are ± one SEM and n=6.

Figure 5. Mean percent change from starting *Chlorella* sp. abundance (cell density and chlorophyll *a* concentration) after seven days of chemical exposure in greenhouse experiment. The black asterisk indicates a change in cell density percent different from the control. Grey asterisks represent differences in chlorophyll *a* concentration relative to the control. Error bars are ± one SEM and n=6.

Tables:

Table 1. Predicted outcomes for mixtures of textile chemicals THPC, ammonium, H_2O_2, and temperature on plankton abundance.

Stressor	Phytoplankton abundance (*Chlorella* sp.)	Citations	Zooplankton abundance (*Daphnia magna*)	Citations
THPC	decrease	WHO 2000	decrease	Osier & Cohen 2020
NH_4	increase	Li et al 2008	decrease	Bartolome 2005 Lyu et al 2013, Cao et al 2014
H_2O_2	decrease	Weenik et al 2015	decrease	Meinertz 2007
THPC+NH_4	antagonism	Liu et al 2015	additive	Osier & Cohen 2020
THPC+H_2O_2	additive		additive	Liu et al 2015
NH_4+H_2O_2	antagonism	Liu et al 2015	additive	Cao et al 2014
THPC+NH4+H_2O_2	additive		additive	

Table 2. Three-factor ANOVA results showing chemical and temperature effects on *Chlorella* sp. cell density and *D. magna* mortality at each time point post-exposure: a. initial, b. 24 hours, c. 48 hours, and d. 96 hours. No *D. magna* mortality occurred at time zero.

a. Initial (0 hours)

		Cell density		Mortality	
	df	F	p	F	p
THPC	1	1.82	0.19	-	-
H_2O_2	1	12.35	**0.002**	-	-
THPC × H_2O_2	1	0.97	0.33	-	-
Temperature	1	2.12	0.16	-	-
THPC × Temperature	1	2.34	0.14	-	-
H_2O_2 × Temperature	1	3.90	0.06	-	-
THPC × H_2O_2 ×Temperature	1	0.45	0.51	-	-

b. 24 hours

		Cell density		Mortality	
	df	F	p	F	p
THPC	1	4.33	**0.048**	0.023	0.88
H_2O_2	1	8.84	**0.007**	1.83	0.19
THPC × H_2O_2	1	0.21	0.64	0.02	0.88
Temperature	1	6.22	**0.02**	6.52	**0.02**
THPC × Temperature	1	0.64	0.43	0.56	0.46
H_2O_2 × Temperature	1	7.87	**0.01**	3.81	0.06
THPC × H_2O_2 ×Temperature	1	6.02	**0.02**	0.023	0.88

c. 48 hours

		Cell density		Mortality	
	df	F	p	F	p
THPC	1	5.09	**0.03**	0.23	0.63
H_2O_2	1	23.38	**<0.0001**	0.23	0.63
THPC × H_2O_2	1	2.43	0.13	0.23	0.63
Temperature	1	136.37	**<0.0001**	1298.08	**<0.0001**
THPC × Temperature	1	0.50	0.49	2.08	0.16
H_2O_2 × Temperature	1	0.001	0.98	0.23	0.63
THPC × H_2O_2 ×Temperature	1	1.34	0.26	2.08	0.16

d. 96 hours

		Cell density		Mortality	
	df	F	p	F	p
THPC	1	11.52	**0.002**	1.92	0.18
H_2O_2	1	0.14	0.71	0.69	0.41
THPC × H_2O_2	1	0.33	0.57	0.08	0.78
Temperature	1	280.44	**<0.0001**	387.77	**<0.0001**
THPC × Temperature	1	7.39	**0.01**	1.92	0.18
H_2O_2 × Temperature	1	6.87	**0.02**	0.69	0.41
THPC × H_2O_2 ×Temperature	1	3.32	0.08	0.08	0.78

Table 3. Water quality for greenhouse experiment.

a. Mean temperature (°C) on each day for each treatment.

Treatment	Day 0	Day 1	Day 3	Day7
Control	21.4±0.07	23.1±0.10	22.8±0.07	26.2±0.11
H2O2	21.1±0.04	23.1±0.08	22.9±0.04	26.4±0.06
NH4	21.0±0.02	23.2±0.04	23.0±0.03	26.6±0.02
THPC	20.8±0.15	23.5±0.08	23.1±0.03	26.8±0.04
H+A	20.8±0.04	23.7±0.06	23.3±0.02	26.9±0.05
T+H	21.1±0.02	23.8±0.04	23.4±0.05	27.1±0.02
T+A	20.9±0.03	24.1±0.04	23.6±0.02	27.2±0.02
T+H+A	20.9±0.05	24.5±0.04	23.8±0.02	27.1±0.04

b. Mean conductivity (µS cm^{-1}) on each day for each treatment.

Treatment	Day 0	Day 1	Day 3	Day 7
Control	243±2.1	240±0.0	240±0.0	230±2.6
H2O2	240±0.0	240±0.0	240±0.0	238±1.7
NH4	243±2.1	242±1.7	240±0.0	240±0.0
THPC	240±0.0	240±0.0	240±0.0	240±0.0
H+A	250±0.0	245±2.2	240±0.0	235±3.4
T+H	245±2.2	242±1.7	240±0.0	240±0.0
T+A	250±0.0	248±1.7	240±0.0	250±0.0
T+H+A	247±2.1	250±0.0	240±0.0	250±0.0

c. Mean dissolved oxygen concentration (mg L^{-1}) on each day for each treatment.

Treatment	Day 0	Day 1	Day 3	Day 7
Control	7.8±0.02	7.2±0.03	7.5±0.13	6.8±0.26
H2O2	7.6±0.04	7.9±0.04	7.5±0.16	6.3±0.11
NH4	8.0±0.09	7.6±0.08	7.4±0.20	6.2±0.12
THPC	7.6±0.08	7.3±0.14	7.1±0.13	6.2±0.04
H+A	8.3±0.03	7.3±0.04	7.2±0.13	5.9±0.10
T+H	8.0±0.05	7.5±0.12	7.1±0.17	5.9±0.02
T+A	8.2±0.06	7.4±0.07	7.5±0.04	5.9±0.05
T+H+A	8.1±0.08	7.2±0.08	7.4±0.13	5.9±0.06

d. Mean pH on each day for each treatment.

Treatment	Day 0	Day 1	Day 3	Day 7
Control	8.3±0.02	8.5±0.00	8.5±0.02	8.5±0.02
H2O2	8.2±0.00	8.4±0.00	8.5±0.00	8.7±0.02
NH4	8.3±0.00	8.4±0.00	8.5±0.00	8.6±0.03
THPC	8.3±0.02	8.4±0.00	8.5±0.00	8.6±0.02
H+A	8.3±0.02	8.4±0.00	8.6±0.02	8.5±0.00
T+H	8.2±0.00	8.4±0.00	8.5±0.00	8.6±0.02
T+A	8.3±0.02	8.4±0.00	8.5±0.00	8.6±0.00
T+H+A	8.2±0.00	8.4±0.02	8.5±0.00	8.6±0.00

Figures:

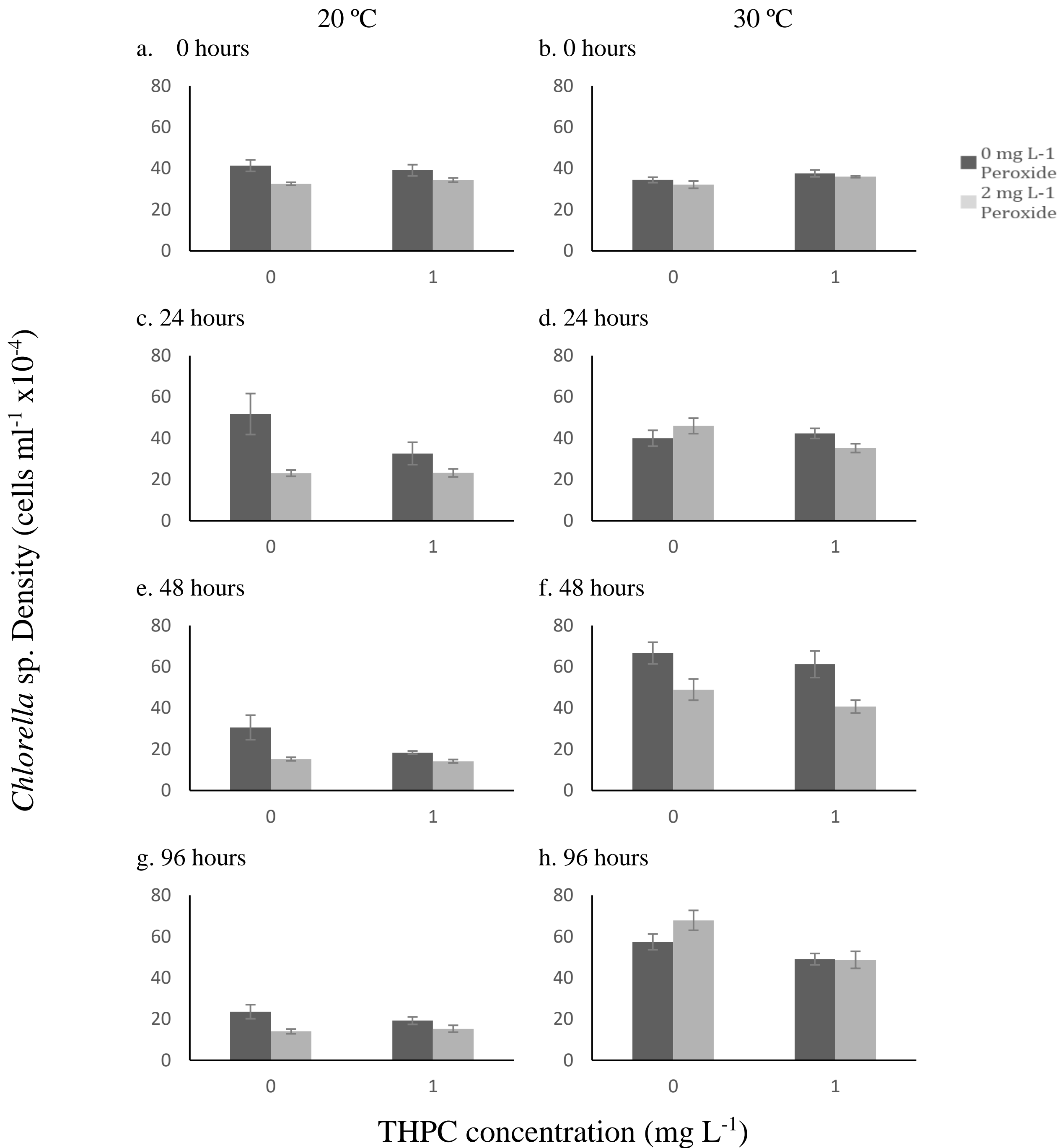

Figure 1. *Chlorella* sp. density at 20 ºC and high 30 ºC after 0 (a,b), 24 (c,d), 48 (e,f) and 96 (g,h) hours of exposure to treatments in the pilot study. Error bars are ± one standard error of the mean (SEM) and n=4.

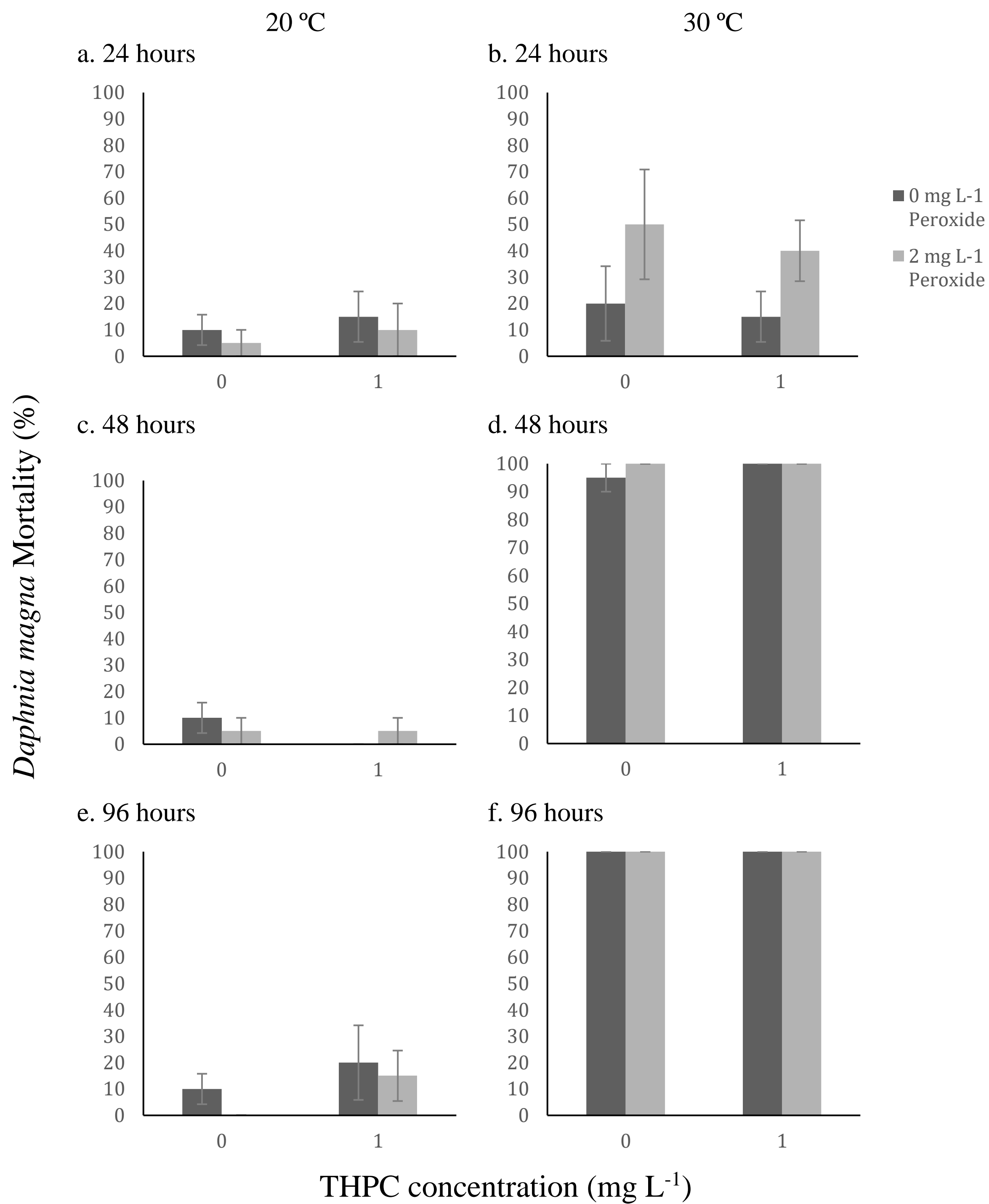

Figure 2. Mortality of *Daphnia magna* in the pilot study at 20 ºC and 30 ºC after 24 (a,b), (c,d), and 96 (e,f) hours of exposure to treatments no mortality occurred at time zero (not shown). Error bars are ± one SEM and n=4.

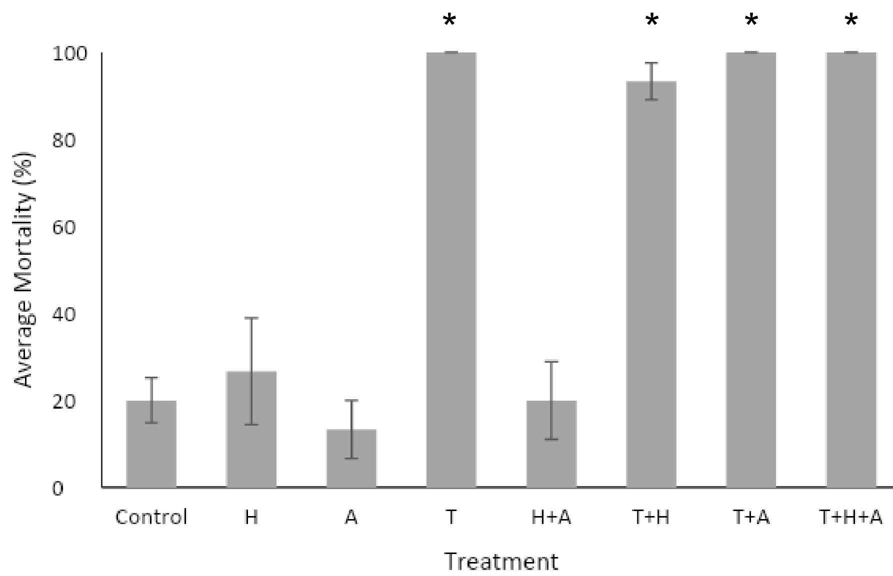

Figure 3. Mean *D. magna* percent mortality after seven days of chemical exposure in the greenhouse experiment. Means with asterisks are significantly different from the control. Error bars are ± one SEM and n=6.

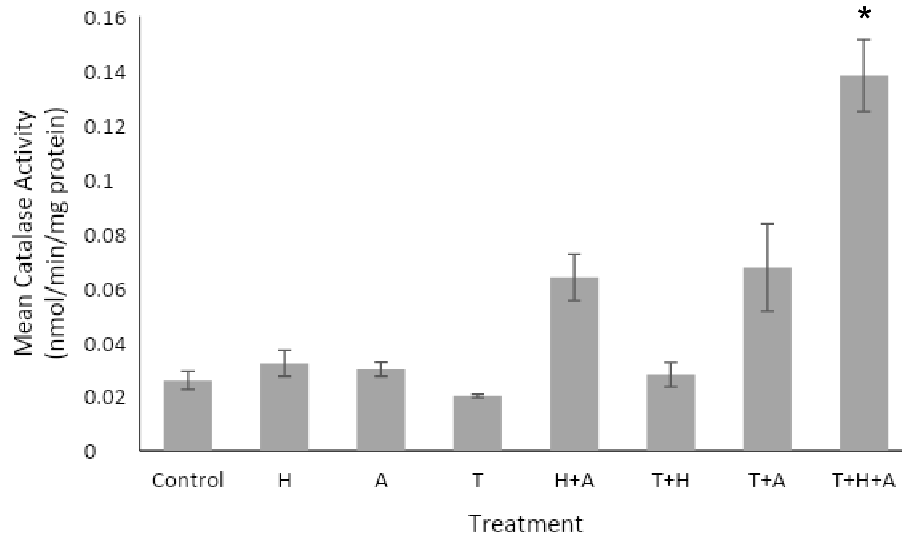

Figure 4. Mean catalase activity for surviving *D. magna* after three days of chemical exposure in the greenhouse experiment. Asterisk indicates significant difference from the control. Error bars are ± one SEM and n=6.

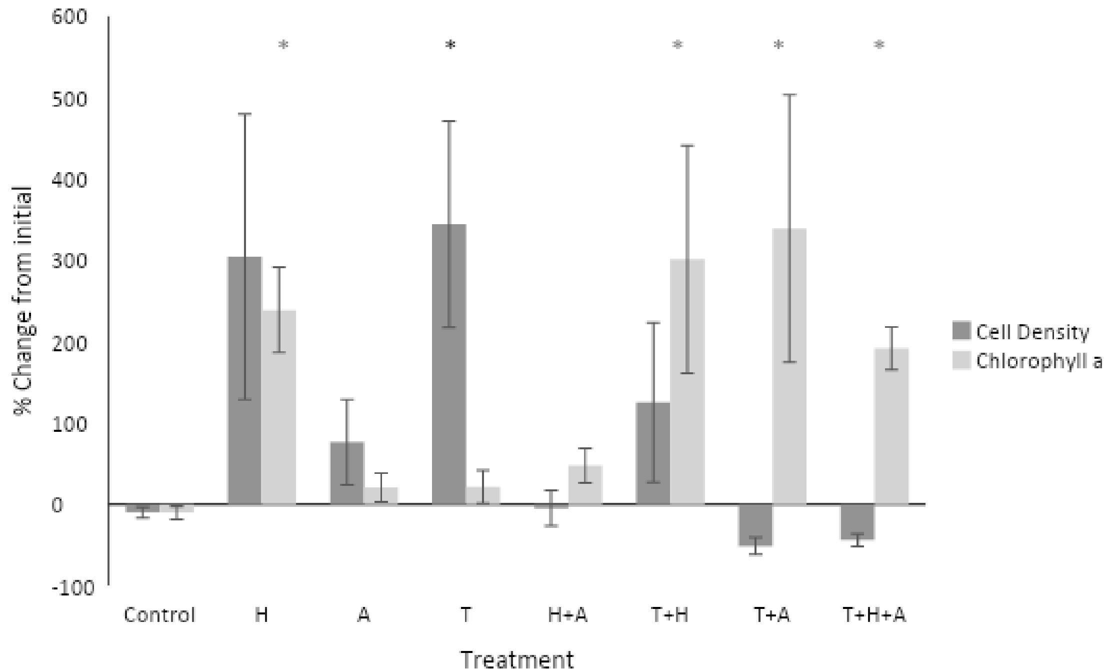

Figure 5. Mean percent change from starting *Chlorella* sp. abundance (cell density and chlorophyll *a* concentration) after seven days of chemical exposure in the greenhouse experiment. The black asterisk indicates a change in cell density percent different from the control. Grey asterisks represent differences in chlorophyll *a* concentration relative to the control. Error bars are ± one SEM and n=6.

Appendix 1:

Table 1. Predicted outcomes for mixtures of textile chemicals THPC, ammonium, H_2O_2, and temperature on plankton abundance.

Stressor	Phytoplankton abundance (*Chlorella* sp.)	Zooplankton abundance (*Daphnia magna*)
THPC	decrease	decrease
NH_4	increase	decrease
H_2O_2	decrease	decrease
Temperature	increase	decrease
THPC+NH_4	antagonism	additive
THPC+H_2O_2	additive	additive
NH_4+H_2O_2	antagonism	additive
THPC+NH4+H2O2	additive	additive
Combinations+temperatures	synergistic	synergistic

Methods:

Two experiments were conducted to examine temperature and textile chemical mixture effects on *Chlorella* sp. abundance and *Daphnia magna* mortality and stress:

a. Laboratory Experiment

A laboratory study was designed to determine the effects of THPC, ammonium, and hydrogen peroxide alone and in all possible combinations at 20 ℃ or 25 ℃ on *Chlorella* sp. and *D. magna*. The experimental treatments consisted of two peroxide treatments (0 or 2.0 mg L^{-1} H_2O_2 (H) (Hydrogen peroxide, Acros Organics, 35 wt% in H_2O_2, CAS: 7722-84-1)), two THPC treatments (0 or 1.0 mg L^{-1} THPC (T) (Tetrakis(hydroxymethyl)phosphonium chloride solution, Aldrich Chemistry, 80% in H_2O_2, CAS: 124-64-1)), two ammonium treatments (0 or 0.5 mg L^{-1} NH_4 (A) (Ammonium chloride, Fischer Scientific, CAS: 12125-02-9)). These chemical treatments were tested in two temperature treatments (20℃ or 25) in a fully crossed design

yielding a total of 16 treatments with three-fold replication. Concentrations of each chemical were selected to reflect concentrations measured in the environment but known to have effects on plankton species abundance (WHO 2000; Xiang et al. 2010; Cao et al. 2014; Meinertz et al. 2007). The concentrations of THPC and peroxide were determined based on results from the preliminary study. This experiment lasted one week to allow the observation of chronic effects on *D. magna*. Measurements of *D. magna* mortality, *D. magna* stress, *Chlorella* sp. cell density, *Chlorella* sp. pigment concentration, and water quality were taken from each treatment (n=3) initially and after 24 h, 72 h, and one week.

The hydrogen peroxide, THPC and ammonium were administered as a single dose at the beginning of the experiment to simulate a discharge event (Constable et al. 2003; Moore and Ausley 2004). Each microcosm consisted of a 250 ml beaker containing 200 ml of treatment solution, five *Daphnia magna* and enough phytoplankton food (*Chlorella* sp. at a density of $1.4*10^6$ cell ml^{-1}) for the duration of the experiment (Martinez-Jeronimo et al. 2000; Krylov and Osipova 2013). The microcosms were maintained in environmental chambers under daylight fluorescent bulbs (average intensity 21.43±0.80 µmol m^{-2} s^{-1}) on a 16h:8h light-dark cycle and randomized by location at 20ºC and 25ºC respectively.

Table 4. Mean temperature (°C) on each day for each treatment of laboratory experiment.

Treatment	Day 0	Day 1	Day 3	Day 7
Control 20	21.4±0.0	19.7±0.23	19.9±0.27	19.9±0.10
H2O2 20	21.2±0.18	19.5±0.27	19.9±0.15	20.3±0.12
NH4 20	21.1±0.07	19.7±0.13	20.4±0.23	20.0±0.15
THPC 20	21.2±0.03	19.5±0.06	20.0±0.15	20.2±0.32
H+A 20	21.2±0.03	19.4±0.33	19.9±0.28	20.3±0.15
T+H 20	21.3±0.03	19.4±0.26	20.0±0.15	20.2±0.24
T+A 20	20.7±0.07	19.7±0.12	19.7±0.23	19.7±0.03
T+H+A 20	21.2±0.00	19.5±0.28	19.8±0.15	20.1±0.07
Control 25	21.4±0.03	24.3±0.24	24.9±0.15	24.8±0.13
H2O2 25	21.4±0.09	24.6±0.23	24.4±0.15	24.8±0.20
NH4 25	21.1±0.06	25.0±0.03	24.7±0.17	24.7±0.06
THPC 25	21.3±0.03	24.0±0.61	24.8±0.09	24.9±0.13
H+A 25	21.1±0.00	24.5±0.12	24.7±0.18	24.7±0.03
T+H 25	21.0±0.17	24.3±0.32	24.6±0.15	24.9±0.09
T+A 25	20.6±0.03	24.5±0.03	24.5±0.09	24.6±0.20
T+H+A 25	21.3±0.03	24.5±0.38	24.5±0.07	24.7±0.03

Table 5. Mean conductivity (μS cm^{-1}) on each day for each treatment of laboratory experiment.

Treatment	Day 0	Day 1	Day 3	Day 7
Control 20	240.0±0.00	250.0±0.00	240.0±0.00	256.7±6.67
H2O2 20	246.7±3.33	240.0±0.00	240.0±0.00	246.7±3.33
NH4 20	250.0±0.00	240.0±0.0	240.0±0.00	250.0±0.00
THPC 20	250.0±0.00	240.0±0.0	240.0±0.00	243.3±3.33
H+A 20	250.0±0.00	240.0±0.0	240.0±0.00	246.7±3.33
T+H 20	250.0±0.00	240.0±0.0	240.0±0.00	246.7±3.33
T+A 20	246.7±3.33	240.0±0.00	240.0±0.00	243.3±3.33
T+H+A 20	250.0±0.00	240.0±0.0	240.0±0.00	243.3±3.33
Control 25	240.0±0.00	246.7±3.33	250.0±0.00	256.7±3.33
H2O2 25	250.0±0.00	240.0±0.00	246.7±3.33	250.0±0.00
NH4 25	250.0±0.00	250.0±0.00	250.0±0.00	250.0±0.00
THPC 25	250.0±0.00	240.0±0.00	246.7±3.33	250.0±0.00
H+A 25	250.0±0.00	240.0±0.0	250.0±0.00	250.0±0.00
T+H 25	250.0±0.00	243.3±3.33	250.0±0.00	250.0±0.00
T+A 25	250.0±0.00	243.3±3.33	250.0±0.00	250.0±0.00
T+H+A 25	250.0±0.00	240.0±0.00	250.0±0.00	250.0±0.00

Table 6. Mean dissolved oxygen (mg L^{-1}) on each day for each treatment of laboratory experiment.

Treatment	Day 0	Day 1	Day 3	Day 7
Control 20	5.8±0.03	8.4±0.03	8.1±0.03	8.1±0.09
H_2O_2 20	5.7±0.13	8.6±0.00	8.0±0.06	7.7±0.07
NH_4 20	5.4±0.09	8.6±0.12	7.9±0.03	7.7±0.03
THPC 20	5.6±0.00	8.4±0.10	8.1±0.07	7.7±0.03
H+A 20	5.4±0.06	8.3±0.06	8.1±0.03	7.6±0.00
T+H 20	5.5±0.10	8.4±0.06	8.1±0.03	7.6±0.03
T+A 20	5.6±0.06	8.3±0.06	8.0±0.09	7.7±0.09
T+H+A 20	5.4±0.03	8.4±0.07	8.0±0.03	7.6±0.03
Control 25	5.8±0.00	7.9±0.03	6.9±0.03	7.1±0.03
H_2O_2 25	5.5±0.07	7.8±0.03	7.3±0.07	6.9±0.09
NH_4 25	5.7±0.10	7.6±0.03	7.1±0.03	7.2±0.06
THPC 25	5.5±0.03	7.3±0.03	7.1±0.03	6.9±0.03
H+A 25	5.4±0.03	7.5±0.03	7.1±0.09	6.8±0.03
T+H 25	5.5±0.06	7.5±0.03	7.2±0.07	6.6±0.09
T+A 25	5.6±0.03	7.2±0.03	7±0.03	6.8±0.07
T+H+A 25	5.4±0.00	7.6±0.09	7.0±0.09	6.8±0.00

Table 7. Mean pH on each day for each treatment of laboratory experiment.

Treatment	Day 0	Day 1	Day 3	Day 7
Control 20	8.5±0.07	8.4±0.03	8.5±0.03	8.4±0.03
H2O2 20	8.4±0.00	8.4±0.00	8.5±0.03	8.6±0.00
NH4 20	8.3±0.00	8.4±0.00	8.5±0.00	8.5±0.03
THPC 20	8.3±0.00	8.4±0.00	8.5±0.00	8.5±0.00
H+A 20	8.3±0.00	8.4±0.00	8.5±0.00	8.5±0.00
T+H 20	8.3±0.03	8.4±0.00	8.5±0.00	8.5±0.00
T+A 20	8.3±0.00	8.4±0.00	8.5±0.00	8.5±0.00
T+H+A 20	8.3±0.00	8.4±0.00	8.5±0.00	8.5±0.00
Control 25	8.4±0.00	8.5±0.03	8.6±0.00	8.7±0.00
H2O2 25	8.4±0.00	8.5±0.00	8.6±0.00	8.6±0.00
NH4 25	8.3±0.00	8.5±0.03	8.6±0.00	8.7±0.03
THPC 25	8.3±0.00	8.4±0.00	8.6±0.00	8.6±0.00
H+A 25	8.3±0.00	8.4±0.03	8.6±0.00	8.6±0.00
T+H 25	8.3±0.00	8.5±0.00	8.6±0.00	8.6±0.00
T+A 25	8.3±0.00	8.5±0.00	8.6±0.00	8.6±0.00
T+H+A 25	8.3±0.00	8.5±0.03	8.6±0.00	8.6±0.00

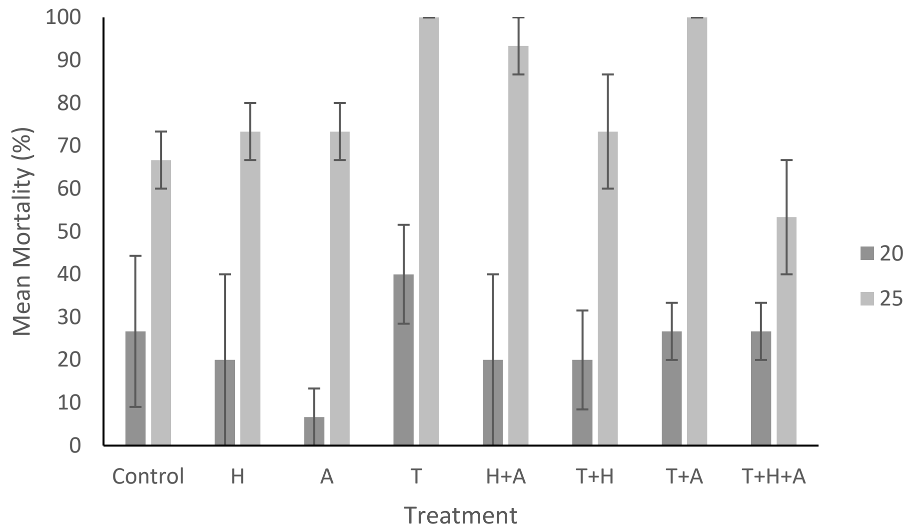

Figure 6. Mean percent mortality of *D. magna* after 7 days of exposure in laboratory study. Error bars are ± one SEM and n=3.

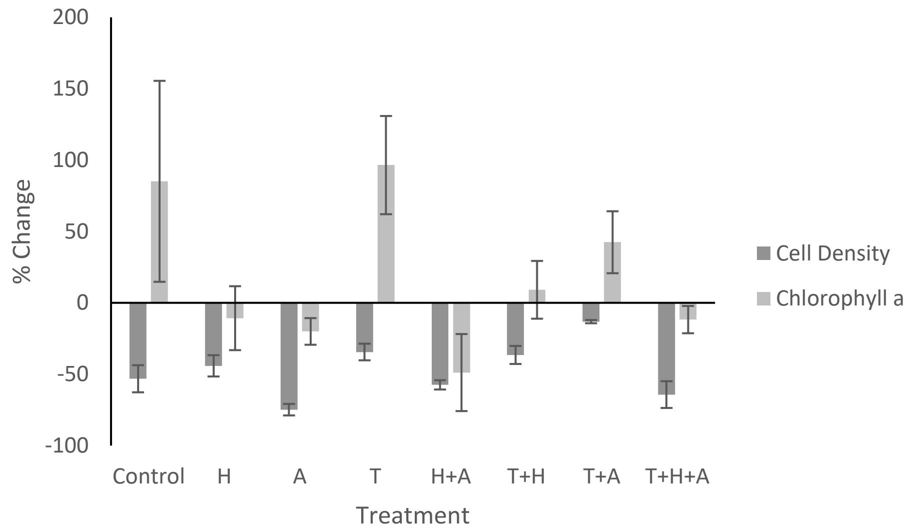

Figure 7. Mean percent change of *Chlorella* sp. abundance from initial time point on Day 7 of laboratory experiment. Error bars are ± one SEM and n=3.