TEMPERATURE-DEPENDENT EFFECTS OF DIBUTYL PHTHALATE IN *DAPHNIA MAGNA*

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I, THE UNDERSIGNED MEMBER OF THE COMMITTEE,

HAVE APPROVED THIS THESIS

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BY

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ABSTRACT

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With the growing human population, the physical and chemical environment of the global marine environment is changing. A recent highlighted threat has included the increasing amount of plastic present or entering into the marine environment. To date, plasticizers are a class of chemicals that are added to plastics in order to make them more malleable and can leach from plastic products once in an aquatic environment. The plasticizer dibutyl phthalate (DBP) was specifically chosen due to its high prevalence in the environment, as well as, its common use in our daily lives. While toxicity tests have been conducted on potentially dangerous effects of DBP, studies have not looked at the combined effects of increased temperature on DBP toxicity. A 7-day toxicity test was conducted to look at the individual and combined effects of DBP with increasing environmentally relevant water temperature on *Daphnia magna*. We found that the only significant result was from chemical concentration alone ($p < 0.01$), where neither temperature alone nor the combined effects of the two factors showed significant results. More research should be conducted on plastic pollution in environmentally relevant temperatures, as

most acute toxicity tests are run at standard 20 °C temperature, which may not show the complete picture of how these two factors interact with organisms in the wild.

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INTRODUCTION

Plastics have been in production since 1907 and their incorporation into our daily lives has increased largely due to their single-use appeal. This increase in utilization has led to excessive plastic dumping in landfills or littering that leads to accumulation in subsequently marine or terrestrial environments[1]. As of 2015, approximately 6,300 million metric tons of plastic waste had been generated globally, of which approximately 9% was recycled, 12% was incinerated, and 79% was accumulated in landfills or the natural environment[3]. Plastics have been shown to be the most abundant type of debris found in the world's oceans[5] and, or other aquatic environments, and can be transported great distances and persists due to their inability to naturally disintegrate [7]. This plastic debris has can accumulate in different aquatic environments including the five oceanic gyres, with one example being the Great Pacific Garbage Patch, first discovered by Captain Charles Moore in 1997[8].

Plastic pollution in the world's oceans was first reported in the 1970's[10] but research has only recently begun to look at the negative effects on the environment. Physical threats of plastics in the environment include the possibility of plastic debris ensnaring animals, plastic particles being ingested, and plastic build up and subsequent disruption of habitat structure. Plastics are also associated with chemical pollutants including ingredients found in so-called virgin plastic material, chemical byproducts from the plastic product manufacturing process and once in the environment plastic debris can sorb and accumulate pollutants found in a given waterbody, such as persistent organic pollutants or metals[11].

Chemical plasticizers are chemicals that are added to virgin plastics in order to make them more malleable or heat tolerant. In some cases, these plasticizers can make up to 40% of

the weight of the final product[6]. Phthalic acid esters (phthalate esters) are widely used in the manufacturing of plastics as nonreactive plasticizers that increase the flexibility and workability of high molecular weight polymers. They are commonly used in polyvinyl chloride (PVC) and are found in plastic tubing, floor tiles, furniture, automobile upholstery, and, to a lesser extent, in insect repellents and cosmetics[11]. The widespread production and use of phthalate esters, combined with the fact that phthalates are not chemically bonded to the polymeric matrix and are able to migrate from the plastic, make their environmental fate and effects a concern.

While many plasticizers have been found to be toxic to a variety of marine organisms in laboratory settings, under standard conditions, many occur in aquatic environments at very low concentrations. Of those documented in the environment to date, the plasticizer dibutyl phthalate (DBP) appears to be detected at relatively high concentrations especially in areas with high populations and near sewage effluents. For example, in multiple studies that were conducted around the world, DBP was detected in almost every sample, both water and sediment, ranging from the Gulf of Mexico to the Netherlands, where levels ranged from 74 – 3,700 ng/L[15,16]. These levels are lower than that currently known to be toxic to organisms, where for example the 48h effective concentration to 50% of a population (EC_{50}) of *Daphnia magna* ranges from 3.0 – 5.2 mg/L[12]. While these concentrations are higher than that occurring in the environment, it is important to keep in mind that animals in the environment are exposed to these toxins for their entire life, not only a short test period, and experience variable environmental conditions that may contribute to induced stress.

This project aims to look at how changes in temperature effects the toxicity of DBP in *Daphnia magna*. *Daphnia magna* is a species of freshwater planktonic flea that is used prominently in toxicology tests due to its rapid reproductive cycle and multiple factors of

evaluation of toxicity, such as mortality, reproductive success, rate of growth, feeding success, and movement or immobility. Because of its simplicity to culture and availability of toxicity data, this was the sample organism that we chose for our study. The study had three objectives (1) determine an environmentally relevant temperature range for local environments, (2) determine if a sole factor of temperature or chemical toxicity has any effect on *Daphnia* and (3) observe the potential combined effects of temperature and chemical toxicity.

METHODS

Determination of Environmentally Relevant Temperature Range

The Southern California Bight is a 78,000 km^2 area of land spanning from Point Conception (34.4486° N, 120.4716° W) to San Diego (32.7157° N, 117.1611° W); in total it measures about 1,000 km in length (see Figure 1). It encompasses estuaries such as the Bolsa Chica conservatory and Colorado Lagoon, as well as highly populated and industrialized areas like the Los Angeles and Long Beach ports and multiple recreational beaches where pollution is likely to enter the ecosystem.

Based on NOAA Regional Climatology Data, ocean temperatures in the Southern California Bight have significantly changed in the past 40 years[18]. Available decadal periods from 1975-2012 were analyzed and the seasonal statistical mean was calculated including temperatures from 0m-10m surface water collected from 32.5° N to 34.5° N. Based on this data (Figure 1), we chose three temperatures for our study: 18°C, 21°C, and 24°C to act as a viable range of average, moderately high, and high ocean temperatures in the southern California region.

The original *Daphnia* cultures were raised at 20°C until the test duration began. Upon initiation of the DBP exposure, organisms were placed into a water bath or incubator at their respective temperature. Our tests were conducted on third-brood neonates.

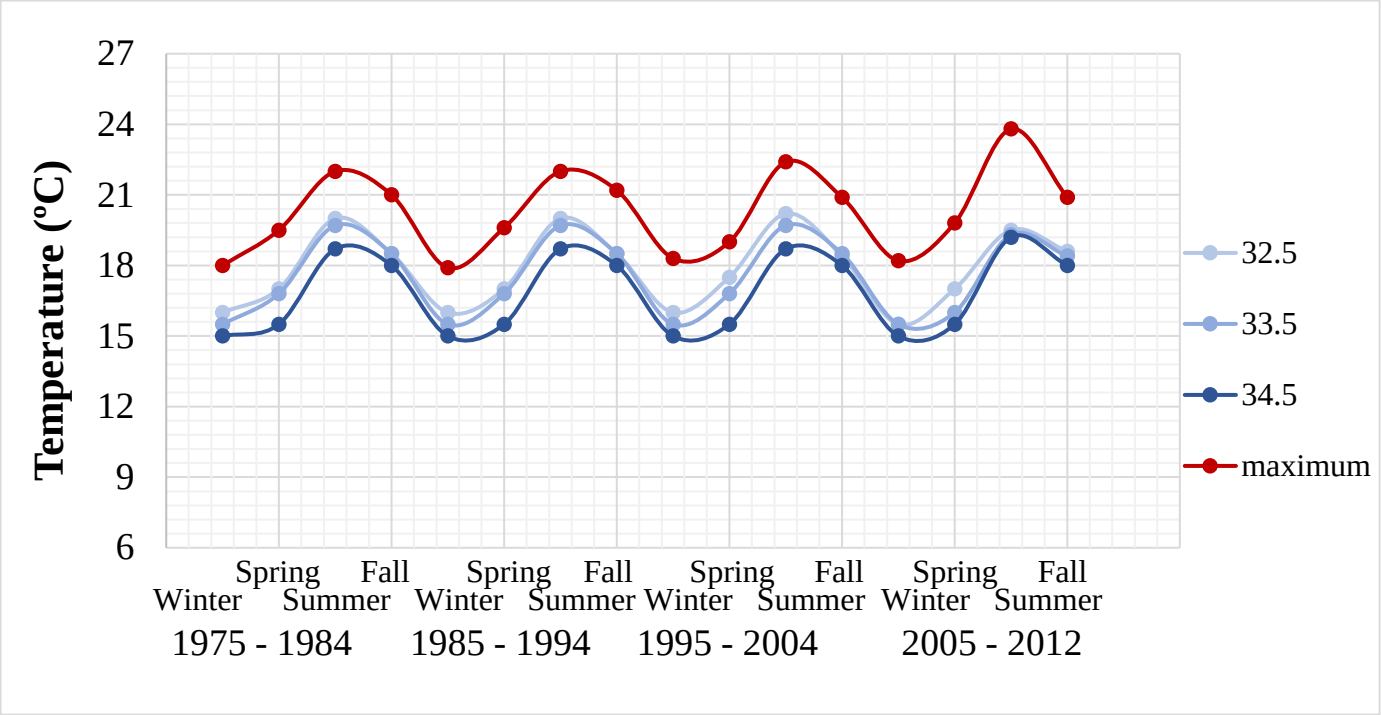

Figure 1: Graph depicting average temperatures at the depicted coordinates. The red line depicts the maximum temperature seen at the measured depth range of 0-5m.

Acute Toxicity Tests

A virgin 20L Nalgene carboy held base culture water; prior to the addition of culture water the carboy was rinsed with water and put into an autoclave for 20 minutes at 120 °C. Autoclaved water was dumped, the carboy was refilled with fresh deionized water and left to sit for one week to continuously leach remaining chemicals from the virgin plastic. After one week, the carboy was emptied, and the stock synthetic freshwater solution was created to EPA standards on Moderately Hard Synthetic Freshwater[21]. Water was prepared by dissolving 3.84g $NaHCO_3$, 2.40g $CaSO_4$, 2.40g $MgSO_4$, and 160mg of KCl in 20L of deionized water to receive a pH level of 7.6-8.0 and a Hardness of 160-180 mg $CaCO_3$/L.

We conducted a 7-day toxicity test that measured lethality. Five ~24h neonates were put in to four replicate 50mL test beakers. The exposures were conducted at a 16h-8h light-dark photoperiod under their respective test temperatures. Every day, each beaker was counted for live animals, any dead *Daphnia* were removed; each beaker was fed 0.1mL of YCT (yeast, cerophyll, and trout chow) and algae. Every other day, approximately 80% of the test water from each beaker was removed and replaced with fresh test water to keep DBP levels consistent. Any water that was added to the test beakers was first brought up to experimental temperature as to not cause a change in temperature. During these water exchanges, analysis was done for temperature, pH, dissolved oxygen, conductivity and hardness before it was utilized and after it was removed from the beakers. This was done to ensure that water quality levels were consistent throughout the test.

We chose three different toxicity concentrations based on their lethality levels. Our maximum concentration, 3.0 mg/L, was used as a lethal concentration. The mid-range concentration, 1.5 mg/L, was used as a sub-lethal concentration. Finally, our lowest concentration, 0.75 mg/L, was utilized as a no-effect concentration (NOEC). Table 1 shows an outline of our experiment, outlining each concentration at each temperature.

Statistical Analysis

We conducted a two-way ANOVA to analyze the individual and combined effects of temperature and chemical toxicity on *Daphnia magna*. Using Excel, morbidity data from the seventh day of testing was analyzed. Results from the ANOVA can be seen in Table 2.

Table 1: Experiment design.

Temperature	**Concentration**				
18 °C (incubator)	Control	Vehicle Control	0.75 mg/L (no-effect)	1.5 mg/L (sub-lethal)	3.0 mg/L (lethal)
21 °C (water bath)	Control	Vehicle Control	0.75 mg/L (no-effect)	1.5 mg/L (sub-lethal)	3.0 mg/L (lethal)
24 °C (water bath)	Control	Vehicle Control	0.75 mg/L (no-effect)	1.5 mg/L (sub-lethal)	3.0 mg/L (lethal)

Table 2: Results of ANOVA test.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Temperature	0.6333	2	0.316	0.8028	0.4544	3.204
Concentration	12.5	4	3.125	7.922	6.358E-05	2.578
Interaction	3.7	8	0.4625	1.172	0.3365	2.152
Within	17.75	45	0.394			
Total	34.58	59				

RESULTS

We determined that DBP treatment concentration showed a significant impact on *Daphnia* survival ($p \leq 0.01$), while temperature alone ($p = 0.46$) and the interaction between temperature and treatment concentration showed no significant impact ($p = 0.34$). Figure 2 shows the combined effects of chemical concentration and increased temperature.

Because temperature was not significant, we decided to isolate all toxicity data irrelevant of temperature. Figure 3 demonstrates the percent lethality observed at various DBP concentrations. Percent lethality at 3.0mg/L DBP was the highest observed in our studies where there was no lethality observed in our controls. This is in line with previous studies addressing the toxicity of DBP.

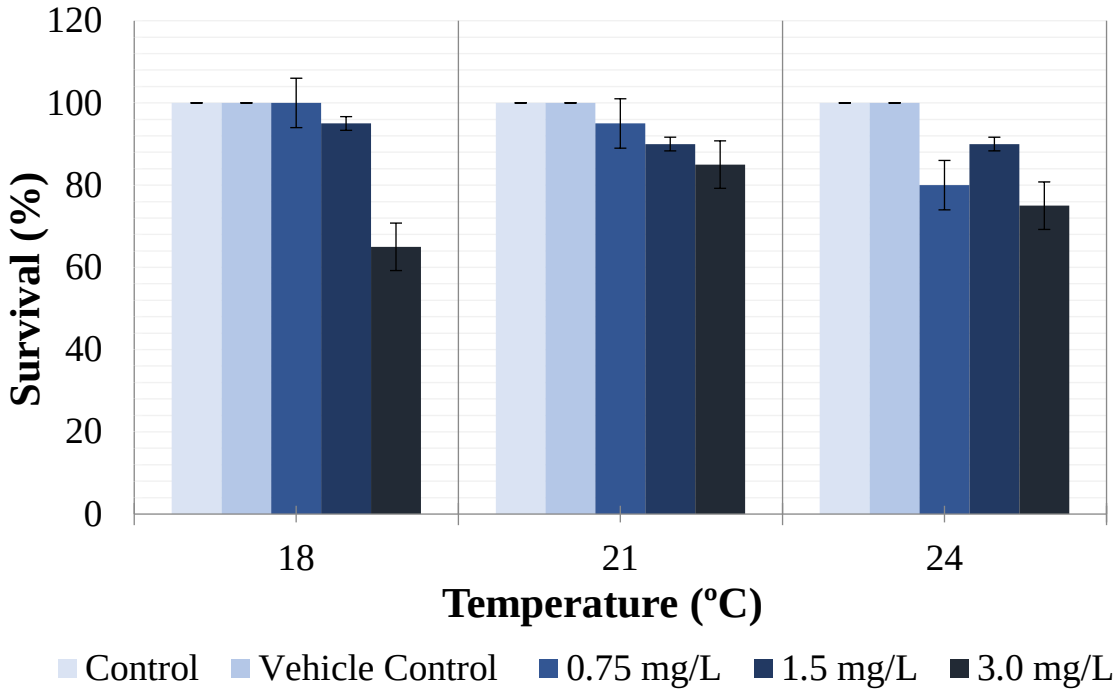

Figure 2: Two-way ANOVA analysis of temperature and chemical concentration.

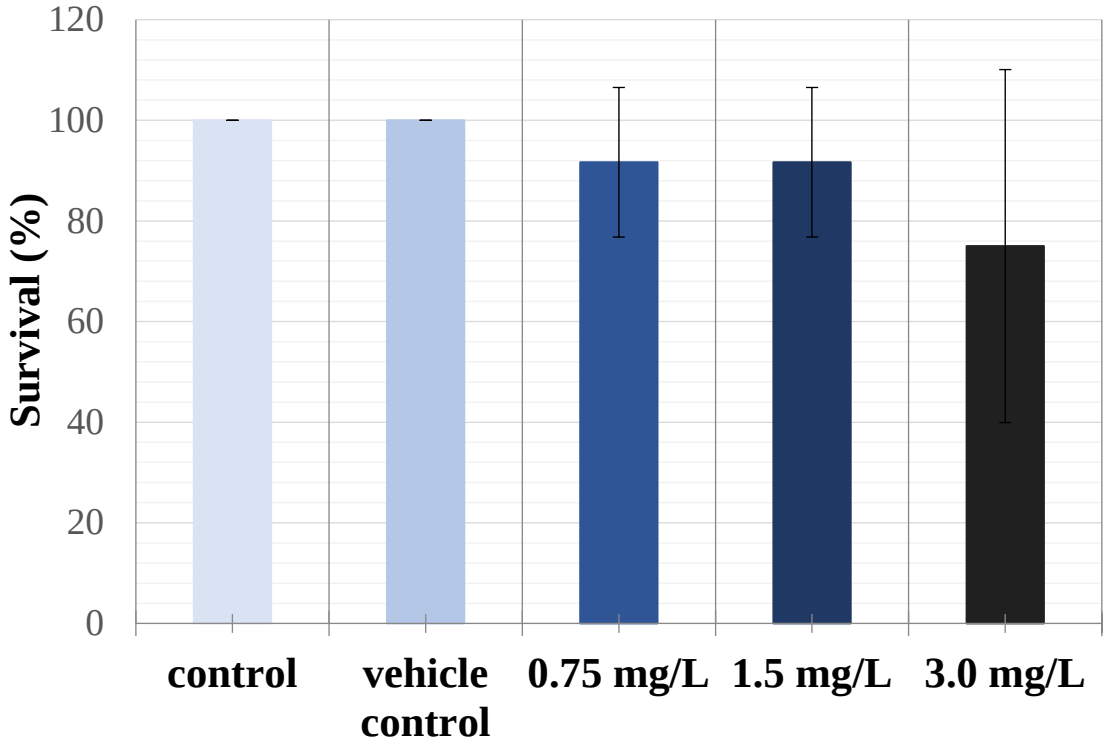

Figure 3: One-way ANOVA analysis of chemical concentration irrespective of temperature.

DISCUSSION

The aim of this study was to observe the effect of DBP when combined with temperature stress, however, we found the main effect to be from chemical concentration alone with no effect from temperature. Because our study design worked with a narrow temperature and chemical ranges, it is thought that including the greater temperature fluctuations seen within a day or shorter time period may have produced more significant results.

We combined a limited temperature stress with a standard acute toxicity test. Other studies have found that the EC_{50} of DBP alone is similar across a variety of acute toxicity tests and a 21-day life history study. Specifically, in a 21-day toxicity test, one paper found an EC_{50} value of 3.9mg/L[22] while that observed for a 48h acute test was found to range from 3.0-5.2 mg/L[12]. While a longer test period may not have shown a significantly different EC_{50} value, it should be noted that wider range of chemical concentrations may have shown a more precise range of how DBP effects on *Daphnia magna*.

Currently research has not addressed the potential damage caused from DBP, or other phthalates, in conjunction with other stressors. However, in a paper analyzing the temperature-dependent toxicity of cadmium in *Daphnia*, it was found that control survivorship and the 48h – LC_{50} began to decrease drastically at temperatures above ~ 26°C[19]. This trend of decreasing LC_{50} values at increasing temperatures has been seen with many toxic chemicals including chlorine, copper, and zinc for a number of aquatic invertebrates[19,23]. It is possible that with larger temperature fluctuations, as may be seen in oceans on a daily, monthly or annual basis, there would be more pronounced effects of toxicity of DBP. Various literature has been conducted looking as to rather organisms should be acclimated to target temperature before conducting

temperature-dependent experiments[19,20]. Being raised at a steadily higher temperature has been shown to increase organism growth and feeding rate, which would cause a mismatch of animal size between the three temperature standards. Because of this, we chose not to acclimate the *Daphnia*, and instead directly transfer them from 20 °C to their test temperature at the start of the test. Most toxicity tests for regulatory purposes are conducted at a standard temperature of 20 °C, which is not always relevant to environmental temperatures.

Oceanic environments are naturally prone to vast temperature changes due to local weather events such as El Nino/ Nina and regular seasonal variations[17]. These fluctuations are suggested to be increasing due to anthropogenically driven climate change [24]. For this study, our temperature range was based on observed decadal average temperatures. This data concluded at 2012, which means that the past six years of ocean data are unaccounted for. These years include a major El Nino year (2015) and the three recorded hottest years on record (2016, 2015, and 2017)[25]. Even through our results were not largely significant, it is still important to consider the final effects that these factors have on organisms. For example, our test species, *Daphnia magna*, are ectotherms and are particularly susceptible to temperature changes because their bodily functions rely on the environment around them.

CONCLUSION

Plastic pollution has been a threat to marine environments since its initial production. Research on the physical and chemical threats of plastics, including plasticizers, have not been adequately studied, particularly when considering the changing water temperatures. Plasticizers are a class of chemicals that are added to plastics in order to make them more malleable and flexible; dibutyl phthalate (DBP) specifically is prevalent in the environment and seen in many common household products. Through conducting a 7-day toxicity test, we found that the only significant impact on *Daphnia magna* was from the concentration of DBP alone, where temperature alone or the combined effects of both factors were not statistically significant. While the results in this experiment had limited success, it is important to take the experiments limitations into consideration and understand that results may be more significant with a wider range of variables. These variables should be studied further in order to determine the true impact we are having on our environment.

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