Four plastic additives reduce larval growth and survival in the sea urchin *Strongylocentrotus purpuratus*

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ABSTRACT

Plastic additives are utilized during the production of plastic to modify the attributes and stability of the polymer. As oceanic plastic waste degrades, these additives can leach, and are harmful to global marine ecosystems. Despite the high abundance of additives leached into the marine environment, little is known about their direct impact on marine zooplankton. Here we test for impacts of four plastic additives, UV-327, Irganox 1010, DEHP, and methylparaben, all commonly used in plastic manufacturing, on purple sea urchin (*Strongylocentrotus purpuratus)* larval growth and survival in a serial dose response for 4 days. Methylparaben, UV-327, and Irganox 1010 significantly reduced larval body length by about 5% for at least one dose. In contrast, all compounds reduced larval survival by 20-70% with strongest effects at intermediate rather than high doses. Our results highlight that plastic additives should be tested for their effects on marine organisms.

KEYWORDS: Di(2-ethylhexyl) phthalate (DEHP), Methylparaben (MeP), [pentaerythritol tetrakis (3-(30,50-di-tert-butyl-40-hydroxyphenyl) propionate] (Irganox 1010), 2,4-Di-tert-butyl-6-(5-chloro-2H-benzotriazol-2-yl) phenol (UV-327), *Strongylocentrotus purpuratus*, plastic additives

ABBREVIATIONS: Di(2-ethylhexyl) phthalate (DEHP), artificial seawater (ASW), Dimethyl sulfoxide (DMSO), polyvinyl chloride (PVC), non-monotonic dose response (NMDR), bisphenol A (BPA), nonylphenol (NP), octylphenol (OP), [pentaerythritol tetrakis (3-(30, 50-di-tert-butyl-40-hydroxyphenyl) propionate] (Irganox 1010), ultraviolet light stabilizer 2,4-Di-tert-butyl-6-(5-chloro-2H-benzotriazol-2-yl) phenol (UV-327), Methylparaben (MeP)

1. INTRODUCTION

To keep on pace with global demand, plastic production has increased exponentially since the start of mass production in the 1950s (Thompson et al., 2009). Global annual plastic production reached 359 million tons in 2018 (PlasticsEurope, 2019), accounting for 10% of global municipal waste (Barnes et al., 2009). The buildup of plastic debris is exacerbated by poor waste management resulting in plastic escaping and collecting in nature (Ejaz et al., 2010). As much as 12 million metric tons of plastic waste entered the ocean in 2010, and with current manufacturing and waste policies, the annual input is expected to increase by an order of magnitude by 2025 (Jambeck et al., 2015). Accumulating marine plastic waste can cause both environmental and biological threats such as fragile ecosystem damage (Sheavly and Register, 2007), wildlife entanglements (NOAA, 2020), and ingestion (Arias et al., 2019; Cole et al., 2011; Hall et al., 2015; Provencher et al., 2014; Shore et al., 2021). These hazards have made plastic in the oceans a major concern as marine ecosystems are important to global health (Perry et al., 2010), primary productivity (Gregory, 2009) and carbon cycling (Roman and McCarthy, 2010). Plastic debris has been found in almost every type of marine ecosystem, from beaches, open oceans, seabeds (Barnes et al., 2009), and deep-sea abysses (Chiba et al., 2018).

The widespread existence of marine plastic pollution is a result of the large variety of plastics manufactured. Plastics are produced with the combination of polymers, or the polymerization of monomers derived from oil or gas, and additives (Thompson et al., 2009). Combinations of additives and polymers create large variation in chemical composition and allow desired physical traits to be incorporated into plastic products (Lithner et al., 2012). Most plastic polymers have high longevity and are designed to be resistant to biological degradation (Moore, 2008). Plastic materials are slow to degrade, however, saltwater accelerates the process and causes plastics to leach toxic substances like antioxidants and commercial preservatives into the marine environment (Rani et al., 2017; Weinstein et al., 2016). An estimated 190 tons of 20 different chemical additives entered the oceans with escaped plastic waste in 2015 (Frond et al., 2019). Plastic additives are not usually bound to polymers, which results in faster leaching from plastic materials (Oliviero et al., 2019). Physical abrasion and turbulence by ocean waves in conjunction with direct exposure to sunlight on beaches degrade and fragment plastic rapidly (Barnes et al., 2009; Browne et al., 2007), releasing plastic additives into the ocean (Gunaalan et al., 2020; Kwan and Takada, 2019). Floating ocean plastics that are exposed to prolonged periods of sunlight can result in UV photo-degradation which initiates photo-oxidative degradation (Andrady, 2011; Moore, 2008). Degradation can also occur thermooxidatively, without further exposure to UV radiation, possibly allowing plastic debris that sinks below the ocean's surface to continue to fragment and leach (Webb et al., 2013). Leached additives have been found to reduce the growth, development, and survival of marine calcifiers such as mussels (Aarab et al., 2006; Baršiene et al., 2006; Canesi et al., 2007a, 2007b; Gandara e Silva et al., 2016; Orbea et al., 2008; Tato et al., 2018), oysters (Gardon et al., 2020), barnacles (Li et al., 2016), clams (Ke et al., 2019), and sea urchins (Arslan et al., 2007b, 2007a; Beiras et al., 2019; Martínez-Gómez et al., 2017; Nobre et al., 2015; Oliviero et al., 2019; Özlem and Hatice, 2008; Rendell-Bhatti et al., 2021), which could hinder population growth.

Of particular interest, additives ultraviolet stabilizer 327 (UV-327; 2,4-di-*t*-butyl-6-(5-chloro-2H-benzotriazole-2-yl) phenol) and antioxidant Irganox 1010 ([pentaerythritol tetrakis (3-(30 ,50 -di-tert-butyl-40 -hydroxyphenyl) propionate]) haven been found to leach from beached plastic debris into synthetic seawater, suggesting these chemicals may have high concentrations in the ocean (Rani et al., 2017, 2015). UV-327 is added to plastics to protect the material against damage from ultraviolet radiation (Liu et al., 2018), and has been discovered in the organismal tissue of clams, hammerhead sharks, oysters, seabirds (Nakata et al., 2009) and finless porpoises (Nakata et al., 2010). Irganox 1010 additive, which is incorporated into food wrappers to minimize degradation, likely has a very high oceanic concentration as food wrappers are one of the most prevalent types of plastic marine debris. (Rani et al., 2017; Schwope et al., 1987). While a few studies identified environmental concentrations of UV-327, it has been found in specific geographic locations (Langford et al., 2015; Nakata et al., 2012), though concentrations are unknown beyond limited sites. Beached plastic debris has been analyzed for chemical additive presence to help determine possible environmental concentrations (Rani et al., 2017), however, to our knowledge, there are no studies that have explored the oceanic concertation of Irganox 1010. Thus, it is important to consider the physiological affects chemical additives like UV-327 and Irganox 1010 may have on wildlife health, particularly for organisms in the marine environment. In addition, other potentially harmful plastic additives of concern include methylparaben (MeP) and Di(2-ethylhexyl) phthalate (DEHP), both of which have been found to bioaccumulate and are widespread in global marine ecosystems (Fossi et al., 2012; Haman et al., 2015; Ye et al., 2014). Parabens are commonly used in the production of cosmetics and some skin care products (Soni et al., 2005), and have affected growth and survival of marine organisms such as copepods (Kang et al., 2019), brine shrimp (Comeche et al., 2017), and oysters (di Poi et al., 2018). DEHP is widely used as a plasticizer in polyvinyl chloride (PVC) formulation (Aignasse et al., 1995), one of the two most common plastic products produced (Hartman et al., 2014), and is also included in the manufacture of construction products, medical devices, pharmaceuticals, and personal care products (Heudorf et al., 2007). DEHP exposure has disrupted endocrine receptors in brackish ricefish (*Oryzias melastigma*) (Ye et al., 2016, 2014), goldfish (Golshan et al., 2015), and zebrafish (Jia et al., 2016), but to our knowledge, not much else is known about the effects of DEHP additives on marine organisms. Due to the widespread environmental existence of both DEHP and MeP (Haman et al., 2015; Hermabessiere et al., 2017; Ye et al., 2014), understanding the effects of these additives is important for the health of marine organisms.

To understand the potential impact of leached plastic additives on marine invertebrate growth and survival, we used the model organism purple sea urchin *Strongylocentrotus purpuratus*. Sea urchins are an excellent model species for ecotoxicological studies in marine animals (Pinsino et al., 2010; Roccheri et al., 2004), as thousands of urchin larvae can be spawned and reared in replicate cultures in abbreviated time periods. More importantly, the developing skeletons of the pluteus-stage larvae are vulnerable to chemical pollutants, allowing the use of larval body length as a measure of compound toxicity (Martino et al., 2017; Özlem and Hatice, 2008). Here, we reared larval urchins in the presence of four plastic additives, UV-327, Irganox 1010, DEHP, and MeP, and tested their effects on early development using a serial dose response and measuring fertilization success, developmental staging at 3 hours post-fertilization, and pluteus body length and survival at 96 hours (4 days) post-fertilization. We tested the hypotheses that the direct exposure to additives that leach from plastics will result in reduced pluteus larval body length, survival, development, and fertilization success in a dose-dependent manner.

2. MATERIALS AND METHODS

2.1 Compound Preparation

To carefully control doses and directly test the impacts of compounds that commonly leach from plastics, we used pure compounds purchased from Sigma-Aldrich (St. Louis, MO). For example, the media used for leaching plastic products (I.e. sea water or fresh water) can influence the volatility of these plastic compounds (Gunaalan et al., 2020). In addition, temperature, UV radiation, and pH are also known to affect plastic deterioration and leachate release (Sajiki and Yonekubo, 2003; Westerhoff et al., 2008), increasing the amount of leachates in the marine environment (Hermabessiere et al., 2017). These factors can make laboratory-simulated leachate extractions difficult or nonuniform in concentration. To alleviate this constraint, we executed our experiments with industrially produced pure compounds to ensure concentrations were uniform. Powdered MeP and liquid DEHP were dissolved and diluted, respectively, in artificial seawater (ASW) made with Instant Ocean salt (Blacksburg, VA) to produce 5.0 mg L^{-1} stock solutions at a salinity of 34 ppt. Serial dilutions were performed to produce 5.0 mg L^{-1}, 1.0 mg L^{-1}, 0.5 mg L^{-1}, and 0.1 mg L^{-1} experimental doses for each compound. DEHP and MeP leachates have been found at high oceanic concentrations, 62.77 μg

L^{-1} and 3.64 - 18.59 μg L^{-1}, in the Mediterranean Sea and Normandy, France, respectively and are both reflective of the doses used in this study (Di Poi et al., 2018; Hermabessiere et al., 2017). Non-water-soluble powdered standards of Irganox 1010 and UV-327 were also obtained from Sigma-Aldrich. To create serial dilutions, 250 mg of each chemical was first dissolved in 10 mL Dimethyl sulfoxide (DMSO) to create a stock solution of 25 mg L^{-1}, then serially diluted to 5.0 mg L^{-1}, 1.0 mg L^{-1}, 0.5 mg L^{-1}, and 0.1 mg L^{-1} experimental doses using seawater. Both Irganox 1010 and UV-327 additives have not yet been quantified in the ocean, thus we chose experimental concentrations that would span the range of the other additives assessed in this study. To control for the potential negative impact of DMSO, a separate control seawater solution was made for Irganox 1010 and UV-327 with the same final concentration of DMSO (0.02%) of the highest treatment dose by combining 0.0002 mL of DMSO with 1.0 mL of ASW.

2.2 Collections, Spawning, and Fertilization

Gravid purple sea urchins (*S. purpuratus*) were collected from Monterey Bay, California by the Monterey Bay Abalone Company in March 2017 and shipped overnight to the University of Vermont. The urchins were kept in recirculating aquaria systems in ASW at 12 °C and fed dried Pacific Kombu seaweed (Shandong Peninsula, China) *ad libitum* until the experiment in June 2017. On the first day of the experiment, *S. purpuratus* were spawned by injecting individuals with 2-5 mL of 0.5 M KCl solution in 34 ppt ASW. Sperm from one male was collected "dry" and kept on ice until fertilization, and eggs from one female were shed into ASW, collected, and rinsed through a 215 μm mesh filter into a falcon tube with 12 °C ASW. The density of the eggs collected was measured using a microscope to determine the volume of ASW-egg solution needed for each replicate. For egg fertilization, 80 mL of each compound solution (i.e., 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1} UV-327 etc.) as well as each control solution were added to separate 150 mL glass beakers, creating 18 fertilization beakers. Approximately 12,000 eggs were added to each beaker. For fertilization, 15 μL of dry sperm was diluted into 10 mL ASW and 50 μL diluted sperm solution was added to each beaker. After 5 minutes, 3 30-μL samples of the ASW control (no chemicals) were taken and counted to ensure 90% fertilization success was reached.

2.3 Fertilization Success

Forty minutes post-fertilization, an 8-mL sample from each of the 18 fertilization beakers was fixed with 2 mL of calcium carbonate-neutralized 10% formalin. The number of embryos displaying a fertilization envelope and the number of unfertilized eggs was counted using a compound microscope and percent fertilization success was recorded for each treatment.

2.4 Development, Body Length, and Survival

Larval development was measured at 3 hours post-fertilization and larval growth and survival was measured at 96 hours post-fertilization for each compound and dose (4 replicates, 2 time points, 4 compounds, and 5 doses, including controls; 160 replicate vials). Prior to fertilization, replicate vials (20-mL scintillation vials) were filled with 14 mL of the compound or control solution and equilibrated to 15 °C. After fertilization, egg density was re-calculated for each fertilization beaker and 150 fertilized eggs were transferred to each vial and incubated at 15 °C. Final volume was adjusted to 15 mL per vial resulting in a density of approximately 10 embryos per mL. At 3 hours post-fertilization, four replicates per compound and per dose, plus controls, were fixed with 5 mL of calcium carbonate-neutralized 10% formalin. To quantify successful growth and development at 3 hours post-fertilization, eggs developing past the fertilization envelope stage to at least one cell division were counted and recorded as the percentage developing (four replicates per compound per dose). The remaining replicates were incubated at 15 °C, opened and inverted once per day for aeration. At 4 days post-fertilization, remaining vials were fixed with 5 mL of calcium carbonate-neutralized 10% formalin. To measure body length and survival, fixed larvae were imaged on a Zeiss Axioscop 2 compound microscope equipped with an Insight FireWire camera and Spot imaging software. Total body length, including the body rod and the postoral arm, was measured using ImageJ software calibrated with a stage micrometer. Dead larvae quickly disintegrate; thus, survival was estimated by counting the number of intact, fixed larvae from a 1 mL sample and comparing to the number of larvae counted in the control conditions.

2.4 Data Analysis

To assess the effect of each compound on body length, an ANOVA analysis was performed using the *aov* function in the R software environment, pairing UV-327 and Irganox 1010 data with the DMSO control and the MeP and DEHP data with the ASW control. Post-hoc Tukey multiple comparisons tests were used to compare the various doses to the respective control for each compound.

To assess the effect of each compound on fertilization success, early development (cell division), and survival, a binomial distribution was used by coding individual embryos or larvae as ‘fertilized,’ ‘divided,’ or ’survived’ as one (1) or ‘not fertilized,’ ‘no division,’ or 'dead’ as zero (0). These binomial data were then analyzed via a generalized linear model using function *glm* from the R package *lme4*. Because dead larvae disintegrate rapidly and because the number of larvae seeded into each replicate vessel was an estimate based on egg density rather than an actual count, we developed a method to estimate the number of dead larvae based on the maximum number of surviving larvae sampled for a given compound. For each replicate, we estimated the number of dead larvae by subtracting the number of surviving larvae from the max number of surviving larvae for that compound. This approach allowed us to account for potential variation among replicate vessels in the initial number of larvae seeded and avoid having survival estimates greater than one. For example, for Irganox 1010, the highest number of surviving larvae (34) was in replicate 3 in the treatment group 0.1 mg L^{-1}. For each of the other Irganox 1010 replicates, the number of surviving larvae was subtracted from 34 to estimate the number of dead larvae (0s) for that replicate. Significant differences were confirmed where $P < 0.05$. All data analyses were conducted using R statistical software version 4.0.2 (https://github.com/PespeniLab/purpuratus_leachates).

3. RESULTS

3.1 Body Length

Three of the four chemical additives resulted in shorter *S. purpuratus* pluteus body length after a 4-day exposure for at least one dose (Fig. 1). Methylparaben (MeP) only reduced body length at the highest dose ($F_{(4,445)} = 5.77$, $P < 0.001$; one-way ANOVA). Individuals exposed to 5.0 mg L^{-1} MeP were 5.4% shorter compared to the control (control: 300.84 ± 31.72 μm; 5.0 mg L^{-1}: 284.73 ± 29.67 μm; Tukey $\text{HSD}_{5\ \text{mg L}^{-1}\ \text{vs control}}$: $P < 0.01$; Figure 1A). DEHP had no effect on larval body lengths at any of the concentrations tested ($F_{(4,377)} = 1.54$, $P = .19$; one-way ANOVA; Figure 1B). Larvae exposed to UV-327 and Irganox 1010 had reduced body length at intermediate doses (UV-327: $F_{(4,418)} = 8.18$, $P < 0.001$; Irganox 1010: $F_{(4,351)} = 4.27$, $P < 0.01$; one-way ANOVA). UV-327 resulted in a 4.6% reduction in body length at 0.5 mg L^{-1} compared to the control (control: 298.41 ± 26.74 μm; 0.5 mg L^{-1}: 284.73 ± 42.53 μm; Tukey $\text{HSD}_{0.5\ \text{mg L}^{-1}\ \text{vs control}}$: $P < 0.001$; Figure 1C), while Irganox 1010 resulted in a 6.5%

reduction in body length at 1.0 mg L^{-1} relative to control (control: 298.41 ± 26.74 μm; 1.0 mg L^{-1}: 279.12 ± 54.49 μm; Tukey $HSD_{1.0\ mg\ L^{-1}\ vs\ control}$: $P < 0.05$; Figure 1D).

3.2 Survival

Larval survival was strongly impacted by each of the four plastic additives after a 4-day exposure (Fig. 2). For Methylparaben, larval survival was reduced by 17.3% in the lowest dose, 0.1 mg L^{-1} MeP (control: 26.0 ± 4.3 survivors; 0.1 mg L^{-1}: 21.5 ± 10.3 survivors; GLM, df = 795, z = -2.10, $P < 0.05$), and reduced by 53.7% in the next, intermediate dose, 0.5 mg L^{-1} MeP compared to the control (control: 26.0 ± 4.3 survivors; 0.5 mg L^{-1}: 15.0 ± 3.6 survivors; GLM, df = 795, z = -4.94, $P < 0.001$; Fig. 2A). In contrast, the highest doses of MeP, 1.0 mg L^{-1} and 5.0 mg L^{-1}, did not result in increased mortality (Fig. 2A).

In contrast to the body length results from DEHP, all doses of DEHP resulted in reduced survival, with the strongest impacts at the intermediate doses (Fig. 2B). Larval survival was reduced by 29.8% in the lowest dose, 0.1 mg L^{-1} DEHP (control: 26.0 ± 4.3 survivors; 0.1 mg L^{-1}: 19.3 ± 6.7 survivors; GLM, df = 565, z = -3.73, $P < 0.001$), and reduced by 61.64% when exposed to 0.5 mg L^{-1} DEHP compared to the control (control: 26.0 ± 4.3 survivors; 0.5 mg L^{-1}: 13.8 ± 8.1 survivors; GLM, df = 565, z = -6.27, $P < 0.001$; Fig. 2B). Additionally, larval survival was reduced by 19.2% when exposed to 1.0 mg L^{-1} DEHP (control: 26.0 ± 4.3 survivors; 0.1 mg L^{-1}: 21.0 ± 7.2 survivors; GLM, df = 565, z = -2.71, $P < 0.01$) and reduced by 20.2% when exposed to 5.0 mg L^{-1} DEHP compared to the control (control: 26.0 ± 4.3 survivors; 5.0 mg L^{-1}: 20.8 ± 2.9 survivors; GLM, df = 565, z = -3.07, $P < 0.01$; Fig. 2B).

Larval survival was reduced by 45.0% after exposure to 0.1 mg L^{-1} UV-327 (control: 27.7 ± 4.0 survivors; 0.1 mg L^{-1}: 17.5 ± 7.3 survivors; GLM, df = 639, z = -4.32, $P < 0.001$; Fig. 2C). In addition, larvae exposed to 0.5 mg L^{-1} UV-327 experienced a 65.6% decrease in survival compared to the control (control: 27.7 ± 4.0 survivors; 0.5 mg L^{-1}: 14.0 ± 7.1 survivors; GLM, df = 639, z = -4.51, $P < 0.001$; Fig. 2C). Similar to MeP, the highest doses of UV-327, 1.0 mg L^{-1} and 5.0 mg L^{-1}, did not result in increased mortality (Fig. 2C).

For Irganox 1010, survival was slightly higher than control at the lowest dose of 0.1 mg L^{-1} (control: 27.7 ± 4.0 survivors; 0.1 mg L^{-1}: 31.3 ± 3.8 survivors; GLM, df = 572, z = 2.24, $P < 0.05$; Fig. 2D). We speculate this anomaly was due to slightly higher mortality in the control replicates, or more larvae were added to the 0.1 mg L^{-1} replicates than originally calculated. For the intermediate doses of Irganox 1010, larval survival was reduced by 39.7% when reared with 0.5 mg L^{-1} Irganox 1010 (control: 27.7 ± 4.0 survivors; 0.5 mg L^{-1}: 18.5 ± 3.3 survivors; GLM, df = 572, z = -3.59, $P < 0.001$; Fig. 2D) and reduced by 70.47% when reared with 1.0 mg L^{-1} Irganox 1010 compared to the control (control: 27.7 ± 4.0 survivors; 1.0 mg L^{-1}: 13.3 ± 6.9 survivors; GLM, df = 572, z = -5.66, $P < 0.001$; Fig. 2D). Finally, larvae survival was reduced by 33.8% when reared with 5.0 mg L^{-1} (control: 27.7 ± 4.0 survivors; 5.0 mg L^{-1}: 18.5 ± 3.3 survivors; GLM, df = 572, z = -2.91, $P < 0.01$; Fig. 2D).

3.3 Fertilization Success and Early Development

All of the additives and doses tested had no effect on fertilization success (Table 1). Similarly, there was no effect for any additive or dose on early larval development using cell division at 3 hours post-fertilization as a proxy (Table 1).

4. DISCUSSION

Our results confirm the hypothesis that MeP, UV-327, and Irganox 1010 can cause reduced body length (~5%) for *S. purpuratus* pluteus larvae (Fig. 1A, C, D). We also confirm the hypothesis that all four additives had major negative impacts on larval survival, 20-70% reduction, at a range of doses (Fig. 2A-D). Interestingly, for both body length and survival, negative effects were strongest at intermediate additive doses (Figs. 1-2). In contrast, during early development, we did not observe any negative impacts on fertilization or early cell divisions (Table 1).

To our knowledge, our results demonstrate for the first time that MeP, UV-327, and Irganox 1010, additives that commonly leach from plastics, can reduce body length in marine sea urchins. The results from this study confirm and expand the results from previous studies that suggest leachates from plastics negatively affect larval growth and development of larval sea urchins (Arslan et al., 2007a, 2007b; Beiras et al., 2019; Martínez-Gómez et al., 2017; Nobre et al., 2015; Oliviero et al., 2019; Özlem and Hatice, 2008; Rendell-Bhatti et al., 2021). Reduced growth in larval urchin *Paracentrotus lividus* after exposure to PVC toy leachates has been observed at high experimental doses of 10 mg $L^{-1}$and 30 mg L^{-1} (Oliviero et al., 2019). In contrast to our results, low doses 0.3, 1.0 or 3.0 mg L^{-1} had no effect on growth (Oliviero et al., 2019), where we found larvae were shorter at a similar range of doses, suggesting that MeP, UV-327 and Irganox 1010 may cause more developmental toxicity to marine organisms than the commonly studied leachate from PVC. We found that body length of larval *S. purpuratus* was reduced after exposure to 5,0 mg L^{-1} MeP (5.4%, Figure 1A), 0.5 mg L^{-1} UV-327 (4.6%, Figure 1C), and 1.0 mg L^{-1} Irganox 1010 (6.5%, Figure 1D). In contrast, Nobre et al. (2015) found larval development in the urchin *Lytechinus variegatus* was reduced by a significantly larger difference of 34.6% after exposure to beach-collected microplastic granules that were suspected to leach during the duration of the experiment. It was not determined what plastic leachates were present in the experimental media, and a dose response was not used (Nobre et al., 2015), which could explain the large difference between the reduction of body length reported here and Nobre et al. (2015).

Similarly, Martínez-Gómez et al. (2017) found that both trials of aged polystyrene microplastics and HDPE fluff, a common plastic additive in food containers (GESAMP, 2016), each caused a reduction in growth and embryo development for the sea urchin *Paracentrotus lividus*. Like Nobre et al. (2015), the concentration of polystyrene leachate was not measured, however, 10^3, 10^4 and 10^5 microplastics L^{-1} were used in experimental replicates, suggesting that there was a high concentration of toxins present (Martínez-Gómez et al., 2017). Surprisingly, the lowest concentration of both trials of polystyrene particles and HDPE fluff caused the highest percent abnormal larvae in *P. lividus,* which the authors explained to possibly be caused by a larger microplastic/water interface due to less particle aggregations relative to those observed in the higher particle concentration trials (Martínez-Gómez et al., 2017). This phenomenon of less concentrated doses having more negative effects was also observed in the study presented here, where two (UV-327 and Irganox 1010) out of the three chemicals displaying a negative effect on body length had the most severe effects displayed in the intermediate doses. These patterns do not follow a classic dose-response curve and are consistent with a NMDR (non-monotonic dose response). NMDRs occur "when dose response curves contain a point of inflection where the slope of the curve changes sign at one or more points within the tested range" (U.S. Environmental Protection, 2013). This phenomenon has been described as occurring when estrogen, androgen, and thyroid pathways are disrupted, and is usually associated with high-dose trials, although low-dose effects should be investigated more thoroughly (Rhomberg and Goodman, 2012). Although we did not test for endocrine disruption here, DEHP is a known endocrine disruptor for aquatic organisms such as brackish ricefish (Ye et al., 2016, 2014), goldfish (Golshan et al., 2015), and zebrafish (Jia et al., 2016), and may be disrupting the urchin endocrine hormones in this study; this could explain the NMDR pattern found in the body length and survival trials presented here.

In contrast to Martínez-Gómez et al. (2017) and the present study, most marine ecotoxicological studies that have tested effects of plastic-related compounds using a range of doses have not found a NMDR pattern. Using concentrations comparable to this study (0.3 – 3.5 mg L^{-1}), Özlem and Hatice (2008) found that bisphenol A (BPA) leachates significantly affected *P. lividus* differentiation at the gastrula stage and skeletal formation; these abnormalities followed a typical dose response curve (monotonic done response), which was not observed in the presented study. Similarly, results from sea urchin larvae, *Arbacia lixula,* that were exposed to plastic additives nonylphenol (NP) and octylphenol (OP) at concentrations ranging from 0.937-18.74 ug L^{-1} and 5-160 ug L^{-1}, respectively, followed a classic dose-response pattern (Arslan et al., 2007a). Additionally, *P. lividus* exposure to NP and OP experienced deformities and arrested larval development during both the blastula and gastrula stages (Arslan et al., 2007b). All leachate assays showed a direct relationship between the increase of leachate dose and developmental arrest (Arslan et al., 2007b, 2007a). Similarly, *P. lividus* exposed to a leached PVC inflatable toy experienced a reduction in body size dependent on the leachate dose (Beiras et al., 2019). There was no effect observed at doses 0.01 and 0.1 g L^{-1}, however 1.0, 10, and 100 g L^{-1} showed a toxic response in larvae (Beiras et al., 2019). These less-concentrated doses are higher than the lowest doses used in the present study, again highlighting that the compounds studied here may be more developmentally toxic than PVC leachates. In another study, urchins (*P. lividus*) exposed to leached PVC, nurdles (plastic resin pellets (Pozo et al., 2020)), and biobeads expressed a dose-dependent delay in larval development and deformities in embryonic tissues ranging from crossed or separated arms to no arms visible during late gastrulation from 24 hours post-fertilization (Rendell-Bhatti et al., 2021).

Early urchin larval development and established energy reserves are crucial for survival through metamorphosis (de La Uz et al., 2013). Stunted larval growth may affect juvenile or adult stage survival, thus possibly reducing population numbers (Byrne et al., 2013). Our results reveal, for the first time to our knowledge, that MeP, DEHP, UV-327, and Irganox 1010 additives reduce larval *S. purpuratus* survival. For MeP, DEHP, and UV-327 treatments, survival was most reduced at the intermediate dose of 0.5 mg L^{-1}, and for Irganox 1010 at 1.0 mg L^{-1} (Fig. 2). Embryonic and larval death was also observed in *P. lividus* after exposure to biobead, nurdle, and PVC leachates at 24 and 48 hours post-fertilization respectively (Rendell-Bhatti et al., 2021). Additionally, newly fertilized *P. lividus* embryos exposed to BPA leachates died at the gastrula stage in increasing numbers starting at 1500 to 3500 μg L^{-1} (Özlem and Hatice, 2008), comparable to the doses used in the present study.

Mirroring the body size results in this study, larval survival also followed a NMDR pattern after exposure to plastic additives. Significant reductions of larval survival at intermediate doses could mean that the larvae activate a physiological response that improves survival only at concentrations above a specific additive threshold. Importantly, death at intermediate doses suggests that concentrations of marine plastic additives do not need to be high to cause harm to marine life; diffuse additive concentrations could result in maximum mortality. DEHP leachates are found at a wide range of concentrations in the Earth's oceans, from 448 x 10^{-6} μg L^{-1} in the Arctic, to 62.77 μg L^{-1} in the Mediterranean Sea (Hermabessiere et al., 2017), encompassing the doses used in this study. DEHP has been found to leach quickly, as high as 2.45% of total additive weight was leached from plastic water bottles into water over a duration of 3 weeks (Kastner et al., 2012). UV-327 additive concentrations were reported to be 3.2 ± 2.6 ng g^{-1} in Ariake Sea sediment samples (Nakata et al., 2009), but have not been measured in the marine water column. Like UV-327, Irganox 1010 additives have not yet been quantified, nor have leaching rates been determined in aquatic environments, thus it is difficult to determine appropriate experimental dose concentrations. Finally, MeP, the most abundant paraben found in aquatic organisms (Comeche et al., 2017), has a global marine presence ranging from Normandy, France (3.64 - 18.59 μg L^{-1}) (di Poi et al., 2018), northwestern Portugal (51 ng L^{-1}) (Haman et al., 2015), the coast of Florida (3.02 - 31.7 ng L^{-1}) (Xue et al., 2017), to untreated wastewater leachates in China (3480 - 7930 ng L^{-1}) (Peng et al., 2014) that has the potential to seep into nearby marine ecosystems. Despite the ubiquitous distribution, to our knowledge, the rate of leaching of MeP from plastics into the oceanic environment has not yet been determined. Our MeP experimental doses are higher than these environmental concentrations, however, there are large gaps on the geographic knowledge of leached oceanic plastic additives (Gunaalan et al., 2020). Nevertheless, our results reveal that intermediate doses of plastic additives can have the highest effect on marine biota, suggesting that oceanic leachates, even when at moderate concentrations, can be detrimental to marine life.

S. purpuratus exposure to MeP, DEHP, UV-327 or Irganox 1010 showed that there was no effect on fertilization success 40 minutes post-gamete mixing (Table 1). There was also no effect of additives on early cell division 3 hours post-fertilization; embryos were able to cleave at least once (Table 1). This could be due to gametes only being exposed to the plastic additives for a short amount of time, and embryo developmental toxicity had not yet occurred. In contrast, *P. lividus* embryos exposed to 30 mg L^{-1} PVC leachates did not develop past the earliest phase of development following fertilization (Oliviero et al., 2019). This dose is much higher than our most concentrated dose, which may explain why early-stage development was not arrested in our study. Similarly, *P. lividus* eggs exposed to aged polystyrene microplastics in doses 10^3, 10^4, and 10^5 microplastics mL^{-1} experienced a 56-58% reduction in fertilization success; the lowest microplastic leachate concentration had the strongest effect on fertilization, again displaying a NMDR pattern (Martínez-Gómez et al., 2017). Fertilized eggs also experienced undeveloped embryos, thickening and proliferation of the ectodermal membrane, as well as reduced arm length immediately following fertilization (Martínez-Gómez et al., 2017), which we also did not see here. This is most likely due to the extremely high concentrations of microplastic particles, and thus high leachate doses, which were much higher than the present study. Additionally, *P. lividus* exposure to NP leachates reduced fertilization success the most at intermediate doses (1.874 $\mu g\ L^{-1}$, 2.811 $\mu g\ L^{-1}$) and the highest dose (18.74 $\mu g\ L^{-1}$), with slight recovery with the stronger of the intermediate doses (Arslan et al., 2007b). This created a non-conforming dose-response curve that may have revealed a NMDR if more doses were included in the assay. High exposure concentrations of NP and OP to *A. lixula* also resulted in early embryonic development arrest due to cells unable to perform mitosis (Arslan et al., 2007a), which we may have observed if we assayed cell division further in the experiment. Finally, BPA exposure reduced fertilization success of *P. lividus* sperm in all doses, thus reducing offspring quality and survival (Özlem and Hatice, 2008). We did not determine if the plastic additives used in this study specifically affected either the eggs or the sperm, which could be a topic for future research. We also suggest the use of multiple urchin families to ensure genetic diversity in future studies.

In summary, this study examined the effects of four common plastic additives, methylparaben, DEHP, UV-327, and Irganox 1010, on the larval sea urchin *S. purpuratus.* Both body length and survival were reduced after additive exposure, while fertilization and early cell divisions were not affected. Our dose-response experimental design revealed a NMDR in both body length and survival trials, suggesting that these plastic additives could be endocrine disruptors for sea urchins. Regardless of whether these under-studied additives are endocrine disruptors, it is evident that they negatively impact larval urchins and should be included in future ecotoxicological research. Comprehensive toxicological dose response assays of plastic additives should be considered in determining the long-term consequences to human and wildlife health that might occur with the continued use of these additives.

FIGURES

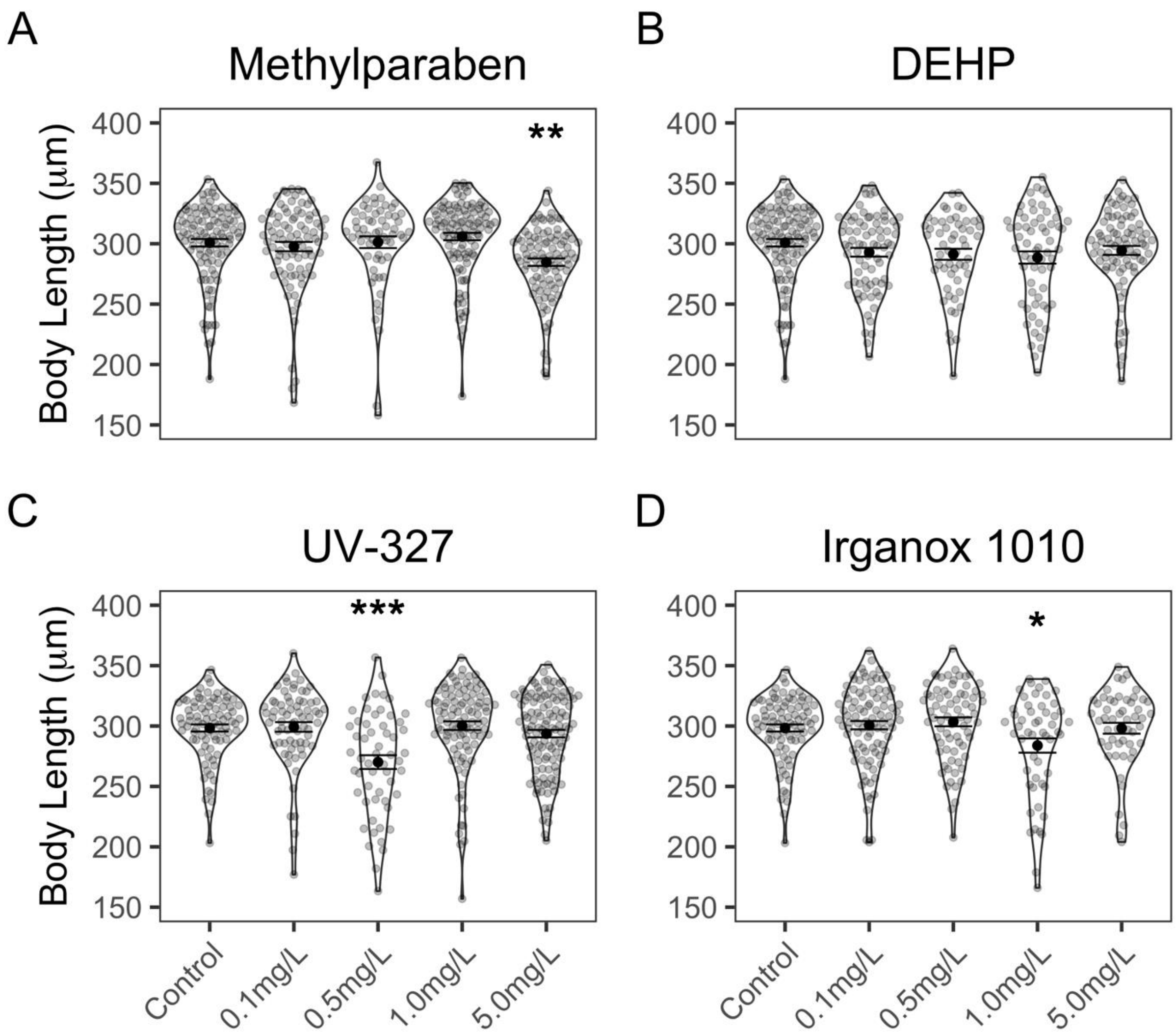

Figure 1: The impact of plastic additives on *Strongylocentrotus purpuratus* pluteus larval body length after a 4-day exposure. (A) Methylparaben; (B) DEHP; (C) UV-327; (D) Irganox 1010. Dots represent measurements from individual larvae; asterisks indicate statistical significance (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

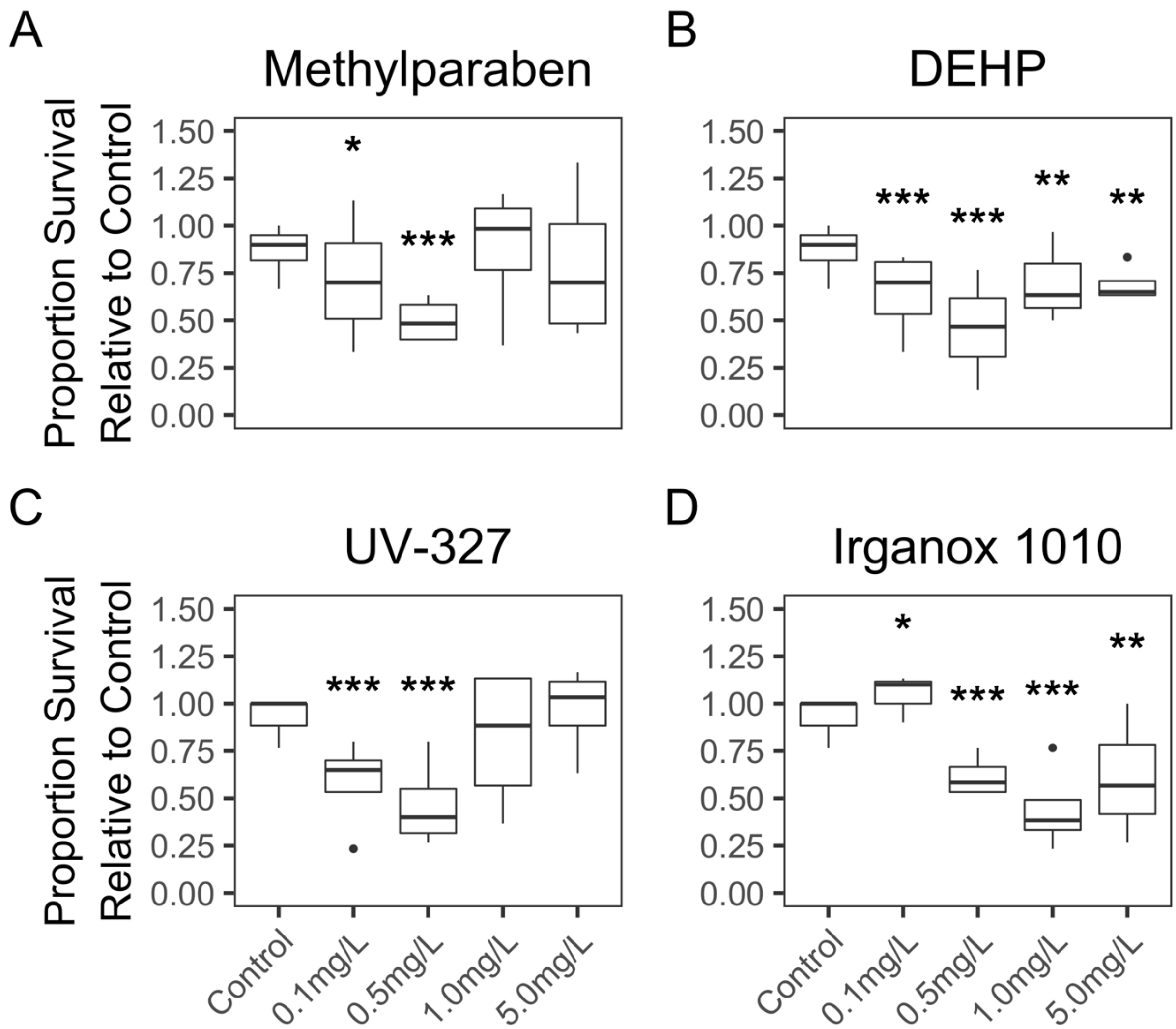

Figure 2: The impact of plastic additives on *Strongylocentrotus purpuratus* larval survival after a 4-day exposure. (A) Methylparaben; (B) DEHP; (C) UV-327; (D) Irganox 1010. Asterisks indicate statistical significance (**P* < 0.05; ** *P* < 0.01; *** *P* < 0.001).

Table 1: Fertilization success and percent developing embryos (cell division) at 3 hours post-fertilization for *S. purpuratus*, across increasing doses of methylparaben, DEHP, Irganox 1010, and UV-327 additives. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Additive	Treatment (mg L^{-1})	Fertilization Success (%)	Cell Division at 3hrs (%)
Methylparaben		-	
	Control (ASW)	99	97
	0.1 mg L^{-1}	100	97
	0.5 mg L^{-1}	99	100
	1.0 mg L^{-1}	100	99
	5.0 mg L^{-1}	99	99
DEHP			
	Control (ASW)	99	99
	0.1 mg L^{-1}	99	99
	0.5 mg L^{-1}	100	100
	1.0 mg L^{-1}	99	99
	5.0 mg L^{-1}	97	98
Irganox 1010			
	Control (DMSO)	99	100
	0.1 mg L^{-1}	99	99
	0.5 mg L^{-1}	97	98
	1.0 mg L^{-1}	99	99
	5.0 mg L^{-1}	99	97
UV-327			
	Control (DMSO)	99	100
	0.1 mg L^{-1}	98	99
	0.5 mg L^{-1}	99	98
	1.0 mg L^{-1}	98	97
	5.0 mg L^{-1}	97	100

Funding

This work was supported by the National Science Foundation (NSF) grants OCE-1559075 (to M.H.P.) and OIA-1736253 (to M.H.P.) and the NSF Graduate Research Fellowship Program DGE-1451866 (to A.D.G.).

Acknowledgements

We thank Pete Halmay and Patrick Leahy for urchin collections and Jeremy Arenos for assistance with imaging and image analysis.

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