

EFFECTS OF BISPHENOL-A AND STYRENE ON FERTILIZATION
AND DEVELOPMENT OF THE PURPLE SEA URCHIN
(*STRONGYLOCENTROTUS PURPURATUS*)

A Thesis
presented to
the Faculty of California Polytechnic State University,
San Luis Obispo

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Biological Sciences

by
Nicole Cathleen Uibel

June 2016

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COMMITTEE MEMBERSHIP

TITLE: Effects of bisphenol-A and styrene on fertilization
and development of the Purple Sea Urchin
(*Strongylocentrotus purpuratus*)

AUTHOR: Nicole Cathleen Uibel

DATE SUBMITTED: June 2016

COMMITTEE CHAIR: Nikki Adams, Ph.D.
Professor of Biological Sciences

COMMITTEE MEMBER: Jennifer Carroll, Ph.D.
Associate Professor of Chemistry

COMMITTEE MEMBER: Sean Lema, Ph.D.
Associate Professor of Biological Sciences

ABSTRACT

Effects of bisphenol-A and styrene on fertilization and development

of the Purple Sea Urchin (*Strongylocentrotus purpuratus*)

Nicole Cathleen Uibel

Plastic waste and debris have greatly increased in the marine environment during the past 50 years. Not only do these plastics entangle and get ingested by marine mammals, turtles, and sea birds, but they also leach chemicals, such as bisphenol-A (BPA) and styrene into the aquatic environment. While some of these chemicals are known to be toxic, few studies have examined their effects on broadcast spawning marine invertebrates, specifically at environmentally relevant concentrations. Purple sea urchins, *Strongylocentrotus purpuratus*, from the central coast of California, were utilized to examine effects of BPA and styrene on fertilization success and early development of resulting embryos. Previous research has demonstrated that BPA and styrene decrease successful fertilization and delay development of sea urchins, though no previous studies have examined effects of continuous chemical treatment on *S. purpuratus* development. We exposed eggs, sperm, or both to environmentally relevant concentrations of BPA or styrene (100 $\mu\text{g/L}$, 500 $\mu\text{g/L}$, and 1000 $\mu\text{g/L}$) to test the hypothesis that continuous treatment would lead to developmental abnormalities. The greatest effect was observed in pluteus larvae development, as the percentage of normal embryos decreased by as much as 80% in high dose chemical treatments ($p < 0.005$). Time to the first cleavage occurred more quickly in exposed vs. unexposed gametes (up to 20%, $p < 0.0001$), however, fertilization was not significantly affected by chemical treatment. Additionally, BPA was demonstrated to have a 20% stronger effect on normal development of pluteus larvae compared to styrene at high (1000 $\mu\text{g/L}$) concentrations ($p < 0.05$). These results indicate that exposure to BPA or styrene has the potential to impact processes crucial to normal development.

ACKNOWLEDGMENTS

Completion of this thesis would not have occurred without the immense help and support of many people. First and foremost, I would like to graciously thank my committee chair and trusted advisor, Nikki Adams. Her overwhelming trust, support, and aide saw this project through from conception to completion despite the numerous hurdles involved in such a novel project. Thank you for never giving up on me, and for allowing me to explore every opportunity possible, especially when I couldn't say "no" to a project. My fellow graduate student, Andrea Debrish deserves applause for her constant positive presence throughout my undergraduate and graduate career, from the field to the lab to the office, everything was more fun, fulfilling, and focused with my partner in crime. Grace Goschke's helpful advice and support was instrumental in keeping me sane throughout this project. I would also like to recognize my committee: Dr. Sean Lema and Dr. Jennifer Carroll, for ideas, advice, criticism, and for pushing me to my best potential. Many thanks to Christoph Pierre for collection of the sea urchins used in this research. Special thanks to Tom Moylan and Jason Felton for use of the Cal Poly Pier and for scouring the coast to collect kelp, to Doug and Rob Brewster for technical advising and shop use, and to my fellow graduate students for comradery in times of great need. Many undergraduate researchers assisted with this project: Yareli Alvarez, Alyssa Eszlinger, Brooke Darrah, Rachael McLellan, Ariana Jensen, and Aparna Bhat. My family and friends deserve special recognition for putting up with my disappearing act for the last three years. Finally, I would like to thank California Polytechnic State University for funding this project through the College Based Fees initiative, and for providing me with the opportunity to receive the best undergraduate and graduate education possible.

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INTRODUCTION

Anthropogenic-related changes to the Earth's oceans have accelerated in the past century. One of the most obvious changes in the last half-century is the increase in marine debris, defined as any man-made material, deposited into the oceans (Barnes et al., 2009; Jambeck et al., 2015). Items such as fishing gear and household consumer products are among the most common marine debris (Barnes et al., 2009; Derraik, 2002), which can contain metal, rubber, wood, paper, and plastic. Among these items or debris, plastic pollution appears to be posing the most relevant threat to ocean ecosystems due to the multiple impacts it may have on wildlife, many of which have not been sufficiently studied.

Plastics make their way into oceans directly or indirectly through land and ocean based sources (Derraik, 2002; Jambeck et al., 2015). Land based sources of plastic pollution include littering, dumping, runoff through storm drains, poor waste management practices, and industrial losses during production, transport, or processing. Ocean-based sources include dumping from vessels, derelict fishing gear, and industrial shipping accidents (Barnes and Milner, 2005; Cozar et al., 2014; Jambeck et al., 2015).

Though plastics typically constitute only 10% of discarded human waste, they represent 60%-80% of all accumulated debris in the oceans (Barnes et al., 2009; Derraik, 2002; Jambeck et al., 2015) and are virtually ubiquitous in the marine environment (Moore, 2008). Since mass production of plastics began in the 1950's, accumulation of plastic debris has accelerated at alarming rates. Current estimates of 5.1 kilograms/kilometer of plastic debris in the oceans are an order of magnitude higher than estimates in the 1980's (Jambeck et al., 2015; Moore, 2008; Ryan et al., 2009), with

highest densities in urban areas and water convergences (Barnes and Miller, 2005; Cozar et al., 2014).

Although the absolute abundance of plastics has increased, the average size of plastic particles in the ocean is decreasing (Barnes et al., 2009; Cozar et al., 2014; Jambeck et al., 2015). Because plastics do not biodegrade, they can persist in the environment for thousands of years, causing the oceans to become reservoirs of plastic waste (Barnes et al., 2009). Many of these plastics undergo a process called photodegradation, where the larger pieces break down into microplastics (<5mm pieces), leachates of plastic chemicals, or other byproducts due to the presence of ultraviolet (UV) light (Ryan et al., 2009).

The majority of research on plastic marine debris has focused on the effects of these macro-plastics including entanglement and ingestion hazards posed to wildlife. Other studies also demonstrate that the abundance and impact of micro-plastics and micro-fibers may be just as damaging (Andrady, 2011; Barnes et al., 2009; Moore, 2008). However, impacts of the leachates and other byproducts of plastics have been largely unstudied.

Common examples of chemicals released through plastic leaching include bisphenol-A (BPA), styrene, phthalates, and vinyl chlorides (Laist, 1987). Some of these leachates are endocrine disruptors, others are carcinogens, and some have not yet been classified (Barnes and Milner, 2005; Barnes et al., 2009; Hirai et al., 2011). We have chosen to focus on the effects of BPA and styrene for this project due to their widespread use in industrial plastic production, ease of use in laboratory settings, as well as their

previously described deleterious effects on wildlife (Crain et al. 2007; Kang et al. 2007; Welshons et al., 2006).

BPA is commonly used in polycarbonate plastic manufacturing as a stabilizer. Polycarbonate plastics include items such as food and beverage containers and epoxy resins on metal dishware (National Institute of Environmental Health Services, 2015).

BPA can be transferred to the environment through multiple vectors, including burning of plastics, and photodegradation of plastics in the ocean (Kang et al., 2007; Teuten et al., 2009). When BPA is released into the air, it degrades rapidly; however, it is more stable and persists longer in water (Moore, 2008). Current aquatic measurements estimate that detectable BPA concentrations in the USA range from 0.5 µg/L in freshwater rivers to 146 µg/L in industrial effluent (Ozlem and Hatice, 2008). Other estimates suggest BPA levels are as high as 283 µg/L in the open ocean, 730 µg/L in coastal areas of Japan (Hirai et al., 2011), and 1000 µg/L in Malaysia (Teuten et al., 2009).

These environmental concentrations of BPA, coupled with evidence for transference to people through the use of food and beverage containers (Von Goetz et al., 2010; Casajuana and Lacorte, 2003), have raised concerns about potential health impacts of BPA exposure. In a study published by the Center for Disease Control and Prevention (CDC), over 93% of urine samples contained detectable levels of BPA, indicating that BPA exposure is widespread in the U.S. (Calafet et al., 2005; Fu and Kawamura, 2010).

While federal agencies such as the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) have deemed levels below 5 mg/kg/day safe levels for consumption (Aungst, 2014), a recent review of published studies on BPA

revealed that 80% of *in vivo* studies found evidence for negative BPA effects, and 30% of those found this evidence below “safe” doses (Vom Saal and Hughes, 2005). This research has prompted the EPA and the FDA to examine effects of BPA in the environment. Within the last year, the EPA has acknowledged the challenge of BP, especially in aquatic environment and has listed BPA as a chemical of concern for additional research.

BPA can have impacts as an endocrine disruptor and carcinogen in mammals and fish by mimicking estrogenic compounds (Welshons et al., 2006). Multiple human health studies have found evidence for hormonal effects of BPA, including advancing puberty rates (Howdeshell et al., 1999), increasing obesity (Jirtle and Skinner, 2007; Carwile and Michels, 2011), and links to disease such as diabetes (Lang et al., 2008). Exposure to high doses of BPA causes malformation of testes and deformation of spermatids at 200 µg/kg in mice (Toyama et al., 2004). Sohoni et al. (2001) similarly found reproductive anomalies in male fathead minnows (*Pimephales promelas*) exposed to BPA long-term, including an inhibition of spermatogenesis at concentrations of 1600 µg/L. Vom Saal et al. (1998) began examining low dose effects of BPA, exposing mice to environmentally relevant concentrations of 2-20µg/L. These low dose exposures caused increased size of preputial glands at 2 µg/L, but decreased spermatid formation at 20 µg/L (Vom Saal et al., 1998).

In addition to low dose effects of BPA, previous studies have demonstrated mixture effects, where two chemicals produce synergistic effects when combined at concentrations below their individual EC₅₀ levels to become toxic to organisms (Wormley et al., 2004). BPA has been demonstrated to display these mixture effects in combinations

with known EDC's such as pentachlorophenols (PCPs), benzophenones, and hydrocarbons in recombinant yeast systems (Silva et al., 2002). These mixture effects have also been demonstrated to delay development and cause morphological abnormalities in zebrafish (*Danio rerio*) embryos at high concentrations (2000 µg/L) of BPA (Duan et al., 2008). Though these results describe the negative effects of BPA at biologically relevant concentrations, there are few other studies, specifically on invertebrate models, which mirror this methodology.

Reports vary on the sensitivity of aquatic invertebrate organisms to BPA. For example, sea urchin (*Hemicentrotus pulcherrimus*) embryos developed normally, but grew significantly smaller adult test sizes compared to controls in BPA concentrations as high as 570 µg/L (Kiyomoto et al., 2006). In contrast, BPA causes developmental abnormalities in abalone (*Haliotis diversicolor supertexta*) at concentrations as low as 50 µg/L (Zhou et al., 2011). Ozlem and Hatice (2008) observed similar developmental abnormalities if the sea urchin *Paracentrotus lividus* embryos were exposed to BPA concentrations of 300µg/L. Higher concentrations of BPA (2mg/L) stimulate asexual reproduction in an aquatic cnidarian, *Hydra oligactis* (Fukuhori et al., 2004), and longer exposures (up to three weeks) to immersed recycled plastics may also be detrimental to fertilization of sea urchin eggs (Weis et al., 1992). These varying results on aquatic organisms indicate there is a gap in our understanding of how invertebrates respond to differential BPA exposures, especially at environmentally relevant concentrations.

Styrene is the most commonly used monomer in polystyrene products, which include Styrofoam containers, plastic utensils, and other packaging materials (National Institute of Environmental Health Services, 2014). Styrene is formed during combustion,

and trace amounts are released when smoking cigarettes, in vehicular exhaust, and even when using a photocopier, and airborne styrene has been linked to respiratory irritation and effects on humans (Date et al., 2002; Saido et al. 2014). Not surprisingly given its ubiquitous presence, the EPA has found presence of styrene in “100% of all samples of human fat” (U.S. EPA, 1994). However, the EPA lists safe levels of styrene at 100 µg/L in air and 100 ng/L in water (ATSDR, 2010) and currently the U.S. Department of Health and Human Services (DHHS) estimates that indoor styrene levels are well below this toxic threshold (Lunn et al., 2008).

Detectable environmental levels of styrene vary widely. For example, a recent study found that styrene concentrations in seawater samples in Japan range from 2 µg/L to 8000 µg/L (Saido et al., 2014). When styrene was measured in air, land, and water samples, researchers found concentrations were an order of magnitude higher on shorelines compared to nearby seawater samples, and seawater samples maintained higher concentrations of styrene compared to air samples at the same location (Saido et al., 2014). These results suggest that styrene may persist longer in terrestrial systems compared to aquatic systems; however, styrene was detected in the aquatic samples, suggesting that styrene is a continued threat in marine environments or organisms (Saido et al., 2014).

Despite its obvious persistence in marine ecosystems, few studies have examined effects of styrene monomers on wildlife. Most studies have focused on vertebrate models and few have focused on environmentally relevant concentrations. Most studies have concluded that styrene can act as both an endocrine disruptor (Colborn et al., 1993) and a carcinogen (Conti et al., 1988); however these studies did not focus on the mechanism of

action behind styrene toxicity. Rats exposed to styrene orally at 400mg/L exhibited impaired spermatogenesis, although a lower dose (200mg/L) of styrene caused no overt signs of toxicity (Srivastava et al., 1989). A study by Pagano et al. (1978) on the sea urchin *Paracentrotus sp.* demonstrated that exposure of sea urchin eggs to high concentrations of styrene (1000µg/L) causes cytolysis and reduced fertilization of eggs. When the sperm of the same species were exposed to similar concentrations of styrene, fertilization was slightly reduced, but no effects were observed at low doses (100µg/L) on either gamete. Additionally, styrene has been demonstrated to reduce reproductive rates in daphnids (*Ceriodaphnia dubia*) at low (1.7 µg/L) concentrations, indicating that these chemicals have the potential to impact population structures of invertebrates (Tatarazako et al., 2002).

BPA and styrene have toxic effects on both vertebrates and invertebrates, however the relative toxicity of the compounds on developing marine invertebrate embryos has, to our knowledge, not been addressed in the literature. There has been evidence that styrene may have a larger effect on reproductive output compared to BPA (Tatarazako et al., 2002), however this research was performed on adult crustaceans (*Ceriodaphnia dubia*). Both compounds can be classified as endocrine disruptors (Colborn et al., 1993; Vom Saal et al., 1998) and as carcinogens (Conti et al., 1988; Keri et al., 2007), however there is a gap in the literature addressing continued exposure to these chemicals in early development at environmentally relevant concentrations.

Sea urchins have been used as model organisms in developmental biology for over 100 years due to their relatively simple early development and the ease of experimentally perturbing this development (McClay, 2011). Taxonomically, they are

closely related to chordates, allowing parallels of early development to be drawn (Talsness et al., 2009), but they are much easier to collect and manipulate. Sea urchins also display synchronous development, meaning one mother's batch of eggs can be separated, experimentally treated, and compared for changes in developmental process or timing.

Sea urchins serve as an important invertebrate model to test effects of chemicals and stress on marine organisms, specifically in the gamete and embryonic life stages. Due to their rapid rate of development until metamorphosis, embryos may be more susceptible to chemical changes in the water column compared to adults. Because this embryonic stage is considered to be the most sensitive life stage, governing agencies such as the EPA frequently utilize sea urchin embryos in toxicology studies (U.S. EPA, 2002). In addition, multiple studies have demonstrated environmental effects on these early life stages of sea urchins, where the embryos become stalled in various developmental stages including fertilization, cleavage, and larval stages (Adams and Shick, 1996, 2001; Kobayashi and Okamura, 2002; Kiyomoto et al., 2006; Ozlem and Hatice, 2008). Utilizing these well-documented developmental stages, we aim to track developmental abnormalities from gamete through larvae after exposure to BPA and styrene.

This study aimed to determine how exposure of gametes to environmentally relevant levels of BPA and styrene impacted fertilization and early embryonic development in *Strongylocentrotus purpuratus*. Through separately exposing the gametes of sea urchins, I aimed to determine a sensitivity response for eggs versus sperm, and aimed to test whether there are additive or synergistic effects of exposing both sperm and eggs together. Based on prior observations by Ozlem and Hatice (2008), I predicted that

sperm will be more sensitive to the chemicals, but that we will see a significant increase in sensitivity when we expose both eggs and sperm.

By utilizing multiple concentrations of BPA and styrene, I also attempted to determine the effect of increasing concentrations of these chemicals on various developmental hallmarks. I predicted BPA and styrene will delay fertilization and time to first cleavage, and that the chemicals will have a dose-dependent negative effect on normal development. Response to plastic-derived chemicals and how they affect reproduction and developmental biology is of utmost importance for broadcast spawning ecologically and economically relevant organism.

MATERIALS AND METHODS

Adult *S. purpuratus* were collected off the coast of Goleta, California, USA, in August 2014 and maintained at the Cal Poly Center for Coastal Marine Science Pier facility in Avila Beach, California, USA. Tanks were constantly supplied with flowing natural seawater and sea urchins were weekly fed ad libitum diets of locally collected kelp (*Macrocystis pyrifera*). Gamete and embryo exposures were conducted in the laboratory on Cal Poly campus.

Stock solutions of each Bisphenol-A (BPA, Acros Organics, Pittsburg, PA, USA) and Styrene (Sigma-Aldrich Chemical Company, Milwaukee, WI, USA) were dissolved in dimethylsulfoxide (DMSO, Fisher Scientific, Chino, CA, USA), an environmentally friendly solvent. All solutions were stored at room temperature until use. Concentrations of each chemical used in experiments were determined based on published data about environmentally relevant concentrations (Hirai et al., 2011; Teuten et al., 2009; Saido et al., 2014). Stock solutions were dissolved into seawater filtered through 0.22 μm paper (filtered seawater, FSW) for final treatment concentrations of 100, 500, and 1000 $\mu\text{g/L}$ of each BPA and styrene.

Spawning of adult sea urchins was induced via intracoelomic injection of 2 ml of 0.5 M KCl. One male and one female were spawned for thirty minutes for each experiment. Eggs were collected into FSW at 12°C. After washing eggs twice in FSW, 20 ml of settled eggs were added to 380 ml FSW to create a 5% egg dilution, which was used in chemical treatments. Sperm was collected “dry” and kept on ice until use. Sperm suspensions of 1:50,000 to 1:100,000 (sperm to FSW ratio) were mixed to activate sperm, and kept on ice until use (immediately in egg exposures, after chemical treatment in

sperm and egg/sperm exposures; see below). BPA or styrene were added to beakers at the appropriate concentrations and mixed. A DMSO control at highest chemical concentration (1000 µg/L) and a FSW control were also prepared.

To assess effects of chemicals on eggs, chemical treatments were added and mixed 30 minutes prior to their fertilization. To assess effects on sperm, chemical treatments were mixed with sperm dilutions 30 minutes prior to fertilization. To assess additive effects of both egg and sperm exposures, the previously described techniques were utilized, and egg batches were fertilized after 30 minutes with complimentary sperm treatments (e.g. control eggs fertilized with control sperm; see Table 1).

Fertilization was assessed using an established fertilization assay (see Belton et al., 2001) where 1 ml samples were preserved with 1% formalin (in FSW) every minute for ten minutes. The first 200 embryos observed were scored for successful fertilization with visual confirmation that the fertilization envelope had raised. All batches reached >80% fertilization within the first 10 minutes' post fertilization (mpf). Delays in fertilization were calculated using methodology described in Adams and Shick (1996; see below), and were compared to DMSO controls.

The time the embryos took to reach the first cleavage was assessed using a cleavage delay assay (CDA as per Adams and Shick, 1996) where 1 ml samples were fixed with 1% formalin every ten minutes until 100% cleavage was observed (approximately 80 mpf-180 mpf). The first 200 embryos observed were scored for successful cleavage. Time to 50% cleavage was measured, and percent cleavage delay (PCD) was calculated for each batch as per Adams and Shick (1996). DMSO was used as control to account for any affect due to dissolution.

$$PCD = \frac{\textit{Time to 50\% of Chemical Treated to Divide} - \textit{Time to 50\% of Control to Divide}}{\textit{Time to 50\% of Control to Divide}}$$

Immediately following sampling for the CDA, 10mL from each treatment group was transferred to glass petri dishes, and diluted with an additional 40 ml FSW appropriately dosed to maintain chemical concentrations for larval rearing.

Developmental hallmarks such as the blastula, gastrula, and pluteus larval stages were examined to determine the effects of prolonged exposure through development. The first 100 blastulae observed (~24 hours post fertilization: hpf), gastrulae (~48 hpf), and pluteus larvae (~72 hpf) were scored live for normal development/morphology. Additional samples were preserved in 1% formalin at each time point for further analysis. Immediately following sampling, 20 ml water changes occurred and samples were again dosed with appropriate chemical treatment to maintain original concentrations.

All data were analyzed using JMP Pro 11.2 (SAS Institute Inc., North Carolina, USA) multi factor analysis of variance (ANOVA) utilizing the general linear model (GLM) function to identify significant differences among parameters. Factors included type of gamete exposed, chemical treatment, and the interaction factor. Additionally, each batch of eggs was included as a random factor to account for variations among individual urchins. Logit percent transformations were performed on normal development data to account for variation within the residual errors (Warton and Hui, 2011). For analysis of fertilization and cleavage delays, chemical treatments were compared to DMSO control. In these cases, the FSW treatment was excluded from the analyses. Tukey's post-hoc tests were utilized to determine difference among treatments. All tests used an $\alpha = 0.05$.

RESULTS

Our results suggest that BPA and styrene cause the greatest effect at the pluteus larvae stage of sea urchin development after continuous exposure to environmentally relevant concentrations. Other larval stages, including the blastula and gastrula stages, were also negatively affected by the chemicals at less extreme rates. Cleavage rates increased after treatment with both BPA and styrene, a novel conclusion in this field. Finally, we found no evidence that fertilization is effected by any chemical treatment.

The time for 50% of sea urchin eggs to be fertilized did not significantly differ among chemical treatments (ANOVA: $F=0.596$, $P=0.76$; Fig 1). There was no significant effect of gamete exposure on time to 50% fertilized (ANOVA: $F=2.084$, $P=0.15$) and there was no effect of the interaction between chemical treatment and gamete exposure (ANOVA: $F=0.373$, $P=0.98$).

The percent fertilization delay between sea urchin embryos did not significantly differ among gamete treatments (ANOVA: $F=1.219$, $P=0.32$). There was no significant effect between chemical treatments on fertilization delay (ANOVA: $F=0.655$, $P=0.65$; Fig 2), and there was no significant interaction effect of chemical treatment and gamete exposure (ANOVA: $F=0.599$, $P=0.83$).

There was a significant effect of chemical exposure on time to 50% cleavage of sea urchin embryos (ANOVA: $F=8.772$, $P=<0.0001$; Fig 3). Embryos exposed to all concentrations of BPA and low doses of styrene cleaved significantly faster compared to control embryos (Tukey's post-hoc: $p=<0.034$); however, mid and high doses of styrene did not significantly affect time to 50% cleavage in embryos when compared to FSW (Tukey's post-hoc: $p=>0.23$); however, high doses of styrene did cleave significantly

faster when compared to DMSO (Tukey's post-hoc: $p=0.019$). There was no significant difference in cleavage between FSW and DMSO (Tukey's post-hoc: $p=0.96$). There was no significant effect of gamete exposure on time to 50% cleavage (ANOVA: $F=0.765$, $P=0.48$) and there was no significant interaction between chemical treatment and gamete exposure (ANOVA: $F=0.933$, $P=0.53$).

There was a significant effect between chemical treatments on cleavage delay (ANOVA: $F=5.191$, $P=0.0009$; Fig 4). Embryos exposed to BPA cleaved 16%-20% faster compared to embryos in DMSO, and those embryos exposed to styrene cleaved 9%-13% faster compared to embryos in DMSO. Low doses of BPA caused significantly faster cleavage compared to mid and high doses of styrene (9-11%; Tukey's post-hoc: $p<0.008$), however there was no evidence for a significant difference between low doses of BPA and low doses of styrene (Tukey's post-hoc: $p=0.10$). The percent cleavage delay of sea urchin embryos did not significantly differ between gamete exposures (ANOVA: $F=0.505$, $P=0.62$). There was no significant interaction between gamete exposures and chemical treatments (ANOVA: $F=1.418$, $P=0.21$).

The percentage of normal development of sea urchin embryos to the blastula stage was significantly affected by chemical treatments (ANOVA: $F=3.294$, $P=0.0088$; Fig 5, 6, 11). While all doses of styrene and low and mid doses of BPA did not significantly impact blastula normal development when compared to controls (Tukey's post-hoc: $P=>0.61$), high doses of BPA caused significant decreases in normal development (Tukey's post-hoc: $P=0.0483$). Additionally, high doses of BPA caused significantly more abnormalities when compared to low doses of both BPA and styrene (Tukey's post-hoc: $P=<0.0483$). There was no significant effect of gamete exposure on normal

development at the blastula stage (ANOVA: $F=0.245$, $P=0.78$; Fig 8,11) and there was no effect of the interaction between chemical treatment and gamete exposure (ANOVA: $F=0.711$, $P=0.73$).

The percentage of normal development of sea urchin embryos to the gastrula stage was significantly affected by chemical treatments (ANOVA: $F=8.306$, $P<0.0001$; Fig 7, 8,11). Though low doses of the chemicals did not significantly affect normal development, mid and high dosed chemically treated embryos showed significantly decreased normality at gastrula stage compared to controls (Tukey's post-hoc: $P<0.04$). In addition, high doses of BPA and styrene caused increased gastrula abnormalities compared to low doses of their respective chemicals (Tukey's post-hoc: $P<0.04$). There was no significant effect of gamete exposure on normality at the gastrula stage (ANOVA: $F=0.362$, $P=0.70$) and there was no effect of the interaction between chemical treatment and gamete exposure (ANOVA: $F=1.492$, $P=0.16$).

The percentage of normal development of sea urchin embryos to the pluteus stage was significantly affected by chemical treatments (ANOVA: $F=13.59$, $P<0.0001$; Fig 9, 10, 11). Mid and high dosed chemically treated embryos showed significantly decreased normal development at the pluteus stage compared to controls (Tukey's post-hoc: $P<0.03$), however low doses of either chemical had no significant effect on normal development (Tukey's post-hoc: $P>0.06$). Doses of BPA caused increased pluteus abnormalities in a dose dependent fashion (Tukey's post-hoc: $P<0.007$), while styrene caused similar levels of abnormalities regardless of the concentration (Tukey's post-hoc: $P>0.03$). High doses of BPA caused an 19.8% increase of abnormalities compared to high doses of styrene (Tukey's post-hoc: $P=0.01$). There was no significant effect of

gamete exposure on development at the pluteus stage (ANOVA: $F=0.082$, $P=0.92$) and there was no effect of the interaction between chemical treatment and gamete exposure (ANOVA: $F=0.856$, $P=0.59$).

DISCUSSION

Our study demonstrated that BPA and styrene can negatively affect the long term development of *S. purpuratus*. Despite these chemicals causing no clearly observable effect on fertilization and an earlier onset of cleavage, the long-term effects of BPA and styrene appear to be detrimental to development of marine invertebrates.

Our data indicate that BPA or styrene do not affect fertilization of sea urchin embryos. This finding is in contradiction to previous studies that reported a reduced or delayed fertilization after BPA exposure in humans (Ehrlich et al., 2012), mice (Vom Saal et al., 1998), and sea urchins (Ozlem and Hatice, 2008). However, our experimental design was not optimal for sensitively examining fertilization impacts of BPA and styrene, and further studies are needed to understand the impact of these chemicals on fertilization.

In the majority of sea urchin fertilization studies, sperm is mixed with eggs within 10 minutes to optimize sperm activity (Christen et al., 1983). We exposed our sperm to chemical treatments for 30 minutes, thus there may have been a compounding effect of decreased sperm activity over time. If this was the case, we would have seen a significant effect of exposing sperm compared to exposing eggs, wherein the sperm exposures would lead to decreased fertilization rates. However, because there was not significant evidence that gamete exposure effected fertilization (ANOVA; $F=2.560$, $P=0.1005$), and because all of our experiments reached >80% fertilization within ten minutes, this methodology does not appear to have affected our results considerably.

Furthermore, because our experiments were designed to examine exposure over longer periods of time, we utilized higher concentrations of sperm (1:50,000-1:100,00,

sperm:FSW) to ensure fertilization success. When utilizing these sperm dilutions, it may be possible that any effect of the chemicals was diminished due to increased sperm concentrations. Further studies are needed to examine differences in fertilization and fertilization delays when sea urchins are exposed to BPA and styrene, possibly by utilizing different sperm dilutions and testing the fertilization response at different concentrations of chemicals (see Belton et al., 2001).

While documenting fertilization data, artificial activation of eggs may have confounded our fertilization results, however this result seems unlikely. More specifically, there is no way to immediately visually distinguish between an egg that has been fertilized (naturally activated) compared to an egg which has been artificially activated, because activated eggs are morphologically identical to a fertilized egg (envelope raised), but remain haploid and unfertilized. Artificial activation can be induced by numerous chemicals including ionophores, which facilitate transport of cations across membranes. One common example is A23187, a calcium ionophore that has been demonstrated to activate sea urchin eggs (Steinheart and Epel, 1974). This ionophore facilitates the release of calcium stores within sea urchin eggs causing the rise of the fertilization envelope. This ionophore was absent from our study, but BPA maintains a negative charge at the pH of seawater (Joseph et al., 2011), and may be facilitating a similar process. However, eggs were checked for activation prior to the addition of sperm, so this hypothesis is unlikely.

Furthermore, had the eggs been artificially activated in any gamete exposure, we would likely have seen a decrease in successful development at the next time point. For example, if there was an increase of activated eggs during fertilization counts, those eggs

would be stalled in development and would cease to continue to cleavage. Because all of our experiments reached >90% cleavage, this suggests that artificial activation was unlikely to have caused the results we witnessed in fertilization, and instead suggest that BPA and styrene may not effect fertilization at these concentrations.

In the vast majority of research on cleavage rates in organisms, any perturbation to the embryos results in a delay in cleavage rates (an increased time to first cleavage) (Adams and Shick, 1996; Kobayashi and Okamura, 2002). In our case, we saw the opposite trend: a faster time to first cleavage compared to the untreated embryos, and a negative cleavage delay (hereby referred to as ‘cleavage facilitation’). While some studies have demonstrated no significant difference in cleavage rates in humans when exposed to BPA (Ehrlich et al., 2012), to our knowledge no studies have observed this facilitation phenomenon with BPA or styrene on any organism.

Though not as potent as other endocrine disrupting compounds (EDCs), both BPA and styrene have been linked to hormone interference (Colborn et al., 1993; Kiyomoto et al., 2005; Rubin 2011; Sugni et al., 2009), including a decrease in time to human puberty (Howdeshell et al., 1999). However, decreased time to earlier developmental hallmarks have not been well documented in human systems.

Both chemicals display carcinogenic properties (Conti et al., 1988; Keri et al., 2007), which can cause increased cell proliferation. However, this proliferation leading to tumor development has only been demonstrated in embryonic cell lines from parents carrying cancer strains (Isoda et al., 2009; Murray et al., 2007), never in embryos exposed de novo to carcinogens. Therefore, BPA may be causing faster cleavage by similar mechanisms; however more research testing this possibility is necessary.

One possible mechanism for this ‘cleavage facilitation’ is that BPA and styrene may be precociously activating key proteins that control cell division, including the mitosis promoting factor (MPF). The MPF is composed of a complex of Cyclin B and phosphorylated CDC2, and controls when a cell enters the division phase. Upstream of the MPF is a cell cycle checkpoint, which effectively can stall mitosis by blocking the MPF if DNA damage is detected (Liu et al., 2000). This G2/DNA damage checkpoint is composed of CDC25, which dephosphorylates CDC2 to activate the MPF, and a number of checkpoint proteins which control the activation of CDC25.

Any number of proteins near this cell cycle checkpoint may cause early activation of the MPF, including Cyclin B, CDC2, or CDC25. It can be postulated that these proteins may be susceptible to influence by either BPA or styrene. For example, the toxins might possibly be causing precocious translation of Cyclin B, dephosphorylation of CDC2, or early activation of CDC25, all of which could directly activate the MPF and propel the embryos into cleavage.

Alternatively, these chemicals may inhibit checkpoint proteins, which normally function to arrest the cell in the presence of DNA damage. For example, CHK1 kinase (CHK1) is a protein that detects DNA damage and will prevent the cell from entering mitosis by inhibiting activity of CDC25 at this G2/DNA damage checkpoint (Liu et al., 2000; Zachos et al., 2007; Zhou and Elledge, 2000). There is evidence that CHK1 can become activated following environmental stress, such as UV radiation, and will cause the cell to become arrested. Active CHK1 delays first cleavage while the damaged DNA is repaired (Goschke et al., 2005; Liu et al., 2000). Based on our results suggesting a ‘cleavage facilitation,’ BPA and styrene may be inhibiting CHK1 (or upstream proteins

controlling) activity, thereby allowing the embryo to begin mitosis without undergoing the proper checkpoints for DNA damage.

If any of the proteins postulated above are affected by BPA or styrene, it might help explain why we saw faster cleavage in our chemically treated embryos. If these embryos are then ill-prepared for mitosis, will not have had a chance to repair DNA damage, and they may not be able to develop normally, which would lend an explanation as to why the developmental abnormalities we saw were extreme. Because our study is the first to suggest this ‘cleavage facilitation,’ further investigation of the activity of cell cycle proteins is warranted.

Throughout the experiments, we observed consistent decreases in normal development of embryos exposed to high doses of both BPA and styrene. It is logical that we observed increased abnormalities the longer embryos were exposed to the chemicals, and that there was a dose dependent increase in abnormalities with increasing concentrations of the chemicals because there have been multiple studies which demonstrated these same findings (Roepke et al., 2005; Ozlem and Hatice, 2008). Based on our proposed hypothesis of cell cycle protein alteration, the embryos would lack the required time to repair DNA damage caused by BPA and styrene if they have bypassed the necessary checkpoints. If these embryos are entering mitosis with damaged (or unrepaired) DNA, it stands to reason they will not be able to develop normally.

Common morphological abnormalities historically seen in *S. purpuratus* exposed to environmental stress include “packed” blastula (Fig 5), exogastrulation (Fig 7), and malformation of spicules (Fig 9; Adams and Shick, 2001). We were able to identify these same abnormalities in larvae exposed to BPA and styrene at each larval stage.

After 24 hours, sea urchin embryos are in the blastula stage, and begin to enter the maternal-zygotic transition (MZT; Schier et al., 2007). Here, embryos transition from utilizing the maternal cytoplasmic RNA bestowed in the egg and begin transcribing the embryonic genome. “Packed” blastula refer to the embryos in which cellular debris fill the hallow blastocoel. While these abnormal blastula were present after exposure to BPA and styrene, the blastula stage was the most resilient to decreases in normal development in our study, likely due to the maternal investment inherent in the eggs.

During the gastrula stage, embryos are undergoing cell migration and changes in cell shape in the form of invagination to form the larval gut, or archenteron. Beta-catenin (β -catenin) expression localized in the vegetal region of the embryo signals the vegetal plate to invaginate (Logan et al., 1999). If the β -catenin signaling pathway is disrupted so that β -catenin expression is uniform throughout the embryo, exogastrulation will occur (Logan et al., 1999). During exogastrulation, the mesenchyme cells will evaginate, and form an external gut structure called an exogastrula. This anomaly is known to be caused in sea urchins by other environmental toxicants such as excessive lithium chloride and polyaromatic hydrocarbons (Logan et al., 1999; Pillai et al., 2003). BPA and styrene exposure led to a significant increase in the number of exogastrula compared to controls, indicating that the toxins may somehow be interfering in the B-catenin pathway.

Normal development in the pluteus larval was most effected after exposure to BPA and styrene. Prior to and during this pluteus larval stage, embryos are beginning to show symmetry, form coelomic pouches, and build spicules. Abnormalities seen in this study included elongated larvae, irregular spicule formation, and delayed development (see Figure 9). These abnormalities are caused when the primary mesenchyme cells are

damaged, which ultimately disrupts spicule formation. These abnormal embryos will then not be able to form normal spicules required for orientation during this pluteus larvae stage and will eventually die (Pennington and Strathmann, 1990; Adams and Shick, 2001).

It is also common for embryos to become delayed in later development (Fig 9), however the current study did not categorize delayed embryos any differently from abnormally developed. Although previous work has validated this method of categorizing retarded (delayed) and malformed embryos as abnormal (Ozlem and Hatice, 2008), additional analysis could be performed to further investigate these morphological differences among chemical treatments.

It is important to note that due to our study design, we cannot make conclusions about stage specific toxicity as they relate to BPA and styrene. Though the blastula stage demonstrated resiliency and the pluteus larval stage showed the greatest decrease in normal development, we cannot separate the temporal aspect of exposure. Whether these effects were the consequence of exposure duration or of embryonic resiliency at each stage is a question for further study.

It is interesting that there is minimal evidence to examine the difference in sensitivity of gamete in any of our developmental time points. Previous studies have demonstrated increased sensitivity of sperm to BPA compared to eggs (Ozlem and Hatice, 2008), but increased sensitivity of eggs to styrene compared to sperm (Pagano et al., 1978). Additional studies utilizing differential sperm dilutions may be more useful to determine the differences between gamete exposures to BPA and styrene.

There was no effect of DMSO compared to FSW at any time point, which is consistent with previous studies (Ozlem and Hatice, 2008); however, these studies used seawater as a control, and did not account for the effect of DMSO on the chemically treated embryos. Though FSW as a control may have yielded more significant results, each chemical was dissolved in DMSO when stock solutions were prepared. Because of this, we chose to compare our chemically treated data to our DMSO control instead of the FSW control. By sacrificing some statistical power, we hoped to examine more accurate impacts of BPA and styrene and control for the impact of DMSO, even if minimal.

To our knowledge, this is the first study to compare the relative toxicity of BPA and styrene on sea urchin embryo toxicity. Our experimental design allowed us to perform this analysis, and though the chemicals were similar in how they affected the embryos, we can see evidence that BPA causes significantly more developmental damage compared to similar doses of styrene by 72 hours of exposure (Fig 7). These results provide evidence for a novel conclusion that BPA may cause more damage to developing embryos when compared to styrene.

Low dose effects, where lower doses of chemicals cause a greater effect compared to higher doses, were not demonstrated in the current study. During cleavage, lower doses of BPA and styrene were slightly faster to reach 50% cleavage compared to higher doses (Fig 3), however this result was not significant. Although these effects have not been demonstrated for styrene, low dose effects of BPA have been repeatedly demonstrated in previous literature (Vom Saal et al., 1998; Vandenberg et al., 2013). These studies utilized relevant low doses (2 $\mu\text{g/L}$) and high doses (20 $\mu\text{g/L}$), however,

most low dose effects have been demonstrated on maternal effects in mammals orally dosed with BPA (Vom Saal et al., 1998).

One hypothesized mechanism for these demonstrated effects is that high doses of BPA may activate negative feedback loops which detoxify the chemical or shut down the physiology of an organism (Myers et al., 2009). It is possible our study did not examine high enough doses of BPA or styrene to detect these effects; however, our results do agree with previous studies which utilized similar biologically relevant concentrations (Roepke et al., 2005; Ozlem and Hatice, 2008). In addition, our study examined external exposure to these chemicals, whereas previous work has examined oral exposures (Vom Saal et al., 1998). Low dose effects are especially important to examine for regulatory agencies in order to determine safe levels of chemicals in the environment, as current “safe levels” of both BPA and styrene are set at disturbingly high levels.

Sea urchins, specifically *S. purpuratus*, are a common intertidal invertebrate on the central coast of California, which play a foundational role in kelp forest ecosystems as both grazers and prey (Pearse 2006). In addition, adult benthic invertebrates such as sea urchins are likely to be exposed to plastic byproducts for long durations, which might affect reproductive success, accumulation of these byproducts in the organism, or adult survival.

As broadcast spawners, *S. purpuratus* are exposed to chemicals in the water column from gamete through juvenile metamorphosis (Russell 1987). As our results suggest, normal development of these sea urchins is significantly impacted by BPA and styrene when gametes and embryos are exposed at environmentally relevant

concentrations. If embryos cannot develop normally in current conditions, it would not be unexpected to begin seeing decreased rates of recruitment of sea urchin juveniles.

If recruitment rates of *S. purpuratus* were to decrease, there would be a devastating declines in populations that might result in overwhelming trophic impacts. As grazers, sea urchins maintain the kelp and algae growth in their ecosystems. By removing that grazing pressure, algal biomass would increase, which could cause eutrophication (Frieder et al., 2012). Alternatively, decreased numbers of sea urchins as prey biomass might affect the nutritional status of organisms at higher level trophic orders such as sea otters (Pearse, 2006).

Due to their herbivorous nature and position in food webs, accumulation of micro-pollutants in the water column becomes incredibly important for economically relevant fisheries of sea urchins (Pearse, 2006). Because sea urchins are harvested for their gonads, bioaccumulation of plastics might fill their gut and slow consumption rates and thus gonadal quality, therefore, BPA and styrene may be of utmost concern to the fishery of sea urchins. Although *S. purpuratus* forms a smaller portion of the sea urchin fishery compared to the closely related red sea urchin (*S. fransiscanus*), the total sea urchin fishery in California amounts to \$7.1 million (Sweetnam et al., 2005). In addition, there is evidence that BPA can accumulate in the gonads of sea urchins (Schweitzer et al., 2000), however additional studies are necessary to determine the link between early developmental exposure of these chemicals and adult gonad concentrations of BPA and styrene.

Our work adds to the growing body of literature that suggests there is a need to increase governmental regulations on these chemicals (or the release of the chemicals

into aquatic environments). Though BPA has been banned in some products in the U.S. (such as baby bottles), environmental levels of BPA and styrene are still expected to increase (Moore, 2008); therefore, this area of research deserves more attention, with a specific focus to understand how marine organisms will be impacted by environmental toxins. Continued research into these chemicals impacts, including lower concentrations, other species of invertebrates and vertebrates, and different life stage sensitivity, will add to our knowledge of BPA and styrene toxicity. Additionally, by including more factors such as increasing temperature, UV radiation, and ocean acidification, we can more specifically understand how these chemicals are affecting marine organisms.

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APPENDICES

Appendix A: Tables

Table 1. *S. purpuratus* gamete exposures detailing if gametes did (+) or did not (-) receive chemical treatments and the sample size (n) of spawning replicated used for each exposure level. Gametes for each exposure group were always exposed to eight chemical treatments.

Gamete Exposures:	Eggs Treated?	Sperm Treated?	n
Eggs	+	-	5
Sperm	-	+	3
Both	+	+	3

Appendix B: Figures

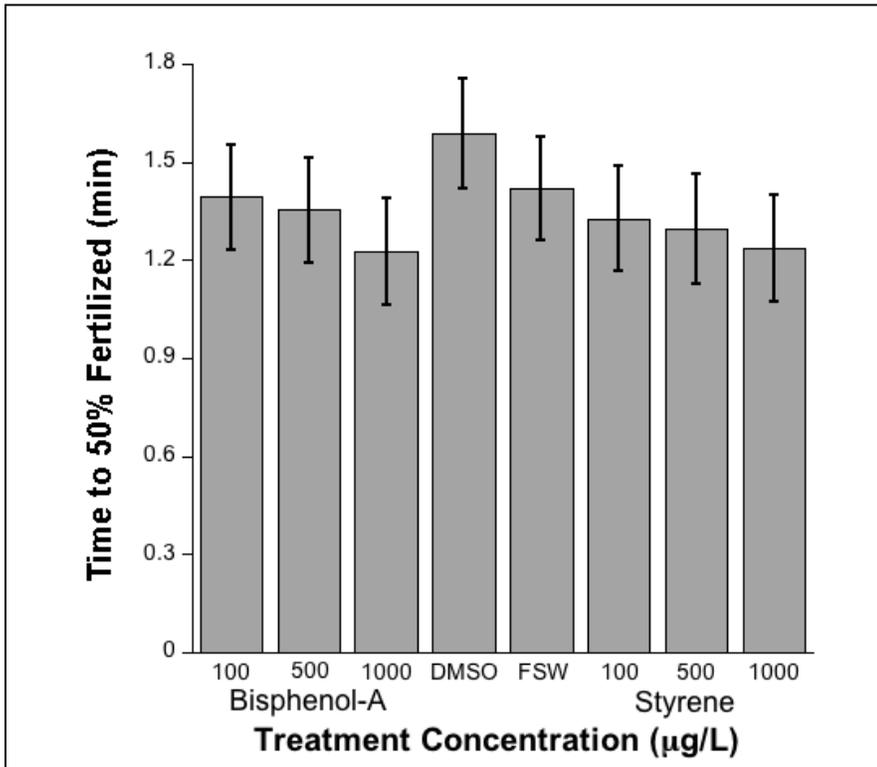


Figure 1. Mean (\pm SE) time (min) to 50% fertilization of *S. purpuratus* embryos after treatment with bisphenol-A and styrene. Dimethylsulfoxide (DMSO) and filtered seawater (FSW) were used as controls. Data shown include all gamete exposures combined (n=11).

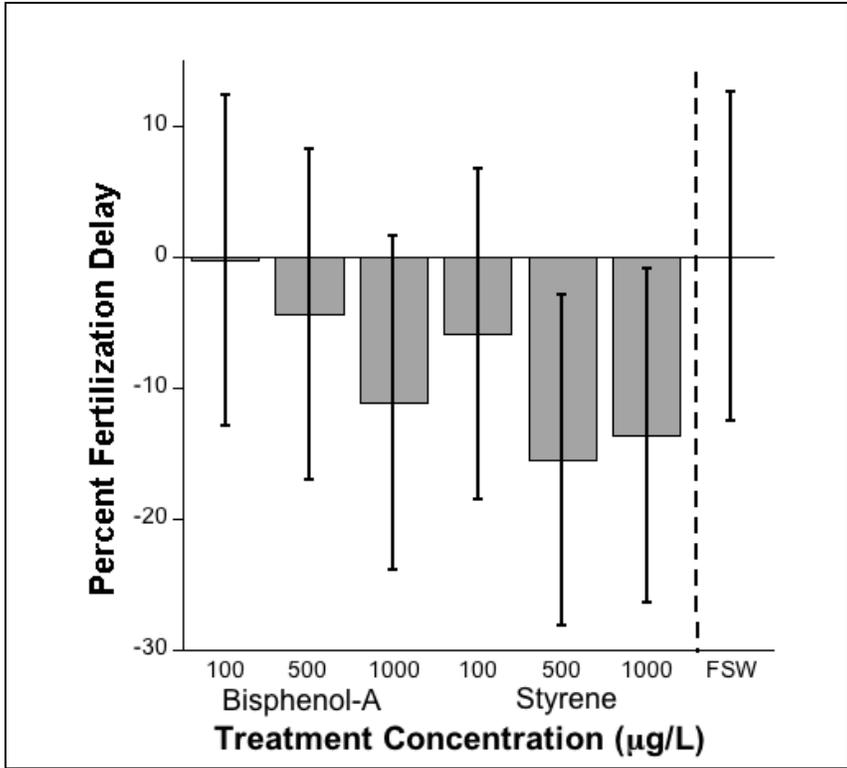


Figure 2. Mean (\pm SE) percent fertilization delay relative to DMSO (control) exposed *S. purpuratus* embryos after treatment with bisphenol-A, styrene, and filtered seawater (FSW). FSW data are displayed for transparency, but were excluded from statistical analysis. Data shown include all gamete exposures combined (n=11).

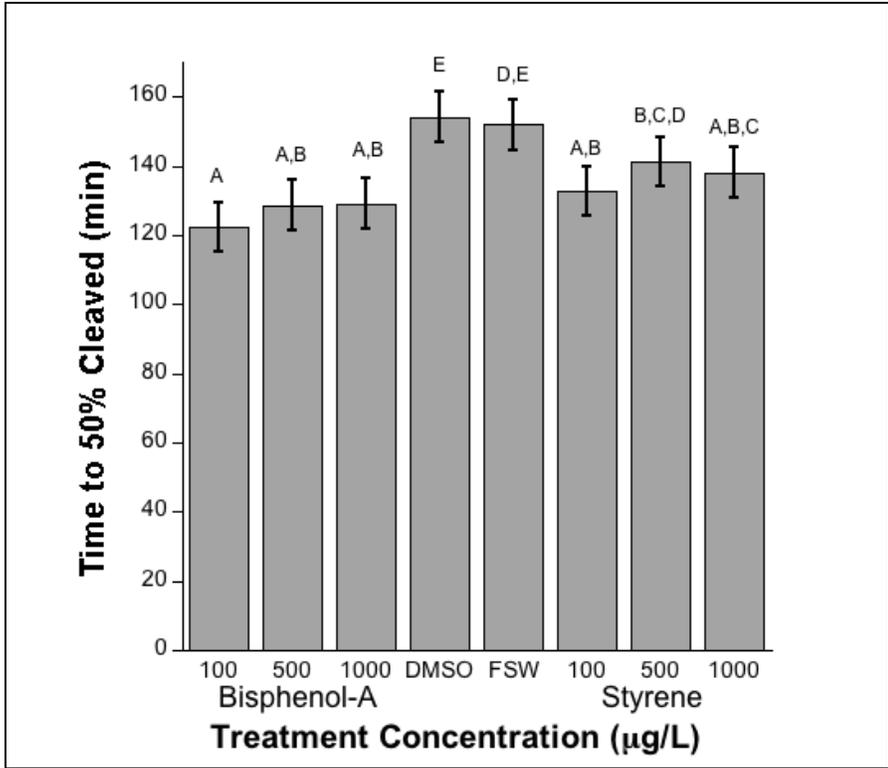


Figure 3. Mean (\pm SE) time to 50% cleaved *S. purpuratus* embryos after treatment with bisphenol-A and styrene at three concentrations, dimethylsulfoxide (DMSO), and filtered seawater (FSW). Means not connected by same letter are significantly different (Tukey HSD test, $\alpha=0.05$). Data shown include all gamete exposures combined (n=11).

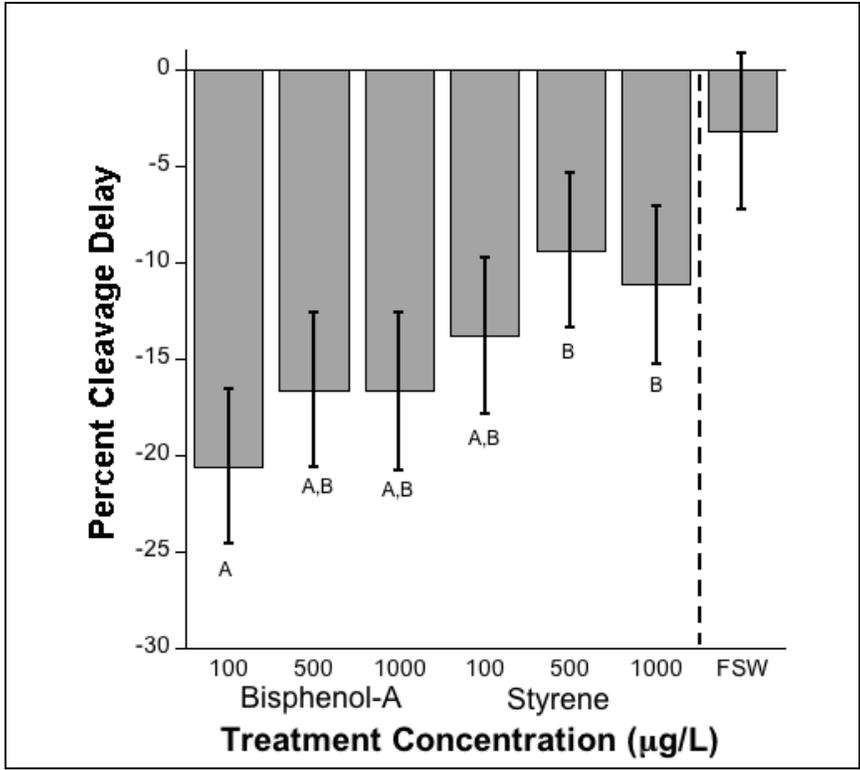


Figure 4. Mean (\pm SE) percent cleavage delay relative to DMSO (control) exposed *S. purpuratus* embryos after treatment with bisphenol-A and styrene at three concentrations. Means not connected by same letter are significantly different ($P < 0.05$). Filtered seawater (FSW) data are shown but were excluded from statistical analysis. Data shown include all gamete exposures combined ($n=11$).

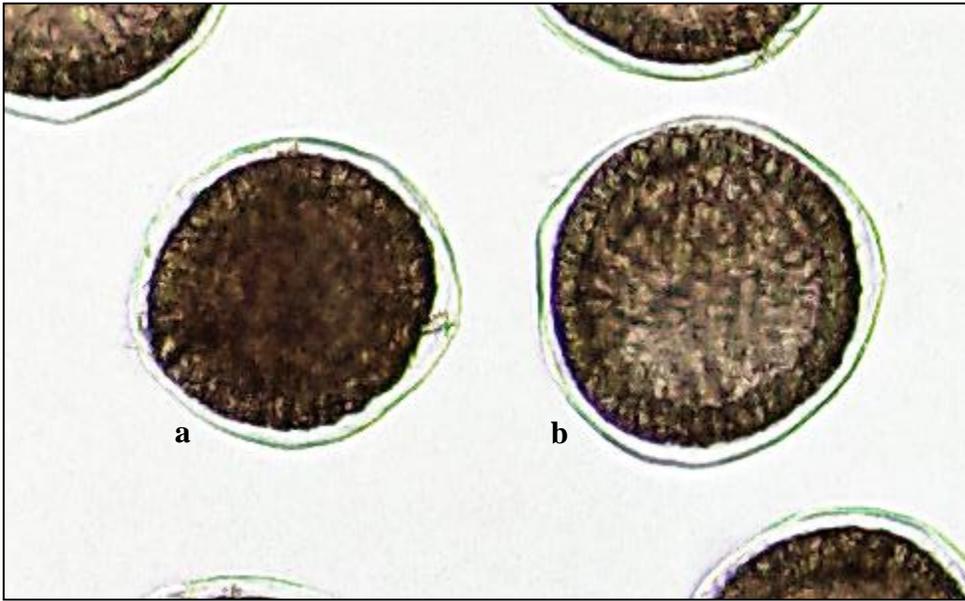


Figure 5. Examples of abnormal packed (a) and normal (b) blastula larvae after exposure to styrene for 24 hours.

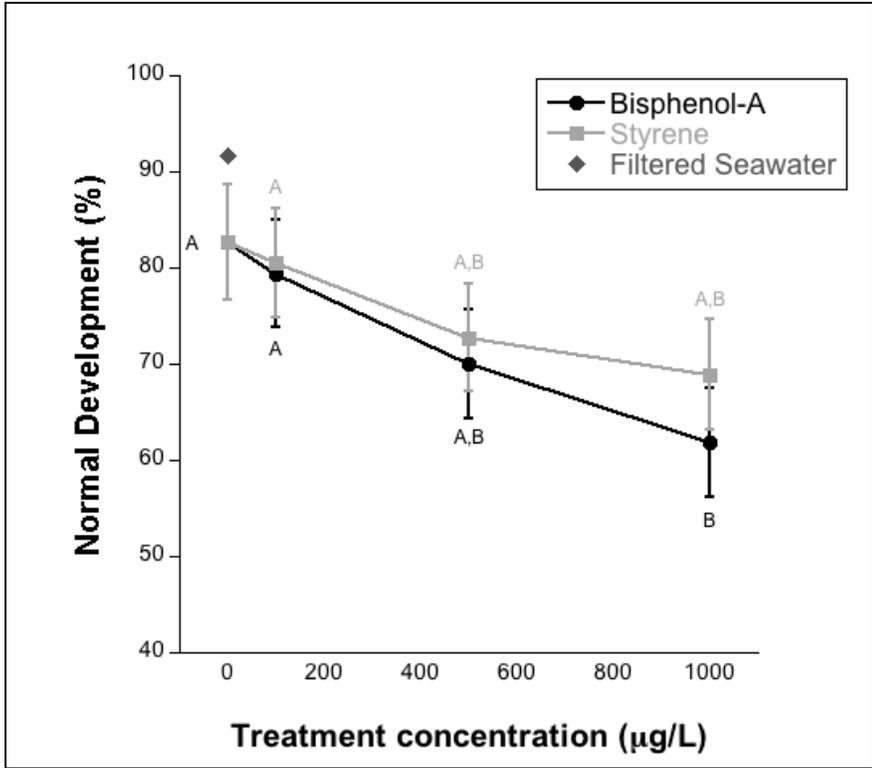


Figure 6. Mean (\pm SE) percent normal development of *S. purpuratus* blastula (~24 hours) by treatment concentration when gamete exposures are combined. Means not connected by same letter are significantly different ($P < 0.05$). Filtered seawater (FSW) included for transparency, but not included in analysis ($n = 11$).



Figure 7. Example of abnormal exogastrula (a) and normal gastrula (b) after exposure to styrene for 48 hours.

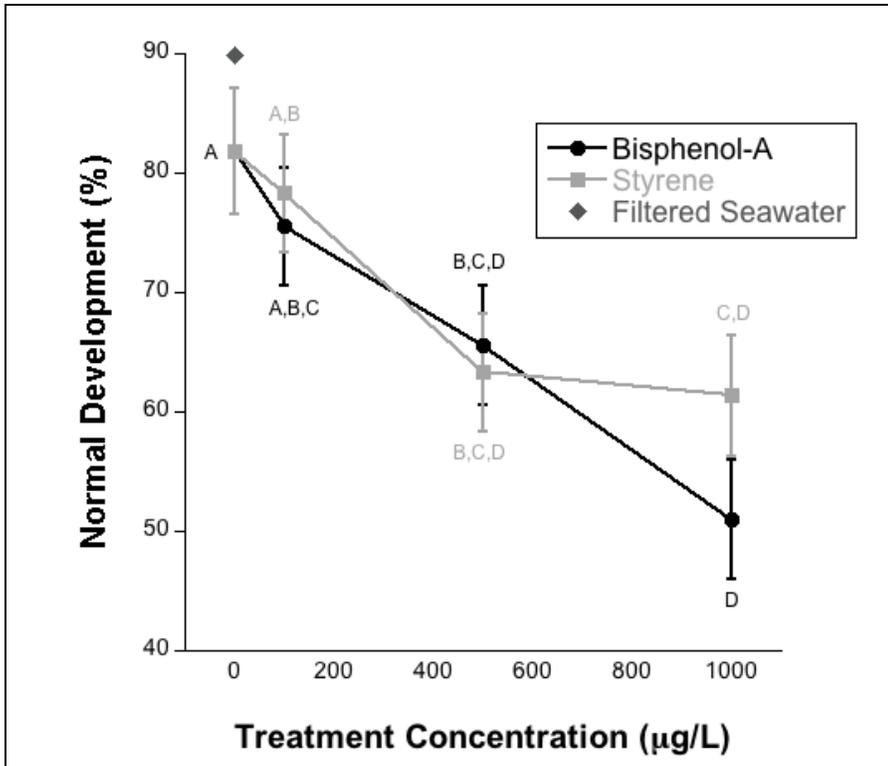


Figure 8. Mean (\pm SE) percent normal development of *S. purpuratus* gastrula (~48 hours) by treatment concentration when gamete exposures are combined. Means not connected by same letter are significantly different ($P < 0.05$). Filtered seawater (FSW) included for transparency, but not included in analysis ($n=11$).



Figure 9. Examples of abnormal spicules (a), delayed development (b), and normal pluteus larvae (c) after exposure to BPA for 72 hours.

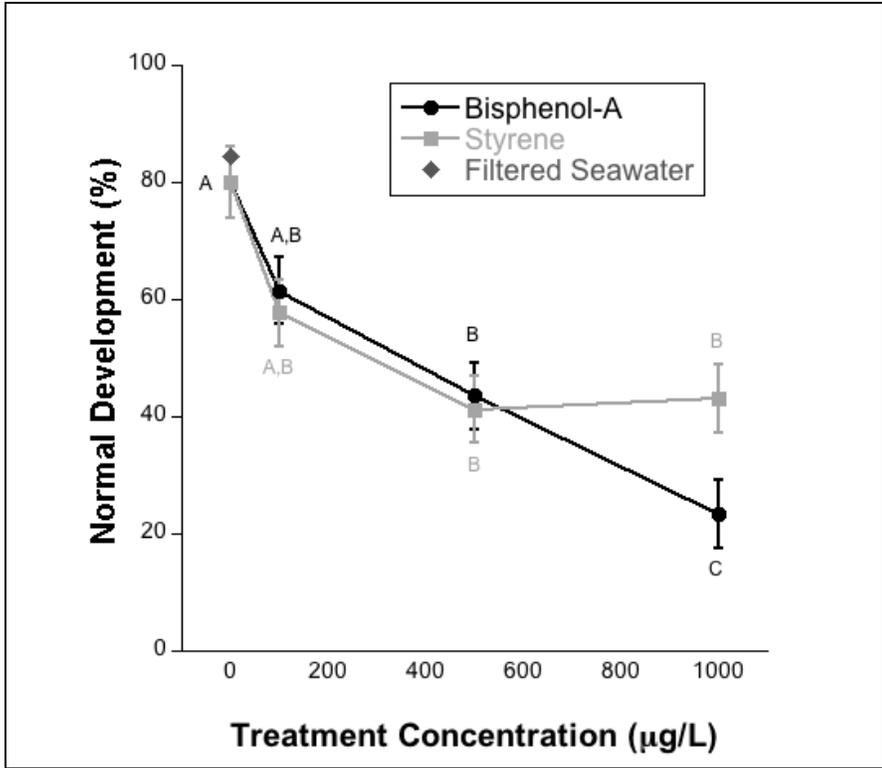


Figure 10. Mean (\pm SE) percent normal development of *S. purpuratus* pluteus (~72 hours) by treatment concentration when gamete exposures are combined. Means not connected by same letter are significantly different ($P < 0.05$). Filtered seawater (FSW) included for transparency, but not included in analysis ($n = 11$).

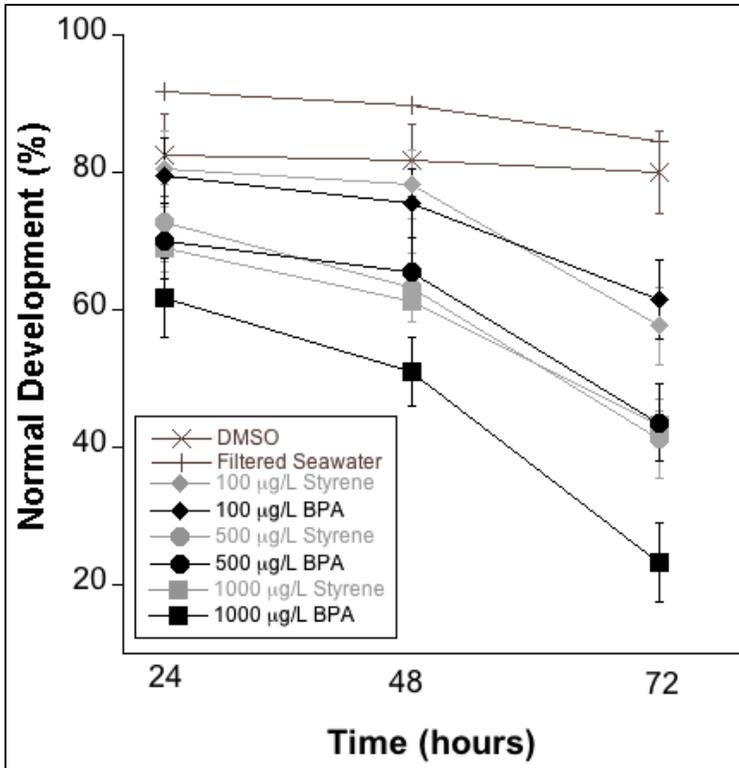


Figure 11. Mean (\pm SE) percent normal development of *S. purpuratus* pluteus by treatment concentration over time (hours). Filtered seawater (FSW) included for transparency, but not included in analysis (n=11).