

ZINC SUNSCREENS AFFECT DEVELOPMENT OF *STRONGYLOCENTROTUS*
PURPURATUS EMBRYOS

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 Science

by

Brittany Cunningham

June 2018

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COMMITTEE MEMBERSHIP

TITLE: Zinc Sunscreens Affect Development of *Strongylocentrotus purpuratus* Embryos

AUTHOR: Brittany Cunningham

DATE SUBMITTED: June 2018

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

COMMITTEE MEMBER: Lars Tomanek, Ph.D.
Professor of Biological Sciences

COMMITTEE MEMBER: Corinne Lehr, Ph.D.
Professor of Chemistry

ABSTRACT

Zinc Sunscreens Affect Development of *Strongylocentrotus purpuratus* Embryos

Brittany Cunningham

The growing popularity of physical sunscreens will also lead to an increased release of the ingredients from zinc oxide (ZnO) sunscreens into marine environments. Though zinc (Zn) is a necessary micronutrient in the ocean, greater than natural Zn concentrations are being released into marine environments by use of sunscreens. The extent of the consequences of the addition of Zn to the ocean are not fully understood. We investigated effects of materials released by zinc oxide (ZnO) sunscreens on the development of California purple sea urchin, *Strongylocentrotus purpuratus*. Embryos developed in various concentrations of Zn, the sources of which included zinc-containing compounds: ZnO and ZnSO₄; and ZnO sunscreens: All Good, Badger, and Raw Elements. ZnO sunscreens were slightly more toxic than ZnO and ZnSO₄, suggesting that the sunscreens may release additional unknown materials that are detrimental to sea urchin embryo development. All concentrations of Zn exposure resulted in significant malformations (skeletal abnormality, stage arrest, axis determination disruption), which were identified using light and fluorescent confocal microscopy. Developing embryos internalize Zn²⁺ in proportion to the concentration of Zn in their environment. Additionally, both ZnO sunscreens and ZnO and ZnSO₄ at 1ppm Zn, significantly increased calcein-AM (CAM) accumulation, indicating decreased multidrug resistant (MDR) transporter activity. This is the first research that we know of to show that ZnO sunscreens release high concentrations of Zn that are internalized by and have detrimental effects on aquatic organisms.

ACKNOWLEDGMENTS

A special thank you to Dr. Gary Cherr and Dr. Cristina Torres-Duarte, for the use of their lab space, supplies, and organisms. Without their incredibly generous support and guidance this thesis would not have been possible. A huge thank you to my fellow biology graduate students Yareli Alvarez and Crystal Castillo, who continuously provided a network of support and encouragement. I could not have asked for two better people to go through this masters program with. Additionally, I am so grateful for my family for their never-ending love and support. I would also like to thank Craig Stubler in the Natural Resources Management and Environmental Sciences department who did the AAS analysis of my sunscreen solutions and Dr. Andrew Schaffner in the Statistics department who helped me run the appropriate statistical analysis for my data. I am grateful to both of these men who were instrumental parts of the data collection and data analysis, respectively. Thank you to my committee members Dr. Lars Tomanek and Dr. Corinne Lehr; and especially my committee chair and advisor Dr. Nikki Adams. I am very appreciative that she generously gave me both the freedom to explore novel research areas and the assistance and guidance to be successful in these endeavors. Finally, thank you to California Polytechnic State University's College Based Fees and the Dr. Earl H. Myers & Ethel M. Myers Oceanographic & Marine Biology Trust for funding this project.

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CHAPTER 1. INTRODUCTION TO THESIS

1.1 Sunscreen types

Topical sun protection products have been in use for almost 100 years. The first sunscreen to feature ingredients that offered protection from both ultraviolet A (UVA, 320-400 nm) and ultraviolet B (UVB, 280-320nm) rays was developed in the 1920s (Urbach, 2001). Chemical sunscreens contain organic UV filters that absorb sunlight, and physical sunscreens, contain inorganic UV filters, titanium dioxide (TiO₂) or zinc oxide (ZnO), that reflect and scatter sunlight. While traditional chemical UV filters are absorbed into the skin, physical UV filters sit on top of the skin (Stiefel and Schwack, 2015), which increases the potential that physical sunscreens will be released into the environment during use. Little research exists on the amount of sunscreen transferred from people to the environment; however, one study on a chemical sunscreen found that over a twenty-minute submersion period about 25% of applied sunscreen is released into the water (Danovaro et al., 2008). Though ZnO is the most common UV filter in physical sunscreens in the US (Stiefel and Schwack, 2015), the quantity of zinc from sunscreen released during submersion has not been investigated.

The “reef safe” labels seen on many ZnO sunscreen brands are not regulated by the FDA. These sunscreens are labeled as “reef safe” or “ocean safe” solely because they do not contain oxybenzone or other chemical UV filters shown to harm reef organisms (Danovaro et al., 2008; Giokas et al., 2007). The FDA's latest ruling on labeling and effectiveness testing addressed issues related to water resistance claims, application

directions, and maximum allowable SPF labels. However, they failed to address the environmental or ocean safety of active sunscreen ingredients (Wang and Lim, 2011).

1.2 Nano verses bulk zinc oxide

Physical, ZnO sunscreens are sold with zinc in both nano and non-nano (bulk) forms. Nano zinc oxide (nZnO) offers transparent UV protection that is aesthetically appealing to many sunscreen users (Moezzi et al., 2012). Such nanoparticles have been used in sunscreen since the 1990s, but it was not until lately that health and environmentally-conscious consumers have begun to question the safety of nanotechnologies (Moezzi et al., 2012). Environmental groups have warned against the use of nanomaterials in personal care products and sunscreens based on studies that showed that use of these products could result in an uptake of zinc-nanomaterials into the human body (Nohynek et al., 2007). Additionally, many studies have investigated the effects that nZnO has on marine invertebrates, demonstrating that nano-materials can have unique toxicities due to their small particle size (Fairbairn et al., 2011; Manzo et al., 2013; Miglietta et al., 2011; Wu et al., 2015).

Oral and topical toxicity studies have found that nano-metals are not more damaging to humans than non-nano metals (Burnett and Wang, 2011; Lademann et al., 2006; Moezzi et al., 2012; Nohynek et al., 2007). However, public distrust of nanotechnology has contributed to the growing popularity of bulk ZnO sunscreens. It has been estimated that approximately 70% of ZnO sunscreens on the market are produced with bulk ZnO (Newman et al., 2009). Still, no studies have investigated the effects of sunscreen-related bulk ZnO that may be discharged into the environment.

1.3 Zinc in the environment

Zinc (Zn) is a trace metal micronutrient that is essential to many ocean organisms (Morel and Price, 2003). In the ocean, Zn distribution follows the pattern of nutrient-type elements like phosphorous, nitrogen, and silicon. It shows low concentrations in surface waters and increased concentrations in deep water (Nozaki, 1997). Though described Zn concentrations in the ocean vary because of differences in terrestrial discharges, values of approximately 0.03 nM to 9 nM have been reported in seawater (Bruland, 1980; Ellwood and Van den Berg, 2000; Jickells and Knap, 1984; Lohan et al., 2002; Yeats, 1988).

1.4 Anthropogenic sources of zinc in the ocean

Release of heavy-metal Zn from natural sources is exceeded by introduction from anthropogenic sources. Though currently domestic wastewater is one of the greatest sources of anthropogenic heavy-metal Zn introduction into aquatic environments, 21,000 to 58,000 metric tons per year (Roney et al., 2005), the impacts of more direct sources of this pollution are growing in importance. The majority of Zn^{2+} in wastewater comes from industrial processes including the production of rubber, paint, brass, bronze, and other alloys (Roney et al., 2005). Another way that Zn^{2+} gets into wastewater and the environment is through the use of personal care products (PCPs) (Díaz-Cruz and Barceló, 2009; Giokas et al., 2007). Many PCPs including sunscreens, skin cream, and make-up products contain ZnO UV filters which could be washed from the body during bathing or swimming (Nohynek et al., 2010). The maximum concentration of ZnO allowed in PCP's by the United States federal government is 25% (Food and Drug Administration, 2017).

Conventional methods of heavy metal removal from wastewater: ion exchange, lime precipitation, chemical precipitation, and solvent extraction, are ineffective, expensive, or generate large quantities of toxic byproducts (Mehta and Gaur, 2005; Wilde and Benemann, 1993). However, wastewater treatments for removing heavy metals continue to improve with innovations in new absorbents and membrane filtration processes (Barakat, 2011; Gunatilake, 2015). For example, a technique called Activated Sludge Biomass can remove 86.5% of Zn^{2+} from wastewater (Ahmad et al., 2010). Another technique using microalgae to remove heavy metals, reduced the amount of zinc in wastewater by 94.1% (Chan et al., 2013). As the removal of dissolved metals from wastewater becomes more efficient, other, unregulated, sources such as direct release from ocean bathers will become responsible for larger percentages of marine ZnO pollution.

Both chemical and physical UV filters can be found in sediment as well as water samples and can have a long life span in these environments (Botta et al., 2011; Giokas et al., 2007). Giokas et al. (2007) found that the concentration of sunscreen filters in the environment varied depending on location, intensity of recreational activity, and the weather, with maximum concentrations released during warmer weather. They measured organic UV filters, from chemical sunscreens, in concentrations from 0.0082 to 0.0197 ppb in salt water (Giokas et al., 2007). It is known that the aging and break down of physical sunscreen containing TiO_2 in an aqueous environment released a significant amount of titanium into the aquatic habitat (Botta et al., 2011). Botta et al. (2011) estimated that in reef areas, 36 to 56 tons of TiO_2 was released from sunscreens. No such study could be found for ZnO release from sunscreen in aqueous environments.

1.5 Zinc toxicity

The toxicity of a sunscreen to aquatic organisms is mostly dependent on the solubility of the sunscreen ingredients in marine waters (Giokas et al., 2007). Attempts have been made to reduce the solubility and consequently the toxicity of ZnO through iron doping. Doping involves adding a metal salt precursor during the synthesis of the nanoparticles. While this was shown to reduce its cytotoxicity in cell culture (George et al., 2009), iron-doped ZnO was found to be just as toxic as non-doped ZnO to sensitive marine embryos in a study by Fairbairn et al. (2011). It is believed that the majority of the toxicity of metal oxides comes from the release of metal ions (Moezzi et al., 2012).

One component that allows embryos to deal with exposure to xenobiotics, such as heavy metals, are multidrug resistant (MDR) transporters (Cole et al., 2013; Hamdoun et al., 2004). These efflux transporters belong to the ATP-binding cassette (ABC) transporter family and play a protective role during early sea urchin embryo development (Litman et al., 2001). If they are inactivated or preoccupied with removing large quantities of a toxicant from the embryos cells, then other ions or compounds may build up to toxic levels within the embryos. Exposure to nZnO (Wu et al., 2015) and other heavy metals in both nano and non-nano forms (Achard et al., 2004; Torres-Duarte et al., 2017) has been shown to impair multidrug resistant (MDR) transporter activity in developing urchin embryos.

The ecotoxicity of both bulk and nano ZnO is attributed to their solubility into Zn^{2+} . A portion of this toxicity is thought to be caused by increased generation of reactive oxygen species (ROS), which can cause lipid peroxidation or DNA damage within an organism (Manzo et al., 2013). The exact mechanism through which increased

internalized Zn^{2+} causes ROS production is not fully known. However, studies on brain cells have found that intracellular zinc can inhibit mitochondrial processes and energy production (Dineley et al., 2003). For example, Zn^{2+} accelerates the oxidation of nicotinamide adenine dinucleotide (NADH) by lipoamide dehydrogenase, forming hydrogen peroxide (Gazaryan et al., 2002). Additionally, intracellular Zn^{2+} has been shown to cause loss of membrane potential and reduced O_2 consumption in mitochondria (Dineley et al., 2005). Many have suggested that internalized Zn^{2+} in sea urchin embryos results in the production of ROS (Fairbairn et al., 2011; Manzo et al., 2013). A recent study found that both nZnO and ZnSO_4 exposure increase the generation of ROS in embryos (Wu et al., 2015).

Overall, the sensitivity and availability of sea urchin embryos, along with the wide body of existing research on the development of their early life stages, make them a good model organism for this type of environmental and developmental research. For marine invertebrate embryos, Zn^{2+} is known to cause animalization, a disruption in the development of the animal-vegetal axis (Timourian, 1968) where endomesoderm formation is prevented (Poustka et al., 2007). This and other abnormalities have been witnessed in sea urchins developing in water contaminated with heavy metals (Kobayashi and Okamura, 2004). Because the common developmental abnormalities of sea urchin embryos exposed to Zn compounds are known (Fairbairn et al., 2011; Kobayashi and Okamura, 2004; Manzo et al., 2013; Miglietta et al., 2011; Timourian, 1968; Wu et al., 2015), ZnSO_4 and ZnO were used as positive controls during this study to assess whether the abnormalities observed in developing sea urchin embryos are likely the result of Zn or some other released sunscreen ingredient.

This study addressed the question of whether ZnO sunscreens are toxic to sea urchin embryos and if this toxicity can be attributed solely to Zn release. In addition, it examined the differential impacts related to stage of embryo at exposure to sunscreen components. Because sea urchin embryos are planktonic and can be found in the water column of shallow coastal oceans (Raff, 1987), they may be exposed to increased Zn concentrations at various stages of development. In order to capture the full range of Zn toxicity, it is important to know if the effects of Zn exposure are dependent on sea urchin embryos' developmental stage.

For this study, purple sea urchin, *Strongylocentrotus purpuratus*, embryos were grown in varying concentrations and formulations of sunscreen solutions or either ZnSO₄ or ZnO as positive controls. We evaluated the experimental embryos for morphological abnormalities and documented their relative concentrations of internalized Zn²⁺. Additionally, this study investigated the effect of these sunscreen ingredients on the activity of MDR transporters to understand developmental physiology of the embryos. I hypothesized that commercially available bulk ZnO sunscreens, release materials into marine environments that have a negative effect on the development of *S. purpuratus* embryos. Embryos exposed to sunscreen solutions were expected to develop abnormally and contain higher than average concentrations of internalized Zn²⁺. The internalized Zn²⁺ was predicted to decrease MDR transporter effectiveness.

CHAPTER 2. MANUSCRIPT

2.1 INTRODUCTION

Topical sun protection products have been in use for almost 100 years. Physical sunscreens contain inorganic UV filters, generally titanium dioxide (TiO₂) or zinc oxide (ZnO) that reflect and scatter sunlight. While traditional chemical UV filters are absorbed, physical UV filters sit on top of the skin (Stiefel and Schwack, 2015). This characteristic increases the potential that physical sunscreens will be released into the environment during use. Little research exists on the amount of sunscreen transferred from people to the environment; however, one study on a chemical sunscreen found that over a twenty-minute submersion period about 25% of applied sunscreen ingredients are released into the water (Danovaro et al., 2008).

Physical, ZnO sunscreens are sold with zinc in both nano and non-nano (bulk) forms. Recently, health and environmentally-conscious consumers have begun to question the safety of nanotechnologies. Many studies have investigated the effects that nano zinc oxide (nZnO) has on marine invertebrates, demonstrating that nano-materials have unique toxicities due to their small particle size (Fairbairn et al., 2011; Manzo et al., 2013; Miglietta et al., 2011; Wu et al., 2015). Oral and topical toxicity studies have found that nano-metals are not more damaging to humans than non-nano metals (Burnett and Wang, 2011; Lademann et al., 2006; Moezzi et al., 2012; Nohynek et al., 2007). However, public distrust of nanotechnology has contributed to the growing popularity of bulk ZnO sunscreens. It has been estimated that approximately 70% of ZnO sunscreens are produced with bulk ZnO (Newman et al., 2009). Still, no studies have investigated the effects of sunscreen-related bulk ZnO that may be discharged into the environment.

Release of heavy-metal Zn from natural sources is exceeded by introduction from anthropogenic sources. Currently, domestic wastewater is one of the greatest sources of anthropogenic heavy-metal Zn introduction into aquatic environments, 21,000 to 58,000 metric tons per year (Roney et al., 2005). Zn^{2+} can also enter wastewater and the environment through the use of personal care products (PCPs) (Díaz-Cruz and Barceló, 2009; Giokas et al., 2007). Many PCPs including sunscreens, skin cream, and make-up products contain ZnO UV filters, which could be washed from the body during bathing or swimming (Nohynek et al., 2010). The removal of dissolved metals from wastewater is becoming more efficient (Barakat, 2011; Gunatilake, 2015); for example, a technique called Activated Sludge Biomass can remove 86.5% of Zn^{2+} from wastewater (Ahmad et al., 2010). Another technique using microalgae to remove heavy metals, reduced the amount of Zn in wastewater by 94.1% (Chan et al., 2013). As these processes become more efficient, other, unregulated, zinc sources such as direct release from ocean bathers will become responsible for larger percentages of marine ZnO pollution.

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sea urchin embryos exposed to Zn compounds are known (Fairbairn et al., 2011; Kobayashi and Okamura, 2004; Manzo et al., 2013; Miglietta et al., 2011; Timourian, 1968; Wu et al., 2015). The sensitivity and availability of sea urchins, along with the wide body of existing research on development of their early life stages, make them a good model organism for this type of environmental and development research.

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2.2 METHODS

2.2.1 Spawning Sea Urchin

Gametes were collected from adult *Strongylocentrotus purpuratus* kept at the University of California Davis Bodega Marine Laboratory (Bodega Bay, CA) in flow-through seawater tanks. They were induced to spawn by intracoelomic injection of 0.55 M KCl. Following collection, eggs were washed in 0.22µm filtered seawater (FSW). Sperm were held “dry” on ice until ready for use. Both eggs and sperm were evaluated for quality and quantity before fertilization. Successful fertilization was verified by the presence of the fertilization envelope on at least 90% of the embryos.

2.2.2 Preparing Sunscreen Solutions

ZnO sunscreen solutions were created from three different sunscreens: Badger (spf 35, 22.5% ZnO), All Good (spf 33, 22.5% ZnO), and Raw Elements (spf 30, 23% ZnO). These were commercially available bulk ZnO sunscreens and were chosen because they contained high amounts of ZnO, near the maximum level (25%) allowed by the FDA (Wang and Lim, 2011). Each had bulk ZnO as its sole active ingredient and was labeled as water-resistant up to 80 minutes. Finally, each of the sunscreens’ labeling included claims of being eco-safe or coral-reef safe.

The procedure used represents maximum breakdown of sunscreen in marine environments. Each sunscreen was combined with FSW and stirred at medium speed over heat (~37°C). The solutions continued to be stirred while cooling. The solution was then filtered through Whatman Filter Paper, with a particle retention of 25µm, to remove large chunks of unincorporated sunscreen. The concentration of Zn^{2+} in these sunscreen

solutions was evaluated using Atomic Absorption Spectrometry (AAS). Knowing the concentrations of Zn^{2+} in the sunscreen solutions allows for the standardization of Zn concentrations and a comparison of embryos treated with sunscreen or positive control treatments, Zinc Sulfate (ZnSO_4) and ZnO.

2.2.3 Assessing Abnormality in ZnO Sunscreen Treated Embryos

Using the above-mentioned sunscreen solutions, we determined the toxicity of the ZnO sunscreens at uniform concentrations of Zn. For each ZnO sunscreen assay, one batch of embryos was exposed to treatments pre-cleavage stage, approximately 1 hour post-fertilization (hpf), and a second set was exposed at hatched blastula stage, approximately 24 hpf. Various volumes of the treatment solution were added to glass vials along with FSW to obtain treatment concentrations ranging from 0.01 ppm to 1 ppm Zn. Embryos developing solely in FSW were used as a control to identify normal development and rates of abnormalities in untreated embryos. Treatments of ZnSO_4 and ZnO were used as positive controls to establish a baseline for developmental morphology typical of sea urchin embryos exposed to Zn. Embryos in treatment vials were incubated at 15°C until the pluteus stage was reached (~96 hpf). The embryos were then preserved with glutaraldehyde (0.1% final concentration). One hundred embryos were assessed from each vial for normal development using an Olympus BH-2 microscope (Tokyo, Japan) at 20x magnification. Embryos were considered abnormal based on skeletal malformations, axial disruption, and stage arrest. Experiments were replicated using six females (n=6), and treatments were always conducted in triplicate for each experiment (Fig.1).

2.2.4 Measuring Internalization of Zn and Multidrug Resistant Transporter Activity

Using a modified version of the methods reported by Santillán-Urquiza et al. (2017): (1) soluble zinc accumulation (Zn^{2+}) by the embryos was determined using the fluorescent probe Newport Green (NPG-Thermo Fisher Scientific (Waltham, MA)) and (2) MDR efflux transporter effectiveness was measured with fluorescent probe Calcein-AM (CAM-Life Technologies (Carlsbad, CA)).

Embryos were cultured at 15°C in 500 mL polymethylpentene containers with 100 mL of the corresponding treatment at 200 embryos/mL. Treatment chemicals were identical to those used in the above-mentioned morphology assay; however, only two treatment concentrations (0.1 and 1 ppm Zn) were used. Embryos were exposed to treatment solutions from 1 hpf to 24 hpf. At the blastula stage (24 hpf), embryos were added to the wells of 96 well plates along with Hoechst 33342 (0.2µM final concentration), a fluorescent probe that stains DNA and shows the presence of nuclei. Wells in four columns of the plate received CAM (2.5µM final concentration) and were incubated for 60 min. Then, wells in four different columns of the plate received NPG (0.2µM final concentration). The plate was incubated for an additional 30 min and fixed with paraformaldehyde (0.1% final concentration). The fluorescence was detected using a Tecan GENios microplate reader (Maennedorf, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Embryos were taken from the plate wells and put on slides. They were viewed using a 20X water immersion lens on an Olympus BX61WI fixed stage upright microscope by using scanning laser confocal microscopy. Experiments were replicated using four females (n=4) (Fig.1).

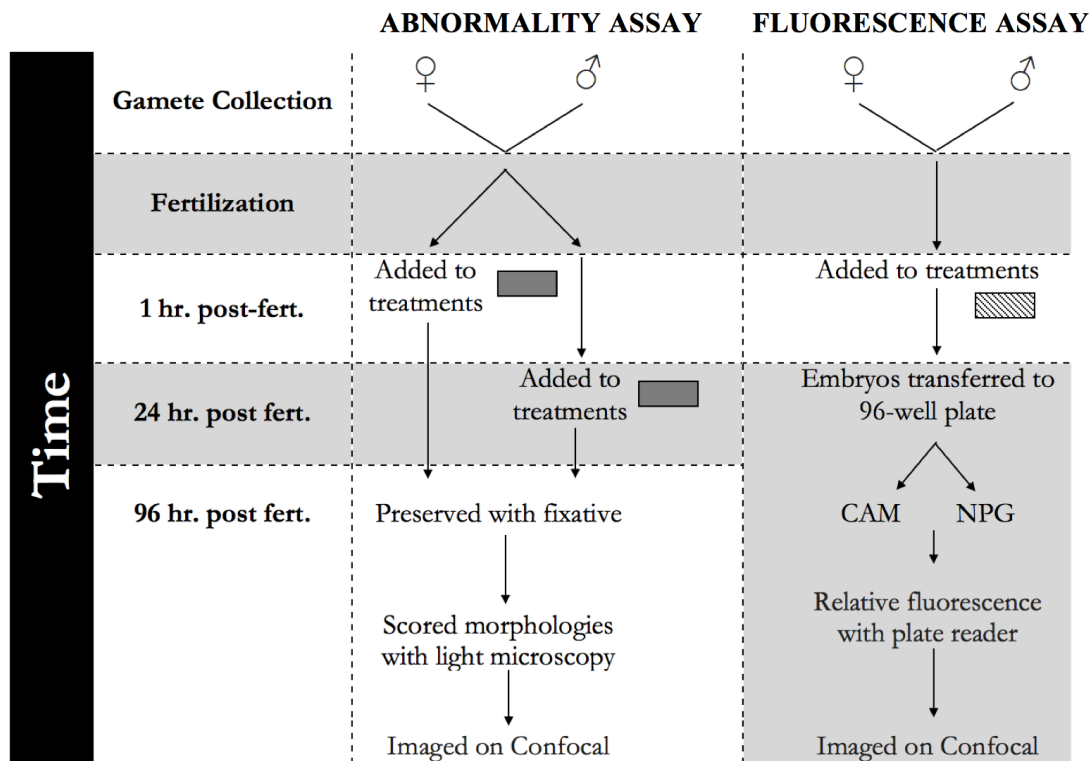


FIGURE 1: Experimental design flow of both abnormality and fluorescence assays for experiments assessing the effects of ZnO sunscreens on *S. purpuratus* embryo development. Shaded and hashed boxes indicate Zn treatments detailed in Table 1. The fluorescence assay uses the fluorescent probes Newport Green (NPG) and Calcein-AM (CAM).

TABLE 1: Experimental concentrations and types of sunscreens used in experiments. The gray shaded areas indicate the treatments to which embryos were exposed. Treatments range from 0 to 1 ppm Zn. Sources of Zn include zinc-containing compounds: ZnO and ZnSO₄; and ZnO sunscreens: All Good, Badger, and Raw Elements. Shaded and hashed boxes are treatments used for both the abnormality and fluorescence assays.

Concentration (ppm)	Zn ²⁺ Source in Treatment					
	FSW	ZnO	ZnSO ₄	All Good	Badger	Raw Elements
0		–	–	–	–	–
0.01	–					
0.05	–					
0.1	–					
0.5	–					
1	–					

2.2.5 Data Analysis

Statistical analyses were performed using JMP Pro 13 (SAS Institute Inc., North Carolina, USA). Results were statistically evaluated by the one-way analysis of variance (ANOVA) test followed by Dunnett's post-hoc test for comparisons to FSW control treatment ($\alpha = 0.05$) for all assays in this study. EC₅₀ values were calculated by fitting a logit model and using an inverse predictor. A 95% confidence interval was calculated by the predictor for each EC₅₀ value. A logit transformation was applied to the morphological abnormality data, with the count data constrained between values of 0 and 100. We used Student's t-tests instead of Tukey post-hoc because not all comparisons were necessary. Student's t-tests gave comparable significance results to Tukey HSD and allowed for clearer analysis of the contributions to significance.

Differences between Zn treatments of different concentrations from the same Zn source, and differences between Zn treatments of the same concentration from different Zn sources, were compared using Student's t tests. Because of the large number of comparisons made using Student's t-tests for analysis of the morphological abnormality data, a Bonferroni correction was used, setting statistical significance at $\alpha = 0.001$. For CAM and NPG fluorescence assays, a Bonferroni correction set the statistical significance at $\alpha = 0.003$.

2.3 RESULTS

2.3.1 *ZnO Sunscreens Affects Embryo Development*

Sea urchin embryos were exposed to zinc-containing compounds: ZnO and ZnSO₄, and ZnO sunscreens: All Good, Badger, and Raw Elements, at either a pre-cleavage, or blastula stage, until the experiment endpoint at pluteus stage. All Zn treatments were toxic to the development and resulted in high levels of abnormality of the embryos, at even the lowest Zn treatment concentration, 0.01 ppm (Fig.2). The EC₅₀ values, the concentration at which 50% of embryos showed abnormal development, for all Zn sources were at concentrations less than 0.04 ppm Zn (Fig.2). Analysis of predicted EC₅₀ values showed no significant differences in the toxicity of the Zn treatment sources or in the exposure stages (Fig.2). However, at both exposure stages, ZnO and ZnSO₄ had slightly higher EC₅₀ values, indicating potentially lower toxicity than the ZnO sunscreens (Fig.2).

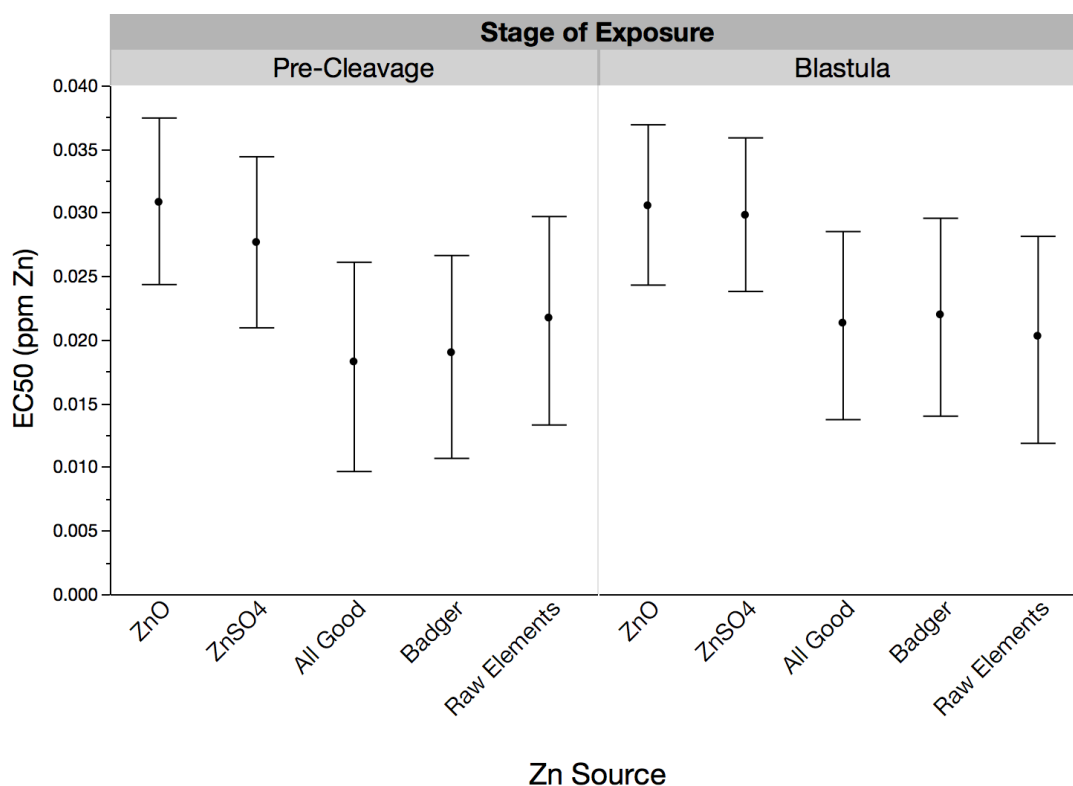


FIGURE 2: EC₅₀ values (ppm Zn) for Zn-containing compounds tested in 96 hpf *S. purpuratus* developmental bioassay. Bars represent 95% confidence intervals (CIs)(n=6).

Following growth in Zn treatments, at 96 hpf, we quantified the proportion of embryos with each type of development, ranging from normal to a variety of documented abnormalities. The percentage of sea urchin embryos that developed normally to the pluteus stage was affected by exposure to all concentrations of all Zn treatments. Abnormalities from exposure to ZnO, ZnSO₄, and ZnO sunscreens were diverse, but resembled those seen in previous studies (Fairbairn et al., 2011; Kobayashi and Okamura, 2004; Mitsunaga and Yasumasu, 1984; Timourian, 1968; Torres-Duarte et al., 2016). The developmental abnormalities observed during this study, in order of increasing severity,

were defined as follows: 1) Delayed, pluteus-like development with smaller size and/or shorter limbs (Fig.3b); 2) Abnormal Skeleton, relatively normal development accompanied by abnormal skeletal rod angle and/or asymmetric limb development (Fig.3c); 3) Larval Arrest, no development beyond normal gastrulation and spicules (Fig.3d); 4) Bell-Shaped, abnormal gut elongation and no skeletal development (Fig.3e/f); and 5) Axis Disruption, no internal gut, spicule, or skeleton development (Fig.3g/h).

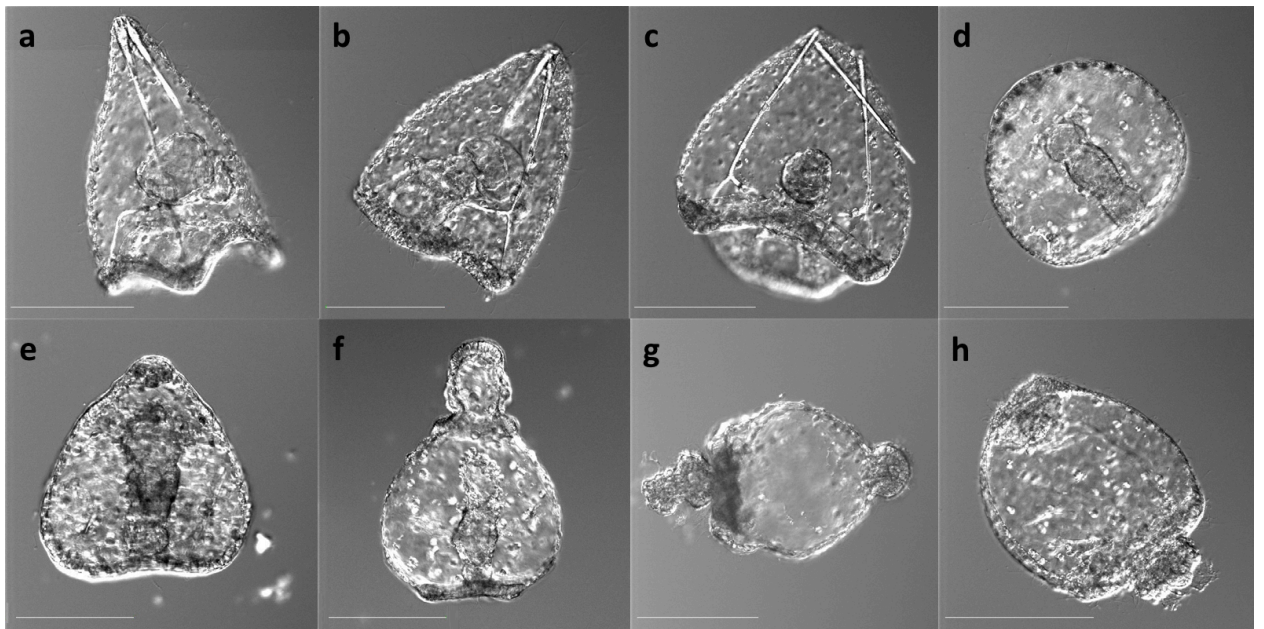


FIGURE 3: Variety of morphologies observed and quantified in *S. purpuratus*, 96 hpf after exposure to Zn. (a) normal pluteus (b) delayed (c) skeletal abnormality (d) larval arrest (e) mild bell-shape (f) severe bell-shape (g/h) axis disruption. Bar = 100 μm.

The percentage of embryos observed with each morphology varied according to both source of Zn and stage of exposure. The proportion of embryos with normal development decreased significantly with each increase in Zn concentration for both exposure stages ($p < 0.001$) (Fig.4a, 5a). No embryos developed as normal plutei when exposed to either 0.5 or 1 ppm Zn from any source (Fig.6). Accompanying the decrease in normal development, the proportion of embryos with Delayed morphology significantly increased as Zn concentration increased ($p < 0.001$), until it peaked at 0.05 ppm Zn (Fig.4b, 5b). The proportion of Delayed embryos remained at peak levels for embryos exposed to 0.1 ppm Zn from the ZnO and ZnSO₄. However, embryos exposed to 0.1 ppm Zn from ZnO sunscreens saw a decrease in the number of Delayed embryos (Fig.4b, 5b) because the majority of these embryos had more severe malformations and never reached a Delayed stage.

The percent of embryos with abnormal skeletal morphology significantly increased as the concentration of Zn increased, and peaked in treatments of 0.1 ppm ($p < 0.001$). Then, at 0.5 and 1 ppm, the proportion of pre-cleavage exposed embryos with abnormal skeletons decreased because most embryos were so severely malformed that they no longer showed skeletal development of any kind (Fig.4c). However, blastula embryos exposed to Zn from ZnO and ZnSO₄ still showed some skeletal development and had peak levels of skeletal abnormality at both 0.1 and 0.5 ppm (Fig.5c).

We observed no significant differences, in the proportions of embryos with larval arrest, between embryos exposed to the FSW control and to the lower concentrations of Zn, 0.01 and 0.05 ppm. These lower concentration Zn treatments were not toxic enough to restrict all skeletal development and result in this severe malformation (Fig.6). The

proportion of embryos displaying larval arrest peaked for ZnO sunscreens at 0.1 ppm, and for ZnO and ZnSO₄ at 0.5 ppm (Fig.4d, 5d). The concentrations of Zn at which the quantity of larval arrest embryos peaked from ZnO and ZnSO₄ exposure, were significantly greater than the ones from the ZnO sunscreen exposures ($p < 0.001$).

The majority of embryos exposed to concentrations of 0.5 and 1 ppm Zn, with the exception of blastula embryos exposed to 0.5 ppm Zn from ZnO and ZnSO₄ (Fig.5c), no longer showed skeletal development of any kind. At these high Zn concentrations, embryos exposed to Zn from ZnO sunscreens displayed a combination of Bell-Shaped and Axis Disruption morphologies (Fig.4e/f, 5e/f)(Fig.6). Pre-cleavage exposed embryos had higher proportions of Axis Disruption than blastula exposed embryos. Furthermore, embryos exposed to Zn from ZnO and ZnSO₄ had a significant increase in Bell-shaped embryos as Zn concentration increased from 0.5 to 1 ppm ($p < 0.001$); with almost all embryos Bell-shaped at 1 ppm (Fig.4f, 5f). Embryos exposed to Zn from ZnO and ZnSO₄ did not display the Axis Disruption morphology until Zn concentration was 1 ppm, and the amounts of embryos displaying Axis Disruption were significantly lower than 1 ppm ZnO sunscreen exposed embryos ($p < 0.001$) (Fig.4f, 5f).

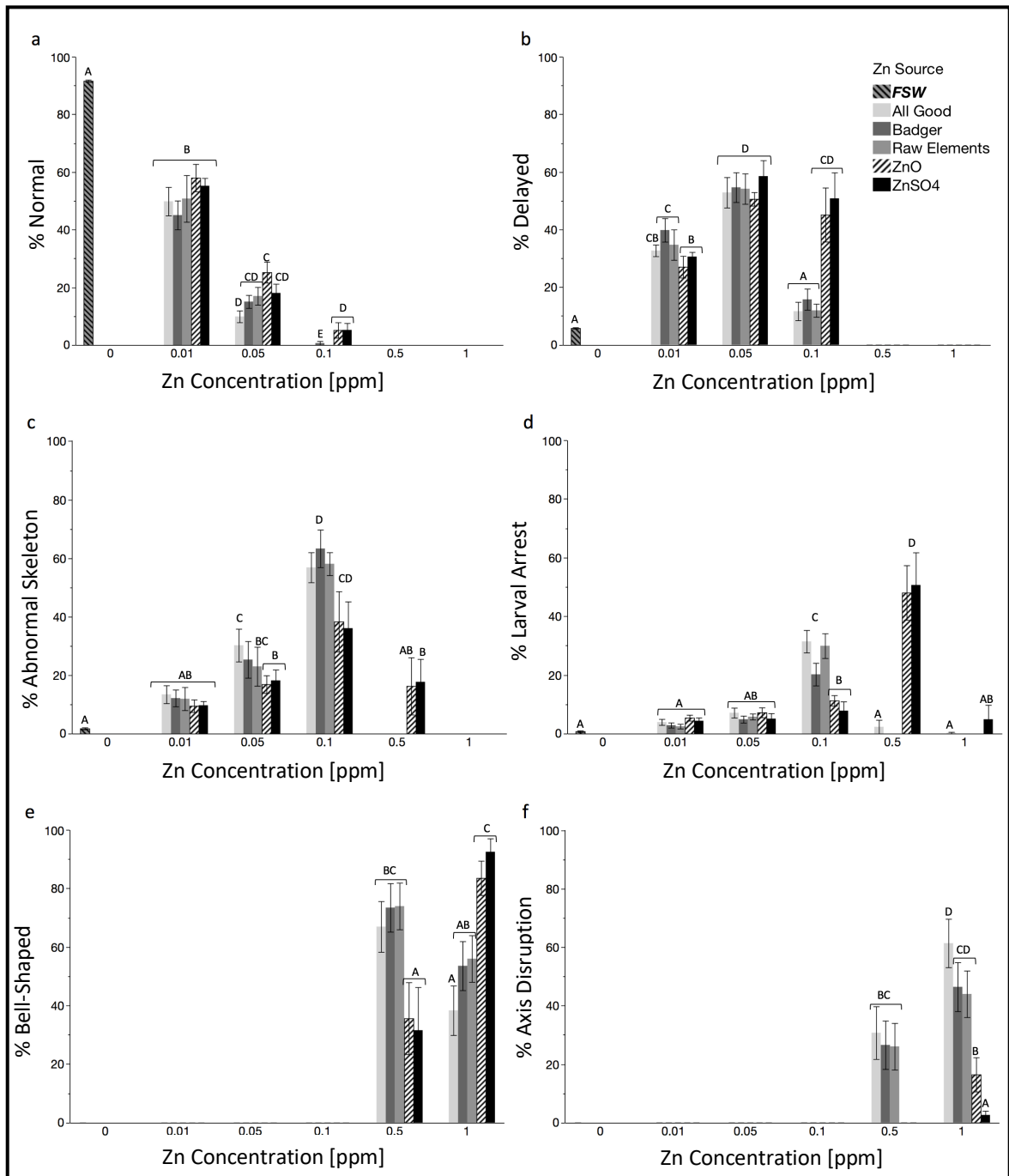


FIGURE 4: Mean percentages (\pm S.E.) of morphologies observed at 96 hpf in *S. purpuratus* embryos exposed at the pre-cleavage stage to Zn concentrations, from various sources, ranging from 0 to 1 ppm. (a) Normal development. (b) Delayed. (c) Abnormal skeleton. (d) Larval arrest. (e) Bell-shaped. (f) Axis disruption. Different upper-case letters represent statistical differences among treatments ($p < 0.0033$) according to Bonferroni corrected Student's t-tests ($n=6$).

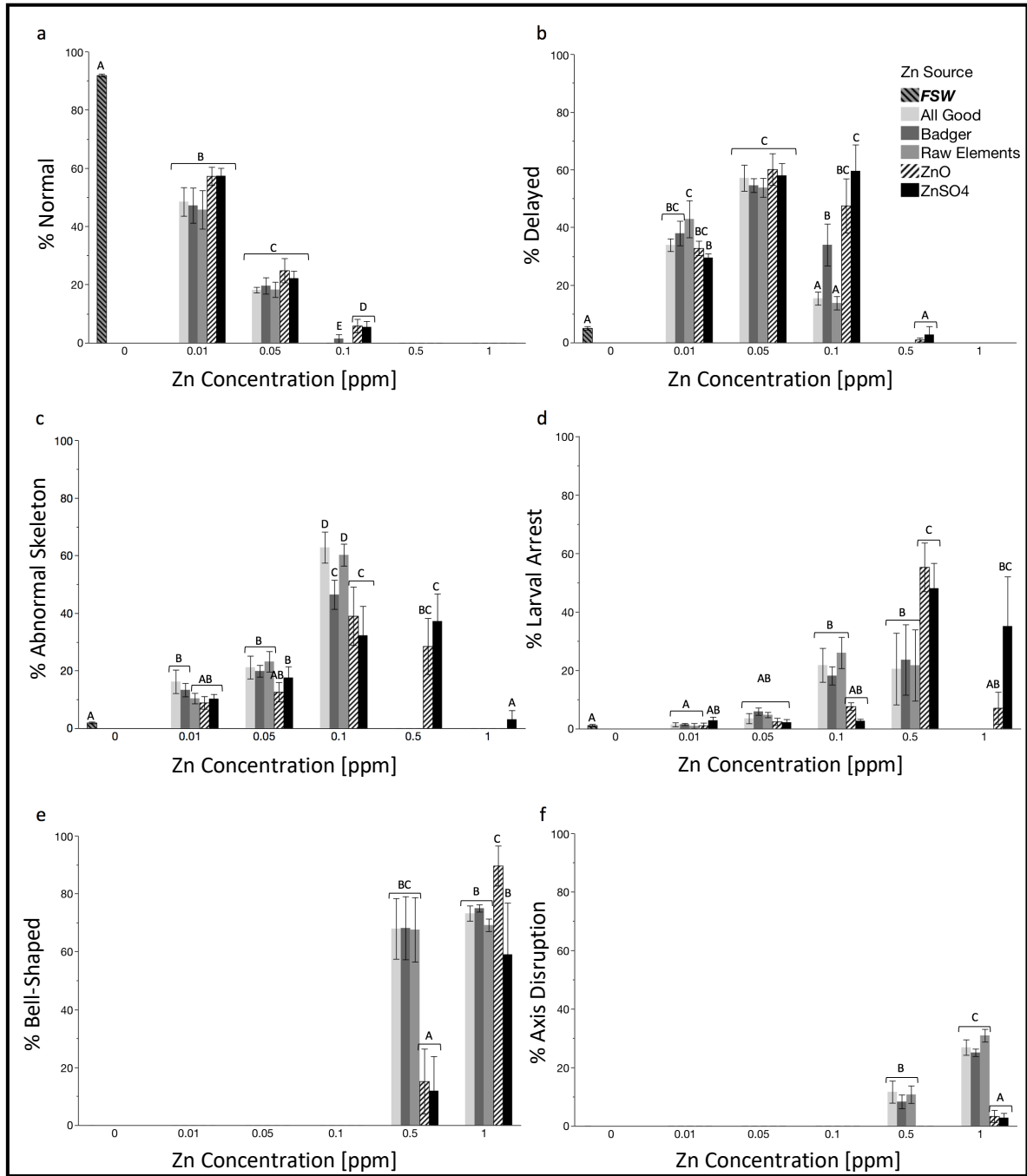


FIGURE 5: Mean percentages (\pm S.E.) of morphologies observed at 96 hpf in *S. purpuratus* embryos exposed at the blastula stage to Zn concentrations, from various sources, ranging from 0 to 1 ppm. (a) Normal development. (b) Delayed. (c) Abnormal skeleton. (d) Larval arrest. (e) Bell-shaped. (f) Axis disruption. Different upper-case letters represent statistical differences among treatments ($p < 0.0033$) according to Bonferroni corrected Student's t-tests ($n=6$).

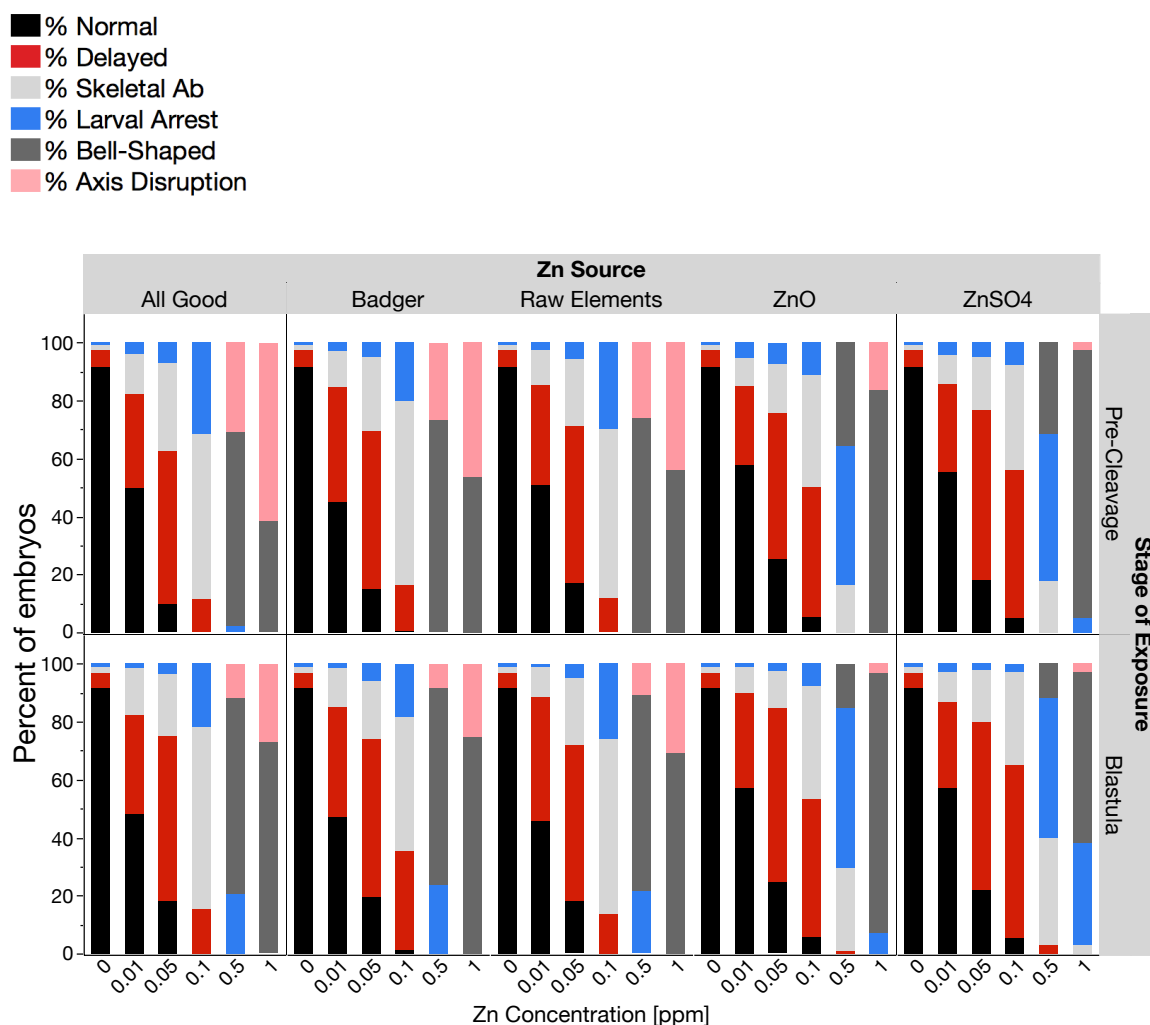


FIGURE 6: Stacked mean percentages of all morphologies observed at 96 hpf for *S. purpuratus* embryos exposed at the pre-cleavage and blastula stages to Zn concentrations, from various sources, ranging from 0 to 1 ppm (n=6). Embryos in concentrations of 0 ppm Zn were grown in FSW.

2.3.2 Embryos Accumulate Zinc from Sunscreens

Newport Green (NPG), a cell-permeant fluorescent probe that selectively binds to Zn^{2+} , was used to visualize the accumulation of Zn^{2+} within embryo cells. Increased NPG fluorescence is associated with increased Zn^{2+} accumulation. Embryos were exposed to either a low (0.1 ppm) or high (1 ppm) concentration of Zn at 1 hpf, while still surrounded by the fertilization envelope, and remained in the Zn treatment until 24 hpf, at the hatched-blastula stage. The source of Zn in each treatment was either a zinc-containing compound: ZnO or ZnSO_4 , or a ZnO sunscreen: All Good, Badger, or Raw Elements.

Regardless of the Zn source, sea urchin embryos exposed to both the low (0.1 ppm) and high (1 ppm) concentrations of Zn showed significant increases in NPG fluorescence compared to the FSW control (0 ppm) ($p < 0.05$), indicating concentration-dependent increases in internalized Zn. Embryos exposed to the high concentration (1ppm) of Zn, from all sources except ZnSO_4 , showed significantly more NPG fluorescence than embryos exposed to low Zn concentrations (0.1ppm) ($p < 0.003$) (Fig.7). For embryos exposed to Zn from all sources except ZnSO_4 , NPG accumulation seemed to increase proportionally with the increase in Zn treatment concentration. These increases are reflected in images taken with a scanning laser confocal microscope (SLCM) (Fig.8). Embryos treated with 1 ppm ZnSO_4 did not differ from any of the sunscreen treatments.

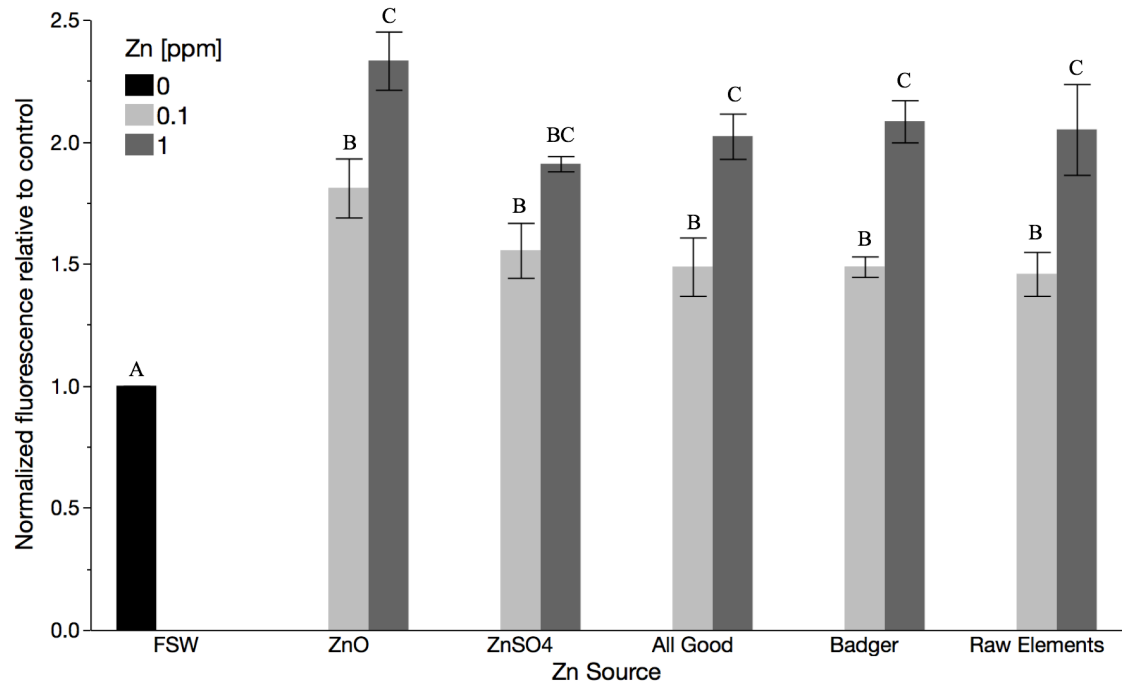


FIGURE 7: Mean Newport Green (NPG) accumulation (\pm S.E.) in *S. purpuratus* embryos after exposure to Zn compounds and ZnO sunscreens. Relative fluorescence is calculated in relation to control, FSW, group. Different upper-case letters represent statistical differences between treatments ($p < 0.0033$) according to Bonferroni corrected Student's t test ($n=4$).

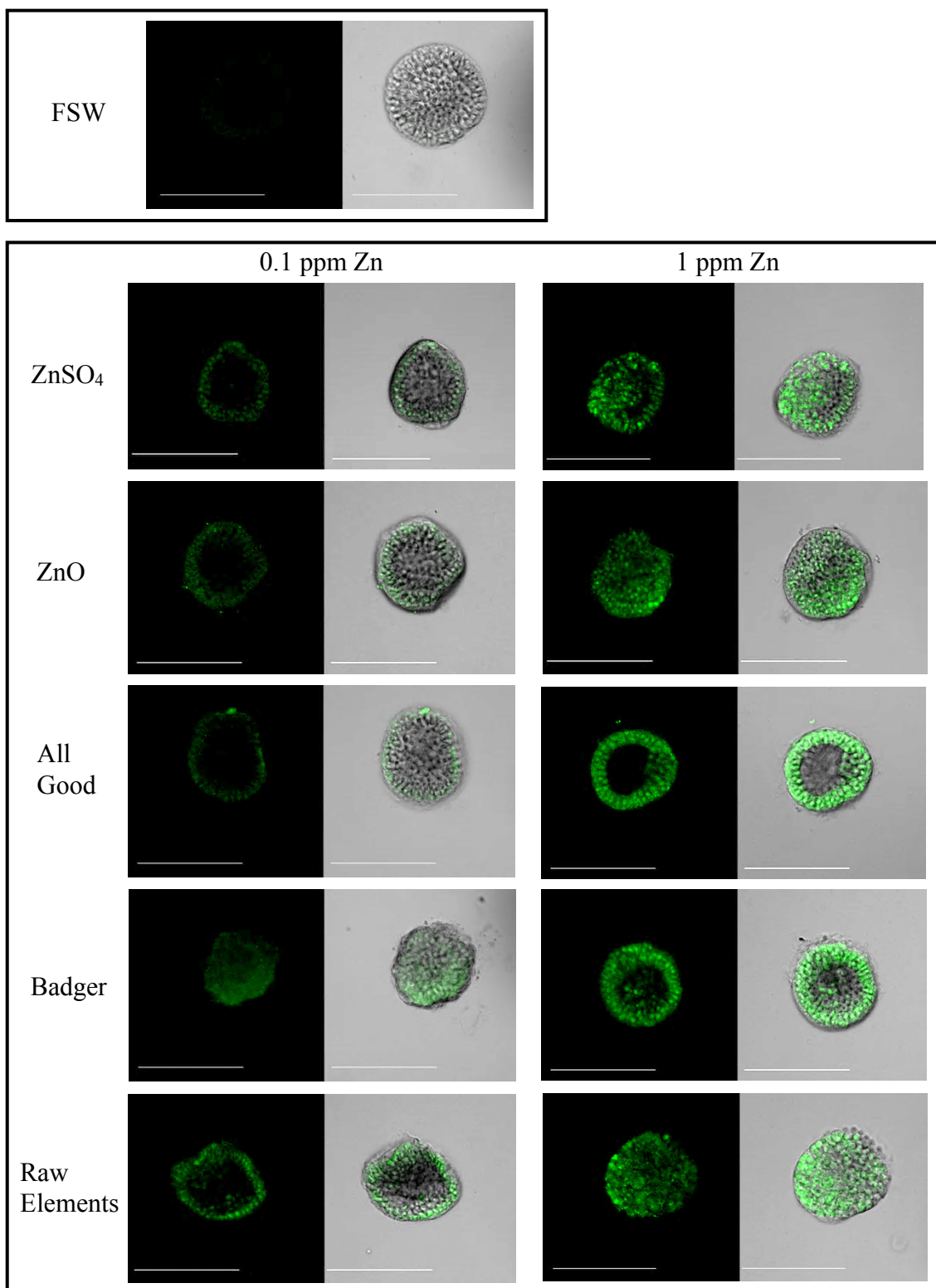


FIGURE 8: NPG accumulation in *S. purpuratus* embryos after exposure to Zn compounds and ZnO sunscreens. Representative dark field and bright field SLCM images of NPG accumulation in blastula stage sea urchin embryos. Bar = 100 μ m.

2.3.3 MDR Transporter Activity

Calcein-AM (CAM) fluorescent probe accumulation was used to investigate the activity of MDR transporters. CAM has previously been used to assess the activity of the multidrug efflux transport activity (Cole et al., 2013; Wu et al., 2015). Cells easily internalize CAM, which does not fluoresce. However, if CAM is not removed from the cell, then esterases cleave it into calcein. Calcein fluoresces and is unable to be effluxed by the cells (Legrand et al., 1998). Therefore, increased fluorescence is associated with increased CAM accumulation and indicates decreased MDR transporter activity. As with the NPG assay, the CAM assay embryos were exposed to either a low (0.1 ppm) or high (1 ppm) concentration of Zn, from all of the above-mentioned sources, between 1 hpf and 24 hpf.

All embryos exposed to the lower Zn concentration (0.1ppm) displayed no significant increases in CAM fluorescence in comparison to the FSW control ($p < 0.05$) (Fig.9). However, embryos exposed to the higher concentration (1ppm) of Zn, from all Zn sources, showed a significant increase in CAM fluorescence ($p < 0.003$) (Fig.9). We did not detect any differences between the three, different-brand sunscreen treatments. These results showed no differences in the way that either the Zn compounds or the ZnO sunscreens affected intracellular CAM accumulation. Images taken with a SLCM support an increase in CAM fluorescence only in embryos exposed to the higher concentration of Zn (Fig.10).

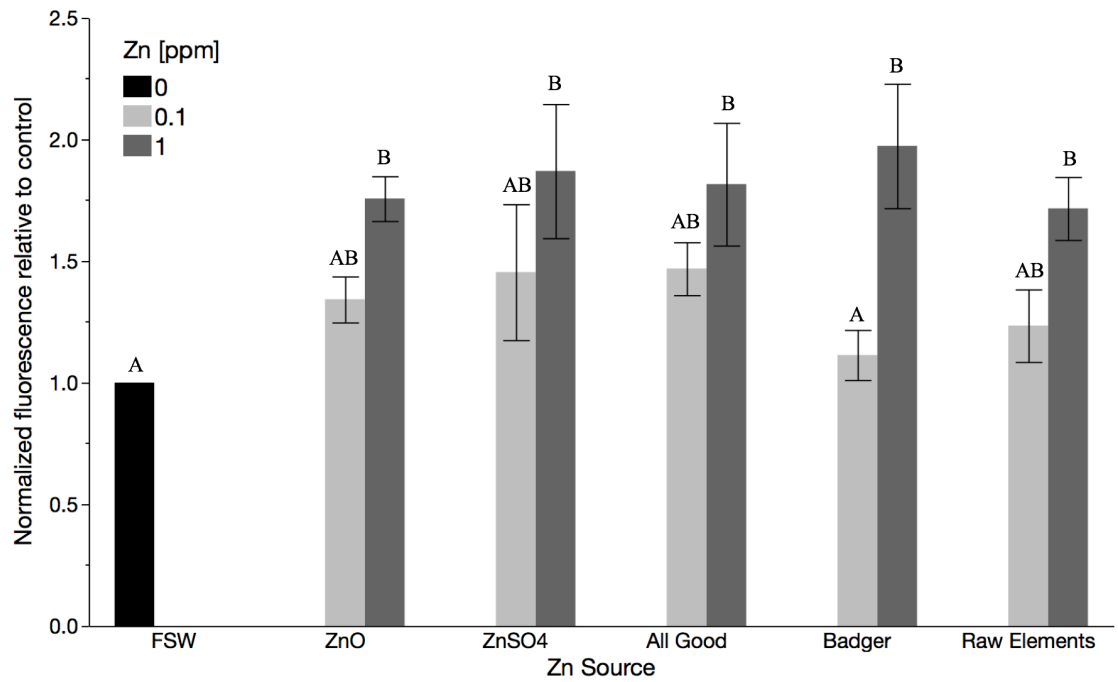


FIGURE 9: Mean Calcein-AM (CAM) accumulation (\pm S.E.) in *S. purpuratus* embryos after exposure to Zn compounds and ZnO sunscreens. Relative fluorescence is calculated in relation to control, FSW, group. Different upper-case letters represent statistical differences between treatments ($p < 0.0033$) according to Bonferroni corrected Student's t test ($n=4$).

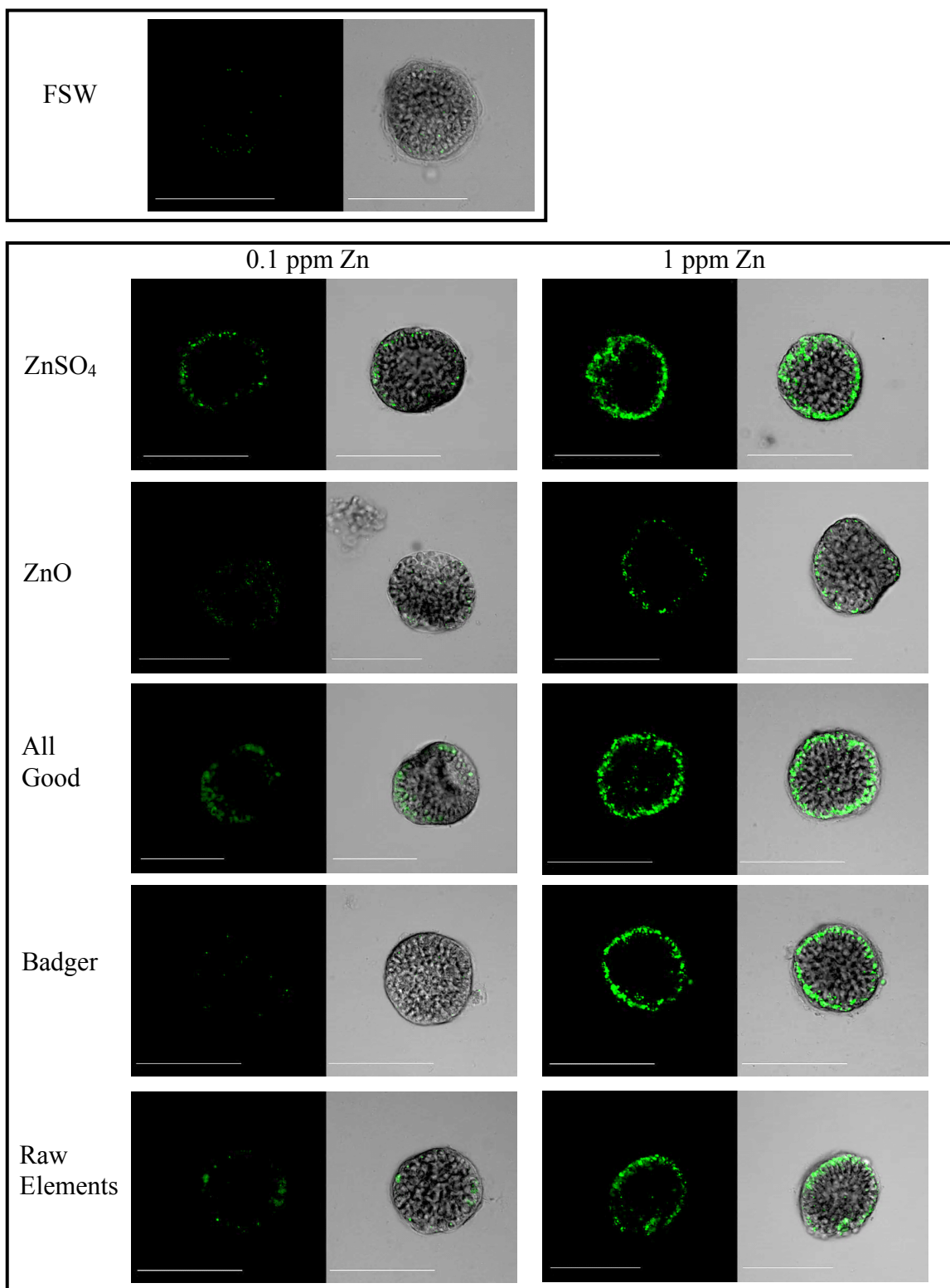


FIGURE 10: CAM accumulation in *S. purpuratus* embryos after exposure to Zn compounds and ZnO sunscreens. Representative dark field and bright field SLCM images of CAM accumulation in blastula stage sea urchin embryos. Bar = 100 μ m.

2.4 DISCUSSION

Exposure of *Strongylocentrotus purpuratus* embryos to zinc-containing sunscreens causes them to develop abnormally in a concentration and sunscreen formulation–dependent fashion. As the concentration of Zn that the embryos were exposed to increased, so did the proportion of abnormal embryos and the severity of the abnormalities. Additionally, embryos exposed to high concentrations of Zn from ZnO sunscreens exhibited significant increases in internalized Zn^{2+} and decreases in MDR Transporter activity. Our results provide clear evidence that the majority of the developmental abnormality witnessed in this study was due to Zn toxicity. Nevertheless, our data also suggest that other sunscreen components may be released and have an effect on sea urchin embryo development. Further studies are needed to confirm this hypothesis.

While, the toxicity of various zinc-containing compounds has been studied in marine invertebrates (Kobayashi and Okamura, 2004; Manzo et al., 2013; Miglietta et al., 2011; Mitsunaga and Yasumasu, 1984; Phillips et al., 2003; Poustka et al., 2007; Timourian, 1968; Wu et al., 2015), the bioavailability and toxicity of materials released from PCPs is a relatively new and important area of research. This is the first research we know of to show that non-nano ZnO sunscreens release materials that have a detrimental effect on the development of a marine organism.

The EC_{50} values for embryos exposed to Zn from ZnO and ZnSO_4 were slightly higher than those for embryos exposed to Zn from ZnO sunscreens. This may suggest that the ZnO sunscreens may be more toxic than pure Zn-containing compounds: ZnO and ZnSO_4 . All calculated EC_{50} 's were at concentrations of Zn less than 0.04 ppm. This is similar to, values reported for *S. purpuratus* (0.0969 ppm of ZnSO_4 , Phillips et al.,

2003), *Paracentrotus lividus* (0.059 ppm of ZnO, Manzo et al., 2003), and *Lytechinus pictus* (0.06 ppm of nZnO, Fairbairn et al., 2011). Though nanomaterials may exhibit different characteristics than their bulk counterpart, Fairbairn et al. (2011) found no differences between the EC₅₀'s of sea urchin embryos exposed to nZnO and bulk ZnO. Slight differences in EC₅₀'s among these studies may be accounted for by species-specific differences among sea urchins, sources of Zn, stages of exposure, and lengths of exposure used in these studies.

Exposure to the lowest concentration of Zn (0.001 ppm) resulted in at least 40% malformations from ZnO and ZnSO₄, and at least 50% malformations from ZnO sunscreens. Embryos exposed to Zn treatments at the pre-cleavage and blastula stages experienced 100% abnormality at 0.1 ppm Zn from the ZnO sunscreens, and 0.5 ppm Zn from the ZnO and ZnSO₄ (Fig.6). A small number of embryos treated with Badger sunscreen displayed normal development at 0.5 ppm (Fig.4a, 5a). Overall, these results are consistent with the Fairbairn et al. (2011) finding of 100% abnormal embryos at 0.2 ppm ZnO and ZnSO₄. However, the differences we observed in the concentration of Zn that caused total embryo abnormality, along with slightly lower EC₅₀ values for ZnO sunscreens, provides evidence that these sunscreens might have higher toxicity than pure zinc-containing compounds.

The toxicity of Zn-containing compounds to developing sea urchin embryos is generally attributed to their quick dissolution into Zn²⁺ (Fairbairn et al., 2011; Kobayashi & Okamura, 2004; Manzo et al., 2013). Fluorescence of NPG, a fluorescent probe that selectively binds to intercellular Zn²⁺, revealed a dose-dependent response in which concentrations of internalized Zn²⁺ increased as the concentration of Zn in their

environment, from either ZnO, ZnSO₄, or ZnO sunscreens, increased. The source of the Zn treatment did not have an effect on the level of internalized Zn²⁺. This is the first research that we know of to show that ZnO sunscreens release high concentrations of Zn that are internalized by aquatic organisms.

NPG fluorescence analysis showed that Zn²⁺ was internalized by sea urchin embryos developing in water containing materials released from ZnO sunscreens. These data, in combination with the observation that the majority of abnormalities caused by the sunscreens were identical to the abnormalities caused by ZnO and ZnSO₄, indicates that Zn toxicity is responsible for most of the malformations in ZnO sunscreen-treated embryos.

The most common malformations observed at low and intermediate Zn-exposure concentrations were “Delayed Development”, “Abnormal Skeletons”, and “Larval Arrest”. Each of these malformations reflects a delay or complete breakdown of normal skeletal development. Primary mesenchymal cells (PMCs) direct the formation of the sea urchin skeleton (Gustafson and Wolpert, 1961). PMCs form spicules at the start of skeletogenesis; then, calcium carbonate is added to these spicules to form the skeletal rods (Guss and Etensohn, 1997). Calcium (Ca) is a necessary component to sea urchin embryo skeletal formation, with over 99% of the skeleton being composed of calcium carbonate that is pulled from the embryos’ environment (Wilt, 1987). In freshwater fish, Zn²⁺, has been shown to compete with and impair the uptake of Ca²⁺ (Hogstrand et al., 1995; Spry and Wood, 1985). Additionally, Zn is a known calcium channel inhibitor (Nikonenko et al., 2005).

It is possible that Zn^{2+} may reduce skeletal calcification by replacing Ca^{2+} within the embryos, similar to what has been suggested for lead (Ghorani et al., 2013) and copper (Torres-Duarte et al., 2016). This Ca shortage may lead to an asymmetric skeleton, shorter limbs or in extreme cases, little to no calcite deposits on the spicules (Byrne et al., 2013; Okazaki, 1956). Additionally, the movement and positioning of PMCs is directed by vascular endothelial growth factor (VEGF) (Duloquin et al., 2007). It has been suggested that abnormal expression or location of VEGF from embryo exposure to heavy metals may result in atypical skeletal shapes (Torres-Duarte et al., 2017).

One of the more severe malformations seen in embryos exposed to higher concentrations of Zn, were bell-shaped embryos. Sea urchin embryos develop along two major axis; the primary axis is the animal-vegetal axis (AV), and the secondary axis is the oral-aboral axis (OA) (Coffman and Davidson, 2001). This abnormality involves a disruption of secondary axis formation. The bell-shaped, or oralized, morphology is commonly seen in developing sea urchin embryos exposed to nickel (Ni) (Agca et al., 2009; Duboc et al., 2004; Ertl et al., 2011; Flowers et al., 2004; Hardin et al., 1992; Ryu et al., 2012) and copper (Cu) (Torres-Duarte et al., 2016; Torres-Duarte et al., 2017; Wu et al., 2015). Ni causes oralization of the ectoderm by influencing nodal expression (Agca et al., 2009; Duboc et al., 2004; Ertl et al., 2011; Flowers et al., 2004; Yaguchi et al., 2008). Exposure to Ni mimics the phenotypes produced by nodal overexpression (Duboc et al., 2004) and the inhibition of dynamin-mediated endocytosis (Ertl et al., 2011). Both Ni and Zn have been shown to affect similar genes involved in endocytosis (Ertl et al., 2011). For OA axis specification in a normally developing embryo, nodal expression is

confined to the future oral ectoderm (Flowers et al., 2004). Zn ions can cause nodal expression to spread to the regions that typically give rise to oral and aboral ectoderm, and suppresses expression of aboral ectoderm genes (Ertl et al., 2011). Therefore, it is likely that the bell-shaped abnormality we observed in sea urchin embryos is caused by the Zn from ZnO sunscreens altering the embryos' normal nodal expression.

The most severe malformation witnessed in embryos from this study was termed, “Axis Disruption”. Embryos with this abnormality showed no skeletal, spicule, or internal gut development. A similar abnormality, resulting from Ni exposure, was termed mushroom shaped by Ryu et al. (2012), though they did not speculate on what may be causing this malformation. It is possible that some of the embryos categorized as “Axis Disruption” were exogastrulated. During gastrulation, the archenteron of exogastrulated embryos forms outside of the embryo (Kobayashi and Okamura, 2004). Exogastrulation can result from Zn^{2+} exposure at certain developmental stages (Mitsunaga and Yasumasu, 1984). However, classically exogastrulated embryos, as seen in those exposed to lithium chloride (LiCl) (Ransick et al., 1993), do not have the additional protrusions from the animal side of the embryo that we often saw in embryos displaying axis disruption (Fig.3g). It is probable that these abnormalities are the result of a disruption in AV axis formation, but this malformation could not be found in the literature. Because the “Axis Disruption” abnormality was observed almost exclusively in embryos exposed to the ZnO sunscreens, it is possible that the abnormality is not the result of Zn toxicity, but rather from exposure to an unknown sunscreen component.

An abnormality expected, but not seen, during this experiment is animalization. This malformation is caused by a disruption in the development of the AV axis. Zn is a

known animalizing agent (Mitsunaga and Yasumasu, 1984; Nemer, 1986; Timourian, 1968). While evidence indicates that Zn prevents endomesoderm formation, the molecular mechanism for this process is not fully understood (Poustka et al., 2007). It is likely that animalization was not observed during this study because the Zn treatment concentrations used were too low for animalization to occur. Previous studies have used much higher concentrations of zinc-containing compounds (~80-120 ppm ZnSO₄) with shorter incubation times, to achieve animalization (Nemer, 1986; Poustka et al., 2007).

The embryos did not differ much in their response to Zn at different developmental stages. Previous studies have found that effects of heavy metals differ based on the developmental stage of the sea urchin embryo and can alter the type and severity of malformation observed. For example, Ryu et al. (2012) found that sea urchin embryos are most sensitive to nickel, and displayed the largest amount of morphological abnormality, at the blastula stage. Additionally, Torres-Duarte et al. (2017) reported that copper exposure at the cleavage stage resulted in the highest level of skeletal abnormality. These studies generally involved instances of shorter (24-30 hours) exposures at various developmental stages (Ryu et al., 2012; Torres-Duarte et al., 2017). Therefore, while it is possible that Zn does not affect sea urchin development in a stage-dependent manner like other heavy metals do, it is more likely that no differences between pre-cleavage and blastula exposed embryos were witnessed because both exposures were at least 72 hours long and embryos were given no recovery period.

Inhibition or preoccupation of MDR transporter activity was investigated as a possible contributor to morphological abnormalities caused by ZnO sunscreens. This study used a CAM fluorescence accumulation assay that has been used in many

published studies, to evaluate the activity of MDR transporters (Cole et al., 2013; Torres-Duarte et al., 2017; Wu et al., 2015). CAM accumulation was assessed in embryos exposed to 0.1 or 1 ppm Zn from all of the sources discussed above. Exposure to the low Zn concentration (0.1 ppm), was not sufficient to increase CAM accumulation from control amounts. Therefore, this low concentration of Zn did not affect the activity of the MDR transporters, even though it affects development slightly. Significant increases in CAM accumulation, indicating decreased MDR transporter activity, were only observed in embryos cultured in treatments of 1 ppm Zn. These findings are consistent with previous work that reported significant increases in CAM after embryo exposure to ZnSO₄ at concentrations upwards of 0.5 ppm (Wu et al., 2015).

Following embryo fertilization, the activity of multidrug efflux transport activity increases. These transporters, part of the ABC transporter family, have a protective role and are necessary to remove foreign or harmful substances from the embryos cells (Hamdoun et al., 2004). It is known that Zn is associated with ABC transporter activity (Achard et al., 2004; Snider et al., 2013; Wu et al., 2015). Snider et al. (2013) report that ABC transporters are involved in Zn homeostasis and can be associated with Zn transport proteins. During the early stages of sea urchin development, Zn can act as a chemosensitizer through competitive inhibition of ABC transporter activity (Wu et al., 2015). Wu et al. (2015) suggest that metals inhibit MDR activity not by preoccupying efflux transport, but rather by impairing the functioning of the transporter. This can chemosensitize the sea urchin embryos and increase their vulnerability to other materials that sunscreens may leach into water.

Our data clearly showed that sea urchin embryos can internalize Zn from ZnO sunscreens in the water; however, it is not yet known what other sunscreen components may also be released and internalized, or how they are affecting embryo development. More research is needed on the potential release and environmental toxicity of PCP's inactive ingredients. Some ZnO sunscreens may release complexing agents that facilitate zinc uptake by embryos, such as organic acids. For example, certain complexing agents and weak organic ligands have been found to enhance Zn uptake in marine phytoplankton (Aristilde et al., 2012). Ours is the first study that we know of to suggest, and possibly provide some evidence of, the interactions between and environmental impacts of ZnO sunscreen components released into marine areas.

2.5 CONCLUSION

Exposure of sea urchin embryos to ZnO sunscreens during early development has significant developmental impacts. This includes malformations that increase in severity in relation to the concentration of Zn in the embryos' environment. Zn toxicity affects the embryos' ability to survive by interfering with skeletal formation and axial determination. We observed that Zn released by ZnO sunscreens is internalized by the embryos in a dose-dependent manner. Additionally, exposure to ZnO sunscreens reduced embryos' ability to cope with added toxicants by reducing MDR transporter activity. To our knowledge, this is the first research we know of to show that non-nano ZnO sunscreens release materials that have a detrimental effect on the development of a marine organism. More research is required to determine if other sunscreen components are released and whether they too have an environmental impact.

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