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Scientific Research

Development of the Sea Urchin *Arbacia Punctulata* in the Presence of the Environmental Toxin Sodium Hypochlorite

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ABSTRACT

Sodium hypochlorite (*NaOCl*) *or bleach, found in effluent from wastewater treatment plants, can act as an environmental toxin. The sea urchin Arbacia punctulata is a common subject of embryological toxicity tests due to its sensitivity to environmental pollutants. Using concentrations of NaOCl that mimic those found in treated wastewater* (0.11 *ppm*, 0.06 *ppm, and* 0.03 *ppm*) *we observed minimal affects on early larval development, though most larvae took longer to develop at higher NaOCl concentration. There was a significant difference in the percentage of non-normal plutei based on concentration* (P = 0.038) *and significant interaction between the percent of each morphology and NaOCl concentration* (P = 0.0027). *The most significant change in non-normal plutei was in the retarded* (*shortened skeletal rods*) *malformation which increased in frequency with NaOCl concentration* (P = 0.001). *There was a significant reduction in skeletal length in both normal and retarded plutei* (P < 0.05) *as NaOCl increased.*

Keywords: *Arbacia*, *Urchin*, *Hypochlorite*, *Development*, *Toxin*

1. Introduction

Sea urchins are a useful indicator species for environmental contamination due to the fact that their sperm, embryos, and larvae are very sensitive to toxins in the water [1-5]. Sea urchins also make an excellent research species because spawning and gamete collection is relatively simple, literature on echinoid embryological development is plentiful, the larvae develop quickly, animals are available year round and are easily maintained under laboratory conditions [1,3,6]. The sea urchin, *Arbacia punctulata* (Lamark, 1816), is readily available and has well documented early development stages as well as established EPA embryological toxicology methods [1,7, 8].

The effects of heavy metals, butylins and other environmental toxins on sea urchin embryos have been well researched. Bioaccumulation [9,10], development and embryotoxicity [11-13], and more recently genotoxicity and genetic mutation [14-16] are the primary areas of concern for these toxins. These toxins can have potent effects on embryos: concentrations of 250 mg·Pb·l^{-1} can cause accumulation of 3 mg·Pb·g^{-1} dry weight [9].

Relatively little work has been done to determine the effects of sodium hypochlorite on sea urchin embryo development. Sodium hypochlorite (NaOCl), (commonly found in household bleach) has wide applications in science, medicine, and especially sewage treatment, where it is used to reduce the number of viable bacteria in effluents [17]. In tests against the budding yeast, *Saccharomyces cerevisiae*, sodium hypochlorite causes induced genotoxic effects [18].

Human activities directly impact the ocean and in particular estuarine environments [19]. With the growing population on our coastlines, estuaries are becoming primary receiving waters for treated wastewater effluent from coastal communities. Therefore, the presence of NaOCl in wastewater, used in the terminal process to kill bacteria, has the potential to affect both geochemical cycles and resident organisms in the estuarine environment [20].

One previous study focused on the effects of wastewater NaOCl on sea urchin fertilization [20]. Even at trace amounts, 0.025 - 0.125 ppm NaOCl, it was found that NaOCl negatively affected sea urchin fertilization success by reducing viability of sperm [20]. Chlorinated

sewage, in comparison to unchlorinated sewage, was a significant and potent fertilization inhibitor though sperm showed sensitivity to either kind of sewage [20].

This study focuses on determining what, if any, developmental effects sodium hypochlorite has on *Arbacia punctulata* embryos and larvae. We tested the effects of sodium hypochlorite exposure at concentrations of 0.00, 0.03, 0.06 and 0.11 ppm NaOCl on sea urchin larvae. From this we were able to determine if there were differences in the pluteus morphology (**Figure 1**), skeletal lengths and the ratio of normal and abnormal sea urchin larvae at each concentration.

2. Materials and Methods

These two experiments were performed in the spring of 2009 and repeated in spring of 2010. A pilot experiment showed that developmental timing was sensitive to low or variable laboratory temperatures causing delays (past 36 h) in pluteus formation. In the work reported below, all samples were monitored for temperature throughout the experiments reported here and were maintained at 20˚C ± 2˚C.

Sodium hypochlorite solutions were prepared using Clorox bleach containing 6.15% sodium hypochlorite or 293 ppm [21]. Solutions were prepared with artificial seawater (originally Instant Ocean) from an established cycling marine aquarium (36 ppt NaCl) filtered through a Whatman Grade 1 Qualitative filter (11 microns), to reduce biologic sources of contamination. The concentrations made were 0.11 ppm, 0.06 ppm, and 0.03 ppm NaOCl mimicking the wastewater hypochlorite concentrations reported by Muchmore and Epel [20]. We tested

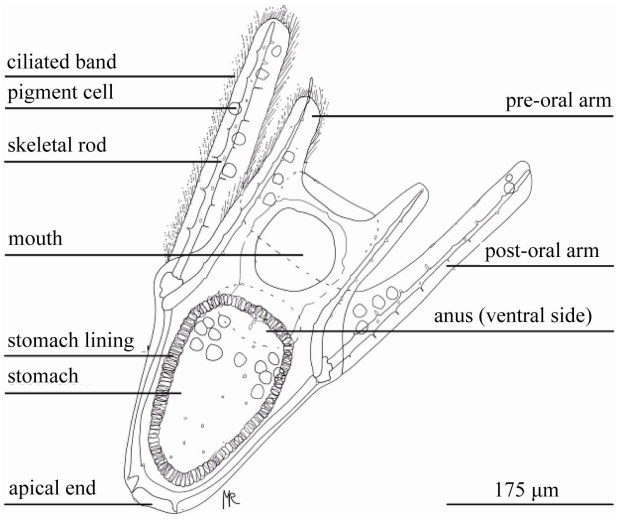

Figure 1. Major characters used to classify pluteus morphologies and length calculations. This illustration is based on a related urchin, *Strongylocentrotus purpuratus*, at ~five days old.

these concentrations after the experiment was complete using Varian ICP-OES and found that they were accurate to 0.005 ppm.

Using standard procedure [1,7-9,20], sea urchins (ordered from Gulf Specimen Marine Lab: Panacea, FL) were spawned with a 0.5 ml intracoelomic injection of 0.5 M KCl. The eggs were collected in sea water and the sperm were stored dry in an ice bath until use. To make a standard sperm suspension, 1 ml of sperm was added to 10 ml of seawater. This was used to fertilize the eggs at a ratio of 2 drops of standard sperm suspension per every 10 ml of eggs and seawater. This was repeated again after 10 min to ensure fertilization.

The egg/sperm mixture was kept at room temperature (20˚C ± 2˚C); at 40 min the eggs were checked for development, then were checked at 10 min intervals until first cleavage was observed in the majority of the cells in the sample (≤60 min). For each replicate one ml of fertilized egg solution was placed in a sterile 50 ml cell culture bottle, and then 9 ml of the respective treated seawater were added. The final NaOCl concentrations were 0.11 ppm, 0.06 ppm and 0.03 ppm NaOCl respectively. The bottles were sealed with parafilm, capped lightly, and left to develop at room temperature in low light. Organisms reached the pluteus developmental stage by 45 h for experiment 1 and 50 h for experiment 2. For each of the four concentrations of NaOCl (0.00, 0.03, 0.06 and 0.11), three replicates were prepared.

When the majority of larvae reached pluteus stage, samples were preserved by adding 1 ml of 37% buffered formaldehyde to each culture bottle and then placing the samples in a 4˚C refrigerator for 24 h. After 24 h in 3% buffered formaldehyde, the samples were rinsed twice with de-ionized water to remove formaldehyde and reduce its degrading effects, then the fluid was serially replaced with ethanol in the following concentrations: 30%, 40%, 50%, 60% and 70%. Between each ethanol rinse the samples stood 10 min, and after the final 70% ethanol rinse the preserved larvae were stored in a 4˚C refrigerator to slow cellular decay.

Counting was performed with a Sedgewick-Rafter counting cell on a Fisher Scientific Micromaster microscope (model CK) at 200x magnification. For each treatment, three individual 1 ml aliquots were tallied until 400 larvae were counted. There were four counting morphological categories used: normal development (N), reduced skeletal rod length or retarded growth (R), malformed plutei (M) and pre-pluteus larvae (P) based on Kobayashi and Okamura [22]. An embryo was counted as Normal (N) when spicule length was longer than one body length (accounting for normal larvae that were shorter) and lacking any obvious physical deformity (**Figure 2(a), (f), Figure 3**). An embryo was counted as

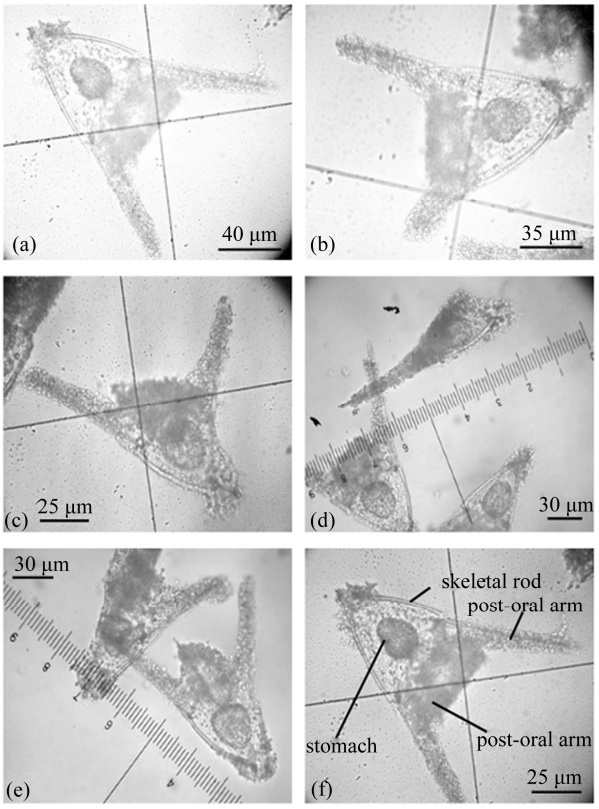

Figure 2. Different kinds of pluteus larvae seen in the experiment (from one 0.11 ppm NaOCl sample in experiment 2): (a) Normal larva; (b) Retarded larva; (c) Malformed (Toothless embryo with torsion); (d) top larva Arabesque malformation, bottom two Normal; (e) upper larva Retarded, lower larva Malformed (Toothless, slightly Vampire); f) Normal larva with labels.

retarded (R) if the skeletal rods length was smaller than a body length (**Figure 2(b), Figure 3**). For a malformed (M) count, any physical deformities present were noted, and when possible, photographed (**Figure 2(c)-(e)**). An embryo was considered pre-pluteus (P) when it did not have any skeletal rods or any of the internal structure expected of a larva at pluteus stage.

Measurements were taken with a marked eyepiece calibrated with a micrometer. Larvae were only measured when it was clear the whole larva was on a parallel plane to the lens and slide, so that the measurements were not distorted by foreshortening. The larva was measured along its whole length from the apical end to the end of one skeletal rod (**Figure 3**).

We used the program PASW Statistics (Version 17) for statistical analysis of the count and length data. Raw count data and length data were analyzed using the generalized linear model (GLM), as well as post hoc Tukey's tests. The count data was normalized using a logit function, ln(count + 1) (**Figure 3**). The +1 was added in order to assess instances where there was not a specific morphology of sea urchin malformation found. The GLM used for count data was as follows: NaOCl was coded as a random factor, to determine if there were differences between treatments in total counts for each malformed morphology. In addition, the average percentage of each type of pluteus (normal, malformed, pre-pluteal, and retarded) was analyzed to better determine how concentration altered the presence of each type using an ANOVA followed by the post hoc Tukey's analysis. The total length of the normal and retarded plutei were analyzed using an ANOVA followed by the post hoc Tukey's analysis.

3. Results

Four malformed morphologies were noted during counting. The first were primarily skeletal rods with most of the cell-tissue missing, these larvae were classified as "Wishbone malformations", and were prevalent in the first experiment and seem to be related to pre-formaldehyde larvae death. The second abnormal morphology, more common in the first experiment than second, was the "Vampire malformation" (**Figure 2(e)**). This occurred when the two front skeletal arms of the larva lacked tissue on the spicule, making them appear as if they were fangs. The lack of tissue while a skeletal rod remains may be an indicator of larval stress. The third notable malformed morphology, and the most common in both experiments, was the "Toothless malformation" (**Figure 2(c) and (e)**) where an larva's smaller skeletal rods were greatly shortened or missing on one or both sides beyond what is expected due to the organism's natural asymmetry. Lastly, the "Arabesque larvae" were noted in the second experiment only; larvae had arms (usually postoral arms) that did not lie in the same plane, were bent at odd angles relative to the body and other arms, or missing altogether (**Figure 2(d)**). Abnormal morphologies were not counted separately, but samples with many of a single malformed morphology were noted. Some forms, especially the toothless malformation, appear similar to those found in centrifuged embryos [23] and embryos exposed to styrene derivatives [24]

We used a general linear model (GLM) to test for any significant differences between experiments; none were found. There was a significant difference in the percentage of each morphology present based on concentration (P = 0.038) and evidence for a significant interaction between the percent for each morphology found and NaOCl concentration (P = 0.0027). This data suggests an increase in the percent of non-normal morphology pluteus found with an increase in the concentration of sodium hypochlorite. We then compared the percent of each larval type present to determine which was contri-

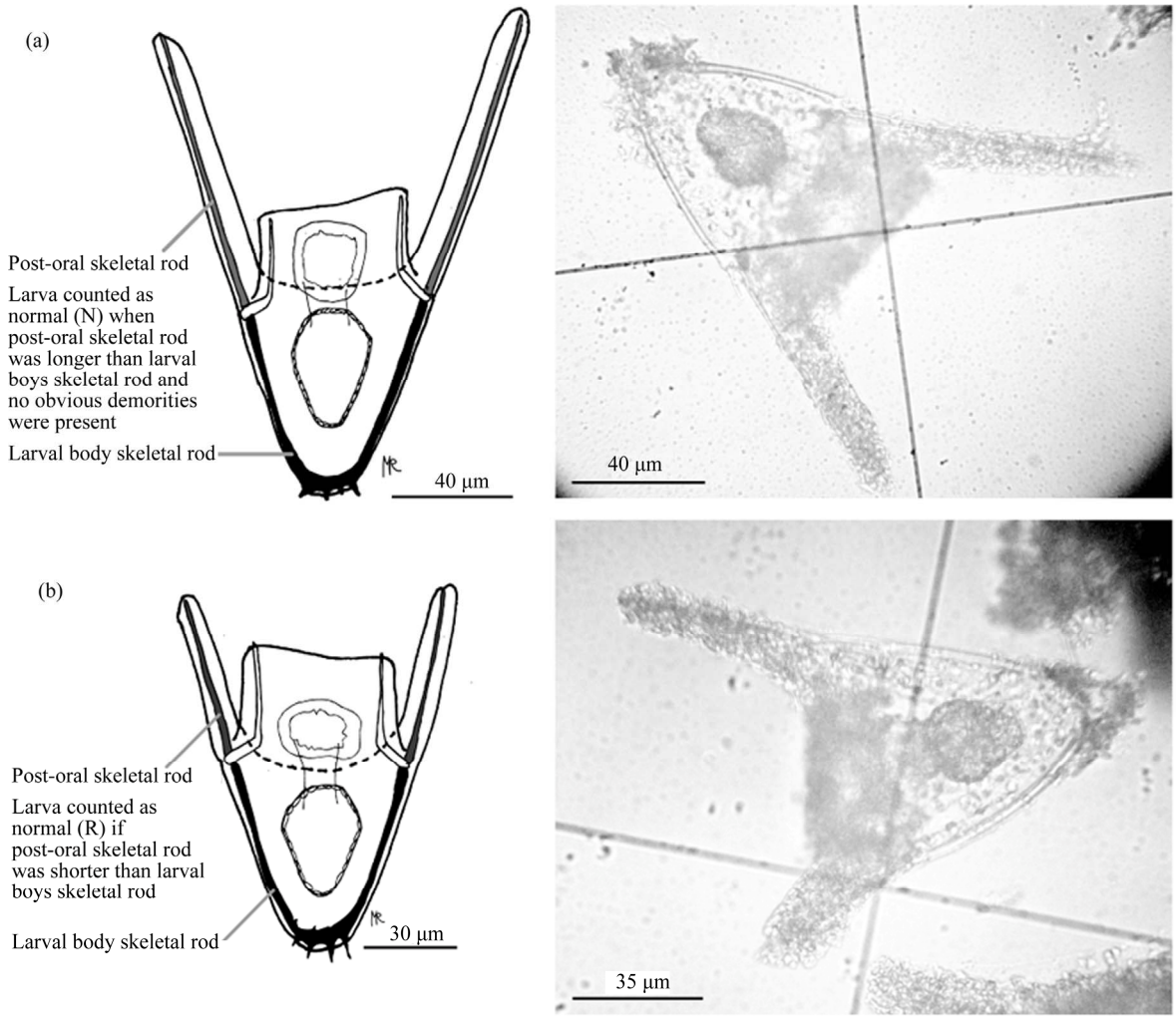

Figure 3. Larval length measurements between (a) Normal larva and (b) Retarded larva. While the total overall length is close, retarded larvae were determined via the body to arm ratio.

buting to the differences found (**Figure 4).** There were no significant differences in the percent of larvae that were normal, malformed or pre-pluteal due to concentration ($P > 0.05$). There was a significant increase in the number of retarded larvae with an increase in NaOCl concentration, both 0.06 and 0.11 ppm ($P < 0.01$), (**Figure 4**).

Given the most significant change in the pluteal morphology was found in the shortening of the skeletal rods (retarded type plutei) body length of the normal plutei and retarded plutei were assessed by concentration and type (**Figure 5**). Total body length is significantly shorter in retarded larvae for all treatments ($P < 0.001$). Total body length, though proportional, was significantly reduced in normal larvae at 0.11 ppm NaOCl ($P < 0.001$). There is suggestive evidence that the body length is shorter in normal larvae in 0.06 ppm NaOCl ($P = 0.06$). The two lower concentrations of NaOCl produced average larval lengths ~25 μm longer than the average length of the larvae in the highest concentration. The increased frequency of retarded plutei resulted in the 0.03 ppm NaOCl treatment having average larval lengths ~30 μm longer than the average larval length of the larvae in the 0.11 ppm NaOCl treatment.

4. Discussion

We found significant effects due to the concentration of sodium hypochlorite on both the observed larval morphologies and on the lengths of normal and retarded plutei. Count data shows that there is a significant difference between concentrations and larval morphologies.

At the highest concentrations of sodium hypochlorite solution there were more developed larvae present. However, in comparison to larvae in the lowest concentration and the control groups, these larvae had a significantly shorter total length, even in normally developed plutei. It

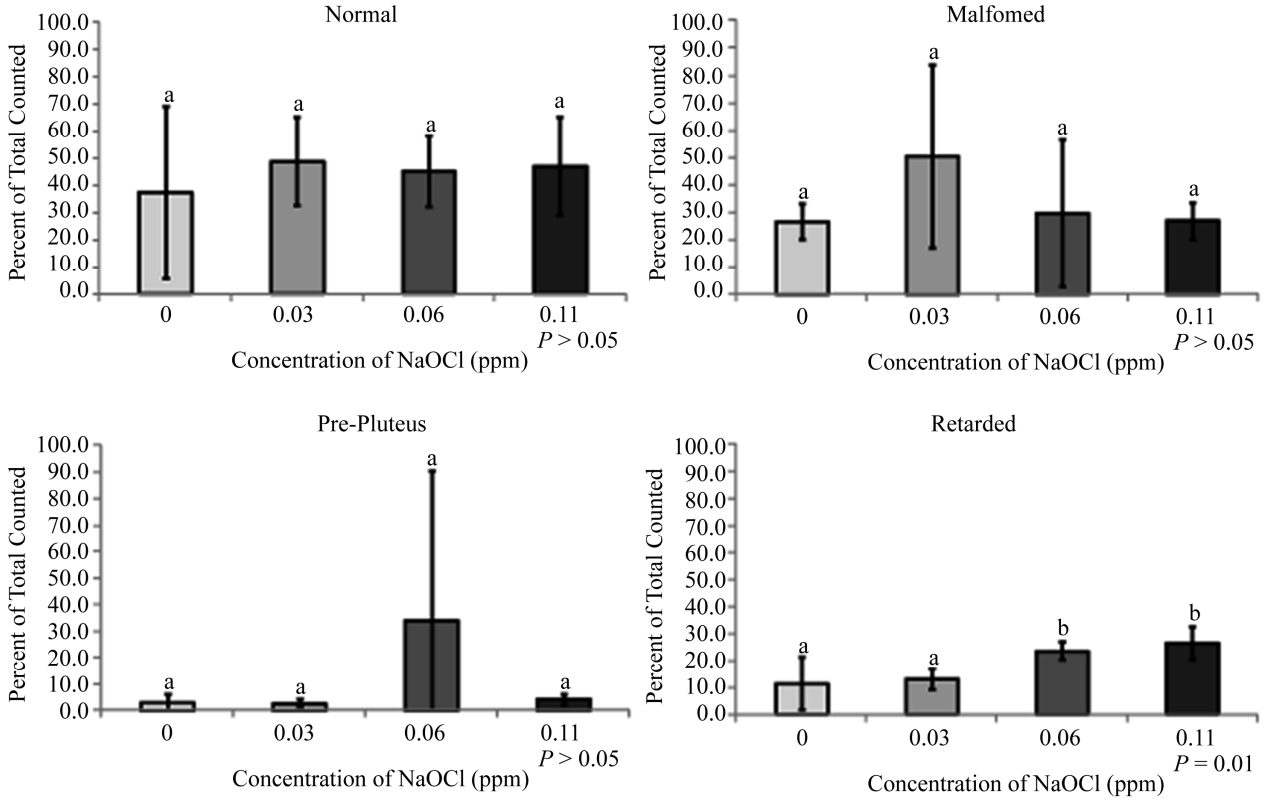

Figure 4. Differences in the *average percent* of each type of pluteal formation (shown with standard deviation error bars) scored for replicate counts during each of the 2 experiments; (a) normal, (b) malformed, (c) pre-pluteal, and (d) retarded. Averages were calculated by averaging the percentage of each type noted in each trial, not by combining the total counts and recalculating a pooled average.

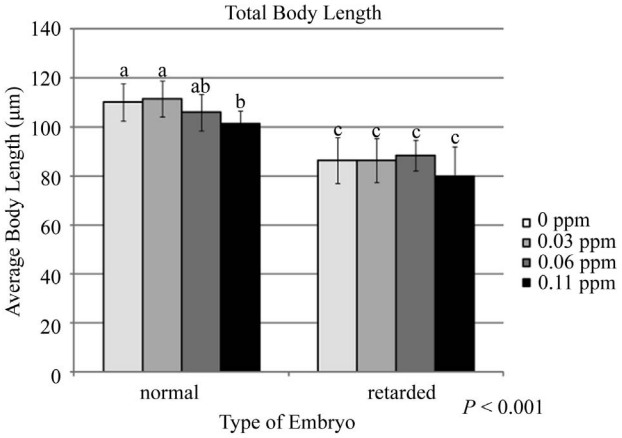

Figure 5. Pooled average larvae lengths in the Normal and Retarded categories with standard error bars. The retarded larvae are significantly shorter than normal morphology for all treatments. There is suggestive evidence that normal larvae exposed to 0.06 ppm are shorter than controls and those exposed to 0.03 ppm NaOCl ($P < 0.06$). Total body length, though proportional, was significantly reduced in normal larvae to 0.11 ppm NaOCl ($P < 0.001$). This suggests that the presence of NaOCl may be affecting the overall growth of plutei.

is possible that, while the larvae were able to develop in the 0.11 ppm NaOCl, their growth was affected by the presence of sodium hypochlorite. These observations may be an indication that development can occur even in higher concentrations of NaOCl, though Muchmore and Epel [20] reported that fertilization at these concentrations was negatively impacted.

There was also a noticeable developmental delay at room temperature (16˚C - 20˚C) for our samples. During the pilot study for this project (data not reported) a delay in development due to temperature was noted. Larvae were preserved after checking for development but because we did not scan all samples, we did not know the extent of the developmental delay. In subsequent experiments samples were checked periodically at and after the 36-h mark using the control treatment to determine if the majority of the larvae were at pluteus stage.

5. Conclusions

We originally hypothesized that there would be fewer developed larvae in the samples with higher levels of sodium hypochlorite and the data is inconclusive to that end, though our results show that there is a difference

between samples by concentration levels, especially at the highest concentrations of NaOCl. At higher levels of NaOCl exposure, *A. punctulata* larvae have shorter skeletal lengths than those larvae in lower levels of NaOCl. The significant increase in retarded type plutei exposed to NaOCl and overall shortening of body length even in normal type plutei, suggests that NaOCl may be impacting the development of the skeletal rods. Further investigation into the physiological mechanisms behind this finding needs to be addressed. Development may be slowed by the presence of NaOCl as noted by the increased number of pre-pluteus larvae present in the 0.11 ppm NaOCl treatment. Further research is necessary to determine the impact NaOCl has on observed larval morphologies.

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