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Describing the qualitative effects of Daconil fungicide in the gametes and fertilization process of sea urchin (*Tripneustes gratilla*)

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Abstract. Surface run-off of chemical pollutants such as fungicide in the aquatic environment might have negative effects on the early life cycle of invertebrates and hence their abundance. In this study, the effects of Daconil fungicide in the gametes and the fertilization process of *Tripneustes gratilla* were evaluated at different concentrations. Fresh and alive *T. gratilla* were obtained and chemically induced to produce gametes. The egg cells and sperm cells were then placed together into medium with different fungicide concentrations (1.0%, 0.1% and 0.01%) to initiate fertilization. Results revealed the fungicide causes destruction of the gametes at 1.0% concentration. Cell lysis in egg cell and absence of sperm cells motility were noted at lower concentrations. Hence, Daconil fungicide had detrimental effects on the gametes of *T. gratilla* which inhibits the process of fertilization to occur.
Key Words: *Tripneustes gratilla,* Daconil fungicide, chlorothalonil, immotility, cell lysis.

Introduction. *Tripneustes gratilla* (Linneaus, 1758) is a shallow water echinoid which is widely distributed in the tropical Indo-West Pacific region (Lawrence & Agatsuma 2001, 2007). Like many other littoral echinoids, this species is of economic and ecological interest since it is edible with good potential for aquaculture and is considered as a primary herbivore in the various habitats it occupies (Lawrence & Agatsuma 2001, 2007; Dworjanyn et al 2007; Unsworth et al 2010). *T. gratilla* generally feeds on available sea grass species; however, in the presence of different types of sea grasses, it showed preference to *Syringodium isoetifolium* (Lyimo et al 2011). Further, Sheppard Brennand et al (2010) revealed that *T. gratilla* is ecologically important as it directly or indirectly recycle nutrients in various habitats (Lawrence & Agatsuma 2001; Sheppard Brennand et al 2010). In the Philippines, *T. gratilla* which is locally called "*swaki*", has higher economic value as compared to other echinoids. Although many other species of sea urchin are considered edible, the *T. gratilla* is the most widely exploited by local communities due to its large gonads that can be eaten either raw or cooked. According to Juinio-Meñez et al (1998), unregulated harvesting of *T. gratilla* as food has led to the dropped of its population density to zero in late 1992 from 2 individuals per m^2 in 1987. Ocean acidification might also contribute to the decline in *T. gratilla* population as it causes reduction in the size of *T. gratilla* larvae which more likely impair their performance (Sheppard Brennand et al 2010). However, aside from over exploitation and ocean acidification, anthropogenic disturbances including surface run-off of chemical pollutants such as fungicides might have also contributed to the reduction of *T. gratilla* abundance.

Daconil is considered as one of the world's leading contact fungicide with a multi-site mode of action and is widely use on over 65 crops for the control of more than 125 diseases throughout the world; chlorothalonil is the active ingredient contained in Daconil responsible for its anti-fungal efficiency (Daconil: The World's Leading Fungicide Partner for Fungal Control Programs 2015). Chlorothalonil is a chloronitrile fungicide with a non-systemic broad-spectrum mode of action and foliar action with some protectant

properties (Leitão et al 2014). It is a multi-site inhibitor that affects the various enzymes and other metabolic processes in fungi and is toxic to fungal cell membranes (Pesticide Info Sheet: Chlorothalonil 2004). Moreover, chlorothalonil acts by preventing spore germination and zoospore motility (Sakkas et al 2002; MacBean 2012). The mode of action of chlorothalonil is not fungi specific; hence, surface run-off of this fungicide into the aquatic environment might also bring devastating effects on the macrobenthic organisms. The ability of chlorothalonil to contaminate water at long distances from where it is used was demonstrated in a U.S. Dept. of Agriculture study in Bering Sea (Chernyak et al 1996). Chlorothalonil was found in several sea water samples that have collected from the Bering Sea (Chernyak et al 1996). Laboratory studies conducted by Syngenta Crop Protection, Inc. showed that chlorothalonil is highly toxic to fish and aquatic invertebrates; however, studies pertaining to the effects of chlorathalonil in the gametes and fertilization process of marine invertebrates were not explored by the company.

Several mechanisms involved in the fertilization process of sea urchin are susceptible to chemical changes in its surrounding environment; hence, their gametes are frequently used as indicator organisms in environmental related studies. In this paper, the effect of Daconil fungicide on the gametes and fertilization process of *T. gratilla* was evaluated. Moreover, different fungicide concentrations were used to assess the possible variation in the response of gametes at different concentration.

Material and Method. Fresh and alive *T. gratilla* were collected along the coastal area in the Municipality of Kauswagan, Philippines on March 30, 2015. A total of 10 individuals were collected to ensure the presence of female and male *T. gratilla.* Samples soaked with seawater were then immediately brought to the biology laboratory at MSU-IIT for processing.

To induce spawning in *T. gratilla,* about 0.1-0.2 ml of 0.5M KCl solution per inch of sea urchin width were injected using a small needle into the surrounding soft tissue of the mouth side. After the injection, the *T. gratilla* were placed in the Petri dish with its mouth side down. At some point later, gametes were then seen extruded from the top of the sea urchin. A white milky substance identifies a male while an orange substance identifies a female. A small amount of these substances were viewed under microscope for validation of gametes. Subsequently, the gametes were collected separately and mixed with filtered seawater.

Different concentrations of Daconil fungicide (10%, 1.0% and 0.1%) were prepared in this experiment for the experimental set-up. About 1.0 mL of each concentrate was added separately into 9.0 mL filter seawater contained in Petri plates which serve as medium for the fertilization process. The concentration of the fungicide in the 3 mediums was further diluted into 1.0%, 0.1% and 0.01% respectively. The fertilization medium for the control set-up purely contained 10 mL filtered seawater.

In each of the experimental and control set-ups, a drop of egg cells and sperm cells were added and were mixed gently to allow fertilization to occur. Each of the mediums was then immediately observed under the microscope to determine and compare the response of the gametes with different concentrations of fungicide. Constant observations were made to detect, if present, the formation of fertilization envelope and subsequent cell cleavage. The outcome obtained from the experimental and control set-ups were then compared. The whole duration of observation lasted for only 6 hours.

Results and Discussion. The effects of Daconil fungicide have been evaluated on the gametes and subsequent fertilization process in *T. gratilla* at increasing concentration. Addition of sperm cells in the medium with 1.0% concentration alters the sperm cells activity making it immotile and eventually lead to its death. Sperm cells can no longer be recognized as it submerged at the bottom together with the insoluble component of fungicide. Presumably, sperm cells were degraded by the chemical components of the fungicide. A more evident adverse effect on the egg cells has also been observed at 1.0% concentration as shown in Figure 1. Observations revealed that eggs cells have expanded and faded relative to its original size and colour (Figure 1a). As with the sperm cells, egg

cells have also sink at the bottom, started to disintegrate (Figure 1b) and eventually vanished leaving a clear spot in the area where the egg cell was once located (Figure 1c). The effects of fungicide at 1.0% concentration are more prominent and conclusive in egg cells compared to the sperm cells due to its large size. Apparently, fertilization was impossible to occur, fertilization envelope was not visible in any of the egg cells and sperm cells were not even seen clustered around the egg cells (Figure 1d).

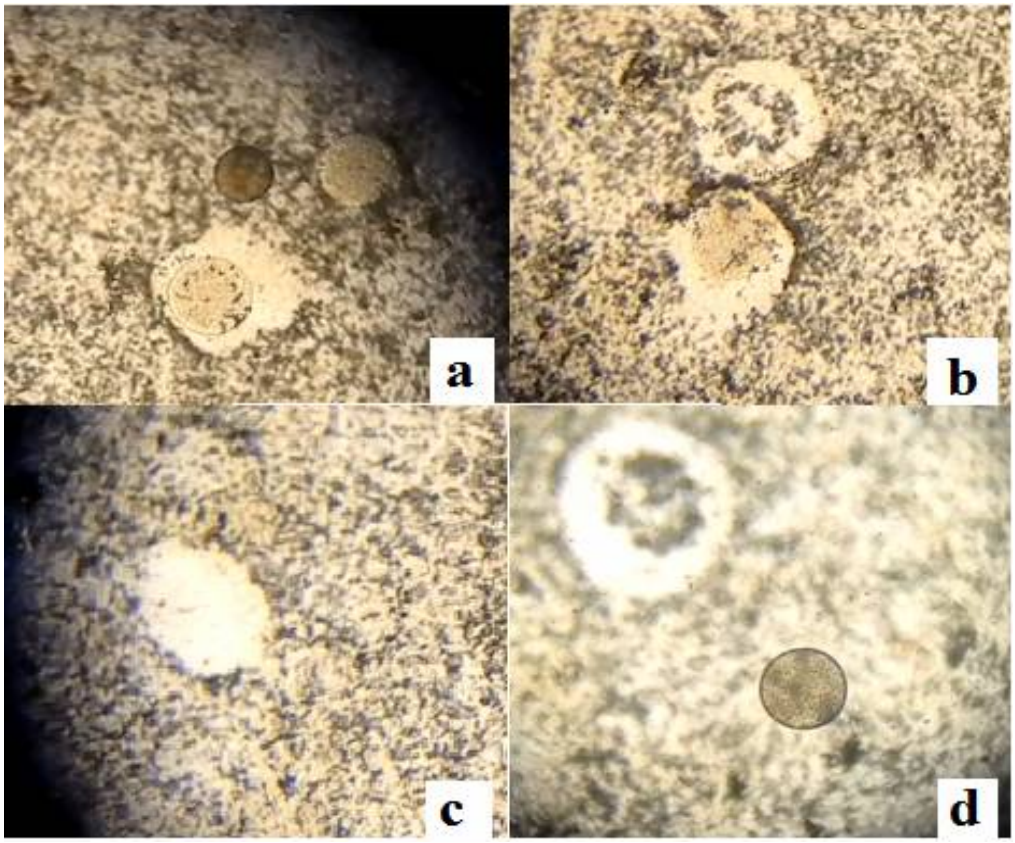

Figure 1. Effect of Daconil fungicide on the gametes of *T. gratilla* at 1.0% concentration.

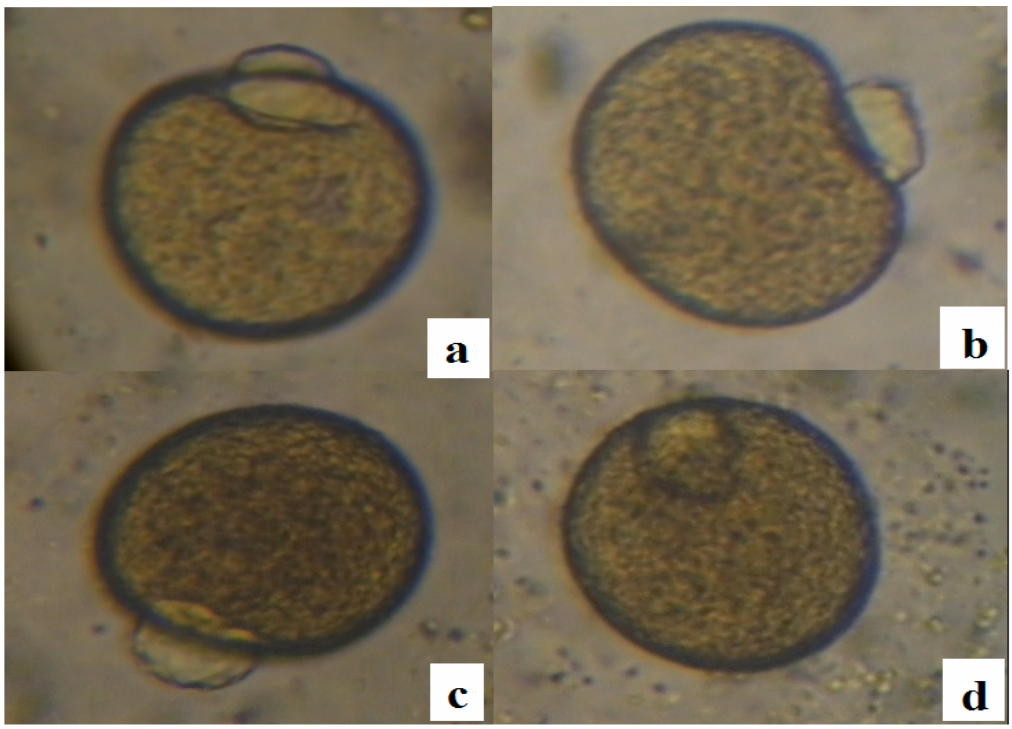

Figure 2. Effect of Daconil fungicide on the gametes of *T. gratilla* at 0.1% concentration.

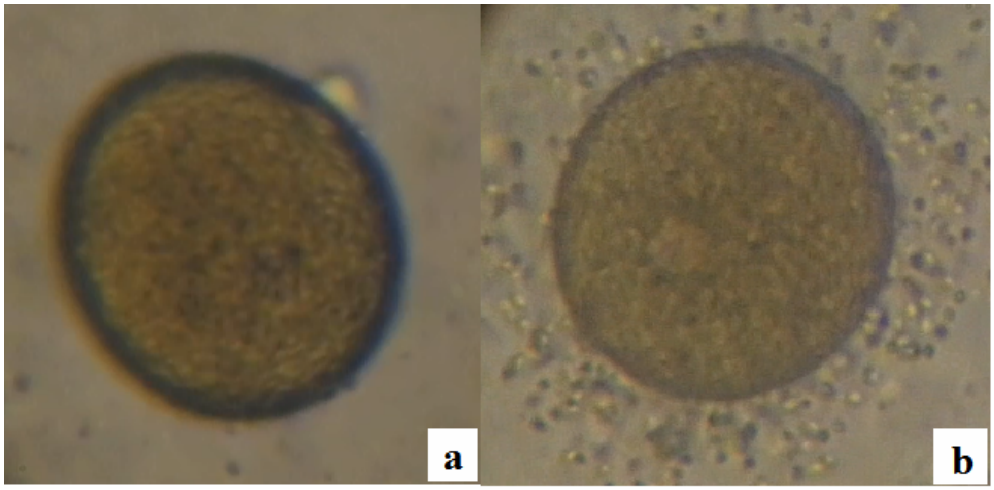

Figure 3. Effect of Daconil fungicide on the gametes of *T. gratilla* at 0.01% concentration.

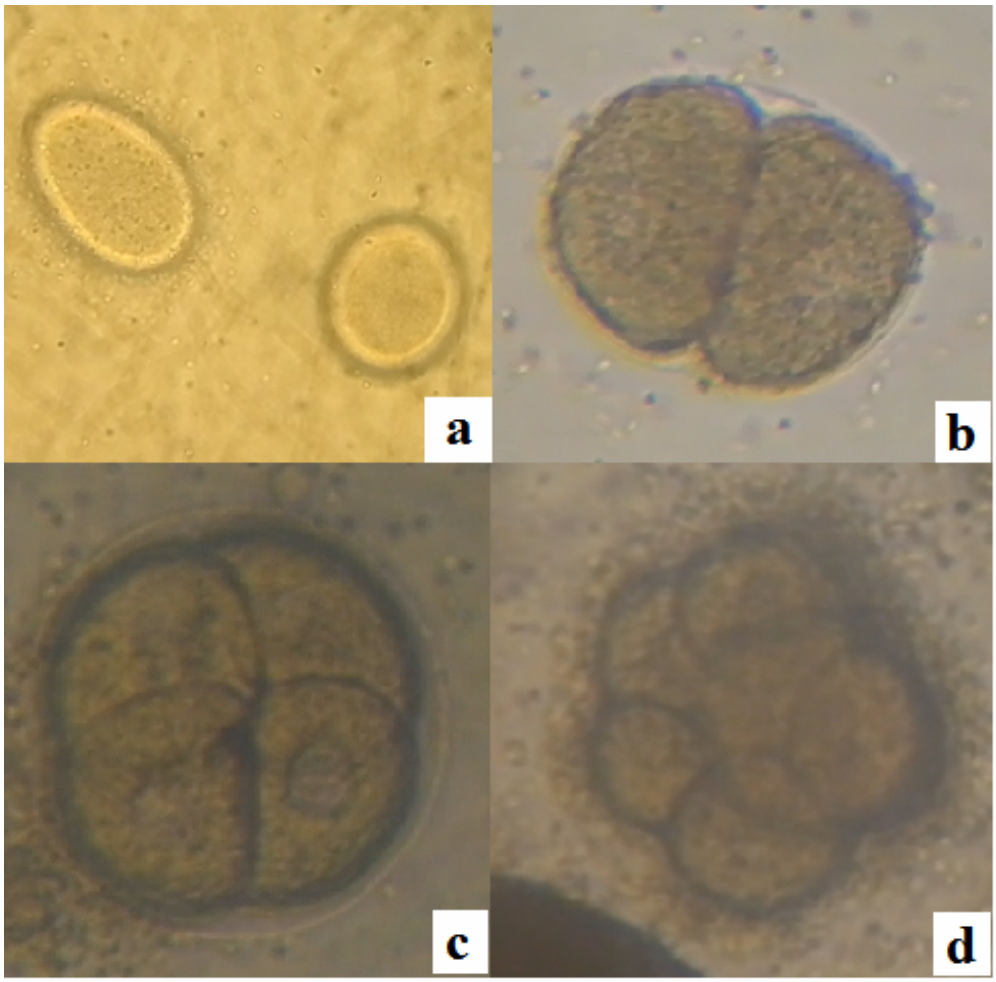

Figure 4. Fertilization and early cleavage stage in *T. gratilla* at the control set-up.

The effect of Daconil fungicide at 0.1% is quite different and to some extent less adverse compared to the former concentration (Figure 2). Though egg cells did not vanish completely, an opening had been observed at one pole of the egg cell (Figure 2a) that allows cytoplasmic content to be extruded (Figure 2a,b,c,d) and altered the shape of the egg cell (Figure 2b). Cell lysis had most likely occurred and although it was not further documented, this event will ultimately lead to cell destruction. Additionally, sperm cells were not seen clustered around the egg cells. Sperm cells were still visible in the medium

after 3 hours as shown Figure 2d but were no longer moving and most appeared to have sunk at the bottom.

At 0.01% concentration, no significant changes in the egg cell's shape have been observed as compared to 1.0% and 0.1% concentration. However, during the last hour of observation, it was noticed that there were very few egg cells that started to release little amount of its cytoplasmic content to the surrounding medium (Figure 3a). The event was similar to what was observed in the egg cells at 0.1% concentration but with less apparent effect. Sperm cells have remained clustered around the egg cell (Figure 3b), very few were observed moving slowly but most have become immotile and sunk at the bottom. Although some sperm cells may still appear to be viable, fertilization envelope was still not seen in all egg cells (Figure 3b) after 6 hours of observation; hence, fertilization is still not possible at 0.01% concentration.

In the control set-up, successful fertilization and early cleavage stage of the embryo have been observed. The successful penetrations of sperm cell through the jelly coat and plasma membrane of egg cell results in the formation of fertilization envelope (Figure 4a). Figure 4 shows the 2-cell embryo resulting the first holoblastic and equal cleavage (Figure 4b), the 4-cell embryo showing the nucleus in each cell (Figure 4c) and the 8-cell embryo (Figure 4d) that were observed at the control medium containing only filtered seawater.

The results of this laboratory experiment demonstrated the detrimental effects of Daconil fungicide in *T. gratilla.* The fungicide appears to have a toxic effect on the gametes that inhibits the fertilization process to occur. In situ, *T. gratilla* along with other species of sea urchin undergoes external fertilization making its gametes vulnerable to various chemical pollutants such as fungicide. The harmful effects of Daconil were more evident at 1.0% concentration. As the fungicide concentration decreases, the effects on the gametes were reduced but fertilization was still impossible.

As mentioned, chlorothalonil is the active ingredient present in Daconil fungicide. Several studies cited by De Lorenzo & Serrano (2003) and Cox (1997) indicated that the chlorothalonil's mode of action involves binding and depleting all available glutathione in the cell leaving enzymes that are dependent on glutathione functionless. As reviewed by Deponte (2013), the reduced form of glutathione serves as a ubiquitous nucleophile in order to convert a variety of electrophilic substances under physiological conditions and glutathione-dependent enzymes significantly accelerate most of these chemical reactions in numerous metabolic pathways. Several enzymes that are important in the process of cellular respiration such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are glutathione dependent (Caux et al 1996; Tillman et al 1973). Cellular respiration is a vital metabolic process in the cell that breaks down macromolecules to generate ATP used by the cell as the source of energy needed to perform other important process essential for survival. The action of chlorothalonil inhibits cellular respiration to occur and thus making cells depleted with energy. ATP is essential for the sperm cell's motility in order to fertilize an egg cell; depletion of ATP brought about by the action of chlorothalonil negatively affects the sperm activity and the sperm cell as a whole. Decreased sperm activity therefore reduces the chance of successful fertilization as sperm cells were probably killed before it could penetrate the egg cell.

The result of this study also reveals that chlorothalonil has a negative effect on the plasma membrane of the egg cells. Although no literatures were available to support the claim, chlorothalonil might have disrupted the layer of phospholipids which is the main structural component of the plasma membrane leading to cell lysis and degradation. In the study conducted by Hosoya & Mikami (2008), Imazalil fungicide was found to have an inhibitory and cytotoxic effect on the early development of fertilized egg of two species of sea urchin (*Scaphechinus mirabilis* and *Strongylocentrotus nudus*). In this present study, however, the effects of Daconil fungicide were evaluated during the pre-fertilization and fertilization stage not on the early development stage. Hence, regardless of the stage, fungicides were proven to have detrimental effects on the early life cycle of sea urchins that reduces its survival rate and abundance. According to Syngenta Crop Protection, studies in the laboratory and field have shown that chlorothalonil dissipates rapidly in the aquatic environment with a half-life of less than 8 hours. Nevertheless, the

success of fertilization in *T. gratilla* still remains at risk as the result of this study showed that the negative effects of Daconil fungicide at the lowest concentration of 0.01% were apparent within the 6 hours of observation. Best management practices must be followed to ensure that surface run-off in the aquatic environment are prevented.

Conclusions. The effect of Daconil fungicide was found to have detrimental effects on the gametes of the *T. gratilla* sea urchin that makes the process of fertilization impossible. The most adverse effect of fungicide on the gametes was observed at 1.0% concentration. The effects of fungicide on the sperm cells at 0.1% and 0.01% concentrations were not clearly evaluated as compared to the egg cells due to its small size. Absence and decreased in motility were the only observations noted in sperm cells. However, due to its nature, sperm cells were deduced be more susceptible to the action of the fungicide compared to the egg cells which greatly contributed to failure in fertilization process. Hence, from the findings in this study, possible run-off of Daconil fungicide in the marine environment could pose a risk in the survival and abundance of *T. gratilla* sea urchin.

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