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In Vitro Fertilization and Embryonic Development of *Sargassum polycystum* C. Agardh (Phaeophyceae) Under Different Salinity, pH, and Temperature Levels

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ABSTRACT

Sargassum species are ecologically and economically important resources in marine environments. Recently, they have been subjected to mass cultivation to avoid overexploitation in the wild. Since physicochemical conditions could easily fluctuate in culture settings, this study evaluated the fertilization and embryonic development of *Sargassum polycystum* under various salinity, pH, and temperature levels. Reproductive organs were subjected to 30 ppt, 35 ppt, and 40 ppt salinity levels, 4.0-5.0, 5.5-6.5, and 7.5-8.5 pH ranges, and 29-30ºC, 34-35°C, and 39-40ºC temperature ranges. Results showed that fertilization and embryonic development of *S. polycystum* are significantly affected by the different treatments. Increased salinity (40 ppt), increased acidity (pH=4.0-5.0 & 5.5-6.5), and increased temperature (34-35°C & 39-40ºC) caused a significant decline in the fertilization and survival of embryos of *S. polycystum*. These results will be helpful for the development of better culture conditions for *S. polycystum.*

Keywords: Acidification; Brown macroalgae; Philippine algae; Seaweed culture

1. Introduction

Sargassum species are brown algae that are widely distributed, mainly in tropical and subtropical seas [1]. It is the largest and most dominant genus in terms of standing crop, percent cover, and height in tropical high subtidal and lower intertidal zones of the marine environment [2]. They are also known to exhibit reproductive allocation and strategy as a form of resource trade-off. Some species of sargassum allocate resources to rhizome production and sexual reproduction as a form of energetic trade-off between growth and reproduction [3, 4].

Sargassum species possess a haplobiontic-diploid type of life history. The

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dominant form is the sporophyte and there is no alternation of generations [5]. Thus, these groups of species do not multiply asexually through spores. Instead, they reproduce by vegetative method and the formation of sex organs. The male gamete is born inside the antheridia, and the female gamete is born inside the oogonia. They can also be termed microsporangia and megasporangia, respectively [6]. The reproductive organ occurs in receptacles, usually found at the apical tips of branches or on lateral branches of the main axis. Each of the many receptacles contains numerous spherical conceptacles anchored to the cortical and epidermal tissue [7] and usually bears only one type of sex cell or gametes [6].

A review from Vadas et al. [8] mentioned that various factors like age-specific survival, growth rate and size, germination and spore viability, attachment setting time, substratum, sediment deposition, water motion, desiccation, nutrients, density, and competition affect the developmental stages of the algae. These are in terms of gamete release, fertilization, dispersal period, settlement, attachment, and subsequent growth [9].

Various species of *Sargassum* have recently been explored for mass cultivation due to their economic potential. For instance, Largo et al. [10] described the process of culturing *S. siliquosum* from hatchery to planting**.** Aaron-Amper et al. [11] likewise described the same culture process for *S. aquifolium.* For *S. polycystum*, Magcanta et al. [12] studied its egg release and fertilization under varying temperature, desiccation, nutrient addition, and salinity levels.

S. polycystum expands widely along the coast of Maitum, Baybay City, Leyte, Philippines. It is an edible seaweed which is a source of several natural products with various medical, environmental, and agricultural applications [13, 14]. It is reported to have antioxidant [15, 16], antibacterial [17], anti-stress [18], anti-inflammatory [19], and anticancer properties [15]. *S. polycystum* could also be potentially used as liquid fertilizer [20] and as a bioremediation agent for the biosorption of toxic heavy metals [21].

S. polycystum is an ideal candidate for mass cultivation because it is naturally occurring in the locality, thereby avoiding the introduction of new species in the area. This study aimed to provide information on how *S. polycystum* may respond to fluctuating salinity, pH, and temperature levels in terms of their fertilization and embryonic development in laboratory settings.

2. Materials and Methods

2.1 Collection of *S. polycystum*

S. polycystum samples were collected in the shallow waters of Maitum, Baybay City, Leyte, Philippines in April 2023. April is the start of the wet case (Type W) pre-summer monsoon days in the country [22]. Identification of *S. polycystum* was based on the morphological analyses of the blades, holdfast, vesicles, and receptacles following the book of Trono [23] and was validated by a phycologist from the Department of Biological Sciences at Visayas State University. The general morphology of *S. polycystum* includes a discoidal holdfast underlining its several primary branches transformed into horizontal stolons, and Y-shaped short processes along the branches [23].

Healthy receptacle-containing thalli were packed in slide lock bags, transported to the laboratory, and washed with seawater to remove surface epiphytes [24].

2.2 *In Vitro* Fertilization and Embryonic Development of *S. polycystum*

Four thalli of *S. polycystum* were collected. From each thallus, five grams of receptacles were picked up randomly for a total of twenty grams for each treatment. This is to ensure a uniform volume of receptacles and to have enough samples for use in the

experiments. Each group of receptacles was placed in a beaker with an aerated 150 ml volume of filtered seawater (FSW) and subjected to different temperature, pH, and salinity treatments at separate set-ups. Factors were independent, and each treatment had three replicates [24, 25].

2.3 Experimental treatments

The levels considered normal in this study were 35 ppt salinity, 29-30ºC temperature range, and 7.5-8.5 pH range. These are based on normal tropical values and optimum culture conditions reported in the literature [11, 26-29].

For salinity treatment, three salinity levels were applied: 30 ppt, 35 ppt, and 40 ppt under 29-30ºC seawater temperature at a pH of 8.0 [26, 29]. The 35 ppt salinity level was the basis for the normal tropical salinity value [28]. In 2011, NASA stated in their review that evaporation of sea water increases its salinity. However, it is continually counterbalanced by processes that decrease salinity such as the continuous input of fresh water from rivers, precipitation of rain and snow, and melting of ice. Meanwhile, PAGASA [30] also predicted heavy daily rainfall to become more frequent across the Philippines in 2020 and 2050. However, the number of dry days was expected to increase more in all parts of the country during the mentioned years. Therefore, it was assumed that there will be both decreasing and increasing salinity levels in the next 30 years, so the 30 ppt and 40 ppt salinity treatments were chosen. Obtaining these salinity levels was done by diluting the filtered seawater with distilled water for 30 ppt, and adding laboratory-grade NaCl solution for 40 ppt [31]. A handheld refractometer (Alla France) was used to estimate the salinity.

For pH stress treatment, three pH ranges were investigated: 4.0-5.0, 5.5-6.5, and 7.5-8.5 under 30ºC seawater temperature [27] and salinity at 35 ppt [26]. Marsh [32] mentioned that according to the report of the Royal Society dated 2005, continued acidification of the oceans was already a crisis due to the emissions of anthropogenic carbon dioxide and also emphasized that the pH of ocean water will decrease by 0.5 by 2100. A study from the American Association for the Advancement of Science (AAAS) led by Zeebe et al. [33] suggested that 40% of anthropogenic carbon dioxide emissions are absorbed by the ocean and remarked that by 2050, seawater pH will decrease by approximately 0.35-0.4. Thus, with the assumptions of pH ranges mentioned above, using an interval of ±0.5 for each century, were used to match the predicted levels for at least two centuries accounting for the increasing amount of CO_2 emissions over the past several decades. A 0.1M HCl solution was used to acidify filtered seawater, and a 0.1M NaOH solution was used as the counterpart [34]; a handheld pH meter (Biobase) was used to measure and regulate the pH values.

For temperature treatments, receptacles were subjected to three ranges: 29-30ºC, 34-35°C, and 39-40ºC, using separate water baths under the normal tropical seawater salinity of 35 ppt and a pH of 8.0 [26]. Temperature values were based on the study by Comiso et al. [27]. That study showed the highest recorded sea surface temperature (SST) of 30.1°C in the Western Pacific near the Philippines in November 2013 as observed by the National Oceanic and Atmospheric Administration (NOAA) contributed to the formation of the destructive Supertyphoon *Haiyan*. Comprehensive and high-quality data collection regarding climate change began to be recorded in 1981, which is also when global sea surface temperature (SST) records started. Moreover, the report also emphasized an average temperature of about 29°C in the Philippines. According to the study of PAGASA [30] regarding climate change, the expected average increase of air temperature in 2050 is 2ºC on the national scale. On the global scale, a maximum

increase of approximately 5.8°C could be seen by 2100 according to the report by the Intergovernmental Panel on Climate Change [35]. Cayan [36] noted that the variances of SST and Surface Air Temperature (SAT) globally were seen to be generally well correlated. Thus, temperature ranges used were 29-30ºC as the average temperature, 34-35°C for 2100 prediction and 39-40ºC for 22nd century as predicted sea surface temperatures.

2.4 Data gathering

From each treatment, five aliquots were obtained. Each aliquot contained 1 ml volume and was examined under a light microscope using a Sedgewick-Rafter counting cell slide. Aliquot sampling was done after 6 hours, the estimated time when the first cell division occurs [25]. For the determination of embryo survival, the living cells or embryos were categorized into four-cell stages to understand their development: 1) fertilized egg (FE); 2) two to six-cell stage (2 to 6); 3) multicellular: more than six-cell stage (>6) but no obvious rhizoid development of the basal cells; 4) multicellular: evident emergence of rhizoids at the basal cells (ER). For mortality, dead embryos were categorized as collapsed or fragmented, as described by Chu et al. [31]. All unusual cells (ruptured, plasmolyzed, deformed, pigmented) or embryos observed were categorized as collapsed. Embryos were considered fragmented when small fragments surrounding the embryo were visible.

2.5 Statistical analysis

All data were processed using StataMP v.14.2 software. To detect any significant effects of the treatments on the response variables, data for each experiment were separately tested using analysis of variance (ANOVA, $P<0.05$) after the test for homogeneity using Levene's test was satisfied. Tukey's test was used for the post hoc comparison of means. The differences were considered statistically significant if the probability value was less than 5% ($P<0.05$).

3. Results and Discussion

As shown in Fig. 1A, the highest percentage of survival of *S. polycystum* embryos under the different salinity levels was found at 35 ppt (77.99%) which is almost four times higher than its mortality (22.01%). It was followed by 30 ppt (69.27%) which is more than 2 times higher than its mortality (30.73%), and the lowest survival was found at 40 ppt (27.41%) which is almost three times lower than its mortality (72.59%). The survival of *S. polycystum* embryos at 35 ppt was not significantly different ($P>0.05$) from that at 30 ppt. In contrast, a significant decrease ($P<0.05$) in embryo survival was found at 40 ppt compared to 30 ppt and 35 ppt.

Fig. 1A also depicts the distribution of the different embryonic stages as they appeared at different salinity levels. There is not much difference in the numbers of fertilized eggs (Fig. 2A), two to six-cell stage embryos, and the multicellular structures of more than 6-cell stage (Figs. 2B-2E) of *S. polycystum* subjected to 30 ppt and 35 ppt salinity levels. Meanwhile, at 40 ppt, the stage of emerged rhizoids (Fig. 2F) was found to be almost two times lower (22.91%), and the multicellular structure of more than 6-cell stage was observed to be almost five times lower compared to 35 ppt (25.54% & 39.18%, respectively).

Chu et al. [31] reported that dead embryos were classified as fragmented or collapsed (Figs. 2I-2R). The most observed dead and fragmented embryos were found at 40 ppt, thus giving the highest mortality (Fig. 1A). These results imply that the survival of *S. polycystum* under hyper-osmotic stress had a significantly greater impact than those subjected to a hypo-osmotic level. These results further suggest that if salinity decreases due to frequent rain, no significant effect will occur, whereas increasing salinity due to high evaporation will significantly

affect survival, especially in the early stages of the development of *S. polycystum*. Additionally, this coincided with the findings of Zou et al. [37], who showed that *S. polycystum* has optimal growth at salinity ranges between 32-36 ppt. Species of the genus *Sargassum* have also been reported to have similar optimal salinity conditions for growth.

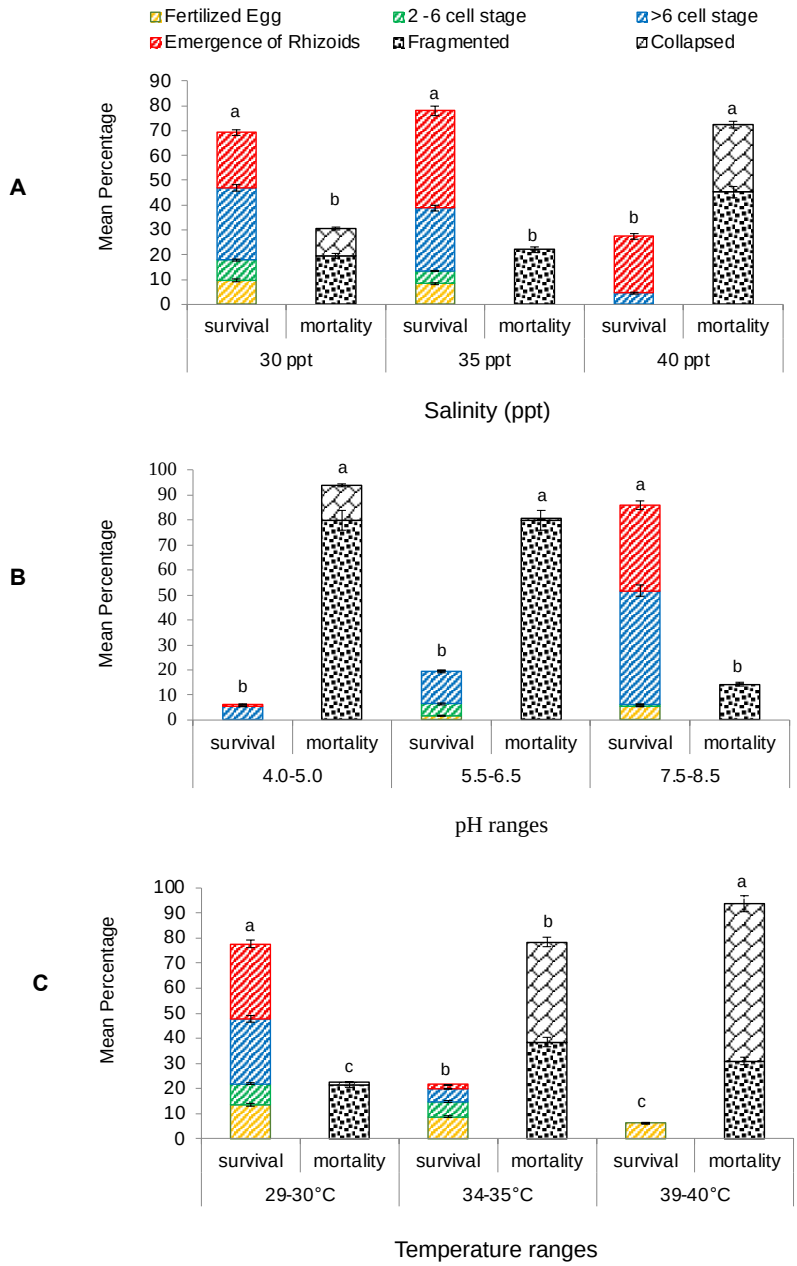

Fig. 1. Survival and mortality of *S. polycystum* embryos as influenced by (A) salinity, (B) pH, and (C) temperature. Percentage breakdown of the different embryonic stages and classified dead embryos are shown under survival and mortality, respectively. Values are means (±SE) in percentage. Letters above the error bars correspond to the post hoc comparison of means at 0.05 level of significance between survival and mortality.

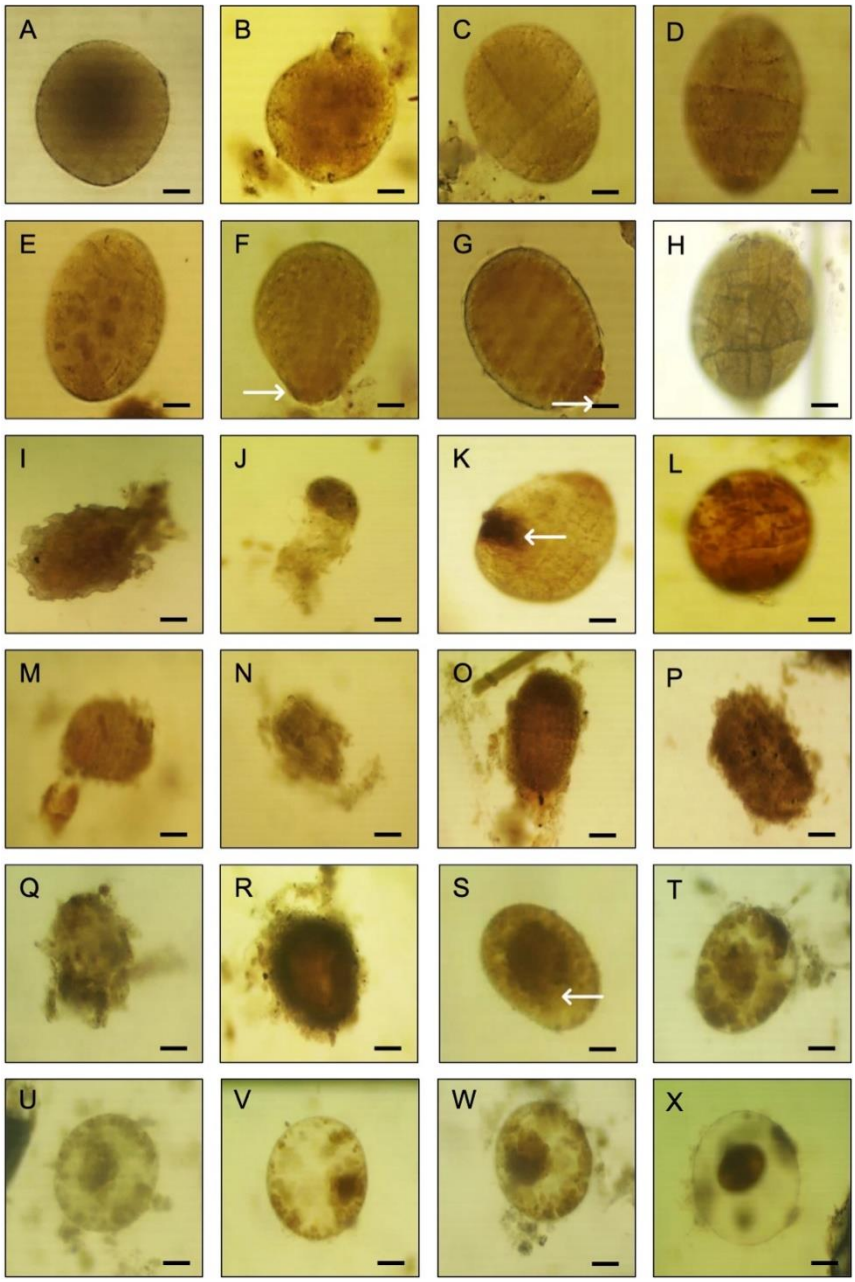

Fig. 2. (A-H) Embryonic stages of development of *S. polycystum* (scale bars*:* 30 µm): A) fertilized egg (FE) with large nucleus, B) 2-cell structure, C) 4-cell structure, D) 5-cell structure, E) multi-cell structure with no evident cells developing into rhizoids, F) Multi-cell structure with the emergence of rhizoids (ER; arrow), G) Multi-cell structure with basal cells starting to develop into rhizoids (arrow), H) embryos showing asymmetrical divisions of cells. (I-L) Dead embryos are classified as collapsed (100X). I) totally plasmolyzed embryo, J) collapsing of the embryo, K) darkened spot (arrow), L) discoloration of embryo turning red. (M-R) Dead embryos are classified as fragmented (100X). M) embryo starts to fragment with highly visible cells, N) fragmentation is more visible, O) starts to fragment, P-R) totally fragmented embryo. (S-X) Ruptured intercellular contents (100X) of fertilized eggs. S) disintegration of cellular contents (arrow) thus starts to rupture, T-W) ruptured internal contents, and X) totally ruptured cellular contents except for the nucleus.

For the pH conditions, the acidification greatly affected the reproductive potential of the *S. polycystum*. As shown in Fig. 1B, the highest survival of embryos was found at pH 7.5-8.5 (85.74%) and the lowest survival at pH ranges 5.5-6.5 (19.44%) and 4.0-5.0 (6.21%). In general, all classified embryonic stages were observed only at pH 7.5-8.5 while at both lower pH range values, almost no further development after fertilization was observed due to embryos being either fragmented or ruptured. Furthermore, fertilization in pH 7.5-8.5 (5.45%) was approximately three times higher than pH 5.5-6.5 (1.71%). The multicellular structure of more than 6-cell stage was significantly higher in pH 7.5-8.5 at almost 3 times higher (45.31%) than pH 5.5-6.5 (13.02%) and was almost 9 times higher than pH 4.0-5.0 (5.48%).

Moreover, the stage where rhizoids have emerged was observed but only at less than 1% in both lower pH range values. This result signifies that no further development occurred in *S. polycystum* embryos due to the low tolerance to acidic environments, resulting in fragmented and ruptured cells or embryos. Therefore, high mortality was recorded in both lower pH ranges. This result is supported by the highly significant difference in mortality and survival between pH treatments ($P<0.05$).

Wahyuningtyas et al. [38] found that acidification caused the bleaching of *Sargassum* thallus. They reported that the lowest color gradation and amount of chlorophyll-*a* was found in *Sargassum* thallus submerged in pH 5. They discussed that in an acidic environment, double-bond oxidation reactions can damage and cause degradation to chromatophores found in *Sargassum* sp. cells. In their natural environments, brown macroalgae species have a special preference for habitats with rocky substrates and a pH of 8 [39]. Thus, seawater acidification may cause a habitat shift in these species and could further result in the species extinction.

For the temperature treatments, as shown in Fig. 1C, the highest survival was found at 29-30°C (77.54%), almost four times higher than its mortality (22.46%). It was followed by 34-35°C (21.57%) which was almost four times lower than its mortality (78.43%), and the lowest survival was found at 39-40ºC (6.22%) which was 15 times lower than its mortality (93.78%).

Furthermore, multicellular structures observed between temperature treatments were significantly different ($P<0.05$). This result denotes that thermal stress would genuinely affect the further development of fertilized eggs as they would undergo bleaching and decaying (Figs. 2S-2X), eventually leading to high mortality. The effect of temperature on mortality came mostly from the rupturing of internal cell contents leaving the nucleus more visible. Mortality at 29-30°C, significantly differs from the other treatments. Fertilized eggs were found to be the lowest at 39-40ºC and no further development was observed at this temperature level. At 34-35ºC, the early stage of 2 to 6-cell was still observable but at a lower percentage (6%) than 29-30°C. At 29-30°C, the embryonic stage of more than six cells was significantly higher ($P<0.05$), and the embryos whose rhizoids have finally emerged were higher than those at 35°C.

These findings suggest that no further development emerged after fertilization due to thermal stress. As Rao and Rao [40] reported, *S. polycystum* experienced death after exposure to thermal stress at 35ºC and beyond. Hence, according to NOAA, the optimum survival rate for *S. polycystum* was found at 29-30ºC, the average normal range for tropical seawater. This result is also in consonance with the findings of Magcanta et al. [12]. They reported that for *S. polycystum*, the optimum conditions for egg release and fertilization is under ambient temperature.

Other observations on the *S. polycystum* embryos are presented in Fig. 2. In Fig. 2L, the complete discoloration of the embryo turning red can also be observed. No

other studies have reported the probable cause of this discoloration. Thus, it is hypothesized that the discoloration may be induced by the unfavorable conditions causing damage to the membrane of the plastids that triggered the release of other colors, red in this case. Another reason could be the increased production of other pigments as influenced by fluctuating conditions. Polo et al. [41] found increased carotenoid content in *S. cymosum* treated with 30 and 40 psu (ppt) as compared to 35 psu. This could be the same phenomenon happening for *S. polycystum* as revealed in this study.

Plasmolyzed cells (Fig. 2I) classified as collapsed were also observed at 40 ppt salinity. Cells with darkened spots (Fig. 2K) were also observed. It is believed to be a form of initial plasmolysis. To date, no significant studies have discussed the degree of tolerance at different cell stages of any *Sargassum* species affected by any physio-chemical disturbances since most of the studies focused only on the success of fertilization and growth under various conditions.

The significant effects of increased salinity, acidity, and temperature on the fertilization and embryonic development of *S. polycystum*, as revealed in this study, portend deleterious consequences to the population of this species in the context of climate change. *Sargassum* species are canopy-forming species that provide shelter and breeding areas for various marine organisms. They also serve as food for various animals and they are effective sequesters of carbon and nitrogen. Thus, their population decline would significantly impact marine biodiversity and ecosystem functioning in a negative manner.

4. Conclusion

Based on the results of this study, *S. polycystum* reproductive capacity is greatly affected by increasing temperature. Elevated temperature to at least 35°C decreased the fertilization and further development of the *Sargassum* embryos. This thermal stress caused the rupture of the cell's internal contents indicating no survival of the embryos or zygotes. Acidification also highly influenced the reproduction of *S. polycystum*. Fragmented dead embryos were commonly encountered at lower pH ranges. Increasing salinity up to 40 ppt plasmolyzed the embryos increasing their mortality. Thus, constant monitoring of these physicochemical conditions during culturing is necessary to prevent mortality. This study also shows that, in the context of climate change, the population of *S. polycystum* in the wild would decline which would negatively affect other marine organisms that depend on them for various purposes.

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