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Effects of Different Disinfectants on Blue Mussel (*Mytilus edulis* L.) Embryo

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Abstract:- Occurrence of diseases caused by bacteria during larval culture is still one of the major constraints in aquaculture. Understanding the host-microbe interactions is certainly relevant to develop disease control systems for the aquaculture industry. Therefore, obtaining test animals free from microorganisms (germ-free or axenic) is necessary, as the presence of naturally occurring microorganisms in the host may lead to false conclusions. The aim of this study is to obtain axenic blue mussel (Mytilus edulis) embryo using different disinfectants. The efficacy of chemicals in reducing the bacterial load associated with mussel eggs and embryos is tested, as well as the resistance of the eggs to these chemicals. For that purpose, fertilized eggs are exposed to different chemicals at different concentrations. The disinfectants tested include hydrogen peroxide, chlorhexidine, and Sanocare HC. All disinfectants are found to be detrimental for the mussel embryo.

Keywords:- *Mytilus edulis* L.*; Blue Mussel; broodstock mussels; Axenic; Germ Free; Bacteria Free; Sterile; Disinfectants.*

I. INTRODUCTION

Aquaculture production is expected to play a crucial role in meeting the growing demand for fishery products since capture fisheries have markedly stagnated. Currently, it is the fastest growing sector in the food production industry with an average yearly growth rate of more than six percent over the past two decades [14]. In 2010, one-third of the world's farmed fish are coming from bivalve production [25]. Bivalve molluscs are important food commodity in the world. Natural population cannot meet the increasing demand due to over-exploitation, which led to development of hatcheries [3]. Nevertheless, mollusc aquaculture growth and sustainability are still hampered by the occurrence of diseases, severely impacting socio-economic development [12]. Various methods have already been developed to control proliferation of pathogens and to maintain a healthy microbial environment in aquaculture systems. Among these are the use of probionts, immunostimulants, vaccines, quorum sensing analysis and antimicrobial peptides [41], [68], [40], [46]. However, the implementation of these alternative techniques should be based on thorough understanding of the mechanisms involved and the putative consequences [46].

It is certainly relevant to have a better understanding on the host-microbe interactions to develop effective solutions of disease control for the aquaculture industry [44]. A powerful tool to study these host-microbial relationships is to define the animal functioning in the absence of all micro-organisms (under germ-free or gnotobiotic conditions) and then evaluate the effects of adding a single or defined populations of microbes or certain compounds [34]. This allows determination of the effects of the tested microbes on the target organisms without interference from unwanted microbial contaminants [21]. Moreover, axenic animals provide a direct means to study the host's reaction to a single species of a pathogenic or parasitic agent [20]. Germ-free culture of animals has also been helpful in defining the nutrient requirements of the organism [54].

The aim of this study is to determine the effects of different disinfectants on the Blue Mussel (*Mytilus edulis*) embryo. To date, few studies are performed with gnotobiotic aquatic animals. This is the first study that attempts to generate bacteria-free mussel larvae (M. edulis) and will therefore provide baseline information for future research.

II. MATERIALS AND METHODS

- *Obtaining Mussel Embryo*

Adult mussels, Mytilus edulis were obtained from the hatchery Roem van Yerseke, The Netherlands. They were thoroughly cleaned and stocked dry at 4°C. Thermal shock technique was then employed to induce the mussels to spawn by submerging them first in warm filtered autoclaved seawater (FASW) at temperatures between 18 to 25°C followed by a cold shock treatment at temperatures between 5 to 10°C. The male and female mussels that started spawning were placed separately in a sterile plastic beaker filled with FASW in order to collect the gametes separately (Figure 1). The adult mussels were removed from their respective spawning beakers when adequate amount of gametes were released. All the eggs were pooled in a one-litre sterile beaker which was topped-up with FASW till one litre. Sperm cells were then added to reach an approximate sperm to egg ratio of 10:1. After fertilization (15 minutes), the eggs were sieved (30µm) and washed with FASW to remove the excess of sperm. All manipulations were carried out under a laminar flow hood. Materials used were autoclaved for 20 minutes at 121^{o}C and 15 psi.

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Fig 1:- Female (left) and Male (right) Mussels Spawning.

➢ *Checking Axenity*

Bacterial contamination was checked by plating 100µl of undiluted culture medium on marine agar (Difco™) plates. Serial dilutions were not done for plating as it was not necessary to count the colonies. Plates were prepared by suspending 55.1g of marine agar (Difco™) in one litre of demineralised water. The solution was autoclaved for 20 minutes at 121°C and 15 psi. Pouring of the solution in the Petri plates was done under a laminar flow hood.

➢ *Checking Larval Survival*

Larval survival was checked by staining the larvae with lugol solution inside the culture recipient and then concentrating the larvae by carefully removing the top layer of the water in the culture medium, after the larvae have sunk to the bottom. Concentrated larvae were transferred to 24-micro well plates and placed under an inverted microscope for observation. If quantitative data were needed, the live/dead ratio was counted of exactly 100 larvae. Live/good larvae were D-shaped larvae. Trochophore larvae (ciliated embryo) were also considered live larvae with delayed development. Empty shells and undeveloped eggs were considered dead.

➢ *Developing a Sterile Culture Procedure for Mussel Embryo Using Different Disinfectants.*

Different experiments were conducted in order to obtain sterile mussel embryo. The efficacy of chemicals in reducing the bacterial load on the mussel eggs and embryos were tested, as well as the resistance of the eggs to these chemicals. The fertilized eggs were exposed to different chemicals at different concentrations and time exposures, and were incubated in sterile plastic vials at densities 20-50 eggs ml^{-1} (Figure 2) in 10ml of FASW without aeration or mechanical shaking. Temperature was maintained at 17°C for all treatments. Axenity, survival and development of mussel larvae were monitored. All treatments with the eggs were replicated thrice and manipulations were done under a laminar flow.

Fig 2:- Vials for incubation of fertilized eggs.

In Experiment 1, the fertilized eggs were incubated for 48 hours in sterile plastic vials with the addition of Sanocare HC in the following concentrations:

Treatment 1 – Sanocare HC - 10µg ml^{-1}
Treatment 2 – Sanocare HC - 50µg ml^{-1}
Treatment 3 – Sanocare HC - 150µg ml^{-1}
Treatment 4 – Sanocare HC - 200µg ml^{-1}
Treatment 5 – control

A stock solution of Sanocare HC (10,000µg ml^{-1} was prepared by mixing 10g of Sanocare HC in one litre of FASW with electric mixer to obtain an emulsion (100x more concentrated).

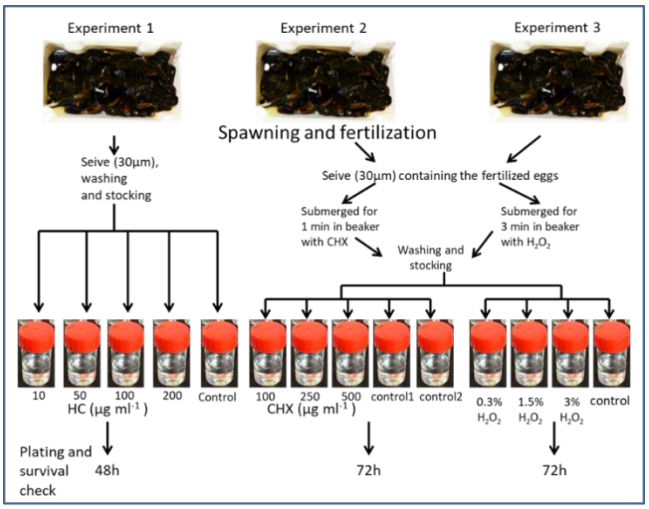
In Experiment 2, fertilized eggs were exposed to chlorhexidine in demineralised water (since chlorhexidine hardly dissolves in seawater), for 1 minute at concentrations of:
Treatment 1 – Chlorhexidine - 100µg ml^{-1}
Treatment 2 – Chlorhexidine - 250µg ml^{-1}
Treatment 3 – Chlorhexidine - 500µg ml^{-1}
Treatment 4 – control 1 - demineralised water
Treatment 5 – control 2 - FASW

Two controls were made to check whether chlorhexidine or the freshwater has an effect on the eggs: for Control 1, eggs exposed for one minute in demineralised water and for Control 2, eggs exposed for one minute in FASW, to check if the handling has a negative effect on the embryos. Five beakers were prepared containing different concentrations of chlorhexidine and the controls. Different concentrations of chlorhexidine (100, 250 and 500µg ml^{-1}) were prepared by adding 100, 250 and 500µg of chlorhexidine to sterile beakers filled with one litre water to obtain the desired concentration. Exposure was done by submerging the sieve (30µm) containing the fertilized eggs in the beaker for one minute. The eggs were then rinsed with FASW and incubated for 72 hours in sterile plastic vials.

Experiment 3, fertilized eggs were exposed to hydrogen peroxide (H_2O_2) in seawater for 3 minutes at the following concentrations:
Treatment 1 – H_2O_2 - 0.3%
Treatment 2 – H_2O_2 - 1.5%
Treatment 3 – H_2O_2 - 3%
Treatment 4 – control

Exposure of the fertilized eggs with hydrogen peroxide was done according to the same procedure described in Experiment 2. The eggs were washed with FASW after exposure and then incubated in the plastic vials for 72 hours.

Axenity, survival and larval development were checked after 48 hours for Experiment 1 while in Experiments 2, and 3 after 72 hours post fertilization. Schematic diagram of the experiments is presented in Figure 3.



HC – Sanocare HC; CHX – Chlorhexidine; H_2O_2 – Hydrogen peroxide

Fig 3:- Schematic diagram of the three experiments

III. RESULTS AND DISCUSSION

➢ *Effects of Different Disinfectants on Mussel embryo*

Sanocare HC® is a product developed by INVE Aquaculture that reduces the development and transfer of putative pathogens associated with live food culture. It is a self-emulsifying product that ensures maximum bacterial suppression during *Artemia* hatching. Different concentrations of Sanocare HC were tested in Experiment 1. Results revealed bacterial contamination at all concentrations, going from 10 to 200µg ml^{-1} (Table 1). After 48 hours of exposure, no D-larvae were observed in the control treatment, treatments 1 (10µg ml^{-1}) and treatment 2 (50µg ml^{-1}). This is due to the fact that it was still too early for the larvae to reach this development stage. Higher concentrations of Sanocare HC, treatments 3 (100µgml^{-1}) and 4 (200µg ml^{-1}) were found to be lethal since the eggs had not developed into trochophore larvae. In the study of [69], Sanocare HC significantly reduced the *Vibrio* loads in *Artemia* hatching water without affecting the embryo. *Artemia* cysts have a chorion that protects the embryo from the chemicals. In contrast, mussel embryos only possess a vitelline coat of 0.5-1.0 µm thick [8] which makes them very vulnerable to chemicals.

Experiment	Treatment	Axenity	Development/Survival (48h after fertilization)
1	1 (10µg ml^{-1} HC)	-	Trochophore
	2 (50µg ml^{-1} HC)	-	Trochophore, Undeveloped eggs
	3 (100µg ml^{-1} HC)	-	Undeveloped eggs
	4 (200µg ml^{-1} HC)	-	Undeveloped eggs
	5 (Control)	-	Trochophore

HC – Sanocare HC, (-) : Bacterial contamination, (+) : axenic

Table 1:- The effects of different concentrations of Sanocare HC on mussel embryos

In Experiment 2, different concentrations of the disinfectant chlorhexidine were evaluated. As shown in Table 2, none of the concentrations did eliminate microbial contaminants. Larval survival and development was checked after 72 hours and D-larvae were observed in the treatment Control 2 (FASW). Total mortality was observed in all of the treatments even in the Control 1 (demineralised water). Chlorhexidine is one of the best and most widely used antiseptics. It is a strong base and is most stable in the form of its salt [29]. Dilutions of chlorhexidine were prepared by mixing in demineralised water since it precipitates in seawater. Exposure to a freshwater solution of chlorhexidine caused the eggs to burst because of osmosis. This in turn leads to leakage of nutrients into the water that would enhance microbial growth and deteriorate the water quality [21].

Experiment	Treatment	Axenity	Development/Survival (72h after fertilization)
2	1 (100µg ml^{-1} CHX)	-	Total mortality
	2 (250µg ml^{-1} CHX)	-	Total mortality
	3 (500µg ml^{-1} CHX)	-	Total mortality
	4 (Control 1)	-	Total mortality
	5 (Control 2)	-	D larvae

CHX – Chlorhexidine, (-) - Bacterial contamination, (+) – axenic

Table 2:- The effects of Different Concentrations of Chlorhexidine on Mussel Embryos

Hydrogen peroxide is a highly reactive, strong oxidizing and bleaching agent [73]. It has long been used as a disinfectant for different species and life stages of fish against organisms that cause diseases such as external parasites, bacteria and fungi [73]. [21] obtained bacteria-free red drum (*Sciaenops ocellatus* L.) larvae by exposing the eggs to hydrogen peroxide (3%) for five minutes. This disinfectant was used in Experiment 3 at different concentrations. Treatments 2 (1.5%) and 3 (3%) showed no evidence of bacterial contamination while treatment 1 (0.3%) and treatment control had bacterial colonies growing on the plates (Table 3). The disinfectant however adversely affected the larval development causing total mortality. This is in contrast to the results of [21] where no adverse effects on the larval survival of red drum (*Sciaenops ocellatus* L.) were observed. However, when the disinfectant was tested on eggs of two other marine fishes (yellowtail snapper, *Ocyurus chrysurus*, and spotted seatrout, *Cynoscion nebulosus* Cuvier), the exposure to different concentrations showed differential toxicity [21].

Experiment	Treatment	Axenity	Development/Survival (72h after fertilization)
3	1 (0.3% H_2O_2)	-	Total mortality
	2 (1.5% H_2O_2)	+	Total mortality
	3 (3% H_2O_2)	+	Total mortality
	4 (Control)	-	D larvae, empty shell

H_2O_2 – Hydrogen peroxide, (-) : Bacterial contamination, (+) : axenic

Table 3:- The Effects of Different Concentrations of Hydrogen Peroxide on Mussel Embryos

IV. CONCLUSION

All disinfectants (hydrogen peroxide, chlorhexidine and Sanocare HC) seriously damaged the embryos resulting in delayed development and high mortalities. Further research and verification are needed, such as finding disinfectants that are not harmful to the mussel embryos and use of antibiotics.

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