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Article

Identification of causative agent for fungal infection and effect of disinfectants on hatching and survival rate of common carp (*C. carpio*) larvae

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Abstract: Common carp (*Cyprinus carpio*) is one of the commercially important and commonly cultured fish. In the hatchery intensive incubation leads to microbial overgrowth in *C. carpio* eggs that hamper egg development, hatchability and larval survivability. The aim of this study is to find out causes of mass mortality in *C. carpio* eggs during peak- breeding season between March to May 2015 at Mafatema fish hatchery, Chanchra, Jessore sadar upazilla. In the present study three disinfectants with three different concentrations in each such as methylene blue 1, 3 and 5mg/L., malachite green 1, 3 and 5mg/L., sodium chloride 1, 2 and 3g/L were used to observe the hatching rate of fertilized eggs and survival rate of larvae. Bacterial load of culture water was examined during the induced breeding of *C. carpio* with mycological examination of egg samples with different disinfectants. The total bacterial count fluctuated from 3.4 x 10^8 CFU/ml to 32.7 x 10^8 CFU/ml during the period of fertilization to 4days of hatching. The fertilized eggs infected by *Saprolegnia* spp. were appeared as tuft hairy like balls with a white cottony envelop. Among all the treatment 1mg/L methylene blue, 3mg/L malachite green and 1g/L sodium chloride showed significantly better ($P<0.05$) hatching rate 95·33±2·08, 88.00±2.64 and 92.33±4.04% respectively. The same concentration of methylene blue, malachite green and sodium chloride showed significantly better ($P<0.05$) better survival rate 95·00±4.35, 75.00±3.00 and 87.00±6.24% respectively. Finally among all the treatment 1mg/L of methylene blue showed significantly better ($P<0.05$) hatching and survival rate 95·33±2·08% and 95·00±4.35 % respectively. So 1mg/L of methylene blue is the best disinfectant for *C. carpio* fertilized egg treatment.

Keywords: common carp larvae; fungal infection; *Saprolegnia* spp.; disinfectants; survival rate

1. Introduction

Common carp are frequently cultured and are of great commercial value as a fish for food, both over their native and introduced range (Aguirre *et al.*, 2000). Nowadays, world-wide production of common carp is 3.2 million tons which is more than twice the Salmonids production (FAO, 2005). Common carp fertilized eggs are spherical, adhesive in nature, small and demersal that hatch within 48hrs at 28-30° C (Padmakumar *et al.*, 1985). Mohán and Shankar (1994) recorded that mass mortality in carp hatcheries resulted from the microbial diseases. Intensive incubation leads to microbial overgrowth in *C. carpio* eggs that hamper egg development, hatchability and larval survivability. Fungi are a group of organisms called heterotrophs that require living or dead matter for growth and reproduction. However, fungi can become a problem if fish are stressed by disease, by poor environmental conditions, receive poor nutrition, or are injured. If these factors weaken the fish or damage its

tissue, fungus can infest the fish. All fungi produce spores--and it is these spores which readily spread disease. The three most common fungal diseases are Saprolegniasis, Branchiomycosis, and Ichthyophonus. Saprolegniasis is a fungal disease of fish and fish eggs most commonly caused by the *Saprolegnia* species called "water molds." Poor water quality (for example, water with low circulation, low dissolved oxygen, or high ammonia) and high organic loads, including the presence of dead eggs, are often associated with *Saprolegnia* spp.infections. Saprolegniasis is often first noticed by observing fluffy tufts of cotton-like material colored white to shades of grey and brown on the fish eggs. These bacterial strains consumed oxygen and/or produce toxic metabolites which lead to mass mortalities of fish eggs (Bergh *et al.*, 1990).The bacteria as *Flavobacterium* spp., *Pseudomonas* spp., *Aeromonas* spp. and *Vibrio* spp. are easily to colonize and developed within hours after fertilization which mainly returned to water bacterial composition (Hansen and Olafsen, 1989; Madsen *et al*., 2005). Up till now, the effect of the total bacterial population in closed common carp production system in Bangladesh is ignored although; it has a vital role in the breeding operation. Changes and deterioration in the aquatic environment cause most of the bacterial disease encountered and environmental effects give rise to many other adverse culture conditions. Different types of disinfectants such as methylene blue, malachite green, sodium chloride are used for carp fish egg treatment to increase hatching rate and survival rate. The therapeutic action of methylene blue on bacteria and other parasites is probably due to its binding effect with cytoplasmic structures within the cell and also its interference with oxidation-reduction processes. It is also used in a single application as an indeterminate bath at 3 ppm. Methylene blue is also effective against superficial fungal infections of fishes. Eggs of *C. carpio* have been treated prophylactically with malachite green to prevent fungal infection. Malachite green is mostly common used as disinfectant for fish eggs as a dip or flush. It has been used in controlling bacteria, fungi, protozoans and monogenetic trematodes on eggs, fry and adult fish (Herwig 1979). Sodium chloride is considered as a safe, common substance which has antimicrobial properties (Kitancharoen*et al.,* 1997). Salt treatment at 20 ppt for 60 minutes was the best to prevent mortality amongst salmon eggs (Edgell *et al.,* 1993). Kitancharoen *et al.,* (1997) used sodium chloride at 20 ppt for 60 minutes two times a week to treat rainbow trout eggs against saprolegniosis and yielded a hatching rate of 46.0 %. The main objectives of this study were to determine the bacterial load before and after the disinfection treatment, identify the causative agent of fungal disease of *C. carpio* fertilized eggs and identify the best disinfection treatment of fertilized eggs for increasing hatching rate and survival rate of *C. carpio*.

2. Materials and Methods

2.1. Study location and time

The study was carried out during peak- breeding season between March to May 2015 at Mafatema fish hatchery, Chanchra, Jessore and laboratory of fisheries and marine bioscience, Jessore University of Science and Technology.

2.2. Sample collection

Water sample was collected in aseptically sterilized glass container (250 ml capacity). Egg samples were collected in a sterilized screw cap tube. Samples were transmitted to laboratory for examination within 2-3 hours of collection.

2.3. Bacteriological examination

Bacterial load of incubation tank water was observed two times. First observation was conducted before transferring the fertilized eggs in to the bottle incubator. Final observation was conducted after four days of disinfection treatments.

2.3.1. Laboratory preparation

2.3.1.1. Preparation of glass and plastic wares

At first glass wares (petridishes, test tubes, L-sticks, mortar , conical flasks, vials, measuring cylinder etc.) were washed very nicely after that dry and sterilized at 170°C for 1 hour by a dry sterilizer. Time was maintained very carefully. The plastic materials were autoclaved at 121°C for 15 minutes.

2.3.1.2. Preparation of physiological saline (PS)

An amount of 0.85g NaCl was weighed and kept in a measuring flask. It was then filled with distilled water to make the volume 100 ml. This was called physiological saline (PS = 0.85% NaCl). Then preparation was mixed nicely by vortex mixer. All the PS was autoclaved at 121°C for 15 min and kept at 4°C for future use.

2.3.2. Preparation of agar plates

TSA media was mainly used as a nutrient medium for bacteria culture. TSA medium was prepared by mixing at the rate of 40 g/L of distilled water in conical flask. Required amount of distilled water was measured in a cylinder. The mixture was heated on a hot plate for few minutes and then autoclave at 121°C. After autoclaving it was placed in clean chamber waited up to cooling to 60°C and then poured to sterile petridishes at an amount of 20 ml. After completion of cooling and solidification, all the TSA Plates were turned upside down.

2.3.3. Diluting the bacteria

Bacteria commonly grow up to densities around 10^9 CFU/ml, although the maximum densities vary tremendously depending on the species of bacteria and the media they are growing in. In the present study a 10-fold serial dilutions of the bacteria was prepared to cover the entire probable range of concentrations. Then 0.1 ml of each dilution was transferred to an agar plate, which in effect makes another 10-fold dilution, since the final unit is CFU/ml and only 0.1 ml was streaked.

2.3.4. Inoculating the plate

Spreading in this technique is done using a bent glass rod. Bacterial suspension at 0.1ml was placed in the center of the plate using a sterile pipette. The glass rod is sterilized by first dipping it into a 70% alcohol solution and then passing it quickly through the Bunsen burner flame. The burning alcohol sterilizes the rod at a cooler temperature than holding the rod in the burner flame. Then streak the rod back and forth across the plate working up and down several times. The cover of the petridishes was marked into three divisions corresponding to the designated serial dilution. Each petridishes was replicated three times.

2.3.5. Incubation of bacteria

The petridishes were incubated in an inverted position at 37°C for 24 hours.

2.3.6. Colony counting

The average count of colonies from the designated division of each of the replicates was taken as mean ± standard deviation.

2.4. Mycological examination

The infected egg was compressed with a drop of normalsaline between two slide examined at low power magnification. Eggs with hyphae were taken for fungal isolation using Potato dextrose agar (PDA). After three days incubation period at 25°C the fungal growth was observed. Wet smears from the isolated colonies were done and stained with Lactophenol cotton blue for microscopic study.

2.5. Disinfection treatment of fertilized eggs of *C. carpio*

A total of 1g of fertilized common carp eggs (1500 egg g-1) was used for each treatment in triplicate. The number of eggs was calculated from the weight of eggs and expressed as number/g eggs. Three different egg disinfectants with their three different concentration treatments and a control treatment were assigned for that experiment. All treatments were 30 minute bath 1 time/day for four days after fertilization. Disinfection treatment trials of fertilized eggs of *C. carpio* are shown in Figure 1 and Table 1.

Figure 1. Disinfection treatment of fertilized eggs.

Table 1. Disinfection treatment trials of fertilized eggs of *C. carpio.*

Name of chemical	Concentration	Replication
		R1
	1mg/L	R2
		R3
		R1
Methylene blue	3mg/L	R2
		R3
		R1
	5mg/L	R2
		R3
		R1
	1mg/L	R2
		R3
		R1
Malachite green	3mg/L	R2
		R3
		R1
	5mg/L	R2
		R3
		R1
	1g/L	R2
		R3
		R1
Sodium chloride	2g/L	R2
		R3
		R1
	3g/L	R2
		R3
		R1
No chemical	control	R2
		R3

2.6. Estimation of hatching rate

All other conditions during the experimentation were maintained in same condition. Then hatching rate was determined by the following formula.

$$\text{Hatching rate (\%)} = \frac{\text{No. of hatchlings} \times 100}{\text{Total number of fertilized eggs}}$$

2.7. Estimation of survival rate

All other conditions during the experimentation were maintained in same condition. After completion of the experiment at 4th day, the survival rate was estimated by using the following formula

$$\text{Survival rate} = \frac{\text{No. of hatchlings alive up to 7th day} \times 100}{\text{Total number of hatchlings}}$$

2.8. Statistical analysis

The results obtained in the experiment were subjected to analysis. Qualitative and quantitative analysis of all kinds of data were carried out. MS Excel was also used for presentation of the tables and graphs obtainable from different types of data. Analysis of variance (One way), Tukey-Kramer Test for differences between means were used to analyze the effect of different disinfectants treatment on hatching and survival rate of common carp (*C. carpio*) eggs by using SPSS 11.5 software. The significance of the data from disinfectants treatment effect were considered significantly different at $P<0.05$.

3. Results

3.1. Identification of bacterial load

At first bacterial load was observed before transferring the fertilized eggs into the bottle incubator. The bacterial load was 3.4×10^8. After 96 hours treatment the load increased gradually. Among all the treatment the bacterial load varies from 5.5×10^8 to 13.2×10^8. But in control the bacterial load was increased rapidly 32.7×10^8 (Table 2).

Table 2. Bacterial load before and after disinfection treatment.

Name of disinfectant	Concentration	Mean bacterial load at 0 hrs CFU/ml	Mean Bacterial load after 96 hrs CFU/ml
Methylene blue	1mg/l		10.2×10^8
	3mg/l		7.5×10^8
	5mg/l		6.5×10^8
Malachite green	1mg/l		13.2×10^8
	3mg/l	3.4×10^8	7.6×10^8
	5mg/l		5.5×10^8
Sodium chloride	1g/l		8.4×10^8
	2g/l		6.7×10^8
	3g/l		5.9×10^8
Control			32.7×10^8

3.2. Mycological findings

The infected fertilized eggs in hatching containers appeared as tuft hairy like surrounded with a white cottony envelope. It didn't hatching and capitulated within 48-50 hrs. Microscopically, the infected eggs showed highly branched non septated hyphae with presence of zoosporongia. The fertilized eggs in the hatching containers (incubators) were surrounded by a cloudy white cottony envelope. All affected eggs appear as hairy balls with tuft hair like outgrowths. Wet mounts of infected egg showing highly branched non septated hyphae with the presence of zoosporongia. That sharply indicated that the fungas was *saprolegnia* spp. (Figure 2).

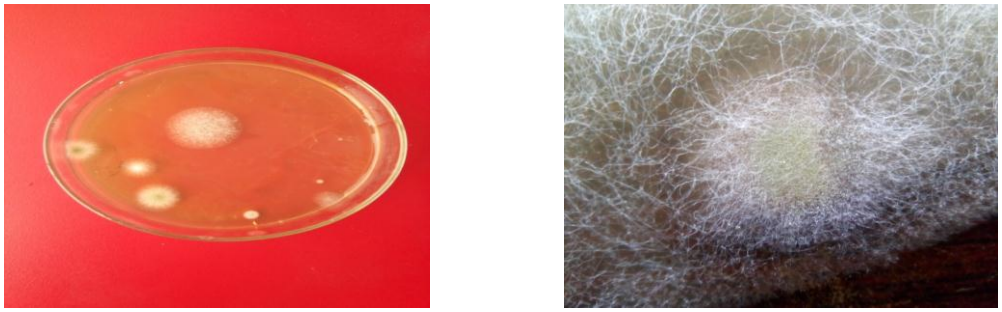

Figure 2. *Saprolegnia* spp.

3.3. Effect of disinfectants on hatching rate of *C. carpio* eggs

C. carpio eggs were disinfected by three types of disinfectant with three different concentrations in each. The hatching rate of *C. carpio* eggs are shown in the following Figure3, 4 and 5. The hatching rate ranged from 82·66±4·50% to 95·33±2·08% for three different concentrations of methylene blue and among the three different concentration of methylene blue 1mg/L showed the highest hatching rate (95·33±2·08%)(Figure 3). Methylene blue at the concentration of 1mg/L has significant difference ($P<0.05$) with 5mg/L concentration of methylene blue. The hatching rate ranged from 79·66±3.51% to 88.00±2.64 % for three different concentrations of malachite green and 3mg/L showed the highest hatching rate (88.00±2.64 %) (Figure 4). Malachite green at the concentration of 3mg/L has significant difference ($P<0.05$) with 5mg/L concentration of malachite green. The hatching rate ranged from 59·66±6.11% to 92.33±4.04% for three different concentrations of sodium chloride and 1g/L showed the highest hatching rate (92.33±4.04%) (Figure 5). Sodium chloride at the concentration of 1g/L has significant difference ($P<0.05$) with 2mg/L and 3g/L concentration of sodium chloride. The hatching rate in the control group was 71·66±2·08%. Methylene blue at 1mg/L treatment showed significantly higher ($P<0.05$) hatching rate (95·33±2·08) (Figure 6).

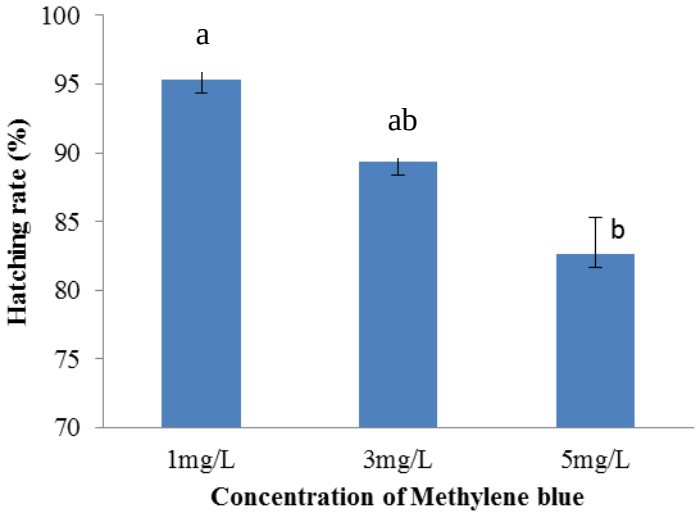

Figure 3. Relationship between concentration of methylene blue and hatching rate of *C. carpio* eggs.

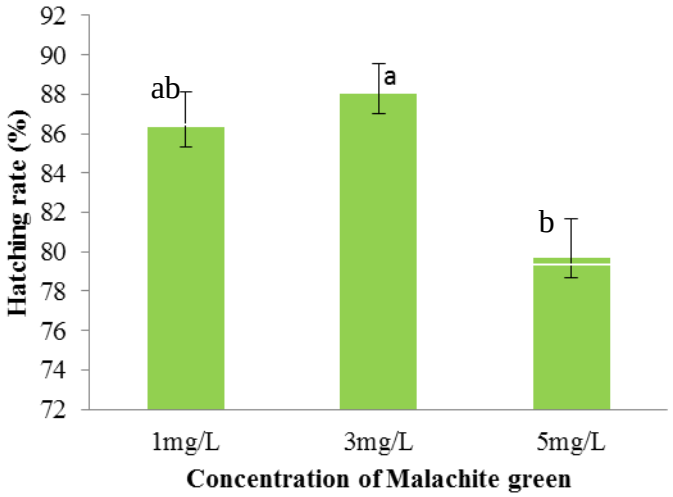

Figure 4. Relationship between concentration of malachite green and hatching rate of *C. carpio* eggs.

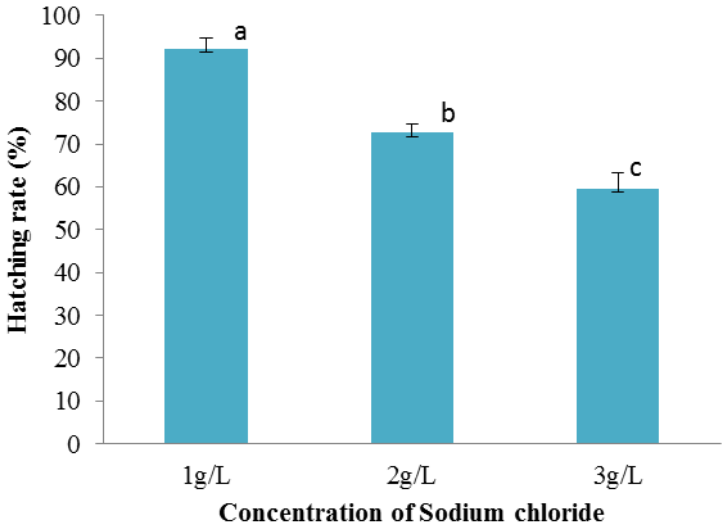

Figure 5. Relationship between concentration of sodium chloride and hatching rate of *C. carpio* eggs.

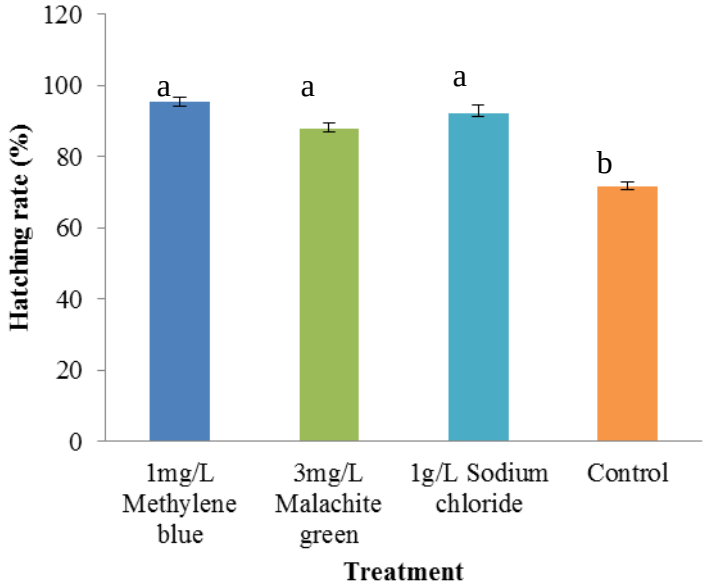

Figure 6. Hatching rate of common carp eggs in different disinfection treatment

3.4. Effect of disinfectants on survival rate of *C. carpio larvae.*

The survival rate ranged from 77·66±6·80% to 95·00±4.35 % for three different concentrations of methylene blue and among the three different concentration of methylene blue 1mg/L showed the highest survival rate (95·00±4.35 %) (Figure 7). Methylene blue at the concentration of 1mg/L has significant difference ($p<0.05$) with 5mg/L concentration of methylene blue. The survival rate ranged from 67.00±2.64% to 75.00±3.00 % for three different concentrations of malachite green and 3mg/L showed the highest survival rate (75.00±3.00 %)(Figure 8). Malachite green at the concentration of 3mg/L has significant difference ($P<0.05$) with 1mg/L concentration of malachite green. The survival rate ranged from 51.66±4.93% to 87.00±6.24% for three different concentrations of sodium chloride and 1g/L showed the highest survival rate (87.00±6.24%)(Figure 9). sodium chloride at the concentration of 1g/L has significant difference ($P<0.05$) with 2g/L and 3g/L concentration of sodium chloride. The survival rate in the control group was 45±1%. Methylene blue at the concentration of 1mg/L treatment showed significantly higher ($P<0.05$) survival rate (95·33±2·08) (Figure 10).

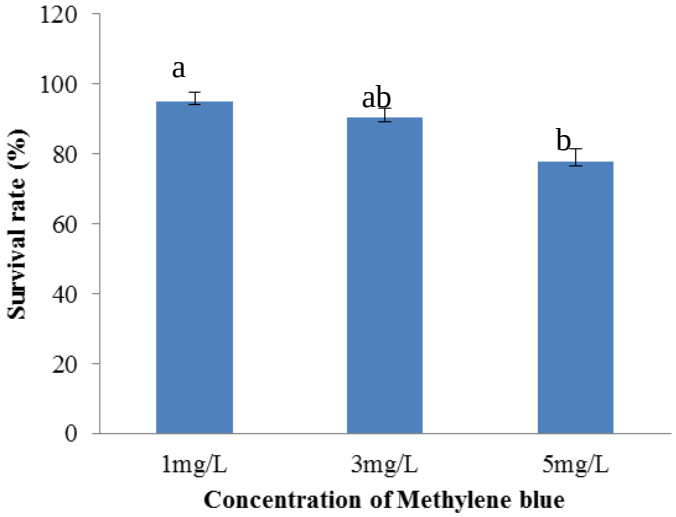

Figure 7. Relationship between concentration of methylene blue and survival rate of *C. carpio* larvae.

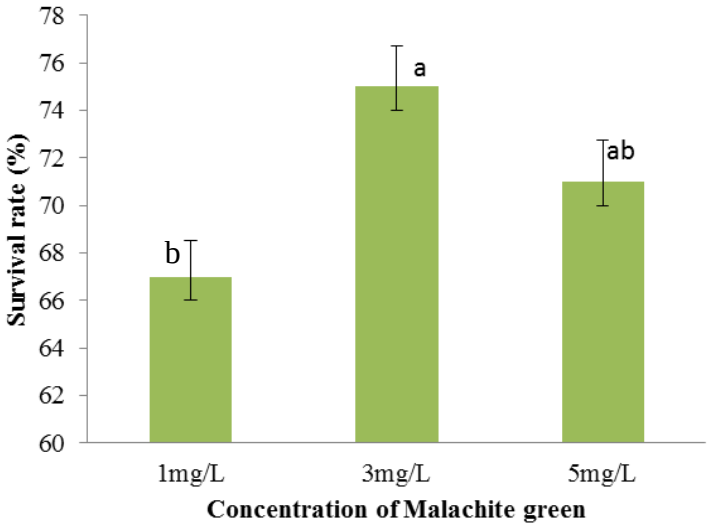

Figure 8. Relationship between concentration of malachite green and survival rate of *C. carpio* larvae.

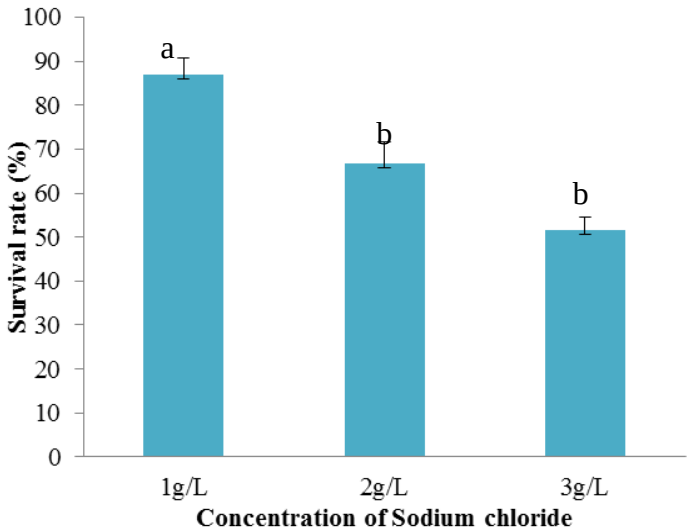

Figure 9. Relationship between concentration of sodium chloride and survival rate of *C. carpio* larvae.

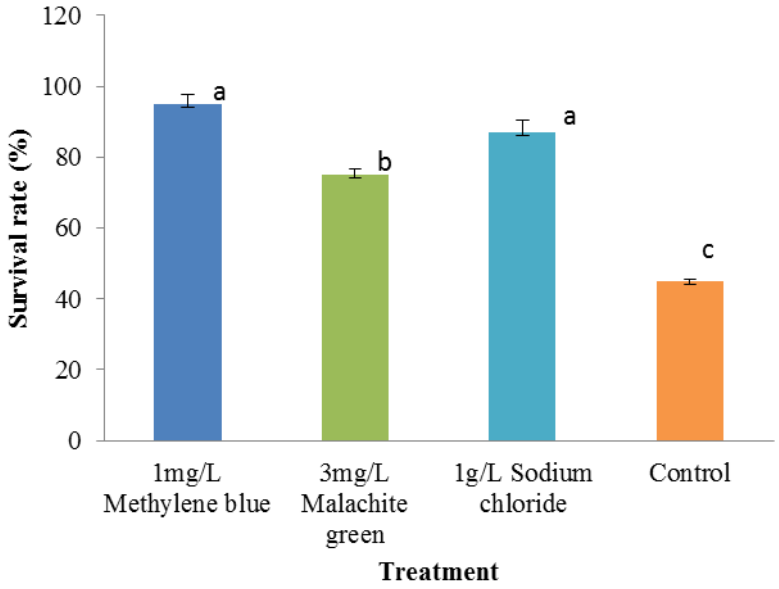

Figure 10. Survival rate of common carp larvae in different disinfection treatment.

4. Discussion

Increasing bacterial load is one of the important factors for decreasing larval survivability. For decreasing bacterial load different types of disinfectants are used. In the present study three different disinfectants with their three different concentrations were used for treatment trial. In the present study, the total bacterial count fluctuated between 3.4×10^8 CFU/ml at initial time of incubation and 32.7×10^8 CFU/ml after 2 days of hatching. There were 8.8 times average increase in bacterial population. It also found in the experiment that CFU/ml decrease with the increase of disinfectants concentration. Disinfectants reduce bacterial load through washing egg and other debris and treated water but due to continuous flowing water in incubation bottle bacterial load reduced as compare to control after four days but was not sufficient as bacterial load was 3.6×10^8 in supply water. Yahya *et al.,* (2014) also found bacterial load 4.2×10^{10}CFU/ml after 24 hours of fertilization and 48×10^{10} CFU/ml after 4 days of incubation till hatching. Mohan and Choudhary (2010) reported that at common carp hatching unit the bacterial count was about $40.66\text{x}10^2$ colonies ml^{-1} and after hatching rose to reach to 846.25×10^2 colonies ml^{-1}. Hansen and Olafsen (1989) found that the viable bacterial counts were 10^3/ml before hatching and rose to 10^6/ml after 2 days of hatching in water pool of hatching unit of *Gadusmorhua* due to hatching egg, debris and release of inorganic and organic substance. While, the total bacterial count was 10^2-10^5cfu/ml in rainbow trout rearing pond water (Diler *et al.,* 2000). Embryos and hatchlings mass mortalities were recorded when the bacterial count more than $1800\text{x}10^2$ colonies ml^{-1}.Fish egg shell is composed mainly of glycol-proteins which are chemo-attractive for bacterial colonization. The massive growth of bacteria on eggs shell result in hypoxia, accumulation of lactic acid and death of fish eggs. Beside, excessive bacterial colonization may penetrate the egg shell or produce toxic metabolites that damage the chorion (Hansen and Olafsen., 1989).

Fungal infection of eggs has been reported in different fish species (Cao *et al.,* 2012; Czeczuga *et al.,* 2005). Padmakumar *et al.,* (1985) found that fungal infected common carp eggs were enveloped with a cloudy cover and did not hatch and succumbed in 12-36 hrs. Thoen *et al.,* (2011) noticed that the live egg contact with dead infected salmon egg had developed foci of white discoloration then death with total whitening. *Saprolegnia* species were grown on a surface of egg shell which affected it by direct adhesion mechanism. The mass mortality was recorded during the hatching period caused by oxygen Withdrawal (Espeland *et al.,* 2004). In the present study it has been confirmed by fungal isolation and microscopic studies where the infected *C. carpio* eggs showed numerous fungal hyphae attached to the outer surface of the whole eggs besides pores in the inner envelope with focally invaded to the cytoplasm. This result is in agreement with the work of Hanjavanit *et al.,* (2008) and Paxton and Willoughby (2000) recorded that histopathological examination of infected eggs showed numerous fungal hyphae on the outer surface of the eggs and may penetrate the egg envelope and in some cases infected eggs showed germinated zoospores in the cytoplasm. This may return to the characterization of damaged dead egg or degradation of the egg envelope structural components by fungi released enzyme that

facilitate hyphae penetrated across the egg envelope and accumulated in the cytoplasm (Rand *et al.,* 1992). In the hatchery, fungal infection could be a significant cause of mass mortality in *C. carpio* eggs especially in the presence of dead eggs and high organic matter which considered as good media for these pathogens. Without disinfectant treatment, these pathogens can rapidly overgrow leading to reduce the hatching rates in hatcheries.

In the present study three different disinfectants with their three different concentrations were used for common carp egg treatment to increase the hatching rate. Among all the treatment 1mg/L methylene blue, 3mg/L malachite green and 1g/L sodium chloride showed better hatching rate 95·33±2·08%, 88.00±2.64 % and 92.33±4.04% respectively. This confirmed the report of Yahya *et al.* (2013) that sodium chloride disinfectant treatment at the rate of 1.5/L showed significantly higher hatching rate (77.17±1.81 %). Sodium chloride is considered as a safe, common substance which has antimicrobial properties (Kitancharoen *et al.,* 1997). Edgell *et al.,* (1993) found that salt treatment at 20 ppt for 60 minutes was the best to prevent mortality amongst salmon eggs. The lists of fungal treatment medicine are also found in Chowdhury *et al.,* (2015). Kitancharoen *et al,*.(1997) used sodium chloride at 20 ppt for 60 minutes two times a week to treat rainbow trout eggs against saprolegniosis and yielded a hatching rate of 46.0 %. Salmonid and coregonid fishes treated by dipping in solutions of 20 to 50 g/l sodium chloride (Citek *et al.,* 1997). Bolivar *et al.,* (2001) worked on effect of methylene blue and sodium chloride on the bacterial load in the transport water with Nile tilapia (*Oreochromis niloticus* L.) fingerlings. In that study they found that the highest percentage survivability (99.6%) was found in 1mg/L of methylene blue treatment, followed by treatment 2 (99.4%), then by treatments 4 and 3 with 99.3% . Treatment 1 had the lowest survival rate with 99.2%.

In the present study among all the treatment 1mg/L methylene blue showed the highest survival rate (95·00±4.35 %) of common carp larvae. This result tallies with the work of Bolivar *et al.,* (2001) that 1mg/L of methylene blue showed the highest survival rate (99·6%) among all the treatment trial. In the present study sodium chloride also show the better survival rate of common carp larvae. Among three different concentration of sodium chloride disinfectants 1g/L sodium chloride show better survival rate (87.00±6.24%). This confirmed the report of Yahya *et al.,* (2013) that sodium chloride disinfectant treatment at the rate of 1.5/L showed significantly higher survival rate (83.98±1.34%). Sodium chloride is considered as a safe, common substance which has antimicrobial properties (Kitancharoen *et; al* 1997). Edgell *et al.* (1993) found that salt treatment at 20 ppt for 60 minutes was the best to prevent mortality amongst salmon eggs. Kitancharoen *et al.*(1997) used sodium chloride at 20 ppt for 60 minutes two times a week to treat rainbow trout eggs against saprolegniosis and yielded a hatching rate of 46.0 %.Salmonid and coregonid fishes treated by dipping in solutions of 20 to 50 g/l sodium chloride (Citek *et al.,* 1997).

5. Conclusions

The fungi*Saprolegnia* spp. fungus commonly attack the *C. carpio* fertilized eggs during the incubation period. High egg densities, organic matter in fish hatcheries beside the regular removal of dead eggs, as they are considered the basic environment for microbial overgrowth which hamper egg development and subsequently affect hatching and larval survival rates. Treatment of methylene blue at 1mg/L, malachite green at 3mg/L and sodium chloride at 1 g/L for 30 min. bath daily for 4 days showed significantly ($P<0.05$) higher hatching rate and survival rate.

Conflict of Interest

None to declare.

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