Evaluation of the effects of different concentrations of neutral anolyte on fungal infected eggs in rainbow trout *(Oncorhynchus mykiss)* in comparison with green malachite

Sharifpour I.[1]*; Kakoolaki S.[1]; Mehrabi M.R.[1]; Gheyasi M.[2]; Najjar Lashkari S.[3]

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Abstract

The objective of this study was to determine the effect of different concentrations of anolyte on saprolegniasis in comparison with green malachite in rainbow trout hatcheries, in the northern part of Iran, Tonekabon. Nearly 5000 green eggs of *Oncorhynchus mykiss* (equivalent to 300 g) were obtained from a private farm in the north of Iran. The study was designed in 5 treatments, 0.25, 0.5, 25, 30 and 100 ppm of neutral anolyte and 2 ppm of green malachite and two controls including positive control, meaning that the green eggs were purposely infected with saprolegnia but without any disinfectant, and negative control, which was implied to as the untreated group, all in triplicate. There was no significant difference ($\alpha>0.05$) in hatchability percent between 2 ppm of green malachite and the group treated with 0.25 ppm of neutral anolyte, contrary to other anolyte concentrations. It is concluded that constant use of 0.25 ppm of neutral electrolyzed oxidized water (NEOW) is a more effective anti-fungal solution with the least side effects in comparison with 2 ppm of green malachite.

Keywords: Neutral anolyte, Fungal infection, Saprolegnia, Green malachite, Rainbow trout *(Oncorhynchus mykiss)*

1-Department of Aquatic Animal Health & Disease, Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization, Tehran, Iran.

2-Caspian Sea Ecology Research Center, Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization.

3-Cold water Fishes Research Center (CFRC), Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization, P. O. Box: 46815-467 Tonekabon, Iran

*Corresponding author's email: isharifpour@yahoo.com

Introduction

Today, rainbow trout (*Onchorhynchus mykiss*) has become the top ranking species among cold water fishes in the world, due to traits such as accessible artificial propagation, a good adaptation to intensive culture conditions with commercial food habits and an appropriate growth rate (Vosoughi and Mostajir, 2001). Based on the annual statistics of Iran Fisheries Organization (IFO, 2000) rainbow trout production increased from 9000 MT in 1990 to 91519 MT in 2000.

Eyed egg fungus infection is one of the worst constraints in rainbow trout hatcheries in which *Saprolegnia* spp. is considered as an invasive pathogen including *S. parasitica, S. diclina, S. ferax* and *S. monica* (Ghiasi, 2008). Saprolegnia can infect the dead eggs and be contagious for live eggs due to a positive chemotaxic potential.

Since the establishment of the fungus on dead eggs, zoospores occur, which result in distribution throughout the dead eggs, rapidly (Espeland and Hansen, 2004).

Over the past decade, this constraint was solved well with a powerful fungicide, malachite green (Pottinger and Day, 1999). It has been used broadly by hatchery owners, throughout the world for many years due to the lack of an approved anti fungal alternative (Fitzpatrick *et al*., 1995; Kitancharoen *et al.*, 1997). Malachite green is known as carcinogenic (Pottinger and Day, 1999), mutagenic and teratogenic (Meyer and Jorgenson, 1983). On the other hand, some researchers (Meinertz *et al*., 1995) found undetectable residues in rainbow trout, which were already exposed to malachite green.

Salt is known as a safe, familiar material, which has been extensively used as a disinfectant in aquaculture. It is more expensive than other routine substances but it is an effective disinfectant which at more than 30 ppt concentration affects the fish osmo-regulation and is harmful for them (Kitancharoen *et al*., 1997). Hydrogen peroxide rapidly disintegrates to water and oxygen when in contact with oxidizable organic matter. There is no proof to show hydrogen peroxide as a carcinogenic or teratogenic material (EMEA, 1996) but its use should be considered in terms of fish species or age and water temperature. Nowadays, one of the most important disinfectants, which has been used in poultry husbandry, is electrolyzed water especially with 0.2 percent of added salt (Yoshida, 2003; Al-Haq *et al*., 2005). Although, hypochlorite which is produced in the electrolyzed water process, is a weak acid it has good disinfectant potentiality for aquaculture use (White, 1992).

The objective of our study was to determine the effect of different concentrations of anolyte on saprolegniosis in comparison with malachite green in rainbow trout hatcheries, in the northern part of Iran, Tonekabon.

Materials and methods

Eggs and protocol

Nearly 5000 green eggs of *O. mykiss* (equivalent to 300 g) were purchased from a private farm in Tonekabon, north of Iran. Our study was designed with 5 treatments, 0.25, 0.5, 25, 30 and 100 ppm of neutral anolyte and 2 ppm of green malachite and two controls including positive control, meaning that the green eggs were purposely infected with saprolegnia but without any disinfectant, and negative control, which was implied to as the untreated group, all in triplicate. The temperature of water was maintained at 14^{o}C and the green eggs were then acclimated with the new water condition before distribution among the 21 troughs (treatments) positioned in one row (Fig. 1).

Figure 1: Californian troughs covered by a layer of solid plastic sheets (carton plast) were used for incubation.

Solution and measurement

For each trough, fifty infected eggs bundled in clean cheesecloth were used to infect the eggs in troughs (Fig. 2). Physico-chemical parameters were the same for all troughs. Envirolit, ela 2000 apparatus, made in Holland, was used to produce neutral anolyte. Anolyte solutions were continuously added to treatments, 0.5 and 1 ppm to obtain the required concentrations and it was extended for 48h before the eggs hatched. In other treatments, solutions were added to the trough water every half an hour in 48h in distinct concentrations. Chlorine measurement was carried out using titration method to record the values for each treatment during the study (Clesceri *et al*., 2005).

Figure 2: A bundle of infected eggs in cheesecloth for each trough.

Mortality percent and disorders

Isolation and counting of dead eggs was done 2 times, in the eyed-egg and yolk sac fry phases, every 3 days.

The former phase started on the 13th and ended after 14 days. Abnormal fry and hatching rate were enumerated 6 days and 72 h after hatching time, respectively (Arndt *et al*., 2001) in each group and replication. Crippled fries were then removed and counted. Caudal fin snail form, yolk sac disorder and angled spine were considered as deformities. The study span took nearly one month.

Mycological examination

The infected eggs were squeezed together with a drop of normal saline between two slides and examined at low magnification. Eggs with hyphae were taken for fungal isolation according to Ghiasi (2008) using sabouraud dextrose agar (SDA) and glucose-yeast extract (GY) agar (Fig. 3). Fungal colonies were countered using digital colony counter (IRMECO: Germany).

Wet smears from the isolated colonies were prepared and stained with lactophenol methylene blue and analyzed under light microscope (Ghiasi, 2008).

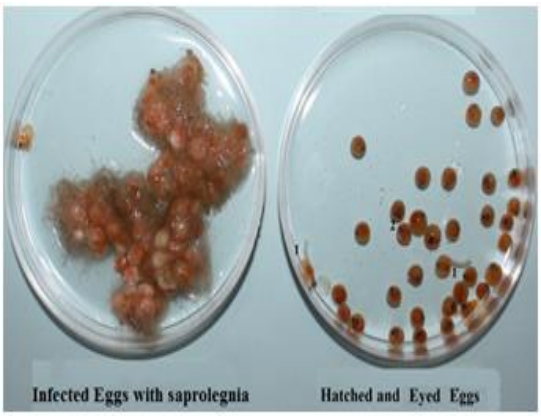

Figure 3: Left: Disinfectant solution did not affect the treated eggs, so saprolegniasis with distinct hyphae recorded; Right: Yolk sac fry (1) and eyed eggs (2) observed when proper dosage of disinfectant solution was used.

Formulae and calculations

The following formulae were used to calculate the indices of the study:

Fungal infected eggs=[no. of infected eggs/total eggs]×100 (Barnes *et al*., 1998)

Eyed egg percent=[no. of eyed eggs/ total eggs-primary mortality]×100

Hatchability percent=[no. of hatched eggs/total eggs]×100 (Arndt *et al*., 2001)

Abnormality percent= [no. of abnormal fries/total hatched eggs]×100 (Arndt *et al*., 2001)

Statistical analysis

Paired sample T- student test was used to determine the differences in the colony quantities before and after use of solutions in different concentrations. Levene's test was used as a pre-condition test along with t-test to find out whether the variances of paired samples were equal. To find out if there were any differences between the different substances a one-way ANOVA was used. The null-hypothesis, which stated that there was no difference between the variables, was rejected at $p<0.05$. Mann-Whitney test was used to show the significant differences between the groups and the ranks between them were evaluated two by two using SPSS software (V. 16).

Results

Values of physicochemical parameters of Tonekabon fish hatchery water samples taken in 2013 are listed in Table 1 and the values of measured colonies are shown in Table 2. In all treatments, significant differences ($\alpha<0.05$) were observed before and after treating (Table 2). Fig. 4 shows sporangia and hyphae in sampled infected eggs.

Table 1: Physico-chemical factors (mean±SD) of the trough water of each group.

	t.1	t.2	t.3	t.4	t.5	t.6	t.7
T^{O}C	15.15±1.18	15.15±1.18	15.15±1.18	15.15±1.18	15.15±1.18	15.15±1.18	15.15±1.18
Chlorine	0.24±0.05	0.51±0.06	33.70±8.35	98.70±11.44			
Hardness	259.3±1.70	261±2.18	263.15±7.50	270.75±17.80	269.80±18.00	270.75±18.80	270.75±17.50
DO	6.95±0.14	6.98±0.17	6.93±0.13	6.84±0.17	6.95±0.16	6.98±0.12	6.99±0.18
pH	7.76±0.04	7.75±0.05	7.73±0.04	7.74±0.06	7.75±0.05	7.75±0.06	

0.25, 0.5, 30 and 100 ppm of neutral anolyte (t.1-t.4), 2 ppm of malachite green (t.5) and positive and negative controls (t.6-t.7)

Table 2: Quantities of fungal colonies from different treatments (n=9; α<0.05)

Treatments	Groups	Mean±SEM	*p* value
t.0.25	1	107.77±5.95	0.000
	2	68.88±3.88	.
t.0.5	1	153.33±15.89	0.000
	2	66.66±5.52	
t.30	1	45.55±6.03	0.004
	2	22.22±3.64	
t.100	1	24.44±4.74	0.041
	2	12.22±2.77	
t.mal.	1	46.66±8.97	0.002
	2	13.33±2.35	

Group 1=before treatment, group 2=after treatment; t.0.5=treatment 1; t.1= treatment 2; t.25= treatment 3; t.100= treatment 4; t.mal= malachite green treatment

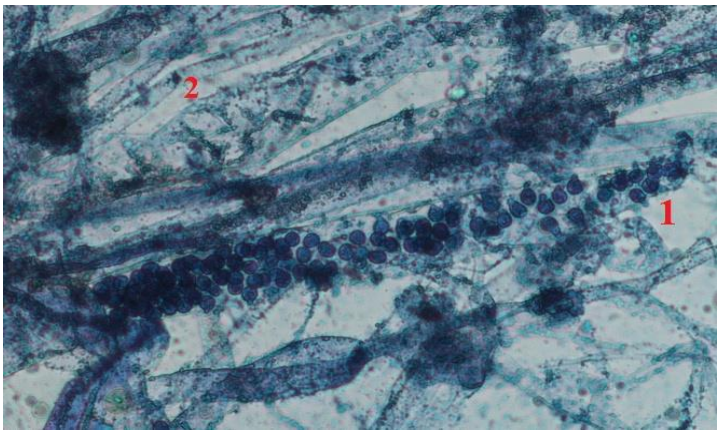

Figure 4: Grown saprolegnia on dead eggs was smeared as wet; sporangia (1) Hyphae (2) of saprolegnia ×1000.

According to the results of Table 3, there was no significant difference (α>0.05) in hatchability percent between 2 ppm of malachite and constant 0.25 ppm of neutral anolyte in comparison to other anolyte concentrations.

Table 3: Analysis of variances among groups in case of some selected growth and health indices (*n*=9, α=0.05).

	0.25	0.5	30	100	Green malachite	F-test	*p* value
Fungal count	68.88±3.88^{a}	66.66±5.52^{a}	22.22±3.64^{b}	11.11±3.09^{b}	11.11±2.60^{b}	47.80	0.000
Hatchability%	88.17±0.85^{a}	0^{b}	0^{b}	0^{b}	87.63±2.57^{a}	11.95	0.000
Eyed-egg%	73.41±12.17^{a}	37.16±7.73^{ab}	38.83±4.78^{acd}	27.08±2.76^{bc}	65.66±5.81^{ad}	4.60	0.009

Different superscripts in each row imply on significant difference

Table 4: The mean rank of anomalies between groups 0.25 of anolyte (1) and green malachite (2); (*n*=3, α=0.05).

	Groups	Mean rank	Sum of ranks	Mann-Whitney U	Asymp. Sig. (2-tailed)
Anomaly	1	5.00	15.00	0.000	0.05
	2	2.00	6.00		

Discussion

Due to the low temperature of water and the long incubation time for eggs, fungal contamination of fertilized eggs of rainbow trout in hatcheries is one of the major casualties in mortality (Ebrahimzadeh Mousavi *et al*., 2004). Many studies showed the effect of electrolyzed oxidized water (EOW) on hyphae and spores of different types of saprophytic fungus (Buck *et al*., 2002). Our results showed a decrease in the fungal colony number (α<0.05) after adding the anolyte or malachite green in selected concentrations. Studies showed that EOW has a proper bactericidal effect on gram negative bacteria, gram-positive and specially mycobacterium (Fenner, 2005). This difference before and after adding solutions were conspicuously higher in 0.25 and 0.5 ppm of the anolyte.

On the other hand, the higher concentrations of anolyte showed side effects on hatchability index and based on our results, no hatch was observed in 0.5, 30 and 100 ppm of anolyte. Kiura *et al*., (2002) demonstrated that EOW in exposure to bacteria i.e. *Bacillus subtilis* results in membrane fracture and bubble creation at higher doses.

In case of hatchability, 0.25 ppm of neutral anolyte and 2 ppm of green malachite had good effective properties on hatching of eggs, in addition to fungal colony reduction. No significant difference was observed in hatchability percent for both solutions. Despite the equality of hatchability percent (Table 3) in the use of green malachite (87.63±2.57) in comparison to the value of 0.25 ppm of neutral anolyte (88.17±0.85) there was higher ranking of group 0.25 ppm of anolyte against the green malachite. Therefore, the constant use of 0.25 ppm of anolyte is more recommended than green malachite due to the lower abnormality percent in the former group.

It is concluded that EOW is a more effective anti-fungal solution with least side effects in lower concentration in comparison to green malachite.

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