.EFFECT OF METHYLENE BLUE AND SODIUM CHLORIDE ON THE BACTERIAL LOAD IN THE TRANSPORT WATER WITH NILE TILAPIA (*Oreochromis niloticus* L.) FINGERLINGS

Remedios B. Bolivar[1], Michael Angelo D. Aragones[1] and Gemerlyn G. Garcia[2]

[1] Freshwater Aquaculture Center-College of Fisheries, Central Luzon State University, Science City of Muñoz, Nueva Ecija 3120, Philippines

[2]College of Veterinary Science and Medicine, Central Luzon State University, Science City of Muñoz, Nueva Ecija 3120, Philippines

Abstract

The study evaluated the effect of two chemicals (sodium chloride and methylene blue) on bacterial load in the transport water with size # 24 *Oreochromis niloticus* fingerlings following concentrations used under farm practice and from the literature. The five treatments used were: Treatment 1 (control), Treatment 2 (1 g/l of sodium chloride), Treatment 3 (2 g/l of sodium chloride), Treatment 4 (3 mg/l of methylene blue), and Treatment 5 (1 mg/l of methylene blue).

The bacterial counts in Treatments 2, 3, 4, and 5 decreased after 12 hour of exposure. Treatment 4 obtained the highest decrease in bacterial count with 2.36×10^8 colony forming units per milliliter (cfu/ml), followed by Treatment 5 with 4.59×10^8 cfu/ml. The bacterial counts in Treatments 3 and 2 decreased to 2.12×10^8 cfu/ml and 2.79×10^8 cfu/ml, respectively. The bacterial count in Treatment 1 increased to 5.5×10^{10} cfu/ml after 12 hours.

Results showed that both methylene blue and sodium chloride were effective in reducing bacterial load during transport of fingerlings. Data analysis revealed that there were significant differences on the bacterial count at 6 and 12 hour of transport in each treatment ($P<0.05$). Linear contrast revealed that there was significant difference on the bacterial counts between Treatment 1 and Treatments 2, 3, 4, and 5 ($P<0.05$). No significant differences were observed in the bacterial counts among Treatments 2, 3, 4, and 5 ($P>0.05$).

Introduction

Tilapia culture is becoming an important part of the local fish culture industry in the Philippines. But as it continues to intensify, outbreaks of tilapia diseases have been observed to cause considerable financial and economical losses and create to tilapia growers. Fish losses due to diseases are now important problems that affect aquaculture venture and threaten the sustainability of the industry as a whole (Lavilla, 2001). According to Alapide-

Tendencia and de la Peña (2001), the control of disease is particularly difficult because fish are often farmed in systems where production is dependent on natural environmental conditions. 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. A constraint on disease control is the relatively limited number of therapeutic agents available for the control of bacterial disease agents.

Fish transport is often the final step in any hatchery operation. There is a need to produce healthy tilapia fry or fingerlings harvested from hatcheries to be delivered to grow-out farms. The ultimate goal of this transport operation is to provide fish in good condition that will survive after stocking in grow-out ponds. However, the survival of fingerlings during transport is very critical to both hatchery owners and grow-out operators. As the demand for tilapia fingerlings increases, it is therefore important for hatchery owners and fish farm operators to be more efficient in their loading and transport operations, taking into consideration the activation of latent disease organism and effect of stress to fish during hauling and transport activities.

To deal with the problem, hatchery owners use methylene blue ($C_{16}H_{18}N_3CIS.3H_2O$) and sodium chloride (NaCl) to prevent the proliferation of bacteria during fish transport. According to Jensen (1990), fish losses from death or injury are the result of the activation of latent disease organisms or osmoregulatory problem, causing mortality during transport and thereby affecting the profit margin. Knowledge on the effect of methylene blue and sodium chloride on the proliferation of bacteria during fish transport is important to both hatchery owners and grow-out operators. This is to prevent the possible spread and outbreak of the disease in the new environment where the fingerlings will be delivered. Moreover, proper hauling and transport of fish should be applied to lessen stress and mortality during transport. Experience and research have shown that fish transport can be improved by the addition of certain chemicals such as sodium chloride and methylene blue (Little, 2002 as cited by Fajardo, 2002).

In line with the development of a viable tilapia industry, effective disinfectant that can lessen or eliminate stress and bacterial load during transport must be identified. In this regard, the study attempted to find out the effect of methylene blue and sodium chloride in the transport water with Nile tilapia (*Oreochromis niloticus*) fingerlings.

Common table salt or sodium chloride is one of the most commonly used drugs in aquaculture and it is sometimes referred to as the "aspirin of aquaculture" (Swann and Fitzgerald, 1993). According to Francis-Floyd (1995), salt has many uses in modern aquaculture although FDA has not approved salt as a "drug" to treat fish, the agency has designated salt as a compound of "low regulatory priority". Salt is inexpensive, readily available, and when properly administered, safe for use in freshwater fish. It can be used to treat many external parasites including *Costia, Epistylis, Trichodina, Chilodonella* and flukes *Dactilogyrus* and *Gyrodactylus* (Swann and Fitzgerald, 1993). Salt may be used in the treatment of sliminess of the skin in the early stages of Pillularis and velvet diseases and against ectoparasites such as skin flukes (*Gyrodactylus*), anchor worms, and fish lice. It can also provide additional treatment in several cases of bacterial diseases (Van Duijn, 1973).

Weaker solutions containing 0.5 to 1.0 percent salt may be used as a bath for several hours to eliminate some freshwater parasites and 0.1 to 0.3 percent maybe used to enhance mucus production and osmoregulation in freshwater fish during handling and transport (Francis-Floyd, 1995). Sodium chloride can also be used in the treatment of freshwater parasite *Oodinium*. A dip of 80 seconds in 35 ppt is recommended or short bath for 5-15 minutes at 30 ppt for fish species capable of tolerating certain salinity levels. It can be used to treat external protozoan parasite and monogenetic trematodes (Plumb, 1992), and as prophylaxis against external parasites of freshwater fishes (Baticados and Paclibare, 1992). The effects of salt on fish are determined both by salt concentration and duration of exposure (Francis-Floyd, 1995). According to Swann and Fitzgerald (1993), the method of salt application depends on the disease organism, fish species, weight and types of aquaculture unit. Salt treatment methods include short-term dips, prolonged baths and indefinite treatment. Dip treatment involves exposing the fish to very strong solution of salt for short periods of time, usually 30 seconds to one minute. Prolonged salt baths are useful for treating fish in small tanks where salt solution can be flushed quickly. Indefinite treatments are used when transporting, handling fish, or when dealing with large volumes of water, such as in ponds.

Methylene blue is a redox dye which raises the oxygen consumption of cells. This means that the hydrogen to be oxidized is passed on to the oxygen. Each molecule of the dye is oxidized and reduced about 100 times per seconds. Thus, while disinfection results from this, methylene blue is also excellent against methemoglobin intoxication (Schaperclaus, 1992). 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 and may be used as an alternative to malachite green for the control of fungus when it is known that the fish to be treated are sensitive. It is safe for use with fish eggs and fry for the prevention of fungal infection. According to Van Duijn (1973), methylene blue may be used for the treatment of *Ichthyophthiriosis* (white spot disease), skin and gill flukes, velvet disease, *Costiasis*, coral fish disease, *Chilodonelliasis* and as a palliative medicine in all cases of disease of the gills, where fishes suffer from difficulty in breathing. According to Schaperclaus (1992), methylene blue acts as an inhibitor of bacteria and fungi, and is well tolerated by most fish species. It is particularly effective against *Saproglenia*, *Costia*, *Trichodina* and *Chilodinella* species by applying 3 milligrams of methylene blue per liter of water for long duration or 30 milligrams of methylene blue per liter of water for short duration bath.

According to Anderson (1992), methylene blue can be used for the treatment of integumentary mycosis on all ages of freshwater fishes in ponds and raceways at an application rate of 2 to 2.5 mg/l in a permanent bath. When used against gill rot and other bacterial disease, the rate is at 8-10 ppm following a bath treatment. It is also applied during quarantine treatment of aquarium fishes especially for breeders by using indefinite bath of 1 ppm. At a concentration of 2-3 ppm, it can be used as an indefinite bath for fish at all ages (Subasinghe, 1992). At 1-2 ppm, it can be used for the treatment of *Icthyopthirius multifilis* (Puwaputanon and Chinabut, 1983 as cited by Tonguthai and Chanratchakool, 1992).

For the chemicals used in the study, dosages of 2 g/l of sodium chloride and 1 mg/l of methylene blue as practiced by the Bureau of Fisheries and Aquatic Resources-National Freshwater Fisheries Technology Center (Morales, pers. comm.) were adopted in the transport of tilapia fingerlings while 1 g/l of sodium chloride (Francis-Floyd, 1995) and 3 mg/l of methylene blue (Schaperclaus, 1992) were the dosages used based from the literature.

This study was conducted at the College of Fisheries and the Freshwater Aquaculture Center (FAC), Central Luzon State University (CLSU), Science City of Muñoz, Nueva Ecija. Philippines. Bacterial analysis was done at the Veterinary Microbiology Laboratory of the College of Veterinary Science and Medicine at CLSU from February 9-13, 2004.

The general objective of this study was to determine the effect of methylene blue and sodium chloride on the bacterial load in the transport water with *O. niloticus* fingerlings. Specifically, the study aimed to investigate the effect of two different concentrations of methylene blue (farm practice and based from the literature) and two concentrations of sodium chloride (farm practice and based on the literature) on the bacterial loads in the transport water with *O. niloticus* fingerlings and to compare which concentration is more effective.

Materials and methods

The study used two chemicals, sodium chloride and methylene blue at concentrations used as farm practice and as based from the literature. The chemicals were added in the transport water. Five treatments were used and were as follows: Treatment 1 (control – no chemicals added), Treatment 2 (1 g/l of NaCl - based on the literature), Treatment 3 (2 g/l of NaCl – based on farm practice), Treatment 4 (3 mg/l of methylene blue - based from the literature), and Treatment 5 (1 mg/l of methylene blue – based on farm practice). Each treatment was replicated three times.

Nile tilapia fingerlings of size # 24 (weight range: 0.055 to 0.096 g) were used as test species. The fingerlings were acquired from the Bureau of Fisheries and Aquatic Resources-National Freshwater Fisheries Technology Center (BFAR-NFFTC), Central Luzon State University, Science City of Muñoz, Nueva Ecija. The test fish were conditioned for one day in a concrete tank with aeration. No feeding was done to prevent the accumulation of feces that can deteriorate the water quality.

Fifteen polyethylene bags each measuring 20 x 30 x 0.003 in, were filled up with four liters of water from the conditioning tank. Obtaining water from the conditioning tank is a practice done by the BFAR and the Phil-FishGen hatcheries when transporting tilapia fingerlings. Thus, the same practice was followed in this study. Each polyethylene bag was lined with another plastic bag of the same dimension for additional protection against leakage.

Before introducing the fingerlings to the polyethylene bags for transport, the desired concentrations of the sodium chloride and methylene blue were mixed to the transport water.

A loading rate of 1,100 pieces of size # 24 Nile tilapia fingerlings per bag was used in this study. Each bag was filled with oxygen immediately after mixing the chemicals to the water and loading the fingerlings.

Fingerlings were subjected to 12 hours transport from CLSU to Pampanga and back to CLSU. Re-oxygenation of the transport water was done after collection of water samples for bacterial counting and for water quality monitoring.

Designated bags win each treatment were opened after 6 hours for water quality monitoring and collection of water samples for bacterial counting. Since the study simulated a transport procedure, contamination of the transport water during the opening of the polyethylene bags for re-oxygenation and collection of water samples was possible especially so that the study was done in a non-sterile environment. Collection of samples was done by getting 100 ml of water from the polyethylene bags using a pipette at every six-hour interval. The water samples were placed in test tubes. Bacterial counting was done using serial dilution techniques by adapting the procedure prescribed by Garcia (2000). The transport water was initially analyzed for temperature and dissolved oxygen using dissolved oxygen meter and pH using a pH meter. The fingerling mortality was determined and gross examination of the physical appearances of the fingerlings was done at the end of the transport.

Serial dilution of bacterial suspensions taken from the water samples was done up to 10^{-7} in sterile distilled water. A volume of 0.01 ml from suspension of 10^{-5}, 10^{-6}, and 10^{-7} dilutions were placed in petri plates containing Tryticasein Soy Agar. The cover of the petri plates was marked into three divisions corresponding to the designated serial dilution. Each petri plate was replicated three times. The petri plates were incubated in an inverted position at 37^{0}C for 24 hours. The average count of colonies from the designated division of each of the replicates was taken as mean ($\pm$ standard deviation). The number of colony forming units per ml (cfu/ml) of bacterial suspension was computed by adopting the following formula:

For 5th dilution = mean x 100 x 10^{5}
For 6th dilution = mean x 100 x 10^{6}
For 7th dilution = mean x 100 x 10^{7}

where:

mean = average count of the designated dilution in the three petri plates
100 = its reciprocal value correspond to the volume of bacterial suspension plated in one designated division (that is 1/100 of the total volume of bacterial suspension from each serially diluted sample).

The aforementioned procedure was undertaken at each indicated time interval. The different data gathered were water quality parameters which included temperature, dissolved oxygen, pH and ammonia; % mortality and survival, and the bacterial counts at every 6-hour interval.

Data were analyzed using analysis of covariance (ANCOVA) to determine the effect of initial bacterial count among treatments. This was done due to the differences in the initial

bacterial count among treatments. If the covariate (bacterial count at 0-hour) showed no significant effect, the data were analyzed using analysis of variance (ANOVA) in completely randomized design (CRD) at 5 percent level of significance. Group of treatments were compared using linear contrast.

Results and discussion

Bacterial counts in the water with size # 24 Nile tilapia fingerlings at 0, 6 and 12 hours of transport are presented in Table 1.

Table 1. Bacterial counts in the water with size # 24 Nile tilapia fingerlings at 0, 6, and 12 hours of transport.

Treatment	Bacterial count (cfu/ml)		
	0-hr	6-hr	12-hr
1	3.5×10^{9}	5.6×10^{9a}	5.5×10^{10d}
2	5.1×10^{8}	3.8×10^{8b}	2.8×10^{8e}
3	4.5×10^{8}	3.5×10^{8b}	2.1×10^{8e}
4	3.3×10^{10}	1.6×10^{10bc}	4.3×10^{8ef}
5	4.7×10^{9}	3.7×10^{9bc}	4.6×10^{8ef}

Mean values with the same superscript letter/s were not significantly different ($P>0.05$).

The bacterial counts in Treatments 2, 3, 4 and 5 decreased after 12 hours. Treatment 4 had the highest initial bacterial count of 3.3×10^{10} cfu/ml and decreased to 4.3×10^{8} cfu/ml after 12 hours while Treatment 5 had an initial bacterial count of 4.7×10^{9} cfu/ml and decreased to 4.6×10^{8} cfu/ml after 12 hours. The initial bacterial counts in Treatment 3 was 4.5×10^{8} while in Treatment 2, the initial bacterial count was 5.1×10^{8}. After 12 hours of transport, the bacterial counts in these treatments reduced to 2.1×10^{8} and 2.8×10^{8}, respectively. The bacterial count in Treatment 1 increased from 3.5×10^{9} to 5.5×10^{10}.

Analysis of covariance at 6 hours and analysis of variance at 12 hours of the bacterial load in the transport water revealed that there were significant differences among treatments ($P<0.05$). Figure 1 shows the different bacterial counts in each treatment at 0, 6 and 12 hours of transport. Figure 2 shows the changes in bacterial count at every six hours interval.

The highest percentage mortality was found in Treatment 1 (0.8%), followed by Treatment 4 (0.7%), then by Treatments 2 and 3 with 0.7% and 0.6%, respectively (Table 2). Treatment 5 had the lowest mortality with 0.4%. Analysis of variance showed that there were no significant differences on the percentage mortality among treatments ($P>0.05$).

Summary of water quality parameters at 0, 6, and 12 hours of transport of size # 24 Nile tilapia fingerlings are presented in Table 3. Initial pH values were both high in Treatment 4 and Treatment 5 with 8.2 and 8.1 respectively, followed by the control with pH value of 8.0, while Treatments 2 and 3 had pH values of 7.6 and 7.7, respectively. After 6-hours, the pH values decreased in all treatments but at 12 hours of transport, the pH values decreased in Treatments 1, 4, and 5 as compared to the start of the transport (0-hour) while

pH value increased in Treatment 2. In Treatment 3, the pH value at 0 and 12 hours of transport were the same. Temperature readings from 0 to 12 hours increased in all treatments. Temperature at 0-hour ranged from 25.2 to 25.8^{0}C and increased from 29.1 to 29.5^{0}C after 6 hours. The temperature ranged from 30.2 to 30.7^{0}C. Treatment 2 had the lowest temperature while Treatment 5 had the highest temperature after 12 hours of transport. Dissolved oxygen concentration ranged from 18.2 to 18.6 mg/l at 0-hour and decreased after 6 hours of transport. At 12 hours, the dissolved oxygen levels were slightly higher than at 6 hours of transport except in Treatment 3.

Table 2. Mortality (number and percent) and percent survival of size # 24 Nile tilapia fingerlings after 12 hours of transport.

Parameter	Treatment				
	1	2	3	4	5
Average no. of Mortality	9	6	7	8	4
Mortality (%)	0.8	0.6	0.7	0.7	0.4
Survival (%)	99.2	99.4	99.3	99.3	99.6

Table 3. Water quality parameters at 0, 6 and 12 hours of transport.

Parameter	Duration of transport (hour)	Treatment				
		1	2	3	4	5
pH	0	8.0	7.6	7.7	8.2	8.1
	6	7.7	7.3	7.5	7.7	7.5
	12	7.7	7.8	7.7	7.7	7.6
Temperature (^{o}C)	0	25.2	25.4	25.5	25.8	25.3
	6	29.4	29.3	29.5	29.1	29.1
	12	30.4	30.2	30.3	30.3	30.7
Dissolved oxygen (mg/l)	0	18.6	18.2	18.5	18.6	18.2
	6	14.7	14.4	14.9	14.7	14.1
	12	15.0	14.5	14.3	15.4	15.4

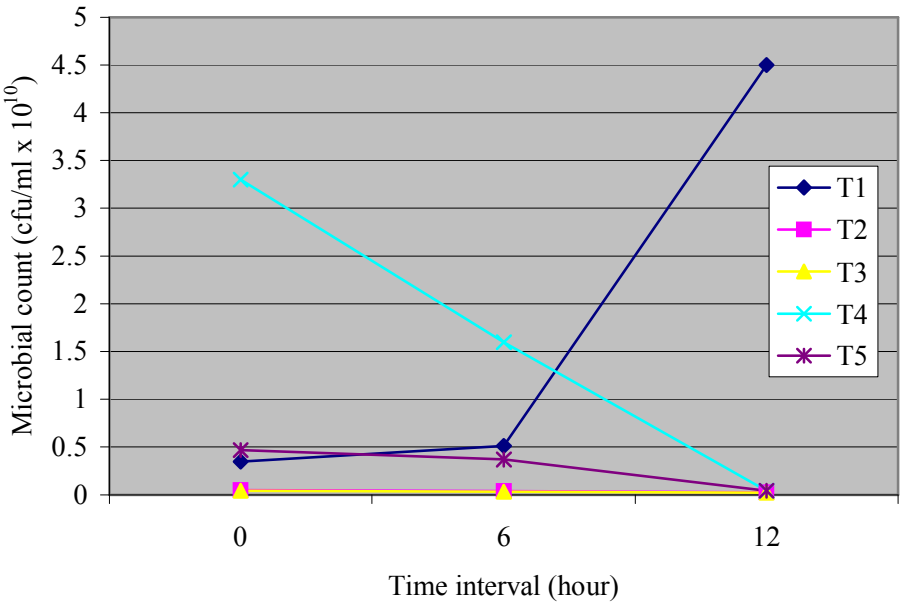

Figure 1. Bacterial count at 0, 6 and 12 hours of transport of size # 24 Nile tilapia fingerlings.

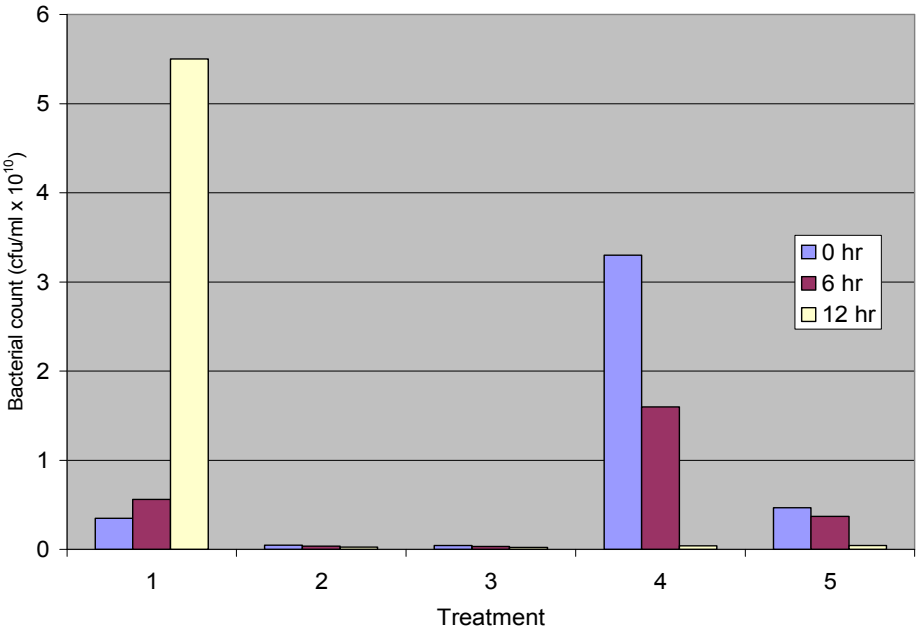

Figure 2. Changes in bacterial count in the five treatments at every 6-hour interval.

Based from the results, both methylene blue and sodium chloride were effective in reducing the bacterial counts in the water during the12 hour transport of size # 24 Nile tilapia (*O. niloticus*) fingerlings. Treatment 4 (3mg/l of methylene blue based from the literature) obtained a higher decrease in bacterial counts compared to Treatment 5 (1mg/l of methylene blue as farm practice). While Treatment 3 (2 g/l of sodium chloride based on farm practice) obtained greater decrease in bacterial count than Treatment 2 (1g/l of sodium chloride based from the literature) after 12 hour of exposure. The highest decrease in Treatments 4 and 3 maybe due to the higher dosages of methylene blue and sodium chloride, respectively used in the study. Mean bacterial counts using methylene blue (both farm practice and based from the literature) showed greater decrease compared to the mean bacterial count when using sodium chloride (farm practice and based from the literature).

Furthermore, data analysis on the bacterial count revealed that there were significant differences among treatments on bacterial count at 6 and 12 hours of transport. Linear contrast among treatment means between Treatments 1 and 2, Treatment 1 and 3, Treatment 1 and 4 and Treatment 1 and 5 showed that there were significant differences in their bacterial counts at 6 and 12 hours of transport ($P<0.05$). Concentration of sodium chloride based from literature (Treatment 2) showed no significant difference with the concentration used based on farm practice (Treatment 3). Concentrations of methylene blue based from literature (Treatment 4) and farm practice (Treatment 5) were not significantly different ($P>0.05$. It was also observed that there were no changes in the physical appearances and behavior of the fingerlings before, during and after the transport.

Summary, conclusion and recommendation

The study evaluated the effect of chemicals (methylene blue and sodium chloride) on the bacterial load in the transport water with size # 24 Nile tilapia (*O. niloticus*) fingerlings at concentrations based from literature and as farm practice. Water quality parameters such as temperature, pH and dissolved oxygen and bacterial count were analyzed at every six-hour interval. Number of mortalities and percentage mortality were determined at the end of the transport.

Highest decrease in bacterial count of the chemical treated treatments was obtained in Treatment 4 with 3.3×10^{10} cfu/ml and decreased to 4.3×10^{8} cfu/ml. After 12 hours, the bacterial count in the control increased from 3.5×10^{9} to 5.5×10^{10} cfu/ml. Treatment 1 had the highest percent mortality with 0.8% followed by Treatment 4 with 0.7% while Treatment 5 obtained the lowest percent mortality with 0.4%.

In this study, methylene blue and sodium chloride were found to be effective in reducing the bacterial load during transport. Furthermore, no significant difference between concentrations of methylene blue based from literature and farm practice was observed ($P>0.05$). For sodium chloride, concentration used in farm practice and based from literature were not significantly different.

It is recommended that a similar study should be undertaken using other chemicals to reduce the bacterial load in the transport water. Also, it is recommended that identification of bacteria present in the transport water should be done in future studies.

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