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Effect of Sanitizer Treatment on Bacteriology of Microcosm Simulating Shrimp Pond Ecosystem

B.M. NAIK, M.N. VENUGOPAL, I. KARUNASAGAR and I. KARUNASAGAR*

Department of Fishery Microbiology
University of Agricultural Sciences, College of Fisheries
Mangalore – 575 002
India

Abstract

The effect of three commonly used water sanitizers, viz benzalkonium chloride (BKC), sodium hypochlorite and iodophor on shrimp pond associated microbiota was studied in laboratory microcosms. Treatment of microcosm with 0.1 ppm BKC did not lower the total heterotrophic bacterial count. With 3.0 ppm, there was an initial reduction of one log unit at 8h followed by an increase in bacterial counts at 18h. Ammonia oxidizers on the other hand were affected even with 0.1 ppm. Sodium hypochlorite at 10 ppm brought about one log reduction in heterotrophic bacterial count and the level of ammonia oxidizers was also reduced. Treatment with 10 ppm iodophor did not affect both autotrophic and heterotrophic bacteria. The results suggest that sanitizers should be used with caution in shrimp ponds due to their deleterious effect on useful microorganisms involved in mineralization.

Introduction

Application of scientific methods in aquaculture has become an important requirement for the sustained commercial production of aquatic

**Corresponding author*

animals such as fish and shellfish for human consumption. Increased export demand for shrimps as well as high economic returns from shrimp culture operations has resulted in the establishment of a large number of shrimp culture farms along the coastal states of India. Emergence of diseases affecting cultured shrimp is adversely affecting the shrimp culture industry and several bacterial, viral and protozoan diseases have been reported in shrimp hatchery and culture systems. The luminous vibrio disease in hatcheries and the white spot syndrome virus (WSSV) disease in culture systems have caused great economic losses in India as in other countries (Karunasagar et al. 1994, Otta et al. 1999). This has resulted in the indiscriminate use of antimicrobials and chemicals to control infection. Emergence of antibiotic resistant bacteria is a major problem in hatcheries (Karunasagar et al. 1994, Plumb 1995) and to overcome the detrimental effect of antibiotics, sanitizers are used. They reduce the microbial population to safe levels as judged by public health requirements and are commonly used in water, in larval tanks, on equipments and to treat eggs. Sanitizers are compounds effective against all types of infectious agents including bacteria, fungi, viruses and protozoa (Pelczar 1996) and differ greatly in their physical, chemical and cidal properties, mode of action, trade names, composition and availability (Hugo and Russel 1982). Commonly used sanitizers in aquaculture include chlorine and iodine based compounds and quaternary ammonium compounds (Plumb 1995). Quaternary ammonium compounds like benzalkonium chloride (BKC) are used for the treatment of columnaris disease in juvenile fresh water fish and bacterial gill disease in juvenile salmonids, enteric red mouth disease of rainbow trout (*Oncorhynchus mykiss*) and vibriosis in shrimp (Anderson and Conroy 1969, Amend 1970, Austin and Stobie 1992, Bullock and Conroy 1971, Kongkaew 1988).

The complete inactivation of planktonic cells of *Vibrio parahaemolyticus* was achieved with a concentration of 0.5 $mg \cdot l^{-1}$ chlorine within 15 min contact period (Venugopal et al. 1999). Sodium hypochlorite at 10 ppm level was able to reduce viable *Aeromonas salmonicida, V. anguillarum* and *V. ordalii* (Ross and Smith 1972).

Use of iodophor 'Beffodyne' and 'Wescodyne' for the treatment of salmonid eggs has been reported by Alderman (1984). Iodophors have demonstrated excellent activity against viruses causing infectious hypodermal necrosis, infectious pancreatic necrosis and viral haemorrhagic septicaemia (Amend and Pietsch 1972) and are used as prophylactics against bacteria such as *A. salmonicida, A. liquefaciens, V. anguillarum* and *V. ordalii* (Ross and Smith 1972, Sako et al.1988).

Since sanitizers are nonspecific antibacterial agents, it is possible that they also kill/affect populations of useful bacteria that are involved in

nutrient cycling in a pond. The effect of sanitizers on nontarget useful bacterial populations in aquaculture systems has never been studied. In this paper, data on the effect of three sanitizers on total heterotrophic and autotrophic bacterial counts in a shrimp pond ecosystem is presented.

Materials and Methods

Three commercially available sanitizers designated A, B and C used by the shrimp farmers were included in the present study: benzalkonium chloride (A), sodium hypochlorite (B) and iodophor (C).

Test organisms

The bacterial cultures used for determining minimum bactericidal concentration (MBC) of sanitizers included the important shrimp pathogens *Vibrio harveyi* (luminescent and non-luminescent strain) and *V. alginolyticus*. They were selected from our laboratory stock cultures of *Vibrio* isolates from shrimp farms maintained in the deep freezer (Sanyo, Japan) at - 80°C in glycerol broth (15% - glycerol, 1% - NaCl and 1% - tryptone). The cultures were activated by subculturing on tryptone soya broth (Hi-media, Mumbai) containing 1% sodium chloride (TSBS) and maintained in tryptone soya agar supplemented with 1% salt (TSAS) slants for further studies.

Determination of minimum bactericidal concentration (MBC) of sanitizers

Available iodine in iodophor and chlorine in hypochlorite was estimated by the iodometric method (APHA 1998). After ascertaining the level of available compound, they were suitably diluted to get the desired concentration.

The MBC of the three different sanitizers to the test organisms was determined in sterile physiological saline (0.85% NaCl dissolved in distilled water, and autoclaved) as well as in TSBS. The test organisms were grown in TSBS for 18h at 30°C to have an activated culture. A graded concentration of the sanitizer was prepared separately in duplicate sets in 5 ml sterile physiological saline and TSBS. After careful mixing, 0.05 ml of the bacterial inoculum was added to these tubes and incubated at 30°C for 24 h. A loopful was then subcultured from the tubes that showed no growth on to TSAS plates and incubated at 30°C for 24 h for confirmation. The MBC was the lowest concentration of sanitizer in which no turbidity was found in the tubes and no colonies were observed on TSAS plates.

Preparation of microcosms

Microcosms simulating shrimp pond conditions were set up in circular plastic tubs of 50 L capacity as follows. The bottom sediment and water was collected from a commercial shrimp farm located near Kundapur, Udupi district Karnataka. The sediment was mixed well, transferred to plastic tubs to give about 15 cm layer and shrimp pond water was added over the sediment gently to all the tubs to have a water column of 20 cm. The experiment was conducted in duplicate for each concentration of sanitizer used.

The enumeration of heterotrophic bacteria from water and sediment in microcosm was carried out at 0, 8 and 18h after adding the sanitizer at the bactericidal concentration to the microcosm. For enumeration, 100 g sediment and 100 ml water was drawn from the tubs and serial ten-fold dilutions were made in 0.85% sterile physiological saline. Appropriate dilutions were spread on Zobell marine agar (Hi-Media, Mumbai) plates, incubated at 30°C for 24 – 48 h and counts reported as colony forming units (cfu^{-1}) ml or g of sample.

Most probable number (MPN) technique was followed for enumeration of autotrophic bacteria and this was done at 0 and 18 h after addition of the sanitizers. Ammonia oxidising and nitrite oxidising bacteria were enumerated using Winogradsky's modified medium (Rodina 1972). Sulphur oxidising bacterial medium and sulphate reducing bacterial medium was used to enumerate sulphur oxidizers and sulphate reducers respectively (Rheinheimer 1992).

Water and sediment samples were used neat or diluted (depending on the load of autotrophic bacteria) before inoculating into the respective liquid media for the enumeration by MPN of autotrophic bacterial groups included in this study. For the enumeration of sulphate reducing bacteria (SRB), the medium was overlaid with sterile liquid paraffin after inoculation of sample to maintain anaerobic conditions. All tubes were incubated at 30°C for 15 days. Presence of SRB was indicated by the development of black colour in the media due to formation of ferrous sulfide.

Results

Minimum bactericidal concentration (MBC) of sanitizers

Minimum bactericidal concentration was determined for the important shrimp bacterial pathogens, *V. harveyi* (both luminescent and nonluminescent) strains and *V. alginolyticus*. As shown in Table 1, *V. harveyi* (luminescent and nonluminescent) strains were killed by BKC at very low

concentration of 0.2 and 0.3 ppm respectively, but *V. alginolyticus* was eliminated with 3 ppm concentration of BKC. The MBC of hypochlorite ranged from 8 to 30 ppm of available chlorine. *Vibrio harveyi* (luminescent and non-luminescent) strains were killed at 8 and 10 ppm of chlorine respectively compared to *V. alginolyticus* which was completely destroyed at a concentration of 30 ppm chlorine. The MBC of iodophor ranged between 15 and 70 ppm of iodine. *Vibrio harveyi* strains were killed at the lower concentration (15 and 50 ppm) compared to *V. alginolyticus* which required a concentration of 70 ppm of iodine for its killing.

Microcosm studies

Effect of benzalkonium chloride on total heterotrophic and autotrophic bacteria

The effect of BKC on total heterotrophic and autotrophic bacteria in water and sediment was studied by using BKC at 0.1 ppm concentration as recommended by the manufacturer for application in shrimp ponds. The study was also carried out using the sanitizer at the MBC of 3 ppm obtained in our experiment. The bacterial load was enumerated at 0, 8 and 18 h after addition of BKC (Tables 2 and 3).

Treatment with 0.1 ppm BKC did not grossly alter the heterotrophic bacterial count in water. The initial heterotrophic count was 2.50 $x10^4$ cfu ml^{-1} and at 8h ,the count was 3.2 x 10^4 cfu·ml^{-1} (Table 2). At 18 h, a slight increase in count (4.05 $x10^5$ cfu·ml^{-1}) was obtained. It was however interesting to note that with 0.1 ppm BKC, the heterotrophic count in sediment was reduced by 2 log units from an initial of 2.23 x 10^7 cfu·g^{-1} to 7.35 x 10^5 cfu·g^{-1} at 18h. With 3 ppm, the heterotrophic count in water showed 1 log reduction to 1.35 x 10^3 cfu·ml^{-1} at 8 h from an initial count of 2.61 x 10^4 cfu·ml^{-1} but increased to 1.07 x 10^6 cfu·ml^{-1} at 18 h. In the case of sediment, there was one log reduction at 8 h and a further one log reduction at 18 h (Table 2).

Table 3 shows that the level of ammonia oxidizing bacteria, *Nitrosomonas* sp in water and sediment was not affected by treatment with 0.1 ppm BKC but on treatment with 3.0 ppm BKC, *Nitrosomonas*

Table 1. Minimum bactericidal concentration (MBC) of sanitizers for the test organisms

Test organisms	Sanitizers (ppm of active component)		
	BKC	NaOCl	Iodophor
Vibrio harveyi (L)	0.2	8.0	15
Vibrio harveyi (NL)	0.3	10	50
Vibrio alginolyticus	3.0	30	70

BKC = Benzalkonium chloride, NaOCl = Sodium hypochlorite, L =Luminescent, NL = Non luminescent

sp. in water decreased from 2.40 x 10^2/100 ml to undetectable levels at 18 h. On the other hand, *Nitrosomonas* in sediment was not affected by BKC treatment even with 3.0 ppm (Table 3).

Results in table 3 show no significant change in the load of nitrite oxidising bacteria, *Nitrobacter* sp. both in water and sediment on treatment with 0.1 and 3.0 ppm BKC. The counts in water remained unchanged at 1.1 x 10^3/100 ml. In sediment there was a slight increase in count from 1.5 x 10^3/100 g to 1.1 x 10^4/100 g at 18 h with 0.1 ppm but with 3.0 ppm, there was no change in count. While BKC had no effect on SRB both in water and sediment with 0.l ppm application, 3.0 ppm BKC reduced the SRB counts in water to undetectable levels. The SOB could not be detected by MPN technique.

Effect of sodium hypochlorite on total heterotrophic and autotrophic bacteria

The heterotrophic bacterial load in water treated with 10 ppm hypochlorite decreased by one log unit at 8 h sampling from the initial count of 5.12 x 10^4 cfu ml^{-1} to 5.52 x 10^3 cfu ml^{-1} and again increased to 2.25 x10^5 cfu·ml^{-1} at 18 h (Table 2). The same trend was observed in sediment. The initial count of 4.6 x 10^5 cfu·g^{-1} reduced to 5.47 x 10^4 cfu·g^{-1} at 8 h and increased to 8.1 x 10^5 cfu·g^{-1} at 18 h. Hypochlorite at 20 ppm reduced the heterotrophic bacteria from an initial count of 9.15 x 10^5 cfu·ml^{-1} to 4.0 x 10^2 cfu·ml^{-1} at 8 h but increased at 18 h to 3.57 x 10^4 cfu·ml^{-1}. In sediment after treatment, the initial and 8 h counts were nearly the same but there was one log decrease in count to 4.9 x 10^4 cfu·g^{-1} at 18 h sampling.

The ammonia oxidising bacteria, *Nitrosomonas* sp. in sediment was not affected significantly by hypochlorite at 10 and 20 ppm level in the microcosm. The counts both at 0 h and 18 h were 2.4 x 10^3/100 g with 10 ppm chlorine treatment, but with 20 ppm chlorine, the count marginally increased to 2.4 x 10^3/100 g from an initial level of 9.30 x 10^2/100 g. In water, the organism was undetectable at 18 h both with 10 ppm and

Table 2. Total heterotrophic bacterial count in water and sediment treated with BKC, hypochlorite and iodophor

Sanitizer used and concentration		Water (cfu·ml)			Sediment (cfu·g)		
		0 h	8 h	18 h	0 h	8 h	18 h
BKC	0.1 ppm	$2.50x10^4$	$3.24x10^4$	$4.05x10^5$	$2.23x10^7$	$1.88x10^6$	$7.35x10^5$
	3.0 ppm	$2.61x10^4$	$1.35x10^3$	$1.07x10^6$	$3.91x10^7$	$1.77x10^6$	$8.20x10^5$
Hypochlorite	10.0 ppm	$5.12x10^4$	$5.52x10^3$	$2.25x10^5$	$4.60x10^5$	$5.47x10^4$	$8.10x10^5$
	20.0 ppm	$9.15x10^5$	$4.00x10^2$	$3.57x10^4$	$5.02x10^5$	$3.05x10^5$	$4.90x10^4$
Iodophor	1.0 ppm	$1.60x10^4$	$2.00x10^4$	$5.60x10^5$	$5.40x10^5$	$7.90x10^5$	$3.70x10^6$
	10.0 ppm	$1.90x10^5$	$1.60x10^5$	$1.20x10^6$	$4.70x10^6$	$1.00x10^6$	$1.90x10^6$

cfu = colony forming unit

20 ppm treatment from an initial count of 9.30 x 10^1/100 ml and 4.30 x 10^1/100 ml respectively.

The level of nitrite oxidisers (*Nitrobacter* sp.) in water decreased by one log unit from 1.10x10^3/100 ml to 4.60 x 10^2/100 ml at both concentrations of chlorine tested. However *Nitrobacter* level in sediment recorded 4.60 x 10^3/100 g both at 0 and 18 h with 10 and 20 ppm chlorine application.

Level of SRB in water was marginally affected by hypochlorite treatment. With 10 ppm application, there was a slight increase in counts at 18 h to 1.66 x 10^2/100 ml from an initial count of 2.30 x 10^1/100 ml. With 20 ppm chlorine application, the SRB counts in water reduced to 0.40 x 10^1/100 ml from 4.30 x 10^1/100 ml after 18 h exposure. In contrast to this, the SRB levels in sediment were not affected by the chlorine treatment. After the exposure period, the SRB counts with 10 ppm and 20 ppm chlorine treatment were 4.60 x 10^3/100 g and 4.30 x 10^2/100 g at 18 h from an initial count of 2.40 x 10^3/100 g and 2.30 x 10^2/100 g respectively.

Table 3. Autotrophic bacterial count in water and sediment treated with BKC, hypochlorite and iodophor

Sanitiser used and concentration	Bacterial groups	Water (MPN·100 ml)		Sediment (MPN·100 g)	
		0 h	18 h	0 h	18 h
BKC	NS	$2.40x10^3$	$1.15x10^3$	$3.30x10^2$	$2.40x10^2$
0.1 ppm	NB	$1.10x10^3$	$1.10x10^3$	$1.50x10^3$	$1.10x10^4$
	SRB	$4.30x10^1$	$9.30x10^1$	$4.60x10^3$	$2.40x10^3$
	SOB	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$	$<0.3x10^1$
3.0 ppm	NS	$2.40x10^2$	$< 0.3x10^1$	$2.30x10^2$	$2.40x10^2$
	NB	$1.10x10^3$	$1.10x10^3$	$9.30x10^2$	$4.30x10^2$
	SRB	$2.30x10^1$	$< 0.3x10^1$	$2.40x10^3$	$2.40x10^3$
	SOB	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$	$<0.3x10^1$
Hypochlorite	NS	$9.30x10^1$	$< 0.3x10^1$	$2.40x10^3$	$2.40x10^3$
10 ppm	NB	$1.10x10^3$	$4.60x10^2$	$4.60x10^3$	$4.60x10^3$
	SRB	$2.30x10^1$	$1.66x10^2$	$2.40x10^3$	$4.60x10^3$
	SOB	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$
20 ppm	NS	$4.30x10^1$	$<0.3x10^1$	$9.30x10^2$	$2.40x10^3$
	NB	$1.10x10^3$	$4.60x10^2$	$4.60x10^3$	$4.60x10^3$
	SRB	$4.30x10^1$	$0.40x10^1$	$2.30x10^2$	$4.30x10^2$
	SOB	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$
Iodophor	NS	$9.30x10^1$	$2.10x10^1$	$4.30x10^2$	$1.50x10^2$
1 ppm	NB	$1.10x10^3$	$1.10x10^3$	$4.60x10^3$	$4.60x10^3$
	SRB	$2.40x10^2$	$2.40x10^2$	$2.30x10^2$	$4.30x10^2$
	SOB	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$
10 ppm	NS	$2.40x10^2$	$4.30x10^1$	$2.30x10^2$	$2.30x10^2$
	NB	$3.50x10^2$	$4.60x10^3$	$1.10x10^4$	$1.10x10^4$
	SRB	$2.40x10^2$	$2.30x10^1$	$2.30x10^2$	$2.30x10^2$
	SOB	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$	$< 0.3x10^1$

NS=*Nitrosomonas* sp(Ammonia oxidizers), NB =*Nitrobacter* sp (Nitrite oxidizers), SRB = Sulphate reducing bacteria, SOB = Sulphur oxidising bacteria, MPN = Most probable number

Results in table 2 of counts taken at 8 h after 1 and 10 ppm iodophor treatment show that the total heterotrophic bacterial load in water as well as sediment was virtually unaffected by the treatment. After 18 h exposure to 1 ppm of iodophor, counts of autotrophic bacteria such as *Nitrosomonas*, *Nitrobacter* and SRB did not show any marked change both in water and sediment (Table 2). Similar results were obtained from sediment after exposure to 10 ppm iodophor but in water, the iodophor brought about 1 log reduction in the count of *Nitrosomonas* from an initial of 2.4 x 10^2/100 ml to 4.3 x 10^1/100 ml and one log reduction in SRB from 2.4 x 10^2/100ml to 2.3 x10^1/100 ml. *Nitrobacter* was not affected by iodophor (Table 3).

Discussion

Use of sanitizers such as benzalkonium chloride (BKC), hypochlorite and iodophor is a common practice in aquaculture. Often, sanitizers are added to the water as a prophylactic measure to combat diseases. However, to our knowledge, no studies have been carried out on the effect of sanitizer treatment on the autotrophic and heterotrophic microflora in the ponds that are extremely important to maintain a healthy environment by carrying out mineralization

The results of this study suggest that the sensitivity of *V. harveyi* and *V. alginolyticus* to sanitizers vary, the former being more sensitive to all the sanitizers included in the study (Table 1). Bacteria vary in their sensitivity to sanitizers and this could be an innate character due to their thin cell wall or outer membrane structure. However, the results suggest that the MBC of sanitizers to *V. harveyi* was much higher than the recommended dose of application by the manufacturers. This raises the question regarding the efficacy of the sanitizers at the recommended concentration in shrimp ponds. The variation in the organic load in water and sediment is expected between different ponds and within ponds in a day and this is bound to have an impact on efficacy.

In microcosms, treatment with BKC at 0.1 ppm failed to significantly lower total heterotrophic bacterial levels in water. Additionally, this concentration of BKC was unable to kill the *Vibrio* sp. tested and therefore it is to be expected that at this level, BKC does not bring about any change in the total microbial load or pathogen load in pond water. Interestingly, however, this level of BKC brought about 1 log reduction in microbial load in the sediment at 8 h and the level at 18 h continued the reduction trend in the counts. This may be due to higher sensitivity of sediment

associated microflora. With 3.0 ppm BKC, the levels of heterotrophic bacteria decreased by about 1 log unit at 8 h, but at 18 h surprisingly there was an increase in the count by about 3 log units. It is possible that the initial decrease could be due to killing of sensitive bacteria and subsequent increase due to multiplication of the BKC tolerant bacteria.

The results of this study show that BKC affected the level of ammonia oxidising bacteria, *Nitrosomonas* in water both at 0.1 and 3.0 ppm levels. This information would be helpful to aquaculturists, because when sanitizers are used, there is a risk of useful bacteria being killed. The results suggest that BKC should not be applied when ammonia levels are significant.

Sodium hypochlorite at 10 ppm brought about 1 log reduction in the heterotrophic bacterial count in water and sediment at 8 h, but the levels returned to original counts or even increased at 18 h (Table 2). As shown in table 1, some *Vibrio* sp. demonstrated sensitivity to 10 ppm hypochlorite but *V. alginolyticus* had an MBC of 30 ppm. Therefore, it can be expected that 10 ppm hypochlorite would bring about only partial reduction of counts at 8 h and multiplication of surviving bacteria without competition from sensitive ones may account for increase in counts observed at 18 h. Almost similar results were observed with 20 ppm hypochlorite.

There was a reduction in the counts of ammonia oxidising bacteria, *Nitrosomonas* in water following treatment with 10 ppm and 20 ppm hypochlorite, but the counts in sediment was not altered at both concentrations of hypochlorite (Table 3). Similar trend was noticed even in the case of nitrite oxidising bacteria, *Nitrobacter*. However, SRB seemed to be relatively more resistant to hypochlorite, as seen from the counts in their levels in both water and sediment subjected to both 10 and 20 ppm hypochlorite treatment.

The reduction in bacterial levels in water following hypochlorite treatment is to be expected. The lack of effect on sediment bacteria could also be due to high organic content in the sediment. It is known that presence of organic matter influences the activity of chlorine compounds (Cords 1983, Moats 1981).

Results in table 3 show that iodophor treatment of the microcosm did not seem to affect the population of heterotrophic and autotrophic bacterial levels both in water and sediment. Results in table 1 show that MCC of iodophor was higher in comparison to hypochlorite. This is significant in view of the fact that the recommended dose for commercial iodophor is much lower. At their recommended concentration, bacterial levels in water is not significantly altered.

Autotrophic and heterotrophic microorganisms play a decisive role in the cycling of organic matter in water. They cause not only reduction and

diverse transformation of organic material as well as its remineralisation, but also the conversion of inorganic compounds, for example, by fixation of free nitrogen, nitrification, denitrification, sulphur oxidation and sulphate reduction or the oxidation of reduced iron and manganese (Rheinheimer 1992).

The results of this study show that hypochlorites should be used with caution since the autotrophic bacteria required for nutrient recycling in the pond are affected by this. However, lack of effect on sediment suggests that though bacteria associated with water column are killed by sanitizer treatment, the sediment associated bacteria can replenish their population after the disappearance of the sanitizers.

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