World Journal of Fish and Marine Sciences 7 (2): 135-140, 2015
ISSN 2078-4589

DOI: 10.5829/idosi.wjfms.2015.7.2.93141

Effects of Antibiotic Treated Fish Diet on Fish Muscle, Bacterial Flora and Water Quality Parameters under Pond Experimental Condition

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Abstract: The present study was aimed to investigate the effects of antibiotic on Sarpunti (*Puntius sarana*) muscle and to determine the microbial load in pond water, sediment, fish gill and intestine and other water quality parameters. Oxytetracycline, the most broadly used antibiotic, was fed to Sarpunti (average body weight 30g) at the rate of 4 g/kg through fish diet and changes in water quality parameters and bacterial content was estimated for a period of 21 days. Pond water, sediment and fish muscle were tested before and after oxytetracycline medicated feed treatment. The initial oxytetracycline accumulation was 5070.0 ppb, which reduced considerably to 1190.0 ppb after 5 days of stopping oxytetracycline treated diets and antibiotic was not detected after 10 days. The withdrawal period of oxytetracycline in Sarpunti muscle was 5-7 days. Before antibiotic treatment, temperature, dissolved oxygen, pH and total hardness of pond water was 25.87±0.015°C, 4.516±0.25 mg/l and 7.413±0.09 and 822.33±1.52 ppm, respectively which reached a value of 27.33±0.015°C, 5.27±0.025 mg/l, 7.773±0.04 and 769.66±2.08 ppm, respectively after 21 days. Before antibiotic treatment, bacterial load of pond water was $9.417\pm0.035\times10^3$ cfu/ml, $7.31\pm0.04\times10^7$ cfu/g in sediment, $6.51\pm0.04\times10^6$ cfu/g in gills and $8.52\pm0.035\times10^7$ cfu/g in intestine, which was reduced significantly to $5.28\pm0.02\times10^3$ cfu/ml in water, $2.91\pm0.02\times10^7$ in sediment, $2.21\pm0.02\times10^6$ cfu/g in gills, $2.39\pm0.02\times10^7$ cfu/g in intestine, respectively.

Key words: Antibiotic · Medicated Feeding Stuff · *Puntius sarana* · Bacterial Load

INTRODUCTION

Aquaculture is growing rapidly in many regions of the world and aquaculture products constitute an important food supply with increasing economic importance. Its contribution to global supplies of several species of fish, crustaceans and mollusks increased from 9% of total production by weight in 1980 to 55% in 2012. It has been estimated that fisheries and aquaculture supplied the world with about 128 million metric tons of food fish per year [1], providing a per capita supply of 18.4 kg (live weight equivalent). Moreover, hygienic shortcomings in fish raising methods, including increased fish population densities, crowding of farming sites in coastal waters, lack of sanitary barriers and failure to isolate fish farming units with infected animals [2], have increased the possibility of rapid spread of infections. To control infectious diseases, similar strategies (eg, vaccination and use of antibacterial agents) are employed in aquaculture as in other areas of animal production. The most used antibiotic in the fish farms is oxytetracyline (OTC) [3]. Use of antibacterial agents in aquaculture has resulted in the emergence of reservoirs of antimicrobial-resistant bacteria in fish and other aquatic animals, as well as in the aquatic environment [4]. Consequently, the use of antimicrobial agents in aquaculture results in a broad environmental application that impacts a wide variety of bacteria [5].

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Intensive use of antibacterial agents in aquaculture provides a selective pressure creating reservoirs of drug-resistant bacteria and transferable resistance genes in fish pathogens and other bacteria in the aquatic environment [6]. From these reservoirs, resistance genes may disseminate by horizontal gene transfer and reach human pathogens, or drug-resistant pathogens from the aquatic environment may reach humans directly. Occurrence of resistance to these antimicrobial agents in human pathogens severely limits the therapeutic options in human infections. Considering the rapid growth and importance of aquaculture industry in many regions of the world and the widespread, intensive and often unregulated use of antimicrobial agents in this area of animal production, efforts are needed to prevent development and spread of antimicrobial resistance in aquaculture to reduce the risk to human health.

Considering the above facts, this study was aimed to determine the residual effects of antibiotic in fish muscle and to determine the changes in bacterial load in water, sediment, gills and intestine of fish before and after administrating of antibiotic treated diets to the fish under experimental pond condition. This study also investigated the changes in water quality parameters in the presence or absence of antibiotic treated diets.

MATERIALS AND METHODS

Experimental Site: The experiment was performed for a period of two months from July to August, 2013 in 4 ponds at the Faculty of Fisheries, Sylhet Agricultural University, Bangladesh. All the ponds were more or less similar in size (1.5 decimal), depth (3.0- 3.5 feet), basin conformation and bottom-soil type. One pond was used as control and other 3 cultural ponds (360cm×240cm×90cm) for replication of the treatment for the experiment. The ponds were prepared accordingly by following FAO guidelines before starting experiment. All parameters and other water quality parameters were controlled accordingly to maintain a same environmental condition.

The Sarpunti (*Puntius sarana*) fingerling was collected from BRAC hatchery, Moulovibazar and the fingerlings were acclimatized and released in each pond at the rate of 160 fish/decimal. Fishes were fed with oxytetracycline medicated feed (4g/kg feed) for 21 days in treatment ponds. Feeds were given twice a day.

Table 1: Samples used for oxytetracycline metabolites and oxytetracycline analysis

Sample no.	Sample Containing
1	Feed containing Oxytetracycline
2	Fish (Before antibiotic treatment)
3	Fish (Continuous antibiotic feeding)
4	Fish (1 day after antibiotic feeding stopped)
5	Fish (5 day after antibiotic feeding stopped)
6	Fish (10 day after antibiotic feeding stopped)
7	Fish (20 day after antibiotic feeding stopped)
8	Fish (30 day after antibiotic feeding stopped)
9	Fish (45 day after antibiotic feeding stopped)

Selection of Feed Stuff: Pelleted feeds containing OTC were prepared in the laboratory using small pellet machine. The ingredients of feed were rice bran (20%), wheat flour (10%), maize flour (14%), soybean meal (15%), fishmeal (35%), molasses (04%), vitamin premix (01%) and salt (01%) where protein percentage was 31.80%. OTC was mixed with the ingredients at the rate of 4g/kg of feed. After preparation of pellets, the feeds were dried and stored in refrigerated temperature (4°C) until use.

Analysis of Pond Water and Sediment for Oxytetracycline: Sarpunti were fed with antibiotic medicated diets for three weeks. After three weeks, Sarpunti samples were collected at definite time interval to determine the residues and retention time. The catch statistics were maintained based on 9 sampling plan. Nine samples were analyzed for the detection of OTC residues using LC-MS/MS in the Fish Inspection and Quality Control (FIQC) laboratory, Department of Fisheries, Bangladesh [7]. Samples used for OTC metabolites and OTC analysis are shown in Table 1.

Estimation of Microbial Load in Fish Muscle, Water and Sediment of the Experimental Ponds: Water and sediment samples were collected from three different locations from each of the pond. Three samples were mixed to make a composite sample for microbial load analysis.

Preparation and Sterilization of Media: For the preparation of cultured media, 23.5g of plate count agar was weighed and suspended into 1.0 L of distilled water in a conical flask. The mixture was boiled on an electric heater to dissolve completely. After that the media was sterilized by placing it into an autoclave for 30 minutes at a temperature of 121°C under 15 lb/sq. inch pressure. Finally it was cooled down to 50°C and was poured into previously sterilized Petri dish.

Sample Preparation and Culture: Standard plate count expressed as Colony Forming Units per gram (CFU/g) of muscle were determined using consecutive decimal dilution technique using spread plates. Five grams of sample was taken for every case of investigations. The sample was homogenized in to 95 ml sterile physiological saline solution (0.85% NaCl) in a sterile blender jar. Then blending was done for 2 minutes and the homogenate was transferred into a sterile bottle. In order to get decimal (10^{-1}) dilution of the original sample solution, 1 ml of sample solution was transferred with a micropipette to a test tube containing 9.0 ml of physiological saline and the test tube was shaken thoroughly on a vortex mixture. Using this process, several dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were made.

Total Plate Count: Sterile plate count agar was poured in to the sterile petri dish in triplicates. From sample solution of different test tubes bearing varying dilution was taken by a micropipette and transferred aseptically in to the pre-prepared agar plates by raising the upper lid sufficient enough to enter the lip of the pipette. The samples were then spread homogenously and carefully by sterile flamed L-shaped glass rod throughout the surface of the media until the sample were dried out. The plates were incubated at 37°C in an inverted position in an incubator. After 48 hours of incubation, the developed colonies were counted following a standard method. Only plates having 30 to 300 colonies were considered for counting in order to get acceptable values. Number of bacteria per gram of the sample (CFU/g) was calculated using the following formula:

$$\text{CFU/g} = \frac{\text{No. of colonies on Petridish} \times 10 \times \text{dilution factor} \times \text{wt. of total sample solution}}{\text{Wt. of fish sample (g)}}$$

Determination of Water Quality Parameters: The water quality parameters were recorded 7 days interval throughout the experimental period. Water quality measurements and samples were collected between 9:30 hours to 11:00 hours on each sampling day. Water samples were collected from each of the pond in the depth of 30 cm. Water quality parameters such as temperature, dissolved oxygen, total hardness and pH were measured by using HACH Test Kit. Model: FF-2, Cat No. 2430-01; Company: HACH Company World Headquarters, Loveland, Colorado.

Statistical Analysis: The data obtained in the experiment were analyzed using one way ANOVA. The mean values compared using Duncan's Multiple Ranged Test (DMRT) as post-hoc test using SPSS (Statistical Package for Social Science, version 11.5) statistical software (SPSS mc; Chicago. USA). Significant differences were determined among treatments at the 5% level ($P < 0.05$).

RESULTS AND DISCUSSION

Accumulation of OTC in Fish Muscle: Initial OTC accumulation in fish muscle was 5070.0 ppb, which reduced considerably to 1190.0 ppb after 5 days of stopping OTC treated diets and the antibiotic was not detected later on (Figure 1). The result of this study indicated that withdrawal period of oxytetracycline in sarpunti muscle was 5-7 days. Paschoal *et al.* [8] reported that the residual effect of OTC was 8 days in tilapia (*Oreochromis niloticus*) under tropical conditions during winter season. On the other hand, withdrawal period of OTC was 10 days in carp muscle Elia *et al.* [9]. The withdrawal period of OTC was 10 days in shrimp Jimenez *et al*. [10]. Result of this study more or less alike with Paschoal *et al.* [8], Jimenez *et al*. [10] and Elia *et al.* [9]. Our result suggests that the OTC treated fish can be consumed after withdrawal period.

Quantitative Analysis of Bacteria: The results of the quantitative estimation of aerobic heterotrophic bacteria in the experimental pond water, sediment, gills and intestine of Sarpunti are given in Table 2. Variations in bacterial load of pond water, gills and intestine of Sarpunti were observed. The highest bacterial load were observed $9.417\pm0.035\times10^{3}$ cfu/ml in water, $7.31\pm0.04\times10^{7}$ cfu/g in sediment, $6.51\pm0.04\times10^{6}$ cfu/g in gills, $8.52\pm0.035\times10^{7}$ cfu/g in intestine of Sarpunti before administrating the

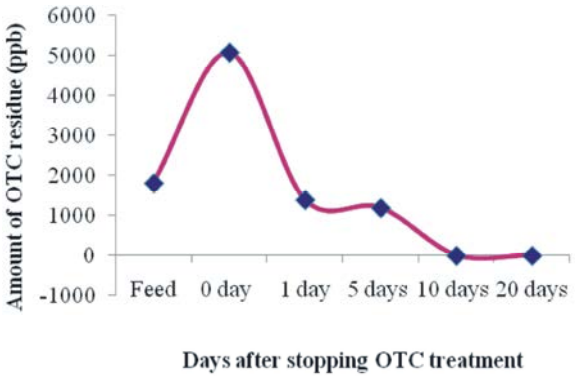

Fig. 1: Withdrawal period of Oxytetracycline

Table 2: Bacterial load in pond water, sediment, gills and intestinal content of Sarpunti fish

	Bacterial load			
Parameters	0 days	7 days	14 days	21 days
Pond water (cfu/ml)	$9.417^{a}\pm0.035\times10^{3}$	$7.41^{b}\pm0.02\times10^{3}$	$6.11^{c}\pm0.025\times10^{3}$	$5.28^{d}\pm0.02\times10^{3}$
Sediment (cfu/g)	$7.31^{a}\pm0.04\times10^{7}$	$5.29^{b}\pm0.03\times10^{7}$	$3.18^{c}\pm0.02\times10^{7}$	$2.91^{d}\pm0.02\times10^{7}$
Gills (cfu/g)	$6.51^{a}\pm0.04\times10^{6}$	$4.24^{b}\pm0.025\times10^{6}$	$3.01^{c}\pm0.03\times10^{6}$	$2.21^{d}\pm0.02\times10^{6}$
Intestine (cfu/g)	$8.52^{a}\pm0.035\times10^{7}$	$5.78^{b}\pm0.02\times10^{7}$	$3.70^{c}\pm0.02\times10^{7}$	$2.39^{d}\pm0.02\times10^{7}$

*Values are (Mean±SD);

Table 3: Water quality parameters with oxytetracycline treatment in ponds for Sarpunti fish

	Antibiotic Treatment			
Parameters	0 days	7 days	14 days	21 days
Temperature (°C)	$25.87^{a}\pm0.015$	$26.40^{b}\pm0.03$	$26.88^{c}\pm0.015$	$27.33^{d}\pm0.015$
pH	$7.413^{a}\pm0.09$	$7.613^{b}\pm0.06$	$7.656^{b}\pm0.04$	$7.773^{b}\pm0.04$
Dissolved oxygen (ppm)	$4.516^{a}\pm0.25$	$4.80^{b}\pm0.026$	$4.94^{c}\pm0.02$	$5.27^{d}\pm0.025$
Hardness (ppm)	$822.33^{a}\pm1.52$	$793.66^{b}\pm1.52$	$778.33^{c}\pm1.52$	$769.66^{d}\pm2.08$

*Values are (Mean±SD);

OTC treated diets. The bacterial load were reduced significantly to $5.28\pm0.02\times10^{3}$ cfu/ml in water, $2.91\pm0.02\times10^{7}$ cfu/g in sediment, $2.21\pm0.02\times10^{6}$ cfu/g in gills, $2.39\pm0.02\times10^{7}$ cfu/g in intestine of Sarpunti after 21 days of antibiotic treatment.

Comparatively higher bacterial load is not harmful for fish culture if the bacteria are nonpathogenic. Higher bacterial abundance of this study may indicate a potential of organic matter recycling, self-cleaning potential and re-mineralization. The composition and quantity of the microorganisms also vary depending on water temperature Al-Harbi and Uddin [11]. High metabolic activity of fish associated with increased feeding rates at higher temperatures might be a cause for high bacterial load in the gills and intestine of fish Al-Harabi and Uddin [12].

The bacterial load in culture ponds were $4.9\pm1.03\times10^{3}$ - $5.75\pm1.0\times10^{3}$ cfu/ml in water, $5.62\pm1.0\times10^{7}$- $6.60\pm1.02\times10^{7}$ cfu/g in sediments, $6.77\pm1.0\times10^{6}$ - $7.57\pm1.0\times10^{6}$ cfu/g in *Labeo rohita* gill, $7.94\pm1.01\times10^{6}$ - $9.12\pm1.0\times10^{6}$ cfu/g in *Hypophthalmichthys molitrix* gill, $6.02\pm1.02\times10^{7}$ - $8.32\pm1.0\times10^{7}$ cfu/g in intestine of *L. rohita* and $5.12\pm1.05\times10^{7}$- $6.60\pm1.0\times10^{7}$ cfu/g in intestine of *H. molitrix* Kashem [13]. He found that the total viable counts in culture ponds were $3.1\pm1.19\times10^{3}$ - $3.1\pm1.20\times10^{3}$ cfu/ml in water; $4.27\pm1.10\times10^{7}$ - $3.1\pm1.13\times10^{7}$ cfu/g in sediments; $5.37\pm1.01\times10^{6}$ - $3.09\pm1.19\times10^{6}$ cfu/g in the gill of *L. rohita*; $3.16\pm1.29\times10^{6}$ - $4.07\pm1.20\times10^{6}$ cfu/g in the gill of *H. molitrix*; $2.69\pm1.12\times10^{7}$ - $4.68\pm1.12\times10^{7}$ cfu/g in the intestine of *L. rohita* and $2.95\pm1.1\times10^{7}$ - $2.63\pm1.17\times10^{7}$ cfu/g in the intestine of *H. molitrix* after antibiotic treatment. He also reported that total viable counts were significantly varied between the control ponds and treatment ponds.

The total viable counts were $6.7\pm2.1\times10^{3}$-$2.7\pm1.1\times10^{3}$ cfu/ml in the pond water Al-Harbi and Uddin, [11]. Total viable counts of bacteria (measured as colony-forming units/cfu) were in the range of $5.6\pm0.8\times10^{3}$ to $2.4\pm1.2\times10^{4}$ cfu/ml in water; $1.2\pm3.1\text{x}10^{6}$ to $7.3\pm1.1\text{x}10^{7}$ cfu/g in sediment; $7.1\pm0.7\times10^{5}$ to $8.7\pm1.1\text{ x}10^{6}$ cfu/g in the gills; and $2.8\pm2.4\times10^{7}$ to $1.0\pm1.6\times10^{8}$ cfu/g in the intestine of tilapia Al-Harbi and Uddin [12, 14]. These results agreed with our findings. The results of Rudrigues and prieto [15], Chowdhury *et al*. [16] was more or less similar to the results of this study.

Water Quality Parameters of Water: Water quality parameters with OTC treated ponds for Sarpunti is shown in Table 3. There was little difference observed in the water temperature of Sarpunti pond. The mean values of water temperature were 26.6°C. The lowest water temperature of pond was recorded (25.87±0.015°C) during the month of July, whereas the highest temperature was recorded (27.33±0.015°C) during the month of August. Similar values were also found in control pond (feeding without OTC treated diets), which indicate that there was no relation between application of OTC treated diets and the increment of water temperature. It was also observed that the temperature of pond water was increased gradually due to environmental conditions not for the application of OTC treated diets.

Water temperature were recorded during the period of 14 days, which ranged from 28.99 to 31.09°C and from 29.45 to 31.97°C before and after antibiotic treatment, respectively in the pond at Mymensingh Kashem [13]. It has been reported that favorable water temperature for fish culture is 20.5 to 30.5°C Haque [17].

Water temperature recorded in this study were 25-27°C, which is more or less similar to the Kashem [13], Mollah and Haque [18] and Aminul [19].

Prior to antibiotic treatment, the lowest DO value was 4.516±0.25 mg/l. In the day of 0-7, 7-14 and 14-21, the DO values were increased to 5.27±0.025 mg/l. The DO content found in the pond water was 4.10±0.10 mg/l and 5.59±0.10 mg/l before and after 20 days of antibiotic treatment, respectively Haque [20]. The DO level from 4 to 8 mg/l was suitable for fish culture DoF [21]. It was also found that the DO ranging from 2.2 to 8.8 mg/l in ponds of BAU campus, Mymensingh Dewan *et al.* [22]. As bacterial biomass was reduced after antibiotic treatment, therefore, the total oxygen requirement of pond also reduced. This might be a potential reason for increasing DO level in the pond after OTC treatment.

Before antibiotic treatment, the lowest pH value was found 7.413±0.09. After antibiotic application, the pH values were also increased and it reached the highest value 7.773±0.04 after 21 days of OTC treatment. There is a positive relationship between DO and pH when DO increased in the pond water at the same time the pH value also increased. Before antibiotic treatment, pH value of pond water was 7.75±0.06, whereas pH value was found 7.86±0.06 after two weeks of antibiotic treatment in the pond water. Changes in pH were not considerable in different treatments except the lime treatment Haque [20]. In this study, pH values varied from 7.41 to 7.77, which was more or less similar to the findings of Ali *et al.* [23], Wahab *et al.* [24] and Kohinoor *et al.* [25].

The highest total hardness of pond water was 822.33±1.52 ppm. After 21 days of antibiotic treatment, total hardness of the pond water was 769.66±2.08 ppm. Total hardness of aquarium water was found 855.00±5.00 ppm and after 3 weeks of antibiotic treatment, it was 710.00±9.30 ppm Haque [20]. In this study, total hardness varied from 822.33±1.52 to 769.66±2.08 ppm, which was more or less alike to the findings of Haque [20].

CONCLUSION

Bacterial loads were reduced significantly in pond water, sediment, fish gill and intestine when antibiotic medicated diets were used. Dissolved oxygen and pH were slightly increased but total hardness was quite reduced after antibiotic treated diets were utilized. The oxytetracycline residual effects presented about 5-7 days just after antibiotic feeding stopped to fish and OTC treated Sarpunti can be consumed after this withdrawal period.

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