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Quantitative analysis of bacterial flora of freshwater prawn (*Macrobrachium rosenbergii*) in fresh and frozen storage condition

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ABSTRACT

The goal of this study was to monitor the quantitative bacterial flora in the freshwater prawn in fresh and in frozen storage conditions for 1 year. This study was also conducted for bacteriological analysis of water and sediment sample to determine the physico-chemical parameters of two prawn ghers from Rupsha and Batiaghata upazilla under Khulna district. The ghers were designated as gher-1 and gher-2 respectively. Duration of the study was April, 2014 to April, 2015. The study included physico-chemical parameters like water temperature, dissolve oxygen (DO), pH and salinity of gher water; bacteriological study like aerobic plate count and presence of enteric bacteria in gher water, sediment and prawn samples in fresh and frozen storage condition. Sampling was done by one month interval with three replication for bacteriological analysis. The samples were collected from three different corner of each gher. Mean water temperature (°C), dissolve oxygen (mg l-1), pH and salinity (ppt) in gher 1 and 2 were 27.85, 27.95; 4.90, 5.0; 6.8, 6.7 and 9.5, 8.5, respectively. In gher-1, total viable bacterial count in water ranged from 8.34×103 to 2.45×104; in sediment ranged from 7.17×108 to 1.51×109. In gher-2, it ranged from 7.75×104 to 1.43×105 in water; 9.87×108 to 8.59×109 in sediment. On the other hand, In fresh and frozen fish, aerobic plate count (APC) ranged from 3.2×107 to 1.4×104 CFU g-1 in the freshwater prawn collected from gher 1 and 7.48×107 to 2.5×104 CFU g-1 in the freshwater prawn from gher-2. APC for all the groups of fish decreased around 2-log cycles after 1 months frozen storage; thereafter, counts slowly declined during frozen storage for 1 year. Gram-negative rods (77%) dominated. Enteric bacteria were also present in both ghers. But in the frozen storage condition, Enteric bacteria were not detected in any of examined prawn samples. The presence of enteric bacteria in the gher waters, sediments and fresh prawn samples suggested that waters were polluted by mammals, bird faeces and other outer sources.

INTRODUCTION

Prawn sector plays a major role in Bangladesh. It contributes a lot in the national economy generating employment and foreign exchange earnings. About two million people are associated with prawn sector for employment and foreign exchange earnings (Azim et al., 2002). The culture of giant freshwater prawn is being practiced on commercial scale in several parts of the world especially in South East Asia. Bangladesh with its favorable resources and agro-climatic conditions is widely recognized as one of the most suitable countries in the world for giant freshwater prawn *Macrobrachium rosenbergii* farming.

In the foreign markets, fresh water prawns of Bangladesh have been rejected mainly due to the contamination of *Salmonella*, and faecal coliforms and rarely for *Vibrio cholerae*, but in the major cases for *Salmonella*. In the fish inspection and quality control laboratory in Khulna under the department of fisheries, in the year 2009, out of 2770 consignments, 46 consignments were rejected due to the presence of *Salmonella*, 123 due to the excess content of total bacterial load (SPC, and faecal coliforms). Among these rejected consignments majority were the consignments of fresh water prawn (*M. rosenbergii*). The rejection of prawn consignment both internally and in the foreign market is involved with huge amount of financial loss to the nation which ultimately severely affects the prawn farmer and all other

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stakeholders. Types and levels of bacterial populations associated with farmed giant fresh water prawn (*M. rosenbergii*), water and sediments are the important indicators for the assessment of quality and safety of prawns. In addition, most diseases in *M. rosenbergii* are caused by opportunistic pathogens which are prevalent in the rearing environment (Janaki and Madhavi, 1999). Therefore the present study was designed to assess the microbiological quantity of raw and frozen freshwater prawn and to make a comparison about microbial load of raw and frozen freshwater prawn.

MATERIALS AND METHODS

Study area

The present study was carried out in two ghers located at Batiaghata and Rupsha upazila under Khulna district during the months of April, 2014 to April, 2015. Both the ghers were supplied with ground water as a major source. Intrusion of saline water was also observed. Gher-2 was comparatively larger (7 acres) than gher-1 (3.5 acres). Both the ghers were surrounded by paddy fields. Higher levels of mud on the bottom were observed (8 cm in gher-1 and 12 cm in gher-2). Use of feed in both prawn gher was common.

Sample collection

Water, sediment and prawn samples were collected from the two ghers mentioned above. Those two gher were designated as gher-1 and gher-2 respectively. The samplings were done three times with one month interval. In each gher three locations were selected for sample collection. Three samplings, one in each gher, were performed at a time to catch prawn by using cast net (15-20 g). Each collected sample was marked properly with a permanent marker pen and wrapped with aluminium foil and transported to the rest room in the gher. Immediately after the catch, one set of samples was subjected to immediate analysis and 12 sample bags from each of the two gher, a total of 24 bags, each containing nine representative (size, condition, sex, etc.) fish were preserved in a refrigerator at temperature of) -20°C in walk-in units. For quantitative bacterial analyses samples were tested monthly. One bag of frozen fish was randomly taken from each unit and evaluated for microbiological quality.

Physico-chemical parameters

During collection of samples surface water temperature and air temperature was measured by a simple thermometer. pH of water and sediment were measured by pH paper stripes. Salinity and dissolved oxygen (DO) were also measured by using laboratory kit and reagents

Preparation of media

The recommended quantities of ingredients were weighed and then dissolved in the required amount of water. The mixture was then boiled on electric heater to mix all the ingredients thoroughly.

Media were sterilized before using them in order to kill any bacterial and fungal cells or spores present in the media or in the glass wares containing them. Sterilization was accomplished by placing the media in an autoclave for 20 minutes at a temperature of 121°C under 15 lbs sq^{-1}. inch pressure. Then it was cooled down to around 50°C and was poured into previously sterilized petridish.

Nutrient Agar media, Plate count agar/ nutrient agar and some selective media viz. Eosin methylene blue (EMB) agar and *Salmonella-Shigella* (SS) agar were used to detect the presence of enteric bacteria.

Bacteriological analysis of fresh sample

After collection of samples from the gher one set of samples were subjected to immediate analysis (bacterial culture in agar plate) which was done in the gher. Though the facility in the gher was so poor, but sufficient precautions were taken. There was no or minimum air flow in the room, two spirit lamp were set. All needed materials like previously prepared agar plate, sterilized physiological saline, sterilized cutting and grinding equipment, sterilized test tubes, sterilized tips, micropipettes etc. were taken to the gher

Sample processing for bacteriological analyisis

Prawn

Nine frozen fish collected from each of two ghers weighing between 15 and 20 g were used in bacteriological examinations. Fresh fish were only tested one time at the beginning of the storage period. Frozen fish were air thawed (10 min) until it was possible to take out the muscle. Three prawn individual were ground and homogenized for each sample preparation. Approximately 5 g of each homogenate was then put in a bottle containing 200 ml of sterile saline solution. One milliliter of each homogenate solution was serially diluted (10^{-1} to 10^{-7}) and treated in the same way as the water samples. Appropriate sample dilutions were made (10^{-1} to 10^{-7}) with sterile physiological saline (0.85% w v^{-1} NaCl). Aliquots of 0.1 ml of the serial dilutions were pipetted out and transferred aseptically to the agar plates by raising the upper lids sufficient enough to admit the tip of the pipette. The samples pipetted were spread by L- shaped glass rods throughout the surface of the media until the samples were dried out and put in incubator. After 48 hours of incubation colonies developed were counted. Only the plates having 30 to 300 colonies were counted. Three samples (in each set of samples) were analyzed.

Water

Gher water samples were collected in sterile glass bottles (250 ml), 15-20 cm below the water surface from three different locations in each gher at every sampling. Three samples were combined to make a composite sample for bacteriological analysis.

Sediment

Bottom sediment samples were collected, with sterile glass bottles submerged to the bottom, from the same three locations in each gher. Five gram of wet sediment was weighed and dissolved in 200 ml physiological saline to make stock solution. One milliliter of the homogenate was serially diluted (10^{-1} to 10^{-9}) and treated in the same way as the prawn samples. Two samples were analyzed.

Bacteriological analysis

Sample preparation and culture

Standard plate count expressed as colony forming units per gram (CFU g^{-1}) were determined by using consecutive decimal dilution technique using spread plate method. Stock solutions were prepared for prawn samples. Five gram of prawn samples were taken (prawn samples were prepared from whole body of three prawns). The samples were mixed in 200 ml sterile physiological saline separately. Then shaking was done properly. One milliliter sample (prawn's stock solution) was transferred with a micropipette to test tube containing 9 ml of physiological saline. The test tube was shaken thoroughly on a vortex mixture in order to get 10^{-1} dilution of original sample solution. Using the similar process several dilutions of 10^{-2}- 10^{-5}, 10^{-2}-10^{-6} and 10^{-2}-10^{-7} were made for prawn respectively.

Aerobic plate count (APC)

All plates in duplicate on sterile petridish were done on sterile nutrient agar media. From sample solution of different dilutions 0.1 ml samples were taken by a micropipette and transferred aseptically into the previously prepared agar plates by raising the upper lid sufficient enough to enter the tips 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. For total heterotrophic aerobic bacterial counts of prawn, all the inoculated plates were incubated at 30°C for 24-48 hours. The colonies units (CFU) were counted under a Quebec dark field colony counter (Leica, Buffalo. NY. USA) equipped with a guide plate ruled in square centimeters. Plates containing 30-300 colonies were used to calculate bacterial load results, recorded as CFU per unit of sample by using following formula:

$$\text{CFU/g} = \frac{\text{No.of colonies on petridish} \times 10 \times \text{dilution factor} \times \text{volume of total stock solution}}{\text{Wt.of prawn sample (g)}}$$

Determination of enteric bacteria

Presence of enteric bacteria was determined by using selective media EMB- agar and SS- agar. From each stock solution of prawn, 0.1 ml samples

were transferred into the selective media. Growth of bacterial colony in EMB-agar and SS-agar media indicate the presence of enteric bacteria.

Isolation of bacteria

Bacterial colonies were isolated from the fresh and frozen prawn of gher at each sampling. For percent composition, the bacterial colonies were divided into different types according to the colony characteristics of shape, size, elevation, structure, surface, edge, color and opacity, and the number of colonies of each recognizable type was counted. Three to five representatives of each colony type were then streaked on agar plates repeatedly until pure cultures were obtained. The streaked agar plates were incubated at 30°C for two days. Discrete colonies from the streaked agar plates were transferred to agar slants.

Gram's staining

A small portion of bacterial culture from freshly prepared agar slants was taken on a grease free clear glass slide with a sterile inoculating loop and prepared smear on the slide. The smear was then dried in air and fixed by passing the slide 3 to 4 times over a spirit lamp flame. Then it was allowed to cool and flooded with crystal violet solution for approximately 1 minute. Then it was washed with tap water and treated with Gram's iodine solution. After 1 minute the iodine solution was tipped off from the slide by tap water. Then the decolorizing agent, 30% acetone and 70% ethanol mixture was applied to the slide and left for 8-10 seconds and was thoroughly washed with water. Then the smear was flooded with counter stain, safranine for about 1 minute and washed with water. The smear was air dried and microscopically examined under the oil immersion objectives.

Maintenance of stock culture

During the experiment it was necessary to preserve the selected isolates for short term periods. For this purpose, after two days of incubation cultures on agar slants were kept at 4°C for stock and these were transferred to new slants at every 6 weeks intervals.

RESULTS AND DISCUSSION

Bacteriological study of gher-1

Physico-chemical parameters

Water temperature (°C), pH, and dissolved oxygen (mg l^{-1}) were recorded. Water temperature in gher-1 ranged from 24.5 to 31.2°C. The air temperature was recorded from 26.4 to 34.2°C. The pH of water ranged from 6.4 to 7.2. The pH of sediment was recorded from 3.4 to 4.1. The salinity of water ranged from 9 to 11 ppt. The dissolved oxygen (DO) of water ranged from 4.55 to 5.26 mg l^{-1} (Table 1).

Bacterial population in gher-1

During the period of study, bacterial loads in gher were 8.34×10^{3} -2.45×10^{4} CFU ml^{-1} in water; 7.17×10^{8}-1.51×10^{9} CFU g^{-1} in sediment when analysis was done by using fresh samples at gher (Table 2).

Presence of enteric bacteria in gher 1 water, sediment, and fresh prawn samples were analyzed and varied in different samplings. but numbers of enteric bacteria in frozen prawn sample was absent in frozen storage condition (Table 3).

Table 1
Physico-chemical parameters of gher-1.

Temperature (°C)		pH		Salinity (ppt.)	DO (mg l^{-1})
Water	Air	Water	Sediment		
27.85	30.3	6.8	3.75	9.5	4.90

*Values given are mean
Table 2

Bacterial load in water and sediment of gher-1 (fresh samples).

Category	Sample	Bacterial Load			Mean
		1st Sampling	2nd sampling	3rd sampling	
Water	Sample 1	1.23×10^4	5.05×10^4	4.13×10^4	
	Sample 2	4.38×10^3	1.64×10^4	7.75×10^3	
	Average	8.34×10^3	3.35×10^4	2.45×10^4	2.21×10^4
Sediment	Sample 1	8.53×10^7	6.25×10^7	2.74×10^9	
	Sample 2	1.35×10^9	9.40×10^8	2.80×10^8	
	Average	7.17×10^8	5.01×10^8	1.51×10^9	9.10×10^8

*Values given are mean

Table 3
Number of enteric bacteria in water, sediment, fresh and frozen prawn sample of gher 1.

Types	In water samples CFU ml^{-1}	In sediment samples CFU g^{-1}	In fresh prawn samples CFU g^{-1}	In frozen prawn samples CFU g^{-1}
In EMB	24.67	97.33	259.67	Absent
In SS	53.00	135.33	108.33	Absent

*Values given are mean

In different samplings the numbers of bacterial isolates were different and in gher 1, the percentage of Gram +ve was 23.68% and Gram – ve was 76.32% (Table 4).

Bacteriological study of gher-2

Physico-chemical parameters

Water temperature (°C), pH, and dissolved oxygen (mg l^{-1}) were recorded. Water temperature in gher-2 ranged from 25.6 to 30.3°C. The air temperature was recorded from 26.9 to 32.3°C. The pH of water ranged from 6.2 to 7.2. The pH of sediment was recorded from 3.7 to 4.0. The salinity of water ranged from 8 to 11 ppt. The dissolved oxygen (DO) of water ranged from 4.75 to 5.25 mg l^{-1} (Table 5).

Bacterial population

During the period of study, bacterial loads in gher were 7.75×10^4-1.43×10^5 CFU ml^{-1} in water and 9.87×10^8-8.59×10^9 CFU g^{-1} in sediment when analysis was done by using fresh samples at gher (Table 6).

Table 4

Presence of enteric bacteria in gher-2 water, sediment and fresh prawn samples were analyzed and varied in different samplings. but numbers of enteric bacteria in frozen prawn sample was absent in frozen storage condition (Table 7).

In different samplings the numbers of bacterial isolates were different and in gher-2, the percentage of Gram +ve was 22.97% and Gram – ve was 77.02% (Table 8).

Comparative study between gher-1 and gher-2

Quantitative analysis of bacteria

Quantitative results of APC in whole prawn at fresh and different storage condition for 12 months are summarized in Table 9. APC decreased from 3.2×10^7 to 1.4×10^4 CFU g^{-1} at gher-1 and 7.48×10^7 to 2.5×10^4 CFU g^{-1} at gher-2 in the whole prawn collected from different sampling during the period of study (Table 9).

The bacterial load in water and sediment of gher-1 and gher-2 were 2.21×10^4, 9.10×10^8, 9.30×10^4 and 3.30×10^9 respectively in case of gher analysis (Table10).

Total number of bacterial isolates in water, sediment, fresh and frozen prawn and percentage of Gram +ve and Gram –ve bacteria at gher-1.

Samples	1st sampling			2nd sampling			3rd sampling		
	Number of isolates	Gram +ve	Gram –ve	Number of isolates	Gram +ve	Gram –ve	Number of isolates	Gram +ve	Gram –ve
Water	4	1(25%)	3(75%)	5	2(40%)	3(60%)	4	1(25%)	3(75%)
Sediment	6	3(50%)	3(50%)	6	2(34%)	4(66%)	5	1(20%)	4(80%)
Fresh &	8	1(12%)	7(88%)	8	1(12%)	7(88%)	7	2(28%)	5(72%)
Frozen prawn	8	2(25%)	6(75%)	8	1(12%)	7(88%)	7	1(14%)	6(86%)
Total	26	7(27%)	19(73%)	27	6(22%)	21(78%)	23	5(22%)	18(78%)

Table 5
Physico-chemical parameters of gher-2.

Temperature (°C)		pH		Salinity (ppt.)	DO (mg l^{-1})
Water	Air	Water	Sediment		
27.95	29.6	6.7	3.85	8.5	5.00

*Values given are mean

Table 6
Bacterial load in water and sediment of gher-2 (fresh sample).

Category	Sample	Bacterial Load			Mean
		1st Sampling	2nd sampling	3rd sampling	
Water	Sample 1	2.70×10^{4}	1.05×10^{5}	2.55×10^{5}	
	Sample 2	1.28×10^{5}	9.30×10^{3}	3.05×10^{4}	
	Average	7.75×10^{4}	5.72×10^{4}	1.43×10^{5}	9.30×10^{4}
Sediment	Sample 1	1.60×10^{9}	6.18×10^{8}	9.79×10^{9}	
	Sample 2	3.74×10^{8}	3.20×10^{7}	7.39×10^{9}	
	Average	9.87×10^{8}	3.25×10^{8}	8.59×10^{9}	3.3×10^{9}

*Values given are mean

Table 7
Number of enteric bacteria in water, sediment, fresh and frozen prawn sample of gher-2.

Types	In water samples CFU ml^{-1}	In sediment samples CFU g^{-1}	In fresh prawn samples CFU g^{-1}	In frozen prawn samples CFU g^{-1}
In EMB	105.33	171.67	191.33	Absent
In SS	135	105.67	145.67	Absent

*Values given are mean
Table 8

Total number of bacterial isolates in water, sediment, fresh and frozen prawn and percentage of Gram +ve and Gram –ve bacteria at gher-2.

Samples	1st sampling			2nd sampling			3rd sampling		
	Number of isolates	Gram +ve	Gram –ve	Number of isolates	Gram +ve	Gram –ve	Number of isolates	Gram +ve	Gram –ve
Water	6	0(00%)	6(100%)	5	2(40%)	3(60%)	4	1(25%)	3(75%)
Sediment	6	2(33%)	4(67%)	6	2(33%)	4(67%)	5	1(20%)	4(80%)
Fresh &	6	1(16%)	5(84%)	7	1(13%)	6(87%)	8	2(12%)	6(88%)
Frozen	6	2(33%)	4(67%)	7	2(28%)	7(72%)	8	1(12%)	7(88%)
prawn									
Total	24	5(21%)	19(79%)	25	7(28%)	18(72%)	25	5(20%)	20(80%)

Graphical presentation of bacterial load in gher-1 fresh and frozen prawn sample

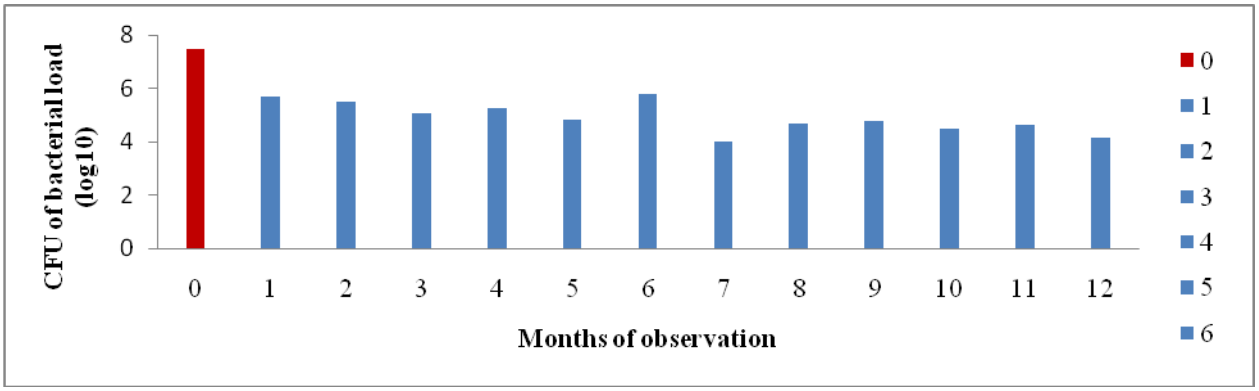

Figure 3
Change in microbiological load in prawn (*Macrobrachium rosenbergii*) under fresh and frozen storage condition at gher-1.

Graphical presentation of bacterial load in gher-2 fresh and frozen prawn sample

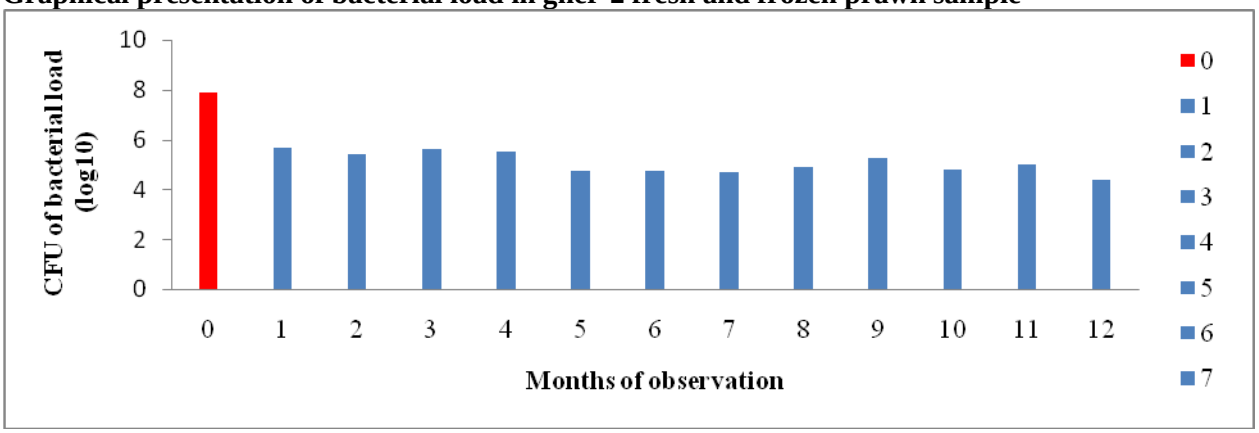

Figure 4
Change in microbiological load in prawn (*Macrobrachium rosenbergii*) under fresh and frozen storage condition at gher-2.

Table 9
Bacterial counts of prawn in fresh and frozen storage for 1 year at gher- 1 and gher-2 (for Monthly interval)
*Values given are mean

No. of gher	Weight of fish (gm)	Length of fish (cm)	Fresh prawn CFU g^{-1}	Month frozen (CFU g^{-1})											
				1^{st}	2^{nd}	3^{rd}	4^{th}	5^{th}	6^{th}	7^{th}	8^{th}	9^{th}	10^{th}	$11th$	12^{th}
1	11.33	17.33	3.2×10^{7}	5.2×10^{5}	3.6×10^{5}	1.2×10^{5}	1.7×10^{5}	6.5×10^{4}	5.9×10^{5}	9.7×10^{3}	4.6×10^{4}	6.3×10^{4}	3.1×10^{4}	4.1×10^{4}	1.4×10^{4}
2	15.6	16.5	7.48×10^{7}	4.9×10^{5}	2.6×10^{5}	4.3×10^{5}	3.1×10^{5}	5.4×10^{4}	5.3×10^{4}	5.2×10^{4}	7.8×10^{4}	1.9×10^{5}	6.4×10^{4}	9.8×10^{4}	2.5×10^{4}

Table 10
Comparative study of bacterial load in water and sediment between gher-1 and gher-2.

Sample	Gher-1	Gher-2
Water	2.21×10^4	9.30×10^4
Sediment	9.10×10^8	3.30×10^9

*Values given are mean

CONCLUSION

Bacterial load reduced significantly during frozen storage of prawn sample. Presence of enteric bacteria in the fresh prawn sample suggested that the ghers were contaminated by mammals, bird faeces and other outer sources;
Gram -ve bacteria were dominant than Gram +ve bacteria in fresh and frozen stored sample;
Sources of contamination should be stopped to keep the water safe for prawn culture.

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