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Full Length Research Paper

Efficacy of probiotics in improving water quality and bacterial flora in fish ponds

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Fish culture aimed at high production through intense culture practices often leads not only to the alteration in water quality but also to severe disease problems. The accumulation of organic wastes deteriorates water quality and encourages the proliferation of pathogenic microorganisms. To avoid these problems, probiotics are used as bioremediation tool. The present study was aimed to know the changing patterns of beneficial and pathogenic bacterial loads and water quality in fish ponds treated with pond probiotics. For this study, three earthen fish ponds, were cultured *Pangasius sutchi*, *Catla catla* and *Labeo rohita*, located in Balliparru near Machilipatnam in Krishna District, Andhra Pradesh, India were selected and studied for a culture period during 2009 to 2010. Two ponds were treated with probiotics having *Nitrosomonas* (1.62 kg/ha) and *Nitrobacter* (0.82 kg/ha) species and one pond was kept as control. During the culture period (August 2009 to July 2010), water quality parameters and the populations of total heterotrophic bacteria (THB), beneficial bacteria (*Nitrosomonas* and *Nitrobacter* species) and pathogenic bacteria (*Pseudomonas*) in water were analyzed. It was observed that in treated ponds, THB and beneficial bacterial load increased and pathogenic *Pseudomonas* load decreased. The bacterial population changed during every fortnight sampling of the culture period. The changing patterns of different bacteria in treated and control ponds were compared and discussed. The concentrations of ammonia, nitrite and phosphates were observed to be low in treated ponds than in the control pond. The present study revealed that the probiotics are instrumental in maintaining good water quality, higher beneficial and lower pathogenic bacterial loads in fish ponds.

Key words: Probiotics, fish ponds, physico-chemical parameters of water, total heterotrophic bacteria, nitrifying bacteria.

INTRODUCTION

Aquaculture has emerged as an important food producing sector in many countries. Like other industries, it constantly requires newly emerging technologies to increase the production yield. Application of probiotics in aquaculture is such a technology and research on probiotics as bioremediation and biocontrol agents is increasing with the demand for eco-friendly aquaculture (Maeda et al., 1997; Moriarty, 1997; 1998; Gatesoupe, 1999; Prabhu et al., 1999; Gomez-Gil et al., 2000; Verschuere et al., 2000; Rao, 2001; Shariff et al., 2001; Irianto and Austin, 2002; Balcázar, 2003; Ali, 2006; Lakshmanan and Sounderpandian, 2008; Sreedevi and Ramasubramanian, 2010; Dimitroglou et al., 2011; Iribarren et al., 2012).

In aquaculture ponds, the quality of water during the culture period will deteriorate mainly due to the accumulation of metabolic wastes, decomposition of unutilized feed and decay of biotic materials. Probiotic bacteria directly uptake or decompose the organic matter or toxic material in the water, thus improving the water quality. In recent years, there is a great interest in the use of probiotic bacteria in aquaculture to

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Table 1. Particulars of inputs used in Pond A, B and C.

Month and Year	Organic Fertilizers				Inorganic Fertilizers				Lime		
	Quality	Quantity (kg/ha)			Quality	Quantity (kg/ha)			Quantity (kg/ha)		
		Pond				Pond			Pond		
		A	B	C		A	B	C	A	B	C
Aug 2009	Poultry manure	1750	2000	2000	Super	4.5	4.5	2.5	--	--	75
Sep 2009	Compost	1675	1800	1800	phosphate	6.5-	6.5	3.5	--	--	--
Oct 2009	Poultry manure	200	200	745	Super	-	--	--	--	--	--
Nov 2009	--	--	--	--	phosphate	--	--	--	--	--	75
Dec 2009	--	--	--	--	--	--	--	--	--	--	--
Jan 2010	Poultry manure	975	975	820	Super	--	--	--	--	--	75
Feb 2010	Cattle dung	1175	1175	1175	phosphate	--	--	--	--	--	25
Mar 2010	--	--	--	--	--	--	--	--	--	--	--
Apr 2010	Poultry manure	--	--	250	--	--	--	--	--	--	--
May 2010	Poultry manure	--	1975	1825	--	--	--	--	--	--	--
Jun 2010	Poultry manure	1975	--	--	--	--	--	--	--	--	--

improve water quality, inhibit pathogens and promote the growth of farmed fish (Verschuere et al., 2000). The use of probiotics which control pathogens through a variety of mechanisms is viewed as an alternative to antibiotics and become a major field in the development of aquaculture. Keeping in view of the beneficial effects of probiotics, some enthusiastic farmers are recently using commercially available probiotics in their fish ponds. However, there is no documented evidence on the efficacy of these probiotics on the ecological processes in fish ponds of this region and so far no attempt has been made to study the role of probiotics in improving water quality and bacterial flora in fish ponds. Hence, the present study was undertaken to know the water quality and the changing patterns of beneficial and pathogenic bacterial loads in fish ponds treated with pond probiotics and compare the results with those of the untreated pond.

MATERIALS AND METHODS

Fish and fish ponds

Three fish ponds located in a farm at Balliparru near Machilipatnam (latitude 16º 09' N, longitude 81° 12' E) in Krishna district, Andhra Pradesh, India were chosen for the present study. The earthen ponds are designated as Pond A, B and C. In the three ponds *Pangasius sutchi*, *Catla catla* and *Labeo rohita* were stocked in the ratio of 4:1:1.5 at the rate of 20,785 fingerling/ ha. The fish were fed with supplementary feed mostly with deoiled rice bran, groundnut oil cake and slaughter waste at the rate of 2% body weight of fish per day. Prior to and after the release of fish, all the ponds were treated with organic manures such as poultry manure, compost and cattle dung, and inorganic fertilizers such as superphosphate. The particulars of fertilizers and lime used in different months in three ponds are given in Table 1.

The study was carried out for a culture period from August 25, 2009 to July 23, 2010. The extent (water spread area) of Pond A, B and C is 3.22, 3.22 and 3 ha, respectively. The depth of the ponds ranged between 1.2 and 1.5 m. Pond A and B were treated with probiotics whereas Pond C was kept as control pond, that is, without probiotics treatment.

Probiotics

In this study, commercially available probiotics with *Nitrosomonas* species and *Nitrobacter* species manufactured by K.C.P Sugar and Industries Corporation Limited, Vuyyuru, Krishna district, Andhra Pradesh, India were used. Probiotics were used in Pond A and B at monthly intervals with *Nitrosomonas* probiotics at 1.62 kg/ ha and *Nitrobacter* probiotics at 0.82 kg/ ha.

Water samples

In the present study, physico-chemical parameters of water and bacterial loads were studied at fortnight intervals by collecting water samples in between 8 and 10 a.m. The physico-chemical parameters such as temperature, transparency, dissolved oxygen, pH, total alkalinity, total hardness, total dissolved solids, nitrite, nitrate, ammonia, phosphorus and iron of water were estimated by following the methods suggested in Golterman and Clymo (1969); Wetzel and Likens (1979); APHA (1999). Primary productivity was determined using the light and dark bottle method described by Vollenweider (1969).

Bacteriological analysis

Bacteriological analysis was carried out for the isolation and

Table 2. Physico-chemical parameters of water (Mean± SD and Ranges) in ponds.

Physico-chemical parameters	Pond-A		Pond-B		Pond-C	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Air temperature(°C)	27.02±3.09	20.50-30.20	26.96 ±3.03	20.90- 31.10	29.06±1.99	25.50-33.00
Water temperature(°C)	29.17±2.32	25.30-31.40	29.66±2.11	25.20-32.70	29.93±1.35	27.60-32.50
Secchi disc transparency(cm)	29.19±1.48	26.10-32.20	28.36±2.15	24.50-31.20	29.37±1.97	26.50-34.20
Dissolved oxygen(mg/l)	4.80±0.81	3.20-6.20	4.09±1.30	2.40-6.40	3.50±0.88	2.00-5.20
pH	7.87±0.43	7.29-8.88	7.92± 0.35	7.50-8.56	7.92±0.40	7.44-8.65
Total alkalinity(mg/l as $CaCO_3$)	148.70±24.68	110-192	150.7±18.13	120-180	134.50±24.50	100-184
Total hardness(mg/l as $CaCO_3$)	153.05±30.79	100-220	164.2±18.51	130-195	147.75±21.97	105-190
Conductivity(mS)	14.55± 6.45	6.72-26.10	13.76±5.78	7.06-26.60	8.53±3.58	3.51-15.80
Total dissolved solids (ppt)	12.48±6.47	4.28-23.60	12.05±6.39	5.24-24.90	7.64±4.37	2.41-16.70
Ammonia-N(mg/l)	0.41±0.07	0.28-0.53	0.38±0.060.	0.27-0.50	0.51±0.080.0	0.38-0.73
Nitrite-N(mg/l)	0.04±0.05	0.08-0.25	03±0.01	0.09-0.07	7±0.02	0.02-0.11
Nitrate-N(mg/l)	0.26±0.09	0.10-0.45	0.26±0.09	0.12-0.46	0.21±0.07	0.09-0.41
Orthophosphates(mg/l)	0.41±0.09	0.25-0.59	0.45±0.06	0.32-0.58	0.56±0.09	0.42-0.75
Iron(mg/l)	0.28±0.06	0.18-0.41	0.27±0.05	0.19-0.36	0.39±0.11	0.10-0.59
Gross Primary Productivity (gc/m^3/h)	0.19±0.19	0.07-0.87	0.14±0.11	0.05-0.40	0.09±0.15	0.02-0.75
Net Primary Productivity (gc/m^3/h)	0.09±0.05	0.02-0.24	0.06±0.10	0.02-0.37	0.09±0.07	0.02-0.26
Community respiration (gc/m^3/h)	0.08±0.13	0.02-0.62	0.07±0.08	0.02-0.38	0.05±0.03	0.02-0.16

enumeration of total heterotrophic bacteria (THB), *Nitrosomonas* species, *Nitrobacter* species and *Pseudomonas* species in three ponds. Samples for bacterial analysis were collected in well cleaned, dried and sterile bottles which were sterilized at 121°C under pressure of 15 lbs for 15 min. Column water samples were collected for the analysis of THB and *Pseudomonas* whereas bottom water samples for *Nitrosomonas* and *Nitrobacter*. After collection, 1 ml of the sample was transferred to sterile conical flask (150 ml) containing 99 ml of sterile distilled water and serial dilution was performed to get 10^{-1},10^{-2},10^{-3},10^{-4} and 10^{-5} samples. THB was enumerated by adopting the spread plate method (Chen and Kueh, 1976; Cappuccino and Sherman, 1992). From the diluents, 0.1 ml was spread in the plates and incubated in an inverted position at 37°C for 20 to 24 h. *Nitrosomonas* species was cultured by using Winogradsky phase-I medium whereas *Nitrobacter* species by Winogradsky phase-II medium. From the diluents, 0.1 ml was inoculated into the medium and plates were incubated at 28±2°C for 48 h. *Pseudomonas* species was cultured by using *Pseudomonas* base medium (Hi-medium Mumbai). From the diluents, 0.1 ml of the sample was inoculated into the medium and incubated at 37°C for 24 to 48 h. All the determinations were carried out in triplicates. Following incubation, plates containing viable colonies were used to calculate bacterial population results. The colonies were counted and expressed as cfu/ml. To study the correlations among physico-chemical parameters of water studied, simple correlation coefficients were calculated and correlation matrix has been developed using SPSS 17.0 software.

RESULTS

Physico-chemical parameters of water

The physico-chemical parameters of water such as temperature, transparency, dissolved oxygen, pH, total alkalinity, total hardness, total dissolved solids, nitrite, nitrate, ammonia, phosphorus, iron and primary productivity were studied at regular fortnight intervals of the culture period. The values of Mean ±SD (n=20) and their ranges in three ponds are shown in Table 2.

Bacterial analysis

Total heterotrophic bacteria (THB) were observed to be high in Pond A and B and low in Pond C. The loads recorded were 5.25×10^5 to 7.80×10^5 cfu/ml in Pond A, 5.42×10^5 to 7.90×10^5 cfu/ml in Pond B and 2.05×10^5 to 4.72×10^5 cfu/ml in Pond C. *Nitrosomonas and Nitrobacter* loads are relatively higher in Pond A and B than in control pond whereas *Pseudomonas* were observed to be higher in control pond than in treated ponds A and B. *Nitrosomonas* loads ranged from 2.01×10^3 to 4.80×10^3 cfu/ml in Pond A, 2.25×10^3 to 4.90×10^3 cfu/ml in Pond B and 1.24×10^3 to 2.00×10^3 cfu/ml in control pond and *Nitrobacter* loads in Pond A, B and C ranged from 2.20×10^3 to 4.95×10^3, 2.10×10^3 to 4.15×10^3 and 1.10×10^3 to 2.15×10^3, respectively. *Pseudomonas* loads in Pond A and B ranged from 1.00×10^5 to 2.96×10^5 cfu/ml and 1.02×10^5 to 2.9×10^5 cfu/ml and in control pond from 2.36×10^5 to 7.25×10^5 cfu/ml. Bacterial populations such as total heterotrophic bacteria, *Nitrosomonas, Nitrobacter* and *Pseudomonas* from three ponds are shown in Figure 1(a-d). The relative bacterial loads during the culture

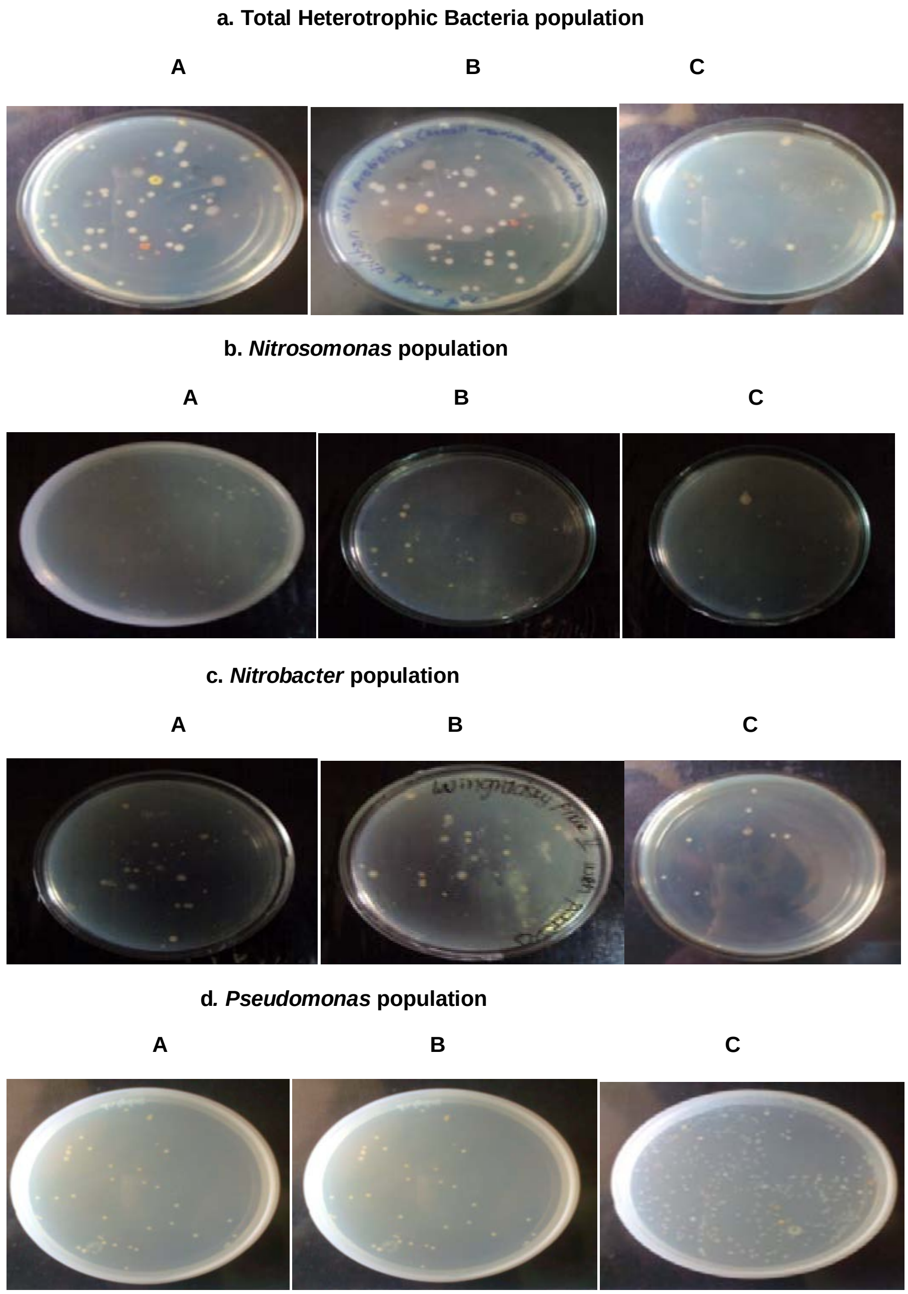

Figure 1. Bacterial populations in ponds A, B and C.

period in the three ponds are shown in Figure 2 (a-d).

DISCUSSION

In intensive and semi-intensive aquaculture practices, high stocking densities of fish along with intense feeding and fertilization often leads to the deterioration of water quality and proliferation of pathogens. These are the major constraints in fish culture. In the present study, water quality parameters of the ponds treated with probiotics were observed to be good which might be because of the various roles played by the microbes. During the period of observation, the amplitude ofvariation in water temperature was very narrow (Table 2) which is considered to be a characteristic of tropical

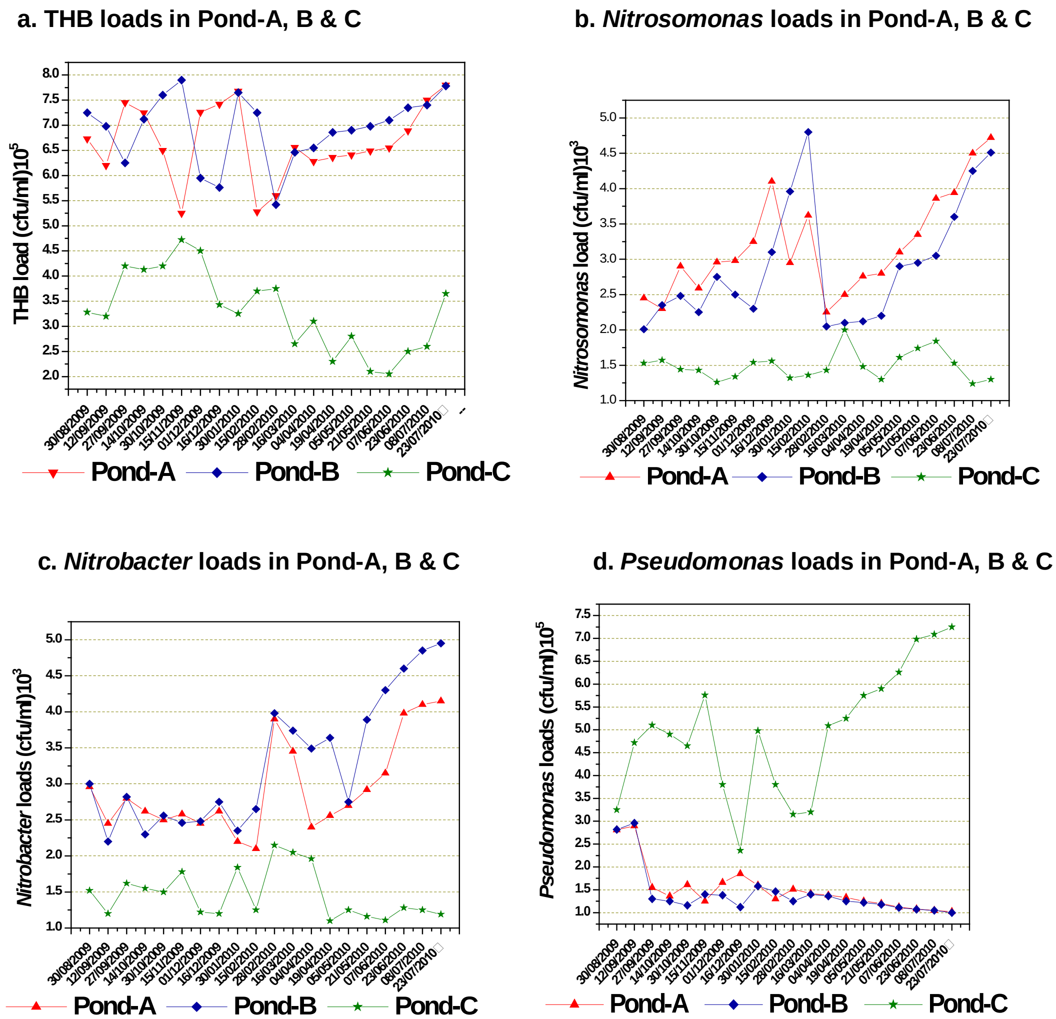

Figure 2. Relative abundance of bacterial loads in the three ponds.

waters (Rahman et al., 1982; Dewan et al., 1991; Wahab et al., 1996; Ahmed et al., 2000). The water temperature in general follows with the pattern of fluctuation of air temperature and showed a strong positive correlation in Pond A ($r = 0.616$; $P < 0.01$), Pond B ($r = 0.857$; $P<0.01$), and Pond C ($r = 0.639$; $P<0.01$). Transparency of water showed an inverse correlation with temperature in Pond A ($r = -0.090$; $P>0.05$), Pond B ($r = -0.509$; $P<0.05$) and Pond C ($r = -0.443$; $P>0.05$). Transparency of water also correlated negatively with dissolved oxygen in Pond A($r = -0.457$; $P<0.05$) and C ($r = -0.326$; $P>0.05$). These relations are interdependent and could be explained by the fact that the high temperatures are conducive to the development of phytoplankton. With the increase in the densities of phytoplankters, a corresponding decrease in the secchi disc transparency and increase in dissolved oxygen content was observed. Hence, a negative correlation between dissolved oxygen and transparency of water was discernible. Relatively higher levels of dissolved oxygen in Pond A and B might be due to the beneficial effect of probiotics which favored the mineralization of organic matter. pH of water has been found to be positively correlated with dissolved oxygen in Pond A ($r = 0.591$; $P<0.05$), Pond B ($r = 0.485$ $P<0.05$) and Pond C ($r = 0.466$ $P<0.05$). Due to intense photosynthetic activity, the free carbon dioxide (if present) or the carbon dioxide of bicarbonate is drawn out by the algae; this results in the decrease of free CO_2 and bicarbonate and increase of carbonate. It is well established that the carbonates increase the pH of water on hydrolysis. Simultaneously, the photosynthetic release of oxygen increase the dissolved oxygen content in the water. The relationship of pH, free carbon dioxide, carbonates and bicarbonates has been discussed by many workers (Seenayya, 1971; Rao, 1972). It was generally noticed that the higher pH values coincided with the period of greater photosynthetic activity of abundant phytoplankton which received support from the relation between primary productivity and pH (r = 0.600 P<0.01).

Total hardness showed a positive correlation with total alkalinity in Pond A (r = 0.853; P<0.01) and Pond B (r = 0.644; P<0.01). According to Boyd (1982), the total hardness is usually related to total alkalinity as the cations of hardness and anions of alkalinity are normally derived from the solution of carbonate minerals. Arce and Boyd (1980) also observed a high positive correlation between total alkalinity and total hardness in pond waters. Conductivity and total dissolved solids represent the mineral content of water and hence they exhibit significant positive relationship in Pond A (r=0.933; P<0.01), Pond B (r = 0.958; P<0.01) and Pond C (r = 0.900; P<0.01). Higher values of conductivity were noticed in the month corresponding to the organic fertilizer application in the ponds. This could be explained by the fact that poultry manure has high soluble inorganic salts and is responsible for the increase of conductivity in the water (Ray and David, 1969).

The nutrients, nitrate-N, nitrite-N, and ammonia-N in the pond water did not follow the same pattern of distribution and the variations may be due to biological or chemical reactions or combination of these two. The application of probiotics, fertilizers, supplementary feeds and metabolites released by the fish might also be responsible for such variations. During the study period, the levels of ammonia and nitrites were relatively low in Pond A and B than in Pond C. This might be because of the use of nitrifying bacteria in the form of probiotics. As these bacteria are known to convert ammonia to nitrite and then to nitrate, low levels of ammonia and nitrite observed in Pond A and B compared to Pond C can be supported. A significant positive correlation could be discernible between nitrate-N and dissolved oxygen in Pond A (r=0.0.462; P<0.05) and Pond B (r = -0.633 P <0.05). The oxidation of various forms of inorganic nitrogen in the well oxygenated surface water might have resulted in the increased concentration of nitrates. A negative correlation has been observed between ammonia and temperature in Pond A (r = -0.448; P<0.01), Pond B (r = -0.373; P>0.05) and Pond C (r = -0.102; P>0.05). This indirect correlation may be explained by the fact that during high temperatures, the intense photosynthetic activity of phytoplankters release oxygen into the pond waters and as such a considerable amount of ammonia might have been converted to oxidized forms in addition to the activity of nitrifying bacteria.

In fish ponds, mineralization of fertilizers, feed wastes and excreta often increases the ammonia concentration, which is harmful to fish above 0.1 mg/l. Hence, it is a critical water quality parameter to be maintained at optimal level in fish ponds. The nitrogen cycle involves the oxidation of ammonia to nitrite by bacteria of the genus *Nitrosomonas* and the subsequent oxidation of the nitrite to nitrate by *Nitrobacter*. Inputs of ammonia cannot be eliminated from the water body. However, it can be converted to non-toxic nitrate by nitrifying bacteria which can be accomplished by means of probiotic treatment. The levels of ammonia and nitrites were relatively low in Pond A and B than in Pond C. This might be because of the use of nitrifying bacteria in the form of probiotics. As these bacteria are known to convert ammonia to nitrite and then to nitrate, low levels of ammonia and nitrite observed in Pond A and B compared to Pond C can be supported.

Phosphorus occurs mainly in the form of phosphate and this element is recognized to be the most important critical factor in the maintenance of pond fertility (Boyd, 1982). It was observed that orthophosphate concentrations were maintained at low levels in probiotic treated ponds than in control ponds. Rao (2001) reported that the probiotic bacteria utilize phosphate for their body metabolic activities and thus diminish this nutrient in pond waters. Orthophosphates showed a significant positive relationship with dissolved iron in Pond A (r = 0.452; P<0.05), Pond B (r = 0.482; P<0.05) and Pond C (r = 0.485; P<0.05). Seenayya (1971) also observed that phosphate and iron were in some way related to each other.

Primary production or the organic carbon fixed through photosynthetic activity by phytoplankton helps in understanding the productive function of aquatic system (Odum, 1971). The organic matter synthesized by primary producers is utilized by the consumers inhabiting the system. The well maintained nutrient levels in probiotic treated ponds A and B support the abundant primary producers and then the next trophic levels like zooplankton and fish. Primary productivity showed positive relationship with dissolved oxygen and pH in the ponds studied.

Probiotic bacteria are known to improve water quality in many ways. Heterotrophic bacteria necessitating some organic sources of carbon in addition to inorganic forms for growth have a significant role in the decomposition of organic matter and production of particulate food materials from dissolved organics (Jana and De, 1990). There are many studies on the relationship between heterotrophic bacteria and water quality (Guo et al., 1988; Fang et al., 1989; Liu et al., 1992). In the present study, total heterotrophic bacteria (THB) were observed to be high in Pond A and B and low in Pond C. The loads recorded were $5.25x10^5$ to $7.80x10^5$ cfu/ml in Pond A, $5.42x10^5$ to $7.9x10^5$ cfu/ml in Pond B and $2.05x10^5$ to $4.72x10^5$ cfu/ml in Pond C. The high levels of heterotrophic bacteria in Pond A and B might be due to the consumption of more organic matter as their sources of carbon and improve water quality. Heterotrophic bacteria are known to utilize nitrogen rich substances and release ammonia or ammonium salts (Jana and Barat, 1983). However, as Pond A and B are treated with nitrifying bacterial probiotics, which convert ammonia to nitrates, relatively low levels of ammonia compared to control pond were observed.

In the present study, *Nitrosomonas* loads in Pond A, B and control ponds ranged from 2.01×10^3 to 4.80×10^3 cfu/ml, 2.25×10^3 to 4.9×10^3 cfu/ml and 1.24×10^3 to 2.00×10^3 cfu/ml, and *Nitrobacter* loads from 2.20×10^3 to 4.95×10^3, 2.10×10^3 to 4.15×10^3 and 1.10×10^3 to 2.15×10^3, respectively. As the Ponds A and B are treated with probiotics having *Nitrosomonas* and *Nitrobacter* species, their abundance in these ponds can be explained. These bacterial loads were also observed to be gradually increasing by the end of the culture period (Figure 2b and 2c). As these bacteria are known to convert ammonia to nitrite and then to nitrate, low levels of ammonia and nitrite observed (Table 2) in Pond A and Pond B compared to Pond C can be supported.

Several species of *Pseudomonas* and *Aeromonas* are pathogenic and reported to cause various kinds of skin ulcerations including the most dreaded Epizootic ulcerative syndrome (EUS), albinoderma, erythroderma, tail and fin rot and hemorrhagic septicemia (Das, 2004). In the present study, *Pseudomonas* loads in Pond A and B ranged from 1.00×10^5 to 2.96×10^5 cfu/ml and 1.02×10^5 to 2.90×10^5 cfu/ml and in control Pond from 2.36×10^5 to 7.25×10^5 cfu/ml (Figure 2d). It was observed that *Pseudomonas* loads showed changing patterns from sampling to sampling with decreasing trend to the end of the culture period. Customarily, the indigenous bad bacteria can cause diseases but when the probiotics are introduced into the ponds, the new comers eliminate the pre-existing bacteria out of the nutrients queue. Thus, the old/bad bacteria, never having had to compete for food, cannot keep pace with the aggressive probiotics. Further, probiotic bacterial excretions make the pond medium less inhabitable for bad bacteria. Not only do probiotic bacteria have terrific appetites; they excrete exo-enzymes as a natural byproduct of their metabolic activity. These enzyme excretions infuse and spread throughout the pond medium, changing its chemistry and destroying bacteria. Thus, the low levels of *Pseudomonas* in probiotic treated Pond A and B can be explained. The general conclusion obtained from the present study is that the probiotics played a major role in maintaining optimum water quality parameters especially dissolved oxygen, ammonia, nitrite, nitrate and phosphates throughout the culture period. It is clear from the bacterial load data that the *Nitrosomonas* and *Nitrobacter* species were dominated and suppressed the *Pseudomonas* species in the probiotic treated ponds when compared to the control pond. Hence by using probiotics, it can be possible to improve water quality and prevent the occurrence of bacterial diseases in fish ponds.

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