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Seasonal variations in abundance of nitrifying bacteria in fish pond ecosystem

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Abstract

Seasonal changes in abundance of nitrifiers (ammonia-oxidizing and nitrite-oxidizing bacteria) in surface and bottom water of freshwater ponds were examined with respect to temperature, DO, pH as well as concentration of ammonia and nitrite. The most probable number (MPN) of ammonia-oxidizers in different ponds varied from 1297±3.6 to 1673.23±0.36 ml^{-1} in bottom and 720.5±8.1 to 955.3±10.8 ml^{-1} in surface water during the rainy season while the MPN ranged from 1074±1.07 to 1372.17±4.6 ml^{-1} in bottom and 515±10.1 to 678±11.8 ml^{-1} in surface water in winter. However, the MPN were greatly reduced in summer and ranged from 435.05±15.7 to 547.54±2.12 ml^{-1} in bottom and 218.7±7.3 to 368.4±9.32 ml^{-1} in surface water. Similar seasonal trends were also observed in MPN of nitrite-oxidizers. Among all the physico-chemical parameters, abundance of nitrifiers was more positively correlated with ammonia and nitrite concentration in all the seasons. The abundance of nitrifiers in surface and bottom water was highest in rainy season followed by winter and modest in summer. The potential nitrification activities and oxidation rates were shown to be linear and activity of ammonia-oxidizing and nitrite-oxidizing bacteria was highest during rainy season.

Key words

Ammonia-oxidizing bacteria, Nitrite-oxidizing bacteria, MPN, Fresh water pond

Introduction

Autotrophic nitrifying bacteria or nitrifiers (ammonia-oxidizing and nitrite-oxidizing bacteria), commonly found in soil, sediment as well as waste, fresh and marine waters, are essential components of nitrogen (N) cycle, linking the most reduced and oxidized forms of inorganic N (Prosser, 1989). Nitrification is microbiological process by which ammonia is oxidized to nitrite by ammonia-oxidizing bacteria (AOB) which is subsequently oxidized to nitrate by nitrite-oxidizing bacteria (NOB) (Siripong *et al*., 2006). Nitrifying bacteria and phytoplankton, which are important primary producers in the aquatic ecosystem, have unique correlation in their use of ammonia and nitrate. These bacteria produce nitrate, which is then utilized by phytoplankton (Feliatra and Bianchi, 1993; Binachi *et al.,* 1994; Feliatra, 2003). In the environments with high inputs of a nutrient such as freshwater fish ponds, mineralization of organic substances as a result of over-feeding and excretion increases the ammonia concentration which is harmful to fish and shrimp (Goldman *et al.,* 1985). Since microbial processes affect water quality parameters such as dissolved oxygen (DO), ammonium, nitrite, nitrate *etc.* (Moriarty, 1996), hence bacteria in ponds play important role in maintaining the water column chemistry. The distribution of nitrifying bacteria in different niches of ponds depends upon the substrate concentration, pH, water stress and other environmental parameters (Belser and Schmidt, 1978). Temperature (Berounsky and Nixon, 1993), light (Horrigan and Springer, 1990), oxygen (Usui *et al*., 2001), ammonia (Magalhaes *et al*., 2005) and sulphide (Joye and Hollibaugh, 1995) are reported to affect the rates of nitrification in different ecosystems. The present study was undertaken to investigate the seasonal variations in abundance of nitrifying bacteria in relation to change in temperature, dissolved oxygen, pH and concentration of ammonia as well as nitrite in the surface and bottom water of fish ponds.

Materials and Methods

Samples of the surface and bottom water were collected from three freshwater ponds (Fwp 1, 2 and 3) of different size at

National Bureau of Fish Genetic Resources, Lucknow, India. Sampling was carried out for a one-year period from August 2008 to September 2009. Size of the fishponds varied from 0.10 to 0.35 ha with stocking density of Indian major carps of 600 to 2100 fingerling ha^{-1}. Ponds were fertilized with raw cow dung and had aeration facility. The fish were fed with supplementary diet of rice bran and mahua oil-cake at a rate of 2-4% of body weight. Enumeration of nitrifying bacteria in the water samples was done by microplate most-probable-number (MPN) method (Rowe *et al*., 1977). Briefly, 50 ml of media was placed into each of the 8/12 wells of a sterile microtitre plate. Aliquots (50 µl) of the bottom and surface water suspension were taken into each of the first eight wells. Two-folds serial dilutions of samples were then prepared across the plate to obtain eight replicates of each dilution. Inorganic ammonium medium for ammonia-oxidizer and nitrite broth for nitrite-oxidizer was placed in the wells (Soriano and Walker, 1978). The plates were covered with parafilm and incubated for 3 weeks. Finally, Greiss reagent and diphenylamine were applied to examine whether nitrite or nitrate was formed. Micro-wells containing dark colored solutions are positive tests for nitrite/ nitrate. Values for P I, P 2, and P 3 were determined as rows: P1 is the number of positive wells in the least-concentrated dilution in which either all the wells are positive or the greatest number are positive, and P 2 and P 3 are the number of positive wells in the next two higher dilutions, respectively. The abundance of nitrifiers was estimated based on classical MPN statistics. The MPN value was then multiplied by the dilution factor.

Nitrification potential was measured using the shaken-slurry method (Hart, 1994). Briefly, 20 ml water was taken into a 250 ml Erlenmeyer flask. 100 ml of 1.5 mM $(NH_4)_2SO_4$ solution was added and the bottle capped with parafilm to allow gas exchange during the potential nitrification assay. All flasks were shaken at 160 rpm for 24 hr. Aliquots of 10 ml were removed from each flask after 2, 8 and 24 hr and centrifuged at 8000 rpm for 5 min. The supernatant was decanted and filtrate was analyzed for the NO_3-N content by spectrophotometer (543 nm). NO_3-N pools increased linearly throughout the 24 hr incubation period.

Table - 1: Ammonia and nitrite concentration of surface and bottom water samples

Parameter	Season	Fwp 1	Fwp 2	Fwp 3
		Surface water		
Ammonia (μmol^{-1})	Rainy	10.8±0.08	13.4±0.08	15.26±0.05
	Winter	13.08±0.03	13.2±0.26	13.6±0.32
	Summer	15.23±0.06	16.2±0.07	16.31±0.11
Nitrite (μmol^{-1})	Rainy	0.17±0.01	0.24±0.01	0.37±0.015
	Winter	0.21±0.09	0.35±0.02	0.48±0.04
	Summer	0.25±0.02	0.58±0.02	0.66±0.02
		Bottom water		
Ammonia (μmol^{-1})	Rainy	16.18±0.15	21.04±0.21	22.59±0.09
	Winter	15.18±0.010	22.17±0.057	22.23±0.17
	Summer	17.6±0.089	23.07±0.16	23.82±0.09
Nitrite (μmol^{-1})	Rainy	0.54±0.046	0.79± 0.001	0.99±0.10
	Winter	0.66±0.010	0.76±0.07	1.44±0.082
	Summer	0.68±0.057	0.80±0.039	3.23±0.02

Values are mean ± SE (standard error) of eight times analysis. Fwp= Fresh water pond

Table - 2: Regression parameter, correlation-coefficient and significant level for abundance of ammonia-oxidizer and nitrite-oxidizer (surface water) with respect to nitrification rate, ammonia and nitrite concentration

X-variables	Y-variables	Season	a	b	r^2	P	N
Ammonia-oxidizer	Ammonia concentration	Rainy	-133.0	0.01±0.001	0.73	< 0 .0001	24
		Winter	-267.5	0.005±0.001	0.46	< 0 .0001	24
		Summer	3.44	62.54±20.49	0.29	< 0 .0001	24
Ammonia- oxidizer	Nitrite	Rainy	-0.43	116.3±78.79	0.90	< 0.0001	24
		Winter	-0.99	464.3±42.2	0.84	< 0.0001	24
		Summer	-0.451	289.7±50.39	0.60	< 0.0001	24
Ammonia-oxidizer	Nitrification rate	All season	-0.4026	343.1±14.94	0.88	< 0.0001	72
Nitrite-oxidizer	Ammonia concentration	All season	21.99	-62.25±13.05	0.24	< 0.0001	72
Nitrite-oxidizer	Ammonia- oxidizer	All season	8.603	0.87±0.27	0.93	< 0.0001	72
Nitrite-oxidizer	All season	Nitrification rate	-0.3474	305.8±14.8	0.85	< 0.0001	72

Regression analyses were performed on seasonal pooled data of the studies sites. a = Intercept, b = Slop, r^2 = Correlation-coefficient, P = probability, N = Number of samples frequency

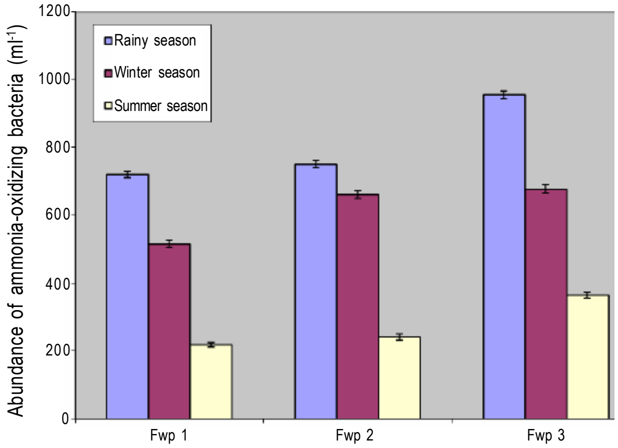

Fig. 1: Abundance of ammonia-oxidizing bacteria in surface water

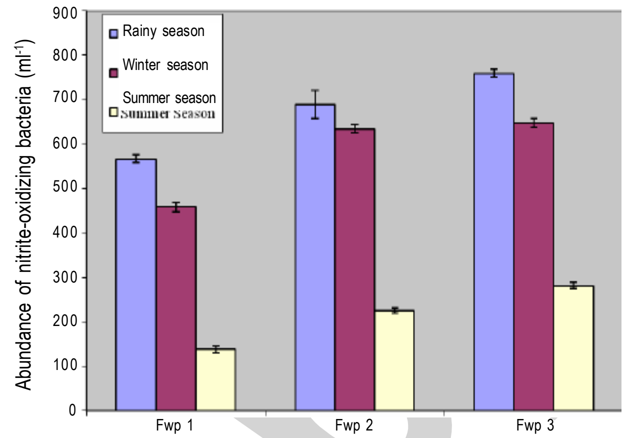

Fig. 3: Abundance of nitrite-oxidizing bacteria in surface water

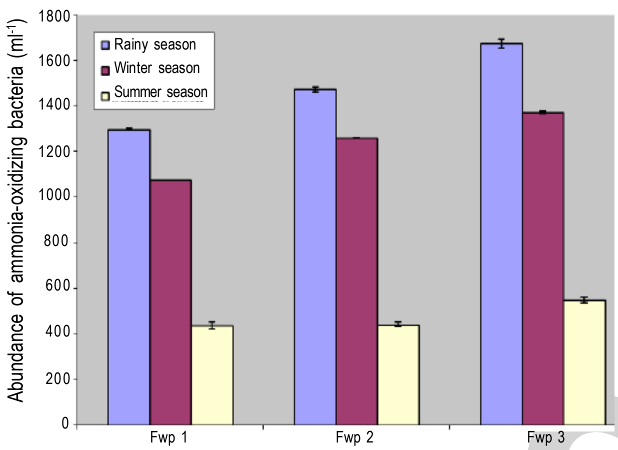

Fig. 2: Abundance of ammonia-oxidizing bacteria in bottom water

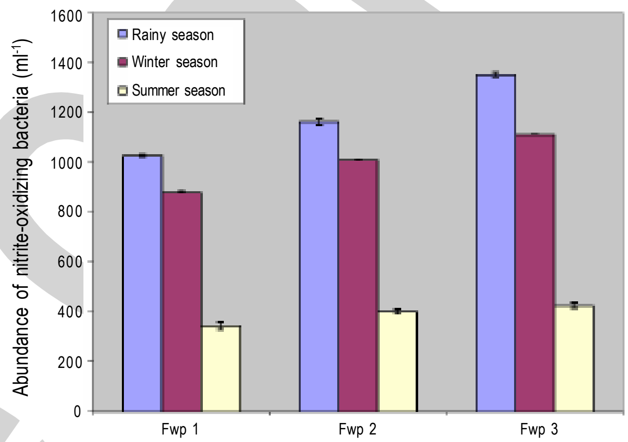

Fig. 4: Abundance of nitrite-oxidizing bacteria in bottom water

DO was measured by Winkler's method while ammonia and nitrite concentration of water samples was estimated by water analysis kit (Hi-Media). The comparison of the physico-chemical properties, nitrification rate and abundance of nitrifiers in the bottom and surface water of ponds was done by one-way ANOVA and estimation of their relationship by linear regression. All statistical analysis was carried out using the SPSS version 10.0 for Windows.

Results and Discussion

Temperature of the pond water ranged between 24.6±0.37-32.1±0.14°C, the highest value was recorded in Fwp 1 during summer season and lowest value during winter season. Dissolved oxygen exhibited highest concentration of 5.5 ±0.23, 5.7±0.07 and 6.02 ± 0.05 mg^{-1}, respectively in Fwp 1, 2 and 3 during rainy season. These values declined considerably in winter, the lowest being in summer. The pond water pH varied from 7.3±0.06-8.4±0.30, the highest being in summer (8.3±0.06-8.4±0.3), followed by rainy (7.6±0.02-7.90.1) and the lowest (7.3±0.064-7.50.04) during winter season.

Concentration of ammonia and nitrite exhibited variations in the surface and bottom water. Ammonia concentration of surface water varied from 10.8±0.08 to 16.31±0.11 μmol^{-1} whereas in the bottom water it was in the range of 15.18±0.010 to 23.82±0.09 μmol^{-1}. Concentration of ammonia was higher in the bottom and surface water during summer in all the Fwp. Pond surface water had lower concentration (0.17±0.01 to 0.66±0.02 μmol^{-1}) of nitrite as compared to bottom (0.54±0.046 to 3.23±0.02 μmol^{-1}). Fwp 3 had the highest concentration of nitrite (3.23± 0.02 μmol^{-1}) during the summer season (Table 1).

Total count of ammonia-oxidizers in the surface water was highest (range 720±8.1- 955.3±10.8 ml^{-1}) during rainy season and lowest (218±7.3-368.4±9.32 ml^{-1}) in summer (Fig. 1). In bottom water also, these counts were significantly higher than surface water with highest value (1297±3.6-1673.23±0.36 ml^{-1}) in rainy season (Fig. 2). Interestingly, the nitrite-oxidizers counts in surface water were lower (Fig. 3) as compared to bottom water during all the seasons (Fig. 4).

Correlation coefficient between the physico-chemical parameters of water and bacterial populations have been summarized in Table 2, 3 and 4. The abundance of AOB was positively correlated with NOB in surface (r^2 = 0.93, p <0.0001) and bottom water (r^2 = 0.99, p<0.0001). Both the bacterial populations were also positively correlated with nitrification rate in all the seasons in bottom (r^2 = 0.92,

Table - 3: Regression parameter, correlation-coefficient and significant level for abundance of ammonia-oxidizer and nitrite-oxidizer (bottom water) with respect to nitrification rate, ammonia and nitrite concentration

X-variables	Y-variables	Season	a	b	r^2	P	N
Ammonia-oxidizer	Ammonia concentration	Rainy	-7.80	53.22±4.3	0.87	<0.0001	24
		Winter	-17.31	33.20±3.3	0.82	<0.0001	24
		Summer	-15.09	12.67±3.4	0.37	<0.0001	24
Ammonia- oxidizer	Nitrite	Rainy	-2.73	399.4±36.7	0.84	<0.0001	24
		Winter	-3.727	265.9±31.4	0.76	<0.0001	24
		Summer	-2.55	115.6±9.9	0.86	<0.0001	24
Ammonia- oxidizer	Nitrification rate	All season	-1.082	340.2±10.3	0.93	<0.0001	72
Nitrite-oxidizer	Ammonia concentration	Rainy	-10.82	3.26±2.1	0.91	<0.0001	24
		Winter	-11.38	0.319±0.003	0.76	<0.0001	24
		Summer	-12.09	11.67±1.44	0.74	<0.0001	24
Nitrite-oxidizer	Nitrite	Rainy	-2.507	339.7±33.0	0.83	<0.0001	24
		Winter	-3.891	208.5±22.7	0.79	<0.0001	24
		Summer	-4.995	322.0±15.3	0.53	<0.0001	24
Nitrite-oxidizer	Nitrification rate	All season	-1.185	266.5±8.9	0.92	<0.0001	72
Nitrite-oxidizer	Ammonia- oxidizer	All season	20.60	1.266±0.011	0.99	<0.0001	72

Regression analyses were performed on seasonal pooled data of the studies sites. a = Intercept, b = Slop, r^2 = Correlation-coefficient, P = Probability, N = Number of samples frequency

Table - 4: Regression parameter, correlation-coefficient and significant level for abundance of ammonia-oxidizer and nitrite-oxidizer with DO, pH and temperature

X-variables	Y-variables	Season	a	b	r^2	P	N
Ammonia-oxidizer	DO	All season	-.01266	136.5 ± 15.76	0.44	< .0001	72
Ammonia-oxidizer	pH	All season	6.253	501.2 ± 64.4	0.38	< .0001	72
Ammonia-oxidizer	Temperature	All season	20.99	-61.25 ± 13.05	0.28	< .0001	72
Nitrite-oxidizer	DO	All season	-0.3610	190.4 ± 25.48	0.44	< .0001	72
Nitrite-oxidizer	pH	All season	6.116	676.3 ± 105.8	0.37	< .0001	72
Nitrite-oxidizer	Temperature	All season	-19.09	60.67 ± 3.4	0.31	< .0001	72

Regression analyses were performed on seasonal pooled data of the studies sites. a = Intercept, b = Slop, r^2 = Correlation-coefficient, P = Probability, N = Number of samples frequency

Table - 5: Potential nitrification rate of surface and bottom water

Surface water	Season	Fwp 1	Fwp 2	Fwp 3
Potential nitrification ($\mu mol^{-1} hr$)	Rainy	1.5 ± 0.04	1.65±0.05	2.2 ± 0 .12
	Winter	0.98 ± 0.23	1.2±0.06	1.6 ± 0.11
	Summer	0.42 ± 0.06	0.54±0.06	0.72 ± .005
			Bottom water	
Potential nitrification ($\mu mol^{-1} hr$)	Rainy	3.20 ± 0.34	3.99±0.03	4.12 ± 0.12
	Winter	2.33 ±0.064	2.48±0.039	2.85 ± 0.07
	Summer	0.277 ± 0.017	0.31±0.010	0.69 ± 0.064

Values are mean of ± SE (standard error) of eight time analysis

< 0.0001) and surface water (r^2 = 0.85, <0.0001). AOB counts in the surface water were positively correlated with ammonium concentration in all the seasons (r^2 = 0.46, 0.73, 0.29 p<0.0001), respectively. AOB counts in the bottom water were also positively correlated with ammonia concentration in winter, rainy and summer (r^2 = 0.82, 0.87, 0.37; p<0.0001), respectively. AOB and NOB counts have positive correlation with nitrite in rainy and winter as compared to summer. These counts were positively correlated with the water parameters such as pH (r^2 = 0.44, p<0.0001 for AOB, r^2 = 0.41, p<0.0001 for NOB), DO (r^2=0.37, p<0.0001 for AOB, r^2 = 0. p<0.0001 for NOB) and temperature (r^2 = 0.28, p <0.0001 for AOB, r^2 = 0.31 p<0.0001 for NOB). By ANOVA also, we found that there was a significant difference (p<0.0001) between abundance of nitrifiers and water quality.

Variations were observed in abundance of nitrifiers in different seasons, the highest value were recorded in the order, rainy > winter > summer in both bottom and surface water samples of freshwater ponds. Singh *et al.* (2007), Jane *et al.* (2003) and Belinda *et al.* (2008) also recorded highest abundance of nitrifiers during rainy season and minimum in summer. The observed differences in abundance of nitrifiers at three Fwp sites with higher values in Fwp 3 than Fwp 2 followed by Fwp 1. This may probably be due to genotypic variations of nitrifiers and different physico-chemical conditions of pond. However, water quality parameters were more optimal for growth of nitrifiers in Fwp 3 and 2 as compared to Fwp 1 (Moriarty, 1997). It appears that the abundance of the nitrifiers is easily influenced by the surrounding environments. There exist reports on the influence of abundance of nitrifiers by sudden change in environmental factors such as temperature, pH, DO, cultured fish species and stocking density (Frank *et al*., 1992; Hynes and Knowles, 1990; Stams and Marnette, 1990; Joye and Hollibaugh, 1995). In other studies too, number of bacteria (microorganisms) in the bottom water was higher than surface water in all the seasons because in the aquatic ecosystem there is competition for consumption of ammonia and nitrite between nitrifiers and primary producers like heterotrophs as well as benthic algae (Moriarty, 1997; James and Embley, 2002; Risgaard-Peterson *et al.*, 2004). But this competition was more pronounced in surface as compared to bottom water (Bernhard and Peele, 1997; Risgaard-Petersen *et al*., 2004; Geets *et al*., 2006).

The temperature of pond water varied from 24.6-32.1°C, the highest during summer in all the ponds though abundance of nitrifiers was lowest in all ponds during this season. In the present study, the nitrifiers were correlated with temperature in both bottom and surface water of the ponds. Observations of the present study suggest that nitrification rate decreases with increases in temperature due to increased respiration of heterotrophic bacteria in sediment and water. This may be the reason of the reduction in the population of nitrifying bacteria in sediment and water during summer (Gunderson and Mountain, 1973). However, it is pertinent to remark that the nitrifying bacteria require optimum temperature between 20 to 28°C for survival and growth (Foch and Versatate, 1977).

Oxygen is essential for both the ammonia and nitrite oxidation process as nitrification take place within DO range of 0.5-2.5 mg l^{-1} (Deri, 1991; Rittman and McCarty, 2001; Martins *et al*., 2003). Though DO level was within the optimum range throughout the seasons in all the ponds, it affected the abundance of nitrifying bacteria because during rainy and winter season, oxygen level was higher as compared to summer. The abundance of nitrifiers was also highest in rainy season followed by winter and lowest during summer. Since the heterotrophic bacteria are generally considered to be the principal consumers of O_2 in any aquatic ecosystems, these along with other microorganisms (zooplankton, protozoa *etc.*) have more competition during summer as compared to rainy and winter seasons (Donderski and Kalwasinska, 2003). In the present study, decrease in DO also coincided with an increase in ammonia concentration in all the ponds suggesting that DO is one of the most important factors in nitrification process. Armin *et al.* (2001) have also remarked that the distribution of nitrifiers corresponds to oxygen level in the aquatic medium.

Since pH of pond was high during summer and abundance of nitrifiers low in this season in surface and bottom water of all the ponds, high pH might be a reason for low abundance of nitrifiers during summer. Wong-Chong and Loehr (1975) have reported that optimum pH for productive growth of nitrifying bacteria is between 7 and 8. Sumartini and Aspriyanto (1996) also found pH to influence activity of ammonia-oxidizers, as the activity of nitrosomonas decreases with increase of pH. Growth of AOB is significantly inhibited at pH <6.5 owing to increased ionization of ammonia (Allison and Prosser, 2003).

Inorganic nitrogen is always a limiting factor in inland aquatic ecosystem. The observed higher ammonia and nitrite concentration of the bottom water in the present study may probably be due to the decomposition of food residues that sink to the bottom as result of over-feeding. Lowest nitrification potential rate and abundance of nitrifiers were recorded in both bottom and surface water of fish pond in summer probably due to heterotrophic bacteria and autotrophic algae which are the more successful in competing for ammonia during summer as they utilize this substrate more efficiently as compared to the nitrifiers. In the rainy and winter seasons, this competition is reduced and the nitrifying bacteria could take up the ammonia even at low concentration (Yoshifomi *et al*., 2008). Carlucci and Strickland (1968) observed increase in activity of ammonia-oxidizer as ammonia concentration increase. Ammonia concentration is likely to play an important role in determining ammonia-oxidizer community structure through potential differences in substrate affinities between different groups (Koops *et al*., 2003).

Olson (1981) reported that the required nitrite concentration of nitrite-oxidizers was minimal at 0.1µmol^{-1}. In the present study, nitrite concentration varied between 0.16±0.09 to 0.66±0.02 µmol^{-1} in the freshwater fish pond with lower values on surface and higher values (0.54±0.01 to 3.23±0.02 µmol^{-1}) in bottom water. Nitrite is a product of ammonia oxidation by ammonia-oxidizers. Binachi *et al.* (1994) also recorded variations in nitrite concentration from 0.2-3.2 µmol^{-1} in Rhone estuary with higher values at bottom than surface water.

The present observations give an explanation for the heterogeneity in nitrification rate frequently observed in all Fwps, through differences in the relative abundance of the ammonia- oxidizer and nitrite-oxidizer bacterial community (Table 5). Potential nitrification rates were variable and usually highest at Fwp 3 with peak value occurring in rainy season. As ammonia concentration, pH, temperature and oxygen supply affect nitrification rate (Stephen *et al*., 1996; Kowalchuk and Stephen, 2001; Jane *et al.*, 2003; Princic *et al*., 1998), the nitrification potential rate was found to be higher at the bottom than in surface water of the fish ponds. Similar findings have also been recorded by Hart (1994) and Allison and Prosser (2003). Our observations showed that abundance of nitrifying bacteria were positively correlated with nitrification rate in all the seasons in the bottom (AOB r^2 = 0.93, NOB r^2 = 0.92) and surface water (AOB r^2 = 0.88, NOB r^2 = 0.85, $p<0.0001$) suggesting higher potential nitrification rate with the maximum abundance of nitrifiers (Jane *et al.*, 2007). Conversely, Belser *et al*. (1978) and Moriarty (1997) remarked that nitrifiers population contribute to the nitrification process at quite diverse rates dependent on type of aquaculture, species cultivated (individual growth rate, niches circumstances) and the economics. In conclusion, the results of our study show that there exist seasonal variations in the population of nitrifiers in freshwater fishponds, suggesting them to be actively involved in nitrification processes. The physico-chemical parameters like dissolved oxygen level, pH, temperature and concentration of ammonia as well as nitrite play regulatory role in controlling rate of nitrification and abundance of nitrifiers in the ponds.

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