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Assessment of nitrogen and sulphur cycle bacteria and shrimp production in ponds treated with biological products

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ABSTRACT

Objective: To study the influence of biological products on the levels of nitrogen and sulphur cycle bacteria in shrimp culture systems of West Bengal, India.
Methods: The pond water and sediment samples were analyzed for physico-chemical parameters as per standard methods. The bacteria involved in ammonification, nitrification, denitrification, sulphate reduction and sulphur oxidation were enumerated by most probable number technique.
Results: The semi-intensive and modified extensive shrimp farms used a variety of biological products during various stages of production. No biological products were used in traditional farms. The water and sediment samples of modified extensive system recorded significantly higher mean heterotrophic bacterial counts. The counts of ammonia, nitrite and sulphur oxidizers, and nitrate and sulphate reducers varied among the systems. The cycling of nitrogen and sulphur appeared to be affected with the intensification of culture practices.
Conclusions: The application of biological products in certain systems helped to maintain the bacteria involved in nitrogen and sulphur cycles and safe levels of ammonia, nitrite and nitrate. An assessment of these metabolically active bacteria in shrimp culture ponds and the application of right kind microbial products would help ameliorate the organic pollution in shrimp aquaculture.

1. Introduction

A massive intensification has been done in shrimp aquaculture throughout the world, where stocking density and rate of application of aqua drugs and biological products have been hugely accelerated[1-4]. The use of aqua drugs has also increased in Indian shrimp culture practices[3,4]. The semi-intensive and modified extensive shrimp farmers of West Bengal, India have been reported to use about 65 different varieties of aqua drugs as therapeutants, disinfectants, soil conditioners, bacterial and enzyme preparations, algaecides, oxygen enhancer, plankton growth promoters, organic matter decomposers and feed additives during various stages of production[3]. In aquatic environments, bacteria are responsible for metabolizing the organic load and inorganic nitrogen compounds mainly from unconsumed or undigested food, dead organisms, *etc*. and incorporating them into the food chain[5]. The concentration, type and distribution of microorganisms *in situ* are strongly related to their ecological function and competitive success, which are strongly influenced by abiotic factors[6]. Microorganisms are involved not only in the production and breakdown of organic matter, but also in the nutrient recycling in aquatic environment. The nitrogen cycle occupies an important place in organic matter recycling and involves nitrogen fixation, ammonification, nitrification and denitrification processes carried out by different microorganisms[7]. Sulphate and hydrogen sulphide are constantly recycled between oxidation and reduction steps, predominantly carried out by sulphate reducers and sulphide oxidizers in sediments. Although large arrays of products are used in shrimp aquaculture, the actual benefits of these products have not been studied in detail. We reported earlier the efficacy of commercial shrimp farm bioremediators[8] and indigenous microflora of traditional shrimp farming system[9] in removing ammonia in microcosm experiments. In the present communication, we report the use of biological products in shrimp farms of West Bengal

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culturing *Penaeus monodon* (*P. monodon*) and their possible effect on the bacteria involved in nitrogen and sulphur cycles and shrimp production.

2. Materials and methods

2.1. Sampling area and sample collection

West Bengal, India has over 40 000 ha of potential area suitable for shrimp farming and commercial culture of *P. monodon* is carried out by traditional (locally called *bhery*), modified extensive and semi-intensive methods. A list of traditional, modified extensive and semi-intensive shrimp farms was prepared from the records of Regional Office of the Marine Products Export Development Authority, Government of India, Kolkata for this study between 2001 and 2005. A total of 45 shrimp farms from three regions, namely, Contai, East Midnapore district, Khariberia, North 24 Parganas district and Malancha, South 24 Parganas district, West Bengal, India were randomly identified from the list. The detailed farming activities, the type of culture and location of the study area are presented in Table 1. Samples of pond water were collected in sterilized polypropylene bottles of 250 mL capacity. Pond sediment samples were collected at four places, *viz.*, near inlet, close to outlet, pond center and close to feeding tray in scientifically managed ponds and/or at four sides of the *bheries* using sterilized plastic corers and transferred immediately to UV sterilized polythene bags. All the samples were placed in insulated containers and brought to the laboratory within 4 h of collection or 24 h in case of outstation samples. All the sampled modified extensive and semi-intensive shrimp ponds of East Midnapore district used at least one biological product during the culture period. Sampling was done in each pond only once preferably after 30 days of culture.

2.2. Enumeration of bacteria

Sediment samples, collected at four places from each pond, were pooled together and mixed thoroughly in a homogenizer. The water and sediment samples of the traditional *bheries* (n = 15) of North and South 24 Parganas, modified extensive (n = 15) and semi-intensive (n = 15) ponds of East Midnapore districts culturing *P. monodon* were ten-fold serially diluted for the enumeration of bacteria involved in nitrogen and sulphur cycles, and heterotrophs. The bacteriological media used were prepared by dissolving known quantity of ingredients in a suspending medium containing 50% distilled water and 50% aged seawater. The salinity of undiluted aged seawater was 20‰. The enumeration of total heterotrophic bacterial counts (THC) was by spread plating on nutrient agar. Ammonia oxidizing bacteria (AOB) in modified Winogradsky medium, nitrite-oxidizing bacteria (NOB) in Winogradsky medium, nitrate reducing bacteria (NRB) in seawater nitrate broth, sulphur oxidizing bacteria (SOB) in SOB medium and sulphate reducing bacteria (SRB) in SRB medium from the water and sediment samples were enumerated by most probable number (MPN) technique as described in Rodina[10] and Abraham *et al*[11].

2.3. Determination of water and sediment quality parameters

The salinity, temperature and pH of pond water were measured by refractometer, mercury thermometer and digital pH meter, respectively. The dissolved oxygen content was determined by Winkler's method. The ammonia (NH_3-N), nitrate (NO_3-N) and nitrite (NO_2-N) levels of pond water were measured by phenate, UV spectrophotometric screening and colorimetric methods, respectively[12]. The sediment pH was measured using a soil pH meter. The total organic carbon content of pond sediment was determined by Walkley and Black[13]. After the log transformation of bacteriological data, the significant difference between the physico-chemical and bacteriological parameters of water and sediment was determined by Two-way ANOVA and Duncan's multiple range tests[14].

3. Results

The list of bacterial, enzymic and plant-based products used in the modified extensive and semi-intensive shrimp farms of West Bengal, India are presented in Table 2. The shrimp farmers of Contai region used about 18 bacterial and enzyme preparations and plant-based products during various stages of production. No biological products were, however, used in traditional shrimp farms during the study period. The physico-chemical characteristics of traditional, modified extensive and semi-intensive shrimp culture systems are presented in Table 3. The levels of water quality parameters such as dissolved oxygen, temperature, pH, NH_3-N, NO_3-N and NO_2-N were well within the optimum values. Salinity levels fluctuated between 1.20‰ and 15.80‰. The traditional system recorded the lowest salinity. The mean ammonia-nitrogen (NH_3-N) level was high in semi-intensive system (0.214 ± 0.120 mg/L) and low in traditional system (0.099 ± 0.039 mg/L).

Table 1

Description of the study area and details of shrimp (*Penaeus monodon*) farm management practices in West Bengal, India.

Type of culture	Study area	Management practices
Traditional (*bhery*)	Malancha, South 24 Parganas district Lat. 22° 30' N; Long. 88°46' E Khariberia, North 24 Parganas district Lat. 22°25' N; Long. 88°35' E	Stocking density ≤ 4 post-larvae (PL)/m^2, no water exchange, no aeration, occasional use of locally prepared feed, water depth 0.75-1 m. Partial stocking and partial harvesting throughout the culture.
Modified extensive	Contai, East Midnapore district Lat. 21°48' N; Long. 87°45' E	Stocking density 8-18 PL/m^2, need based water exchange, aeration by pumping, use of commercial pellet feed and microbial products, with or without reservoir, water depth 1-2 m.
Semi-intensive	Contai, East Midnapore district Lat. 21°48' N; Long. 87°45' E	Stocking density > 18 PL/m^2, frequent water exchange, aeration by aerators, use of commercial pellet feed and microbial products, reservoir for water treatment, water depth 1 m.

Table 2

Biological products used in the modified extensive and semi-intensive shrimp farms of West Bengal, India.

Microbial products / Commercial name	Dose
Aqualact	10 g/kg feed
Biocult	12.5 kg/ha
Bottom lact / Pond lact	10 kg/ha once in 15 days
Cashmin	10 kg /ton feed
Environ-C	75 kg/acre
Farm yeast	30-50 L/ha
Growel	5-7 g/kg feed
Proburst-A	2-3 g/kg feed
Spilac	1 kg/ton feed
Super NB	0.5-1.5 mg/L once in 7 days
Super PS	0.5-2.0 mg/L once in 7days
Ultrazyme-P-FS	3-5 g/kg feed
Plant based products	
Herbal sanitizer	1-3 L/ha
Neem oil cake	34 kg/ha
Tea seed powder	200 kg/ha
Zeolite + neem (1000 mg/L)	During pond preparation: 1000 kg/ha;
Geolite plus	After stocking: 500 kg/ha for every month
Composition of locally made farm yeast	
Bakers' yeast	200 g
Ground nut oil cake	5.0 kg
Molasses	5.0 kg
Polished rice	5.0 kg
Super PS	750 mL

Table 3

Physico-chemical characteristics of shrimp culture systems of West Bengal, India.

Parameters	Culture system		
	Traditional	Modified extensive	Semi-intensive
Salinity (‰)	5.130 ± 3.940^{ab}	11.170 ± 3.360^{a}	12.670 ± 3.087^{b}
Temperature (°C)	25.720 ± 2.610	29.910 ± 0.890	29.460 ± 0.835
Dissolved oxygen (mg/L)	5.202 ± 0.964	6.350 ± 0.391	7.515 ± 0.314
Water (pH)	7.890 ± 0.220	7.970 ± 0.210	8.320 ± 0.271
Ammonia (mg/L)	0.099 ± 0.039^{a}	0.111 ± 0.049^{b}	0.214 ± 0.120^{ab}
Nitrite (mg/L)	0.022 ± 0.009^{a}	0.016 ± 0.006^{ab}	0.027 ± 0.008^{b}
Nitrate (mg/L)	0.010 ± 0.006	0.005 ± 0.002	0.006 ± 0.001
Sediment (pH)	9.018 ± 0.180	8.590 ± 0.288	8.830 ± 0.153
Total organic carbon (%)	0.442 ± 0.312	0.491 ± 0.327	0.466 ± 0.295

a, b: Values sharing common superscripts within the rows differ significantly ($P < 0.05$).

The results of the metabolically active bacterial counts of shrimp pond water and sediment samples from modified extensive and semi-intensive farms that used biological products and also the traditional farms that received no biological products are presented in Table 4. The water and sediment samples of modified extensive system recorded the highest mean THCs. The mean AOB counts were high in traditional system. The recorded mean NOB counts of pond water were higher than in sediment from modified extensive and semi-intensive systems that received biological products. The shrimp production in traditional *bheries* of North and South 24 Parganas districts, and modified extensive and semi-intensive shrimp farms of East Midnapore district was in the range of 214-806 kg/ha per year, 1000-3480 kg/ha per crop and 3560-6300 kg/ha per crop, respectively.

4. Discussion

In aquaculture, the incorporation of nutrients into the water column is made by heterotrophs, chemolithotrophic ammonium-oxidizers, nitrifiers and many other bacteria involved in nitrogen and sulphur cycles. These processes are affected by intensification in shrimp aquaculture, which necessitated the application biological products to augment these processes and to reduce disease outbreaks[1-4]. The present study recorded a large array of biological products used by the shrimp farmers of West Bengal as advised by the marketing personals. The use of 'farm yeast' and vitamin C was more in both semi-intensive and modified extensive systems towards the end of the study period, *i.e.*, 2004 and 2005. The application of farm yeast (locally called as *juice*) was common to increase the productivity of pond water. The ingredients were allowed to ferment for a period of 7 days and applied into the ponds prior to stocking. This was because of the fact that the farmers were not benefited from the use of chemical products and aqua drugs to prevent disease outbreaks[3,4].

All the physico-chemical parameters except salinity and sediment pH were well within the optimum levels recommended for shrimp culture[15], thereby, revealing the overall well being of the systems. The mean salinity in all the systems were generally low (< 12.6‰) compared to the optimum levels (15‰-25‰). The pH values above

Table 4

Counts of metabolically active bacteria in shrimp pond water and sediment samples from modified extensive and semi-intensive farms that used biological products and the traditional farms that received no biological products.

Samples and bacterial group	Counts of bacteria					
	Traditional system (n = 15)		Modified extensive system (n = 15)		Semi-intensive system (n = 15)	
	Range	Mean	Range	Mean	Range	Mean
Pond water						
Total heterotrophic count (Log CFU/mL)	4.00-6.15	$5.49^{a,b}$	5.82-6.51	6.18^{a}	5.36-7.00	6.01^{b}
Ammonia oxidizing bacteria (Log MPN/100 mL)	0.00-4.06	2.77	0.00-3.60	2.40	0.00-3.40	2.33
Nitrite oxidizing bacteria (Log MPN/100 mL)	0.00-4.90	$2.75^{a,b}$	1.70-4.95	4.08^{a}	1.30-4.95	3.16^{b}
Nitrate reducing bacteria (Log MPN/100 mL)	2.18-5.85	3.90	2.08-4.74	3.99	0.00-4.04	2.61
Sulphur oxidizing bacteria (Log MPN/100 mL)	0.00-2.30	0.71	0.00-4.54	0.71	0.00-2.04	1.01
Sulphate reducing bacteria (Log MPN/100 mL)	2.70-5.40	4.13^{a}	0.00-4.63	3.64	1.70-4.54	3.42^{a}
Pond sediment						
Total heterotrophic count (Log CFU/g)	5.00-7.02	6.27	5.86-7.23	6.71	5.18-6.78	6.35
Ammonia oxidizing bacteria (Log MPN/g)	1.36-4.20	2.96	2.36-4.96	3.36	1.36-4.20	2.91
Nitrite oxidizing bacteria (Log MPN/g)	0.00-3.96	2.32^{a}	0.00-4.83	$3.79^{a,b}$	0.30-3.38	2.19^{b}
Nitrate reducing bacteria (Log MPN/g)	0.00-3.96	3.16^{a}	1.45-4.96	$3.86^{a,b}$	1.38-3.96	2.88^{b}
Sulphur oxidizing bacteria (Log MPN/g)	0.00-1.30	0.05	0.00-3.96	0.46	0.00-1.78	0.30
Sulphate reducing bacteria (Log MPN/g)	3.54-4.38	4.02^{a}	3.15-5.04	4.09^{b}	2.23-4.54	$3.67^{a,b}$

a,b: Mean values sharing common superscripts within the row differed significantly ($P < 0.05$); CFU: Colony forming unit.

9.0 were recorded in traditional system; while in other systems, the mean sediment pH was above 8.59. The NH_3-N levels of semi-intensive system were high and differed significantly ($P < 0.05$) from those of other two systems probably due to high stocking and feed input. The NH_3-N exceeding 1.0 mg/L has been reported in Indian shrimp culture ponds[1]. Nevertheless, the levels of NH_3-N recorded in the present study were far less than the safe level (< 1 mg/L)[15]. It is possible that the reduced NH_3 builds up due to it being oxidized to NO_2 by resident NH_3 oxidizers and/or bioremediators. The nitrite-nitrogen (NO_2-N) levels were high in semi-intensive system and low in traditional system. The NO_2-N levels, although differed significantly ($P < 0.05$) were well below the safe level, *i.e.*, < 0.25 mg/L[15] in all the systems. The traditional system recorded the highest NO_3-N levels and the differences in their levels among the systems were insignificant ($P > 0.05$). The observed values of the present study were comparatively lower than the levels reported in other parts of India[1]. The TOC values were in the range of 0.44%-0.49%, which differed insignificantly among the culture systems ($P > 0.05$). These results corroborate the findings of Priyadarsani and Abraham[16] and defer with those of Avnimelech and Ritvo[17] who reported higher TOC in shrimp pond sediment. Sudthikaran *et al*. opined that the NH_3 release and pH increase in sediments are to some extent determined by the organic matter deposits in shrimp aquaculture[18].

The mean heterotrophic bacterial counts of pond water were always close to or above 6.0 log CFU/mL except the traditional system. The results possibly suggested abundant availability of nutrients derived from excess feed, shrimp excreta and other dead and decaying organic matter for bacterial growth. There existed significant differences in THCs among the systems ($P < 0.05$), thus, revealing the influence of management practices on the bacterial populations of shrimp culture systems. The differences in THCs of semi-intensive and modified extensive systems were, however, insignificant ($P > 0.05$). The results corroborate the earlier observations[2,4,11,19]. The mean THCs of pond sediment were always above 6.0 log CFU/g sediment and differences among the systems were insignificant ($P > 0.05$). These results perhaps suggest that the THCs of pond sediment are not influenced significantly by the type of culture.

The counts of AOB were higher in pond sediment than in pond water and did not vary much ($P > 0.05$) among the systems. Nevertheless, the mean AOB counts were high in traditional system, possibly because of the favorable environmental conditions compared to the modified extensive and semi-intensive systems. Rao *et al*.[1] in India and Devaraja *et al*.[2] in Malaysia recorded comparatively lower AOB counts in pond water and sediment than the present study. Ammonia oxidizers are sensitive to sudden changes in temperature, available nutrients, drugs and hydrogen sulphide (H_2S) and these factors could have played a role for the varying levels of their counts and metabolites in different shrimp culture systems of West Bengal. Amano *et al*. demonstrated the occurrence of potential anammox activity in subtropical mangrove-aquaculture ecosystems[20]. It possibly suggested that diverse species of uncultured anammox bacteria could also contribute to the nitrogen cycle in aquaculture systems.

Nitrifying bacteria have a great ecological importance because they convert ammonium into nitrate via an intermediate step of nitrite production[21]. The NOB counts were normally high in water than in sediment samples of modified extensive and semi-intensive systems that received biological products. Their counts in water and sediment differed significantly among the systems ($P < 0.05$). The modified extensive system recorded the highest NOB both in water and sediment and lower NH_3-N compared to semi-intensive system. The difference in NH_3-N of modified extensive and traditional systems was insignificant, thereby suggesting that the biological products are, to some extent, effective in preventing the NH_3-N accumulation. In a study by Devaraja *et al*. in Malaysia, the commercial probiotic products applied ponds recorded comparatively low levels of NOB than the present study[2]. The mean NRB was comparatively low in pond water and pond sediment samples of semi-intensive system. Significant differences ($P < 0.05$) in NRB counts were observed between the pond water samples of semi-intensive system and other two systems. Likewise, the sediment NRB counts of modified extensive system differed significantly ($P < 0.05$) from those of other two culture systems. This could be attributed to the differences in the availability of NO_3-N and organic carbon, and also due to the differences in temperature and limiting levels of oxygen.

In pond water of the traditional system, the SRB count was generally high probably because of the use of sewage water from the Kolkata metropolitan for the polyculture of shrimp and fish. The SRB counts of water differed significantly ($P < 0.05$) between the traditional and semi-intensive the systems. The SRB, despite being anaerobes, were recorded considerably in pond water samples probably due to the presence of reduced microniche in sediment particles suspended in water. The aerobic heterotrophs consume O_2 in these environments and keep the center of the particle anaerobic, thereby allowing the SRB to flourish. The sediment SRB counts differed significantly ($P < 0.05$) among the systems, probably due to the varying levels of substrate availability for their growth and varying degrees of anaerobiosis. The mean SRB was, however, low in pond water and pond sediment of semi-intensive system. The active growth of fermentative bacteria in anaerobic pond sediment release reduced acids, alcohols, carbon dioxide and hydrogen, which are then used by the SRB. The results of the present study, although high, are comparable to those of Rao *et al*.[1] and Devaraja *et al*[2]. The SOB counts of pond water were higher in semi-intensive and modified extensive systems probably a result of addition of biological products in those systems. The counts of SOB were nil in pond sediment samples of few modified extensive and traditional system during the initial periods of culture due to the non-availability of substrates. The results, thus, implied that H_2S would be converted effectively to sulphur compounds. Oxidation of H_2S is very important in ponds, which otherwise lead to blackening of pond bottom due to H_2S. Earlier studies reported comparatively high SOB counts in pond water as well as sediment in shrimp farms using biological products[1,2].

The shrimp production in traditional *bheries* was in the range of 214-806 kg/ha per year due to the low stocking density and occasional feeding. The shrimp of traditional ponds sustained mainly on natural foods. The ranges of shrimp production in modified extensive and semi-intensive shrimp farms of East Midnapore district were 1 000-3 480 kg/ha per crop and 3 560-6 300 kg/ha per crop, respectively obviously because of high stocking densities, use of pellet feed and intensive management practices. The common bioproducts used in those farms include bottomlac, super PS, spilac, aqualact, environ C and yeast. The production of shrimp in the modified extensive ponds of East Midnapore district was comparable to those of Ananda Raja *et al*[4]. They reported the use of vide range of probiotics and immunostimulants during the culture period and productions in the range of 3000-7000 kg/ha in ponds with stocking densities 14-16 PL/m^2 in Sunderbans, West Bengal.

It appears from the results of this study that the natural processes of cycling of nitrogen and sulphur were not affected by the traditional shrimp farming. The bacteria involved in the cycling of nitrogen and sulphur were, to some extent, affected by the intensification of culture practices and that necessitated the application of biological products. Such practices enabled the shrimp farmers to maintain the nitrogen and sulphur cycle bacteria and the levels of NH_3, NO_2 and NO_3 well within the safe limit in modified extensive and semi-intensive culture systems of West Bengal. Nevertheless, the shrimp farm effluents with high bacterial load may modify the diversity of natural bacteria in the discharge area, which could affect natural populations and the microbial balance of the receiving area as has been observed earlier[16,22,23]. Screening of metabolically active bacteria involved in ammonification, nitratation, nitritation, denitrification, sulphur oxidation and sulphate reduction and their distribution in shrimp culture ponds is suggested for the successful microbial manipulation. Such information would help select right kind of microbial products for the amelioration of organic pollution in shrimp aquaculture.

Conflict of interest statement

We declare that we have no conflict of interest.

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