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Evaluation of the Physiological Bacterial Groups in a Tropical Biosecured, Zero-Exchange System Growing Whiteleg Shrimp, *Litopenaeus vannamei*

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Abstract

To elucidate the individual and multiple roles of physiological bacterial groups involved in biogeochemical cycles of carbon, nitrogen, phosphorus and sulfur, the changes in the abundance of aerobic bacteria (heterotrophs, methane oxidizers, ammonia oxidizers, sulfur oxidizers, phosphate solubilizers, phosphate accumulators) and anaerobic bacteria (total anaerobes, nitrate reducers, denitrifiers and sulfate reducers) were investigated in a biosecured, zero-exchange system stocked with whiteleg shrimp, *Litopenaeus vannamei* for one production cycle. Key water quality parameters during the 96-day production cycle fell within the normal range for *L. vannamei* culture. Results of Spearman's correlation matrix revealed that different sets of variables correlated at varying levels of significance of the interrelationships between bacterial abundances and water quality parameters. The three nitrogenous species (ammonia, nitrite and nitrate) strongly influenced the physiological bacterial groups' abundance. The strong relationship of bacterial groups with phytoplankton biomass and abundance clearly showed the trophic interconnections in nutrient exchange/recycling. Canonical correspondence analysis performed to assess the total variation revealed that the three dissolved nitrogen species followed by salinity, temperature, phytoplankton biomass and pH collectively accounted for as much as 82% of the total variation. In conclusion, the results of the study revealed that the major drivers that interweaved biogeochemical cycles are the three dissolved nitrogen species, which microbially mediated various aerobic-anaerobic assimilation/dissimilation processes in the pond ecosystem. Considering the pond microbial ecology becoming an important management tool where applied research could improve the economic and environmental sustainability of the aquaculture industry, the findings of the present study are practically relevant.

Keywords Nutrients · *Litopenaeus vannamei* · Shrimp aquaculture · Bacterial groups · Canonical correspondence analysis · Water quality

Introduction

The ever increasing demand for animal protein has resulted in the tremendous expansion of the shrimp culture industry and has now become an activity of economic importance in many countries of the world [1]. With a total production of 4.97 million tonnes, the whiteleg shrimp, *Litopenaeus vannamei*, has emerged as the highest harvested crustaceans in the world, contributing to ~ 53% of the total shrimps and prawns production (~ 9.39 million tonnes) in 2018 [2]. Remarkable attributes of *L. vannamei* such as faster growth rate, high density tolerance, adaptability to grow and survive under varying salinity and temperature regimes and cultivation in both indoor (tanks or recirculating aquaculture systems) or outdoor facilities (ponds) have made *L. vannamei* as the most choice shrimp species for commercial culture in subtropical and tropical regions [3, 4]. The high-density and high-yield culture of *L. vannamei* has largely been attributed to rapid transformation from "open systems" (frequent water discharge) to "closed systems" (minimal or zero-water discharge).

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Biosecured, zero-exchange systems offer apparent advantages such as better control over water quality, limited land use, reduced pollution discharge and water usage, and risk of disease transmission between wild and captive stocks (5–7).

The major problem associated with the zero-exchange systems, however, has been the accumulation of nitrogenous waste (especially ammonium and nitrite) and organic matter (derived from the excess feed, feces and metabolites) with the progress of the culture thereby affecting the water quality and productivity [5–8]. It has been reported that only 15% of the applied feed (amounting 24–37% of nitrogen and 11–20% of phosphorus) is consumed, assimilated and retained as shrimp biomass, while the remainder is released to the water and sediment [9]. The success of zero-exchange shrimp culture systems therefore depends on striking a balance between waste production and assimilation capacity, by taking into account the waste impact on the growth of cultured organisms, the mortality of the cultured stock and overall expansion of total biomass [10]. In this context, the role played by physiological bacterial communities in maintaining the water quality, by assimilation and mineralization of excess organic load (uneaten feed and particulate matter) from the pond ecosystem while maintaining the optimum shrimp growth is of paramount importance [11].

Different groups of bacteria and phytoplankton communities recycle nutrients and degrade organic matter (uneaten feed, excreta, sloughed exoskeletons and other debris) in the shrimp ponds. Excess nitrogenous compounds are the major contributors to eutrophication thereby disrupting the aquatic ecosystem balance and could lead to massive mortality of cultured organisms [12]. In natural ecosystems, microbes are quintessential in nutrient recycling and maintain a balanced concentration of each nitrogen species, thereby maintaining water quality. In shrimp ponds, the microbial metabolism of the nitrogen (N) species is carried out mainly by ammonia-oxidizing bacteria (AOB), nitrifying bacteria, heterotrophic bacteria (THB), nitrate-reducing bacteria (NRB), denitrifying bacteria (DNB) and phytoplankton [13].

Similarly, the excess dissolved phosphate could also trigger eutrophication affecting the shrimp pond ecosystem. Polyphosphate-accumulating bacteria (PAB) and phosphate-solubilizing bacteria (PSB) involved in uptake and release of phosphorus (P) respectively, are important players in the P cycle. In addition to dissolved nutrients, organic matter, majorly the carbon (C) accumulated in the shrimp pond, is oxidized by sulfate-reducing bacteria (SRB) resulting in the release of toxic hydrogen sulfide (H_2S), particularly under anoxic conditions [14]. On the other hand, by assimilating H_2S as a substrate, the sulfur-oxidizing bacteria (SOB) mitigates the toxicity in the pond ecosystem [15]. The prevalence of anaerobic conditions in superintensive and intensive zero-exchange systems may lead to the rapid release of greenhouse gases such as methane [1]. This methane is mitigated by methane-oxidizing bacteria (MOB) by assimilating it for its growth [14, 16].

Despite their ecological significance, the interrelationships between the physiological bacterial groups involved in elemental cycles of C, N, S and P, and environmental variables in the shrimp ponds have not been addressed holistically. In this context, the present study was conducted with an overall aim to evaluate the changes in the abundance of different physiological bacterial groups and their interrelationships with environmental variables during a production cycle of *L. vannamei* in the zero-exchange system. Considering shrimp aquaculture as a significant economic activity worldwide, the conclusions drawn from the study would advance the current understanding of the roles played by different physiological groups, which may aid in sustainable expansion of the industry.

Materials and Methods

Experimental Pond and Farm Management

The shrimp farm located near Alvekodi village, near Kumta town (Karnataka state) on the west coast of India (14.42° N lat. 74.40° E long.), was considered for the study. The farm is tide-fed from an adjoining creek located about 3 km from the coast. Investigations were carried out in a pond having a water spread area of ~ 0.84 ha and a pond depth of 1.2 m. Farm management practices as applicable to biosecure, zero-exchange shrimp farming (prevention of entry of carriers/vectors including crabs and birds, use of dechlorinated water for pond filling, healthy certified seeds, stringent feed management, periodic water quality monitoring, application of probiotics, maintenance of high standards of hygiene of personnel and equipment) were followed. The pond preparation practices (sun drying, fertilization and liming) were carried out 1 month before the post-larval stocking. The pond was then filled with dechlorinated seawater from reservoir pond followed by an application of inorganic fertilizer (4 ppm; urea:single super phosphate, 1:1). For enhancing the pond natural productivity, the farm-made liquid mixture containing wheat bran, jaggery, yeast and a protein source (groundnut oil cake, old feed, buttermilk, vinegar) was added (100 to 150 l) during the first 15 days of culture (DoC). Two weeks post-pond preparation, stocking with seeds was carried out.

White spot syndrome virus (WSSV) negative post-larvae (PL_{18}) of *L. vannamei* as confirmed by polymerase chain reaction (PCR) test was procured from a nearby shrimp hatchery (Skyline Aqua Hatchery) and was transported to the pond site in oxygen-filled bags. The actively motile post-larvae with no visible signs of disease or morbidity were stocked at a stocking density of 16 PL_{18}/m^2 during early morning hours.

Commercially, formulated pellets (CP Aquaculture; proximate composition: 38–40% crude protein, 5% lipid, 3% fiber) were used to feed the shrimps three times in a day. With the increase of shrimp biomass, a progressive decrease in the feeding rate from 10 to 2% of body weight was followed. However, based on the survival and shrimp biomass, feeding rates were adjusted.

To buffer the wide fluctuations in pH, the hydrated lime and pH fixers were applied to the pond throughout the production cycle that lasted for 96 DoC with zero-water exchange. Fresh dechlorinated water to compensate evaporation and seepage losses was added on a fortnightly basis to maintain minimum water depth (~ 1.0 m). Optimum levels of dissolved oxygen (DO) in the pond were maintained by deploying paddle-wheel aerators with an aeration protocol of 8, 12 and 16 h/day during 0–50, 51–80 and 81–96 DoC respectively. The liquid and powdered probiotics were applied mixed with the feed as well as directly added to the pond water. The liquid probiotic "Super PS" (*Rhodobacter* spp. and *Rhodococcus* spp., ~ 10^9 CFU/ml) was applied periodically to prevent the formation of hydrogen sulfide (H_2S), increase DO levels, promote the non-pathogenic bacteria, digest the accumulated organic substances (protein, carbon and lipids), and prevent disease occurrence. For supporting the growth and survival rates of shrimp, the commercial feed-based probiotic containing *Bacillus subtilis* (10^8 CFU/ml) and vitamin-mineral feed additive were also applied periodically.

Sampling Methodology and Analysis

Sampling started from the larval stocking day (0 DoC) till 96 DoC (harvesting day). Water samples (in triplicate) were collected from three pre-determined sampling points in the pond. The collected water samples were transported aseptically to the laboratory in an ice box for immediate processing and analysis. Temperature, pH and salinity were measured *in situ* with the help of a standard thermometer, a portable pH meter (ColeParmer-99361-12) and a hand-held refractometer (Atago) respectively. All the physicochemical parameters were analyzed in triplicate. The concentration of DO was estimated by Winkler's method [17] and biochemical oxygen demand (BOD_5) loading was determined as per the methods described in Parsons et al. [17]. The water samples collected in acid-washed polyethylene bottles (in triplicates) were analyzed for nutrients following the protocols outlined in Parsons et al. [17]. Total suspended solids (TSS) content was also estimated [17]. Water samples for estimation of chlorophyll *a* (Chl *a*) and phaeophytin (Phaeo) were analyzed spectrophotometrically [17].

Bacterial Enumeration

In the present study, culturable aerobic and anaerobic bacterial groups involved in four elemental cycles were enumerated. Total heterotrophic bacteria (THB) in pond water were enumerated by the spread plate technique on nutrient agar plates [18]. Similarly, the total anaerobic bacteria (TAB) were enumerated by using anaerobic agar medium (HiMedia, India), sulfur-oxidizing bacteria (SOB) with modified Lieske's medium [19], sulfate-reducing bacteria (SRB) using modified Hatchikan's medium [19], methane-oxidizing bacteria (MOB) on NMS medium [20], polyphosphate-accumulating bacteria (PAB) in acetate mineral medium [21], polyphosphate solubilizers (PSB) in hydroxyl apatite medium [22], ammonia-oxidizing bacteria (AOB) in nitrifying medium [23] and, denitrifying bacteria (DNB) and nitrate reducing bacteria (NRB) [19] were enumerated, respectively, on specific agar media [18, 24].

Statistical Analysis

Before subjecting the data for statistical analysis, all raw values of bacterial and environmental variables were normalized using log $(x + 1)$ and square root transformations, respectively. To analyze the interrelationships within bacterial groups, as well as between bacterial groups and environmental variables, the data were subjected to Spearman's correlation analysis using Statistica 12.0 version [25]. Three levels of significance ($p < 0.05$, $p < 0.01$ and $p < 0.001$) were reported. Canonical correspondence analysis (CCA) was performed to decipher the role of environmental variables in controlling the abundance of different physiological bacterial groups. CCA—a multivariate data reduction technique was employed mainly to understand the major driving factors contributing to the changes in the abundance of (i) aerobic bacterial groups (ABG), (ii) anaerobic bacterial groups (ANBG) and, (iii) all the bacterial groups (ABG and ANBG), separately, by using canonical community ordination (CANOCO) software for Windows v.4.5 [26, 27]. Monte Carlo test was performed with the maximum number of possible random permutations (999) for statistical interpretation of the data.

Results

Water Quality

Key water quality parameters (water temperature, pH, salinity and DO) recorded during the production cycle fell within the acceptable levels for the culture of penaeid shrimps [28]. During the culture period, the water temperature ranged from 25 to 32 °C (mean ± SD; 29.4 ± 1.85 °C). Throughout the cultivation period, near alkaline conditions (7.91 ± 0.08) were

maintained by periodic application of lime and pH fixers. Salinity increased from 32 (0 DoC) to 44 (96 DoC) with the progress of the culture. Optimum concentrations of DO (5.50 ± 1.14 mg/l) were maintained with the help of paddle-wheel aerators. Values of BOD_5 (3.21 ± 1.26 mg/l) throughout the production cycle were within the threshold level for the shrimp culture. The recorded survival rate was 78%. The average body weight of the harvested shrimps was 20.83 ± 0.5 g.

Concentrations of NH_4^+ peaked on 41 DoC (19.48 ± 0.14 μM) and thereafter decreased (Fig. 1). Concentrations of both NO_2^- (13.66 ± 0.49 μM) and NO_3^- (122.76 ± 27.97 μM) showed a similar trend with the highest levels recorded on 12 DoC and decreased progressively thereafter until 96 DoC (Fig. 1). The temporal variation in concentrations of PO_4^{3-} did not show any particular trend until 78 DoC and then increased drastically towards the end of the culture period (19.14 ± 1.56 μM) (Fig. 1).

Changes in Bacterial Abundance

The abundance of different physiological bacterial groups showed variable trends during the production cycle (Fig. 2). The abundance of THB was an order higher on 12 DoC (5.5×10^6 CFU/ml) than on 0 DoC (1.5×10^5 CFU/ml) and later dropped by two orders (3.3×10^4 CFU/ml) at the time of harvest (96 DoC). TAB were more abundant (2×10^6 CFU/ml) during the initial phase of culture (< 30 DoC), decreased progressively thereafter, and reached to the lowest abundance on 96 DoC (1.1×10^4 CFU/ml). MOB was 5.28×10^3 CFU/ml on 0 DoC, with a peak of 1.73×10^4 CFU/ml on the 68 DoC followed by a decrease to 6.8×10^3 CFU/ml on 96 DoC. In the case of AOB, from the initial culture period, it attained a two order increase on 55 DoC (1.19×10^4 CFU/ml), and similar counts were recorded until 96 DoC (1.2×10^4 CFU/ml). Anaerobic bacterial groups, DNB (9.8×10^4 CFU/ml) and NRB (3.3×10^4 CFU/ml) involved in nitrogen assimilation had a highest count at 41 DoC and decreased subsequently (DNB: 1.8×10^3 CFU/ml; NRB: 1.4×10^4 CFU/ml) towards the last phase of culture (96 DoC). In the case of the aerobic oxidizer SOB, it was 1×10^4 CFU/ml on 0 DoC, peaked on 41 DoC (5.6×10^6 CFU/ml) and dropped to 1.3×10^5 CFU/ml on 96 DoC. In contrast, the abundance of SRB decreased progressively from 4.8×10^4 CFU/ml (0 DoC) to 9.2×10^3 CFU/ml (96 DoC). The abundance of physiological bacterial groups involved in phosphorus cycle (PAB and PSB) showed an increasing trend from 0 to 96 DoC (PSB: 2.7×10^2 to 1×10^4 CFU/ml; PAB: 1.1×10^3 to 1.75×10^4 CFU/ml).

Interrelationships Between Bacterial Abundance and Water Quality

Spearman's Correlation Matrix

The result of Spearman's correlation matrix of different bacterial groups and their significant interrelationships with water quality parameters (physical, chemical, and biological) confirmed the existence of tight coupling between bacterial abundance and dissolved nutrients (Tables 1 and 2). Spearman's correlation matrix also indicated that many physiological bacterial groups (except PSB) showed a significant positive relationship, particularly with one or more dissolved nitrogen species (NH_4^+, NO_2^-, NO_3^-) along with multiple bacterial interrelationships (Tables 1 and 2). Spearman's correlation matrix results and scatter plot graph reaffirmed a strong positive relationship between THB and dissolved nitrogen species ($p < 0.001$) as well as a strong negative correlation with phytoplankton pigments ($p < 0.001$). In addition, results of Spearman's correlation matrix revealed that MOB negatively correlated with NH_4^+ ($p < 0.01$), NO_2^- ($p < 0.001$) and THB ($p < 0.001$) (Tables 1 and 2). Abundance of AOB showed a strong positive relationship with PO_4^{3-} and Chl *a* and, negative relationship with NO_3^-. The results of the correlation

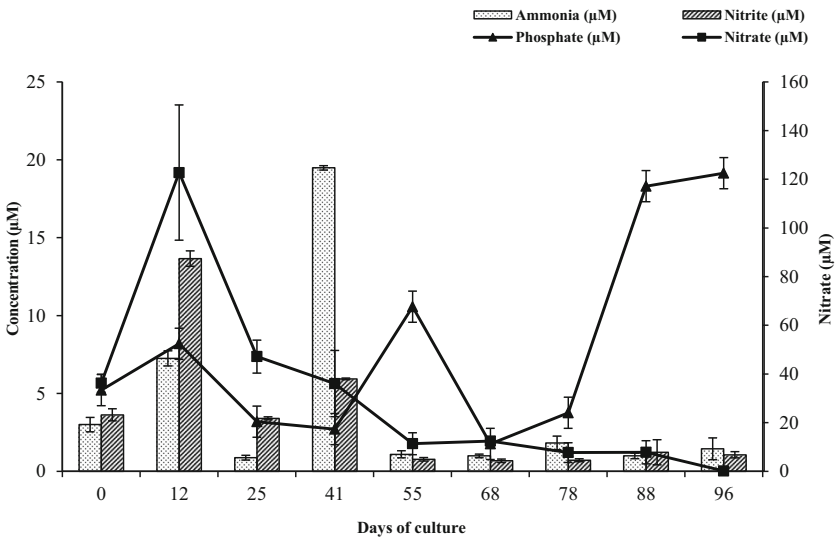


Fig. 1 Temporal variation in concentrations (μM) of dissolved nutrients (ammonia, nitrite, nitrate and phosphate) during the cultivation of *Litopenaeus vannamei* in zero-exchange shrimp system. Data is expressed as mean ± SD ($n = 3$)

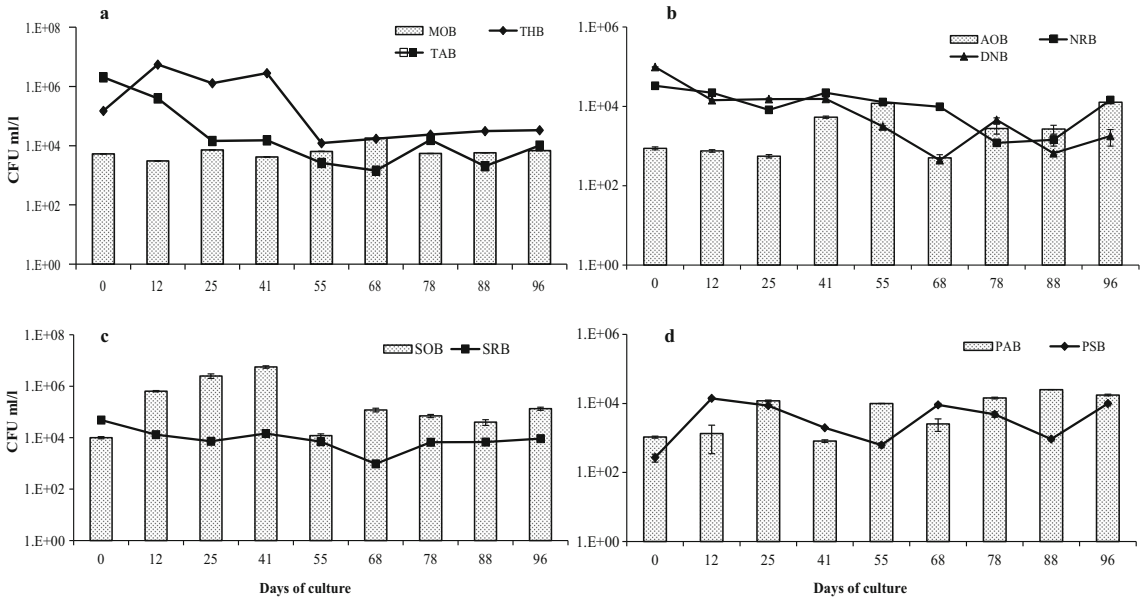


Fig. 2 Temporal variation in the abundance (CFU/ml) of physiological bacterial groups: (**a**) total heterotrophic bacteria (THB), total anaerobic bacteria (TAB) and methane oxidizing bacteria (MOB); (**b**) ammonia-oxidizing bacteria (AOB), nitrate-reducing bacteria (NRB) and denitrifying bacteria (DNB); (**c**) sulfur-oxidizing bacteria (SOB) and sulfate-reducing bacteria SRB; (**d**) phosphate-accumulating bacteria (PAB) and phosphate-solubilizing bacteria (PSB) during the cultivation of *Litopenaeus vannamei* in zero-exchange shrimp system. Data is expressed as mean ± SD ($n = 3$)

matrix also confirmed that DNB strongly correlated with NO_2^-, NO_3^- and TAB ($p < 0.001$) and correlated with NRB and SRB ($p < 0.01$) implying the multiple substrate utilization. Similarly, NRB positively correlated with all the three nitrogen species and with SRB, albeit with a lower p value (0.05). Whereas NRB, SOB and SRB showed a strong negative relationship with Chl *a* ($p < 0.001$). SOB and SRB also significantly correlated with nitrogen species (NH_4^+ and NO_2^-). PAB correlated positively with PO_4^{3-} ($p < 0.01$), Chl *a* ($p < 0.001$) and negatively with all the three nitrogen species and also with TAB, NRB and DNB (Tables 1 and 2).

Canonical Correspondence Analysis

For assessing the degree of interrelationships between environmental variables and culturable bacterial groups, CCA was performed separately for all bacterial groups (ABG and ANBG). The results of CCA are depicted in ordination biplots

Table 1 Spearman's correlation matrix of physiological bacterial groups ($n = 27$). Significant correlations are in italics (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Bacterial groups	THB	TAB	MOB	SOB	SRB	NRB	DNB	AOB	PSB	PAB
THB	1.00									
TAB	0.54	1.00								
MOB	*− 0.60***	− 0.58	1.00							
SOB	*0.77***	− 0.02	− 0.19	1.00						
SRB	0.45	*0.80***	*− 0.77***	− 0.06	1.00					
NRB	0.47	0.53	− 0.13	0.19	*0.40**	1.00				
DNB	0.64	*0.86***	− 0.59	0.18	*0.83****	*0.54***	1.00			
AOB	− 0.35	− 0.34	− 0.20	− 0.18	0.16	− 0.05	− 0.22	1.00		
PSB	0.27	− 0.17	0.16	0.60	− 0.49	− 0.06	− 0.29	− 0.24	1.00	
PAB	− 0.58	*− 0.58***	0.23	− 0.27	− 0.32	*− 0.72****	*− 0.56***	0.36	0.11	1.00

THB, total heterotrophic bacteria; *TAB*, total anaerobic bacteria; *MOB*, methane-oxidizing bacteria; *SOB*, sulfur-oxidizing bacteria; *SRB*, sulfate-reducing bacteria; *NRB*, nitrate-reducing bacteria; *DNB*, denitrifying bacteria; *AOB*, ammonia-oxidizing bacteria; *PSB*, phosphate solubilizing bacteria; *PAB*, phosphate accumulating bacteria; *Temp*, temperature; *TSS*, total suspended solids; NH_4^+, ammonia; NO_2^-, nitrite; NO_3^-, nitrate; PO_4^{3-}, phosphate; *DO*, dissolved oxygen; *BOD*, biochemical oxygen demand; *Chl a*, chlorophyll *a*; *Phaeo*, phaeophytin

Table 2 Spearman's correlation matrix of physiological bacterial groups and environmental variables ($n = 27$). Significant correlations are in italics (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Bacterial groups	Water quality parameters												
	pH	Salinity	Temp	TSS	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	Chl *a*	Phaeo	Phyto
THB	0.04	− 0.37	*− 0.86***	0.25	*0.71***	*0.92***	*0.86***	− 0.24	− 0.13	0.25	*− 0.77***	*− 0.70***	*− 0.45**
TAB	− 0.34	− 0.62	− 0.39	− 0.22	0.35	0.67	0.61	− 0.12	− 0.05	0.07	− 0.62	− 0.71	− 0.66
MOB	− 0.00	0.26	0.34	− 0.14	*− 0.57***	*− 0.69****	− 0.52	− 0.24	0.14	− 0.37	0.35	0.34	*0.43**
SOB	0.37	0.17	− 0.67	0.50	*0.59****	*0.53***	*0.47**	− 0.31	− 0.35	0.03	*− 0.38**	− 0.25	0.13
SRB	− 0.17	− 0.42	− 0.32	0.01	*0.41**	*0.49***	0.34	0.17	− 0.09	0.34	*− 0.41**	*− 0.50***	− 0.66
NRB	0.30	− 0.52	− 0.35	0.14	*0.45**	*0.52***	*0.45**	− 0.15	0.18	0.32	*− 0.59****	*− 0.62****	− 0.30
DNB	− 0.13	− 0.66	− 0.61	0.05	0.46	*0.61****	*0.60****	− 0.28	0.08	0.38	− 0.73	− 0.79	− 0.62
AOB	0.63	0.47	0.53	0.37	0.11	−0.35	*− 0.55***	*0.49***	− 0.16	0.25	*0.57***	*0.57***	0.41
PSB	0.33	0.43	− 0.05	− 0.07	− 0.01	0.20	0.19	− 0.13	− 0.40	− 0.42	0.06	0.19	0.50
PAB	0.01	0.63	0.51	− 0.40	*− 0.72****	*− 0.66****	*− 0.62****	*0.49***	− 0.16	− 0.13	*0.69****	*0.72****	0.29

THB, total heterotrophic bacteria; *TAB*, total anaerobic bacteria; *MOB*, methane-oxidizing bacteria; *SOB*, sulfur-oxidizing bacteria; *SRB*, sulfate-reducing bacteria; *NRB*, nitrate-reducing bacteria; *DNB*, denitrifying bacteria; *AOB*, ammonia-oxidizing bacteria; *PSB*, phosphate solubilizing bacteria; *PAB*, phosphate accumulating bacteria; *Temp*, temperature; *TSS*, total suspended solids; NH_4^+, ammonia; NO_2^-, nitrite; NO_3^-, nitrate; PO_4^{3-}, phosphate; *DO*, dissolved oxygen; *BOD*, biochemical oxygen demand; *Chl a*, chlorophyll *a*; *Phaeo*, phaeophytin

(Fig. 3). The forward selection of the water quality parameters retained 9–12 variables that explained the significance of each factor with respect to the bacterial groups (Table 3).

In the first CCA biplot for all bacterial groups (Fig. 3a), two axes, Ax1 and Ax2, explained 81.9% of the total variance contained in all bacterial abundance and the water quality data. Individually, Ax1 and Ax2, accounted for 65.7% and 16.2% respectively, of the total variation (Table 3). The three dissolved nitrogen species (NH_4^+, NO_2^-, NO_3^-), temperature, salinity, phytoplankton pigments (Chl *a* and Phaeo) and pH were the main factors controlling the bacterial abundance. The ordination plot showed the close relationship of DO and BOD with all the four groups of bacteria.

The first two axes (Ax1 and Ax2) in the second CCA biplot of ABG (Fig. 3b) accounted for 86% of the total variance. Ax1 and Ax2 explained 58.3% and 27.7% respectively, of the total variation of aerobic bacterial groups with the environmental variables. The three nitrogen species, temperature and salinity were the main factors controlling the total variation in the abundance of ABG (Fig. 3a). On the Ax1, all the three nitrogen species negatively correlated with ABG and on the Ax2, pH, TSS and NH_4^+ showed significant correlations ($p < 0.05$) (Table 3). The influence of PAB, THB and phytoplankton on the variability of dissolved phosphate is discernible from the ordination biplot.

In the third biplot for ANBG (Fig. 3c), Ax1 and Ax2 explained 94.8% of the total variance of anaerobic bacterial groups with the water quality data (Table 3). Ax1 and Ax2, accounted for 63.2% and 31.6% respectively, of the total variation. The three dissolved nitrogen species, temperature, salinity, phytoplankton pigments (Chl *a* and Phaeo) and PO_4^{3-} were the main factors controlling the variation in the abundance of anaerobic bacterial communities.

Discussion

Microbes, especially bacteria, form an integral component in shrimp pond ecosystems and play a critically important role in the cycling of nutrients as well as in biotransformation of xenobiotics, mediating the balance of biogeochemical cycling processes (C, N, P and S) and thus maintaining the ecosystem health [29–31]. In zero-exchange shrimp aquaculture systems, degradation of accumulated organic matter (uneaten feed, feces, and other debris) lead to an increase in the concentrations of inorganic nutrients [9]. Assimilation and mineralization of excess nutrients by dense bacterial communities facilitate the growth of bacteria and phytoplankton communities concurrently leading to temporal variations in water quality parameters (pH, DO, TSS) [32]. For optimization of the culture environment to achieve higher production, farmers operating zero-exchange systems therefore often rely on the application of several farm inputs in the form of probiotics (*Aeromonas* spp., *Bacillus* spp., *Lactobacillus* spp., *Roseobacter* spp.), disinfectants, environmental modifiers, immunostimulants and antimicrobials [14]. Recognizing the role of bacterial communities involved in biogeochemical cycles (C, N, P and S) in aquaculture systems, some studies have been conducted previously, as C-N [33, 34], C-N-S [31, 35, 36], N [37–40], N-S [41], S [42–44], C-N-P [45–47] and P [48]. However, the focus of these studies has been limited to fewer nutrient cycles and microbial interactions. In this context, the present study

Table 3 Summary for the 2 axes (Ax1 and Ax2) of canonical correspondence analysis with 12 selected environmental variables. Percentage variance of species-environment, cumulative percentage variance of species-environment relation; eigenvalues; sum of eigenvalues and canonical eigenvalues. Significant values are represented in italics

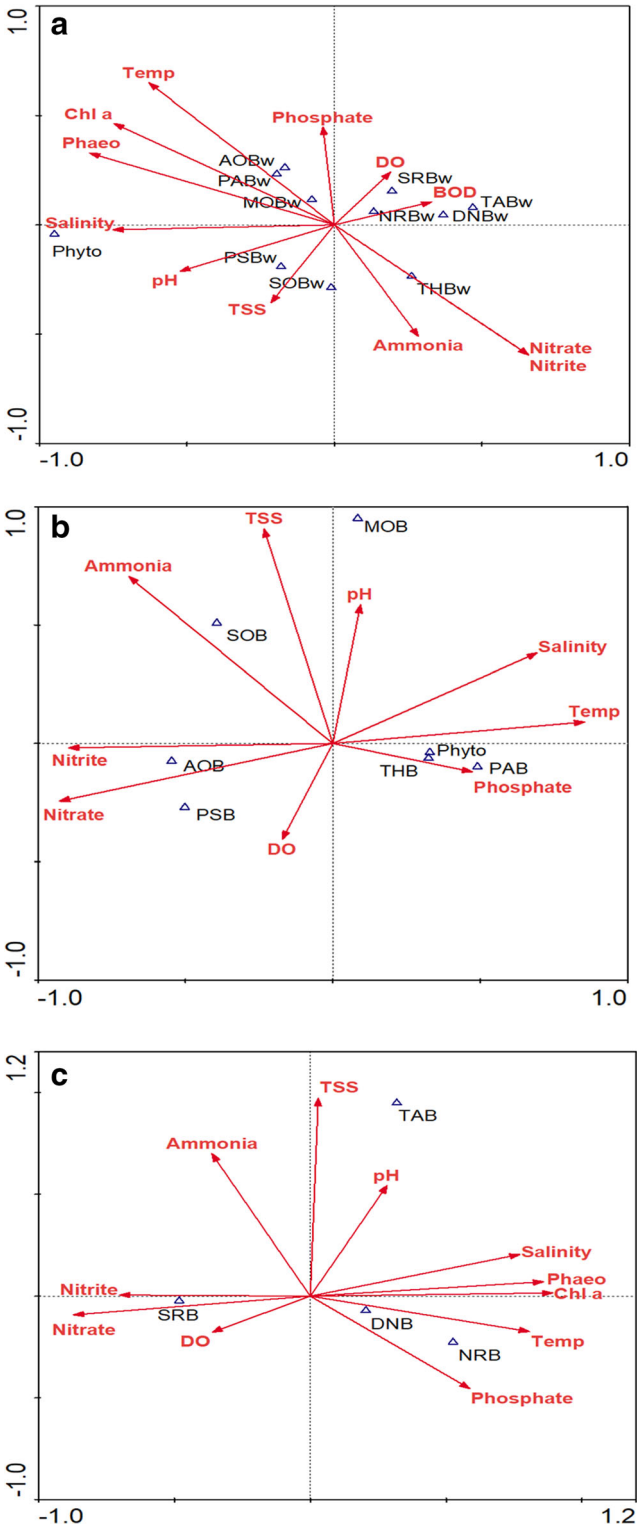
	All bacterial groups		Aerobic bacteria		Anaerobic bacteria	
Variable	Ax1	Ax2	Ax1	Ax2	Ax1	Ax2
pH	*− 0.52*	− 0.21	0.093	*0.582*	0.277	*0.542*
Salinity	*− 0.75*	− 0.02	*0.686*	0.38	*0.757*	0.205
Temperature	*− 0.62*	*0.64*	*0.846*	0.09	*0.791*	− 0.174
Total Suspended Solids	− 0.21	− 0.35	− 0.232	*0.902*	0.028	*0.972*
Ammonia	0.28	*− 0.50*	*− 0.686*	*0.701*	− 0.355	*0.698*
Nitrite	*0.65*	*− 0.58*	*− 0.888*	− 0.018	*− 0.688*	0.006
Nitrate	*0.65*	*− 0.58*	*− 0.918*	− 0.243	*− 0.855*	− 0.092
Phosphate	− 0.04	0.44	0.468	− 0.119	*0.575*	− 0.455
Dissolved oxygen (DO)	0.19	0.24	− 0.17	− 0.403	− 0.354	− 0.177
Biochemical oxygen demand (BOD)	− 0.33	0.10	-	-	-	-
Chlorophyll *a*	*− 0.74*	0.46	-	-	*0.875*	0.015
Phaeophytin	*− 0.82*	0.32	-	-	*0.841*	0.07
Eigenvalues :	0.023	0.006	0.023	0.011	0.029	0.015
% var group-env	*65.7*	*81.9*	*58.3*	*86.0*	*63.2*	*94.8*
Total inertia		0.036		0.041		0.047
Sum eigenvalues		0.036		0.041		0.047
Sum of canonical eigenvalues		0.035		0.040		0.046

deciphers the ecological roles of culturable bacterial groups involved in four biogeochemical cycles (C, N, P and S) as well as their interactions with environmental parameters in the presence of probiotics in a zero-exchange system, comprehensively.

Nutrient recycling has been recognized as one of the crucial processes governing pond dynamics. In a complex pond ecosystem, photoautotrophic, autotrophic and heterotrophic bacterial communities are involved in nitrogen cycle pathways [49]. These bacterial communities also depend upon the availability of carbon source (inorganic or organic) derived from the feed, fecal matter, or carbohydrate supplements to meet their metabolic requirements [49]. THB is known to influence the levels of key water quality parameters (NH_4^+, NO_2^-, NO_3^- and DO) and primary production through nutrient recycling [50]. The peaking of NO_3^- concentrations on 12 DoC may be attributed to its utilization via heterotrophic denitrification [51]. This is corroborated by a strong positive correlation between THB and the three nitrogen species (Tables 1 and 2). A relatively higher degree of negative relationship between THB and Chl *a* ($p < 0.001$) when compared between THB and phytoplankton abundance ($p < 0.05$) indicates that THB are more efficient in the acquisition of inorganic nutrients than phytoplankton [52].

The major factors that affect the rate of nitrification also play a dominant role in heterotrophic bacterial growth. Furthermore, sustenance of a high abundance of THB during the initial phase of the culture (Fig. 2) may be attributed to the heterotrophic ammonia assimilation in building cellular protein [6]. The elevated levels of dissolved phosphate recorded during the final phase of the culture period might have stimulated the phytoplankton growth and abundance [33]. A strong positive relationship between phosphate concentrations and phytoplankton pigments (Chl *a* and Phaeo; $p < 0.01$) corroborates such a relationship. The increase in PO_4^{3-} concentration could be attributable to the accumulation of uneaten feed with the progress of the culture [12]. Furthermore, increased concentrations of Chl *a* and higher phytoplankton abundance attributing to increased phosphate concentrations towards the end of the culture period has also been recently reported [3]. The elevated concentrations of PO_4^{3-} and higher abundance of PAB towards the final phase may indicate phosphate utilization as corroborated by a significant relationship between PAB and PO_4^{3-} ($p < 0.01$). Similarly, a positive relationship between PAB and Chl *a* ($p < 0.001$) was also discernible. The luxuriant growth of phytoplankton and higher abundance of PAB towards the end of the culture concomitant with elevated concentrations of PO_4^{3-} imply that the phosphate may act as a common substrate.

By virtue of enhanced capabilities of biological phosphorus removal, PAB is known for its important role in bioremediation and phosphate mineralization [53]. Furthermore, PAB plays a dual role by participating both in N (nitrite reduction and/or denitrification) and P cycles (PO_4^{3-} accumulation) [54–56]. Strong negative correlations between PAB and, the three nitrogen species and TAB observed during the present study support the multiple roles of PAB. The existence of possible competition for substrate between PAB and ANBG



◀ **Fig. 3** Canonical correspondence analysis ordination diagram for water quality parameters with (**a**) cultivable bacterial groups, (**b**) aerobic bacterial groups and (**c**) anaerobic bacterial groups during the cultivation of *Litopenaeus vannamei* in zero-exchange shrimp system. Results are for axis 1 (horizontal) and axis 2 (vertical); arrows represent forward selected environmental variables (Temp, temperature; TSS, total suspended solids; DO, dissolved oxygen; BOD, biochemical oxygen demand; Chl *a*, chlorophyll *a*; Phaeo, phaeophytin; Phyto, phytoplankton count). Arrow length indicates the strength of that variable explaining the distribution during the culture period; arrow direction suggests the approximate correlation to the ordination axes

(TAB, NRB and DNB) [53] was supported by a significant negative correlation. On the other hand, PSB, which are known for facilitating P release in aquatic systems [57, 58], varied within a narrow range except for a small peak on 12 DoC and did not show any significant relationships with the environmental variables as well as with other bacterial groups. The absence of such a significant relationship indicates that a complex array of factors might be controlling their abundance in shrimp culture systems and hence warrants further investigation.

Besides PO_4^{3-}, the modulation of NH_4^+ is important for pond health. In pond systems, NH_4^+ production is controlled through oxidation of NH_4^+ to NO_2^- and subsequent oxidation to NO_3^- by autotrophic bacteria and assimilation of ammonia directly into algal biomass by photoautotrophic processes [59, 60]. Overall, NH_4^+ levels in the present study were within the safe limits [28]. However, the peaking of NH_4^+ during the mid-culture period (41 DoC) could mainly be attributed to either one or all three factors: (i) the reduction of nitrogen species, (ii) the leaching out of NH_4^+ by the breakdown of protein from the uneaten feed [61], (iii) from the feces [32]. Despite an increase in shrimp biomass and accumulation of organic matter, lower concentrations of NH_4^+ observed post 41 DoC could be attributed to its efficient uptake by AOB and phytoplankton. A steady increase in the abundance of AOB and phytoplankton with the progress of the culture and a strong relationship observed between AOB and Chl *a* ($p < 0.01$) supports this contention [62]. Furthermore, aerating the pond to maintain the optimum DO levels might have further enhanced the rate of microbial activity [3].

In aquaculture systems, the toxicity of NH_4^+ and NO_2^- is controlled by microbial N_2 production, which is important in maintaining optimum levels of dissolved nitrogen species [63]. The involvement of NRB and DNB in the removal of NO_3^- through biological denitrification is considered as one of the most effective methods [64, 65]. ANBG (TAB, DNB and NRB) involved in nitrogen assimilation were relatively high during the initial stages of the culture (up to 41 DoC). Sustenance of such significantly high abundance ANBG might be responsible for the maintenance of water quality by the removal of excess nitrogen [39]. Although denitrification is known to occur in anaerobic conditions as well, but it is not an anoxic process *per se* as nitrogen oxide reductases are expressed in the presence of oxygen [66]. In the present study, NRB significantly correlated ($p < 0.05$) with all the three nitrogen species and SRB (Tables 1 and 2). Similarly, DNB also significantly correlated with NO_2^- ($p < 0.001$), NO_3^- ($p < 0.001$), TAB ($p < 0.001$), SRB ($p < 0.001$) and NRB ($p <$

0.01). This testifies the active roles played by DNB and NRB in regulating the levels of nitrogen species (denitrification or dissimilatory nitrate reduction to ammonium) and other anaerobic microbial interactions involving sulfur cycle [67].

In the light of recent reports that aquaculture ponds acting as potential sources of CH_4 emissions [16, 68], the relationship between MOB and environmental variables was evaluated as methane oxidizers are the major regulators controlling the CH_4 emissions [69]. The observed significant negative relationship of MOB with NH_4^+ ($p < 0.01$) and NO_2^- ($p < 0.001$) may explain their competitive inhibitory effect on methane monooxygenase (MMO)—the major enzyme involved in methane oxidation [70]. By virtue of its competition for CH_4 as the substrate by MOB, NH_4^+ is presumed to be a major influential factor in methane oxidation [68]. The complex role of NH_4^+ in methane oxidation (inhibitory, stimulatory, or, no effect) and evolutionary relatedness between MOB and AOB have also been reported [71, 72]. MOB showed a positive relationship with the phytoplankton abundance ($p < 0.05$). Dimethylsulfoniopropionate (DMSP)—a constituent of phytoplankton leachates and debris, acting as a substrate for production of methane (methanogenesis) and its subsequent oxidation by MOB has been reported [73].

Forms of suspended organic matter as microniches aid in the proliferation of anaerobes such as SRB in aquatic ecosystems [74]. With the progress of the production cycle, production of H_2S gas by SRB is likely to occur in high density zero-exchange shrimp culture systems. On the other hand, obnoxious H_2S gas, thus formed, is assimilated by SOB. The predominance of SOB over SRB observed throughout the production cycle may be related to the routine application of "Super PS" probiotic (*Rhodococcus* spp. and *Rhodobacter* spp.) in addition to *in situ* SOB populations. In contrast, the lower abundance of SRB during the production cycle could probably relate to varying levels of substrate availability and maintenance of requisite concentrations of DO in the pond by artificial aeration [30].

Positive relationships between SOB and the three nitrogen species ($p < 0.05$) were discernible. This indicates the effective utilization of nitrate/nitrite as electron acceptors for energy conservation and growth by autotrophic denitrifying SOB [75]. A strong relationship between SOB and THB ($p < 0.001$) indicates the detoxification of sulfide by SOB and the utilization of produced organic substrates by THB [75]. Therefore, it appears that the process of chemolithotrophic denitrification coupled with sulfur oxidation, carbon and nitrogen metabolism as documented in nutrient-rich coastal ecosystems [41, 76, 77], might be also prevalent in shrimp culture systems. Sulfide produced by SRB acting as a controlling factor during the dissimilatory nitrate reduction by producing ammonium has also been reported [67]. Sulfide and organic carbon have significant effects on the nitrogen cycle, especially involving NH_4^+. As reported previously [78, 79], SRB in the present study also showed positive relationship with NH_4^+ ($p < 0.05$), NO_2^- ($p < 0.01$), and MOB ($p < 0.001$). This confirms the significant role played by SRB in the coupled biogeochemical cycling of C, N and S [36, 80]. Effective utilization of NO_3^- as substrate by THB, DNB, SRB and PAB might have prevented it reaching to alarming concentrations as excess NO_3^- is known to induce toxicity in shrimps causing the reduction in growth and survival [81].

The structure and function of microbial communities in aquatic systems are influenced by environmental factors [82]. The succession of microbial communities in response to combinations of Chl *a*, total nitrogen, PO_4^{3-}, C/N ratio [83], total phosphate, chemical oxygen demand [84] and addition of feed sources [85] have been reported. The results of CCA performed to understand the major environmental determinants controlling the variation in the abundance of physiological bacterial groups (C, N, P and S cycles) revealed significance of the three dissolved nitrogen species along with temperature and salinity, phytoplankton biomass and pH. Collectively, these parameters explained as much as 82% variation in all the studied bacterial groups. A negative correlation of the three nitrogen species with Chl *a* and temperature supports the dynamic removal of N species from the pond. The progressive decline in the abundances of TAB, SRB, NRB and DNB with the progress of the culture signaling a shift in the bacterial communities from anaerobic to aerobic is supported by the first quadrant of the CCA ordination plot.

An assessment of the environmental factors shaping the structure and function of microbial communities in shrimp culture enclosure systems has been investigated recently by Hou et al. [86]. Based on CCA results, they concluded that salinity, total phosphate, total nitrogen, temperature and pH were the most important factors shaping microbial community structure in enclosed culture systems growing *L. vannamei*. The CCA inference combined with Spearman's correlation matrix in our study highlighted the significant interrelationship between nitrogen species and different aerobic and anaerobic bacterial groups thus, interlinking it with biogeochemical cycles. The aerobic, anaerobic bacterial and phytoplankton communities are directly or indirectly influenced by the nutrients, temperature and salinity. There exists strong interrelationships between the elemental cycles within the shrimp culture ecosystem.

Conclusion

In conclusion, the study provides comprehensive information on the abundance and temporal changes in physiological bacterial communities involved in C, N, P and S cycling and the delineation of principal environmental variables affecting their variability in zero-exchange shrimp culture. The results of the study (Spearman's correlation matrix and CCA)

conclusively showed that the three dissolved nitrogen species (NH_4^+, NO_2^-, NO_3^-) followed by salinity, temperature, phytoplankton biomass and pH are the major environmental determinants accounting as much as 82% of the total variation in bacterial abundance. The inferences and conclusions drawn in the present study are based on the enumeration of physiological bacterial groups using conventional techniques for one shrimp production cycle. On the other hand, molecular methods (high-throughput 16S RNA, NGS analysis) generate robust information. Nevertheless, the results provide baseline information for predicting the changes in abundance of physiological bacterial communities in response to environmental parameters. Follow-up studies comparing the bacterial communities in closed shrimp culture systems with varying stocking densities, with or without probiotics and farm management practices using modern molecular methods, would provide greater insights into the precise role of physiological bacterial groups.

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