Research Article

Molecular characterization of bacteria and archaea in a bioaugmented zero-water exchange shrimp pond

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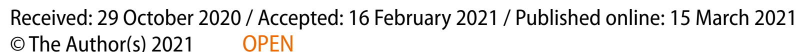

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Abstract

In the zero-water exchange shrimp culture pond maintained with the application of indigenous bioaugmentor, low levels of total ammonia–nitrogen were reported, indicating the relevance of indigenous microbial communities. Sediments (0–5 cm layer) were sampled from the pond (85th day) and the bacterial and archaeal communities; specifically, the ammonia oxidizers (ammonia-oxidizing bacteria, ammonia-oxidizing archaea, and anaerobic ammonia-oxidizing bacteria) in the sediment metagenome of the pond were analysed using the 16S rRNA and functional genes. Bacterial and archaeal 16S rRNA genes showed the relative abundance of Delta-Proteobacteria and Bacteroidetes groups performing sulphur respiration and organic matter degradation, archaeal groups of anaerobic sulphur respiring *Crenarchaeotae*, and chemolithoautotrophic ammonia oxidizers belonging to *Thaumarchaeota*. The presence of these diverse bacterial and archaeal communities denotes their significant roles in the cycling the carbon, nitrogen, and sulphur thereby bringing out efficient bioremediation in the bioaugmented zero-water exchange shrimp culture pond. Similarly, the functional gene-specific study showed the predominance of *Nitrosomonas* sp. (ammonia-oxidizing bacteria), *Nitrosopumilus maritimus* (ammonia-oxidizing archaea), and *Candidatus* Kuenenia (anaerobic ammonia-oxidizing bacteria) in the system, which points to their importance in the removal of accumulated ammonia. Thus, this study paves the way for understanding the microbial communities, specifically the ammonia oxidizers responsible for maintaining healthy and optimal environmental conditions in the bioaugmented zero-water exchange shrimp culture pond.

Keywords 16S rRNA gene · *Amo*A gene · Ammonia oxidizers · Archaea · Bacteria · Sediment metagenome

1 Introduction

Zero-water exchange (ZWE) shrimp culture ponds are environmentally sustainable, bio-secured systems developed as an alternative to traditional open aquaculture production. In open culture systems, water quality is maintained through frequent exchange of water [110]. Frequent release of water into the nearby aquatic systems often causes eutrophication due to excess nutrient contents [93], and the uptake of water for culturing from the neighbouring water bodies also leads to horizontal transfer of pathogens causing subsequent disease outbreak and mortality of shrimp [24]. ZWE aquaculture ponds have been developed to abate the negative impacts of open shrimp farming on the environment. The problem faced by ZWE ponds is the build-up of high organic matter and toxic inorganic nitrogen composed of unutilized feed, fertilizers, and metabolic waste [10]. The concentrations of ammonia–nitrogen (ammonia-N) above the recommended safe levels [13, 14] induce less oxygen transport and stress in

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shrimps, which in turn decrease the immunity leading to morbidity, mortality, and loss of crop [15]. The bioaugmented ZWE shrimp culture ponds with bioaugmentor "Detrodigest™" (a preparation containing euryhaline *Bacillus cereus* MCCB 101, GenBank accession no: EF062509), developed, implemented, and validated by the National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology (CUSAT), Cochin, India [34], proved to maintain optimal and stable environmental parameters required for shrimp culturing, notably low or negligible concentration of ammonia throughout the culture period [34, 44]. *Bacillus cereus* MCCB 101, the sole component of a commercial product Detrodigest™, is an indigenous Gram-positive bacterium isolated from shrimp pond sediment, with hydrolytic potential, used in aquaculture ponds for detritus degradation.

Bioaugmentation is the process of adding specially formulated microorganisms to improve the water quality through the degradation of organic matter and altering the microbial ecology [68, 87]. Microorganisms, whether they occur naturally or added artificially, are of great importance and play significant roles in pond ecosystems, particularly for its productivity, nutrition of the cultured animals, nutrient cycling, disease control, water quality, and environmental impact [68]. Knowledge of microbial interactions is essential for successful management of the aquaculture practices. The application of bioaugmentor has shown to enhance mineralization of organic matter [62], reduction of nitrogen and phosphorus from the systems, improving the survival and productivity of the cultured animals [117]. The maintenance of optimal and stable environmental parameters in this bioaugmented pond indicated the presence of active indigenous microbial communities in the sediment [68].

Bacterial and archaeal communities play a vital role in oxidative and reductive processes of nitrification [36]. The major challenge faced by aquaculture ponds is the accumulation of toxic ammonia, which at high concentrations can decrease the rate of outward diffusion from the animal, and toxicity exceeds tolerable level [91]. But the ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) of shrimp ponds could oxidize this toxic ammonia to nitrite and then to less toxic form nitrate [50]. Similarly, chemoautotrophic nitrifier, ammonium-oxidizing archaea (AOA) [108], and bacteria performing anaerobic ammonia oxidation (anaerobic ammonium-oxidizing (anammox)) (single-step conversion of ammonia to nitrogen gas) [40, 99] are observed [4, 60, 119] in aquaculture ponds. Therefore, identification of bacterial and archaeal ammonia oxidizers is an important step in understanding the biogeochemical processes.

In general, ninety-nine percent of microbes are unculturable, and especially, the nitrifying bacteria are slow growers and are highly sensitive to environmental conditions; so, it is difficult to isolate them in pure culture [32]. Therefore, for a true understanding of these microbial communities and nitrifiers in an ecosystem, a culture-independent metagenomic approach is appropriate [32, 79]. The availability of molecular tools and gene sequencing has made it possible to explore the slow-growing or uncultivable bacterial and archaeal species in different environments [55]. PCR amplification and clone libraries of highly conserved regions (bacterial or archaeal 16S rRNA genes and functional genes) are widely used to describes the microbial community [114] in varied ecosystems such as freshwater [107], seawater [94], marine sediments [30], farm sediment [63], zero-discharge mariculture, and aquaculture systems [50]. For the microbial community 16S rRNA gene profiling and diversity, a molecular fingerprinting technique ARDRA (amplified ribosomal DNA restriction analysis) was used [64, 78].

The addition of "Detrodigest™" [34, 44, 84] degrades the organic matter and stimulates the natural microbial communities, which in turn help to maintain the optimal environmental conditions for shrimp growth, and therefore, it is important to study the microbial communities and ammonia oxidizers in bioaugmented ZWE shrimp pond. Thus, the present study highlights 1) the bacterial and archaeal 16S rRNA gene-based community structure and 2) the functional gene-based characterization of ammonia oxidizers like AOB and AOA, and anammox specific 16SrRNA gene based chareacterizaiton of anammox bacteria in the ZWE shrimp culture pond from the sediment metagenome collected from a bioaugmented ZWE shrimp pond in Kodungallur, India (10.254639°N, 76.20991°E) on 85th day of culturing (25th April 2008), with the consent of the farmer. The research was carried out at National Centre for Aquatic Animal Health (NCAAH), CUSAT, Cochin, India, during March 2008 to February 2011.

2 Materials and methods

2.1 Study site, sample collection, and physicochemical characterization

The sediment samples were collected from a bioaugmented ZWE shrimp culture pond (10,117 m^2) with 1 m depth located at Kodungallur, India (10.254639°N, 76.20991°E), on 25th April 2008 on the 85th day of culture (prior to harvest). The samples were collected on the 85th days of the culture, the time most intense microbial activity, when there were maximum biomass, feed input, and faecal matter output, but the ammonia concentrations were negligible. The pond was maintained by the

application of indigenous bioaugmentor "Detrodigest™", every 7 days.

The top 0–5 cm layer of sediment was collected from five different sites of the pond and maintained in refrigerated condition. Composite sediment samples were prepared and stored at – 80 °C for DNA extraction.

The analysis of water quality parameters was carried out in triplicates. The salinity was measured using refractometer, pH using digital pH metre (Scientific Tech, India), alkalinity and hardness by titrimetric method [5]., and ammonia–nitrogen (ammonia-N) by phenol hypochlorite method [95]. Briefly, 10 mL of water sample was incubated with 0.4 mL phenol (20 g of crystalline phenol dissolved in 95% V/V ethyl alcohol), 0.4 ml sodium nitroprusside (1 g dissolved in 200 mL distilled water), and 1.0 mL oxidizing solution (alkaline reagent (100 g sodium citrate and 5 g sodium hydroxide dissolved in 500 mL distilled water) and sodium hypochlorite (4:1)). After 1 h, the absorbance was read at 630 nm using UV–Vis spectrophotometer (Shimadzu, 1650 PC Japan). The factor value was calculated by preparing a series of standards at a concentration of 10 to 60 µg ammonia-N (a standard solution of 10 µg mL^{-1} ammonia-N was prepared by dissolving 4.714 mg ammonium chloride in 100 mL double-distilled water). Nitrite nitrogen (nitrite-N) was analysed by incubating 10 mL water sample using 0.2 mL sulphanilamide (5 g dissolved in a mixture of 50 mL concentrated hydrochloric acid and 450 mL distilled water) and 0.2 mL N-(1-naphthyl)-ethylene diamine dihydrochloride (NED dihydrochloride) (0.5 g dissolved in 500 mL distilled water) [8]. After 8 min, the absorbance was read at 543 nm using UV–Vis spectrophotometer (Shimadzu, 1650 PC Japan). The factor value was calculated by preparing a series of standards at a concentration of 10 to 60 µg nitrite-N (a standard solution of 10 µg mL^{-1} nitrite-N was prepared by dissolving 4.925 mg sodium nitrite in 100 mL double-distilled water). Nitrate nitrogen (nitrate-N) [8] was estimated by incubating 10 mL water sample by adding 0.4 ml phenol–sodium hydroxide solution (1:1), and hydrazine sulphate–copper sulphate solution (1:1) in dark for 18 to 24 h. Phenol–sodium hydroxide solution is prepared by mixing phenol solution (46 gm dissolved in 1 L distilled water) and sodium hydroxide (30 g dissolved in 2 L distilled water), and hydrazine sulphate–copper sulphate solution was prepared by the mixing hydrazine sulphate (14.5 g hydrazine sulphate dissolved in 1 L distilled water) and copper sulphate (0.1 g copper sulphate dissolved in 1 L distilled water). After incubation, 0.4 mL acetone, 0.2 mL sulphanilamide, and 0.2 mL NED dihydrochloride were added. Absorbance was measured after 8 min at 543 nm using UV–Vis spectrophotometer (Shimadzu, 1650 PC Japan). The factor value was calculated by preparing a series of standards at concentrations of 10 to 60 µg nitrate nitrogen (a standard solution of 10 µg mL^{-1} nitrate–N was prepared by dissolving 6.0707 mg sodium nitrate in 100 mL double-distilled water). The efficiency of the reaction was determined to measure the percentage of nitrate converted into nitrite. The inorganic phosphate was measured by ascorbic acid method [98] by adding 1.6 mL combined reagent (5 mL 5 N sulphuric acid (14 mL concentrated sulphuric acid diluted to 100 mL distilled water), 0.5 ml potassium antimonyl tartrate (0.686 g dissolved in 250 mL distilled water), 1.5 mL ammonium molybdate (4 g dissolved in 100 mL distilled water), and 3 mL ascorbic acid (1.76 g dissolved in 100 mL distilled water)) to 10 mL water sample. Absorbance was measured at 880 nm after a 10-min incubation using UV–Vis spectrophotometer (Shimadzu, 1650 PC Japan). The factor value was calculated by preparing a series of standards at a concentration of 50 to 600 µg PO_4^{3-} mL^{-1} (54.87 mg anhydrous potassium dihydrogen phosphate dissolved in 250 mL double-distilled water gave 50 µg PO_4^{3-} mL^{-1}). All the analyses were performed with triplicate samples with distilled water as blank, and values were represented as mean value.

3 Metagenomic DNA isolation

The metagenomic DNA was isolated from sediment sample (250 mg) using Power soil DNA isolation kit following manufacturer's instructions (MoBio Laboratories Inc., USA), and the concentration of the DNA was estimated using a UV spectrophotometer (Hitachi U-2800, Hitachi Corp, Japan) by measuring absorbance at 260 nm.

4 Polymerase chain reaction (PCR) amplification and library preparation

PCR amplification was carried out to analyse the bacterial and archaeal 16S rRNA genes, bacterial and archaeal ammonia monooxygenase A (*amo*A) functional genes, and 16S rRNA gene-specific to anammox bacteria. The details of primers and the annealing temperature used in this study are specified in Table 1. The PCR reactions were processed in a thermal cycler (Eppendorf, Germany). All the genes were amplified using Master Taq DNA amplification kit (5 PRIME, USA), which included 5X Taq Master PCR Enhancer (1X), 10X Master Taq buffer with Mg^{2+} (1X), 10 mM dNTP (0.2 mM) of forward and reverse primers, Taq DNA polymerase (0.04 U), and metagenomic DNA (100 ng). Triplicate PCR products were pooled, electrophoresed on 1.5% (w/v) agarose gel (Sigma-Aldrich, USA), and purified using the GenElute™ Gel Extraction Kit (Sigma-Aldrich, USA). The purified PCR products were cloned into the pGEM-T ®-T Easy Vectors (Promega, USA) for library

Table 1 PCR primers used for the amplification of bacterial and archaeal 16S rRNA genes and functional genes of ammonia oxidizers (AOB, AOA, and anammox)

Name	Primer sequence (5′ to 3′)	Annealing (°C)	Product Size (bp)	Reference
Bacterial 16SrRNA	Bac-fD1-GAGTTTGATCCTGGCTCA Bac-rP2-ACGGCTACCTTGTTACGACTT	58	1500	[114]
Archaeal 16SrRNA	Arch-21F-TTCCGGTTG ATCCYGCCGGA Arch-958R-YCCGGCGTTGAMTCCAATT	55	950	[19]
AOB	amoA-1F-GGGGTTTCTACTGGTGGT amoA-2R-CCCCTCKGSAAAGCCTTCTTC	60	491	[86]
AOA	Arch-amoAF-STAATGGTCTGGCTTAGACG Arch-amoAR-GCGGCCATCCATCTGTATGT	53	635	[26]
Anammox	Amx 368F-TTCGCAATGCCCGAAAGG Amx 820R-AAAACCCCTCTACTTAGTGCCC	56	478	[3]

preparation and transformed, and the screened positive clones were selected for plasmid extraction using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, USA).

5 Amplified ribosomal DNA restriction analysis (ARDRA)

Using the purified plasmids (1:200 dilutions), the bacterial and archaeal 16S rRNA genes were re-amplified with bacterial- and archaeal-specific 16S rRNA gene primers as mentioned earlier (Table 1), using the same PCR mix and conditions mentioned in the previous section. The re-amplified PCR products were subjected to ARDRA using two tetra cutter restriction enzymes *Alu*I and *Hae*III (New England Biolabs, USA). The restriction digestion was carried out separately with two units of each restriction enzymes for 3 h in a twenty micro-litre reaction volume of re-amplified PCR product 5 µl, incubation buffer (New England Biolabs, USA) 2 µl, and Milli-Q water 12.8 µl. The PCR products were run on 3% agarose (Sigma-Aldrich, USA) gel in 1X TAE buffer for 3 h at 100 V and visualized under UV excitation in a gel documentation system (Bio-Rad, USA). Using the Quantity One (Bio-Rad, USA) software, the band patterns on the gel were scored for the absence (0)/presence (1) of individual loci and analysed using the NTSYSpc (version 2.02i, Applied Biostatistics Inc., USA). Further, using NT edit programme, the data matrix prepared in MS-Excel spreadsheets was converted to a proprietary matrix file, and the rectangular data matrix generated was analysed by NTSYSpc (version 2.02i, Applied Biostatistics Inc., USA). Similarities and clustering were performed using a simple matching coefficient SAHN (Sequential, Agglomerative, Hierarchical, and Nested) statistical module and UPGMA (unweighted pair group method with arithmetic mean) clustering method.

6 Sequence analyses and community structure

The representative plasmids selected from each 16S rRNA ARDRA analysis cluster were sequenced using Applied Biosystems ABI 3730 × 1DNA analyser at SciGenom Labs Pvt. Ltd., Kochi, India. The sequences were screened for vector sequences using "VecScreen" (www.ncbi.nlm.nih.gov/VecScreen) and further edited and assembled using Gene Tool Lite1.0 (BioTools Incorporated). The sequence similarity search to database DNA sequences was performed in BLAST (www.ncbi.nlm.nih.gov/BLAST) [2]. The gene sequences were aligned using ClustalW, and the evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model. The phylogeny was analysed in MEGA 6.06 software [101] with 1000 bootstrap. Though the clones were sequenced based on ARDRA clusters, the number of operational taxonomic units (OTUs) was assessed based on 97% sequence similarity criterion using Mothur v.1.12.3 [90]. The taxonomic composition of ZWE shrimp culture pond was visualized using Geneious R8 [47]. The gene sequences of bacterial and archaeal 16S rRNA in Geneious R8 were analysed using the RDP Classifier (Ribosomal Database Project Database) [111], and Krona visualization tool [73] represented a hierarchical graph showing the diversity and relative abundance. The taxonomic composition of 16S rRNA genes was calculated using VITCOMIC (VIsualization tool for Taxonomic COmpositions of MIcrobial Community) software [65]. The visualization tool plots a single figure from all sequences indicating the relative evolutionary distances. Each phylum name was designated with different font colour, and circles indicated similarity of sequence to the sequences in database (80%, 85%, 90%, 95%, and 100% BLAST similarity) and size of the dots indicated relative abundance.

For the analyses of ammonia oxidizers (functional genes for AOA and AOB and group specific 16SrRNA gene for

anammox), clones (~ $n=15$) were randomly sequenced on Applied Biosystems ABI 3730 × 1DNA analyser at SciGenom Labs Pvt. Ltd., Kochi, India. The sequences obtained were vector screened using "VecScreen" tool (www.ncbi.nlm.nih.gov/VecScreen), further edited and assembled using Gene Tool Lite1.0 (BioToolsIncorporated). The sequence similarity search to database DNA sequences was performed in BLAST server (www.ncbi.nlm.nih.gov/BLAST) [2]. Phylogenetic analysis was carried out using ClustalW multiple alignments of gene sequences, and the Maximum Likelihood method phylogenetic tree (1000 bootstrap) was built in MEGA version 6.06 [99]. The genetic distance was calculated using Kimura 2-parameter model.

7 Nucleotide sequence accession numbers

The GenBank accession numbers of genes are bacterial 16S rRNA gene (JF428815–JF428837, JF428842–JF428862), archaeal 16S rRNA gene (JF428780 to JF428804), bacterial *amo*A gene (JF428805–JF428814, JX524531, and JX524532), archaeal *amo*A gene (JX524533–JX524543), and anammox bacteria-specific 16S rRNA gene (KC499605–KC499609, KC499611, and JX524516–JX524530).

8 Results and discussion

8.1 Physicochemical properties of the study site

The environmental parameters of the bioaugmented ZWE shrimp pond are summarized in Table 2. Among the two species of ammonia–nitrogen [85], unionized ammonia (NH_3-N) is more toxic. The recommended safe and tolerable ammonia-N levels for post-larvae, nauplius, and adolescents of shrimps are 1.15 mg L^{-1} (0.10 mg L^{-1} NH_3-N), 0.13 mg L^{-1} (0.01 mg L^{-1} NH_3-N), and 4.26 mg L^{-1} ammonia-N (0.08 mg L^{-1} NH_3-N), respectively [13, 14]. If the ammonia conversion to nitrate is prevented, significant concentrations of nitrite are accumulated in the environment, and it may cause a decrease in the immunity, and increased susceptibility to infection [105]. A suggested safe level of N-NO_2 is 0.09 mg L^{-1} [21], and similarly, safe levels of nitrite-N (0.0412 ± 0.0014 mg L^{-1}) were observed in the bioaugmented shrimp pond on the 85th day of culture. Prior to the harvest (85th day), when there were maximum nutrient and biomass load, an interesting observation was the low level of total ammonia–nitrogen (TAN). The low levels of TAN obtained in the present study comply with previous observations of low levels of TAN in bioaugmented ZWE shrimp culture pond throughout the culture period [34, 44]. The low level of TAN was attained by the microbial degradative processes being carried out by the microbial community in the sediment of the ZWE pond. Therefore, bio-geochemically ammonia-oxidation gains a vital role in the ZWE system. In the ZWE system, microbial-mediated processes and players acquire more importance to maintain low levels of ammonia, and in this context, understanding microbial players in ammonia oxidation from the sediment will be helpful to highlight the role of microorganisms in bioaugmented ZWE shrimp culture ponds.

Table 2 Physiochemical properties of bioaugmented zero-water exchange shrimp culture ponds ($n=5$)

Parameters	Values
pH	7.6 ± 0.043
Salinity	17 PSU
Alkalinity	65 mg L^{-1}
Total hardness	3100 mg $CaCO_3$ L^{-1}
Ammonia-N	0.212 ± 0.0706 mg L^{-1}
Nitrite-N	0.0412 ± 0.0014 mg L^{-1}
Nitrate–N	0.00545 ± 0.0008 mg L^{-1}

9 Bacterial and archaeal 16S rRNA gene analysis of ZWE pond

Metagenomic DNA of good yield and purity with a concentration of 35 μg mL^{-1} was obtained from the ZWE pond sediment sample (Fig. S1). The amplification of bacterial 16S rRNA (1500 base-pair (bp)) (Fig. S2a) and archaeal 16S rRNA (950 bp) genes (Fig. S2b) were successful. Similarly, the PCR analysis of functional genes of bacterial *amo*A gene (491 bp) (Fig. S3a), archaeal *amo*A (635 bp) (Fig. S3b), and planctomycetes group-specific 16S rRNA gene (478 bp) (Fig. S3c) was also successful, confirming the existence of all the three ammonia oxidizers in tropical shallow ZWE shrimp culture pond. The clone libraries of the 16S rRNA genes of bacterial and archaeal communities and ammonia oxidizers were successfully generated and similar approach of PCR and clone library analyses were carried out to study estuary [82], OMZ (oxygen minimum zone) of ocean [53, 54], and varied environments.

The ARDRA of 128 bacterial (Fig. S4a, b) and 44 archaeal (Fig. S4c, d) positive clones showed diverse banding profiles, indicating the level of molecular heterogeneity among the bacterial and archaeal populations in the sediment. The banding patterns were analysed and grouped using NTSYSpc. Each group represents a specific banding pattern. Dendrogram of bacteria (Fig. S5) and archaea (Fig. S6) based on ARDRA profiling were generated. ARDRA of 128 bacterial clones resulted in a dendrogram

consisting of 25 groups and few individual representative clones, from which 33 representative clones were selected and sequenced. Similarly, ARDRA of 44 archaeal clones resulted in 9 groups and individual representatives, from which 25 representative clones were selected for sequencing. The distance coefficient of the dendrogram of bacterial 16S rRNA gene clone library ranged from 0.87 to 1.00, and the archaeal 16S rRNA clone library ranged from 0.82 to 1.00. ARDRA profiling was used as a tool to analyse the genetic diversity of microbial communities from different environments [64, 78].

10 Phylogenetic analysis of bacterial and archaeal 16S rRNA genes

Based on a 3% cut-off, 33 bacterial clone sequences obtained from shrimp pond sediments were clustered into 29 OTUs (Fig.S7). Phylogenetic analysis (Fig. 1) of bacterial clones revealed Delta-Proteobacteria and Bacteroidetes as the most important bacterial communities in the bioaugmented ZWE shrimp pond. The clone sequences also showed similarity to the earlier reported uncultured Gamma-Proteobacteria [43], uncultured *Nitrospira* bacterium clone [1], uncultured Planctomycetales group, uncultured deep-sea bacterial clones, sulphate-reducing *Olavius* sp. [1, 49], uncultured *Chloroflexi* [103], Acidobacteria [1], *Crocinitomix* sp. [41], purple-sulphur bacteria (PBS)-*Thiohalocapsa* sp. [52, 74], Spirochaeta [35], *Desulfuromonas*, and *Desulfucoccus*. The bacterial communities in the bioaugmented ZWE shrimp pond showed similarity to mostly sulphur-oxidizing and reducing bacteria.

A total of 25 representative clones from the archaeal library clustered within 16 OTUs based on a 3% cut-off (Fig.S7). Phylogenetic analysis (Fig. 2) of archaeal clones showed close affiliation to uncultured archaeal clones. To acquire an accurate description of phylogenetic relationships of ZWE shrimp pond sediment clones, we included representative sequences of both cultivated and uncultivated archaeal clones (showing maximum similarity) in the analysis. Based on their phylogenetic affiliation (Fig. 2), archaeal 16S rRNA gene sequences were seen distributed among *Crenarchaeota* and *Thaumarchaeota*. The isolated clones from ZWE pond showed similarity (> 95%) to sequences from habitats such as marine sediment [25, 38, 61], mangrove and estuarine sediments [42, 94, 115], and thermal spring [113].

The Geneious R8 biodiversity map of the bacterial 16S rRNA gene (Fig. 3) showed the presence of three proteobacterial classes and nine other major phyla. The bacterial communities in the ZWE pond were belonging to Proteobacteria, Bacteroidetes, Ignavibacteriae, Firmicutes, Gyanobacteria, Planctomycetes, Spirochaetes, Chloroflexi, Hydrogenedentes, and Acidobacteria. The relative abundance of Proteobacteria accounts for 39%, with a predominance of Delta-Proteobacteria (27%), followed by Gamma-Proteobacteria (9%) and Alpha-Proteobacteria (3%). The Delta-Proteobacteria comprised the major representatives with 3% Desulfuromonadaceae, 6% Deferrisoma, 6% Cystobacteraceae, 3% Desulfobulbaceae, and 9% Desulfobacteraceae. The second relatively abundant Gamma-Proteobacteria represented 3% Coxiellaceae, 3% Chromatiaceae, and 3% Ectothiorhodospiracaea. Rhodobacteraceae (3%) was represented in the Alpha-Proteobacteria. Bacteroidetes (24%) was another major representative followed by Proteobacteria, which showed the presence of Flavobacteria (9% Cryomophaceae, and 6% Flavobacteriaceae), Bacteroidia (6% Prolixibacteraceae), and Cytophagia (3% Flammeovirgaceae). Ignavibacteriaceae (12%) was the third dominant group of bacteria in ZWE ponds. The remaining bacterial groups present in the bioaugmented ZWE pond were 3% Thermoanaerobacterales and 3% Clostridiales of Firmicutes, 3% Gyanobacteria, 3% Phycisphaeraceae of Planctomycetes, 3% Spirochaetes, 3% Dehalococcoidaceae of Chloroflexi, 3% Candidatus *Hydrogenedenes* of Hydrogenedentes, and 3% Gp23 genus of Acidobacteria Gp23. The VITCOMIC plot of the overall taxonomic composition of bacterial communities (Fig. 4) demonstrated the predominance of Proteobacteria and Bacteroidetes, with ≤ 5% relative abundance.

The presence of relevant microorganisms in the sediment metagenome signifies their contribution to the sulphur and nitrogen cycle in the ZWE shrimp pond. The presence of these microbial communities reflects the ecological functions that they could perform in the system. In the overall diversity of the sediment metagenome, the substantial fraction was comprised of Delta-Proteobacteria. They are predominantly anaerobic genera containing sulphate and sulphur-reducing bacteria. The sequences affiliated to *Desulfuromonas* genera, *Deferrisoma* sp., Myxococcales, and Desulfobacterales order were detected. Genera *Desulfuromonas* (family Desulfuromonadaceae) are anaerobic bacterium, performing organic matter degradation and sulphur respiration [22, 23, 76]. *Deferrisoma* sp., a thermophilic, anaerobic, mixotrophic bacterium [75], could oxidize the electron donors completely into CO_2 and H_2O with elemental sulphur and iron (III) as electron acceptors [96]. *Anaeromyxobacter* sp., an anaerobic myxobacterium within the order Myxococcales, grows anaerobically with acetate oxidation and reduction of electron acceptors (oxygen, nitrate, nitrite, and fumarate). They are physiologically adapted to both oxic and anoxic conditions and found distributed throughout soil and sediment environments [102, 104]. Sulphate-reducing bacteria, Desulfobacterales, included among the Delta-Proteobacteria reduces sulphates to sulphides

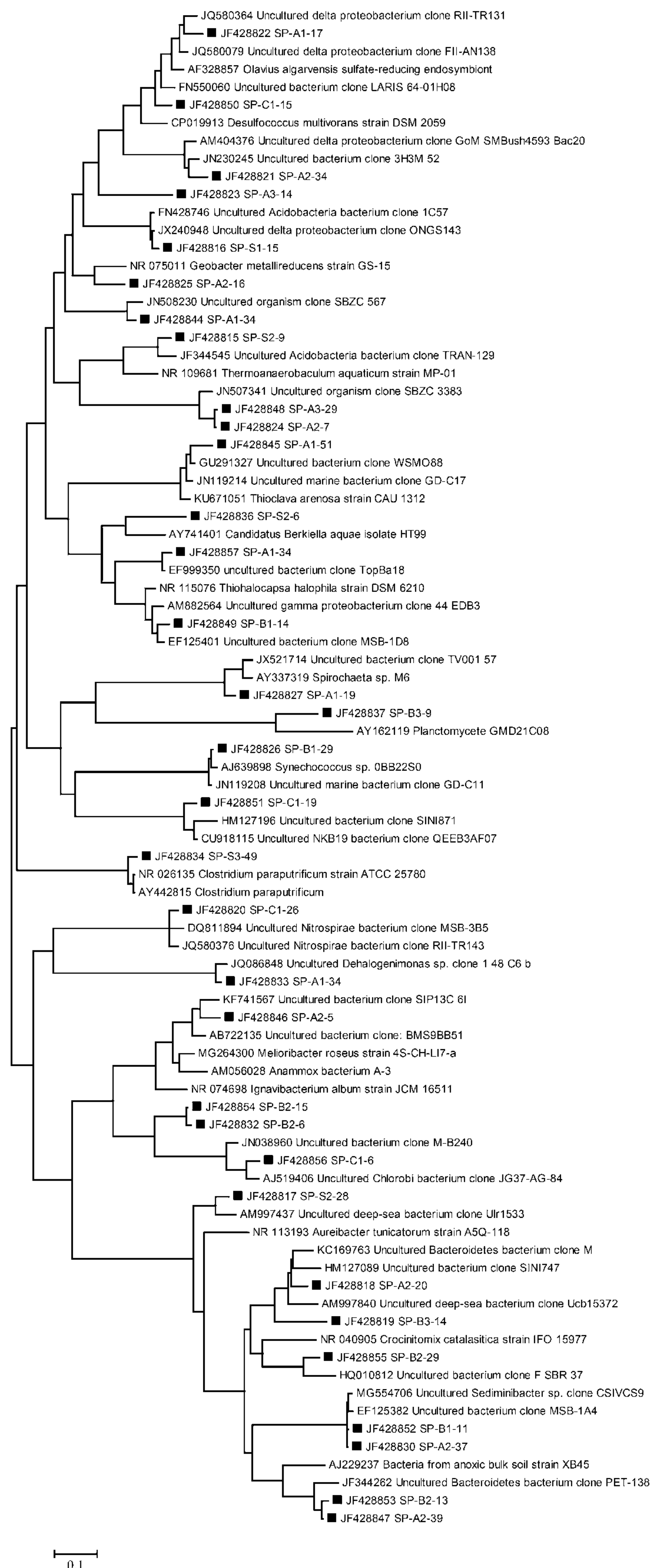

Fig. 1 Phylogenetic tree of bacterial 16S rRNA genes from the sediment metagenome of bioaugmented zero-water exchange shrimp pond constructed using MEGA version 6.06 software with Maximum Likelihood method and Kimura 2-parameter model (1000 Bootstrap). Sequences of bacterial 16S rRNA gene obtained from the present study are represented using the symbol (■) in the phylogenetic tree

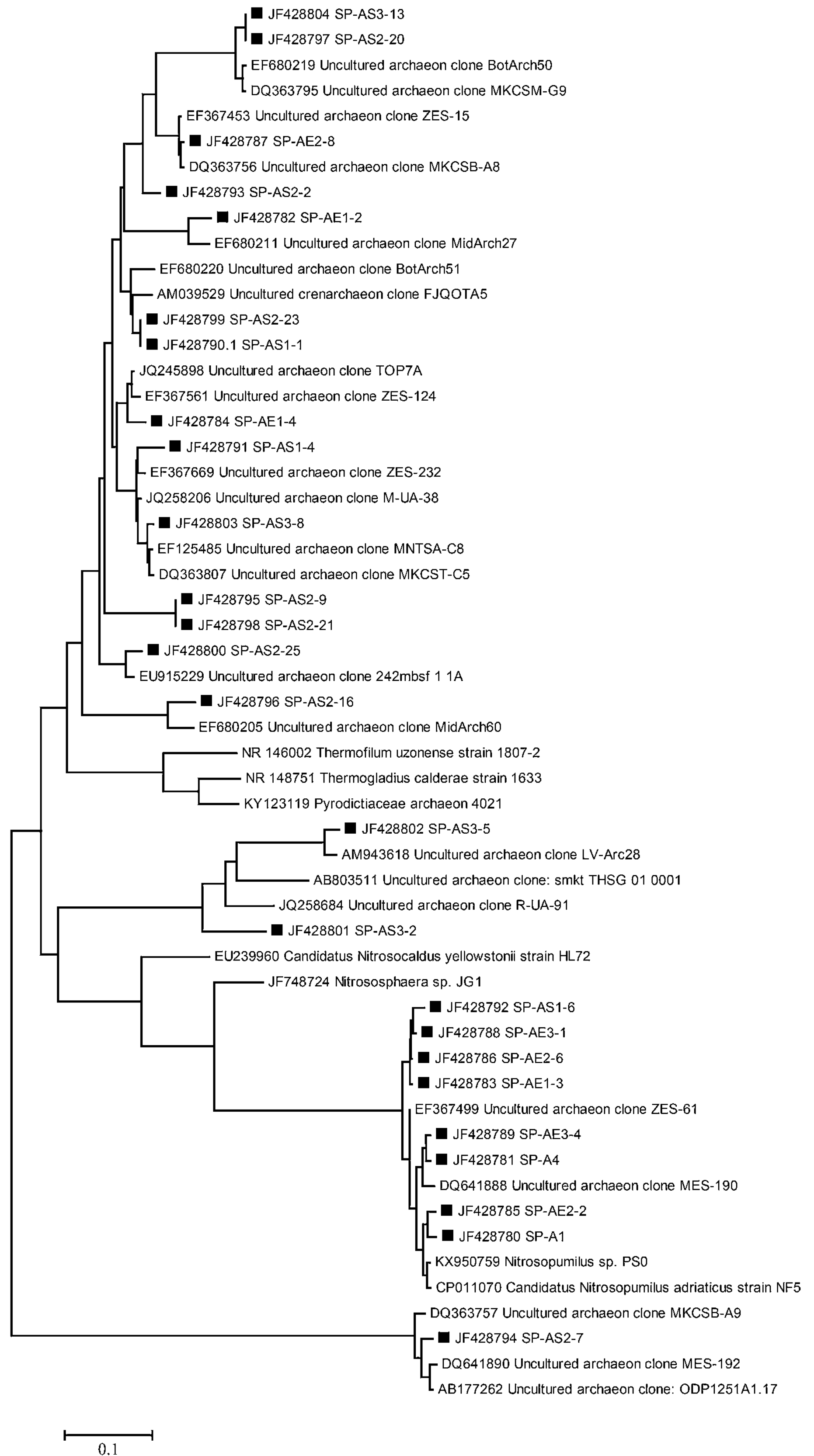

Fig. 2 Phylogenetic tree of archaeal 16S rRNA genes from the sediment metagenome of bioaugmented zero-water exchange shrimp pond was constructed using MEGA version 6.06 software with Maximum Likelihood method and Kimura 2-parameter model (1000 Bootstrap). Sequences of archaeal 16S rRNA gene obtained from the present study are represented using the symbol (■) in the phylogenetic tree

Fig. 3 Geneious map showing the distribution pattern of bacterial groups within the sediment metagenome of bioaugmented zero-water exchange shrimp pond

to obtain energy. Further, sequences associated with *Thiohalocapsa* sp. (family Chromatiaceae) and *Thiogranum* sp. (family Ectothiorhodospiraceae) of order Chromatiales belonging to Gamma-Proteobacteria were observed in the clone library analyses, denoting the prominent role of Gamma-Proteobacteria in sediments. The families of Chromatiaceae and Ectothiorhodospiraceae are found in marine ecosystems and consuming sulphide originating from active sulphate reduction as an electron donor [12]. *Thiohalocapsa* sp. is phototrophic, purple-sulphur bacteria (PSB) found in anoxic zones of a marine aquaculture pond sediment [52], and *Thiogranum* sp. of Ectothiorhodospiraceae are obligately chemolithoautotrophic, sulphur-oxidizing bacteria [67]. In addition, Rhodobacteraceae family of Alpha-Proteobacteria was observed in the clone library. This Rhodobacteraceae family comprises of

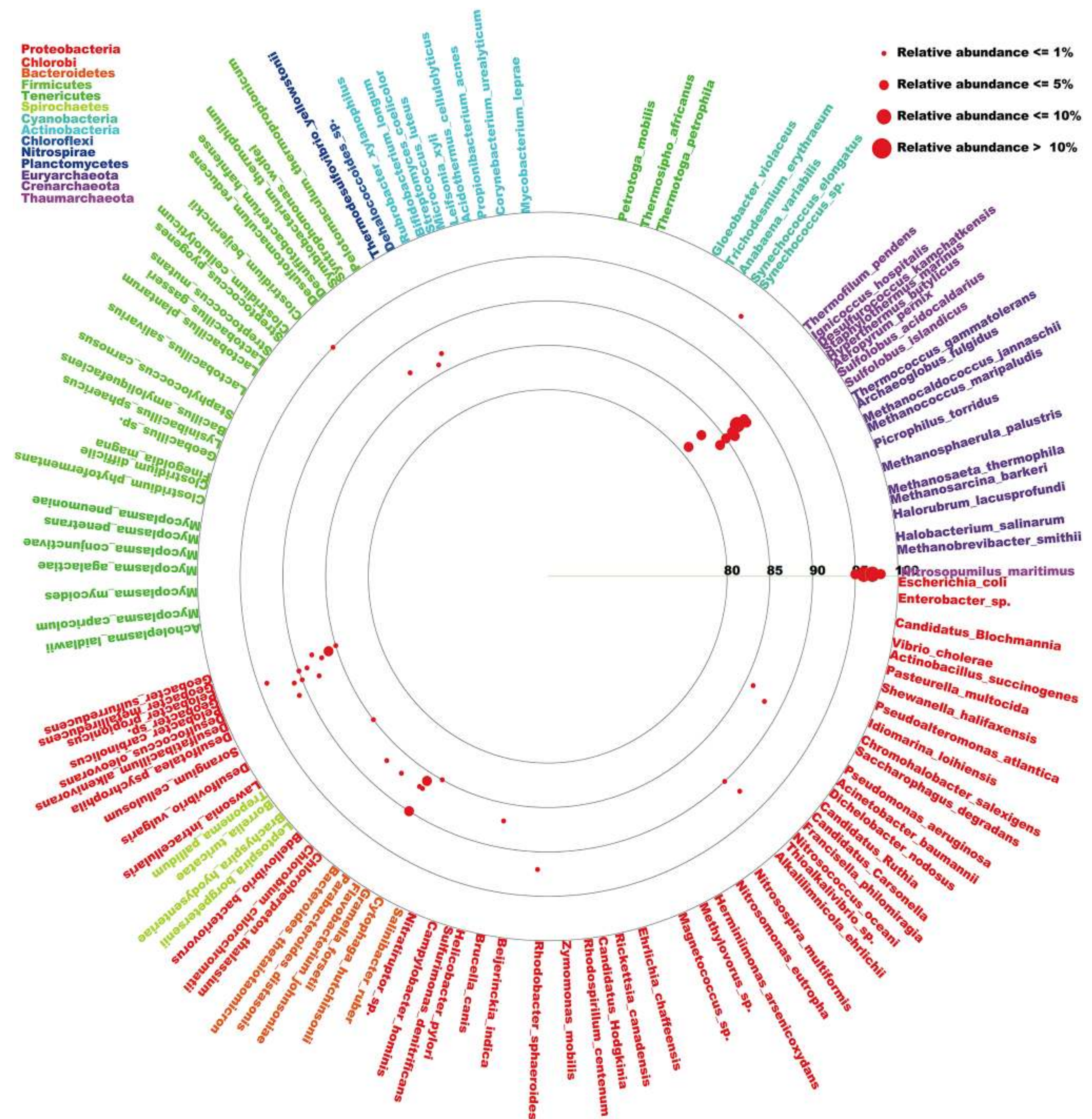

Fig. 4 VITCOMIC merged map results of bacterial and archaeal groups within the sediment metagenome of bioaugmented zero-water exchange shrimp pond

photoheterotrophs and chemoorganotrophs capable of nitrogen fixation and metabolize various sulphur-containing compounds [80].

The clone library also comprised sequences related to Bacteroidetes. Most of the sequences within Bacteroidetes were closely affiliated with Crymorphaceaea. Members of this group play a significant role in secondary production in aquatic ecosystems [9], playing a potential role in organic matter degradation [118]. The sequences related to *Sediminibacter* sp. of Flavobacteriaceae were detected. They are chemoheterotrophic bacterium [48], and some members of the Flavobacteria have potential to degrade complex carbon [18]. Few sequences were affiliated to *Mangrovibacterium* sp. (Prolixibacteraceae family), a nitrogen-fixing bacterium [37], and some sequences to a marine bacterium *Aureibacter* sp. (Flammeovirgaceae

family) [116]. Sequences affiliated to *Melioribacter* sp. (class *Ignavibacteria*) were observed. *Melioribacter* sp. has glycoside hydrolases and transferases genes for polysaccharide degradation and synthesis, and they also possess genes encoding superoxide dismutase and catalase to detoxify the oxygen respiration by-products [45, 77]. The sequence similarity to Firmicutes delineated the association with *Thermanaeromonas* sp. and *Clostridium sensustricto*. *Thermanaeromonas* sp. are anaerobic, thermophilic bacteria, which use thiosulphate as an electron acceptor and can reduce nitrate and nitrite for growth [46, 66]. *Clostridium sensustricto*, abundant Firmicutes in RAS (recirculating aquaculture systems), are reported to have the potential in removing the accumulated nitrate [31, 83]. Very few sequences of GpIIa genus belonging to Cyanobacteria family II [20] were also found. The 16S rRNA gene sequences associated with facultative anaerobe *Phycisphaera* sp. (third class of Planctomycetes) were present in the clone library; they perform nitrate-to-nitrite reduction under anaerobic conditions [27], also associated with fermentation at the sulphate–methane transition zone in marine sediments [33]. Species-specific sequence similarity to *Spirochaeta* genus was observed, which were ubiquitous inhabitants of many aquatic environments [74, 88], can colonize and form flocculent matter, and can metabolize carbohydrates [109]. Dehalococcoidaceae (Chloroflexi family) contains dehalogenation complex [122] and is significant indicator of the re-oxidation stage [56]. *Acidobacteria* Gp23 (Acidobacteria) are capable of nitrate and nitrite reduction. The presence of metabolically diverse bacterial groups in the sediment metagenome suggested their efficiency in cycling the carbon, nitrogen, and sulphur loads and thereby bringing out efficient bioremediation in the bioaugmented ZWE shrimp pond.

The Geneious R8 biodiversity map of archaeal 16S rRNA gene (Fig. 5) revealed the distribution patterns of archaea by demonstrating the presence of two major taxonomic classes, Thermoprotei of Phylum Crenarchaeota and Nitrosopumilales of phylum Thaumarchaeota. Thermoproteals, Desulfurococales, and Acidobales orders belonging to the Thermoprotei class (Phylum Crenarchaeota) were present. Phylogenetically, Thermoproteales included 44% *Thermofilum* genus, and Acidobales included 8% *Caldisphaera* genus. Desulfurococcales included 4% of *Hyperthermus* genus belonging to Caldisphaeraceae family and 12% of *Stetteria* genus belonging to Desulfurococcaceae family.

Phylum Crenarchaeota was found in the sediments of ZWE pond, with the archaebacterium *Thermofilum*, a genus of the thermophilic, anaerobic sulphur respiring Thermoproteales, relatively abundant in the archaeal clone library. The growth of these archaebacterium is dependent on peptides, sulphur, and H_2S [120]. Similarly, the genus *Caldisphaera* (family Acidobales), a thermoacidophilic crenarchaeote, was present which was reported to grow anaerobically and heterotrophically and can be stimulated by the presence of sulphur [39]. Desulfurococcaceae are a family of anaerobic microorganisms belonging to the order Desulfurococcales. *Stetteria* is a genus (Desulfurococcaceae family), mixotrophic sulphur-dependent Crenarchaeotae, which need elemental sulphur as an external electron acceptor, and dependent on the presence of H_2 for its growth. *Hyperthermusbutylicus* sp. is a hyper-thermophilic, sulphur-reducing archaebacterium [121]. The detection of these species in the archaeal clone library denoted the importance of archaea in sulphur cycling of the ZWE shrimp pond. *Nitrosopumilus* genus belonging to Thaumarchaeota accounted for 32%, which are chemolithoautotrophic ammonia oxidizers. There are reports showing *Nitrosopumilus* sp. as the prominent AOA in the biofilter of shrimp RAS and aquaculture ponds [11, 72]. The taxonomic composition of archaeal communities using VITCOMIC (Fig. 4) confirms that *N. maritimus* belonging to Thaumarchaeota was the most abundant archaeal group with a relative abundance $\leq 10\%$, and similarity $> 95\%$. *N. maritimus* was reported as the major archaeal ammonia-oxidizer in marine sediment samples and zero-water exchange shrimp culture systems [19, 72].

Microorganisms are of great importance in pond ecosystem as it increases the self-purification capacity, in turn, improving the water quality and enhancing the growth and immunity of the cultured animals. Bacterial and archaeal clone library dictates the relative abundance of phyla related to sulphur geochemical cycling. Sulphur cycling is important in these ponds as un-ionized dissolved hydrogen sulphide is toxic, and sulphate reduction can account for over 50% of organic matter degradation [69]. The phyla Bacteroidetes was high in clones underscoring the enhanced capacity for organic matter degradation. Bacterial clone library also indicated the presence of a diverse bacterial group capable of nitrogen fixation, nitrate and nitrite reduction, and the archaeal clone library indicated the presence of ammonia oxidizers. Bioremediation of nitrogenous compounds is important as it removes the potentially toxic compounds ammonia and nitrite. Thus, the results of ARDRA and phylogenetic analysis (Mega 6.06, VITCOMIC, and Geneious map) depict the taxonomic composition of microbial community in the bioaugmentation ZWE shrimp pond sediment when there were the maximum biomass, inputs of feed, and metabolic wastes.

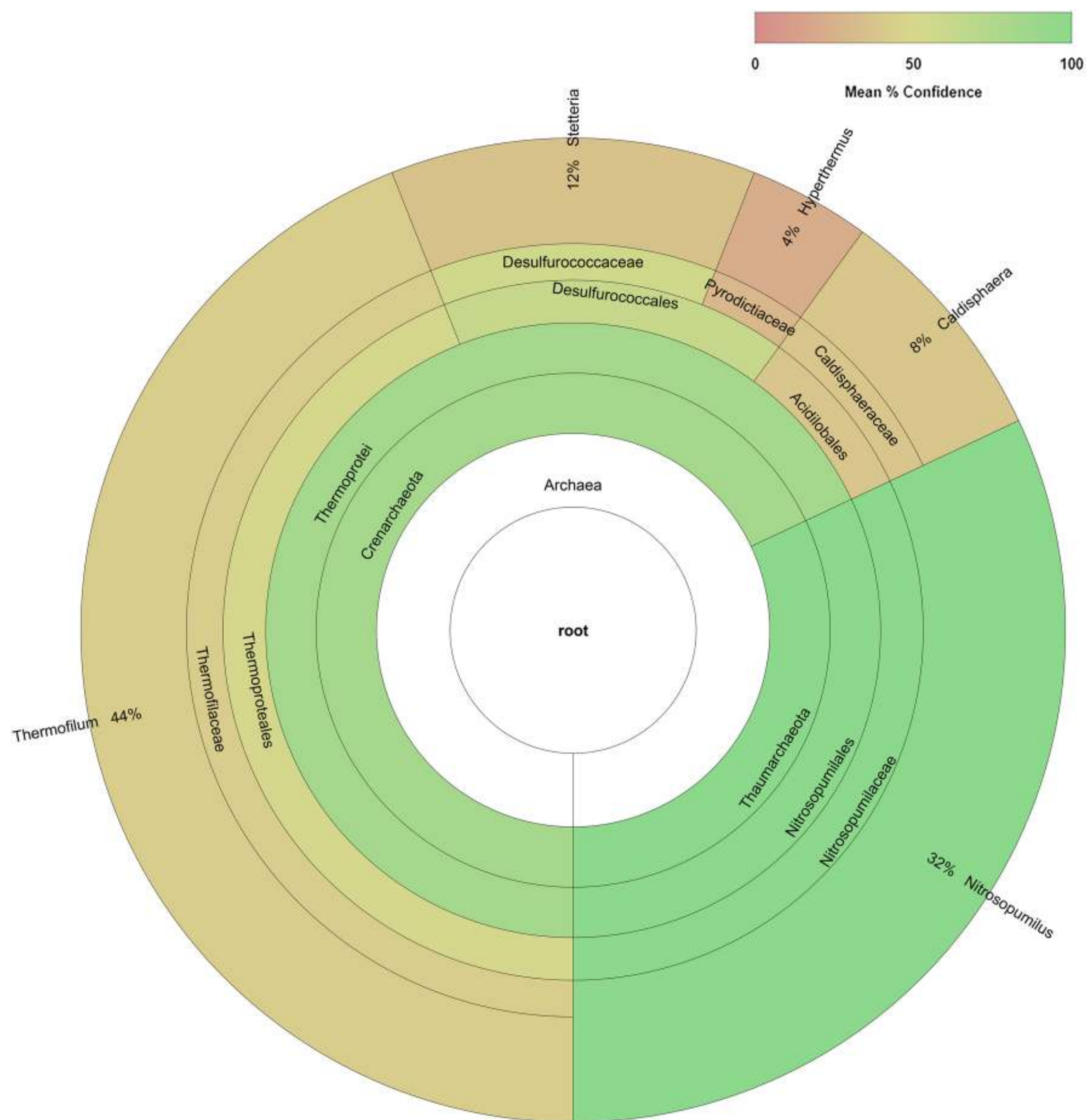

Fig. 5 Geneious map showing the distribution pattern of archaeal groups within the sediment metagenome of bioaugmented zero-water exchange shrimp pond

11 Functional gene analyses of ammonia oxidizers in ZWE pond

In this ZWE pond and earlier reports [34, 44], the ammonia concentration was observed to be at a low level. Hence, the analysis of three major ammonia oxidizers (AOB, AOA, and anammox) was conducted using bacterial- and archaeal-specific functional gene (*amo*A) and anammox-specific 16S rRNA gene primer.

Nitrosomonas and *Nitrosospira* are the two major genera of AOB [81]. Ammonia-rich ecosystems generally demonstrate the habitual predominance of *Nitrosomonas* sp. [6, 51]. The bacterial *amo*A gene sequences showed 99% identity to uncultured AOB. Phylogenetic analysis (Fig. 6) showed similarity to the bacterial *amo*A gene of *Nitrosomonas nitrosa* [29]. Sequences also showed similarity to other uncultured bacterial *amo*A clones retrieved from bioreactors [28]. Based on the metagenomic sequence analysis, in the ZWE ponds, *Nitrosomonas* sp. represents the major AOB.

BLAST analyses of archaeal *amo*A gene sequences were similar to the ammonia monooxygenase gene of uncultured archaeon clone with an identity in the range of 96.7–99%. The phylogenetic analyses (Fig. 7) of the archaeal *amo*A clones showed identity to *amo*A gene of uncultured crenarchaeote obtained from tropical

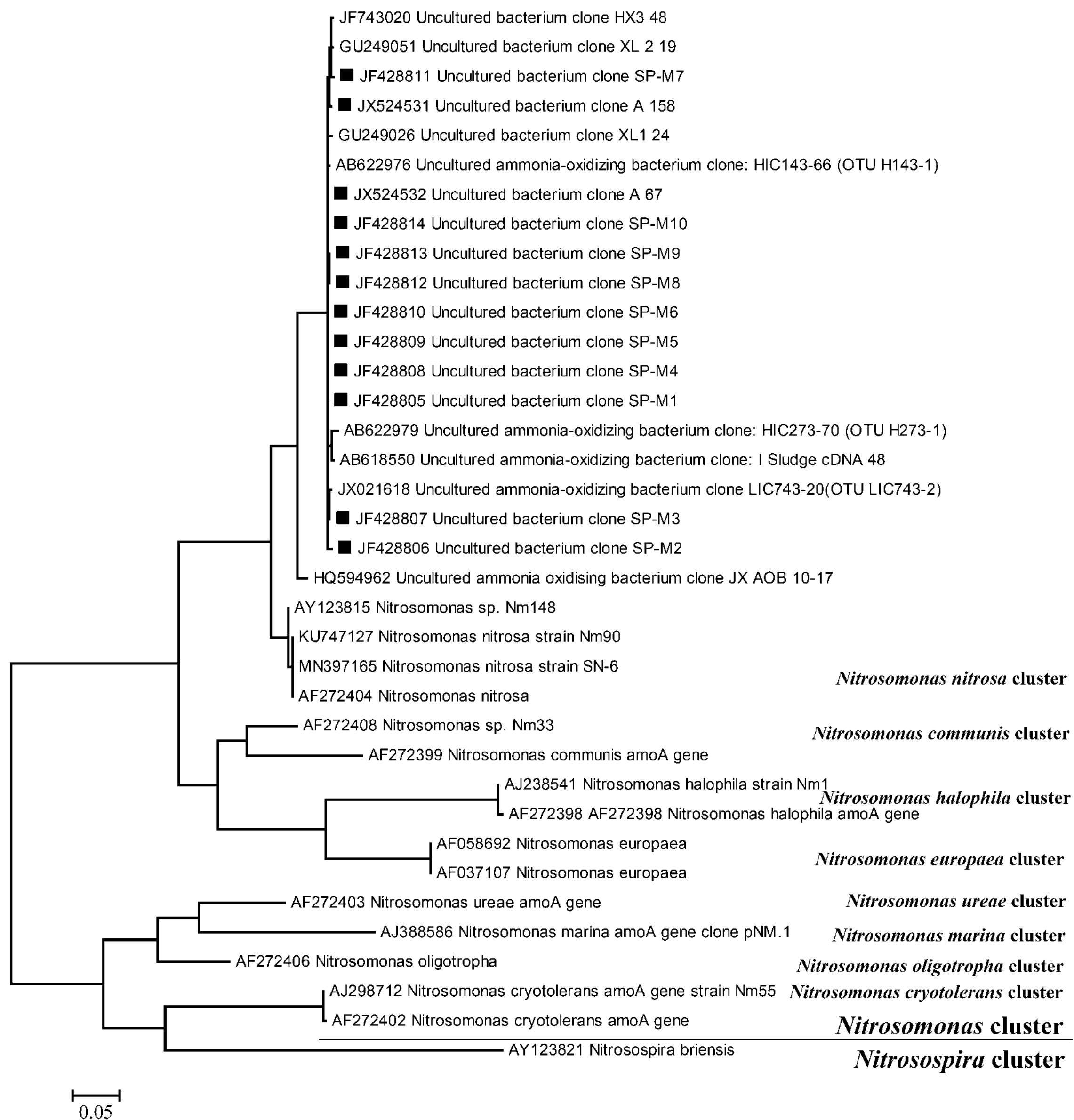

Fig. 6 Phylogenetic tree of uncultured AOB clones obtained from the sediment metagenome of bioaugmented zero-water exchange shrimp pond constructed using MEGA version 6.06 software with Maximum Likelihood method and Kimura 2-parameter model (1000 Bootstrap). Sequences of bacterial *amo*A obtained in the present study are represented using the symbol (■) in the phylogenetic tree

estuarine sediment [94], intertidal [7, 70], and mangrove [59] ecosystems. The archaeal *amo*A clones formed two distinct clusters, and the clone JX524541 was seen widely separated in the phylogenetic tree. Phylogenetic analyses with pure cultures excluding the uncultured environmental sequences showed 90% similarity with 99% coverage to *Nitrosopumilus maritimus amo*A gene of Thaumarchaeota phylum. These sequences were also similar to the *Nitrosopumilus* sequences retrieved from a biofilter of a shrimp RAS [11], except for clone JX524541. The clone JX524541 formed a distinct cluster that showed 81% similarity with 99% coverage to Candidatus*Nitrososphaera gargensis* Ga9.2, belonging to phylum Thaumarchaeota of *Nitrososphaera* genus [97]. Based on the metagenomic sequence analysis, *Nitrosopumilus* sp. was the major AOA present in the ZWE ponds.

The Planctomycetae-specific 16S rRNA gene sequences were identical to uncultured Planctomycetes, Candidatus *Kuenenia stuttgartiensis*, and uncultured anammox bacteria, with a sequence similarity ranging from 98 to 99%. Phylogenetic analysis (Fig. 8) showed similarity to the Candidatus *Kuenenia* from the sediments of mangrove,

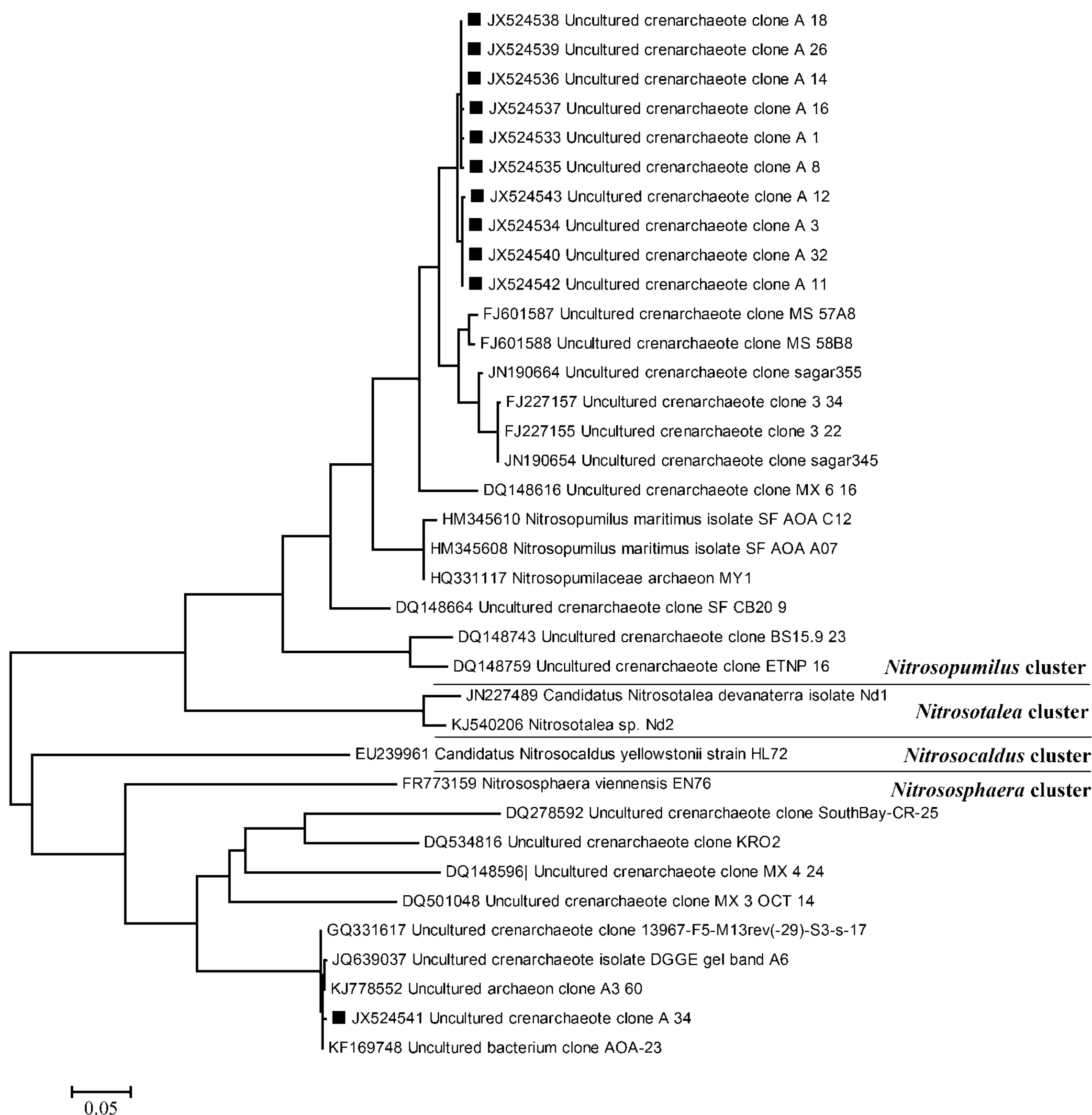

Fig. 7 Phylogenetic tree of uncultured AOA clones obtained from the sediment metagenome of bioaugmented zero-water exchange shrimp pond constructed using MEGA version 6.06 software with Maximum Likelihood method and Kimura 2-parameter model (1000 Bootstrap). Sequences of archaeal *amo*A obtained in the present study are represented using the symbol (■) in the phylogenetic tree

estuary, and high-temperature reservoirs [16, 57, 112]. The presence of anammox in the pond bottom is very significant, as they play an active role in the conversion of ammonia to maintain it at low levels. There are reports on the survival of anammox in natural environments such as coastal, marine, natural freshwater wetland, aerated marine RAS, and anoxic zones of nitrifying biofilters [40, 92, 100, 106].

Nitrosomonas sp. (AOB), *Nitrosopumilus sp.* (AOA), and Candidatus *Kuenenia* (anammox) were predominant in the bioaugmented pond. All the three groups of ammonia oxidizers co-existed in the system, with distinctly different mechanism of ammonia oxidation, substrate complementation, and metabolic interaction [71, 72]. In oxic environments, ammonia is quickly oxidized by AOB and AOA [89]. Anammox bacteria have the ability to oxidize ammonia and reduce nitrite into N_2 gas under anaerobic conditions, and their presence is widely seen in wastewater treatment plants, coastal marine sediments, estuaries, terrestrial habitats, oceanic and freshwater oxygen minimum zones [17, 54]. The anaerobic ammonia-oxidation process by anammox is important to the aquaculture, as it removes both ammonia and nitrite, which is toxic to aquatic animals.

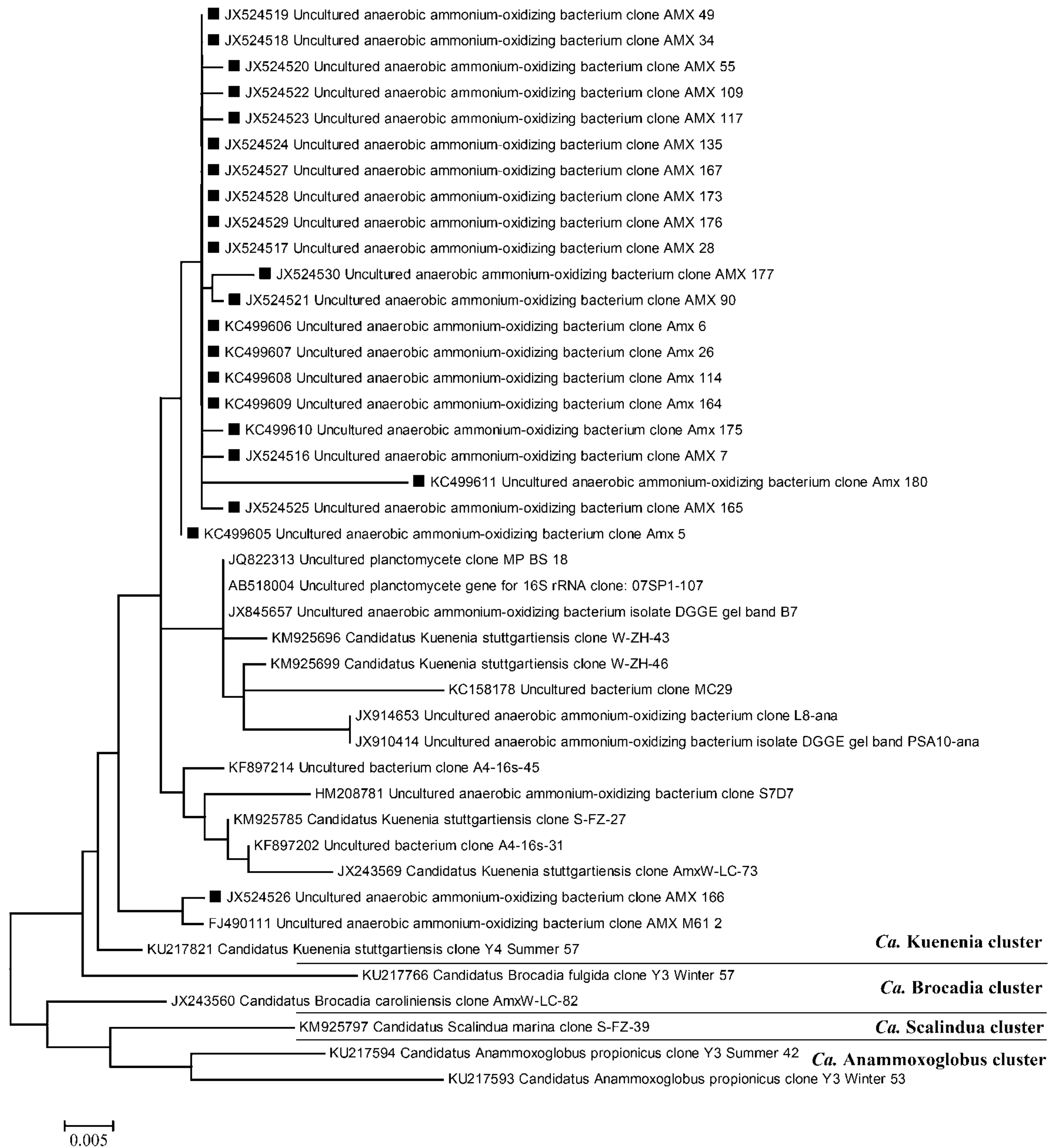

Fig. 8 Phylogenetic tree of uncultured anammox clones obtained from the sediment metagenome of bioaugmented zero-water exchange shrimp pond constructed using MEGA version 6.06 software with Maximum Likelihood method and Kimura 2-parameter model (1000 Bootstrap). Sequences of anammox 16S rRNA gene obtained in the present study are represented using the symbol (■) in the phylogenetic tree

Usually, anammox bacteria are inhibited by the presence of oxygen, but can be detected in the aerated systems due to anoxic zones created by oxygen consumption and limited penetration of oxygen. The presence of AOB and AOA near anammox bacteria has a second great benefit since these organisms supply the nitrite by the oxidation of ammonia. Nitrite produced is required by anammox bacteria to oxidize ammonia [99]. The coexistence of AOB, AOA, and anammox would be ideal in aquaculture

systems, to remove both ammonia and nitrite which are toxic to aquatic animals [53, 54, 71, 72].

12 Conclusions

Bacterial and archaeal communities involved in the sulphur, carbon, and nitrogen cycles were observed in ZWE shrimp culture pond sediment metagenome, which are critical in the maintenance of environmental quality and sustainability of the culture system. The phylogenetic analyses targeting ammonia oxidizers showed the presence of all the three communities (AOB, AOA, and anammox), indicating the key role of microbial communities in maintaining permissible or low levels of ammonia in the ZWE shrimp culture pond. The present study revealed the capability of the pond sediment to act as a bioreactor augmenting the removal of ammonia and nitrite from the culture systems, thus maintaining the optimal conditions required for aquaculture. The addition of the bioaugmentor, which is not a nitrifier, helped to degrade the organic matter and improve the environmental conditions to stimulate growth and activity of naturally occurring microbial communities. Altering the microbial ecology plays significant roles in nutrient cycling, thereby improving the survival and productivity of the cultured animals. Further knowledge on relevant microbial interactions and the overall ecology of these systems is essential for the successful management of the aquaculture systems.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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