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Inorganic Nitrogen Conversion and Changes of Bacterial Community in Sediment from Shrimp Pond after Methanol Addition

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Abstract: Effect of methanol on inorganic nitrogen conversion enhancement and bacterial diversity in sediment from shrimp pond was investigated. Sediment from shrimp pond in Pathum Thani, Thailand, was packed in 40 cm height cylinder shape chamber, filled with 1.4 L of 20 g L^{-1} seawater and incubated under dark and aerated conditions for 48 days. The experimental units consisted of (1) control chambers without methanol addition, (2) treatment-1 chambers supplemented with methanol at C:N ratio of 2:1 and (3) treatment-2 chambers with methanol at C:N ratio of 4:1. It was found that, methanol addition could stimulate ammonification process so ammonia was released from sediment at higher rate than control. Thereafter, concentration of nitrogenous wastes i.e., ammonia, nitrite and nitrate were decreased due to sequential nitrification-denitrification processes. However, methanol addition seemed to inhibit nitrification process since treatment chambers took longer time to reduce ammonia and nitrite. Nitrogen conversion pattern were similar in both treatments with C:N ratio of 2:1 and 4:1. Bacterial community in sediment, as analyzed by PCR-DGGE of 16S rRNA gene fragments, showed that DNA profile at the beginning and the final day of the experiment was clearly changed, while bacterial profiles of treatment 1 and 2 at day 48 was almost similar. On the other hand, addition of methanol at the high C:N ratio reduced the bacterial diversity in which the Shanon-Weaver index was found lowest. DNA sequencing results indicated that denitrifying bacteria such as *Vibrio* sp., *Psuedomonas* sp., *Planococcus* sp., *Streptomyces* sp. and *Thioalcalovibrio* sp. were stimulated after methanol addition.

Key words: Nitrogenous waste, ammonification, nitrification, denitrification, aquaculture, methanol, bacterial community, PCR-DGGE

INTRODUCTION

In shrimp culture pond, bottom soil is generally a sink of large amounts of organic matters derived from uneaten feed, fertilizers and excretory products of aquatic organisms (Kassila, 2003). It is known that the major input of nitrogen waste comes from protein in feed pellets in which approximately 15-30% of nitrogen in feed is converted into shrimp biomass, while the rest is accumulated in high organic content sediment at the pond bottom (Hargreaves, 1998). Release of toxic inorganic nitrogen compounds especially ammonia is from the decomposition process of organic matters and conversion of ammonia to other nitrogenous compounds of nitrogen cycle is regulated by microorganisms in the sediment.

In general, aerobic decomposition of organic waste especially at the pond bottom results in ammonia-nitrogen release. The major sources of organic matter for ammonification are uneaten feed, animal excretion and microorganism debris. In nature, toxic inorganic nitrogen compounds i.e., ammonia and nitrite are converted to low toxic nitrate-nitrogen by nitrification process. High concentration of nitrate in aquaculture pond can result in eutrophication, however, this is rarely occurred since nitrate is eliminated from the pond by denitrification process in sediment (Boyd, 1995; Burford and Lorenzen, 2004).

To enhance denitrification process, various organic carbon compounds such as cellulose, molasses, hydrolyzed corn straw, acetic acid, propanol, butanol,

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glycol, methane, glucose, ethanol and methanol have been used as the substrates for denitrifying bacteria (Sobieszuk and Szewczyk, 2006; Shrimali and Singh, 2001; Ferguson, 1994; Mateju *et al*., 1992). Methanol is reported as one the most effective and the most economical carbon source for denitrifying bacteria especially in aquaculture systems (Hamazah and Ghararah, 1996; Menasveta *et al*., 2001). Based on the stoichiometric relationship of heterotrophic denitrification, the methanol to nitrate ratio have been reported in term of C:N ratio. The C:N ratio for complete denitrification process with methanol as a carbon source was between 3 to 10 (Mateju *et al*., 1992; Ebeling *et al*., 2006).

Present preliminary study (Kutako *et al*., 2005) showed that organic nitrogen waste in shrimp pond sediment was removed by a series of ammonification, nitrification and denitrification processes in which the accumulation of ammonia or nitrite to toxic levels could be found if there was high loading of organic nitrogen. With our attempt to develop the Recirculating Aquaculture System (RAS) in lining pond (pond without bottom soil) incorporated with nitrification biofilters, accumulation of nitrate is commonly found after long usage of the RAS. There are several techniques available to remove high nitrate in aquaculture pond including plant uptake and several types of denitrification reactors. However, these options are not suitable due to problems with cost and operation complexity, thus, nitrate removal can be practically achieved only by water discharge. Recently, enhance denitrification process in soil by organic carbon addition i.e., acetate and ethanol was reported by Martin *et al*. (2009). Another preliminary study by present group (Malapol *et al*., 2008) found that denitrification rate of the sediment increased from 380 mg-N/m^2/day in control without methanol to 5400 mg-N/m^2/day in sediment tank with methanol addition. Therefore, high nitrate water from RAS could be removed by tank or pond containing sediment layer. This leaded to the development of nitrate removal technique using enhanced denitrification in sediment tank system. However, carbon addition might affect the balance between nitrogen removal processes in which the inhibitory effect of organic carbon on nitrification process has been reported (Strauss and Lamberti, 2000). The objective of this study was to obtain more information on effect of methanol additions on nitrogen transformation through nitrification process and changes of bacterial diversity in sediment from shrimp pond. A better understanding of these topics will be useful in improving techniques to remove nitrate from closed system aquaculture pond using enhanced denitrification pond systems.

MATERIALS AND METHODS

Sediment sampling and sediment chambers set up: Shrimp pond in Pathum Thani Province, Thailand, was chosen for sediment sampling. This shrimp pond was closed-system farm culturing white shrimp (*Penaeus vannamei*). Shrimp pond sediment with approximately 5 cm in depth was collected by grab sampler during May-June 2007. Concentration of inorganic nitrogen compounds (ammonia, nitrite and nitrate) in the water column, pore water and extracted soil are shown in Table 1. Soil texture of sediment sample was 58% clay, 36% slit and 6% sand. Sediment sample was immediately transferred to the laboratory (Center of Excellence for Marine Biotechnology, Chulalongkorn University) in dark and cold condition, where it was immediately packed into sediment chambers. The sediment chambers were made of acrylic plastic tube with 11 cm in diameter and 40 cm height (Fig. 1). Sediment was packed to the thickness of 10 cm in the chamber then the

Table 1: Concentration of ammonia, nitrite and nitrate in the water column, pore water and extracted soil from shrimp pond

Inorganic nitrogen compounds	Water column (mg N L^{-1})	Sediment (mg N g^{-1} wet sediment)	
		Pore water	Extracted sediment
Ammonia	0.26±0.04	1.13±0.12	10.61±1.29
Nitrite	0.02±0.00	0.09±0.01	0.07±0.00
Nitrate	1.08±0.02	3.52±0.25	1.73±0.11

Mean±SD, n = 3

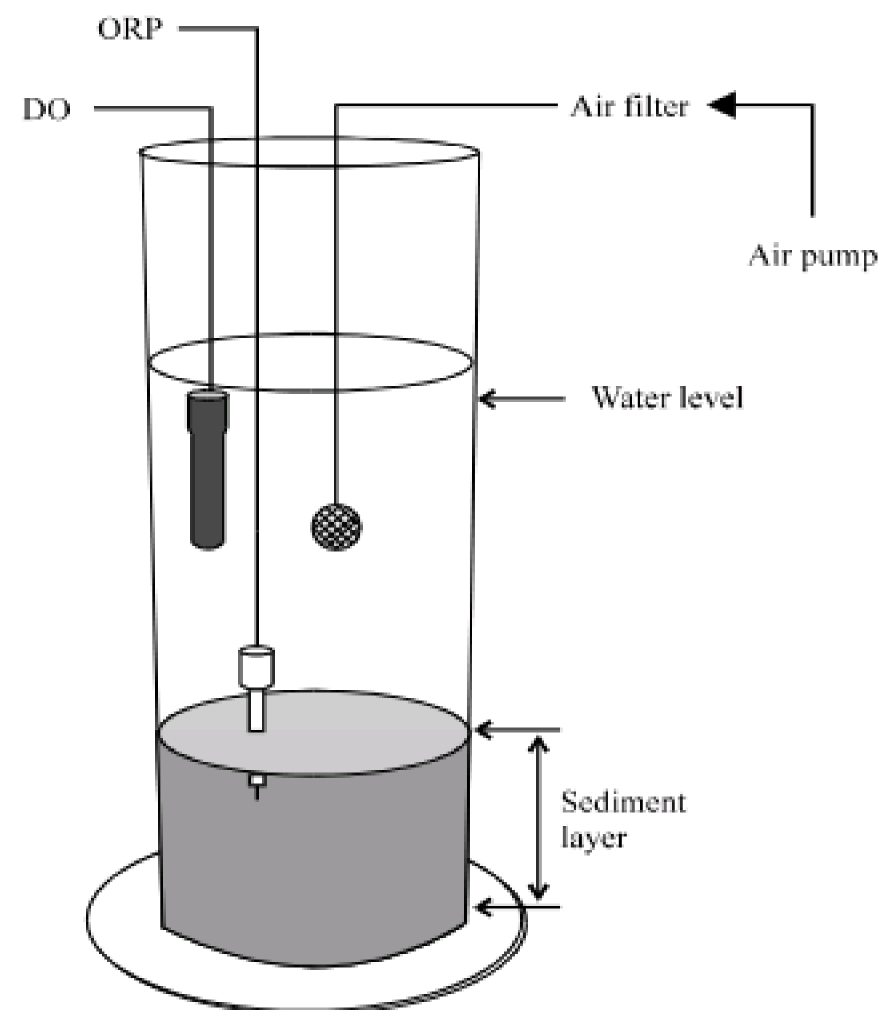

Fig. 1: Schematic diagram of the sediment chamber used in this study

chambers were filled with 1.4 L of 20 g L^{-1} seawater and incubated under dark condition with continuous aeration through air stone.

Experimental procedures: To test the effects of organic carbon addition on inorganic nitrogen conversion and microbial community, the experimental units were separated into three groups i.e., (1) control chambers without organic carbon addition, (2) treatment-1 in which chambers were supplemented with methanol at C:N ratio of 2:1 (1.85 mL methanol L^{-1}) and (3) treatment 2 that methanol concentration was increased to C:N ratio of 4:1 (3.7 mL methanol L^{-1}). All treatments were performed with 3 replicates. The experiment was conducted in dark condition to eliminate the activity of photosynthetic microorganisms. During the experimental period, water samples were collected and analyzed in order to monitor the changes of inorganic nitrogen compounds. At the initial day (day 0) and final day (day 48), sediment samples were collected and changes of sediment bacterial communities were evaluated using PCR-DGGE technique.

Water analysis: Water samples in the experimental chambers were collected at 10 cm below the water surface following by filtered through a GF/C filter and kept in refrigerator prior to analysis. Ammonia (mg NH_4 N L^{-1}) and nitrite (mg NO_2 N L^{-1}) were analyzed using Phenate method and Diazotization method, respectively (Strickland and Parson, 1972). Nitrate (mg NO_3 N L^{-1}) was measured by the ultraviolet spectrophotometric method at 220 and 275 nm (APHA, 1992). Other water quality parameters included alkalinity (titration method: APHA (1992)), dissolved oxygen (digital DO-meter model HI91410 (Hanna Instrument, Portugal)), temperature and pH (pH meter model pH 10 (YSI Inc., USA)).

Sediment analysis: During the experiment, Oxidation-Reduction Potential (ORP) at 2 cm below soil surface was continuously monitored using HI98240 ORP meter (Hanna Instrument, Portugal). Organic matter in sediment was determined using combustion method (Kim *et al*., 1997). On weekly basis, sediment in the chamber was collected using 1 cm diameter core made of clear acrylic tube. The core was gently penetrated into the sediment layer and only top 2 cm of sediment layer was collected for further analysis. Sediment samples were placed in a micro-centrifuge tube and spin at 3000 rpm for 20 min to separate pore water. Consequently, 1 g of wet sediment was extracted with 10 mL of 2 M KCl solution at 25°C and shaken for 30 min at 120 rpm (Schinner *et al*., 1996). The soil mixture was then centrifuged and filtered with GF/C filter in order to obtained extracted sediment solution. Finally, both pore water and extracted sediment were analyzed for inorganic nitrogen compounds.

Bacterial diversity analysis

Genomic DNA extraction and PCR-DGGE analysis: Sediment samples were persevered at -20ºC for bacterial diversity analysis. Bacterial genomic DNA was extracted directly from 0.25 g of the sediment using a protocol of the Fast DNA spin kit for soil (Qbiogene, USA). Total DNA was subsequently verified by electrophoresis in 1% agarose gel for 20 min at 100 V. Gel was stained with ethidium bromide and visualized by gel documentation (Dolphin-Doc Plus, USA). The 16S rDNA was amplified using PRBA338f primer attached with GC clamp (40 bp) at 5' end (5' CgC CCg CCg CgC gCg gCg ggC ggg gCg ggg gCA Cgg ggg gAC TCC TAC ggg Agg CAg CAg 3') and PRUN518r primer (ATT ACC gCg gCT gCT gg) (Muyzer *et al*., 1993). This primer set is specific to all bacterial groups. Polymerase chain reaction mixture (30 µL total volume) was composed of 5 µL of genomic DNA solution, 7 µL of dH_2O, 1.5 µL of each primers (10 µM) and 15 µL of Taq PCR Master Mix kit (QIAGEN, Valencia, USA). The PCR protocol was initial denaturation at 92°C for 2 min followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 55°C for 30 sec and extension at 72°C for 1 min. PCR cycling was completed by a final extension at 72°C for 6 min. The quality of PCR product was examined by gel electrophoresis with 2% (w/v) of agarose in 1xTAE buffer.

Nucleotide fragments of the PCR products were separated by DGGE (Dcode system, Bio-Rad, USA). A duplicate analysis of DGGE pattern was performed within the gel. Twenty microliter of the PCR products was run on polyacrylamide gel (8% of a 37.5:1 acrylamide-bisacrylamide mixture solution in 1xTAE buffer). Denaturing gradients of 30 to 60% (100% denaturant contained 7M urea and 40% formamide) was used. The electrophoresis was operated at 130 V for 6 h in 1xTAE buffer at a constant temperature of 60°C. Thereafter, DNA bands were stained with silverstar® staining kit (Bioneer, Korea) following the manufacturer's protocol.

Bacterial diversity analysis of DGGE

Shannon-Weaver diversity index: Number of DNA bands and their relative intensity within each lane in the gel were counted. This data was then used to calculate the diversity indices including species richness (R), evenness (E) and the Shannon-Weaver index (H) (Shannon and Weaver, 1949). Species richness was determined from the bands number in each lane. The Shannon-Weaver index was used as a diversity index and it was calculated as follows:

$$H = -\Sigma(P_i)(\log P_i)$$

where, P_i is the importance probability of the bands in each lane, calculated from n_i/N, where n_i is the peak height of a band and N is the sum of all peak heights in each lane. Species evenness was calculated using the equation (Eichner *et al*., 1999):

$$E = H/\ln R$$

Cluster analysis: Similarity between the DNA banding pattern generated by PCR-DGGE of the sediment samples were analyzed using Nei and Li's coefficients. The UPGMA (unweighted pair-group method with arithmetic averages: Nei and Li (1979)) with BIO-1D software (Vilber Lourmat, France) was used for cluster analysis and dendrogram generation.

DNA sequencing and phylogenetic tree analysis: The interested DNA bands were cut from DGGE gel, immersed in 50 μL sterilized water and kept at 4°C for 16 h. Eluting DNA solution was re-amplified, purified using a QIAquick purification kit protocol (QIAGEN, Valencia, USA) and sequenced (Tech Dragon, Hongkong). DNA sequences were analyzed using Seqmatch program of the Ribosomal Database Project II (RDP II) (http://rdp.cme.msu.edu/). Distance matrix analyses were performed with a Jukes-Cantor model. With RDP II database, 16S rRNA sequences were aligned with the nearest neighbors and a phylogenetic tree was then constructed by neighbor-joining method.

Nucleotide sequence accession numbers: The 16S rRNA gene partial sequences obtained in this study have been submitted to the GenBank under accession numbers, FJ386435 through FJ386437 and FJ665406 through FJ665419.

RESULTS

Inorganic nitrogen compounds conversion: During 48 days experiment, DO in water column of all experimental units was almost constant at 6.5±1.8 mg O_2 L^{-1} due to continuous aeration. Change in inorganic nitrogen compounds in the water column is shown in Fig. 2. It was found that, nitrification process with both ammonia oxidation and nitrite oxidation steps was somewhat inhibited by methanol addition in which ammonia and nitrite took longer time to be eliminated. For more detail, between initial day to day 9th, ammonia in control chambers (without methanol) was gradually released to the highest concentration of 8.74±2.16 mg N L^{-1} (Fig. 2A) and following by subsequently decreased to

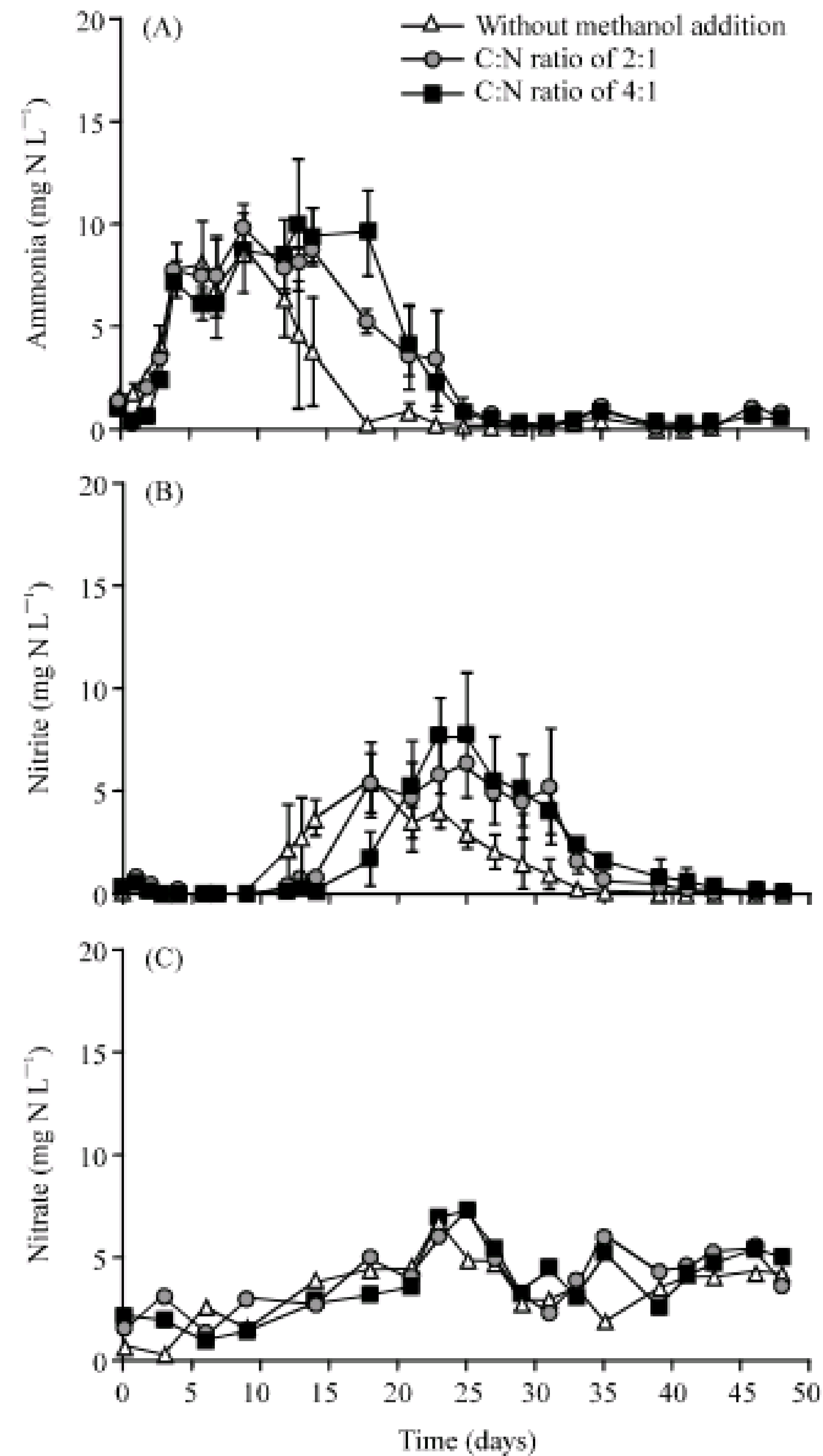

Fig. 2: Changes in (A) ammonia, (B) nitrite and (C) nitrate in water column of the control (without methanol) and treatment (with methanol addition at C:N = 2:1 or 4:1) chambers during 48 days incubation.

0.29±0.05 mg N L^{-1} at day 18th. With treatment 1, after adding methanol to the C:N ratio of 2:1, ammonia was continuously released to the highest concentration of 9.73±2.17 mg N L^{-1} at day 9th and it was completely removed from the water in day 27th. A similar result was found in treatment-2 which supplemented with higher methanol (C:N ratio of 4:1) (Fig. 2B). Increases of nitrite in all experimental units were found after ammonia oxidation (Fig. 2B). At day 46, nitrite was finally removed by the nitrite oxidation process that converts nitrite to nitrate (Fig. 2C).

Results in Fig. 2 also indicated that inhibition level of nitrification process was according to the concentration of methanol. This was due to the progression of ammonia to nitrite was quickest in control chambers. Figure 3A-C show that, ammonia in pore water of all chambers was

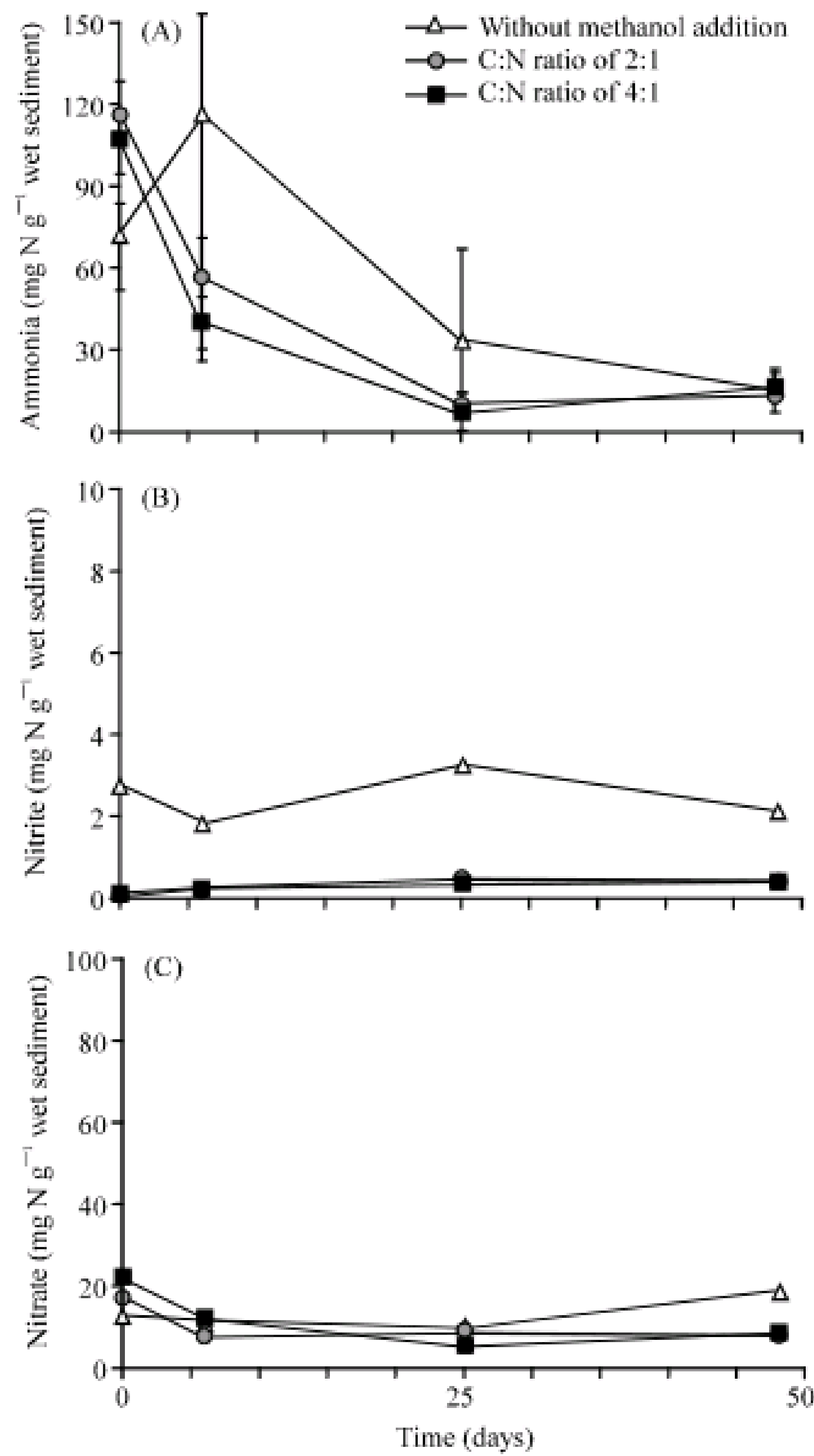

Fig. 3: Concentrations of (A) ammonia, (B) nitrite and (C) nitrate in pore water from sediment chambers during 48 days of incubation

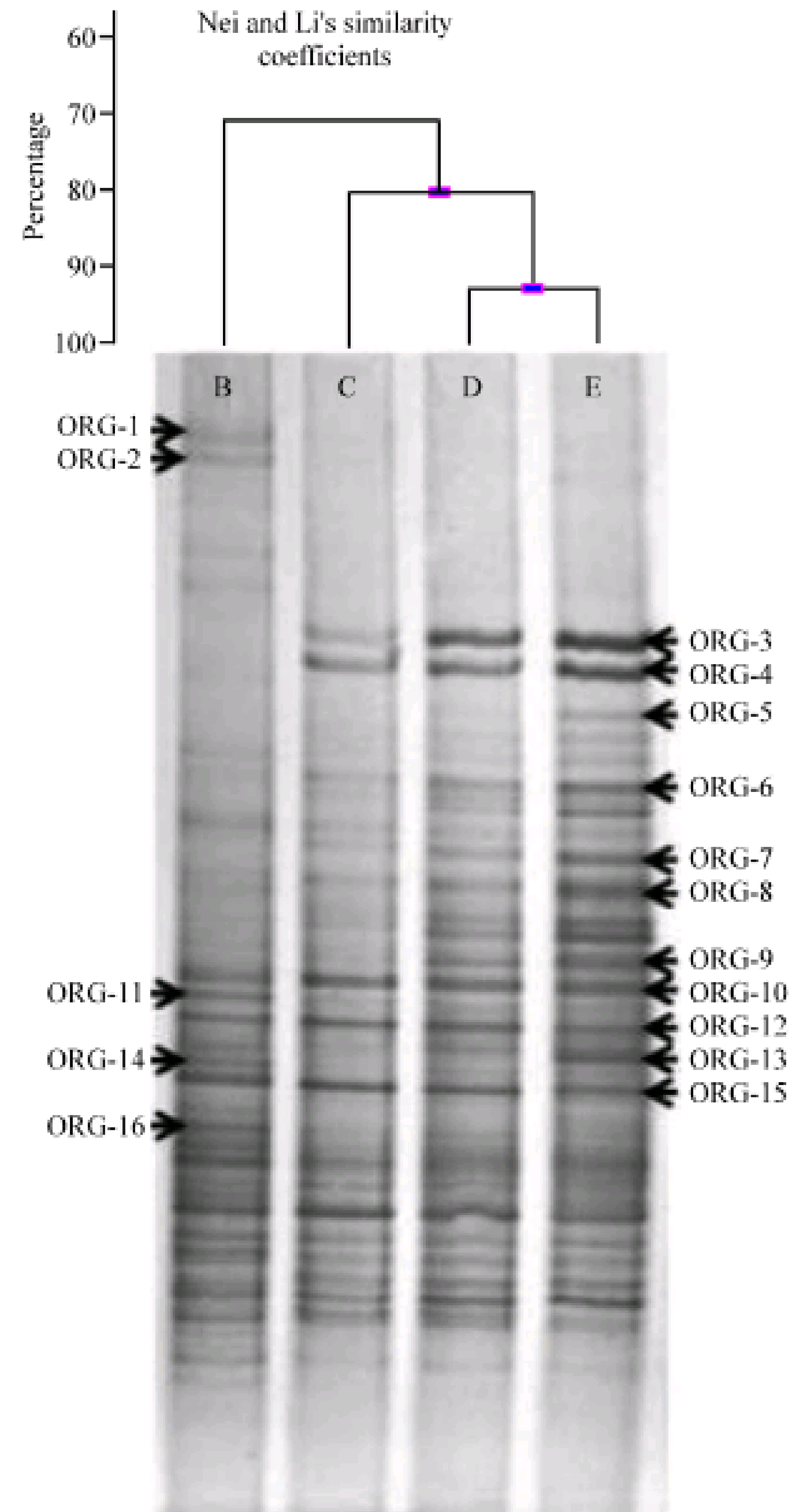

Fig. 4: 16S rDNA-based Denaturing Gradient Gel Electrophoresis (DGGE) profiles with cluster analysis of the sediment bacterial community in sediment chambers. Lane B denotes sediment sample at day 0 and lanes C to E are at day 48. Lane C represents control (without methanol addition), lane D is treatment-1 (C:N ratio of 2:1) and lane E is treatment-2 (C:N ratio of 4:1). Arrows indicate DNA band for further sequencing

high at the initial day then it was decreased to lower than 15 mg N g^{-1} wet sediment. The results indicated that decomposition of organic matter in the sediment rapidly released ammonia to the water column. Over 48 days incubation, concentration of nitrite in pore water of all experimental units were constantly low concentration (Fig. 3B) and nitrate in pore water was also constant (Fig. 3C).

Sediment bacterial community: Denaturing gradient gel electrophoresis profile of 16S rRNA gene showed that bacterial community in the sediment was distinctly changed during 48 days study period (Fig. 4). At least 29 major bacteria species indicated by intense DGGE bands were found at the initial day. After 48 days incubation, at least 4 new bacteria species were found while at least 6 species were disappeared from control chamber. At day 48th, different DNA banding patterns were observed in control chamber and all treatment chambers.

With total number of DNA bands and their relative intensities, bacterial diversity indexes were calculated and the results are in Table 2. It was found that, after 48 days incubation, the Shannon-Weaver index and species richness were higher in control chambers than treatments, while the diversity index was slightly lower. The lowest diversity index was found in high organic carbon addition (treatment 2).

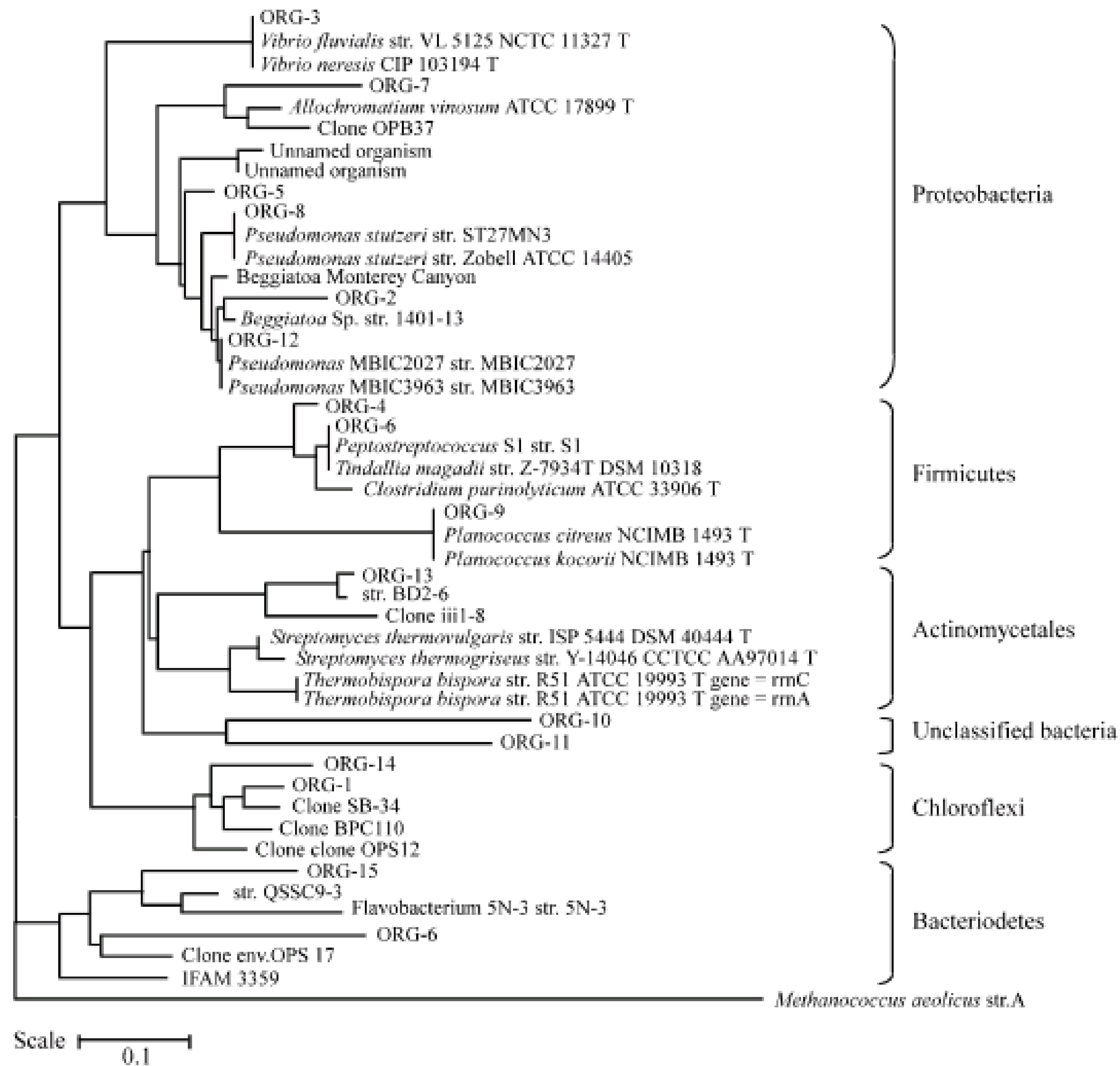

Fig. 5: Phylogenetic tree generated from the alignment of partial 16S rRNA gene sequences from sediment bacteria. DGGE bands analyzed in this study were assigned as ORG-1 to ORG-16. The scale bar corresponds to 10 nucleotide substitution per100 nucleotide position

Table 2: Bacterial diversity in sediment chambers calculated from DGGE patterns

Experimental unit	Species richness (R)	Species evenness (E)	Shannon-Weaver diversity index (H)
Sediment at day 0	15.13	0.97	1.43
Sediment from control at 48 days incubation (without methanol addition)	15.42	0.96	1.40
Sediment from treatment 1 at 48 days incubation (C:N ratio of 2:1)	14.03	0.94	1.38
Sediment from treatment 2 at 48 days incubation (C:N ratio of 4:1)	14.66	0.93	1.38

To identify bacterial species in the DGGE gel, a total of 16 interested DNA bands (Fig. 4) were excised, re-amplified and sequenced. It was found that DNA bands ORG-10, ORG-12 and ORG-15 was appeared in all sediment chambers during the incubation period. With RDP II database, these were closely related to *Thermobispora*, *Pseudomonas* sp. and bacteria clone env. OPS17. In Fig. 4, bands ORG-1, OGR-2, ORG-11, ORG-14 and ORG-15 appeared only day 0. Since ammonia and nitrite was removed in day 48, it was found the succession of new bacterial species, as represented in DNA bands ORG-3, ORG-4, ORG-6 to ORG-9. Intensity of ORG-5 was increased in sediment chamber which methanol supplemented to the C:N ratio of 4:1. This bacterium was identified as *Thioalcalovibrio denitrificans* (Gammaproteobacteria) which is a denitrifying bacterium that coexist with nitrifiers due to the oxygen gradient in sediment chamber and organic carbon source (methanol). Hence, it could be assumed that methanol could promote growth and activity of denitrifying bacteria in the sediment chamber (Fig. 2C).

Phylogenetic distance tree for DNA bands ORG-1 to ORG-16 is shown in Fig. 5. Six lineages were evaluated in sediment reactor including phylum Proteobacteria, Firmicutes, Actinomycetales, Unclassified bacteria, Chloroflexi and Bacteriodetes. DNA bands ORG-3, ORG-5, ORG-7, ORG-8, ORG-2 and ORG-12 were grouped into Proteobacteria while all of them were most closely related to class Gammaproteobacteria which comprised of facultative anaerobic and fermentative gram-negative bacteria.

DISCUSSION

As the aim of this research was to enhance nitrogen removal by sediment from shrimp pond, effect of methanol addition on ammonification, nitrification and denitrification processes was testified. However, since the detection of each process is rather complicate, this study hence evaluated the nitrogen conversion processes by monitoring the changes of inorganic nitrogen compounds in the water. With this assumption, ammonia in water column is continuously released from the decomposition of organic matter in sediment under aerobic condition. This process is so called the ammonification or aerobic decomposition. If the nitrification and denitrification processes occurred without any limitation, low concentration of ammonia, nitrite and nitrate would have been detected in the water because the removal rates are higher than emerging rate. On the other hand, accumulation of ammonia, nitrite or nitrate illustrated as peak concentrations in the water during the experiment was assumed as an inhibition of the conversion process. For example, a peak of nitrite found in water column of the sediment chamber was due to an inhibition of nitrite oxidizing process in which ammonia could be converted to nitrite but nitrite could not be converted to nitrate. Results in Fig. 2 showed that release of ammonia in the sediment chamber was due to the aerobic decomposition process (ammonification) of high organic matter sediment (Avnimelech and Ritvo, 2003; Moriarty, 1997). Visscher and Duerr (1991) reported that decomposition of organic matter usually encounters in the flocculent sediment layer, approximately 2 cm above the sediment surface. In general, nitrification can be found in both water column and at sediment surface (Boyd, 1995; Kim *et al*., 1997). As the activity of nitrifying bacteria were mostly found in biofilm, nitrification process was hence targeted mostly at sediment-water interactive zone with a minor portion in the suspended particles.

As shown in Fig. 2C, nitrate concentration in the chamber was almost constant throughout the experiment. Here, it was probably due to the coupling nitrification-denitrification processes in which nitrate from nitrification was further reduced by denitrification process in sediment layer. Other possibility was the anaerobic ammonia oxidation (anammox) process that ammonia and nitrite are converted to nitrogen gas in anoxic sediment (Rich *et al*., 2008). However, anammox activity in natural condition with much lower ammonia and nitrite concentration than that found in wastewater treatment plant was rarely reported. With this study, denitrification occurred in both control and treatment chambers throughout the study period. This was indicated by ORP values between -265 to -364 mV in the sediment which was the potential levels of nitrate reduction (Li and Irvin, 2007). At an anoxic sediment layer, nitrate was reduced to nitrogen gas through a series of intermediate gaseous nitrogen oxide products (Ebeling *et al*., 2006). Several studies suggested that addition of organic carbon could simulate denitrifying bacteria and other heterotrophic bacterial activities due to the fact that heterotrophic bacteria grew faster than autotrophic bacteria (Moriarty, 1997; Avnimelech and Ritvo, 2003; Cho and Molof, 2004; Ebeling *et al*., 2006). Although, methanol is one of the most economical carbon sources which effective in supporting denitrification process, higher concentration of nitrite in treatment chambers hence suggested that an addition of methanol into the sediment chamber promoted the ammonification process rather than the denitrification process.

With present concept of the sediment tank system for nitrogen treatment in the recirculating aquaculture systems, the results suggested that methanol could be applied to enhance the efficiency of denitrification process for nitrate removal. However, since it was found that methanol addition also inhibited nitrification process, sediment tank should be separated from animal culture tank and methanol residue in the sediment tank must be avoided. Moreover, this technique could also be applied for nitrogen removal in outdoor earthen aquaculture pond. In normal practice, water in the aquaculture pond is drained out after animal harvest. Adding methanol at appropriate concentration to the pond after water drainage possibly enhances ammonification and denitrification processes hence organic nitrogen in the sediment can be eliminated. Nevertheless, further intensive studies with organic carbon addition for nitrogen removal in outdoor aquaculture pond are therefore required.

Results from DGGE analysis illustrated that number of dominant bacterial species in bottom soil was reduced after incubation with high methanol additions since methanol addition could stimulate growth of specific heterotrophic bacteria group (Moriarty, 1997). Hence, bacterial diversity in treatment chambers were differ from that found in control chambers. This was probably due to the effect of methanol on the progression of bacterial community (Lucas and Hollibaugh, 2001).

Cluster analysis of the DGGE patterns with UPGMA method is shown in Fig. 4 and three major clusters were illustrated. The results suggested that bacterial community in sediment chamber strongly differed after 48 days of incubation. In control chamber, similarity within the bacterial community was decreased from 100% at day 0 to 71% at day 48. On the other hand, bacterial community in treatment 1 and 2 was rather similar.

CONCLUSION

Methanol addition at C:N ratio of 2:1 and 4:1 could enhance heterotrophic decomposition (ammonification) and denitrification in sediment chambers under laboratory conditions. However, methanol addition was also inhibiting nitrification process since treatment chambers took longer time to reduce ammonia and nitrite concentrations. Methanol addition also promoted growth of some heterotrophic bacteria. With PCR-DGGE analysis of 16S rDNA, bacterial diversity indicated by Shannon-Weaver index, species richness, species evenness and cluster analysis was clearly changed after adding methanol and incubated for 48 days. Results from DNA sequencing analysis suggested that high C:N ratio stimulated growth of denitrifying bacteria i.e., *Vibrio* sp., *Psuedomonas* sp., *Planococcus* sp. and *Thioalcalovibrio* sp. The implementation of methanol-enhanced denitrification in sediment tank must be used with special attention since high concentration of methanol could affect natural nitrification process in the tank.

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