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Bioaugmentation with nitrifying and denitrifying microbial consortia for mitigation of nitrogenous metabolites in shrimp ponds

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ABSTRACT

Accumulation of nitrogenous metabolites is considered as one of the limiting factors in the intensification of shrimp aquaculture worldwide. The present study reports the development and evaluation of microbial consortium containing ammonia oxidising bacteria (AOB), nitrite oxidising bacteria (NOB) and denitrifying bacteria (DNB) to mitigate total ammonia nitrogen (TAN) in commercial *Penaeus vannamei* shrimp farms. Oxidation rates of NH_3-N and NO_2 due to with AOB and NOB enrichment in the spiked media was 1.57 ppm day^{-1} and 1.46 ppm day^{-1} respectively under experimental conditions. The nitrite reduction rate due to *Marinobacter* spp. in the DNB consortia was 3–4.5 ppm h^{-1}. The AOB and NOB consortia carried out ammonia oxidation and nitrite oxidation at salinities between 2 and 35‰ while DNBs were active above 15‰ salinity. DGGE analysis of the consortium showed the presence of eight AOB and 11 NOB and two DNB groups and metagenome analysis revealed the presence of more than 3000 OTUs at 97% similarity index. Eubacteria dominated the enrichment (>95%) while Proteobacteria were the predominant phylum. *Nitrosomonas* and *Nitrosococcus* (AOB) and *Nitrospira* (NOB) and ammonia oxidising archaea like *Nitrosopumilus* and *Nitrososphaera* were detected in addition to several unclassified species. Significant reduction in the levels of TAN ($p < 0.05$) was observed following application (at 5 L ha^{-1} $week^{-1}$) of the microbial consortia (formulation) in four commercial *P. vannamei* shrimp farming sites having the salinity between 15 and 62‰. Results of the study suggests that the AOB, NOB and DNB formulation developed microbial formulation developed in the study could efficiently mitigate the toxic ammonia species in commercial *P. vannamei* shrimp culture farms.

1. Introduction

Shrimp along with salmon and bivalves constitute a third of the annual international fish and fishery products trade (USD 152 billion), and more than half of the fishery produce is contributed by developing countries (FAO, 2018). In the last two decades, global shrimp culture has recorded an annual increase of close to 3 million tonnes (FAO, 2019). Intensification of aquaculture relies on increased stocking density per unit area of the culture species and use of high protein diets. Shrimp feed is rich in protein (crude protein 30–40%). Generally, the aquatic animals including fish and shrimp accumulate about 20–25% of protein fed into their body and the rest is released to the pond as ammonium and organic nitrogen(Boyd and Tucker, 1998). It is estimated that ~50 g ammonia-N is generated per kg of feed consumed (Ebeling et al., 2006). Further, the accumulation of dead decomposing phytoplankton and faeces in the pond bottom add to the release of toxic metabolites like, ammonia, nitrite and hydrogen sulphide (Boyd, 1995; Valencia-Castañeda et al., 2018). Build-up of these toxic metabolites in the aquaculture ecosystems is a major limiting factor in achieving enhanced productivity (Boyd and Tucker, 1998; Zhou and Boyd, 2015).

The toxicity of nitrogenous wastes to aquatic animals has been reported extensively (Handy and Poxton, 1993; Romano and Zeng, 2013). Higher pH and temperature increases the concentration of highly toxic unionised ammonia and nitrite beyond their reported safe levels of 0.08–2.47 mg L^{-1} and 0.53–3.8 mg L^{-1}, respectively (Chen et al., 1990; Lin and Chen, 2003; Li et al., 2007). By diffusing through the gill

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membranes, ammonia reduces oxygen carrying capacity of haemolymph and disrupts the metabolic function of internal organs, suppresses immune system, affecting growth and survival (Randall and Tsui, 2002; USEPA, 2013). Nitrite at higher concentration forms methaemoglobin/methemocyanin which binds to molecular oxygen leading to hypoxia (Cheng and Chen, 2002; Jensen, 2003; Romano and Zeng, 2013). Thus, detoxification of nitrogenous forms to non-toxic form is an important step in water quality management in aquaculture systems.

Role of microbial communities in maintaining water quality in both natural and cultural environments (Boyd and Tucker, 1998; Mischke, 2003) and their community disruption in intensive aquaculture practices have been extensively reported (Gräslund et al., 2003; Schiller, 2011; Arias-Moscoso et al., 2018). In the microbial nitrification process, chemoautotrophic ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB) convert ammonia to nitrite and to nitrate respectively. While heterotrophic denitrifying bacteria (DNB) reduce nitrate, nitrite, nitric and nitrous oxides to nitrogen gas as end product (Ward, 2010, 2013; Zhao et al., 2012). Formulations form such ammonia metabolising microbes could be exploited for the mitigation of toxic metabolites in shrimp culture operations (Silapajarn et al., 2006; Li and Boyd, 2016; Zorriehzahra et al., 2016).

Several studies have reported successful removal of nitrogenous wastes under laboratory conditions through the application of microbes involved in nitrogen cycle (Abraham et al., 2004; Rejish Kumar et al., 2009; Kuhn et al., 2010; Karthik et al., 2016; Lei et al., 2019). However, scientific studies unequivocally demonstrating the beneficial effects of such microbes or their formulations in commercial farming conditions are scanty (Chiayvareesajja and Boyd, 1993; Queiroz and Boyd, 1998; Mischke, 2003). It is essential to identify the suitable combination of effective microbes and their enrichments adapting to environmental conditions of intended use.

Thus, there is a need to evaluate microbes that effectively mitigate toxic nitrogenous metabolites in the culture ponds and to support the efforts of sustainable intensification of shrimp farming. We recently reported the development of AOB and NOB enrichments and their efficacy in reducing ammonia and nitrite levels under the laboratory conditions (Baskaran et al., 2020). The present study reports the formulation of these nitrifying and denitrifying bacterial consortia and their efficacy to mitigate ammonia, nitrite and nitrate in commercial shrimp culture operations in different geographical locations in India.

2. Materials and methods

2.1. Nitrifying and denitrifying microbial consortia

The consortium of ammonia oxidising bacteria/archaea (AOB/AOA) and nitrite oxidising bacteria (NOB) developed (Dineshkumar, 2013) and maintained at Aquatic Animal Health and Environment Division, Central Institute of Brackishwater Aquaculture (CIBA), India were used in the study. The individual enrichments (n = 66 sediments/n = 15 ponds) were revived (Watson and Waterbury, 1971; Koops et al., 1976, 2006) and respective consortia were pooled to make AOB/AOA and NOB formulation. The denitrifying bacterial isolates (*Marinobacter* spp. cb7 & cb25) stored as 20% glycerol stocks at −80 °C in the laboratory were revived (Matsuzaka et al., 2003; Li et al., 2013) and used in the study.

2.2. Oxidation rate and effect of salinity of nitrifying and denitrifying microbial consortia

2.2.1. Oxidation rate of AOB/AOA and NOB and effect of salinity.

Ammonia oxidation rate was estimated by inoculating 100 mL of AOB/AOA consortia into 900 mL of sterile seawater spiked with 15 mg L^{-1} of ammonia-N and incubated at 30 °C in a shaker incubator at 150 rpm. Similarly the nitrite oxidation rate was estimated by inoculating 100 mL of NOB consortia into 900 mL of sterile seawater spiked with 16 mg L^{-1} of nitrite-N and incubation at 30 °C in a shaker incubator at 150 rpm. The effect of salinity on ammonia and nitrite oxidation rates of AOB and NOB consortia was studied in seawater with salinity 2, 5, 15, 25 and 35‰ spiked with ammonia-N (15 mg L^{-1}) and nitrite-N (16 mg L^{-1}) respectively. Samples were collected every 24 h for a period of 14 days and ammonia-N and nitrite-N levels were measured by Nessler' reagent and sulphanilamide/N-ethylene- di hydrochloride method respectively (APHA, 2012).

2.2.2. Denitrification rate of DNB and effect of salinity.

Rate of denitrification was studied by separately inoculating 1 mL of overnight grown DNB cultures (cb7 and cb25) to make 100 mL of sterile denitrification medium (Matsuzaka et al., 2003) followed by incubation at 30 °C in a shaker incubator at 150 rpm. Similarly, the effect of salinity on denitrification rate was estimated at 2, 5, 15, 25, 30 and 35‰. Bacterial growth (OD_{600}) and the concentrations of nitrite-N were monitored periodically.

2.3. Preparation of formulation

The cultures were initiated by starting from 1 l of the enrichment gradually increased to 5, 10 and 20 l respectively which has been used for mass production of the consortium. The enriched microbial consortium were developed into formulation with the pH being maintained at 7.0–8.5, comprising enriched ammonia oxidising bacteria (AOB) and ammonia oxidising archaea (AOA), enriched nitrite oxidising bacteria (NOB) and denitrifying bacteria (DNB) in the microbial consortium formulation at a ratio of 30:60:10 (v/v) respectively.

2.4. Microbial community profiling of the formulation.

2.4.1. PCR-DGGE analysis and sequencing

DNA was extracted from AOB/AOA and NOB consortium, DNB isolates and the final formulation using DNA Xpress kit (Himedia, India). The extracted DNA was used as a template to amplify 16S rRNA gene with the universal primers 357f-GC and 907r (Muyzer et al., 1993). Amplification was carried out with initial denaturation at 95 °C for 4 min and 10 touchdown cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s (with the annealing temperature decreasing by 1 °C in each cycle), extension at 72 °C for 1 min, followed by 20 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min (iCycler - BioRad, USA). The 16S rRNA PCR products were subjected to DGGE analysis (Muyzer et al., 1993) in 8% polyacrylamide gel (acrylamide: bis-acrylamide, 37.5:1.0) with a denaturant gradient of 30–70% (with 100% denaturant consisting of 7 M urea and 40% formamide) using DCode apparatus (BioRad, USA). Electrophoresis was carried out in 1× TAE buffer at 100 V for 17 h at a constant temperature of 60 °C. The gel was stained for 20 min with SYBR gold nucleic acid stain (1:10,000×) in 1 × TAE buffer and visualised using gel documentation system (BioRad, USA). The resulting DGGE bands were excised and target PCR fragments were eluted by TE buffer and re-amplified with GC clamp free primer pairs and the amplified DNA was sequenced.

2.4.2. Metagenomic sequencing of the formulation.

The DNA from the formulation consisting of AOB/AOA, NOB, DNB was extracted by using Xpress DNA reagent kit (Himedia, India) and quantity of DNA Was found to be 34 ng/μl. Metagenomic sequencing library was prepared by amplifying V3-V4 region of 16S rRNA gene. Cluster generation and sequencing were done with Illumina MiSeq platform using tape station profile (Eurofins, India). Sequences were clustered and operational taxonomic units (OTUs) were generated with ≥97% sequences similarities using QIIME and aligned with PYNAST using Greengenes database. The sequences of the formulation determined by NGS were deposited in NCBI SRA database under Bioproject id PRJNA542210 (#SRX5815866).

Table 1
Characteristics of sites selected for the field trial.

Trial sites	Days of culture at the start of the application	Stocking density nos. m^{-2}	Number of farms		Salinity range (‰)	pH (range)
			Treatment	Control		
Site 1 (C1 & T1)	58	55	4	4	13–25	7.5–7.8
Site 2 (C2 & T2)	80	50	4	4	50–62	7.4–8.0
Site 3 (C3 & T3)	42	60	3	6	20–25	8.0–8.3
Site 4 (C4 & T4)	62	40	3	6	30–38	7.5–8.0

2.5. Mass production of the microbial consortia and shelf life

Consortia of AOB/AOA were raised in media (80 L) containing (g L^{-1}) NH_4Cl 0.5 (~160 ppm); K_2HPO_4 0.05; $CaCO_3$ 5; NaCl 20; trace elements solution 1 mL and the NOB enrichments were raised in 80 L media containing (g L^{-1}) $NaNO_2$ 0.069 (~46 ppm); $MgSO_4.7H_2O$ 0.1; $CaCl_2.2H_2O$ 0.006; K_2HPO_4 0.002; NaCl 20; trace elements solution 1 mL [trace element solution (g L^{-1}): EDTA 2.06; Con. HCl 83 mL; $FeSO_4.7H_2O$ 1.54; $MnCl_2.4H_2O$; $Na_2MoO_4.2H_2O$ 0.1; $ZnSO_4.7H_2O$ 0.1; $CuSO_4.5H_2O$ 0.02; $CoCl_2.6H_2O$ 0.002]. Media was filtered through hollow fibre membrane filter (0.45 μm) and the respective bioreactors were inoculated with 20 L of AOB and NOB enrichments. The cultures were incubated at room temperature under constant aeration. Microbial growth was monitored by analysing the levels of ammonia and nitrite at 24 h intervals. Cultures were transferred to mixing chamber when concentration of ammonia and nitrite reached below 40 ppm and 12 ppm respectively in AOB/AOA and NOB reactors. Later, 10 L overnight culture (~10^8 CFU mL^{-1}) of DNBs [media (g L^{-1}): $NaNO_2$ 0.069; K_2HPO_4 1; $MgSO_2.7H_2O$ 1; $CaCl_2.2H_2O$ 0.2; tri-sodium citrate 8.5 g NaCl; 20 trace element solution 1 mL] was prepared for inclusion in final liquid formulation. A liquid formulation was prepared by mixing the AOB/AOA, NOB and consortia and DNB mixture as described in Section 2.3. The microbial formulation was distributed into 100 mL air tight containers and stored at room temperature for estimation of shelf life. Oxidation and reduction efficiency of the samples were made on 15, 30, 60, 90 and 120th day of storage. Efficacy of the formulation was confirmed by ammonia and nitrite oxidation tests following the procedure mentioned in Section 2.2.1.

2.6. Performance evaluation in commercial shrimp farms

To evaluate the performance of the formulation, four farm sites culturing *P. vannamei* shrimp located in different shrimp farming regions of the country were selected. During the study period basic farm level parameters like stocking density, salinity and pH were recorded (Table 1). Similar farm management practices were followed in both control and test ponds except the application of formulation at the rate of 5.0 L ha^{-1} $week^{-1}$ throughout the study in test ponds. One litre of representative sub-surface water sample was collected from the centre of the pond at weekly interval from each of the test and control ponds and total ammonia nitrogen (TAN) was analysed and pH and salinities recorded.

2.7. Statistics

Statistical analysis was performed employing SAS 9.3. Significance of difference between different treatments was determined by Analysis of Variance - Least Significant Difference (ANOVA-LSD) and the difference was considered significant at $p < 0.05$. The oxidation rate of ammonia and nitrite was determined by regression analysis and goodness of fit of the analysis was determined by checking correlation. The sample mean with standard error were depicted in the graphs.

3. Results

The study reports the formulation and evaluation of consortium of nitrifying and denitrifying bacteria to mitigate ammonia and nitrite toxicity in shrimp culture ponds. The formulation comprises of enrichments of AOB/AOA and NOB and isolates of DNB from brackishwater environments.

3.1. Oxidation rate of ammonia and nitrite

The AOB enrichment was found to oxidize 15 mg L^{-1} of ammonia in 10 days in the spiked seawater incubated under laboratory conditions at a rate of 1.57 ppm day^{-1} with a correlation of 0.95 (Fig. 1a). Similarly NOB enrichment oxidized 16 mg L^{-1} in 12 days at an oxidation rate of 1.46 ppm day^{-1} with correlation of 0.98 (Fig. 1b).

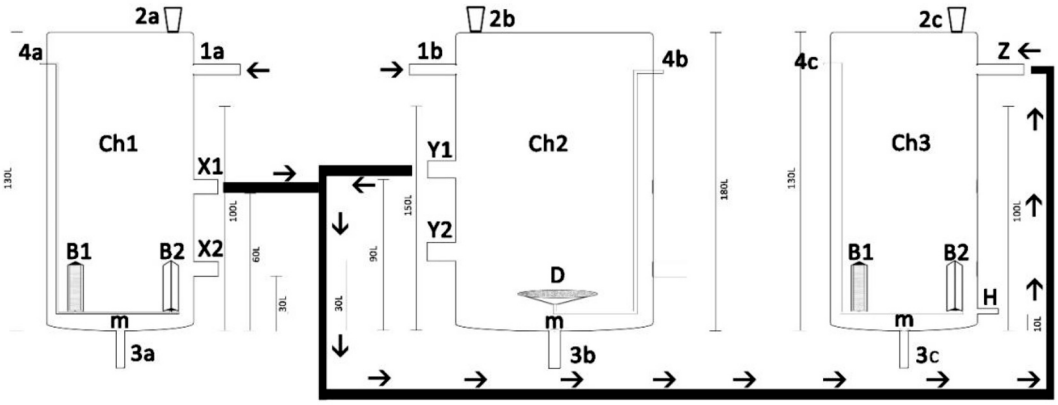

Fig. 1. Illustrative model of 3 inter connected chambers used for the production of formulated consortia Ch1, Ch2 & Ch3 – Chambers 1, 2 & 3, X1, Y1 – Primary outlet port from Ch1 & Ch2, X2, Y2 – Secondary outlet port from Ch1 & Ch2, Z – Inlet port connected to Ch3, B1 – Band diffuser facing front, B2 – Band diffuser facing back, D – Disc diffuser, H – Harvest valve, M – SS mesh (mesh size 4–5 mm), 1a, 1b and 1c – inlet port – filtered water inlet from source, 2a, 2b & 2c – media inlet port, 3a, 3b & 3c – drain valve.

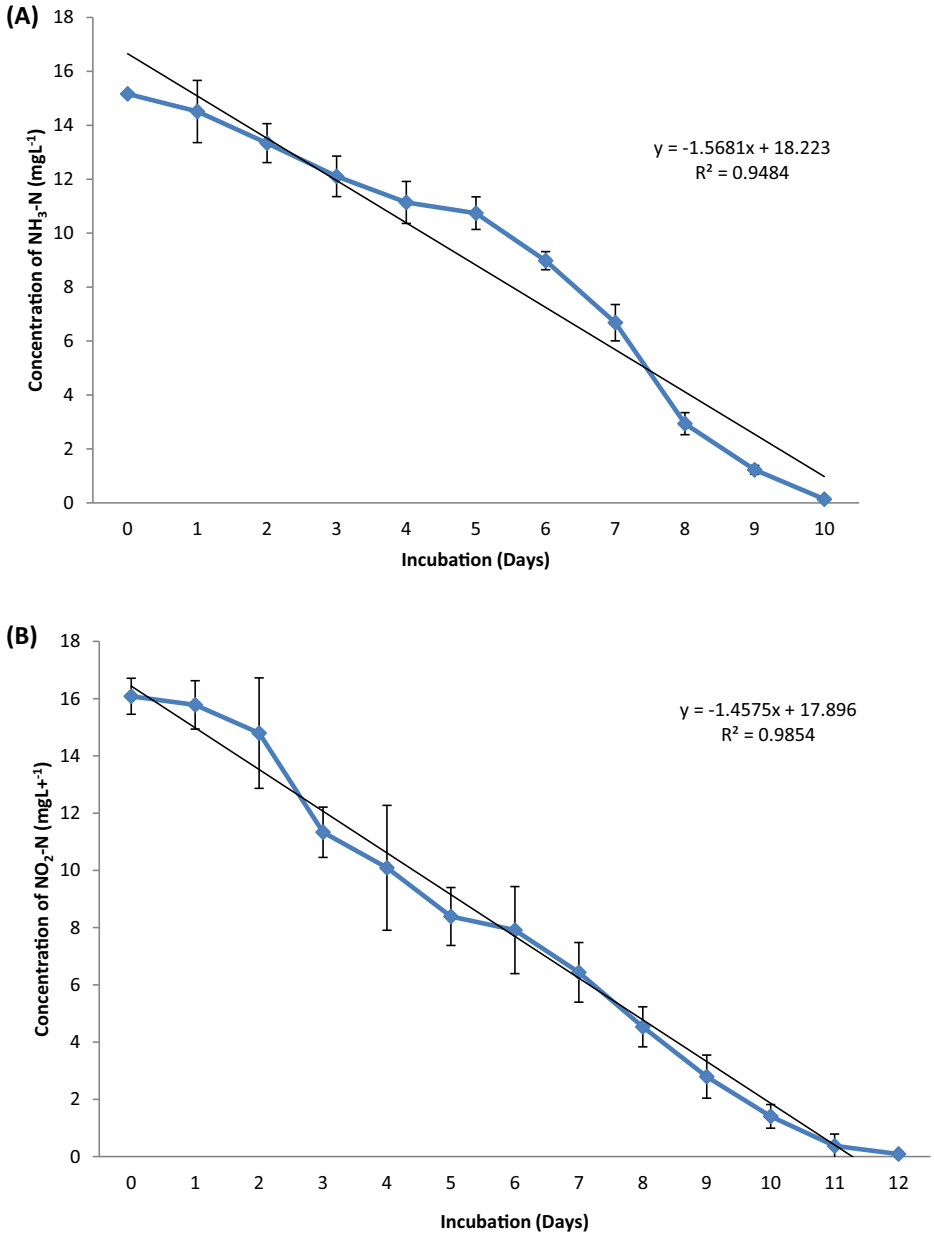

Fig. 2. (A) *In vitro* Ammonia oxidation rate by AOB/AOA enrichment, (B) *In vitro* nitrite oxidation rate by NOB enrichment.

3.2. Effect of salinity on the rate of ammonia and nitrite oxidation

Among the salinity range tested (2‰ to 35‰), it was observed that rates of ammonia and nitrite oxidation was proportional to salinity. The AOB/AOA consortia took 9 days to completely oxidize ammonia at 2‰ while the same quantity of ammonia was oxidized in 8 days when the salinity was above 25‰. Similarly, the NOB consortia took 13 days to completely oxidize the nitrite at 2‰ while the same quantity of nitrite was oxidized in 11 days when the salinity was above 15‰. Ammonia and nitrite were more efficiently oxidized by AOB/AOA and NOB consortia at 25‰ and 15‰ respectively (Fig. 2a and b). The repeated measure ANOVA revealed a similar rate of oxidation of ammonia and nitrite respectively by AOB/AOA and NOB, while at lower salinities; the consortia required 24 h and 48 longer for oxidising the respective metabolites in the spiked seawater.

3.3. Growth of denitrifying bacteria and rate of nitrite reduction

Two isolates of *Marinobacter* spp. cb7 and cb25 reached the maximum growth of 0.65 and 0.30 OD respectively at 9th h (Fig. 3). Complete reduction of nitrite (16 ppm of nitrite-N) in the medium was observed at 9th h of incubation at a rate 2.91 ± 0.32 ppm h^{-1} and 4.57 ± 1.77 ppm h^{-1} respectively for cb7 and cb25.

3.4. Effect of salinity on denitrification

Denitrification rate of *Marinobacter* spp. cb25 and cb7 isolates was shown to be salinity dependent as no activity could be detected at 2‰ (Fig. 4a and c). *Marinobacter* sp. cb7 showed varying growth at salinity range between 5 and 35‰ while *Marinobacter* sp. cb25 could grow only at the salinity range of 15–35‰ (Fig. 4b and d). *Marinobacter* sp. cb25 exhibited uniform growth and denitrification rate whereas, *Marinobacter* sp. cb7 exhibited differing growth pattern with variations in salinity. As the salinity increases, the growth of *Marinobacter* sp. cb7 was found to

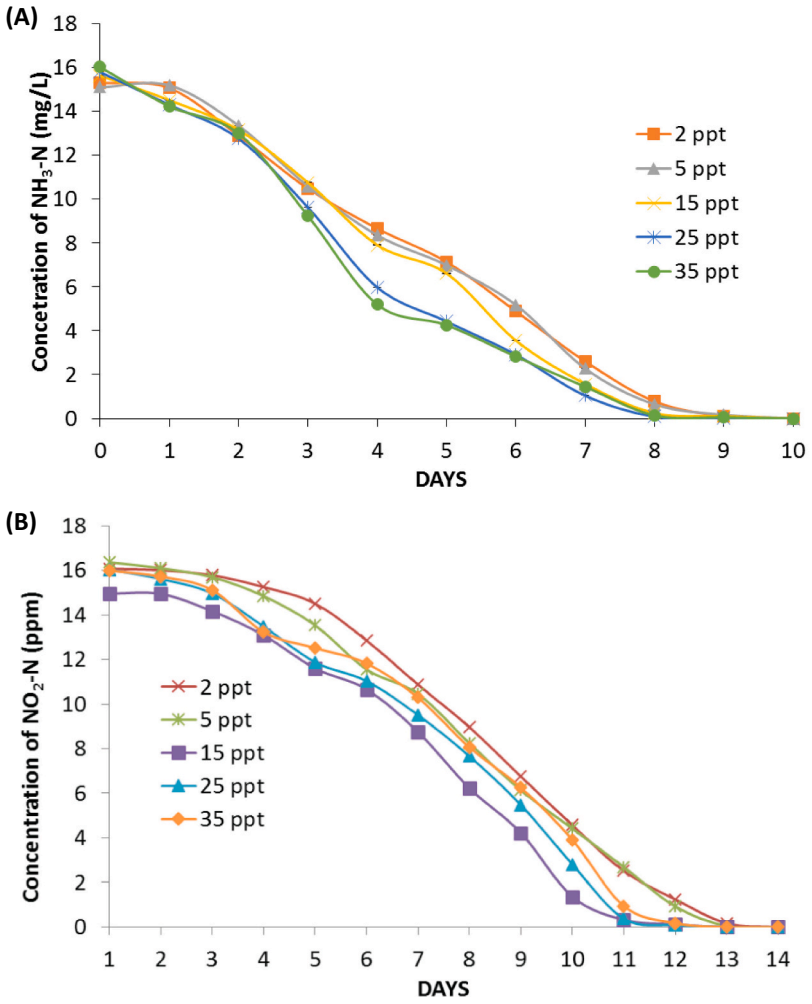

Fig. 3. Effect of salinity on (A) aammonia oxidation rate by AOB/AOA enrichment and (B) nitrite oxidation rate by NOB enrichment.

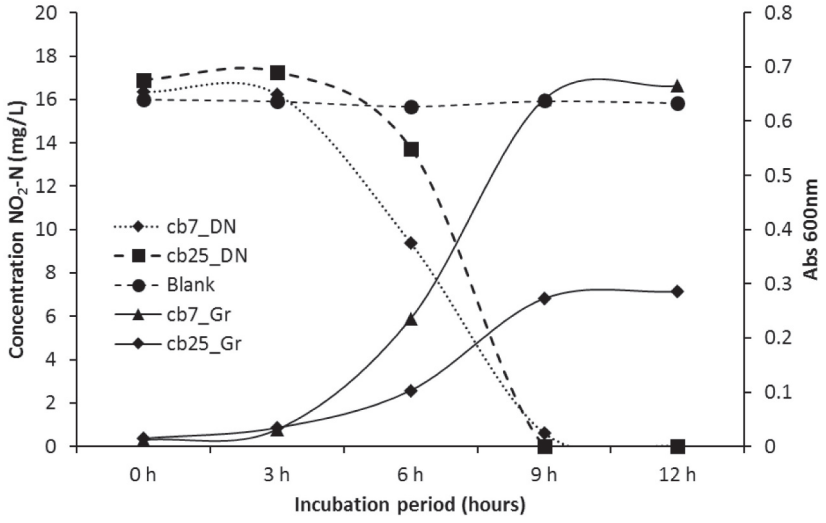

Fig. 4. Denitrification rate and growth curve of isolates denitrifying bacterial isolates. (DN – denitrification rate, Gr – growth rate of the isolates).

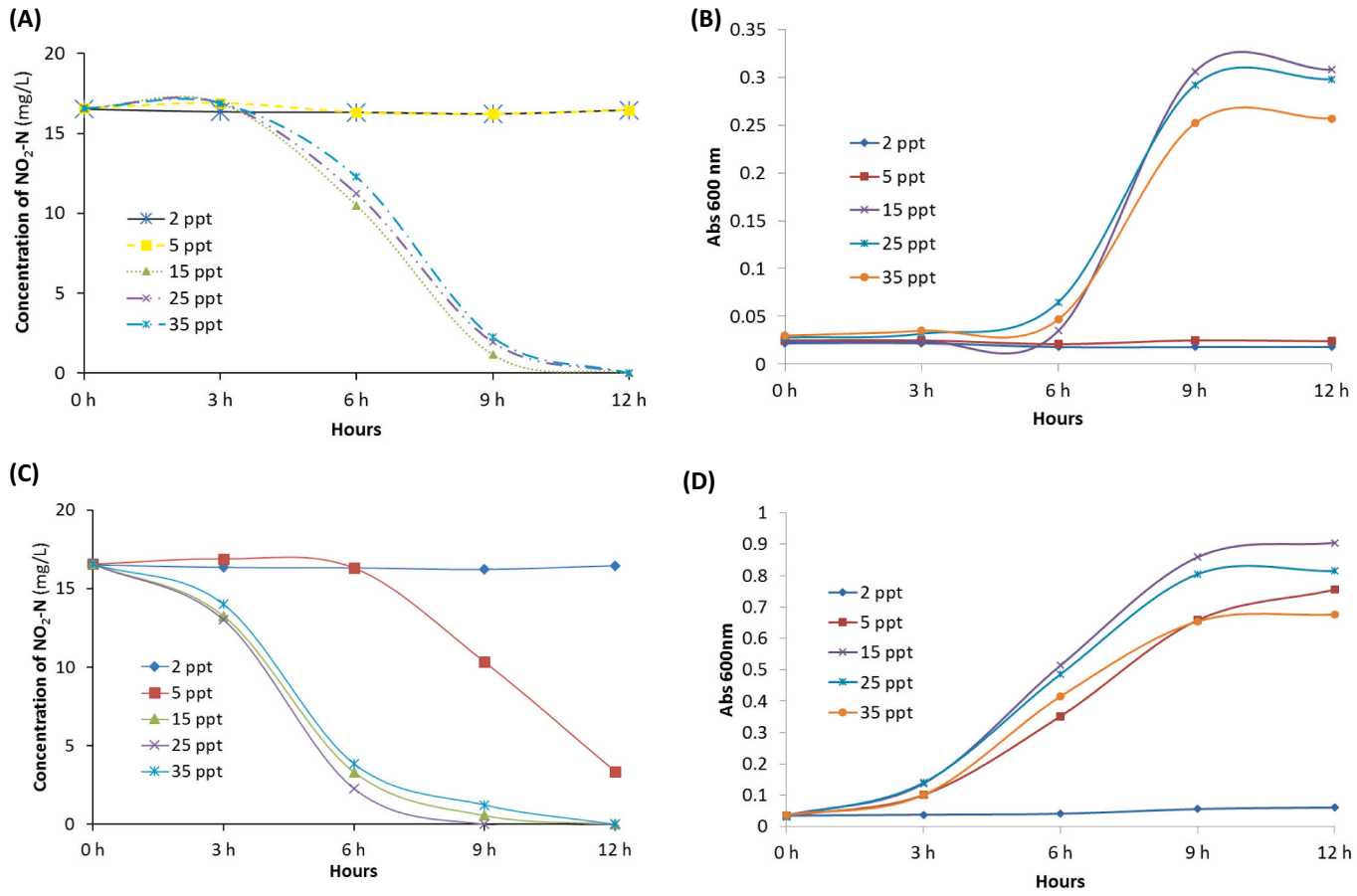

Fig. 5. (A) Denitrification rate of denitrifying bacterial isolate 'cb25' at different salinities, (B) growth rate of isolate denitrifying bacterial isolate 'cb25' at different salinities (C) denitrification rate of denitrifying bacterial isolates 'cb7' at different salinities, (D) growth rate of denitrifying bacterial isolate 'cb7' at different salinities.

decrease, but the denitrification rate remained constant except at 35‰.

3.5. DGGE analysis

DGGE analysis of each of AOB/AOA, NOB and DNB (cb7 and cb25) showed 8, 11 and 2 distinct bands respectively. These bands representing dominant microbial groups were also found in the formulation as indicated by DGGE, used for farm trials (Fig. 5).

3.6. Metagenome analysis of microbial formulation

The AOB, NOB and DNB formulation was analysed for total microbial diversity by metagenomic sequencing analysis. The analysis revealed the presence of more than 3000 OTUs at 97% similarity index. Eubacteria (>95%) was found to dominate the enrichment with Proteobacteria (62.7%) being the predominant phylum, followed by Bacteroidetes (13.9%), Spirochaetes (5.3%) and Planctomycetes (4.3%) (Fig. 6). A detailed study on distribution of microbial communities in AOB/AOA and NOB consortia were described in our earlier study (Baskaran et al., 2020). The study revealed the presence of known bacterial genera like *Nitrosomonas (120otu),* and *Nitrosococcus (107otu)* (AOB) and *Nitrospira* (184 otu) (NOB) in addition to several unclassified species in the formulation. Apart from Eubacteria, ammonia oxidising archaea like *Nitrosopumilus* (8otu) and *Nitrososphaera(4otu)* were also found in the formulation. (See Fig. 7.)

3.7. Mass production and shelf life

Shelf-life of the formulation stored at room temperature (28–32 °C) was monitored for a period of 120 days from the date of packaging. Samples collected periodically during the storage were found to oxidize ammonia and nitrite in 10 and 12 days in AOB/AOA and NOB media spiked with ammonia and nitrite respectively (data not shown). The formulated consortium was used to evaluate its efficacy under field conditions.

3.8. Efficacy of the formulation in commercial shrimp farms

The trial in site 1 ($n = 8$), consisted four treatment and four control ponds, started from 58 days of culture (DOC), with average TAN level of 0.8 ± 0.02 ppm. TAN level at day zero was considered as base value (100%) and subsequent changes in the TAN level was depicted as relative percentage change from zero days. In the following weeks of culture, the TAN level in control ponds gradually percentage increase was 153 ± 0.5 ppm by 4th week of observation. In comparison, the TAN level in the treatment pond reduced to 53 ± 0.2 ppm following application of formulation for 3 weeks. Salinity of these ponds varied between 13 and 14‰ with pH 7.5–7.8 throughout the study period.

The trial in site 2 ($n = 6$), consisted of three treatment and three control ponds at DOC 80 with TAN level at 5.45 ± 0.3 ppm. Treatment ponds showed reduction in TAN level by 47% while no significant variation in TAN level was recorded during the study period in control ponds. Salinity of these ponds during the study period was highly variable between 50 and 62‰ with pH range 7.4–8.0.

The trial in site 3 ($n = 9$), consisted of three treatment ponds and six control ponds at DOC 42 with TAN level at 97 ± 0.3 ppm. Pond treated with microbial formulation showed reduction in TAN levels upto 51% at the end of 3rd week of study period while levels of TAN in control ponds

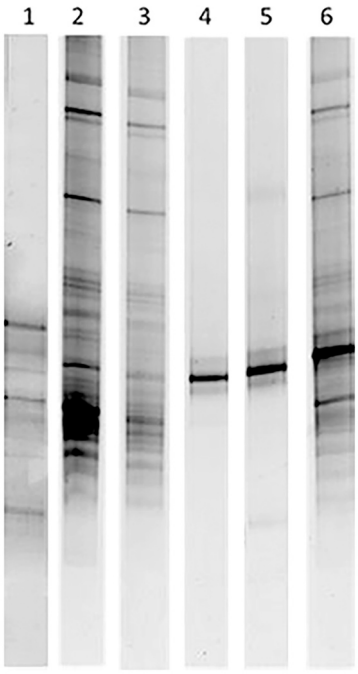

Fig. 6. Denaturing gradient gel electrophoresis (DGGE) analysis of (1) Ammonia oxidising bacterial (AOB) consortia; (2) Nitrite Oxidising Bacterial (NOB) consortia; (3) Mixture of AOB and NOB consortim; (4) Denitrifying bacterial isolate 'cb25' (5) - Denitrifying bacterial isolates 'cb7'; (6) – Formulation containing AOB and NOB consortium and DNB isolates.

showed no significant variation. Salinity of ponds at this site ranged between 20 and 25‰ with pH range of 8.0–8.3.

The site 4 (n = 9) had three treatment ponds and six control ponds at DOC 62 with TAN levels at 53 ± 0.33 ppm. Ponds treated with microbial formulation showed an average of 90% reduction in TAN levels while control ponds showed reduction of 46% at the end of study period. Salinity of culture ponds P4 ranged from 30 to 38‰ and pH 7.5–8.0. The statistical analysis confirmed significant difference ($p < 0.05$) in the levels of TAN between the control and treatment ponds (Fig. 8).

4. Discussion

Maintenance of water quality in semi-intensive and intensive aquaculture ecosystems is of the most important for ensuring optimal production. In semi-intensive and intensive shrimp farming systems, toxic metabolites like ammonia, nitrite and nitrate are issues that need to be mitigated (Colt and Armstrong, 1981; Boyd and Tucker, 1998; Zhou and Boyd, 2015). Several physical and chemical measures such as aeration, application of yucca extract, zeolite *etc.* are being practiced for management of stress due to such metabolites in the aquaculture systems. Bioremediation through microbial amendments was suggested to be the sustainable option for mitigation of toxic metabolites in the intensive shrimp aquaculture (Bernhard and Bollmann, 2010; Kuhn et al., 2010; Dhanasiri et al., 2011; Scarascia et al., 2017). Nitrifying enrichments and denitrifying bacteria from brackishwater ecosystems were isolated in the laboratory and were found to be efficient in removal of ammonia and nitrite under control conditions and were characterized (Dineshkumar et al., 2014; Baskaran et al., 2020). Present study reports the performance of the microbial formulation developed from the AOB, NOB and DNB consortia enriched in specific media under commercial shrimp grow-out farming systems.

Slower growth, high oxygen demand and requirement of attaching surfaces to propagate are few factors hindering the development of efficient microbial formulations containing nitrifyers (Boyd and Tucker, 1998; Fumasoli et al., 2017; Wang et al., 2018; Yun et al., 2019). We designed a specialized bioreactor to achieve higher microbial yield and upscaling production of AOB and NOB consortia. The formulation was prepared from microbial consortium with efficient ammonia and nitrite/nitrate oxidising activity. Specially designed bioreactor ensured the production of high yield autotrophic microbial consortium in bulk quantities. In addition to several groups of microbes, the high-throughput 16S rDNA analysis confirmed the presence of nitrifying bacteria like *Nitrosomonas* spp., *Nitrosospira* spp., *Nitrosococcus* spp. and *Nitrospira* spp. and ammonia oxidising archaea like *Nitrosopumilus* spp. and *Nitrososphaera* spp. in the microbial enrichment (Baskaran et al., 2020). Commercial products containing *Nitrosomonas* spp. and *Nitrobacter* spp. are predominately being applied in aquaculture ponds to control accumulation of ammonia, nitrite and nitrate while

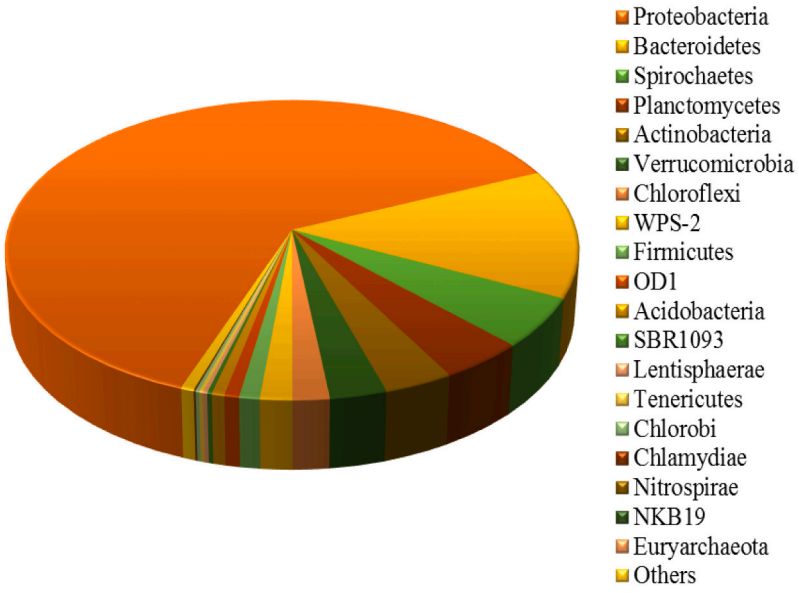

Fig. 7. Percentage distribution of bacterial phylum in the final microbial formulation containing AOB and NOB consortia and DNB isolates assessed by metagenomic analysis.

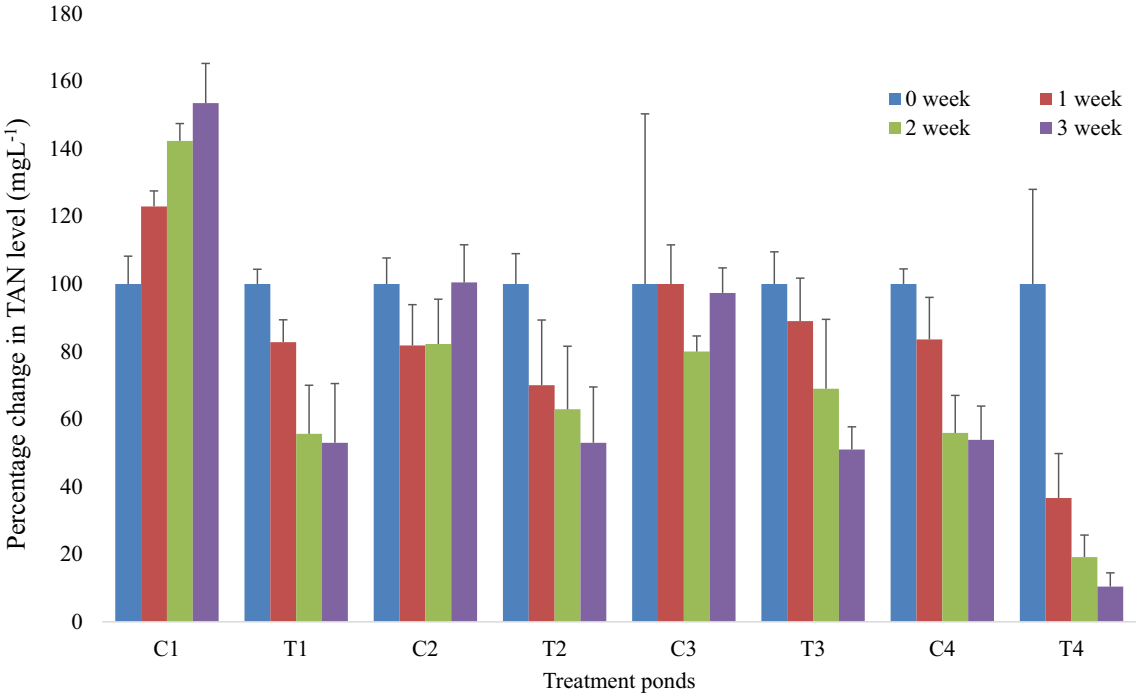

Fig. 8. Total Ammonia Nitrogen levels in the pond water samples from commercial farms of *P. vannamei* grow-out cultures before and after the application of microbial consortia.

Nitrosococcus spp., *Nitrosospira* spp. and *Nitrospira* spp. are reportedly effective nitrifyers under laboratory conditions (Juretschko et al., 1998; Hoang et al., 2014; Yao et al., 2016; Fumasoli et al., 2017).

During the enrichment of denitrifyers, two strains of *Marinobacter* spp. were also isolated which reportedly grow under oxic, anoxic and oxygen tolerant conditions (Zheng et al., 2012; Dineshkumar et al., 2014; Liu et al., 2016). Both the strains of *Marinobacter* spp. showed similar denitrification efficiency while observed variation in their growth was similar to previous observations (Kong et al., 2018). The strains were able to grow under salinity range of 5 to 35‰, similar to that reported for the *Marinobacter* spp. in treatment of saline wastewater earlier (Zheng et al., 2012). In addition to denitrification, sulphur-oxidising activity of these microbes have been documented previously (Choi et al., 2009). These studies support our observation on the utility of *Marinobacter* spp. in denitrification, final stage where the non-toxic gaseous nitrogen is released into the environment (Dineshkumar, 2013).

Salt concentration plays a critical role in the ability and metabolic activity of microbes including nitrification and denitrification. Since salinity reportedly affects the efficiency of microbial nitrification (Gonzalez-Silva et al., 2016; Bakke et al., 2017; Kinyage et al., 2019), the ability of microbes to adapt to the changing osmotic conditions is crucial. In the present study the denitrification activity of *Marinobacter* spp. was shown to be salinity dependant and the activity improved as salt concentration increased as reported in an earlier study (Li et al., 2013). The observed efficiency of the consortia in the oxidation of ammonia and nitrite under wider salinity range could be attributed to the origin of microbes from brackishwater ecosystems.

The formulation developed in the study comprises physiologically different group of microbes which are involved in conversion of ammonia to nitrite and to less-toxic nitrate finally releasing the non-toxic gaseous nitrogen. This combination of nitrifyers with denitrifyers offers unique advantage (Kuai and Verstraete, 1998; Dineshkumar et al., 2014; Liu et al., 2016) where complete removal of nitrogen from the system could be achieved. Stability of the formulation is important to maintain the effectiveness as it contained three physiologically distinct microbial groups *viz.*, AOB, NOB and DNB. The observed stability of these individual groups of microbes in the formulation was possibly due to conversion of residual ammonia in the media by AOBs to nitrite and its conversation to nitrate by NOBs. This process could be occurring at very low scale in oxygen limited conditions in the closed storage containers while the specific heterotrophic DNBs survived due to their ability to grow at different oxygen tolerant conditions using carbon source from organic matter of the dead AOBs and NOBs in the system (Kindaichi et al., 2004; Dolinsek et al., 2013; Ge et al., 2015; Zheng et al., 2019). The microbial formulation prepared in the study was found to be stable at room temperature for 120 days.

Though several commercial products containing microbial consortia are being used in aquaculture for water quality management, scientific reports on their utility in improving the environmental quality are highly inconsistent (Boyd et al., 1984; Shariff et al., 2001; Timmersons and Gerard, 1990). Significant reduction in the levels of TAN in *P. vannamei* farms was observed in the present study, following the application of microbial formulation developed in the study. Reported inconsistency in achieving the expected improvement in the water quality parameters in the previous studies could be attributed to the other prevailing conditions in the commercial ponds like pH, salinity, temperature, DO, sulphur, nitrogenous metabolites as environmental microbes are highly sensitive to these conditions (Zhang et al., 2009; Zheng et al., 2017, 2019; Brailo et al., 2019). Since quality of pond water depends on several parameters and specific microbial metabolic pathways are involved in the regulation of different metabolites, hence it is necessary to understand the group of microbes used for administration in the reported studies needs to be considered. The metagenomic analysis of the individual AOB, NOB and DNB consortia used in the formulation in the present study indicated the presence of several bacterial genera known to be involved in nitrogen recycling in the nature and are extensively reported from sewage treatment plants, deep sea and other waterbodies (Baskaran et al., 2020). Significant reductions observed in the levels of TAN in our study ponds implies the importance of nature of enriched microbes incorporated in the formulation and targeted monitoring for specific environmental parameters in the culture ponds. Further, the ability of microbes to grow under wider range of salinity was confirmed by their efficacy in *P. vannamei* grow out farms, where the salinity ranged from 13 to 62‰.

In conclusion, the microbial formulation containing consortia of AOBs, NOBs and DNBs was developed and bulk production was demonstrated in a specially designed bioreactor. The formulation was stable in room temperature for 120 days and was effective in removing TAN in shrimp ponds under wide range of salinities. The formulation was found to be effective in the reduction of TAN from intensive *P. vannamei* shrimp culture ponds located in three different geographical locations (Tamil Nadu, Andhra Pradesh and Gujarat) of India. The microbial consortium formulated could have potential application in effluent treatment plants located in saline environments and recirculating aquaculture systems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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