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Study of effect of various temperatures on the abundance of ammonia oxidizing archaea and bacteria

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ABSTRACT

Temperature plays significant role in the oxidation of ammonia in filtration units of recirculating aquaculture system. The impact of temperature on the abundance of ammonia oxidizing archaea and bacteria, and the expression of ammonia oxidizing gene (*amoA*) at specific temperature was evaluated. The broken earthen pot pieces used as filter bed materials of recirculating system, showing the presence of microorganisms were introduced in glass containers (5 pieces/5l) filled with synthetic wastewater and exposed to four different temperatures of 10, 20, 30 and 40°C for 40 days. The ammonia oxidation rate was minimum at 10°C. In 20, 30 and 40°C treatments, 99% ammonia was reduced on day-18, 8 and 18, respectively compared to the initial day. Fresh ammonium chloride (2 mM) was added twice to maintain the ammonia concentration in all treatments, except 10°C one. Nitrite-N level was < 1 mg/l at 10°C. The level was highest on day-22 at 20° and 40°C and on day-12 at 30°C. The nitrification was 10 days delayed at 20°C and 40°C compared to 30°C treatment. Concentration of nitrate-N was lowest at 10°C. Highest concentration of nitrate-N was observed on day-40 at 20°C and 40°C and day-26 at 30°C. Highest copy number of bacterial *amoA* was recorded at 30°C (2.59×10^7) followed by 20°C (4.08×10^6), 40°C (1.45×10^6) and 10°C (5.664×10^3). Archaeal *amoA* was highest at 30°C (7.47×10^3) followed by 40°C (2.98×10^2) and 20°C (46.8) treatments. Hence it may be concluded that 30°C temperature was optimum for the efficient and faster oxidation of ammonia in the present recirculating system.

Key words: Ammonia, *amoA* gene, Archaea, Bacteria, Recirculating system, Temperature

In the global nitrogen cycle, microorganisms played significant role as the oxidation of ammonia, the key process for maintenance of nitrogen cycle depends on them. It determined the balance between oxidized and reduced forms of nitrogen in terrestrial and aquatic ecosystems. The oxidation of ammonia, the first step of nitrification was considered as the rate-limiting step because of slow growth rate of ammonia oxidizing bacteria and their response to changing environmental conditions (Wagner *et al.* 1995). Various studies showed the influence of temperature on the nitrification rate. It was influenced by environmental temperature and ammonium-N concentration (Hoilijoki *et al.* 2000). In environment, nitrification rate increased gradually from 10°C to 30°C (Stark 1996, Thamdrup and Fleischer 1998). The ammonia oxidization rate dropped as temperature decreased from 30°C to 10°C (Lei *et al.* 2012). The community structure of the microorganisms was also influenced by the environmental temperature. The diversity decreases at low temperatures. A direct relationship was found between ammonia oxidizing archaea (AOA)/ bacteria (AOB) and abundance of ammonia oxidizing genes (*amoA*) with ammonia oxidation rate depth profiles (Santoro *et al.* 2010, Newell *et al.* 2011). Abundance of ammonia oxidizing bacteria and environmental temperature both influenced the lag period and rate of ammonia oxidation (Lee *et al.* 2011). Each AOB lineages had specific temperature range that controlled the biogeograpic distribution of individual AOB (Avrahami *et al.* 2011, Fierer 2009).

Like ammonia oxidizing bacteria, the activities of AOA are also influenced by temperature. The diversity of AOA decreased at low temperature (Urakawa 2008). Some AOA preferred elevated temperature (Tourna *et al.* 2008). The optimal growth of *Nitrososphaera viennensi* was found at 37°C and *Nitrosocaldus yellowstonii* grew up to 74°C (Tourna *et al.* 2011). The effect of temperature was reflected on the production of nitrous oxide in soil samples incubated for 5 days at different temperatures. Minimum and maximum production were found at 4°C and 37°C, respectively, intermediate production of nitrous oxide was observed at 15°C and 25°C (Gödde and Conrad 1999). Seasonal variation in ammonia oxidation rate was also reported, minimum and maximum rates were recorded in cold and warm seasons, respectively (Kim 2013). The toxicity level of ammonia varied with temperature. A direct

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relationship was found between the percent of un-ionized ammonia and temperature. Increase of temperature from 10°C to 20°C raised the percent by a factor of 1.3–1.6 depending on pH (http://www.water-research.net/index.php/ammonia-in-groundwater-runoff-and-streams). Therefore, temperature plays significant role in intensive aquaculture, like the recirculating system as the rate of conversion of toxic ammonia is temperature dependent.

In recirculating aquaculture system, high stocking densities are used that enhance production of ammonia from excess food and excreta. The microorganisms present in the filter bed of recirculating system help to reduce the ammonia into less toxic nitrite. Although there are reports on the influences of temperature on activity, growth and community structure of microorganisms from various habitats, there is dearth of information on the role of temperature on the development of biofilm on the filter bed of recirculating system. In a multi-seasonal country like India, the functioning of natural biogeochemical-cycles is influenced by the seasonal changes leading to the imbalance of the nitrogen-metabolites. The present investigation aimed to evaluate the impact of temperature on the activity and abundance of ammonia oxidizing archaea and bacteria on the biofilter of recirculating aquaculture system. This will help the aquaculturists to select optimum temperatures for the maintenance of water quality in recirculating aquaculture system.

MATERIALS AND METHODS

Experimental conditions: Freshly designed broken earthen pot pieces (BEP, 5 cm × 5 cm) were incubated in the filtration unit of an established recirculating system for 90 days. Earlier study confirmed the presence of uncultured clones of thaumarchaeote and Nitrosomonadaceae in the filtration unit (Khangembam *et al.* 2017). Five pieces were randomly collected from the filtration unit to confirm the growth of AOA and AOB. Total genomic DNA was extracted and amplified using archaeal *amoA* and bacterial *amoA* primers (Rotthauwe *et al.* 1997, Francis *et al.* 2005).

Once the growths of ammonia oxidizing microbes were confirmed, the broken earthen pot pieces were introduced into the jars (5 pieces/5l) filled with synthetic wastewater (Table 1). The wastewater was prepared following the method of Munz *et al.* (2010) with some modification. This synthetic wastewater was supplemented with 2 mM ammonium chloride. These containers were exposed at four different temperatures of 10°C, 20°C, 30°C and 40°C. Three replicates were used for each treatment and were placed in an aquarium containing clean water for the maintenance of uniform temperature. As the ambient temperature was 25°C, the target temperatures were achieved and maintained with the help of external chillers and heaters. Chiller (Hailea Chiller HC-300, China) was used to achieve 10°C and 20°C temperatures and heater (Sera Aquarium Heater 300, Germany) was used to obtain 30°C and 40°C. A motor was employed in the aquarium to circulate the water and maintain uniformity in all the replicates (Fig. 1a). Dissolved oxygen level was maintained 5±0.5 mg/l with the help of aerator. The pH of culture medium ranged from 7.4 to 8.3 throughout the study period. The initial pH was 8.3 but as the reaction proceeded it became acidic. Sodium hydroxide (5 M) solution was used to maintain the pH of the culture medium. Ammonium chloride solution (2 mM) was added when 99% of ammonia level reduced compared to the initial concentration. The solution was added twice in 20°C, 40°C and 30°C (Fig. 1b). Duration of experiment was 40 days.

Table 1. Composition of synthetic wastewater

Composition	Quantity (mg/l)
Beef extract	90
Yeast extract	90
$MnSO_4$	1.22
$FeSO_4$	10.1
KCl	3.125
K_2HPO_4	87.6
$NaHCO_3$	163.5
$CaCl_2$	1055
$MgSO_4$	10.88
NH_4Cl	106.9

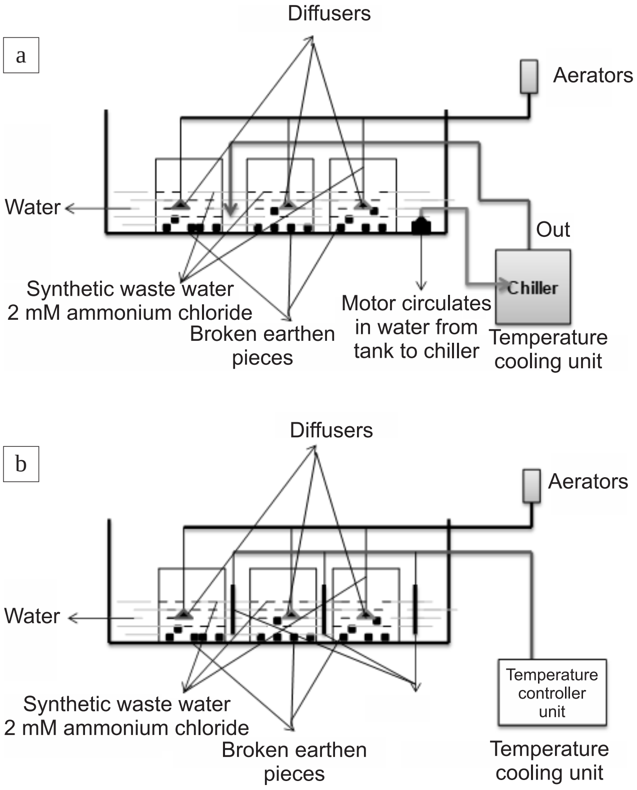

Fig.1. Experimental layout (a) cooling system for 10°C and 20°C and (b) heating system for 30° and 40°C.

Water quality: Dissolved oxygen level of each treatment was monitored regularly using portable oxygen meter with luminescence based probe (IntelliCAL LDO101, Hach, USA). pH was measured using probe (IntelliCAL PHC101, Hach). The rate of change of ammonia (NH_3-N), ammonium (NH_4-N), nitrite (NO_2-N) and nitrate (NO_3-N) was monitored every other day. Ammonia and ammonium were monitored using probes (HQ40d Multiparameter, Hach). Nitrite and nitrates were measured following the methods of APHA (2012).

DNA extraction and PCR: The broken earthen pot pieces were collected after 40 days, the biofilm was removed from the surfaces by scrapping outer layers uniformly. Total genomic DNA was isolated using PowerMax® DNA isolation kit (MoBio Inc., USA) as per manufacture's recommendation. The isolated DNA was checked with agarose gel (0.8%). The concentration was measured using Nanodrop 1000 (ThermoScientific, USA). Amplification of DNA was performed with peQSTAR 2 × double block thermocycler, peQlab under the following conditions. The reaction mixture (50 µl) consisted of 25 µl 2 × mastermix (ThermoScientific, Lithuania), 25 ng DNA templates, 2.5 µl forward (FW) and 2.5 µl reverse (RV) primers of bacterial *amoA* - F: 5´GGGGTTTCTA-CTGGTGGT3´ and R: 3´CCCCTCKGSAAAGCCTT-CTTC5´ (Rotthauwe *et al.* 1997) and archaeal *amoA* F: 5´STAATGGTCTGGCTT-AGACG3´ and R: 3´GCGGCCATCCATCTGTATGT5´ (Francis *et al.* 2005). The rest volume was made up with nuclease free water. The amplification program of archaeal *amoA* was as follows: 95°C for 4 min, 30 cycles consisting of 94°C for 45 sec, 53°C for 45 sec, 72°C for 60 sec followed by 72°C for 15 min. The amplification of bacterial *amoA* was performed as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 51°C for 30 sec and extension at 72°C for 30 sec, final extension at 72°C for 10 min. All PCR results were confirmed with 1.2% (w/v) agarose gel electrophoresis.

Absolute quantification of amoA genes: Copy numbers of archaeal *amoA* and bacterial *amoA* genes were determined in triplicate for both samples by quantitative real-time PCR (ViiA-7^{TM} real-time PCR system, Applied Biosystems, USA). The assay was performed in 96-well reaction plate (MicroAmp® Fast Optical) with optical adhesive cover. New primer sets were designed from the sequences of submitted clones of archaea-F: 5´ CATCCTAGAGCGGCAAAGGT3´and R: 3´ACCCCAA-GTGGGCAAATTCT5´ and bacteria-F: 5´TGGCTCGTG-ACAGCGTTAAT3´ and R: 3´TACGATTGGCAAGTG-GGTCG5´ (Accession no. KP272121 and KP259843) using Primer 3 software. In real-time PCR, amplifications were carried out together with standards, constructed on the dilutions of plasmids of archaeal *amoA* (1.01×10^8–1.01×10^4) and bacterial *amoA* (9.5×10^{10}–9.5×10^3). A uniform sample concentration (20 ng/ µl) was used in real-time PCR. The composition of reaction mixture (10 µl) was as follows: 1 µl genomic DNA, 0.5 µl FW and RV primers (2.5 µM each), 5 µl 2× Power SYBR® Green PCR mastermix and 3 µl PCR-grade H_2O (Merck, Germany). The amplification was performed under the following conditions: 50°C for 2 min, initial denaturation at 95°C for 10 min, this was followed by 40 cycles of 15 sec at 95°C and at 60°C for 1 min. The negative control was prepared without DNA. The amplification efficiencies of archaeal and bacterial *amoA* gene primers were 93.8 and 104.9%, respectively. To confirm the single target fragment of the PCR amplified products, dissociation curves were analyzed and plotted at the end of every quantitative real-time PCR reaction.

Statistical analysis: Statistical analysis was carried out using one way ANOVA (SPSS version 16) with post-hoc DMR analysis.

RESULTS AND DISCUSSION

The role of environmental temperature on the oxidation of ammonia was evaluated in the present study. The rate of oxidation was minimum at 10°C (Fig. 2a) and the concentration reduced by 80% on day-18 compared to the initial value on day-0. Then concentration increased gradually and highest concentration was observed on day-40. Decrease of temperature affected the number of microorganisms. Therefore, the oxidation rate was reduced at 10°C treatment in the present study. Generally, the microbial activity increased with reaction temperature and nitrification rate reduced at temperature below 10°C (Nalco 2009). The activity of microorganisms at 20°C treatment was higher compared to the 10°C, as the ammonia level reduced (99%) on day-16 compared to day-0 (Fig. 2b). Then fresh ammonium chloride solution (2 mM) was added. The concentration again reduced (99%) on day-34 and fresh solution was added. The highest oxidation (99%) rate was recorded at 30°C treatment on days-10 and 30 (Fig. 2c). Fresh ammonium chloride solution was added in this treatment twice. The oxidation rate of ammonia at 40°C treatment was similar to the 20°C treatment (Fig. 2d). In the present study, ammonia oxidation rate was minimum at 10°C and the rate increased up to 30°C and then decreased at 40°C. Jiao *et al.* (2010) found that the nitrification rate increased to an apparent maximum at 30°C and beyond this the rate decreased.

There was also variation in the rate of change of ammonium-N in four different temperatures. Although, ammonium-N is not the direct substrate of ammonia oxidizing microbes, its equilibrium to NH_3-N, changes with the variation of pH. The oxidation rate at 10°C was lowest (15%, on day-14) compared to the other treatments (Fig. 3a). Ammonium oxidation rates were 72, 88 and 91% at 20°C, 30°C and 40°C treatments, respectively (Figs 3b-d). The ammonia oxidizing bacteria (AOB) grew over a wide range of temperature (Koops *et al.* 2006). It consisted of various lineages, with different optimum temperature for growth, *viz.* pure culture of *Nitrosomonas communi* required 30°C and for *Nitrosomonas europea* optimum temperature was 35°–40°C (Tokuyama *et al.* 2004). The optimal temperature for pure culture was 25°–30°C (Koops *et al.* 1991, Odell *et al.* 1996).

Nitrite-N level was minimum on day-0 regardless of treatments (Figs 4a-d). Gradually, the level increased at 20°–40°C treatments. This indicated the first step of nitrification. In 10°C treatment, the level was always less than 1 mg/l

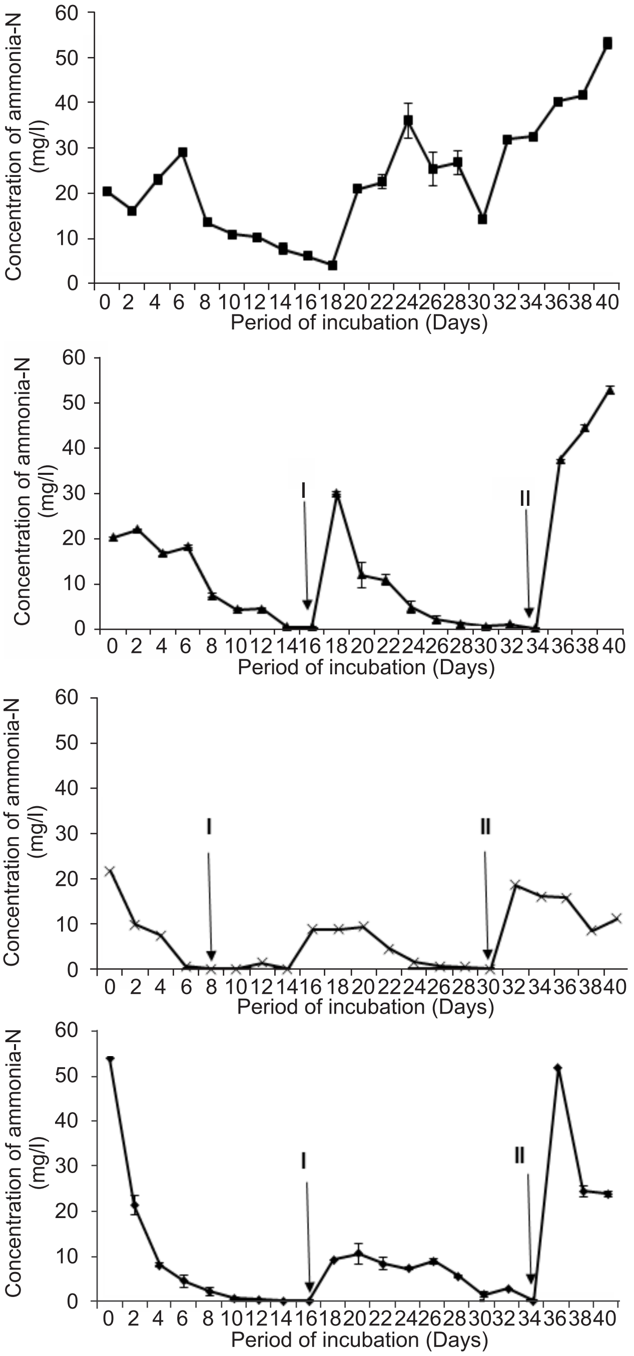

Fig. 2. Rate of change of ammonia-N at (a) 10°C (b) 20°C (c) 30°C and (d) 40°C during 40 days of incubation. Values are given as Mean±S.E. (n=3). Arrow indicates the lowest concentration and I and II indicates addition of fresh ammonium chloride solution (2 mM).

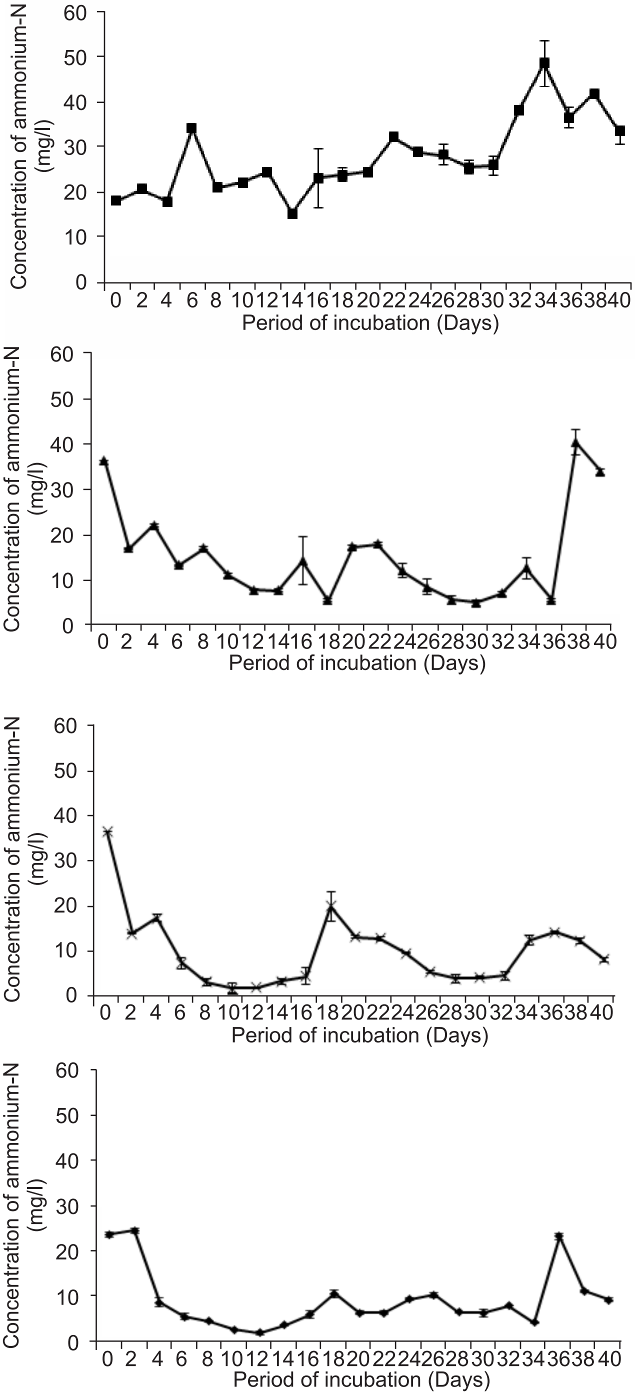

Fig. 3. Rate of change of ammonium-N at (a) 10°C (b) 20°C (c) 30°C and (d) 40°C during 40 days of incubation. Values are given as Mean±S.E. (n=3).

showing poor nitrification rate at this temperature. Highest level of nitrite-N was observed on day-22 at 20°C and 40°C treatments, while the highest rate was observed at 30°C treatment on day-12. This indicated that nitrification was 10 days delayed at 20°C and 40°C compared to the nitrification at 30°C in the present study. The highest level of nitrite was observed after first supplementation of fresh ammonium chloride regardless of treatments. Nitrite rarely seems to accumulate in nature. In the present study, it was accumulated in various treatments at the initial period of incubation, except at 10°C. Limited oxidation of ammonia and lesser activity of nitrite oxidizing bacteria could be the reason of low level of nitrite-N and nitrate-N at 10°C (Spieck and Lipski 2011). Temperature not only influenced AOB but also nitrite oxidizing bacteria (NOB). *Nitrospira* and *Nitrobacter* were mainly cultivated in the laboratory at 28°C (Bartosch *et al.* 1999).

Nitrate-N, the final product of nitrification, depend on

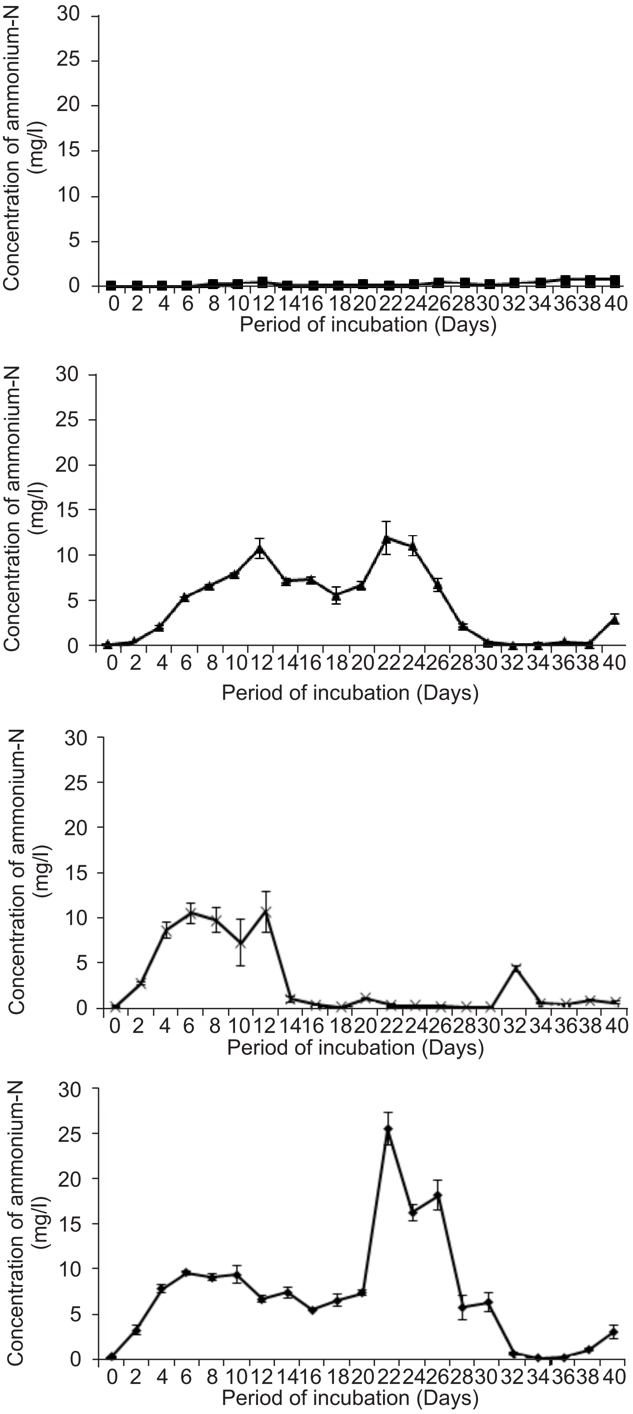

Fig. 4. Rate of change of nitrite-N at (a) 10°C (b) 20°C (c) 30°C and (d) 40°C during 40 days of incubation. Values are given as Mean±S.E. (n=3).

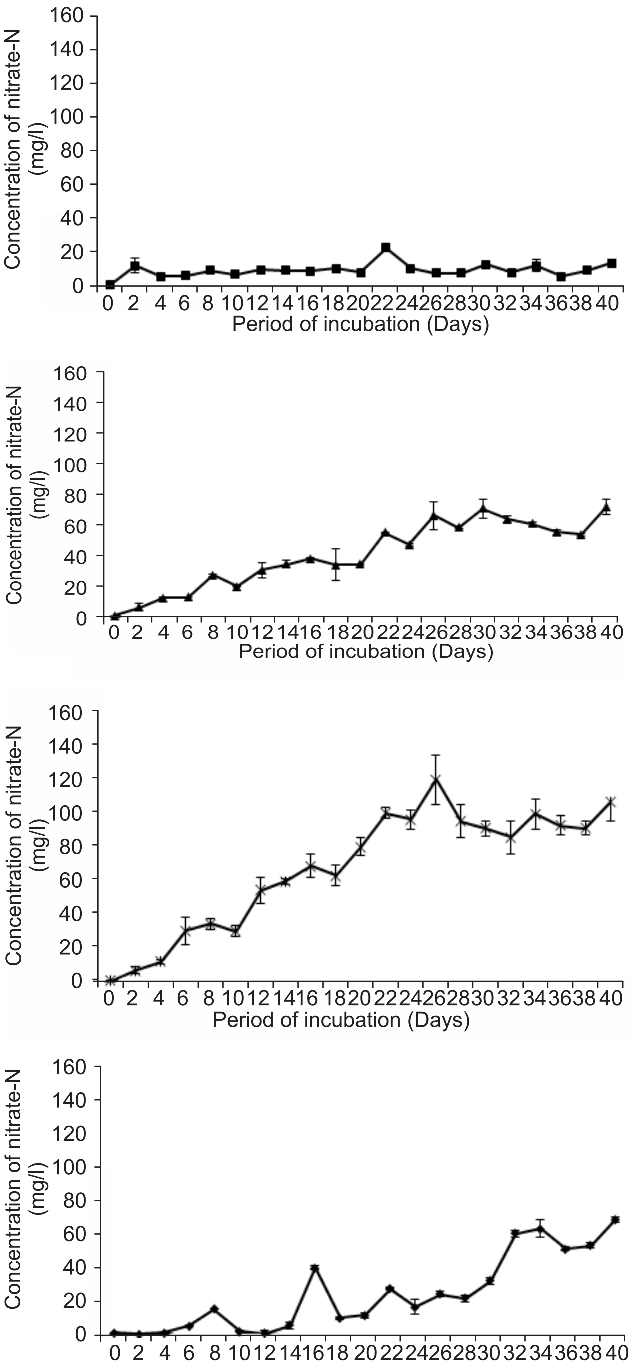

Fig. 5. Rate of change of nitrate-N (a) 10°C (b) 20°C (c) 30°C and (d) 40°C during 40 days of incubation. Values are given as Mean±S.E. (n=3).

the activity of nitrite oxidizing bacteria. Highest concentration of nitrate-N with reference to its initial concentration was observed at 30°C on day-26 (99.6%). This was followed by 20°C and 40°C (99%) on day-40, while the level was minimum at 10°C (Figs 5a-d). The change in the concentration of nitrogen metabolites showed that the bio-kinetics of nitrification was sensitive to elevated temperature.

Abundance of amoA gene: Highest copy number of bacterial *amoA* was recorded at 30°C (2.59×10^7) followed by 20°C (4.08×10^6) and 40°C (1.45×10^6). The lowest value was recorded at 10°C (5.664×10^3), this was lower compared to the initial value (7.1×10^5). At 30°C, the value was 20-fold higher compared to the initial one. At 20°C and 40°C treatments, 2.4 and 0.8-fold higher values were recorded compared to the initial concentration (Fig. 6a). The copy number of bacterial *amoA* gene increased with increasing soil temperature (Szukics *et al.* 2010). Among the four

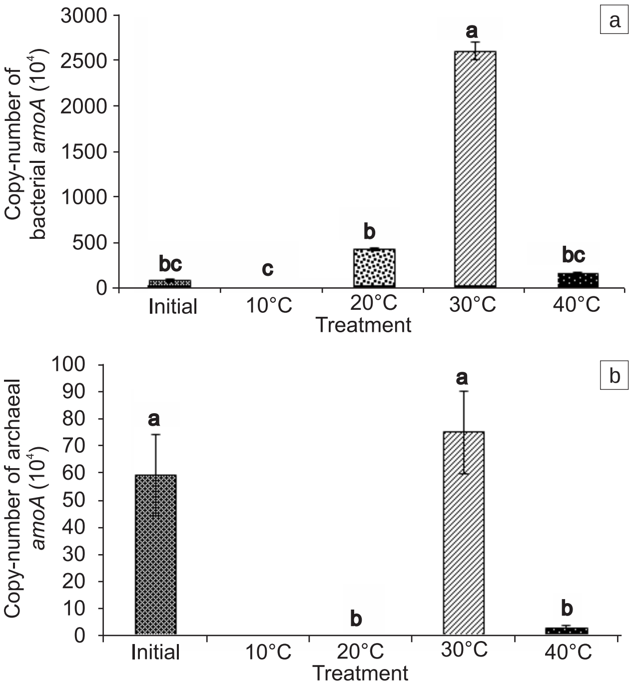

Fig. 6. Abundance of (a) bacterial *amoA* and (b) archaeal *amoA* genes before and after exposure at 10°, 20°, 30° and 40°C. Vertical bars with different superscripts are significantly ($P<0.05$) different ($n=3$).

different treatments, archaeal *amoA* was highest at 30°C (7.47×10^3). This was 1.3-fold higher compared to the initial value (5.9×10^3). This was followed by 40°C (2.98×10^2) and 20°C (46.8) treatments. At 10°C treatment, gene was under determination (Fig. 6b). Highest copy number of bacterial *amoA* was recorded at 30°C. It was followed by 20°C, 40°C and lowest at 10°C. The initial copy number was higher compared to the 10°C treatment. The reduction in abundance of *amoA* copy number at 40°C might be due to elevated temperature.

Like AOB, temperature also affected the growth, activity, abundance and community structure of AOA (Tourna *et al.* 2011). In aquarium biofiltration, abundance and diversity of AOA was minimum at 5.5°C (Urakawa *et al.* 2008). In marine environment, the abundance of *amoA* genes appeared to be correlated with ammonia oxidation (Wuchter *et al.* 2006). The relative abundance of marine and subsurface associated archaea increased with temperature compared to the soil archaea (Tourna *et al.* 2011). Leininger *et al.* (2006) found that in soil the quantity of crenarchaeota population slightly affected by temperature. The Chao1 richness indexes of the archaeal *amoA* gene in sediments of three hot springs (42°–87°C) was recorded and higher values were found at temperature below 75°C compared to the above one (Zhao *et al.* 2011). This indicated that AOA favoured the moderately high temperature environment. In freshwater lake, the abundance of archaeal *amoA* gene reduced at 37°C after four weeks of incubation (Wu *et al.* 2013). In our study, the archaeal *amoA* was under detection at 10°C and the highest abundance was found at 30°C.

In conclusion, the ammonia and nitrite oxidations were directly depended on temperature. The optimum temperature for the growth of microorganisms was 30°C in the filter bed materials of present recirculating aquaculture system as efficient and faster oxidation of ammonia was recorded. The abundance of AOA and AOB was equally affected by the change of temperature. Seasonal fluctuations of environmental temperature should be considered for the proper functioning of the recirculating aquaculture system.

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