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Study on nitrifying bacteria as bioremediator of ammonia in simulated aquaculture system

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Abstract
In intensive aquaculture system, ammonia nitrogen is a key limiting factor. Removal of unionized ammonia (NH3) and nitrite (NO2) through biological activity is thus an important tool for changing such ecosystem. Nitrifying bacterial inoculants are the biologically active materials which may be used in intensive aquaculture for bioremediation. In all, 12 treatments were used with two replications factorial Completed Randomized Design (CRD) to assess the effects on different physio-chemical conditions of water. Decrease of ammonia nitrogen concentration from 10 mg L^{-1} to below the minimum limit (0.3 mg L^{-1}) was obtained within 3 days after inoculation of microbial inoculums with aeration in water. Rate of nitrification was very slow in tanks without aeration. Soil at the bottom was not found to affect the nitrification process. Aeration and microbial application played an important role in increasing the nitrification. After acclimation phase nitrification rate was found to be increased. Therefore, it may be concluded that application of bioremediators (nitrifiers) decreased ammonia and nitrite nitrogen.

Keywords: Nitrifying bacteria, bioremediation, simulated aquaculture, biochemical oxygen demand (BOD) and chemical oxygen demand (COD)

Introduction
Global aquaculture is changing from extensive to intensive system. There is a tendency to increase the inputs i.e. over stocking, overfeeding, more use of fertilizers and various chemicals (antibiotics, herbicides, pesticides etc.). These inputs may change the aquatic environment and lead to negative impact on living organisms resulting into mortality in fish population. The major changes in water quality are increasing the biochemical oxygen demand (BOD) and chemical oxygen demand (COD), increase in ammonia nitrogen (NH_3-N), nitrite-nitrogen (NO_2-N), increase in available phosphate (PO_4^-), accumulation of hydrogen sulfide (H_2S) at pond bottom, accumulation of residues of management chemicals, decomposition of dead organisms and decay of fecal matter. In addition to this, urban ponds are under the pressure of growing population adding tons of sewage kitchen waste and detergents in to the water.
The recent approach to improve water quality in aquaculture is the application of microbes/enzymes to the ponds, known as ‘bioremediation’ which involves manipulation of microorganisms in ponds to enhance mineralization of organic matter and get rid of undesirable waste compounds and there by toxic effect. Bacteriological nitrification is the most practical method for the removal of ammonia from closed aquaculture systems and it is commonly achieved by setting of sand and gravel bio-filter through which water is allowed to circulate. The ammonia oxidizers are placed under five genera, *Nitrosomonas, Nitrosovibrio, Nitrosococcus, Nitrolobus* and *Nitrospira*, and nitrite oxidizers under three genera, *Nitrobacter, Nitrococcus* and *Nitrospira*. Nitrifiers in contaminated cultures have been demonstrated to nitrify more efficiently. Nitrification not only produces nitrate but also alters the pH slightly towards the acidic range, facilitating the availability of soluble materials. The vast majority of aquaculture ponds accumulate nitrate, as they do not contain a denitrifying filter. Denitrifying filters helps to convert nitrate to nitrogen. It creates an anaerobic region where anaerobic bacteria can grow and reduce nitrate to nitrogen gas.
Therefore, to nullify or eliminate the pollutant/toxicants from the aquatic environment, the ecosystem needs remediation. Nitrification is a natural process, which occurs in pond ecosystem but during several occasions, this may not be reaching a higher order of magnitude. Application of nitrifying bacterial consortium to growth artificially and reduce toxicity of ammonia in aquatic system is used in this experiment as a tool for bioremediation. Several bioremediators are developed by scientists, those are below mentioned.

1.1 Bioremediators Developed

Identity of the bioremediator	Used on culturable species	Method of application	References
Bacillus sp.	*Centropomus undecimalis*	Added to water; reduced salinity	Blain *et al*., 1998 [1]
Bacillus sp	Penaeids	Spread in pond water	Moriarty 1998 [2]
Aeromonas media	*Crassostrea gigas*	Spread in pond water	Gibson *et al*. 1998 [3]
Aeromonas CA2	*Crassostrea gigas*	Spread in pond water	Douillet and Langdon 1994 [4]
Nitrosomonas and *Nitrobacter*	Cyprinus carpio communis	Simulated condition	Barik *et al*. 2005 [5]
Roseobacter sp. BS 107	*Oncorhynchus mykiss*	Spread in pond water	Ruiz-Ponte *et al*., 1999 [6]

2. Materials and methods

Experimental design

Three factorial completely randomized design (CRD) was used with two replications and twelve treatments as follows:

$T_1= I_1+S_1+A_1$	$T_5= I_2+S_1+A_1$	$T_9 = I_3+S_1+A_1$
$T_2= I_1+S_1+A_2$	$T_6= I_2+S_1+A_2$	$T_{10}= I_3+S_1+A_2$
$T_3= I_1+S_2+A_1$	$T_7= I_2+S_2+A_1$	$T_{11}= I_3+S_2+A_1$
$T_4= I_1+S_2+A_2$	$T_8= I_2+S_2+A_2$	$T_{12} = I_3+S_2+A_2$

I_1= With microbial inoculum @ 2.5 µl L^{-1} (*Nitrosomonas* sp. and *Nitrobacter* sp.each)

I_2= With inoculum @ 5.0 µl L-1 (*Nitrosomonas* sp. and *Nitrobacter* sp.each)

I_3= Uninoculated

S_1= With soil base @100g/aquarium, S_2=Without soil base

A_1=With Aeration, A_2= Without aeration

Study was carried out in twenty-four aquaria (volume 75 x 45 x 30 cm^3) containing 90l water under indoor conditions at room temperature in the aquaculture laboratory. Twelve aerators (vibrator type-100 watt) were used in aquaria for aeration. Aeration in aquaria tanks were achieved by passing of the air from air pump through a submerged block of porous material called as air stone along with regulators. Water was artificially polluted in a 1000 liter cemented pool through application of raw cow dung and urea.A layer of 6cm of sand bed with 100g of soil was provided in the aquaria[as per the treatment] Aquarium tanks were filled with diluted water up to 90 liters. Ten common carp (*Cyprinus carpio var. communis*) fry were stocked in each aquarium. The mean length and weight of fry was 28mm and 0.283 g respectively. Microbial inoculums were developed by isolation of local *Nitrosomonas* and *Nitrobacter* strains and multiplied in the laboratory (Barik *et al*. 2005) [5]. Both the inoculums of *Nitrosomonas* and *Nitrobacter* were mixed with distilled water 1:200 (inoculums: water) and stirred at every 5 minute's interval for a period of 30 minutes. The Nitrifying bacteria were activated by shaking the diluted inoculums on a rotator shaker at 200 rev/minutes for 24 hours. After activation, the diluted media (slurry) was sprinkled uniformly over the surface of aquarium. Pelletier feed (mixture of oil cake and rice bran @ 1:1) was given to fry 2 per cent of the body weight, twice a day at 8 A.M. and 4 P.M. respectively. Water samples were collected from aquaria just prior to the application of inoculums and every day during experimentation for further follow up. The temperature of water and air were measured by using a Celsius mercury thermometer. Water pH was measured with the help of a pen pH meter (Scan-2-Eutech, Cybernetics Private Limited, Singapore). All physico-chemical parameters were estimated by the methods of APHA (1989) [7]. Dissolved oxygen (DO) was measured by Winkler's (Azide modification) method. Alkalinity of water was measured by electrometric titration (manual) method. Calcium hardness and total hardness were measured by EDTA titration method. Ammonia nitrogen (NH_4-N) was estimated through distillation at high pH (9.5) followed by titration in the presence of boric acid. Nitrite-nitrogen (NO_2-N) was measured by colorimetric method (diazotization). Nitrate nitrogen (NO_3-N) was measured after distillation of ammonia. Residual sample was again distilled with Davarda's alloy followed by titration in the presence of boric acid. Iodometric method was used for determination of Hydrogen sulphide (H_2S). Winkler's (Azide modification) method was used for estimation of BOD. Dichromate reflux method was used for estimation of COD.

3. Results

Effect of various treatments on ammonia nitrogen is shown in Table-1 and Fig.1. Nitrifying bacteria when inoculated 2.5 µl L^{-1} decreased ammonia nitrogen from 10.0 mg l^{-1} to minimum level (0.34 mg l^{-1}) within 5 days after inoculation (DAI). However, when water is aerated with the same microbial level, the decrease of ammonia nitrogen is very fast (3 DAI). Application of soil in the aquaria has no significant effect in the reduction of ammonia nitrogen. Bacterial inoculums 5 µ l L-1 were found to be significantly superior over the inoculums 2.5 µ l l^{-1}. However, both the doses took 5 DAI to reduce the ammonia nitrogen to minimum level. If, the water is not inoculated with nitrifying bacteria and soil and without aeration, the reduction in ammonia nitrogen to a minimum level took eight DAI. Soil bed without microbial inoculums and aeration do not effect the reduction in ammonia nitrogen at any DAI. However, aeration played a significant role in reducing the ammonia nitrogen. Just passing the air without any application of microbes or soil decreased ammonia-nitrogen to the minimum level in five DAI (two days) more than the microbial plus aeration affects. Interaction of the microbial application and aeration was found to be significant i.e., decrease in ammonia nitrogen to a minimum level was achieved in 3 DAI instead of 5 DAI. Soil interaction with microbial application had not found to be significant at all DAI.

Effect of various treatments on nitrate nitrogen is shown in (Table-2 and Fig-2). Nitrifying bacteria when inoculated 2.5 µ l l^{-1} without soil and aeration increased Nitrate nitrogen from 8.00 mg l^{-1} to maximum level (11.00 mg l^{-1}) at 4 DAI and fluctuation of nitrate concentration was observed on all days onwards. In aerated and inoculated water the increase of nitrate nitrogen to (11.1 mg l^{-1}) took 3 days and it was also found to increase regularly. Bacterial inoculums @ 5 µ l l^{-1} were found to increase significantly over the inoculums' 2.5 µ l l^{-1} with soil bed. Soil bed does not affect the nitrate nitrogen at any DAI. Aeration played a significant role in increasing the nitrate-nitrogen, Nitrate concentration was comparatively very low at any DAI in the tanks without aeration. Interaction of soil and aeration significantly increased the nitrate concentration at any DAI. However, the increase was very low compared with the inoculums of nitrifying bacteria with aeration. Nitrate concentration changed after interaction of microbial inoculums and soil though it was very low. However, the interaction of microbial inoculums and aeration was highly significant at any DAI and increase was observed on all DAI. Nitrate concentration was very low in tanks without aeration. Highly significant increase of nitrate-nitrogen was observed at higher inoculums dose @ 5 μ l l^{-1} with aeration in the absence of soil inoculums.

4. Discussion

Nitrification has been reported to get inhibited in polluted waters (Gruditz and Dalhammar, 2001) [8]. Many commercial probiotics are used now a days especially in shrimp aquaculture *viz*. Epigreen, Epicin, Environ AC Super bug to hasten this process (Pradeep *et al*. 2003 and Prabhu *et al*. 1999) [9, 10]. Many studies were conducted on these commercial probiotics which suggest that they improve the water quality parameters in culture ponds (Li *et al.* 2001 and Shariff *et al.* 2001) [11, 12]. Water probiotics/bioremediators consisting of nitrifiers have been used to remove excess ammonia nitrogen from aquaculture system by Prabhu *et al.*, 1999 [10] Shariff *et al.*, 2001 [12] and Sambasivam *et al*. (2002) [13].

Not only the nitrification process gets inhibited but VanRijn *et al.* (1984) [14] have found very high ammonia nitrogen level in polluted ponds (up to 20 mg l^{-1}). However toxic levels reported for ammonia nitrogen to aquatic life is more than 2-3 mg l^{-1}.Thus, high ammonia nitrogen needs to be corrected in intensive aquaculture or in organically polluted water bodies. High concentration of ammonia causes poor growth and survival of fish and shrimp. Ammonia nitrogen exists in water in two forms: ammonia ion (NH_4^+) and unionized ammonia (NH_3). Both ionized and unionized are toxic to aquatic life. This may happen because unionized form is readily soluble in lipids of cell membrane and fast taken up by the gills. The results from the present investigation suggest that as compared to control, ammonia nitrogen decreased significantly by the remediation with nitrifying bacteria. Grommen *et al.* (2002) [15] have shown that an improved nitrifying enrichment containing suspended nitrifying cells (ammonia binding inoculums liquid, ABIL) @ 5 mg l^{-1} decreased the ammonia concentration from 10 mgl^{-1} to below the detection limit within 4 days. However, in present studies it took 3- 5 days when inoculated @ 5.0 µl l^{-1} with or without aeration respectively. In this study the factors responsible to decrease the ammonia level are: nitrification, quantum of ammonia loss thorough volatilization, Heterotrophic consumption of ammonia and other unknown factors. Nitrifying bacteria not only convert ammonia to nitrate but also reduce carbon dioxide to organic matter (carbohydrate), obviously these chemoautotrophic bacteria use energy released by the oxidation of ammonia to nitrate and reduce carbon dioxide to organic carbon. However the amount of organic matter synthesized by chemoautotrophic bacteria is very small in comparison to the quantum produced by photosynthesis.(Boyd *et al* 1987) [16] In the present investigation, non-aerated treatments remain at very low oxygen levels (around 1.0 mg l^{-1}). All continuously aerated treatments had sufficient oxygen levels. (around 6.0 mg l^{-1}) Nitrogen transformations were recorded to be deferent under non-aerated and aerated conditions. Rate of nitrification was so fast in the aerated aquaria that almost all ammonia nitrogen was oxidized within 3 days. No nitrification could occur in unaerated aquaria and therefore the mineralized nitrogen was accumulated as ammonia ions.

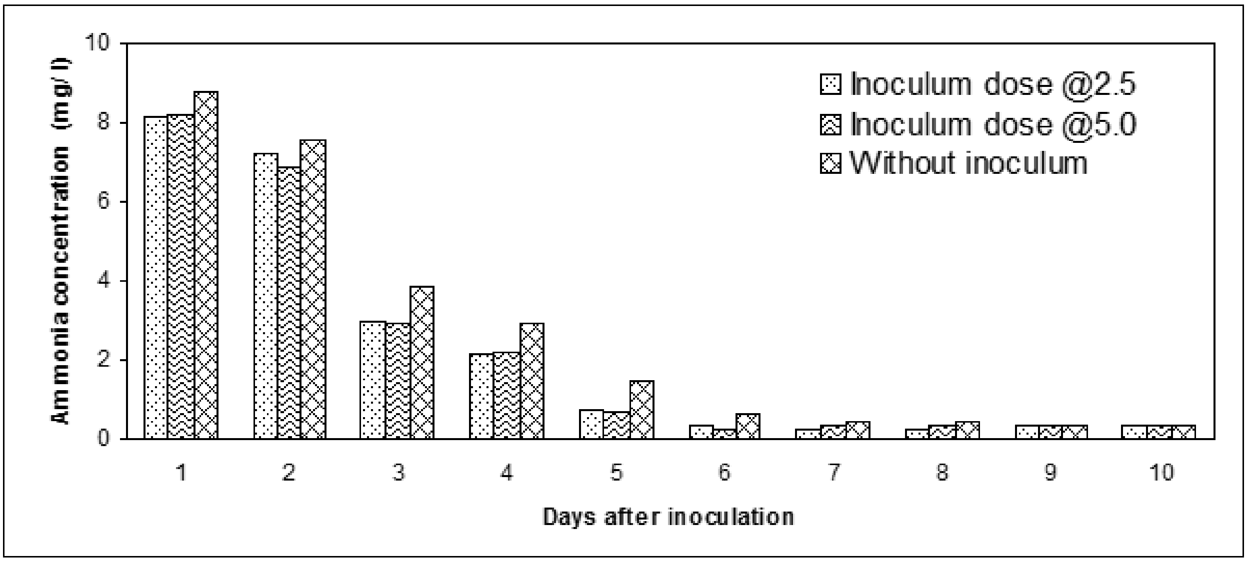
Nitrate is an end product of nitrification and feed for denitrifying heterotrophs. Nitrate is negligibly toxic compared to ammonia and nitrite. It is toxic in aquaculture system when its concentration is beyond 200 mg l^{-1}. Nitrate on reduction converts to nitrogen or back to ammonia. The pathway which leads to the formation of gaseous nitrogen, is the most ideal in a culture system, as in the ammonia production which otherwise tends to enhance the toxicity further. Such organisms can also be mass-produced and introduced in to the system. According to Bohn *et al.* (2001) [17] under low oxygen conditions nitrate nitrogen (NO_3-N) can be used as alternative electron acceptor by heterotrophs. Present investigation also reveals that initial nitrate levels were very low in the polluted water. This is due to poor nitrification (as a result of poor nitrifiers' population, poor dissolved oxygen and high COD, which slowly improved because of nitrifier's inoculation and aeration. Consequently denitrification also gets held up because of aerobic heterotrophs thereby buffering nitrogen status (Table-2 and Fig. 2). Higher nitrate nitrogen recovery in aerated aquaria in comparison to unaerated aquaria further confirms higher nitrification rate with sufficient dissolved oxygen. Nitrification-denitrification however, reaches to equilibrium in all aerated and non-aerated aquaria after 3 to 8 day's (Table-2 and Fig. 2) It is probable that nitrification-denitrification sequence occurred in all aquaria.

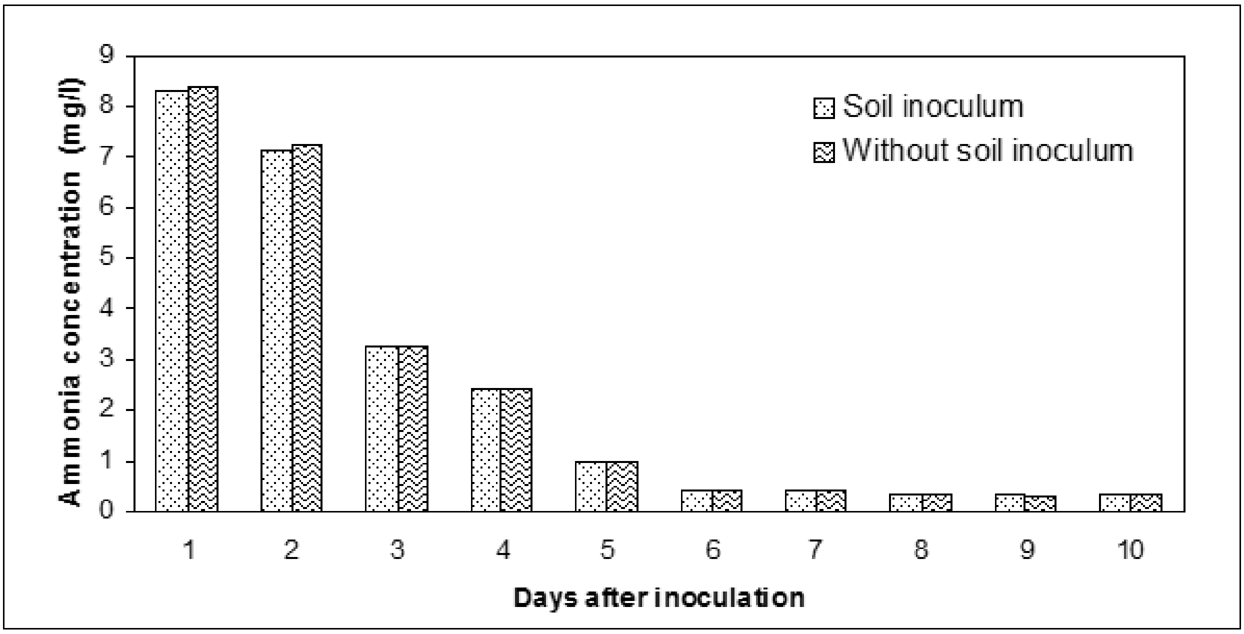
Table 1: Changes in ammonia nitrogen concentration at various days after inoculation in simulated pond systems

Effect of treatments	Ammonia nitrogen concentration(mgI-^{1})									
	Days After Inoculation									
	1	2	3	4	5	6	7	8	9	10
T1= I1+ S1+A1	7.58	6.7	0.31	0.28	0.33	0.3	0.3	0.28	0.28	0.3
T2=I1+S1+A2	8.56	7.5	5.6	4	1.15	0.33	0.34	0.33	0.32	0.33
T3= I1+ S2+A1	7.8	7	0.34	0.28	0.33	0.32	0.32	0.29	0.28	0.3
T4= I1+S2+ A2	8.4	7.5	5.56	4	1.14	0.34	0.34	0.3	0.33	0.33
T5=I2+S1+A1	7.62	6.3	0.33	0.33	0.28	0.22	0.3	0.28	0.28	0.3
T6=I2+S1+A2	8.56	7.2	5.56	4.11	1.15	0.36	0.33	0.3	0.33	0.32
T7= I2+S1+A1	7.8	6.46	0.34	0.33	0.3	0.24	0.32	0.29	0.28	0.3
T8= I2+S2+A2	8.64	7.5	5.48	4.12	1.16	0.36	0.33	0.33	0.3	0.32
T9= I3+S1+A1	8.56	7.3	0.7	0.56	0.38	0.36	0.33	0.34	0.33	0.3
T10= I3+S1+A2	8.9	7.8	6.8	5.28	2.6	0.9	0.78	0.56	0.34	0.33
T11= I3+S2+A1	8.6	7.3	0.89	0.58	0.38	0.36	0.34	0.33	0.32	0.3
T12=I3+S2+A2	8.96	7.84	6.83	5.26	2.57	0.89	0.8	0.56	0.33	0.33
SEM+_	0.0073	0.0071	0.0097	0.0058	0.0058	0.0072	0.0043	0.005	0.0058	0.0041
CD(5%)	0.023	0.0218	0.0298	0.17	0.178	0.0221	0.0132	0.0154	0.178	0.0126
Cumulative Effect of Microbial inoculation										
I1	8.085	7.175	2.954	2.14	0.737	0.323	0.326	0.293	0.302	0.315
I2	8.115	6.865	2.927	2.222	0.722	0.295	0.302	0.3	0.298	0.31

I3	8.775	7.565	3.825	2.92	1.482	0.629	0.563	0.448	0.336	0.315
SEM+_	0.0038	0.0035	0.0048	0.0029	0.0029	0.0036	0.0022	0.0025	0.0029	0.002
CD(5%)	0.01165	0.0107	0.0147	0.0089	0.0089	0.0110	0.0067	0.0077	0.0089	0.0061
Cumulative Effect of soil inoculation										
S1	8.297	7.137	3.231	2.427	0.982	0.412	0.398	0.343	0.313	0.313
S2	8.367	7.267	3.24	2.428	0.98	0.419	0.408	0.35	0.307	0.313
SEM+_	0.0031	0.0029	0.004	0.0024	0.0024	0.0029	0.0018	0.002	0.0024	0.0017
CD(5%)	0.0095	0.0089	0.0012	0.0073	0.0073	0.0089	0.0055	0.0061	0.0073	0.0052
Cumulative Effect of aeration										
A1	7.993	6.843	0.499	0.393	0.333	0.301	0.318	0.302	0.295	0.3
A2	8.67	7.56	5.972	4.462	1.628	0.53	0.488	0.392	0.325	0.327
SEM+_	0.0031	0.0029	0.004	0.0024	0.0024	0.0029	0.0018	0.002	0.0024	0.0017
CD(5%)	0.0095	0.0089	0.0123	0.0073	0.0073	0.0089	0.0055	0.0061	0.0073	0.0052
Interaction of microbial inoculums and aeration effect										
I1+A1	7.69	6.85	0.328	0.28	0.33	0.31	0.31	0.285	0.28	0.3
I1+A2	8.48	7.5	5.58	4	1.145	0.335	0.343	0.3	0.325	0.33
I2+A1	7.71	6.38	0.335	0.33	0.29	0.23	0.31	0.285	0.28	0.3
I2+A2	8.6	7.35	5.52	4.115	1.155	0.36	0.33	0.315	0.315	0.32
I3+A1	8.58	7.3	0.835	0.57	0.38	0.363	0.335	0.335	0.325	0.3
I3+A2	8.93	7.83	6.815	0.27	2.585	0.895	0.79	0.56	0.335	0.33
SEM+_	0.0054	0.005	0.0068	0.0041	0.0041	0.0051	0.0031	0.0035	0.0041	0.0029
CD(5%)	0.0166	0.0154	0.0209	0.0126	0.0126	0.0157	0.0095	0.0107	0.0126	0.0089

I1= Inoculam@2.5µL-1,I2= Inoculam@5µL-1,[I] I3= Uninoculated, S1=Soil Inoculum@100g soil/Aquarium, S2=Without soil, A1= Aeration,A2= without aeration





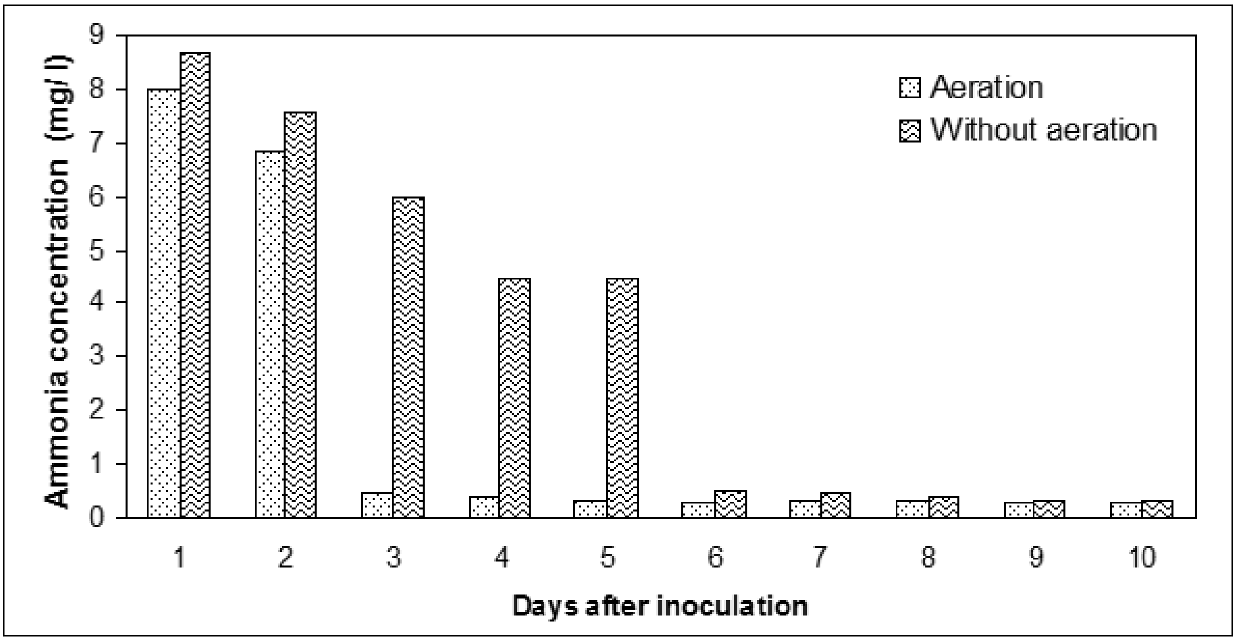


Fig 1: Changes in Ammonia nitrogen concentration at various days after inoculation in simulated pond eco-system

Table 2: Changes in nitrate nitrogen concentration at various days after inoculation in Simulated pond systems

Effect of treatments	**Nitrate nitrogen concentration (mg l^{-1})**							
	Days after inoculation							
	3	4	5	6	7	8	9	10
T1= I1+ S1+A1	11.2	12.14	12.2	12.31	12.56	12.7	13.1	13.2
T2=I1+S1+A2	8.28	11.2	10.2	10.02	10.27	10.28	10.2	10.1
T3= I1+ S2+A1	11.2	12.2	12.3	12.34	12.48	12.68	13.12	13.26
T4= I1+S2+ A2	8.3	11	10.2	10.09	10.28	10.33	10	10.2
T5=I2+S1+A1	11.28	12.2	12.5	12.5	12.5	12.8	13.12	13.24
T6=I2+S1+A2	8.26	11.14	10.3	10.02	10.28	10.3	10.2	10
T7= I2+S1+A1	11	12.3	12.6	12.56	12.46	12.7	13.16	13.26
T8= I2+S2+A2	8.3	11.2	10.24	10.2	10.2	10.33	10.22	10.2
T9= I3+S1+A1	10.2	11.2	11.205	11.56	11.14	11.14	11.3	11.8
T10= I3+S1+A2	8	9.8	10.2	10.2	10.2	10.33	10.2	10.2
T11= I3+S2+A1	10.05	11	11.3	11.2	11.02	11.2	11.2	11.6
T12=I3+S2+A2	8.2	9.6	10.3	10	10.28	10.3	10.2	10.6
SEM+_	0.016	0.005	0.006	0.0058	0.0065	0.0058	NS	0.005
CD(5%)	0.049	0.0154	0.0184	0.178	0.02	0.178	NS	0.0154
Cumulative Effect of Microbial inoculation								
I1	9.695	10.4	11.225	11.19	11.397	11.497	11.605	11.69
I2	9.71	11.71	11.41	11.32	11.36	11.533	11.675	11.675
I3	9.113	10.4	10.751	10.74	10.66	10.742	10.725	10.99
SEM+_	0.0079	0.0025	0.003	0.0029	0.0032	0.0029	NS	0.0025
CD(5%)	0.0243	0.0077	0.0092	0.0089	0.0098	0.0089	NS	0.0077
Cumulative Effect of soil inoculation								
S1	9.537	11.28	11.101	11.102	11.158	11.258	11.353	11.423
S2	9.475	11.217	11.157	11.065	11.12	11.257	11.317	11.48
SEM+_	0.0065	0.002	0.0024	0.0024	0.0026	0.0024	NS	0.002
CD(5%)	0.02	0.0061	0.0073	0.0073	0.008	0.0073	NS	0.0061
Cumulative Effect of aeration								
A1	10.788	11.84	12.018	12.078	12.027	12.203	12.5	12.727
A2	8.223	10.657	10.24	10.008	10.252	10.312	10.17	10.177
SEM+_	0.0065	0.002	0.0024	0.0024	0.0026	0.0024	NS	0.002
CD(5%)	0.02	0.0061	0.0073	0.0073	0.008	0.0073	NS	0.0061
Interaction of microbial inoculums and aeration effect								
I1+A1	11.1	12.17	12.25	12.325	12.52	12.69	13.11	13.25
I1+A2	8.29	11.1	10.2	11.055	10.275	10.305	10.1	10.15
I2+A1	11.14	12.25	12.55	12.53	12.48	12.75	13.14	13.25
I2+A2	8.28	11.17	10.27	10.11	10.24	10.315	10.21	10.1
I3+A1	10.125	11.1	11.253	11.38	11.08	11.17	11.25	11.7
I3+A2	8.1	9.7	10.25	10.1	10.24	10.315	10.2	10.28
SEM+_	0.0345	0.0035	0.0034	0.0041	0.0046	0.0041	NS	0.0035
CD(5%)	0.028	0.0107	0.0104	0.0124	0.0141	0.0126	NS	0.0107

I1= Inoculam@2.5µL-1,I2= Inoculam@5µL-1,[I] I3= Uninoculated, S1=Soil Inoculum@100g soil/Aquarium, S2=without soil, A1= Aeration, A2= without aeration

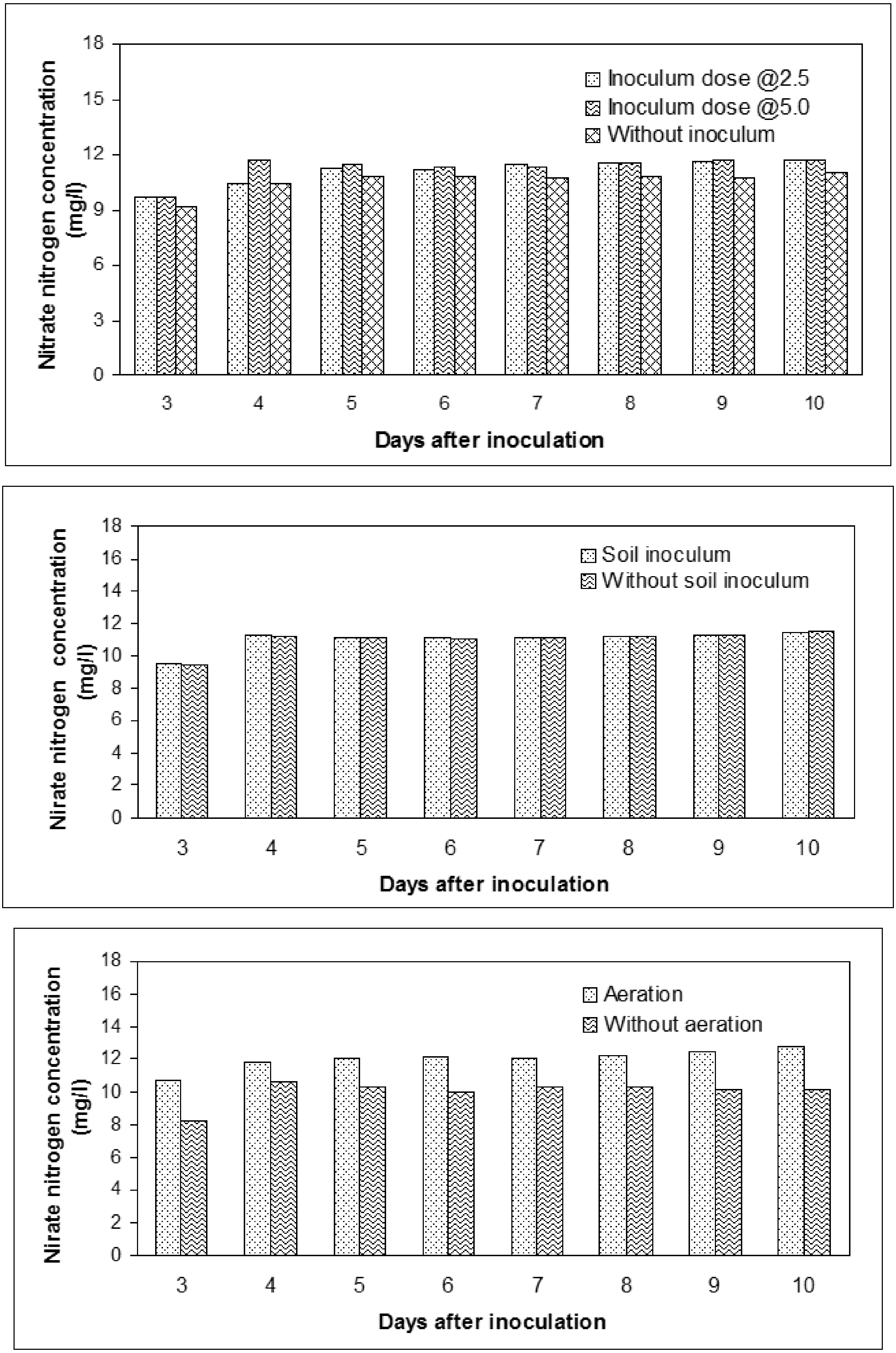


Fig 2: Changes in Nitrate- nitrogen concentration at various days after inoculation in simulated pond system

5. References

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