

Original Research Article

<https://doi.org/10.20546/ijcmas.2017.612.354>

Bioremediation of High Strength Ammonia Laden Fertilizer Industry Effluent with Extremely Low C: N Ratio Using Simultaneous Aerobic Nitrification and Denitrification

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ABSTRACT

Keywords

Simultaneous nitrification and denitrification, Industrial effluent, Ammonia oxidizing bacteria, Nitrite oxidizing bacteria, Aerobic denitrifiers, Wastewater treatment.

Article Info

Accepted:

21 October 2017

Available Online:

10 December 2017

In the present study, autotrophic ammonia- and nitrite- oxidizing bacteria (AOB and NOB respectively) were enriched and methodically added to treat ammonia laden effluent (700 ppm) of a fertilizer industry with exceedingly low C: N ratio. An open aerobic reactor system of 5.3 L was developed and run in the batch mode for the first 30 days to acclimatize the biomass with increasing effluent concentration. A linear correlation was observed during the batch mode between the amount of ammonia oxidized and the ammonia loading rate (R^2 0.79). On making the system continuous, 99% NH_4^+ -N removal was achieved along with complete utilization of indigenous carbon without accumulation of NO_2^- -N and NO_3^- -N. Molecular microbial analysis showed presence of AOB belonging to *Nitrosomonas* sp., NOB belonging to *Nitrobacter* sp. and aerobic denitrifiers mainly *Achromobacter* sp., *Sphingomonas* sp., *Pusillimonas* sp., *Mesorhizobium* sp. and *Alcaligenes* sp. coexisting in the system. Their concerted growth and simultaneous aerobic nitrification denitrification (SND) activity led to efficient removal of nutrients from the effluent and suggested high potential of the developed system in bioremediating industrial effluents with low C: N ratio.

Introduction

India is amongst the top two farm producers in the world implying that agriculture plays a vital role in India's economy (Arjun, 2013). Soil yield depends on nitrogen, phosphorous and potassium (fertilizing elements) and Indian soil which is generally deficient in these elements requires them to be given externally. Owing to the increased demand for fertilizers, a rapid growth has been observed in the fertilizer industries (Smriti Chan, Growth and Distribution of Fertilizers

Industry in India, article). These industries generally lack proper waste management systems. Untreated wastes when channeled into aquatic environment causes extensive damage to the water quality and ecology of the environment specifically when microbial degradation activities cannot cope up with the fast removal of the toxic pollutants (Obire *et al.*, 2008). Very few bioremediation studies have been carried out for the treatment of wastewater from fertilizer industry. George *et*

al., (1997) used immobilized urease enzyme to develop an efficient system that could operate at lower residence time but its application at large scale would become a costly affair. Hence, there is an urgent need to develop microbial systems that can sustain toxic compounds and can lead to rapid, cost effective remediation of the waste generated from fertilizer industries.

Recently, unusual behavior of nitrifiers and denitrifiers has been observed. Production of nitrous oxide by autotrophic ammonia oxidizing bacteria (AOB) through nitrifier denitrification had been reported by Hayatsu *et al.*, (2008). Denitrifying enzymes are sensitive to oxygen, was a long established fact but lately *Paracoccus denitrificans*, *Thiosphaera pantotropa*, *Mesorhizobium sp.*, *Burkholderia cepacia* and others are reported to denitrify under aerobic conditions (Hayatsu *et al.*, 2008). This indicates that conversion of ammonia to molecular nitrogen can be carried out either by a single kind of bacteria or nitrification and denitrification can occur concurrently in a single reactor under identical operating conditions (Daigger and Littleton, 2000). In a study carried out by Keluskar *et al.*, (2013), partial nitrification, anammox and denitrification was employed for the removal of ammonia from fertilizer industry under anoxic conditions. Anammox bacteria have very slow grown rate compared to heterotrophs and other autotrophic ammonia oxidizing microorganisms (Strous *et al.*, 1999). Therefore, application of this process (though highly efficient) at large scale would be time consuming especially during the start-up phase. Very few studies have focused on the use of nitrification along with aerobic denitrification for the treatment of high strength ammonia containing wastewater (Gupta and Gupta, 2001).

The objective of the present study was to develop a microbial system for

bioremediating ammonia laden effluent from a fertilizer industry through simultaneous aerobic nitrification and denitrification. The developed system contained AOB, NOB and aerobic denitrifiers which collectively removed nutrients from the effluent without accumulating nitrite and nitrate.

Materials and Methods

Enrichment of autotrophic ammonia and nitrite oxidizing bacteria (AOB and NOB)

Enrichment of AOB and NOB was carried out from different soil and sludge samples by inoculating 5% sample in inorganic medium described by Hyman and Arp (1992) for AOB and for NOB, ammonium sulphate in the media was replaced with 0.1% sodium nitrite. They were incubated in a shaker incubator at 30 °C temperature for 1 month and were subcultured in the same media to examine their ammonia and nitrite oxidizing activity. Ammonia oxidizing activity was measured in terms of ammonia removed and nitrite produced and similarly for nitrite oxidizing activity, nitrite removed and nitrate formation was measured. Samples showing maximum activity were selected amongst the enriched AOB and NOB.

Characteristics of the fertilizer industry effluent

Fertilizer industry effluent was collected from site and brought to the experimental laboratory in a closed container and stored at 4° C till the end of the study. The effluent contained high ammonia concentration ($\text{NH}_4^+\text{-N}$: 675 ± 38 mg/L), whereas $\text{NO}_2^-\text{-N}$ was around 41 ± 1.1 mg/L and $\text{NO}_3^-\text{-N}$ was below detectable limit. pH of the effluent was high (pH 9.6). Biological oxygen demand (BOD), chemical oxygen demand (COD) and dissolved oxygen (DO) were 29 mg/L, 46.66 mg/L and 8 mg/L respectively. The effluent

also contained metals like nickel (Ni) 1.16 mg/L and iron (Fe) 0.07 mg/L.

SND type reactor set-up and operational conditions

A 5.3 L open aerobic reactor (16 x 26 x 39 cm) was operated continuously for the removal of ammonia from effluent of a fertilizer company. A settler tank was attached to the reactor at 45° angle (Figure 1). The reactor was kept on a magnetic stirrer for agitation at 240 rpm. Dissolved oxygen (DO) in the reactor [measured by YSI DO meter (YSI200 portable DO meter, USA)] was also maintained using an external aerator. Aeration and agitation were switched on and off intermittently at 24 h interval. Enriched seed biomass was added such that the MLVSS in the reactor at the start of the run was 8.2 g/L. The reactor was run initially in the batch mode for first 30 days and was then made continuous with a flow rate of 32.7 ml/h for the next 45 days. Levels of ammonia, nitrite, nitrate, pH, DO, mixed liquor volatile suspended solids (MLVSS), mixed liquor suspended solids (MLSS), sludge volume index (SVI) were measured as per the methods described in APHA standard methods (APHA, 2005). Chemical oxygen demand (COD) was measured according to IS 3025 (Part 58): (2006). Ammonia conversion efficiency was calculated according to Daverey *et al.*, (2012).

Evaluation of the microbial biomass developed in the reactor

Microorganisms present in the reactor were identified by 16S rRNA gene amplification, cloning and sequencing. Sludge (1ml) was taken from the settler tank and centrifuged at 19,200 X g for 15 min. DNA was extracted according to Schmidt *et al.*, (1991). 16S rRNA gene was amplified using universal primer 27F 5' AGAGTTTGATCCTGGCT

CAG 3' and 1541R 5' AAGGAGGTGATC CAGCCGCA 3' according to Keluskar *et al.*, (2013) and cloned in pTZ57R/T vector, using INSTA cloning kit (Fermentas). From the 16S rRNA gene clone library, 50 clones were randomly selected and subjected to amplified ribosomal DNA restriction analysis (ARDRA) using *AluI* restriction enzyme to avoid redundant sequencing.

Nine different patterns were observed through restriction analysis and representative 16S rRNA gene of the 9 different patterns (data not shown) was sequenced commercially by Single Pass Analysis (Bangalore Genei, India). The sequences determined in the study were submitted in GenBank (accession numbers KY704099 to KY704107). Blast analysis of the obtained sequences was carried out to know the identity of the microorganisms.

Results and Discussion

Development of seed consortium

AOB and NOB were enriched individually from different soil and sludge samples. Amongst the enriched AOB and NOB, PF (paddy field soil sample) showed maximum ammonia oxidizing activity (216 ± 0.3 mg/L NH_4^+ -N removed and 103.68 ± 0.18 mg/L NO_2^- -N produced) as well as nitrite oxidizing activity (1.094 ± 0.22 mg/ml NO_2^- -N removed and 0.67 ± 0.054 mg/ml NO_3^- -N formed) in their respective media and were therefore selected for further studies (Table 1). A longer lag period was observed in the nitrite oxidizing activity of PF-NOB whereas PF-AOB showed increase in ammonia oxidizing activity almost parallel to its growth (Figure 2A and B respectively). Specific growth rate constants for the PF-NOB and PF-AOB enriched biomass were 0.384 and 1.24 with doubling time of 1.8 and 0.56 days respectively. A seed consortium was prepared

for inoculating the aerobic reactor by mixing AOB and NOB in the ratio 1:4.

Removal of ammonia from fertilizer industry effluent

A 5.3 L open aerobic reactor was designed for the simultaneous growth of AOB and NOB. The reactor was run in the batch mode for first 30 days with increasing NH_4^+ -N load. Four dilutions (1:5, 1:3, 1:1 and 1:0.5) of the effluent were used to increase NH_4^+ -N load in the influent (Figure 3A). Around 90% NH_4^+ -N removal was achieved without significant build-up of NO_2^- -N and NO_3^- -N along with 82.68 mg/L biomass accumulation by the end of the run in the batch mode (Figure 3A, B and C). A linear correlation (R^2 0.799) existed between the ammonia load and the amount of ammonia removed in the system (Figure 4A).

During the start of the continuous mode, some amount of biomass was washed away and the MLVSS value dropped to 31.56 mg/L which then steadily increased to around 116 mg/L by 55th day and remained constant thereafter, with more than 90% NH_4^+ -N removal (Figure 3A and C). By the end of the run, 99% reduction in the level of NH_4^+ -N was observed (Figure 3A). A distinct drop in the pH from 8.0 to 6.8 of the system, during the batch mode, was indicative of the nitrifying activity in the reactor. Ammonia conversion efficiency was between 56.7 to 28% during this period also suggesting nitrifying activity in the system (Figure 4B). During continuous mode of operation, pH of the system remained between pH 7.4 to 7.8 (Figure 3C). Level of COD of the effluent reduced from 46 mg/L to below detectable limits (p-value < 0.005). Ammonia conversion efficiency was very low during the steady state (56-75th day) which suggested high denitrifying activity during this period (Figure 4B). Nitrogen formed in the system could not be monitored

as it was an open reactor. Dissolved oxygen concentration fluctuated between 7.6 and 7.9 mg/L during the aeration and agitation period whereas it decreased to 5.7 ± 0.4 mg/L in the off mode. SVI and food to mass ratio of the system were 22.4 ml/g and 0.45 respectively.

Molecular microbial analysis showed presence of nitrifying and denitrifying bacteria in the system. Amongst the identified microorganisms, two groups of AOB were present which had sequence identity with *Nitrosomonas* sp. ENI11 (99%) and *Nitrosomonas* sp. DYS317 (99%). NOB in the system belonged to *Nitrobacter* sp. (99%). Presence of heterotrophic denitrifiers like *Achromobacter* sp., (99%), *Sphingomonas* sp. (99%), *Pusillimonas* sp. (99%), *Mesorhizobium* sp. (99%) and *Alcaligenes* sp. (99%) was also observed in the system.

Wastewater from the fertilizer industry contained very high levels of ammonical nitrogen and negligible amounts of organic carbon owing to which nitrifying microbial community were employed for its treatment. A syntrophic association is known to exist between AOB and autotrophic nitrite oxidizing bacteria (NOB) and they can therefore coexist in biofilms or in aggregates in wastewater treatment plants (Schramm *et al.*, 1999; Sliemers *et al.*, 2005; Siripong and Rittmann, 2007). AOB are described to be present in the outer regions of the biofilms or aggregates and provide nitrite (nitrogen source) to NOB residing in their close vicinity in the inner portion of the biofilms or aggregates (Schramm *et al.*, 1999). Keeping this in mind a seed consortium containing AOB and NOB was developed. As AOB and NOB enriched from PF (paddy field soil sample) showed highest nitrifying activity, they were selected for seed consortium development (Table 1).

Table.1 Ammonia and nitrite oxidizing activity of enriched AOB and NOB measured after 1 week of incubation from varied sources

Sr. No.	Sample	Description	Enrichment of AOB		Enrichment of NOB	
			NH ₄ ⁺ -N removed (mg/L)	NO ₂ ⁻ -N formed (mg/L)	NO ₂ ⁻ -N removed (mg/ml)	NO ₃ ⁻ -N formed (mg/ml)
1	N1	Common effluent treatment plant, Nandesari	17.1±0.25	8.0±0.02	1.092±0.05	0.53±0.08
2	N2	Sludge from Nandesari (Deepak nitrite)	4.67±0.07	2.9±0.04	0.22±0.03	0.31±0.06
3	N4	(Municipal waste water treatment plant, Nandesari)	186±0.091*	99.5±0.87*	0.27±0.09	0.2±0.023
4	GSFC	Fertilizer industry sludge sample, Vadodara,	18.55±0.86	12.0±0.12	0.0±0.002	0.12±0.09
5	DNRB	(DNR-B reactor), Sludge sample GNFC, Bharuch,	134±0.5*	97.3±0.19*	1.092±0.14	0.45±0.06
6	WC	Winogradsky's column	10.97±0.49	3.6±0.23	1.092±0.54*	0.62±0.103*
7	NW	Narol, wheat bulk soil	3.2±0.082	0.11±0.05	1.093±0.2	0.54±0.014
8	PF	Mandya, paddy field soil	216±0.3*	103.68±0.18*	1.094±0.22*	0.67±0.054*
9	ECP	Jowar soil (Effluent channel project) Ahemdabad,	5.8±0.037	0.13±0.021	1.093±0.9*	0.61±0.043*
10	VW	Wheat soil, Vinjal	13.94±0.56	0.12±0.003	1.092±0.19	0.55±0.108

*indicates significant reduction in ammonia or nitrite concentrations and nitrite or nitrate formation. (p<0.05)

Fig.1 A 5.3 L open reactor used in the study for the treatment of fertilizer industry effluent and the sludge retained in the settler

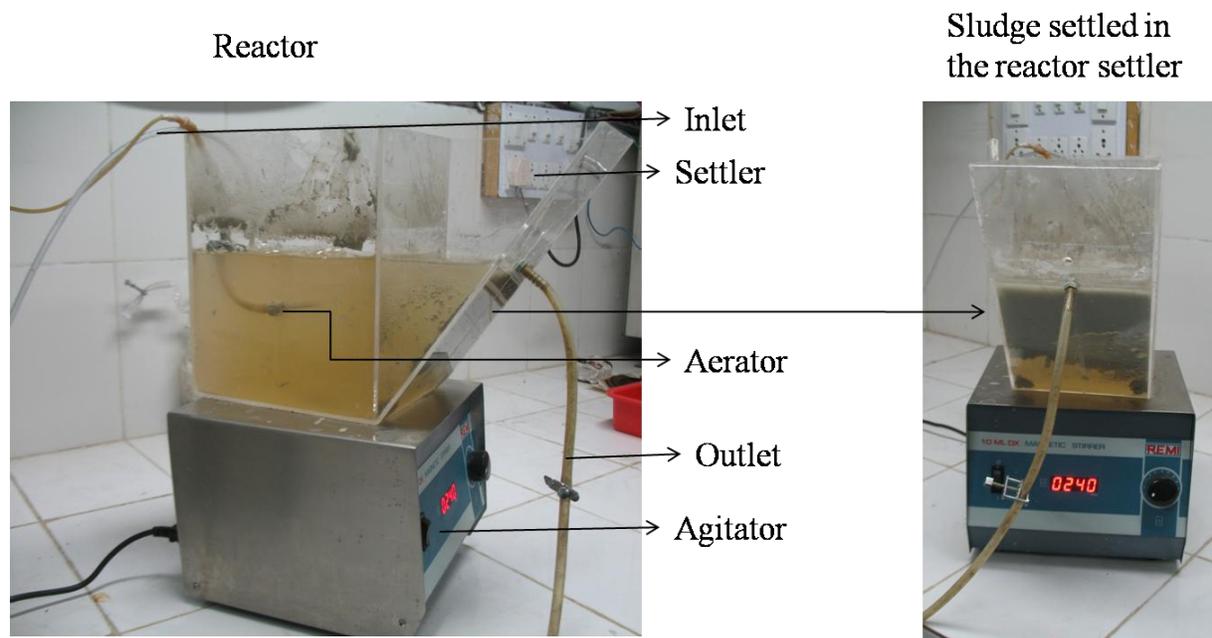


Fig.2 Growth and nitrifying activity of enriched biomass A) PF-NOB B) PF-AOB

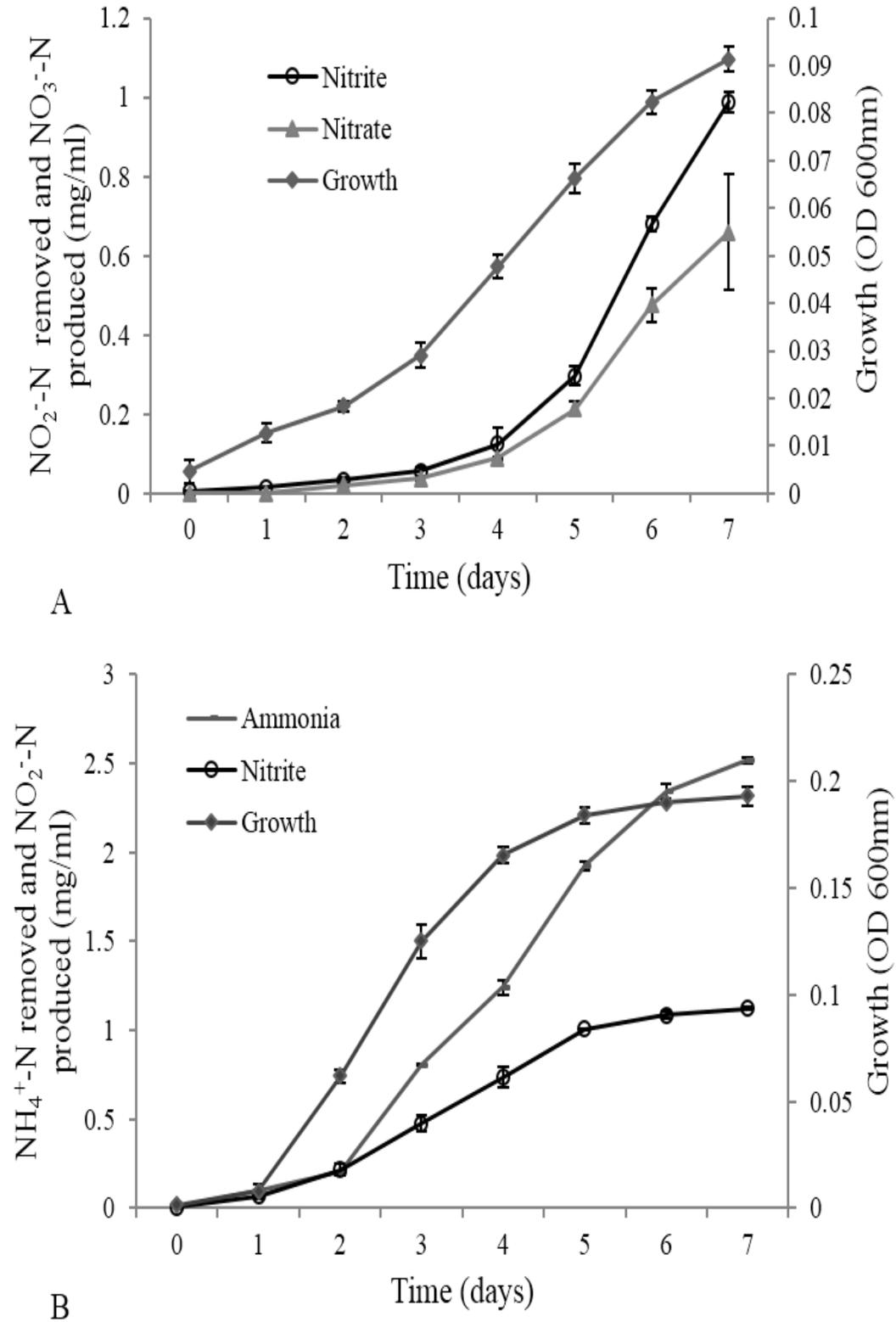


Fig.3 Nitrogen removal performance by the SND type reactor. Change in the levels of A) $\text{NH}_4^+\text{-N}$ and Ammonia removing efficiency B) $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ C) pH and MLVSS. Values mentioned in the box are the ratio of effluent dilution with inorganic media without nitrite or ammonia

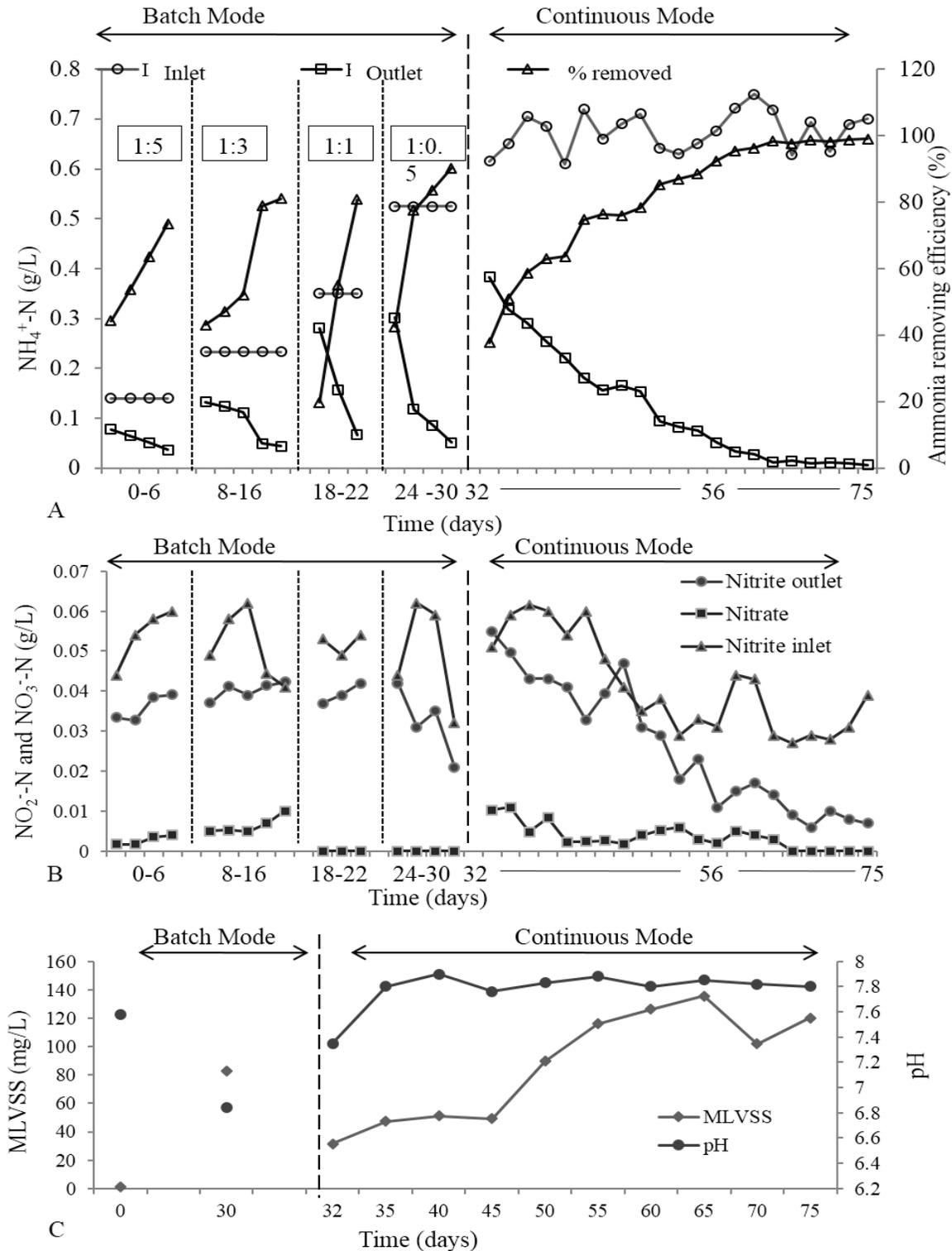
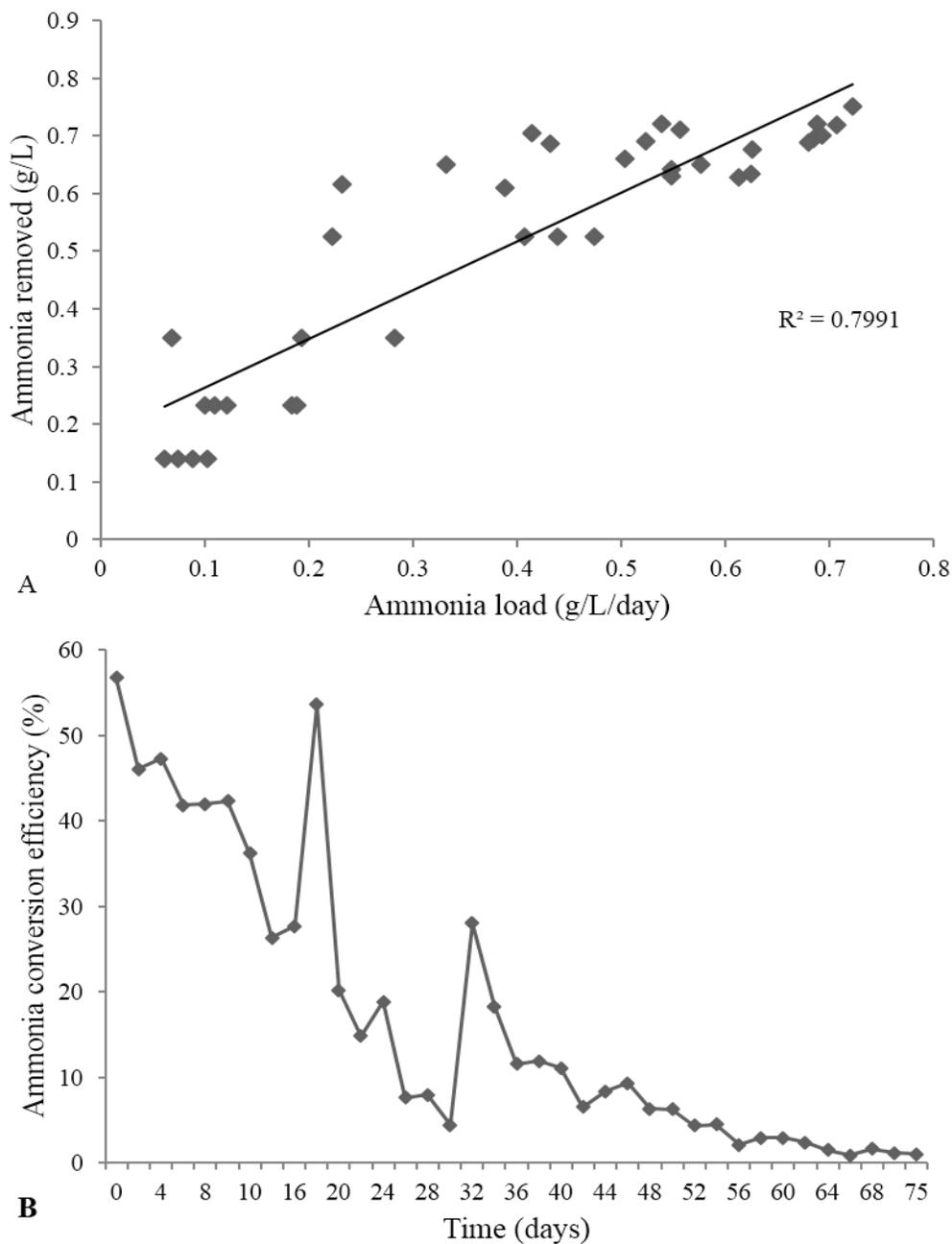


Fig.4 Stability and efficiency of the system A) Correlation between the concentration of ammonia oxidized (g/L) to the applied ammonia load (g/L/day). B) Ammonia conversion efficiency



The growth of PF-AOB (specific growth rate constant 1.24 and doubling time of 0.56) was 3.214 times higher than that of PF-NOB

(specific growth rate constant 0.384 and doubling time of 1.8 days). To get optimal removal of ammonia from the effluent, they

were mixed such that NOB/AOB ratio was 4. This was in the range 1.72 - 5.87 reported in wastewater treatment plants and aerobic granular sludge (Mari *et al.*, 2012; Yao and Peng, 2017).

In municipal wastewater treatment plants, Harms *et al.*, (2003) found that the number of NOB could be three times higher than AOB. However, there are reports which state that for balanced nitrifying system ratio of NOB: AOB should be 1:2- 1:2.7 (Mari *et al.*, 2012).

Ammonium removal from fertilizer industry effluent

The seed consortia were then exploited for their ability to remediate high strength ammonia containing effluent from fertilizer industry in an open aerobic reactor. Oxygen and ammonia concentration play an important in co-culturing AOB and NOB.

In the presence of low oxygen concentrations, co-cultures of AOB and NOB is possible but levels of ammonia in the system should also be low (Sliemers *et al.*, 2005). In the presence of high ammonia and low oxygen concentrations, NOB would be washed out of the reactors and ammonium would be converted to mainly nitrite, nitric oxide and nitrous oxide by *Nitrosomonas* sp. and other related bacteria (Sliemers *et al.*, 2005; Keluskar *et al.*, 2013). Ammonia levels were exceedingly high in the fertilizer effluent used in the present study, hence for simultaneous growth of AOB and NOB under identical conditions in a single reactor, oxygen concentration was also kept high to allow stable nitrification to occur in the system.

Results indicate that the developed aerobic suspended growth in the reactor system was highly efficient in removing ammonia up to 99% and indigenous carbon from the effluent without accumulating nitrite or nitrate (Figure 3). It was therefore interesting to know the microorganisms present in the system as it would shed light on the metabolic activities

going on in the reactor. Blast analysis of 16S rRNA gene showed presence of AOB belonging to *Nitrosomonas* sp. which are mainly responsible for the reduction in the level of ammonia from the system. Presence of NOB, belonging to *Nitrobacter* sp., was also observed in the reactor.

They utilized nitrite present in the system and hence accumulation of nitrite was not observed (Figure 3B). Amongst NOB, *Nitrobacter* sp. are reported to have higher growth rate and affinity for nitrite and oxygen compared to other NOB like *Nitrospira* sp. (Schramm *et al.*, 1991; Nogueira and Melo, 2006). Thus *Nitrobacter* sp. might have enriched from the paddy field soil sample and were consequently present in the system. Apart from AOB and NOB heterotrophic aerobic denitrifiers like *Achromobacter* sp., *Sphingomonas* sp., *Pusillimonas* sp., *Mesorhizobium* sp. and *Alcaligenes* sp. were also found to be present in the reactor (Patureau *et al.*, 2000; Hayatsu *et al.*, 2008; Srinivasan *et al.*, 2010).

Achromobacter sp., is reported to denitrify at DO levels between 3 to 10 ppm (Zhu *et al.*, 2012). These denitrifiers are commonly found in wastewater treatment plants and can also carry out heterotrophic nitrification (Hayatsu *et al.*, 2008). COD and DO play an important role in the denitrifying performance of reactor (Van Rijn *et al.*, 2006). Heterotrophic denitrifiers utilize nitrite and nitrate produced by AOB and NOB as nitrogen and organic matter present in the effluent as carbon source. Therefore nitrite and nitrate accumulation was not observed and COD reduced from 46 mg/L to below detectable limits. Dependence of denitrifiers on the internal carbon source also restricts over growth of denitrifiers and helps in maintaining stable conditions in the reactor. High dissolved oxygen observed during the aeration and agitation period would favor nitrification whereas low DO observed during the off mode would favor aerobic denitrification, thus maintaining conditions for both the reactions to occur simultaneously in the same reactor. High HRT provided sufficient time for both

nitrification and aerobic denitrification to occur effectively. Sludge settleability is an essential parameter in wastewater treatment plants (Tchobanoglous *et al.*, 2003). Higher sludge retention can result in flocs of larger size and density which is extremely desirable for the SND process to proceed steadily (Zaizon, 2012). Settler tank attached to the aerobic reactor helped in retaining microorganisms in the present study. Compact sludge formed during the process (SVI was 22.4 ml/g). Flocs formed in the reactor settled on the walls of the settler (Figure 1) and clear, odorless treated effluent was drained out of the reactor. Food to mass ratio in the present study was 0.45, which was in the range (0.2 and 0.6) reported for maintaining a proper balance in the system (Tchobanoglous *et al.*, 2003). As the amount of food is less than the microorganisms, the C and N present in the system would be immediately utilized.

Efficient simultaneous removal of nutrients was achieved with the concerted growth and activity AOB, NOB and denitrifiers in the reactor without addition of an external carbon source. Aerobic SND processes thus have high potential to be effectively applied in the bioremediation of industrial effluent with extremely high ammonia concentrations. The only limiting factor in its application at large scale is the cost for aeration.

Acknowledgements

The author would like to thank Prof. Anjana Desai for her valuable guidance.

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How to cite this article:

Radhika Priyadarshan Keluskar. 2017. Bioremediation of High Strength Ammonia Laden Fertilizer Industry Effluent With Extremely Low C: N Ratio Using Simultaneous Aerobic Nitrification and Denitrification. *Int.J.Curr.Microbiol.App.Sci*. 6(12): 3029-3039.
doi: <https://doi.org/10.20546/ijcmas.2017.612.354>