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Effect of potential electron acceptors on anoxic ammonia oxidation in the presence of organic carbon

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ABSTRACT

A novel route of anoxic ammonia removal in the presence of organic carbon was identified recently from ecosystems contaminated with ammonia. Sequencing batch reactor (SBR) studies were carried out in anoxic condition at oxidation–reduction potential varied from −185 to −275 mV for anoxic ammonia oxidation with adapted biomass (mixed culture). SBR studies were carried out in absence and in the presence of externally added organic carbon and/or in the presence of inorganic electron acceptors like NO_2^-, NO_3^- and SO_4^{2-}. The results showed anoxic ammonia oxidation to nitrate (in contrast to reported anammox process) in the presence of organic carbon available through endogenous respiration whereas anoxic ammonia oxidation was effective in the presence of externally added organic compound for nitrogen removal. The presence of externally added inorganic electron acceptors like NO_2^-, NO_3^- and SO_4^{2-} was effective in anoxic ammonia oxidation, but failed to follow the reported anammox reaction's stoichiometry in nitrogen removal in the presence of organic carbon. However, the presence of NO_2^- affected best in total nitrogen removal compared to other electron acceptors and maximum ammonia removal rate was 100 mg NH_4^+/g MLVSS/d. Based on the results, it is possible to suggest that rate of anoxic ammonia oxidation depends up on the respiration activities of mixed culture involving organic carbon, NO_2^-, NO_3^- and SO_4^{2-}. The process shows possibilities of new pathways of ammonia oxidation in organic contaminated sediments and/or wastewater in anoxic conditions.

1. Introduction

A number of new processes and configurations for ammonia removal from wastewaters have been developed recently with a potential to meet stringent discharge norms. Processes such as ANAMMOX (anaerobic ammonia oxidation), SHARON (single reactor high activity ammonia removal over nitrite), CANON (completely autotrophic N removal over nitrite), de-ammonification, and the nitrification–denitrification by methanotrophs, have emerged as promising technologies. A comprehensive review and descriptions of above new ammonia removal processes are available in the literature [1,2]. Among the above processes, anammox process is emerging as potential process for removal of ammonia from wastewaters at higher concentration. But reported anammox process is an autotrophic process with NO_2^- as the preferred and optimal electron acceptor [3,4].

Many ammonia containing wastewaters are not free from organic carbon and purely autotrophic anammox is not suitable in such cases [5]. An unusual process of coupled anoxic nitrification/manganese reduction in marine sediments has been reported [6] in the absence of molecular oxygen. Plants releasing oxygen in root zones may provide aerobic niches in anoxic sediments and soils for ammonia oxidizing bacteria [7]. Here, it is argued that the conservation of the nitrifying capacity during anoxic periods and the ability to react instantaneously to the presence of oxygen are important traits of nitrifiers in fluctuating oxic–anoxic environments such as the root zone of aerenchymatous plant species. The ammonia oxidation in such anoxic conditions was a result of oxygen released by such plants. Substantial nitrogen losses have been reported recently for both mixed and pure cultures of *Nitrosomanas eutropha* grown under oxygen limiting conditions, and for pure cultures of *Nitrosomanas europaea* in anoxic conditions [8]. But, the source of O_2 for oxidation of ammonia under anoxic conditions remained unknown. As early as in 1932, it was reported that nitrogen gas was generated via an unknown mechanism during fermentation in the sediments of lake Mendota, USA [9]. Very recently similar observations were found for the direct formation of nitrogen gas from ammonium (in the absence of oxidized nitrogen compounds) in fresh water sediments [10]. The above observations show a possibility of different nitrogen removal mechanism in the presence of organic carbon than reported anammox. Recently, Sabumon [11,12] reported anoxic ammonia oxidation to nitrate in the presence of organic carbon at an oxidation–reduction potential (ORP) of -248 ± 25 mV. Here, it is hypothesized that the oxygen required for oxidation of ammonia

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under anoxic condition was available from the reduction of H_2O_2 by catalase enzymatic activity of facultative anaerobes. H_2O_2 formation might have happened via bacterial metabolism in oxidative stress and/or anoxic conditions of respiration. However, there was not much work done for anoxic ammonia oxidation in the presence of organic carbon available through endogenous respiration, which can encounter in sludge digesters, in anoxic zones in wastewater treatment plants and in sediments.

The objective of this work was to study biologically mediated anoxic ammonia oxidation in the presence of organic carbon available through endogenous respiration under two different conditions, but in the absence of Fe and Mn oxides. The conditions of study were (i) anoxic ammonia oxidation in the absence of oxidized form of nitrogen (i.e., NO_2^- and NO_3^-) and (ii) anoxic ammonia oxidation in the presence of externally added electron acceptors like NO_2^-, NO_3^- and SO_4^{2-}. The first condition gives similar conditions involved in sludge digesters, in anoxic zones in wastewater treatment plants and in sediments. The second condition gives similar conditions involved in oxic–anoxic interfaces where reported anammox process could work by availability of both NH_4^+ and electron acceptors. Also, an additional study was conducted to know the effect of combination of inorganic electron acceptors and/or externally added organic compound in anoxic ammonia oxidation. The results of this study will help in analyzing whether anoxic ammonia oxidation in the presence of organic carbon can occur via reported anammox or not.

2. Materials and methods

2.1. Mineral media for anoxic ammonia oxidation

The mineral media used was same as reported for anaerobic ammonia removal study [11]. Predetermined amount of ammonia (using NH_4Cl) and selected electron acceptor (either NO_2^-, NO_3^- and SO_4^{2-}) were added as per requirement in each experiment. All chemicals were analytical reagent (AR) grade supplied by 'Qualigens' (India). Clean 'Borosil' (India) make glasswares were used for reagents preparation and volume measurements.

2.2. Adapted biomass

Anaerobically digested cow dung sludge and flocculent type extended aeration process sludge employed for the treatment of tannery effluents were screened for potential anoxic ammonia oxidation in the presence of organic carbon [11,12]. The screened sludge was adapted separately in fill and draw batch mode for anoxic ammonia oxidation with external feeding of NH_4^+ and NO_2^- in mineral medium. The oxidation–reduction potential (ORP) maintained in fed batch reactors was -220 ± 25 mV. The adapted biomass was used in the study.

2.3. Batch studies

2.3.1. Anoxic ammonia oxidation process in absence and in the presence of various inorganic electron acceptors

Batch studies were carried out to find the effect of various electron acceptors like NO_2^-, NO_3^- and SO_4^{2-} in anoxic ammonia oxidation and in the absence of NO_2^- and NO_3^-. For this, the adapted biomass of anaerobically digested cow dung and flocculent type extended aeration process sludge was subjected to 3 cycles of anoxic ammonia oxidation process in SBR mode of operation. Experiments were conducted in anoxic condition in 250 mL capacity Erlenmeyer conical flasks, suitably tight fitted with butyl rubber cork. Weekly analyses for electron donor (NH_4^+) and electron acceptors (NO_2^-, NO_3^- and SO_4^{2-}) were carried out. Details of the experimental method followed in each cycle are given below.

2.3.1.1. First cycle. Nine numbers of 250 mL capacity Erlenmeyer conical flasks were selected for conducting batch reactor studies under anoxic conditions. Before start of experiment, the adapted biomass was added to 100 mL mineral medium, with selected electron acceptor and known concentrations of ammonia (electron donor), and then purged with pure N_2 gas for 3 min. Resazurin indicator was added to all reactors to monitor whether anoxic condition was prevailed and crosschecked by measuring oxidation–reduction potential (ORP). The start-up conditions of first cycle are shown in Table 1. In Table 1, CD-Blank and TR-Blank were chosen to study anoxic ammonia oxidation process in the presence of organic carbon (available through endogenous respiration) without any external supply of inorganic electron acceptors (like NO_2^- and NO_3^-). A blank reactor was chosen to study the abiotic loss of ammonia.

Batch experiments were conducted for 2 weeks in an orbital shaker incubator (Remi Equipments LTD, India) at 120 rpm and at 30 °C. Representative samples of adapted digested cow dung sludge and flocculent type extended aeration process sludge used in batch studies at start-up were dried at 103 °C, powdered and preserved in a desiccator for elemental carbon analysis.

2.3.1.2. Second cycle. Centrifuged biomass from the respective first cycle batch reactors were added to mineral media and purged with N_2 free from molecular oxygen. All the experimental conditions were the same as in first cycle (Table 1), except MLSS. The start-up MLSS concentrations in second cycle were 1250, 1100, 700, 850, 1350, 1500, 1500, 1500 mg/L in CD-Blank, CD-SO_4, CD-NO_3, CD-NO_2, TR-Blank, TR-SO_4, TR-NO_3, TR-NO_2 reactors, respectively. Experiments were conducted for 1 week in an orbital shaker incubator at 120 rpm and at 30 °C.

2.3.1.3. Third cycle. Centrifuged biomass from the respective second cycle batch reactors were added to mineral media and purged with N_2 free from molecular oxygen. All the experimental conditions were the same as in first cycle (Table 1), except MLSS. The start-up MLSS concentrations in third cycle were 950, 700, 750, 800, 850, 1350, 1400, 1450 mg/L in CD-Blank, CD-SO_4, CD-NO_3, CD-NO_2, TR-Blank, TR-SO_4, TR-NO_3, TR-NO_2 reactors, respectively. Experiments were conducted for 3 weeks at room temperature with occasional mixing.

NH_4^+, NO_2^-, NO_3^-, SO_4^{2-}, pH, soluble COD and MLSS were monitored weekly to see the kinetic behavior of the process in third cycle. At the end of the experiment, 10 mL representative samples were drawn from all reactors; centrifuged, dried at 103 °C and preserved for elemental analysis of carbon in biomass.

2.3.2. Anoxic ammonia oxidation process in combination of inorganic electron acceptors and/or externally added organic compound

After the completion of third cycle, stored and settled biomass from all adapted cow dung (CD) tagged sequencing batch reactors (Table 2) are transferred to a conical flask and purged with nitrogen free from molecular oxygen for uniform mixing. After uniform mixing, each 10 mL of mixed sludge was then transferred to four numbers of serum bottles (100 mL capacity) containing mineral medium in anoxic condition with resazurin indicator. The start-up conditions of CD-Blank, CD-1, CD-2 and CD-3 are given in Table 3. CD-Blank was maintained for anoxic ammonia oxidation under endogenous respiration where as CD-3 was maintained for anoxic ammonia oxidation with the externally added organic compound (sucrose). CD-1 was maintained for anoxic ammonia

Table 1
The start-up conditions of first cycle for effect of various electron acceptors.

Batch tag	pH	NH_4^+, mg/L	NO_2^-, mg/L	NO_3^-, mg/L	SO_4^{2-}, mg/L	MLSS, mg/L
CD-Blank	8 ± 0.05	100 ± 0.3	0	0	87 ± 0.2	1485 ± 20
CD-SO_4	8 ± 0.05	100 ± 0.3	0	0	380 ± 1	1485 ± 20
CD-NO_3	8 ± 0.05	100 ± 0.3	0	133 ± 0.7	87 ± 0.2	1485 ± 20
CD-NO_2	8 ± 0.05	100 ± 0.3	133 ± 2.2	0	87 ± 0.2	1485 ± 20
TR-Blank	8 ± 0.05	100 ± 0.3	0	0	87 ± 0.2	1507 ± 20
TR-SO_4	8 ± 0.05	100 ± 0.3	0	0	380 ± 1	1507 ± 20
TR-NO_3	8 ± 0.05	100 ± 0.3	0	133 ± 0.7	87 ± 0.2	1507 ± 20
TR-NO_2	8 ± 0.05	100 ± 0.3	133 ± 2.2	0	87 ± 0.2	1507 ± 20
Blank (abiotic)	8 ± 0.05	100 ± 0.3	0	0	87 ± 0.2	60 ± 20

CD: adapted anaerobically digested cow dung sludge, TR: adapted flocculent type extended aeration process sludge employed for the treatment of tannery effluent, MLSS: mixed liquid suspended solids with volatile fraction = 65–75%.

Table 2
Effect of various electron acceptors in anoxic ammonia oxidation and in total nitrogen removal.

Batch tag	Cycle no.	Final pH	Final NH_4^+, mg/L	Final NO_2^-, mg/L	Final NO_3^-, mg/L	Final SO_4^{2-}, mg/L	% Removal of NH_4^+	% Removal of N	Ratio of EA/ED	% Reduction of carbon from Initial biomass	Remarks at end of cycle
CD-Blank	1	7.4	58.9	0	3.6	250.8	41.1	40.1	0		Increase of SO_4^{2-} concentration
	2	7.8	11.8	0	3.3	122.5	88.2	87.2	0		Increase of SO_4^{2-} concentration
	3	5.8	29.2	0	248	131.4	72.7	0	0	11.7	Increase of SO_4^{2-} and NO_3^- concentration
CD-SO_4	1	7.2	21.3	0	2.35	89.9	78.7	78	1.4		Decrease of SO_4^{2-} concentration
	2	7.4	0	0	19.5	347.3	100	94.3	0.12		Decrease of SO_4^{2-} concentration, increase of NO_3^- concentration
	3	5.8	22.9	0	294.1	382.3	77.1	0	0	35.6	Increase of NO_3^- concentration
CD-NO_3	1	6.3	9.8	0	119.3	88.9	90.2	67.9	0.05		
	2	7.3	0	0	302.5	96.5	100	36.6	0		Increase of NO_3^-
	3	5.4	54.1	0	380.2	107.5	45.9	0	0	33.3	Increase of NO_3^- and SO_4^{2-}
CD-NO_2	1	7	13.6	0	5.2	91	86.4	90	0.6		
	2	7.6	0	0	8.6	83.3	100	98.4	0.5		
	3	5.8	19.3	0	187.8	99.6	80.7	51.4	0.65	21.3	Increase of NO_3^- and SO_4^{2-}
TR-Blank	1	6.9	0	0	205.1	97	100	40.4	0		Increase of NO_3^-
	2	7.9	30.8	0	80.7	89.3	69.2	45.7	0		Increase of NO_3^-
	3	6.6	17.6	0	213.6	148.3	82.4	20.4	0	23.4	Increase of NO_3^- and SO_4^{2-}
TR-SO_4	1	7.5	49.9	0	21.5	364.6	50.1	43.8	0.12		Decrease of SO_4^{2-}
	2	7.9	28.2	0	75.2	356.5	71.8	50	0.13		Increase of NO_3^-
	3	6.8	24.6	0	162.3	374.8	75.4	25.7	0.03	19.9	Increase of NO_3^-
TR-NO_3	1	7.0	0	0	313.2	95.4	100	34.3	0		Increase of NO_3^-
	2	7.9	33.2	0	208.6	86.6	66.8	32.3	0		Increase of NO_3^-
	3	6.7	38.7	36.4	233.3	87.4	61.3	12.9	0	18.9	Increase of NO_3^- and nitrite
TR-NO_2	1	8.0	68.1	12	10.7	98.4	31.9	50.1	1.5		Increase of SO_4^{2-}
	2	8.0	34.9	0	9.7	85.5	65.1	75.2	0.8		Decrease of SO_4^{2-}
	3	6.7	0	0	162.1	95.7	100	69	0.52	17.7	Increase of NO_3^- and SO_4^{2-}
Blank (abiotic)	1–3	8.0–8.1	78–81	0	0	87–90	19–21	19–21	0		Nitrogen removal by chemical precipitation and/or volatilization at high pH

Maximum standard deviation is less than 0.485% of given values. EA: externally added electron acceptors (NO_2^-, NO_3^-, and SO_4^{2-}) and ED: electron donor (NH_4^+); initial carbon content in cow dung based sludge = 33.50%, initial carbon content in flocculent extended aeration process based sludge = 27.48%.

Table 3
The start-up conditions for anoxic ammonia oxidation in combination of inorganic electron acceptors and externally added organic compound.

Batch tag	Initial pH	NH_4^+, mg/L	NO_2^-, mg/L	NO_3^-, mg/L	SO_4^{2-}, mg/L	MLSS, mg/L	COD, mg/L
CD-Blank	8 ± 0.05	100 ± 0.3	0	0	87 ± 0.2	400 ± 20	0
CD-1	8 ± 0.05	100 ± 0.3	350 ± 6	210 ± 3.6	270 ± 0.6	400 ± 20	0
CD-2	8 ± 0.05	100 ± 0.3	350 ± 6	210 ± 3.6	270 ± 0.6	400 ± 20	350 ± 18
CD-3	8 ± 0.05	100 ± 0.3	0	0	87 ± 0.2	400 ± 20	350 ± 18
TR-Blank	8 ± 0.05	100 ± 0.3	0	0	87 ± 0.2	400 ± 20	0
TR-1	8 ± 0.05	100 ± 0.3	350 ± 6	210 ± 3.6	270 ± 0.6	400 ± 20	0
TR-2	8 ± 0.05	100 ± 0.3	350 ± 6	210 ± 3.6	270 ± 0.6	400 ± 20	350 ± 18
TR-3	8 ± 0.05	100 ± 0.3	0	0	87 ± 0.2	400 ± 20	350 ± 18

CD: mixed adapted cow dung sludge from all reactors after finishing the cycle number 3 mentioned in Table 2; TR: mixed adapted flocculent sludge after finishing the cycle number 3 mentioned in Table 2. The externally added organic compound (sucrose) is measured as COD.

oxidation under endogenous respiration with combination of inorganic electron acceptors where as CD-2 was maintained for anoxic ammonia oxidation in combination of inorganic electron acceptors and externally added organic compound. Experiments were conducted for 2 weeks in an orbital shaker incubator at 120 rpm and at 30 °C. The MLSS concentration maintained in each serum bottle was 400 ± 20 mg/L. Experiments were conducted in similar way for the adapted flocculent type sludge after mixing all the TR tagged sequencing batch reactor biomass after third cycle. The start-up conditions of TR reactors (four numbers) are given in Table 3. NH_4^+, NO_2^-, NO_3^-, SO_4^{2-} and soluble COD were monitored at end of 2 weeks from all eight reactors.

2.3.3. Kinetics of anoxic ammonia oxidation with NO_2^- as electron acceptor

Batch experiments were carried out in 100 mL serum bottles in anoxic conditions in mineral medium to find out the kinetic nature of anoxic ammonia oxidation with NO_2^- as electron acceptor using adapted anaerobically digested cow dung sludge and flocculent type extended aeration process sludge (collected from stock batch reactors maintained with feeding of NH_4^+ and NO_2^-). The initial (start-up) concentrations of NH_4^+ and NO_2^- in serum bottles were 150 and 520 mg/L, respectively. Experiments were conducted for 8 days in an orbital shaker incubator at 120 rpm and at 30 °C. NH_4^+, NO_2^-, NO_3^-, SO_4^{2-} and MLSS were monitored daily to see the kinetic behavior of the process. Soluble COD was also monitored during initial and final phases of experiment.

2.4. Analytical techniques

All physical–chemical parameter analyses were conducted as per standard methods [13]. NH_4^+, NO_2^-, NO_3^- and SO_4^{2-} were analyzed by Ion chromatography (DIONEX, USA) with ED50 electrochemical detector and the results were processed by in-built 'Chromeleon' software. COD was determined by closed reflux method using HACH (Loveland, USA) COD digester. Elemental carbon analysis of dried biomass samples was carried out using PerkinElmer (USA) 2400 series CHNS/O analyzer. ORP was measured using double junction platinum ORP electrode connected to a calibrated CyberScan pH (1100) meter in mV mode (EUTECH Instruments, Singapore). ORP electrode was calibrated using Quinhydrone 86. Catalase enzyme activity of microbes from all batch reactors was qualitatively tested by the procedure suggested by Dubey and Maheswari [14]. The quantification of catalase enzyme activity in the extracted enzyme (at 4 °C) from the mixed culture was determined by continuous spectrophotometric rate determination. In this procedure [15], presence of catalase shows decrease in rate of absorbance at 240 nm with respect to the blank test. The enzyme extraction procedure was followed by transferring the biomass to 50 mM monobasic phosphate buffer at pH 7 and centrifuged at 4 °C for 10 min at 10,000 × *g*. Then centrifuged biomass was re-suspended in 50 mM monobasic phosphate buffer at pH 7 (1 g biomass in 6 mL buffer). The biomass was lysed by sonication at 5 cycles of 60 s on and 60 s off at 175 W (Sonics vibra cell) in ice bath. Then the suspension was centrifuged again at 4 °C for 10 min at 12,000 × *g* to get clear suspension of protein for immediate catalase enzyme activity measurements. Triplicate samples were analyzed for specific parameters and showed as average values with standard deviations in Tables 1, 3 and 4. However, the figures were plotted with average values.

3. Results and discussion

3.1. Effect of electron acceptors in anoxic ammonia oxidation process

Effect of individual electron acceptors (NO_2^-, NO_3^- and SO_4^{2-}) was studied in SBR operation to verify whether anoxic ammonia oxidation in the presence of organic carbon available through endogenous respiration was occurred as per following reported stoichiometry of anammox reactions (1)–(3) [16–18].

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O;\ \Delta G^\circ = -357\ \text{kJ/M} \quad (1a)$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 0.26NO_3^- + 1.02N_2 + 066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1b)$$

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+;\ \Delta G^\circ = -297\ \text{kJ/M} \quad (2)$$

$$2NH_4^+ + SO_4^{2-} \rightarrow S + N_2 + 4H_2O;\ \Delta G^\circ = -48\ \text{kJ/M} \quad (3)$$

Table 2 shows the results of 3 cycles of sequencing batch operation for anoxic ammonia oxidation. From the stoichiometric ratios of anoxic ammonia oxidation in the present experimental system (Table 2, column 10), it is found that though ammonia oxidation occurred effectively, the respective stoichiometric ratio of reported anammox reactions was not satisfied in all batch reactors where inorganic electron acceptors (NO_2^-, NO_3^- and SO_4^{2-}) were added externally. As the cycles progressed, an interesting and an unusual observation had that there was accumulation of nitrate in all batch reactors except in abiotic blank reactor. So these results show an alternate pathway of anoxic ammonia oxidation.

Another important fact to note that in the absence of externally added electron acceptors (i.e., CD-Blank and TR-Blank reactors in Table 2) ammonia oxidation occurred. Here, the ammonia oxidation was unusual as the reactors were maintained in anoxic condition without external addition of NO_2^- or NO_3. All the batch reactors were maintained in anoxic conditions at oxidation–reduction potentials ranging from −185 to −275 mV and verified by blue color of resazurin indicator.

There are reports of anoxic ammonia oxidation to nitrate observed in marine sediments by the reduction of oxides of Mn [6,19–21] as per the thermodynamically feasible reaction (Eq. (4)). Here, the only source of oxygen for nitrification in the reaction is

Table 4
Effect and percentage removals during anoxic ammonia oxidation in combination of inorganic electron acceptors and externally added organic compound.

Batch tag	Percentage removal, %					Final NO_3^-, mg/L	Final SO_4^{2-}, mg/L	Remarks at end of study
	NH_4^+	NO_2^-	NO_3^-	SO_4^{2-}	COD			
CD-Blank	29.4	0.0	0.0	0.0	0.0	10 ± 0.2	120 ± 0.3	Increase of SO_4^{2-} and NO_3^- concentration
CD-1	37.2	88.6	18.1	0.0	0.0	172 ± 2.9	291 ± 0.7	Increase of SO_4^{2-} concentration
CD-2	38.7	100	93.7	0.0	89.7	13.3 ± 0.2	296 ± 0.7	Increase of SO_4^{2-} concentration
CD-3	43.1	0.0	0.0	71.3	94.9	0.0	25 ± 0.1	Decrease of SO_4^{2-} concentration
TR-Blank	31.7	0.0	0.0	0.0	0.0	6.7 ± 0.2	126 ± 0.3	Increase of SO_4^{2-} and NO_3^- concentration
TR-1	45.0	86.4	12.8	0.0	0.0	183 ± 3.1	304 ± 0.8	Increase of SO_4^{2-} concentration
TR-2	59.2	100	100	0.0	90.8	0.0	309 ± 0.8	Increase of SO_4^{2-} concentration
TR-3	39.4	0.0	0.0	25.3	94.9	0.0	65 ± 0.2	Decrease of SO_4^{2-} concentration

the associated oxygen with Mn. However, the exact mechanism of oxygen association for ammonia oxidation is not yet clear.

$$4MNO_2 + NH_4^+ + 6H^+ \rightarrow 4Mn^{2+} + NO_3^- + 5H_2O \quad (4)$$

The above-mentioned anoxic nitrification is possible and could be happening in the ecosystem in the presence of oxides of Mn. Since the product formed (nitrate) can get easily denitrified in reducing environment in the presence of required electron donors (which will be normally available in unlimited quantity), the usual observation of anoxic nitrification in ecosystem is rare, unless we closely and precisely monitor it.

In the present experimental system anoxic nitrification was observed, though there was no source of oxides of Mn. Since ammonia oxidized anoxically to nitrate in the experimental system (Table 2, column 6), the role of proteobacterial ammonia oxidizers are expected in the system. The proteobacterial ammonia oxidizers can obtain their energy for growth from both aerobic and anaerobic ammonia oxidation. However, the first step of ammonia oxidation (either by oxic or anoxic condition) is initiated by the enzyme ammonia mono oxygenase (AMO), that oxidizes ammonia to hydroxylamine as per Eqs. (5) or (6) [2].

In aerobic condition:

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O;\ \Delta G^\circ = -120\,kJ/M \quad (5)$$

In anaerobic condition:

$$NH_3 + N_2O_4 + 2H^+ + 2e^- \rightarrow NH_2OH + 2NO + H_2O;\ \Delta G^\circ = -140\,kJ/M \quad (6)$$

Here, it is to be noted that there was no external addition of electron acceptors (either NO_2^- or NO_3^- to get the dimmer N_2O_4) in CD-Blank and TR-Blank (Table 1) reactors for anoxic ammonia oxidation as per reaction (6). The possible explanation for anoxic ammonia oxidation occurred is hypothesized as per Eqs. (7) and (8).

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad (7)$$

The oxygen generated via Eq. (7) could have used for initiating ammonia oxidation by Eq. (5) and further nitrification by overall reaction (8).

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+;\ \Delta G^\circ = -349\,kJ/M \quad (8)$$

H_2O_2 could be produced as a result of bacterial respiration in anoxic condition involving organic carbon and/or any electron acceptor. The catalase enzyme can readily break H_2O_2 in to water and O_2. This oxygen generated in the microenvironment could have efficiently utilized by ammonia oxidizing bacteria for nitrification [7] in competition with other facultative heterotrophs. The pertinent microbes could have the specific enzyme system for competent nitrification with the oxygen produced by the catalase enzyme. There was reduction of pH in all cycles, as a result of nitrification (Table 2, column 3).

Bacterial cultures in the entire batch reactors were tested positive for catalase enzyme activity. The catalase enzyme activity was estimated to be 30 and 26 units/mg protein/minute for adapted anaerobically digested cow dung sludge and flocculent type extended aeration process sludge, respectively. One unit of catalase enzyme activity can decompose 1.0 μmol of H_2O_2 per minute at pH 7.0 at 25 °C. Even though the cultures showed good catalase activity, the amount of oxygen produced in the system depends on the amount of H_2O_2 produced in the system which might depends up on the respiratory activity of mixed culture in various conditions. The serum bottles tests with substrates for anoxic ammonia oxidation with biomass were showed presence of oxygen release in anoxic conditions by changing the color (resazurin indicator) from blue to pinkish at intervals and thus showing the evidence of reaction (7), where as the blank bottle (without biomass) was remained in blue color at same experimental conditions. This ensures absence of adequate oxygen diffusion from atmosphere for aerobic nitrification in experimental batch reactors. The quantification of H_2O_2 produced was not easy as a result of spontaneity of reaction (7) at cellular level. The H_2O_2 formation is possible in the presence of trace amount of oxygen (oxidative stress) by the use of oxidative enzymes of facultative organisms. The trace amount of oxygen (below detectable level) could be available at ORP of −185 to −275 mV in the experimental system.

Blokhino et al. [22] reported many ways of formation of reactive oxygen species (ROS) in oxidative stress conditions. Of the ROS, H_2O_2 and superoxide (O_2^-) are both produced in a number of cellular reactions including the iron-catalysed Fenton reaction and by various enzymes such as lipoxygenases, peroxidases and NADPH oxidase. Hydrogen peroxide accumulation under hypoxic conditions has been shown in the roots and leaves of Hordeum Vulgare and in wheat roots. The superoxide radical and the hydrogen peroxide are inevitable and reactive byproducts of biological metabolism and must be eliminated as soon as possible [23]. The presence of enzymes (superoxidase, peroxidase and catalase) defending the cells of anaerobic bacterium against the reactive oxygen species (ROS) is recorded recently [24–27]. The presence of such antioxidant defense of anaerobic bacterium shows that the superoxide radical and the H_2O_2 could be produced during anaerobic/anoxic metabolism in a complex environment. The following Eq. (9) shows how in acidic environment superoxide radical can decompose [28] to H_2O_2 and O_2. The H_2O_2 can be further decomposed to O_2 by the action of catalase enzyme as per Eq. (7).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (9)$$

From the overview of results presented in Table 2, it is observed that both in absence and in the presence of externally added inorganic electron acceptors, anoxic ammonia oxidation occurred. However, the total nitrogen removal was not matched in

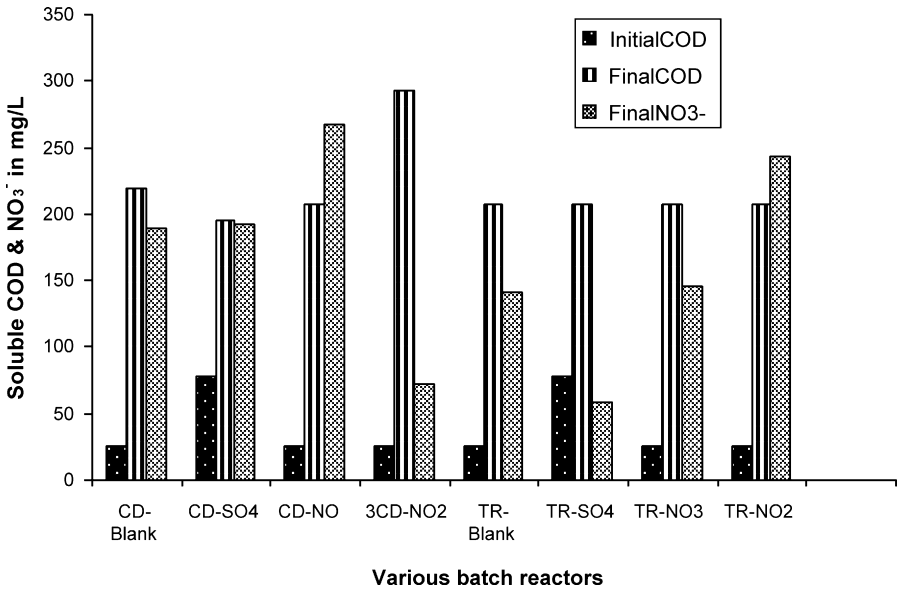

Fig. 1. Concentrations of COD and NO_3^- in third cycle.

correspondence with the ammonia oxidation. This miss-match in the total nitrogen removal was increased when batch reactor operation progressed from first cycle to third cycle. The reason for such miss-match could be due to accumulation of nitrate when the batch progressed in cycles. The nitrate accumulation could be due to exhaustion of internally stored substrates and/or lack of electron donors for denitrification when the batches were progressed in cycles. Limitation of the electron donor could have occurred due to the exhaustion of internal stored substrates, when the same biomass was subjected to endogenous respiration for a prolonged time in the sequential batch reactor (SBR) [29,30]. The denitrification using internally stored substrates as carbon and energy source in the absence of externally supplied substrates is reported [30]. Recently, there have been reports on the accumulation of nitrate by nitrifiers in SBRs operating with limited external carbon source [31]. There was loss of carbon content from the biomass as a result of endogenous respiration when the cycles were progressed (Table 2, column 11), resulting organic carbon contribution to aqueous phase. This organic carbon could also have been utilized for denitrification as per Eq. (10) for nitrogen removal.

$$2NO_3^- + 1.25CH_3COOH \rightarrow 2.5CO_2 + N_2 + 1.5H_2O + OH^-;\ \Delta G^\circ = -528\,kJ/M \quad (10)$$

There was decrease and increase of SO_4^{2-} concentrations observed in many cycles (Table 2, last column). The decrease in concentration of SO_4^{2-} could be as a result of sulphate reduction as per Eq. (11) and increase of SO_4^{2-} could be as a result of *Thiobacillus* denitrification as per Eq. (12). There was H_2S smell during the sampling and can be considered as evidence of reaction (11).

$$CH_3COOH + SO_4^{2-} \rightarrow H_2S + 2HCO3-;\ \Delta G^\circ = -43\,kJ/M \quad (11)$$

$$8NO_3^- + 5HS^- + 3H^+ \rightarrow 4N_2 + 5SO_4^{2-} + 4H_2O;\ \Delta G^\circ = -465\,kJ/M \quad (12)$$

Acetic acid (CH_3COOH) is considered as a typical organic acid formed as a result of biomass hydrolysis and acidification in anaerobic environment. However other organic compounds could also be present for sulphate reduction. Sulphate reduction and sulphide oxidation (as a result of *Thiobacillus* denitrification) could be considered as dynamic reactions in the batch reactors depend up on the availability of organic acids and nitrate. The presence of heterotrophic denitrifiers and *Thiobacillus denitrificans* in the adapted mixed culture of anaerobically digested cow dung and flocculent type extended aeration process sludge employed for treating tannery effluent used in this study were verified by conducting separate batch experiments [11,12]. The presence of both heterotrophic denitrifiers and *T. denitrificans* in the adapted mixed culture were responsible for denitrification of nitrate formed in the reactors in the presence of suitable electron donor.

Among the presence of various electron acceptors studied, NO_2^- was found to be the best electron acceptor in total nitrogen removal. Also in reported anammox process [16], NO_2^- was the preferred electron acceptor for optimum nitrogen removal.

The results from abiotic reactor showed 19–21% of ammonia removal (Table 2, last row). This ammonia removal might be due to the precipitation of an unknown compound and/or possible volatilization of ammonia at around pH 8 and above. Since the final pH in the reactors with biomass was less than 8, ammonia oxidation could have occurred by biochemical routes.

From the results presented above, it is possible to conclude that anoxic ammonia removal is possible in the presence of organic carbon (available through endogenous respiration) either in the presence of externally added inorganic electron acceptors or in the absence of inorganic electron acceptors.

3.2. Third cycle and kinetic study

The kinetic behavior of the system in third cycle of SBR operation with presence of various inorganic electron acceptors and without inorganic electron acceptors was studied. In all batch reactors, ammonia removal occurred but more amount of nitrate was accumulated with time. In few batch reactors, there was no net nitrogen removal in third cycle, though there was comparatively lesser nitrogen removal with respect to first and second cycles in other batch reactors. The nitrogen removal occurred could have happened via denitrification. The electron donors for denitrification might have available through reduced organic carbon (by endogenous respiration) or reduced sulphur compounds which could be generated inside the system as a result of sulphate reduction. There was gradual increase of COD in the liquid phase in correspondence with nitrate accumulation. This residual COD might not be readily used up for denitrification as it might have contained difficult to degrade

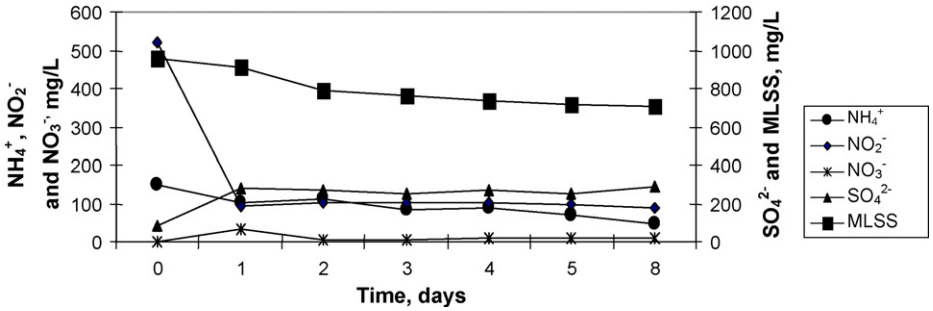

Fig. 2. Kinetics of anoxic ammonia oxidation with adapted cow dung sludge.

cell components. There was also decrease in percentage of carbon content of biomass with time of operation (Table 2, column 11), which shows loss of organic carbon to liquid phase measured as COD. Fig. 1 shows the concentrations of soluble CODs and nitrate accumulated at the end of third cycle. The kinetic results of this study showed a feasible anoxic ammonia oxidation to nitrate in the presence of organic carbon (available through endogenous respiration) and total nitrogen removal depends up on the presence of reduced biodegradable organic and/or sulphur compounds in the system.

The kinetics of anaerobic ammonia oxidation process in the presence of SO_4^{2-} showed higher removal of NH_4^+. This trend was observed in all 3 cycles of SBR operation with SO_4^{2-} as electron acceptor. The presence of sulphate might have triggered more intensive sulphate reduction respiration cycle and there by more H_2O_2 formation. Kenger et al. [32] reported that two NADH oxidase encoding genes noxA-1 and noxB-1, cloned from a strict sulphate reducer *Archaeoglobus fulgidas*, were found to produce predominantly H_2O_2 instead of water. Their report also shows that involvement of NADH oxidase in electron transport chain reactions involving sulphate respiration.

The kinetics of anaerobic ammonia oxidation process in the presence of NO_3^- shows similar trends of other batch reactors. However, the percentage removal of NH_4^+ was less compared to SO_4^{2-} as electron acceptor.

The kinetics of anoxic ammonia oxidation process in the presence of NO_2^- also shows similar trends of other batch reactors. However, the percentage removal of NH_4^+ and total nitrogen was highest. Until 2 weeks, percent removal of total nitrogen was closer to percent removal of ammonia. However, the role of reported anammox process in the presence of NH_4^+ and NO_2^- to give a better performance compared with other batches was not clear. The identification of bonafide anammox bacteria by FISH technique could not be carried out due to lack of facilities. But, certainly there was accumulation of nitrate in third cycle and that showed possibility of anoxic ammonia oxidation to nitrate by catalase enzyme based route.

The trend of kinetic behavior of anoxic ammonia oxidation process with adapted flocculent type extended aeration process sludge (tannery sludge) under various conditions was same as that of cow dung sludge. However, the difference between percentage removal of ammonia and total nitrogen was less in tannery sludge. This could be due to less amount of nitrate accumulation compared to cow dung sludge. In tannery sludge based batch reactor also maximum removal of NH_4^+ and total nitrogen was occurred, when NO_2^- was used as electron acceptor.

From the results of above kinetic study, it is possible to confirm and conclude that anoxic ammonia removal is possible biologically either in the presence of inorganic electron acceptors like NO_2^-, NO_3^- or in the absence of oxidized form of nitrogen in the presence of organic carbon in liquid media available through endogenous respiration. It is hypothesized that ammonia was oxidized to NO_3^- anoxically. The oxygen required for anoxic nitrification might have produced in the anoxic respiration cycle involving NO_2^-, NO_3^-, and SO_4^{2-} in the presence of organic carbon. The amount of anoxic ammonia removal might depend on the activity of various microbial respiration cycles with various electron acceptors in oxidative stress conditions.

3.3. Anoxic ammonia oxidation in combination of inorganic electron acceptors and/or externally added organic compound

Table 4 shows the results of anoxic ammonia oxidation in combination of inorganic electron acceptors and/or externally added organic compound. The results of CD-Blank and TR-Blank reactors, where there was no addition of either inorganic or organic compounds, showed anoxic ammonia removal confirming the earlier batch results under endogenous respiration. Here also, there was an increase in concentrations of nitrate and sulphate as observed in third cycle of SBR operation. So these results confirmed the potential of anoxic ammonia oxidation under endogenous respiration.

The results of CD-1 and TR-1 reactors, where there was external addition of combination of inorganic electron acceptors (NO_2^-, NO_3^- and SO_4^{2-}), showed an improvement in anoxic ammonia oxidation compared to the absence of inorganic electron acceptors. Nitrite was consumed more compared to nitrate. There was increase of sulphate in both reactors and could be as a result of *Thiobacillus* denitrification. Adapted flocculent sludge (TR-1) was performing better than adapted cow dung sludge (CD-1).

The results of CD-2 and TR-2 reactors, where there was external addition of both organic compound and inorganic electron acceptors, showed enhanced anoxic ammonia oxidation. The percentage ammonia removals obtained in CD-2 and TR-2 were 38.7 and 59.2, respectively. There was 100% removal of nitrite in both reactors where as nitrate and COD removals were more in TR-2 reactor. There was increase of sulphate in both reactors and could be as a result of *Thiobacillus* denitrification. The results showed a definite benefit of presence of both inorganic electron acceptors and organic compounds in anoxic ammonia oxidation in contrast to the reported anammox process.

The results of CD-3 and TR-3 reactors, where there was external addition of organic compound without inorganic electron acceptors like NO_2^- and NO_3^-, showed enhanced anoxic ammonia oxidation compared to CD-Blank and TR-Blank reactors. However, CD-3 showed better ammonia removal compared to TR-3, which is in contrast with the trend where TR tagged reactors were performing better in this experiment. This could be because of enhanced sulphate reduction respiration (71.3%) observed in CD-3 reactor compared to less sulphate reduction (25.3%) in TR-3 reactor. The externally added organic compound could have used by mixed cultures for anoxic respiration of both denitrification and sulphate reduction. The COD removals (94.9%) in both reactors were very good. The results of this study conclude that it is possible to have

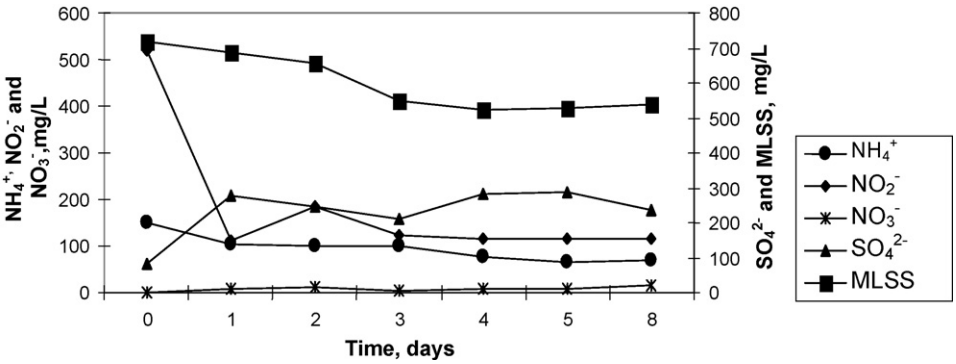

Fig. 3. Kinetics of anoxic ammonia oxidation with adapted flocculent type extended aeration process sludge.

anoxic ammonia oxidation in the presence of externally added organic compounds. This information may be useful for anoxic ammonia removal from organically contaminated wastewaters.

3.4. Kinetics of anoxic ammonia oxidation with NO_2^- as electron acceptor

In all the batch studies, net nitrogen removal was high when nitrite was used as electron acceptor though the process was not followed strictly as per reported anammox. Therefore kinetic studies were conducted with adapted biomass with nitrite as electron acceptor. Fig. 2 shows the kinetics of anoxic oxidation of ammonia in adapted cow dung sludge with electron acceptor NO_2^-. From Fig. 2, it is seen that within 1 day, NH_4^+ was oxidized and NO_2^- reduced to a great extend. The stoichiometric ratio of NO_2^-/NH_4^+ within this 1 day was 3.6, which is not matched with reported autotrophic anammox reaction (1.32) as per Eq. (1b) [16]. There was overutilization of NO_2^- than the requirement of reported anammox reaction. The NO_2^- might have respired for highly feasible both heterotrophic and autotrophic *Thiobacillus* denitrification reactions as per Eqs. (10), (12) and (13). Occurrence of autotrophic *Thiobacillus* denitrification was evident from increase of sulphate within 1 day (Fig. 2). The source of sulphur for *Thiobacillus* denitrification reaction as per Eq. (13) might be from earlier precipitated metallic sulphides or sulphur granules present in the mixed culture (during adaptation process). Sulphur granules formation is possible by the oxidation of sulphides by nitrate formed in the stock culture during adaptation. The anoxic formation of sulphur from sulphide by nitrate is recently reported [33].

$$NO_3^- + 1.1S + 0.4CO_2 + 0.76H_2O + 0.08NH_4^+ \xrightarrow{\textit{Thiobacillus denitrificans}} 0.5N_2 + 1.10SO_4^{2-} + 1.28H^+ + 0.08C_5H_7O_2N \quad (13)$$

There was formation of NO_3^- in anoxic serum bottle like that observed in earlier sequential batch operation cycles. The presence of this NO_3^- shows the possibility of anoxic oxidation of ammonia by catalase enzyme route. Further monitoring of batch reactor showed a decline in MLSS level and NO_3^- concentration. This might be due to sludge hydrolysis and there by organic carbon entered to bulk solution. The COD available could be used for sulphate reduction and/or for denitrification. The sulphate increase could be due to autotrophic *Thiobacillus* denitrification. So the present kinetic data showed complex reactions by mixed culture. The possible reactions are anoxic ammonia oxidation to nitrate, hetero and/or autotrophic denitrification, sulphate reduction and/or anaerobic fermentation.

In the overall outlook in to the batch kinetics, it was observed that ammonia removed in anoxic condition when the batch progressed as a result of anaerobic/anoxic respiration involving NO_2^-, NO_3^-, SO_4^{2-} and organic carbon. The soluble COD analysis at the start and end of kinetic study showed that, there was generation of difficult to degrade COD (41–207 mg/L) as the batch progressed as a result of endogenous respiration for heterotrophic carbon demand of various microbes involved in the mixed culture. The average anaerobic ammonia oxidation capacity of mixed culture was found to be 20 mg NH_4^+/g MLVSS/d. However, the rate of oxidation of ammonia was found to be 72 mg/g MLVSS/d in the first day where maximum utilization of NO_2^- was observed. Such rate of ammonia oxidation was assumed to be happened via catalase enzyme route to nitrate, because there was increase of sulphate as a result of autotrophic *Thiobacillus* denitrification.

Similar trend of anoxic ammonia oxidation and fluctuations of anions (NO_2^-, NO_3^-, and SO_4^{2-}) were observed in the kinetic study of adapted flocculent type extended aeration process sludge (Fig. 3). The average anoxic ammonia oxidation capacity of mixed culture was found to be 23 mg NH_4^+/g MLVSS/d. However, the rate of oxidation of ammonia was found to be 100 mg NH_4^+/g MLVSS/d in the first day where maximum utilization of NO_2^- was observed. Therefore flocculent type extended aeration process sludge was better in anoxic ammonia removal compared to cow dung sludge. The COD analysis at the start and the end of batch kinetic study showed that there was generation of difficult to degrade COD (21–145 mg/L) as the batch progressed.

The results of above kinetic studies showed that anoxic ammonia removal was possible in the presence of organic carbon (available through endogenous respiration) and oxidized forms of nitrogen and sulphur. But the pathway followed is different from reported anammox process. This new pathway of nitrogen removal might be helpful in oxic–anoxic interfaces where possibility of microbial production of H_2O_2 can be high due to oxidative stress conditions. So the results present in this paper may help in understanding nitrogen removal in benthic deposits of lakes and ocean floor and in low-oxygen systems of sludge flocs/biofilms in wastewater treatment plants. However, the anoxic ammonia removal by catalase enzyme route is not suitable for removing higher concentrations of ammonia economically.

4. Conclusions

Anoxic ammonia removal is possible biologically in the presence of organic carbon and either in the presence of inorganic electron acceptors like NO_2^-, NO_3^-, SO_4^{2-} or in the absence of oxidized form of nitrogen. It is hypothesized that ammonia was oxidized to NO_3^- anoxically. The oxygen required for anoxic nitrification could have produced in the anoxic respiration cycle involving NO_2^-, NO_3^- and SO_4^{2-} in the presence of organic carbon. The nitrogen removal was by denitrification process and depends up on the availability of electron donor. Nitrite was the best electron acceptor in anoxic ammonia oxidation and the rate of ammonia oxidation was found to be maximum of 74 mg NH_4^+/g MLVSS/d and 100 mg NH_4^+/g

MLVSS/d for adapted cow dung and flocculent type extended aeration process sludge, respectively. In order to improve the anoxic ammonia oxidation rate, rate of microbial oxygen generation rate by catalase enzymatic route need to maximized in treatment systems.

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