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Nitrate removal from high strength nitrate-bearing wastes in granular sludge sequencing batch reactors

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A 6-L sequencing batch reactor (SBR) was operated for development of granular sludge capable of denitrification of high strength nitrates. Complete and stable denitrification of up to 5420 mg L^{-1} nitrate-N (2710 mg L^{-1} nitrate-N in reactor) was achieved by feeding simulated nitrate waste at a C/N ratio of 3. Compact and dense denitrifying granular sludge with relatively stable microbial community was developed during reactor operation. Accumulation of large amounts of nitrite due to incomplete denitrification occurred when the SBR was fed with 5420 mg L^{-1} NO_3–N at a C/N ratio of 2. Complete denitrification could not be achieved at this C/N ratio, even after one week of reactor operation as the nitrite levels continued to accumulate. In order to improve denitrification performance, the reactor was fed with nitrate concentrations of 1354 mg L^{-1}, while keeping C/N ratio at 2. Subsequently, nitrate concentration in the feed was increased in a step-wise manner to establish complete denitrification of 5420 mg L^{-1} NO_3–N at a C/N ratio of 2. The results show that substrate concentration plays an important role in denitrification of high strength nitrate by influencing nitrite accumulation. Complete denitrification of high strength nitrates can be achieved at lower substrate concentrations, by an appropriate acclimatization strategy.

[**Key words:** Carbon to nitrogen ratio; Denitrification; Granular sludge; High strength nitrate; Nitrite accumulation]

Nitrate is one of the most widespread contaminants of natural water resources. During the past many decades, anthropogenic activities have greatly contributed to the increase in nitrate concentrations in surface and groundwater (1–3). Nitrate contamination of aquatic environments is a concern as it has been linked to ecosystem well-being and public health (4). For example, nitrate contamination causes eutrophication of water bodies and blue-baby syndrome in infants (2,5). Due to potential risks on ecosystem and public health, nitrate is listed as a priority pollutant under US Environmental Protection Agency (6). The maximum contaminant level (MCL) for nitrate (NO_3) in drinking water supplies has been set at 45 mg L^{-1} by US EPA, WHO and Bureau of Indian Standards. The European Union has prescribed 50 mg L^{-1} as the limit for nitrate and 0.1 mg L^{-1} for nitrite in drinking water (7).

Two main point sources for nitrate contamination are sewage and industrial effluents. High strength nitrate effluents are generated in fertilizer, ammunition, pharmaceutical, metal finishing and nuclear industries (8–10). In nuclear industry, nitric acid is used in various processing steps of nuclear fuel fabrication, dissolution and reprocessing of spent nuclear fuel, resulting in production of low and high strength nitrate-bearing effluents (8,11). The acidic nitrate effluents thus generated are neutralized with hydroxide and stored under safe conditions for treatment and disposal in future.

Biological denitrification for treatment of high strength nitrate effluents has been widely studied, particularly in the case of wastes generated in nuclear fuel cycle operations. It is acknowledged that biological treatment of high strength nitrate wastes typical for industrial effluents is challenging (8,9,11–14). One of the problems commonly encountered during denitrification of high strength nitrates is accumulation of high levels of nitrite (8,11,12,14). Accumulation of high concentrations of nitrite can lead to inhibition of the whole denitrification process (8). Two main factors that could influence nitrite accumulation during denitrification are electron donor supply and composition of the microbial community (15). Nitrate effluents generated in the nuclear industry are often devoid of electron donors. Dosage of electron donor in terms of C/N ratio has been studied for minimizing nitrite accumulation and achieving complete denitrification (12). Regarding the microbial community, it has been reported that biomass in the form of granular sludge may be better for overcoming the inhibitory effects of nitrite concentrations encountered during high strength nitrate denitrification (10). The objectives of this study, therefore, were (i) cultivation of granular sludge for the denitrification of high strength nitrate wastewater and (ii) understanding the effect of C/N ratio on the denitrification process.

MATERIALS AND METHODS

Seed sludge Activated sludge collected from the outlet of an aeration tank of an operating municipal wastewater treatment plant at Kalpakkam, India was used as

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inoculum for the sequencing batch reactors (SBRs). The activated sludge was black in color, contained filamentous microorganisms and exhibited poor settling characteristics (16,17). After bringing to the laboratory, the sludge was washed a few times with deionized water and stored at 4°C. Prior to use, the activated sludge was washed with ultrapure water and was placed on a tissue paper to remove excess water. The wet sludge was weighed and transferred to the SBR.

Cultivation of denitrifying granular biomass A glass tank (height: 61 cm, diameter: 15 cm) with 6 L working volume was operated in a sequencing batch mode. SBR operation was chosen as it allows formation and selection of granular sludge. The SBR was inoculated with activated sludge (3 g mixed liquor suspended solids L^{-1}) and operated with 24 h cycle period. The SBR cycle consisted of the following: 5 min fill, 23 h reaction, 5 min settle, 10 min decant and 40 min idle. The SBR was fed with simulated nitrate waste prepared in deionized water and consisting of the following ingredients (g L^{-1}): $MgSO_4.7H_2O$ 0.04, KCl 0.02, K_2HPO_4 0.03, KH_2PO_4 0.01, $CaCl_2.2H_2O$ 0.02. Trace elements were supplied by adding 0.1 mL of trace element mix per 1 L of simulated nitrate waste (18). Sodium acetate and sodium nitrate were added to the simulated nitrate waste, as per requirement (Table 1). The SBR was operated at room temperature (~30°C) with 50% volumetric exchange ratio. Mixing was provided at a fixed speed of 100 rpm using a stirrer (Eltek, India). Effluent was drawn from a port located 17 cm above the bottom. The SBR was operated for more than 8 months in order to monitor the stability of denitrification process.

Microscopy of denitrifying sludge Morphology of the denitrifying sludge was documented using a DP70 camera (Olympus, Japan) attached to SMZ1000 stereozoom microscope (Nikon, Japan) (16). The denitrifying granular sludge was stained with *Bac*Light bacterial viability kit (Molecular Probes, USA) (19). A 200 μL of *Bac*Light stain mixture (SYTO 9 and propidium iodide) was added to 1.5 mL Eppendorf tube containing the sludge and incubated on shaker set at 100 rpm. After 15 min, the sludge was washed twice with ultrapure water. The stained granules were imaged using a confocal laser scanning microscope TCS SP2 AOBS (Leica Microsystems, Germany) attached to an inverted microscope (Leica DMIRE2). Images were collected using a 63x 1.2 NA water immersion objective (20). Sample was excited using a 488 nm Ar laser line. The fluorescence emission was collected between 500 and 520 nm for SYTO 9 and between 600 and 680 nm for propidium iodide.

DNA extraction and PCR-DGGE Activated sludge and denitrifying granular sludge (0.2 g wet weight) collected from the SBR were homogenized by vortexing with glass beads (21). Genomic DNA from the homogenized samples was extracted using DNA isolation kit (Qiagen, Germany). The extracted DNA was verified by 1% (w/v) agarose gel electrophoresis. The extracted genomic DNA was stored at −50°C. The V3 region of the bacterial 16S rRNA gene was amplified using primers PRBA338f (5′CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGGACTCCTACGGGAGGCA GCAG 3′) and PRUN518r (5′ ATTACCGCGGCTGCTGG 3′) (19). Polymerase chain reaction (PCR) was performed using 50 μL reaction mixture using a Mastercycler gradient thermal cycler (Eppendorf AG, Germany) as described earlier (19). The PCR amplified fragments were separated by denaturing gradient gel electrophoresis (DGGE) using an INGENYphorU system (Ingeny International BV, The Netherlands). The DGGE was performed using 40%–70% urea-formamide denaturant gradient gel [8% (w/v) acrylamide solution (40% acrylamide and bisacrylamide in 37.5:1 ratio)] in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 8.0) as described earlier (20,22,23). After electrophoresis, the gel was stained with ethidium bromide (0.1 mg L^{-1}) for 30 min and destained for 15 min in ultrapure water. The stained gel was documented with INFINITY gel documentation system (Vilber Lourmat, France) and diversity indices were determined as Ward's Dice similarity coefficients with the Quantity One version 4.6 (Bio-Rad, USA).

Analytical procedures Liquid samples collected at regular time intervals during the SBR cycle period were monitored for nitrite and nitrate. Nitrate was analyzed by high performance liquid chromatography (HPLC) (Dionex Ultimate 3000) fitted with an Acclaim OA column using a UV–vis detector set at 210 nm (10). Dilute H_2SO_4 (0.003 N) was prepared in deionized water and used at a flow rate of 0.7 mL min^{-1}. Nitrite was estimated spectrophotometrically by reaction with *N*-(1-naphthyl) ethylenediamine dihydrochloride (24). Mixed liquor suspended solids (MLSS) and total organic carbon (TOC) were occasionally analyzed as per standard methods (24).

TABLE 1. The simulated nitrate waste was prepared by varying the amounts of sodium acetate and sodium nitrate.

Constituent (g L^{-1})	Feed NO_3–N (mg L^{-1})			
	1354	2710	4060	5420
$NaNO_3$	8.22	16.43	24.86	32.86
CH_3COONa (3:1)[a]	13.89	27.77	41.66	55.55
CH_3COONa (2:1)[a]	9.26	18.52	27.77	37.03

[a] Sodium acetate was added to give a C/N mass ratio of 3 or 2, i.e., 3 or 2 g of carbon for g of nitrogen.

RESULTS AND DISCUSSION

Denitrification performance The SBR was inoculated with activated sludge and fed with simulated waste containing 1354 mg NO_3–N L^{-1} at a C/N ratio of 3. It may be noted that the starting concentration in the reactor would be 677 mg NO_3–N L^{-1}, because the SBR retains 50% of waste from the previous batch (50% VER), with no nitrates in it. The pH of simulated nitrate waste was 7.5 before feeding to the reactor. The pH observed in the reactor at the end of cycle was 9.5, the increase being due to denitrification. In subsequent cycles of operation, the pH in the reactor stabilized at ~9.5 (Fig. S1). The simulated waste prepared in deionized water and the dissolved oxygen (DO) content before feeding was noted to be 6.0 mg L^{-1}. The DO rapidly decreased to below 0.08 mg L^{-1} within a few minutes of addition to the reactor, due to aerobic microbial respiration. The reactor tank was open to atmosphere and the mixing was provided by means of bottom stirring at 100 rpm. Under these conditions, the DO in the SBR cycle period was always below 0.08 mg L^{-1}. The reactor operating conditions were conducive for heterotrophic denitrification, observed from cycle 1 onwards (Fig. 1). By keeping the C/N ratio constant at 3, feed nitrate-N was increased to 2710 and then to 5420 mg L^{-1}. Complete denitrification was observed at the increased feed nitrate concentrations of 2710 and 5420 mg NO_3–N L^{-1}. The effluent nitrate and nitrite concentrations were invariably less than 10 mg L^{-1} during long term operation of the SBR, except during a few occasions (Fig. 1).

Denitrification of feed NO_3–N concentrations of 1354, 2710 and 5420 mg L^{-1} was completed within the first 4, 6 and 8 h, respectively (Fig. S2). Typical denitrification profiles consisted of nitrate removal, accumulation of nitrite and nitrite removal. Nitrite accumulation reached a maximum value of approximately 250 mg L^{-1} NO_2–N at 1354 mg L^{-1} nitrate-N in the feed.

In order to study the denitrification at reduced substrate concentration, the reactor was subsequently fed with 5420 mg L^{-1} NO_3–N at a C/N ratio of 2. The change in C/N ratio caused inhibition of denitrification, resulting in incomplete denitrification and accumulation of nitrite-N as high as 3500 mg L^{-1} (Fig. 2). SBR operation in the subsequent cycles consistently showed the buildup of the high concentrations of nitrite and nitrate. Inhibition of the denitrification process was probably due to the toxicity caused by high nitrite levels.

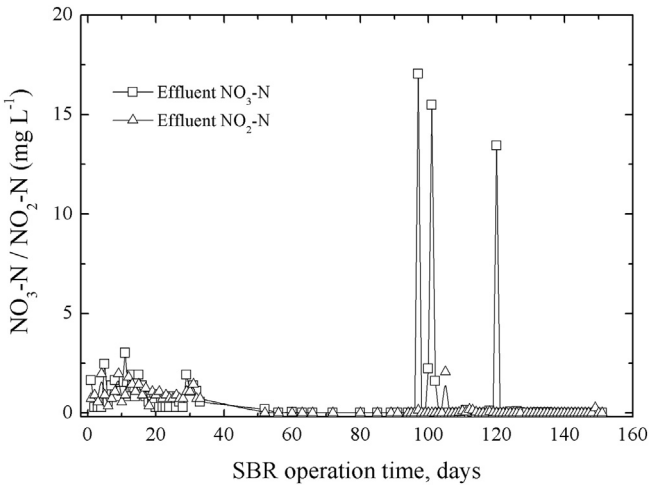

FIG. 1. Effluent nitrate and nitrite levels during 150 days of operation denitrifying sequencing batch reactor. The reactor was fed with simulated nitrate waste containing 1354, 2710, 4060 and 5420 mg L^{-1} NO_3–N at a C/N ratio of 3.