



Continuous biotransformation and removal of nitrophenols under denitrifying conditions

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Abstract

The effect of COD/NO₃⁻-N ratio on the biotransformation and removal of 2-nitrophenol (2-NP), 4-nitrophenol (4-NP), and 2,4-dinitrophenol (2,4-DNP) was studied in bench scale upflow anaerobic sludge blanket (UASB) reactors. Sodium acetate and sodium nitrate were used as electron donor (substrate) and electron acceptor, respectively. Nitrate nitrogen loading was increased from 0.098 to 0.6 kg/m³ d in order to keep COD/NO₃⁻-N ratio as 20.8, 14.3, 9.8, 5.0, 4.0 and 3.33. Throughout the study, input nitrophenolic concentration and hydraulic retention time (HRT) were kept constant as 30 mg/l and 24 h, respectively. 2-Aminophenol (2-AP), 4-aminophenol (4-AP) and 2-amino,4-nitrophenol (2-A,4-NP) were found as the major intermediate metabolite of 2-NP, 4-NP and 2,4-DNP, respectively. Removal of all the three nitrophenols increased with lowering of COD/NO₃⁻-N ratio. However, nitrophenols removal got adversely affected when COD/NO₃⁻-N ratio was reduced below 5. Maximum removal achieved were 91.63%, 90.17% and 86.10% for 2-NP, 4-NP and 2,4-DNP, respectively at a COD/NO₃⁻-N ratio of 5. Simultaneous denitrification and methanogenesis was observed in all the reactors throughout the study.

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1. Introduction

Nitrophenols are frequently used as an intermediate in the production of explosives, pharmaceuticals, pesticides, pigments, dye, wood preservatives and rubber chemicals [1]. Nitrophenols also result from microbial activity and natural processes in the biosphere. They are produced by microbial hydrolysis of several organophosphorous pesticides, such as parathion, or by photo-

degradation of pesticides that contain nitrophenol moiety [2]. 4-Nitrophenol may get produced in the atmosphere through the photochemical reaction between benzene and nitrogen monoxide and has been detected in rainwater in Japan [3].

The annual production of 4-nitrophenol alone is 20,000 metric tonne [4]. Since these chemicals are frequently used for industrial, agricultural and defence purposes, usually they find their way into the effluents from these sources. Nitrophenols have been found in about 14 out of 1177 hazardous waste sites on the National Priority List [1]. These compounds pose significant health risks as they are physiologically active and potentially carcinogenic. Among the nitrophenols, 2-NP, 4-NP and 2,4-DNP are listed on the US Environmental Protection Agency's (USEPA's) "Priority Pollutants List". The US EPA recommends restricting their concentrations in natural waters to below 10 ng/l [2].

Abbreviations: 2,4-DNP: 2,4-Dinitrophenol; 2-A,4-NP: 2-Amino,4-Nitrophenol; 2-AP: 2-Aminophenol; 2-NP: 2-Nitrophenol; 4-AP: 4-Aminophenol; 4-NP: 4-Nitrophenol; COD: Chemical oxygen demand; Eff.: Effluent; HRT: Hydraulic retention time; Inf.: Influent; NO₃⁻-N: Nitrate nitrogen; pH: Potential hydrogenium; UASB: Upflow anaerobic sludge blanket

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Both aerobic as well as anaerobic biodegradation process have been used to treat nitrophenolic wastewater [4–8]. However, electron-withdrawing groups inherent to nitroaromatics reduce the electron density of the aromatic ring and inhibit oxidative attack by the electrophilic oxygenases [9]. Furthermore, the conjugation of unstable nitroso and hydroxyamino intermediates results into the formation of complex azo or azoxy compounds under aerobic conditions. However, under anaerobic conditions nitrophenols readily get transformed to their corresponding amines. On average, aromatic amines are 500-fold less toxic than their corresponding nitroaromatics [4]. This suggests that anaerobic condition will at least detoxify nitrophenolic wastewater if not completely mineralize them.

Biodegradation of nitrophenols under denitrifying condition is of particular interest because the electron acceptor needed are highly soluble in water and many times coexists into the wastewater along with nitrophenolic compounds [10]. Spain et al. [11] identified 2-nitrophenol oxygenase enzyme from *Pseudomonas putida*, capable of removing nitrite group from 2- and 4-nitrophenol.

The present paper includes the effect of different COD/NO₃⁻-N ratios on the biotransformation and removal of nitrophenols under denitrifying conditions. It further discusses simultaneous methanogenesis and denitrification in a upflow anaerobic sludge blanket (UASB) reactor and the physical characteristics of the granular sludge.

2. Materials and methods

2.1. Experimental set-up

The continuous experiments were conducted in four identical bench-scale UASB reactors (R1, R2, R3 and R4) having 12.5 l working volume. Inner dimension of the reactors was 10 cm × 10 cm and a height of 1.2 m with 15 cm long hopper bottom. On the top of the reactor, there was a 2 l gas–liquid–solid separator. The reactors were made of transparent acrylic plastic sheet of 6.0 mm thickness and maintained at room temperature (27 ± 4°C). Fig. 1 shows the schematic diagram of the experimental set-up.

2.2. Chemicals used

Nitrophenols (2-NP, 4-NP and 2,4-DNP) used in the study were analytical reagents (99.9%) from Sigma-Aldrich, Germany. Sodium acetate and sodium nitrate were laboratory reagents from West Coast Laboratories, India. Standard gases for methane (2.5% v/v), CO₂ and N₂ were purchased from Span Gas, India. All other chemicals were procured from E. Merck, India.

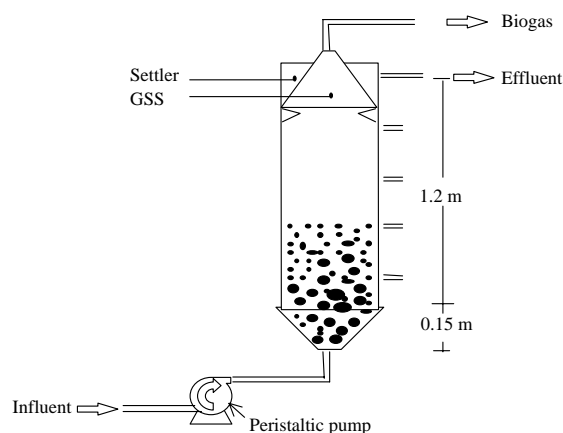


Fig. 1. Schematic diagram of the experimental set-up.

2.3. Experimental design

Four bench scale reactors (R1, R2, R3 and R4) having a working volume of 12.5 l were inoculated with 5 l anaerobic granular sludge (VSS = 25 g/l, granule size = 0.25–4 mm, sludge volume index = 40 ml/g) collected from a bench-scale UASB reactor treating chlorinated aliphatic compounds under methanogenic conditions. Reactor R1 was kept as control, whereas R2, R3 and R4 were fed with 2-NP, 4-NP, and 2,4-DNP, respectively, along with the substrate and nutrients.

During acclimation phase sodium acetate was used as carbon source and sodium nitrate was used as NO₃⁻-N source. Reactors R2, R3 and R4 received 2 mg/l concentration of 2-NP, 4-NP and 2,4-DNP along with sodium acetate, sodium nitrate and nutrients, respectively. Subsequently nitrophenols concentration was increased in steps to 5, 10, 20 and 30 mg/l, keeping hydraulic retention time (HRT) constant as 24 h. At each increment, the reactors were acclimated for 30–53 days to achieve nitrophenols removal of more than 75%. Initially, influent COD was kept as 1000 mg/l. After 45 days of acclimation, influent COD was increased to 1500 mg/l and after next 40 days influent COD was further increased to 2000 mg/l. COD/NO₃⁻-N ratio was kept constant as 20. After 45 days of acclimation, the dry weight fraction of granular sludge inside the reactors having settling velocity of more than 15 m/h were about 45% (w/w) of the total sludge, which increased to about 75% after 80 days of continuous operation. The weight fraction was determined by allowing granular sludge samples to settle through a 75 cm long and 7.5 cm diameter glass column (filled with tap water) for 3 min. The settled fractions were collected and dried at 105°C to calculate dry weight. Visually granules were black in color and size was varying between 0.1 and 2 mm. Details of the reactors performance during acclimation phase has been given elsewhere [12].

Table 1
Composition of the synthetic feed used

| Compounds | Concentration (mg/l) | | | |
|---------------------------------------|----------------------|----------|----------|----------|
| | R1 | R2 | R3 | R4 |
| Sodium acetate (Trihydrated) | 4000 | 4000 | 4000 | 4000 |
| Sodium nitrate | 667–4000 | 667–4000 | 667–4000 | 667–4000 |
| Ammonium chloride | 120 | 120 | 120 | 120 |
| KH ₂ PO ₄ | 200 | 200 | 200 | 200 |
| K ₂ HPO ₄ | 49.8 | 49.8 | 49.8 | 49.8 |
| NaHCO ₃ | 267 | 267 | 267 | 267 |
| CaCl ₂ · 2H ₂ O | 200 | 200 | 200 | 200 |
| MgCl ₂ | 250 | 250 | 250 | 250 |
| KCl | 300 | 300 | 300 | 300 |
| 2-Nitrophenol | — | 30 | — | — |
| 4-Nitrophenol | — | — | 30 | — |
| 2,4-Dinitrophenol | — | — | — | 30 |

The composition of the synthetic wastewater used in the present study has been given in Table 1. Trace metals solution was prepared in distilled water by dissolving per liter 5 g MgSO₄ · 7H₂O, 6 g FeCl₂ · 4H₂O, 0.88 g CoCl₂ · 4H₂O, 0.1 g H₃BO₃, 0.1 g ZnSO₄ · 7H₂O, 0.05 g CuSO₄ · 5H₂O, 1 g NiSO₄ · 8H₂O, 5 g MnCl₂ · 4H₂O and 0.64 g (NH₄)₆Mo₇O₂₄ · 4H₂O [13]. One ml of this solution was added per liter of the feed solution. The influent alkalinity and pH was 1100 ± 50 mg/l (as CaCO₃) and 7.23, respectively.

All the four reactors (R1, R2, R3 and R4) were operated at 24 h HRT keeping influent COD concentration as 2000 ± 48 mg/l (Organic loading rate 2 ± 0.04 kg COD/m³ d). Sodium acetate was used as carbon source and sodium nitrate was used as NO₃⁻-N source. Reactor R1 is kept as control while reactors R2, R3 and R4 were fed with 30 mg/l of 2-NP, 4-NP, and 2,4-DNP, respectively. Nitrate nitrogen loading was increased in steps (0.096, 0.140, 0.204, 0.406, 0.50 and 0.60 kg/m³ d) in order to keep COD/NO₃⁻-N ratio as 20.8, 14.3, 9.8, 5.0, 4.0 and 3.33. The reactors were operated for 20–25 days under steady-state conditions at each COD/NO₃⁻-N ratio. Steady state was arbitrarily considered as variation of nitrophenol and COD concentration in the effluent, and biogas production within 15% of the average value [2].

The analytical procedures for all tests were as outlined in the Standard Methods for the Examination of Water and Wastewater [14], unless specified otherwise. Daily measurements were taken for the rate of gas production, influent and effluent pH, COD, NO₃⁻-N. Influent and effluent samples were analyzed for nitrophenols and aminophenols on alternate day. Volatile fatty acids (VFAs), alkalinity and gas composition were analyzed once a week. The sludge samples were analyzed biweekly

for suspended solids (SS) and volatile suspended solids (VSS).

The specific gravity of the sludge was determined by comparing the mass of a known volume of a homogeneous sludge sample at a specific temperature to the mass of the same volume of distilled water at 4°C [15]. Temperature correction factor was applied for the measured temperature as per Standard Methods [14]. Oxidation–reduction potential (ORP) inside the reactors were monitored using an ORP meter (Model-108, Orion, USA).

Nitrophenols, aminophenols, phenol and aminobenzoate were analyzed by injecting 25 µl filtered liquid samples to high-pressure liquid chromatograph (Shimadzu, LC-6A, Japan) equipped with UV–VIS detector (SPD-6AV) and C₁₈ reverse-phase column (250 mm × 4.6 mm, 5 µ ODS, Hypersil, UK). The detection wavelengths used were 254 (for 2-NP and 2-AP) and 280 nm (for phenol, aminobenzoate, 4-NP, 4-AP, 2,4-DNP and 2-A,4-NP). Mobile phase was 1:1 deionized water and HPLC grade methanol at a flow rate of 1 ml/min [1].

Methane and nitrogen percentage in the biogas was analyzed by injecting 1 ml headspace sample to gas chromatograph (GC) equipped with thermal conductivity detector (TCD). The analysis was done at an oven temperature of 40°C, injector temperature of 100°C and detector temperature of 180°C, using SS Molecular sieve M16, 3 × 1/8" column. The carrier gas was helium applied at a flow rate of 30 ml/min. TCD filament current was kept around 300 mV.

3. Results and discussion

3.1. Effect of COD/NO₃⁻-N ratio

After acclimation reactors were continuously operated and steady state was achieved at COD/NO₃⁻-N ratio of 20.8, 14.3, 9.8, 5 and 4. Steady state could not be achieved at COD/NO₃⁻-N ratio of 3.33 as incomplete denitrification and endogenous metabolism of biomass was observed because of insufficient carbon source inside the reactors. Average steady-state data of this study is summarized in Tables 2–5. At first step, nitrophenols were metabolized into their respective aminophenols. 2-Aminophenol, 4-AP and 2-A,4-NP were found as the main intermediate metabolites in case of 2-NP, 4-NP and 2,4-DNP, respectively. Overall removal of 2-nitrophenol and 4-nitrophenol was found more than 99% at every COD/NO₃⁻-N ratio. 2,4-DNP removal was also more than 99% at all COD/NO₃⁻-N ratios except at 20.8 and 4. Out of the total input nitrophenolic concentration about 42.83–17.99% of 2-NP, 68.76–46.9% of 4-NP, 46–23.50% of 2,4-DNP (on molar basis) were recovered in the form of their

Table 2

Average steady-state data of R1 (Blank reactor) at different COD/NO₃⁻-N ratio

| COD/NO ₃ ⁻ -N ratio | NO ₃ ⁻ -N loading rate (kg NO ₃ ⁻ -N /m ³ d) | %COD removal | Eff. pH | Eff. total alkalinity (mg/l) | Biogas production | | |
|---|---|--------------|---------|------------------------------|-------------------|-------------------|------------------|
| | | | | | l/d | % CH ₄ | % N ₂ |
| 20.8±1.2 | 0.096±0.008 | 97.3 | 8.6 | 1450 | 7.9 | 68.6 | 9.4 |
| 14.3±0.63 | 0.140±0.010 | 98.5 | 8.8 | 1556 | 6.5 | 66.0 | 17.5 |
| 9.8±0.22 | 0.204±0.012 | 98.7 | 9.0 | 2226 | 7.4 | 58.0 | 34 |
| 5.0±0.08 | 0.406±0.009 | 98.8 | 9.2 | 2893 | 6.8 | 22.5 | 71.5 |
| 4.0±0.04 | 0.500±0.010 | 97.7 | 9.3 | 3490 | 6.5 | 0.5 | 94.5 |

Inf.—Influent; Eff.—Effluent.

Table 3

Average steady-state data of R2 (2-NP fed reactor) at different COD/NO₃⁻-N ratio

| COD/NO ₃ ⁻ -N ratio | NO ₃ ⁻ -N loading rate (kg NO ₃ ⁻ -N /m ³ d) | 2-NP (mg/l) | | Eff. 2-AP (mg/l) | COD removal (%) | Eff. pH | Eff. total alkalinity (as CaCO ₃ mg/l) | Biogas production | | |
|---|---|-------------|------|------------------|-----------------|---------|---|-------------------|-------------------|------------------|
| | | Inf. | Eff. | | | | | l/d | % CH ₄ | % N ₂ |
| 20.8±1.2 | 0.096±0.008 | 30.0 | <0.5 | 10.2 | 88.9 | 8.5 | 1373 | 6.4 | 64 | 11 |
| 14.3±0.63 | 0.140±0.010 | 30.8 | <0.5 | 7.2 | 85.6 | 8.8 | 1700 | 4.4 | 65 | 21 |
| 9.8±0.22 | 0.204±0.012 | 30.3 | <0.5 | 6.5 | 94.4 | 9.0 | 2250 | 4.8 | 54 | 39 |
| 5.0±0.08 | 0.406±0.009 | 30.2 | <0.5 | 2.0 | 94.6 | 9.2 | 2940 | 5.1 | 15.5 | 79.5 |
| 4.0±0.04 | 0.500±0.010 | 30.1 | <0.5 | 4.3 | 91.9 | 9.3 | 3500 | 4.8 | 0.5 | 96.2 |

Inf.—Influent; Eff.—Effluent.

Table 4

Average steady-state data of R3 (4-NP fed reactor) at different COD/NO₃⁻-N ratio

| COD/NO ₃ ⁻ -N ratio | NO ₃ ⁻ -N loading rate (kg NO ₃ ⁻ -N /m ³ d) | 4-NP (mg/l) | | Eff. 4-AP (mg/l) | COD removal (%) | Eff. pH | Eff. total alkalinity (as CaCO ₃ mg/l) | Biogas production | | |
|---|---|-------------|------|------------------|-----------------|---------|---|-------------------|------------------|-----------------|
| | | Inf. | Eff. | | | | | l/d | %CH ₄ | %N ₂ |
| 20.8±1.2 | 0.096±0.008 | 30.0 | <0.5 | 16.4 | 88.0 | 8.4 | 1375 | 6.6 | 67.5 | 8.4 |
| 14.3±0.63 | 0.140±0.010 | 30.5 | <0.5 | 4.6 | 92.8 | 8.6 | 1555 | 5.0 | 67.5 | 19 |
| 9.8±0.22 | 0.204±0.012 | 30.2 | <0.5 | 2.5 | 95.3 | 8.9 | 2255 | 6.1 | 53.5 | 40 |
| 5.0±0.08 | 0.406±0.009 | 30.1 | <0.5 | 2.3 | 94.3 | 9.1 | 2940 | 5.1 | 17 | 77.5 |
| 4.0±0.04 | 0.500±0.010 | 30.3 | <0.5 | 12.7 | 95.4 | 9.3 | 3540 | 5.2 | 0.5 | 96.5 |

Inf.—Influent; Eff.—Effluent.

respective amino derivatives at 20.8–4.0 COD/NO₃⁻-N ratio. In other words, out of the total input nitrophenolic concentration (30 mg/l) about 12.85–5.4 mg/l of 2-NP, 20.62–14.07 mg/l of 4-NP, 13.80–7.05 mg/l of 2,4-DNP were present in the effluents in the form of their respective amino derivatives at COD/NO₃⁻-N ratio of 20.8–4. The remaining amount of input nitrophenols got further degraded or came out with the effluents.

Literature suggests that under methanogenic condition nitroaromatic compounds initially get reduced to their respective amino derivatives [4,8]. Further meta-

bolisation of aminophenols can take place via deamination to phenol [8], or by carboxylation dehydroxylation to 3-aminobenzoate [16]. But in the present study no peak other than aminophenol was observed during HPLC analysis. Therefore, it seems that the remaining fraction of nitrophenols got further transformation and degraded. Further degradation of aminophenols in the present study was probably caused by denitrifiers, otherwise aminophenols have been observed to accumulate under methanogenic conditions [26]. On the other hand, denitrifiers (*Pseudomonas*) produce

Table 5

Average steady-state data of R4 (2,4-DNP fed reactor) at different COD/NO₃⁻-N ratio

| COD/NO ₃ ⁻ -N ratio | NO ₃ ⁻ -N loading rate (kg NO ₃ ⁻ -N / m ³ d) | 2,4-DNP (mg/l) | | Eff. 2-A, 4-NP (mg/l) | COD removal (%) | Eff. pH | Eff. total alkalinity (as CaCO ₃ mg/l) | Biogas production | | |
|---|--|----------------|------|-----------------------|-----------------|---------|---|-------------------|------------------|-----------------|
| | | Inf. | Eff. | | | | | l/d | %CH ₄ | %N ₂ |
| 20.8 ± 1.2 | 0.096 ± 0.008 | 30.0 | 2.6 | 10.7 | 83.6 | 8.5 | 1385 | 5.8 | 65.5 | 14.4 |
| 14.3 ± 0.63 | 0.140 ± 0.010 | 30.2 | <0.5 | 4.9 | 84.6 | 8.8 | 1607 | 5.5 | 62.5 | 24.5 |
| 9.8 ± 0.22 | 0.204 ± 0.012 | 29.9 | <0.5 | 4.3 | 92.8 | 8.9 | 2260 | 5.4 | 49 | 45 |
| 5.0 ± 0.08 | 0.406 ± 0.009 | 29.7 | <0.5 | 3.5 | 90.7 | 9.2 | 2940 | 4.9 | 15 | 81 |
| 4.0 ± 0.04 | 0.500 ± 0.010 | 29.8 | 6.4 | 4.7 | 93.0 | 9.5 | 3500 | 5.2 | 0.5 | 97.5 |

Inf.—Influent, Eff.—Effluent.

2-nitrophenol oxygenase enzyme capable of removing nitrite group from 2-NP and 4-NP [11]. Data presented in Tables 3–5 show that the fraction of influent nitrophenols recovered in effluents, in the form of aminophenols, decreased with decreasing COD/NO₃⁻-N ratio. In other words, nitrophenols degradation increased with decrease in COD/NO₃⁻-N ratio. But COD/NO₃⁻-N ratio below 5 had adverse effect on the removal of nitrophenols.

In order to assess the biosorption of 2-NP, 4-NP and 2,4-DNP on granular biomass, batch experiments were carried out using 300 ml serum bottle using granules collected from blank reactor. The results of biosorption and desorption studies have been reported elsewhere [17,18]. Sorption of nitrophenols on the granular sludge obeyed both Freundlich and linear isotherms. 2-NP, 4-NP and 2,4-DNP exhibited a Freundlich constant, K as 19.12×10^{-3} , 23.26×10^{-3} and 26.52×10^{-3} l/g, respectively. Whereas the exponent, $1/n$ was calculated as 1.013, 1.017 and 1.06 for 2-NP, 4-NP and 2,4-DNP, respectively. Using Freundlich isotherm, it was estimated that for an equilibrium nitrophenolic concentration of 0.5 mg/l (equal to the concentration inside the continuous reactors), sorption of 2-NP, 4-NP and 2,4-DNP would be only 8.42 µg/g VSS, 10.20 µg/g VSS and 6.80 µg/g VSS, respectively [17]. Since the UASB reactors were having a long sludge retention time (more than 40 days), the contribution of biosorption was found insignificant as compare to the total removal of nitrophenols.

3.2. Denitrification and methanogenesis

In the reactors treating nitrate-containing wastewater, methanogens and denitrifiers compete for electrons, producing nitrogen and methane, respectively. The fraction of electron flow to methanogenesis and denitrification depends upon the COD/NO₃⁻-N ratio. Because denitrification is thermodynamically more favourable than methanogenesis, denitrifiers out compete methanogens and only the electrons left are utilised

by methanogens [19]. In the present study, at high COD/NO₃⁻-N ratios there were abundant supply of substrate and thus both denitrification and methanogenesis co-existed. Synchronous production of nitrogen and methane gases gave the evidence of simultaneous denitrification and methanogenesis.

NO₃⁻-N removal was more than 99% in all of the four reactors even when the NO₃⁻-N loading was as high as 0.5 kg/m³ d corresponding to COD/NO₃⁻-N ratio of 4. Nitrite concentration was never detected in the effluents. Fig. 2 illustrates the relationship between the quantities of nitrogen gas production and nitrate reduction during COD/NO₃⁻-N ratio study. None of the nitrophenols had adverse effect on the denitrification [17,12]. Therefore, theoretically there should not be any difference in nitrogen gas production in the reactors. However, slight variation in nitrogen gas production as shown in Fig. 2 was probably caused by contamination by atmospheric nitrogen while sampling and analysis. At COD/NO₃⁻-N ratio of 4 and below, reduction in biomass concentration was observed in all the four reactors. Further reduction of COD/NO₃⁻-N ratio to 3.33 resulted in incomplete denitrification and sludge bed rising. At COD/NO₃⁻-N ratio of 3.33 reduction in VSS (by 0.74–0.9 g/d) was observed inside the reactors. This suggests endogenous denitrification inside the reactors and hence steady state could not be achieved. Sludge reduction in the reactors cannot be attributed to washout as VSS in the effluent was only up to 30–40 mg/l. Fang and Zhou [19] reported that denitrifying 1 g of nitrate nitrogen chemically requires 2.86 g COD. However, the actual required COD/NO₃⁻-N ratio for complete denitrification in a bioreactor would be greater than 2.86 because extra COD is required for cell growth. Therefore, from the above results it can be concluded that the optimum COD/NO₃⁻-N ratio for complete denitrification was 5, which is within the wide range of reported data, varying from 3.45 to 5.34 [20].

Denitrification resulted in the generation of alkalinity. Thus the effluent from all the four reactors was having alkalinity more than that of influent. As the

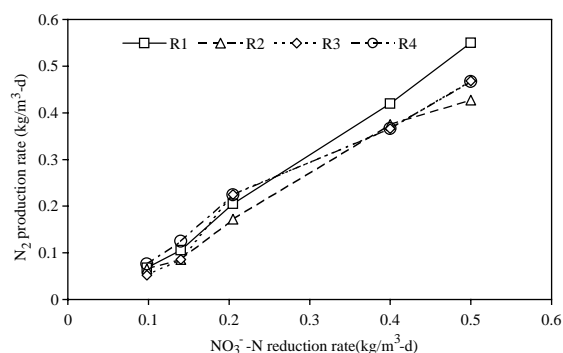


Fig. 2. Conversion of nitrate to nitrogen gas at different COD/NO₃⁻-N.

COD/NO₃⁻-N ratio was reduced, the effluent alkalinity level increased further (Table 2–5). For calculating alkalinity generation per g of NO₃⁻-N denitrification data corresponding to COD/NO₃⁻-N ratio of 4.0 was used, as during this period very small amount of carbon is used for methanogenesis. The alkalinity generated per g NO₃⁻-N reduction was 4.54, which is higher than the theoretical value 3.57. Higher alkalinity generation can be attributed to CO₂ (byproduct) conversion to bicarbonate due to high pH condition in the reactors. Similar conversion of CO₂ (byproduct) to bicarbonate under high pH condition inside a UASB reactor has been reported by Chui et al. [21].

3.3. Characteristics of the granular biomass

Granulation or granule formation is the process of microbial adhesion, which involves attachment of a cell to the surface of another cell. Doling [22] reported that granules are microbial conglomerates with dense structure having well defined appearance.

Initially, granular biomass inside the reactors were of typical black in colour at COD/NO₃⁻-N ratios of 20, 15 and 10, but got changed to light brown at lower COD/NO₃⁻-N ratios of 5.0, 4.0 and 3.33. It may be because of the increased growth of denitrifiers on the surface of the granules at low COD/NO₃⁻-N ratios. The granular sludge developed inside the systems had density of 1017–1049 kg/m³, which is comparable with the observation (1064–1059 kg/m³) made by Hendriksen and Ahring [23].

The average settling velocity of granules was 46.87–54 m/h. About 60% of the granules were of 0.20–0.5 mm size, and maximum size of the granule was 2.6 mm. As the nitrate loading was increased corresponding to lowering of COD/NO₃⁻-N ratio, granules grew more fluffy, filamentous and less dense and tend to washout of the reactor. Similar granular characteristics have been reported by Hendriksen and Ahring [23].

In the present study, oxidation–reduction potential inside the reactors were observed as varying between –210 mV and –250 mV, which depicts prevailing anaerobic condition inside the reactors. However, it is important to note that neither cysteine nor sulphide was used in the influent feed to consume the originally present dissolved oxygen in the water being used for feed preparation. Therefore, it is possible that the presence of dissolved oxygen in the feed (data not available) might caused growth of facultative anaerobes [24] and possibly a three electron acceptors (O₂, NO₃⁻, CO₂) microbial environmental developed at the bottom of the reactors. The densely grown denitrifiers and facultative anaerobes on the surface layer could serve as a barrier and interior of the granules becomes strictly anaerobic zone, favourable for the growth of methanogens [25]. Probably this is how denitrifiers and methanogens co-existed in the granular sludge and simultaneous denitrification/methanogenesis was achieved. This is further proved by the synchronous nitrogen and methane production at 3.33 COD/NO₃⁻-N ratio, when denitrification was incomplete and nitrate was present in the effluents.

4. Conclusions

The present study showed that the removal of nitrophenols improved with the lowering COD/NO₃⁻-N ratio. Specific conclusions are as follows:

1. At first step, nitrophenols got reduced to their respective aminophenols. During HPLC analysis 2-AP, 4-AP and 2,4,4-NP were found as only major intermediate metabolite of 2-nitrophenol, 4-nitrophenol and 2,4-dinitrophenol, respectively.
2. Better removal of nitrophenols was achieved with lowering COD/NO₃⁻-N ratio. However, nitrophenols removal got adversely affected when COD/NO₃⁻-N ratio was lowered below 5.
3. Denitrification and methanogenesis synchronously took place in UASB reactor fed with synthetic wastewater containing acetate, nitrate and nitrophenols. Thus demonstrating the compatibility of denitrifiers and methanogens. NO₃⁻-N removal was more than 99% even when the NO₃⁻-N loading was as high as 0.5 g/l-d corresponding to COD/NO₃⁻-N ratio of 4. Methane gas production was observed even when nitrate nitrogen was present in the effluent (incomplete denitrification at COD/NO₃⁻-N ratio of 3.33). This confirmed the co-existence of denitrifiers and methanogenesis in the granular sludge.
4. At COD/NO₃⁻-N ratio 4 and below endogenous metabolism and sludge bed rising was observed in all the four reactors, may be due to insufficient supply of the substrate. On further lowering of COD/NO₃⁻-N ratio to 3.33 incomplete denitrification was observed. From these findings it can be concluded that the

optimum COD/NO₃⁻-N ratio for complete denitrification was 5.

5. The characteristics of the granules grown inside the reactors were comparable to the other denitrifying granules reported in literature. As the nitrate loading was increased corresponding to lowering of COD/NO₃⁻-N ratio, color of the granules changed from black to light brown and the granules grew more fluffy filamentous and less dense.

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