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Simultaneous biodegradation of nitrogen-containing aromatic compounds in a sequencing batch bioreactor

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Abstract

Many nitrogen-containing aromatic compounds (NACs), such as nitrobenzene (NB), 4-nitrophenol (4-NP), aniline (AN), and 2,4-dinitrophenol (2,4-DNP), are environmentally hazardous, and their removal from contaminated water is one of the main challenges facing wastewater treatment plants. In this study, synthetic wastewater containing NB, 4-NP, 2,4-DNP, and AN at concentrations ranging from 50 to 180 mg/L was fed into a sequencing batch reactor (SBR). Analyses of the SBR system indicated that it simultaneously removed more than 99% of the NACs at loading rates of 0.36 kg NB/(m^3·d), 0.3 kg 4-NP/(m^3·d), 0.25 kg AN/(m^3·d), and 0.1 kg 2,4-DNP/(m^3·d). Bacterial groups of *Bacteriodetes*, Candidate division TM7, α-*Proteobacteria*, and β-*Proteobacteria* were dominant in the clone libraries of 16S rRNA genes retrieved from the microbial communities in the SBR system. "Cycle tests" designed to alter feeding and aeration parameters of the SBR system demonstrated that the resident microbial biome of the SBR system responded rapidly to changing conditions. Consumption of O_2 was concomitant with the apparent mineralization of NACs. Aromatic ring-cleaving dioxygenase activities suggested that (1) AN and NB were degraded via catechol 2,3-dioxygenase; (2) 4-NP was degraded via 1,2,4-benzentriol 1,2-dioxygenase; and (3) 2,4-DNP was degraded via an unresolved pathway.

Key words: nitrogen-containing aromatic compounds; biodegradation; sequencing batch reactor; Candidate division TM7

Introduction

Nitrogen-containing aromatic compounds (NACs) such as nitrobenzene (NB), nitrophenols, and anilines (ANs) are produced in large quantities and are widely used as starting materials for the syntheses of pesticides, dyes, polymers, and pharmaceuticals. NACs have been shown to be toxic or mutagenic, and their release into the environment is subject to governmental regulation. NB, 4-nitrophenol (4-NP), and 2,4-dinitrophenol (2,4-DNP) are listed as "Priority Pollutants" (Tomei *et al*., 2004; She and Liu, 2005). For wastewater discharge in China, NB and AN are required to be lower than 2 and 1 mg/L, respectively (GB 8978-1996, National Standard of China). Bacteria have evolved various pathways for degrading NACs (Nishino and Spain, 1993; Spain and Gibason, 1991; Spain, 1995; Haigler *et al*., 1994; Dickel *et al*., 1993). These metabolic capabilities have been applied in the treatment of various NACs-containing wastewaters, e.g., sequencing batch reactor (SBR) (Tomei *et al*., 2004; Tomei and Annesini, 2005), upflow anaerobic sludge blanket reactor (Karim and Gupta, 2001, 2003), anaerobic baffled reactor (Kuscu and Sponza, 2005), and combinations of different bioreactors (Dickel *et al*., 1993; Sponza and Kuscu, 2005; Majumder and Gupta, 2003). Of these wastewater treatment processes, SBR has proved to be an efficient process and has generated extensive interest. The dynamic operational conditions selectively enrich microbial communities that are optimized to degrade and mineralize NACs (Ellis *et al*., 1996). Furthermore, SBR systems have high operational flexibility under both O_2-enriched and O_2-depleted conditions (Tomei and Annesini, 2005).

Although many studies have been undertaken to resolve the mechanism by which NACs is degraded, most of these assessments were under conditions different from those encountered in wastewater treatment plants (i.e., they utilized pure bacterial strains rather than complex microbial communities, or targeted a single nitrogen-containing aromatic compound) (Tomei *et al*., 2004; Dickel *et al*., 1993; Tomei and Annesini, 2005; Karim and Gupta, 2001, 2003; Majumder and Gupta, 2003). In this study, an SBR system was used to treat synthetic wastewater containing multiple NACs (i.e., NB, 4-NP, 2,4-DNP, and AN). Here we present the results of the performance and microbial

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community of an SBR reactor system.

1 Materials and methods

1.1 Chemicals

NB, 4-NP, 2,4-DNP, and AN were supplied by Beijing Chemical Reagents Company (Beijing, China; AR, 99.9%).

1.2 SBR reactor, start-up, and operation

The experimental set-up is illustrated in Fig.1. The SBR reactor used in this study is a 10×30×30 (cm) rectangular Plexiglas tank and had total volume of 9 L and working volume of 6 L. There was a cover on the top of the tank with a water seal that prevented the volatilization of NACs. Effluent was drawn from a port at 10 cm above the base and was controlled by an electromagnetic switch. The reactor was equipped with a dissolved O_2 sensor (Rex, JPSJ-605, Japan) for online monitoring. Feeding was performed by a peristaltic pump (Pulsafeeder, 150 series). Two stirrers were employed for mixing. Air was supplied with a flow-variable air compressor through 4 gas diffusers fixed at the base of the reactor. The operational parameters were controlled with 4 timers that were programmed to regulate the influent (water-in), aeration, stirring, and effluent (water-out), respectively (Table 1).

The SBR was initiated with 6 L of activated sludge from wastewater treatment plant of Nanjing Chemical Factory (Nanjing, China), which is one of the largest producers of NB, chlorinated NB, and other NACs in China (this factory produces 100000 t of chloranitrobenzene annually). The seed sludge had a mixed liquor suspended solids (MLSS) concentration of 5.0 g/L.

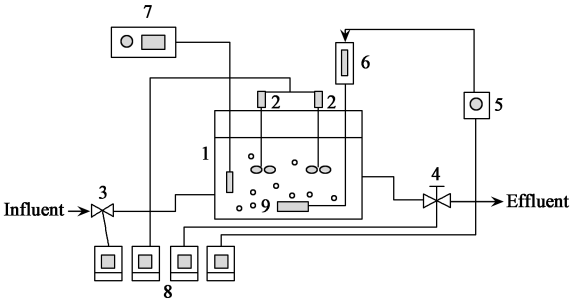

Fig. 1 Pilot experimental system. (1) reactor; (2) stirrer; (3) filling pump; (4) electromagnetic switch; (5) air pump; (6) flow meter; (7) dissolved O_2 meter; (8) programmable timer; (9) air diffuser.

Table 1 Operational parameters of the sequencing batch reactor

Duration (d)		0–118	119–141	142–240
Cycle length (min)		720	480	360
Hydraulic retention time (d)		1	0.67	0.5
Fill (water-in) time (min)	(1)	0–15	0–15	0–15
	(2)	120–135	120–135	60–75
Aerated time (min)	(1)	0–60	0–60	0–120
	(2)	120–180	120–180	150–210
	(3)	240–300	240–360	270–300
	(4)	360–420	390–420	
	(5)	480–540		
Anoxic time (min)	(1)	60–120	60–120	120–150
	(2)	180–240	180–240	210–270
	(3)	300–360	300–360	
	(4)	420–480	360–390	
	(5)	540–600		
Sludge settling time (min)		600–690	420–450	300–330
Draw (water-out) time (min)		690–720	450–480	330–360

1.3 Composition of synthetic wastewater

The reactor was fed with synthetic wastewater that contained (per liter): 100–180 mg NB, 100–150 mg 4-NP, 25–50 mg 2,4-DNP, 50–100 mg AN, 50 mg KH_2PO_4, 12 mg K_2HPO_4, 10 mg yeast extract, and 1 ml of mineral salts solution (Karim and Gupta, 2003). The composition of this synthetic wastewater was based on the analysis of the wastewater discharged by Nanjing Chemical Factory, which contained (per liter): 100 mg NB, 100 mg 4-NP, 25 mg 2,4-DNP, and 50 mg AN.

1.4 Analytical methods

MLSS was measured by standard methods (APHA, 1998). NACs were determined with a high performance liquid chromatograph (HPLC) (Hewlett-Packard 1050 series) equipped with a C18 reverse-phase column (ZORBAX Extend-C18, Agilent) and a UV detector set at 280 nm. The mobile phase was 60% HPLC grade methanol plus 40% deionized water at a flow rate of 1 ml/min. Total organic carbon (TOC) was measured with a TOC analyzer (SHIMADZU, TOC-5000). Dissolved O_2 (DO) concentrations was measured on-line with a DO sensor (Rex, JPSJ-605) at 2 min intervals, and the data were collected with a computer.

1.5 Cycle tests

To study the mechanism by which NACs were degraded in the SBR system, aromatic ring-cleavage dioxygenase activities, as well as changes of various NACs and TOC concentrations during two operational cycles were examined on day 250 and 290. Stable operating parameters were maintained for at least 2 weeks before conducting the cycle tests; the cycle tests were repeated 3 times.

1.6 Preparation of cell-free lysates and enzyme assays

Cell-free lysates of sludge were prepared by subjecting sludge to 90 cycles of sonication in an ice bath, each cycle consisting of 3 s sonication at 200 W and 5 s without sonication. Debris and particulate matter were removed by centrifugation at 12000 ×*g* for 20 min, and the resulting supernatant fluid was used for enzyme assays. The following aromatic ring-cleaving dioxygenases were assayed according to the cited methods: catechol 1,2-dioxygenase (Srachan *et al*., 1998), catechol 2,3-dioxygenase (Akiko *et al*., 2003), 1,2,4-benzentriol 1,2-dioxygenase (Travkin *et al*., 1997), protocatechuate 3,4-dioxygenase (Iwagami *et al*., 2000), 2-aminopheonl 1,6-dioxygenase (Wu *et al*., 2005), and gentisate 1,2-dioxygenase (Werwath *et al*., 1998). One unit of ring-cleavage dioxygenase activity is defined as the amount of enzyme required for the conversion of 1 mmol/min of substrate or the formation of 1 mmol/min product.

1.7 DNA extraction, construction of 16S rRNA gene library, DNA sequencing, and PCR

A bead-beating method (Oved *et al*., 2001) was used for extraction of total DNA from sludge samples. Amplification of 16S rRNA genes from sludge samples was facilitated by PCR with general primers (27f and 1492r) (Lane, 1991). The amplified 16S rRNA genes were inserted into pGEM-T easy vectors (Promega, WI, USA) and transformed into *E. coli* JM109. The diversity of clone libraries of 16S rRNA genes were examined by endonucleatide restriction digestion, and at least 100 clones from each clone library were sequenced at both 3′- and 5′-ends (each approximately 500 bp) by Beijing Genome Institute (Huada Corp., Beijing, China). Chimera sequences were detected by the Chimera function at the RDP site (http://rdp.cme.msu.edu/), and all chimera sequences were eliminated.

2 Results and discussion

2.1 SBR performance

The operation and performances of the SBR system are illustrated in Fig.2. The entire operation period consisted of three stages, each determined by operational parameters and loading rates. During Stage 1 (day 1 to day 118), (1) loading rates of AN, NB, 4-NP, and 2,4-DNP were low (<0.1 kg/(m^3·d), Fig.2d), (2) the hydraulic retention time (HRT) was long (12 h), (3) the sludge retention time (SRT) was 30 d, and (4) the performance and removal rates for all compounds were minimal (Fig.2c). However, at the end of Stage 1, performance improved significantly. 99% or greater amounts of all NACs were degraded (AN, NB, 4-NP, and 2,4-DNP were below detection limits in the effluent). This result appeared to be attributed to the acclimation of sludge to NACs. During Stage 2 (day 119 to day 142), (1) loading rates were increased to approximately 0.15 kg/(m^3·d) for NB and 4-NP by reducing the HRT from 12–8 h, (2) biomass in the reactor, as indicated by sludge concentration (Fig.2a), increased rapidly, and (3) the efficiency of removal of all NACs was high (>99% depletion) (Fig.2c). During Stage 3 (day 142 to day 225) the loading rates were increased to approximately 0.36 kg NB/(m^3·d) and 0.30 kg 4-NP/(m^3·d) by reducing the HRT from 8–6 h and increasing the influent concentrations of NB and 4-NP to 180 and 150 mg/L, respectively (Fig.2b). At the beginning of Stage 3, a significant increase of biomass resulted in sludge concentration as high as approximately 8000 mg/L, and the SRT was decreased to 15 d (in contrast to 25 d in Stage 2) by daily discharge of sludge. Amounts of NACs removed exceeded 99%. Most previous studies have been conducted with a single nitrogen-containing aromatic compound (Tomei *et al*., 2004; Dickel *et al*., 1993; Tomei and Annesini, 2005; Karim and Gupta, 2001, 2003; Majumder and Gupta, 2003), and thus do not simulate industrial wastewaters that contain multiple NACs. The present study demonstrates that industrial wastewater that contains four major NACs can be remediated by SBR. The capacity to simultaneously remove multiple NACs may be due to the oxic/anoxic cycles of the SBR reactor that yielded a relatively low DO (DO was lower than 0.5 mg/L), i.e., the oxic/anoxic cycles and low DO might provide

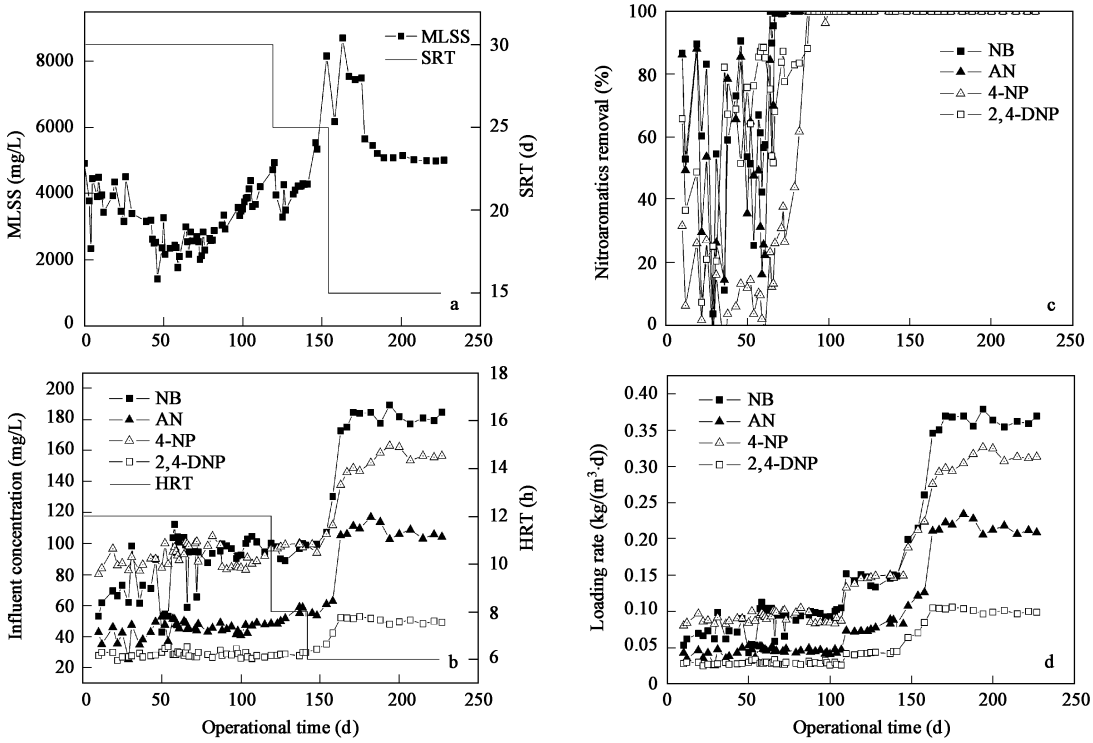

Fig. 2 Operational parameters and performance of the sequencing batch reactor system. (a) sludge concentration (MLSS) and solid retention time (SRT); (b) influent concentrations (mg/L) and hydraulic retention time (HRT); (c) removal efficiency (%); (d) loading rates (kg/(m^3·d)) for aniline (AN), nitrobenzene (NB), 4-nitrophenol (4-NP), 2,4-dinitrophenol (2,4-DNP).

more suitable conditions for the microbial degradation of NACs.

2.2 Bacterial communities at various stages of operation

Four 16S rRNA gene libraries were constructed from sludge samples collected at days 1, 90, 150, and 190 (Fig.3). Analysis of the gene libraries and partial sequences of 16S rRNA genes retrieved by DGGE (data not shown) indicated significant time-dependent changes in the detected microbial communities. Based of the number of detected sequences, the seed sludge (Fig.3, day 1) was dominated by γ-*Proteobacteria* (44%) and *Firmicutes* (33%); *Bacteroidetes* (11%) was a less dominant population. The initial microbial community was not well adapted to the NACs-enriched wastewater, as indicated by the poor SBR performance (Fig.2c). The adapted sludge at day 190 was dominated by bacterial populations of *Bacteriodetes* (47%) and β-*Proteobacteria* (36%); Candidate division TM7 (9%) was also present at day 190 (Fig.3). The apparent shift in the detected populations suggested that *Bacteriodetes*, β-*Proteobacteria*, and perhaps Candidate division TM7 were contributing to the degradation of NACs. α-*Proteobacteria* (12% at day 90) appeared to be a transiently dominant bacterial population. Detected sequences indicative of *Actinobacteria* (3%–4%) remained stable throughout SBR operation. In contrast, detected sequences indicative of γ-*Proteobacteria* and *Firmicutes* decreased significantly by day 150, and those indicative of Candidate division TM7 increased from 1% at day 1 to 44% at day 150 (as noted above, this population decreased to 9% at day 190) (Fig.3).

Bacteria phylogenetically related to the poorly understood Candidate division TM7 occur in soil and activated sludge (Dunbar *et al*., 1999; Bond *et al*., 1995), and appear to be associated with activated sludge bulking (Thomsen *et al*., 2002). In the present study, detected members of Candidate division TM7 increased significantly during SBR operation. However, sludge bulking was not observed, indicating that the Candidate division TM7-related population did not produce sludge bulking under the conditions used in this study. The *in situ* function(s) of this bacterial group in the NACs-degrading SBR is under investigation.

2.3 Cycle tests

Cycle tests (designed to alter feeding and aeration parameters of the SBR system) were conducted to evaluate the microbial processes ongoing in the SBR system (Figs.4 and 5a). Concentrations of AN, NB, 4-NP, and 2,4-DNP decreased quickly due to dilution and microbial degradation as soon as they were fed into the reactor (the influent concentrations of AN, NB, 4-NP, 2,4-DNP were 100, 180, 150, and 50 mg/L, respectively). Low DO concentrations

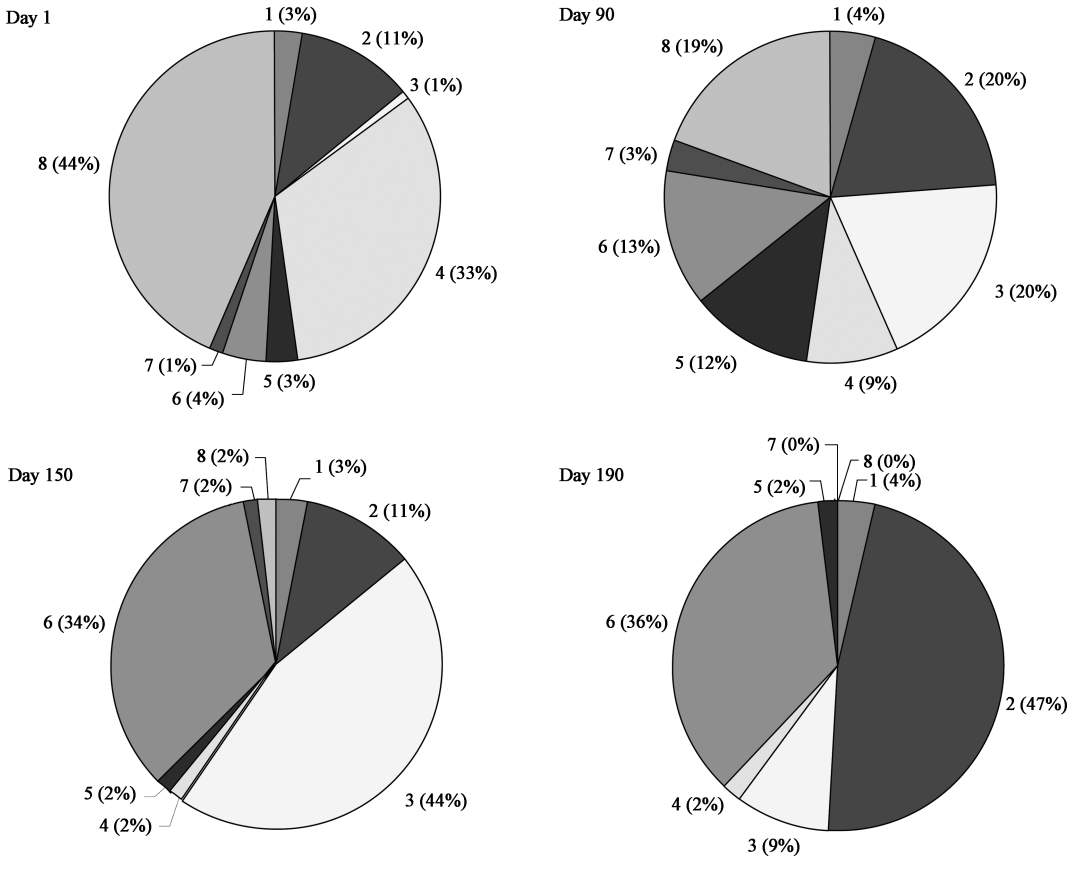

Fig. 3 Bacterial communities in sequencing batch reactor system. (1) *Actinobacteria*; (2) *Bacteriodetes*; (3) Candidate division TM7; (4) *Firmicutes*; (5) α-*Proteobacteria*; (6) β-*Proteobacteia*; (7) δ-*Proteobacteria*; (8) γ-*Proteobacteria*. One sequence of Candidate division OP10 and one sequence of *Planctomycetales* (day 1) were also detected, but these bacterial populations are not shown in the figures.

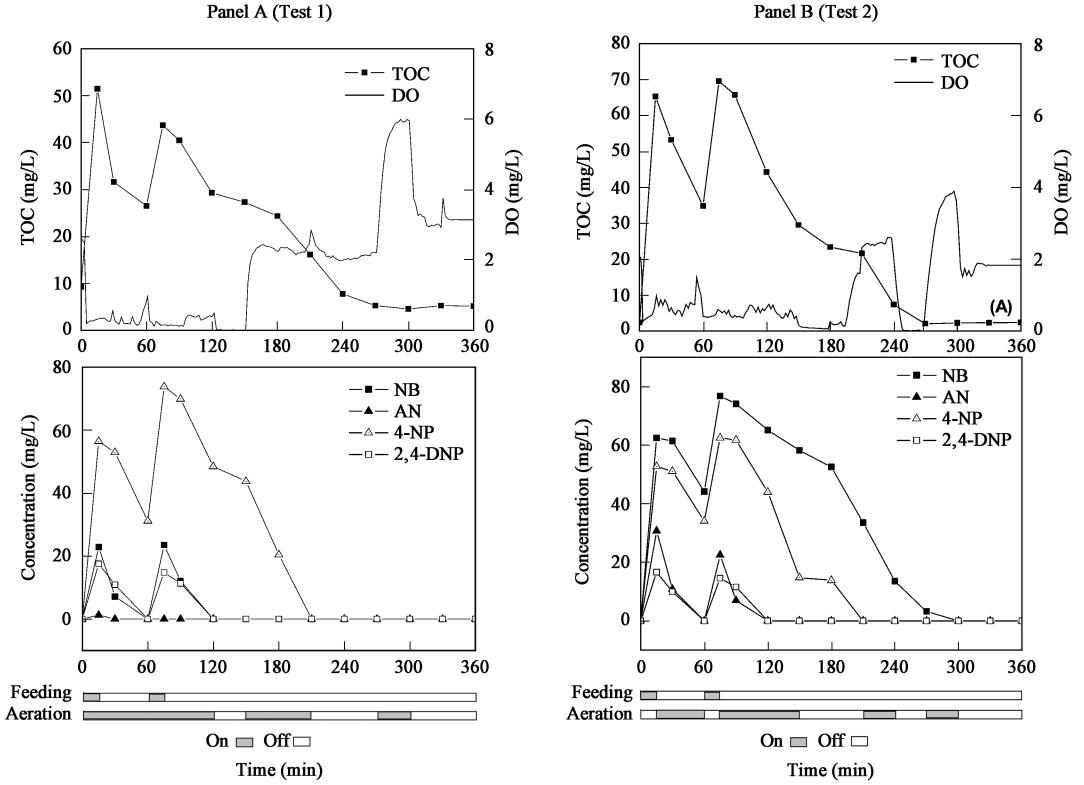

Fig. 4 Profiles of total organic carbon (TOC), dissolved O_2 (DO), nitrobenzene (NB), aniline (AN), 4-nitrophenol (4-NP), 2,4-dinitrophenol (2,4-DNP) and operational parameters during "cycle tests".

(<0.5 mg/L) were correlated to high consumption rates of O_2 during the degradation of NACs. DO concentrations increased significantly only when the concentrations of NACs decreased to very low levels (Fig.4, period after 150 and 200 min for Test 1 and Test 2, respectively). TOC was analyzed to investigate overall removal rates and determine if NACs were converted to intermediates. TOC concentrations at the end of Tests 1 and 2 were lower than 10 mg/L (Fig.4), indicating that overall TOC removal exceeded 95%. HPLC analysis did not reveal any metabolic intermediates (data not shown). These collective observations suggested a nearly complete mineralization of all NACs by the SBR-associated microbial biome.

2.4 Aromatic ring-cleaving dioxygenases and putative pathways for degradation of NACs

Catechol 2,3-dioxygenase, 1,2,4-benzentriol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase activities were prominent during all SBR operational periods (Fig.5a). In contrast, catechol 1,2-dioxygenase, gentisate 1,2-dioxygenase, and 2-aminophenol 1,6-dioxygenase activities were not detected.

To more clearly establish links between dioxygenase activities and the degradation of NACs, each dioxygenase activity was determined when the SBR system received different combination of NACs (i.e., when one of the four NACs was omitted) (Fig.5b). When all four NACs were provided, catechol 2,3-dioxygenase, 1,2,4-benzentriol 1,2-dioxygenase, and protocatechol 3,4-dioxygenase activities were 0.62, 0.13, and 0.14 U/mg SS, respectively. Assuming that these activities are 100% of the maximum possible for each enzyme, the activity of catechol 2,3-dioxygenase

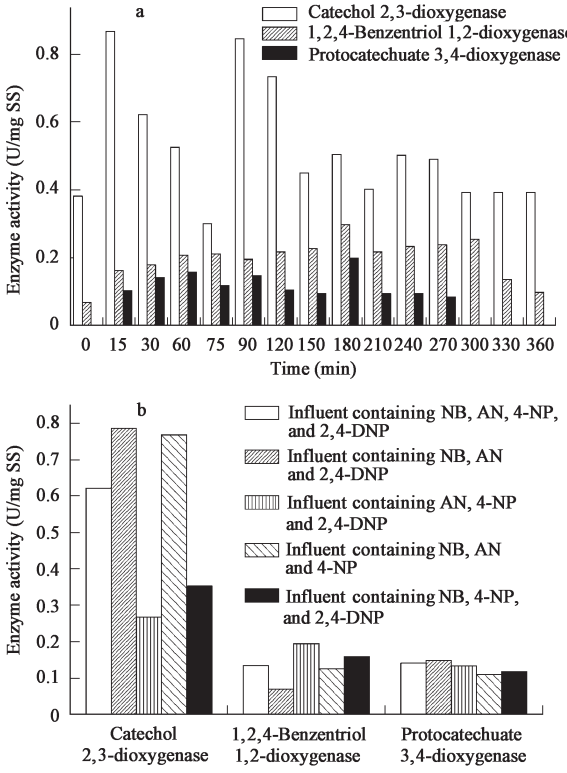

Fig. 5 Aromatic ring-cleaving dioxygenases detected during the degradation of various NACs in the sequencing batch reactor system. See text for details. Abbreviations: nitrobenzene (NB), 4-nitrophenol (4-NP), aniline (AN), 2,4-dinitrophenol (2,4-DNP).

decreased by 62% and 48% when NB and AN were deleted, respectively. This result indicated that catchol 2,3-dioxygenase was involved in the degradation of NB and AN. Similarly, 1,2,4-benzentriol 1,2-dioxygenase activity decreased by 43% when 4-NP was deleted, implying that 4-NP was mainly degraded via a 1,2,4-benzentriol 1,2-dioxygenase-dependent pathway. The data also indicated that each of these dioxygenases could be induced by a specific nitrogen-containing aromatic compound. Protocatechuate 3,4-dioxygenase activity did change significantly in response to the availability of different NACs, and it is therefore not possible to correlate this enzymatic activity to the degradation of any of the tested NACs.

3 Conclusions

In this study, a SBR system for degrading NACs in simulated NACs-contaminated wastewaters was developed. The SBR system simultaneously removed more than 99% of nitrobenzene, nitrophenol, aniline, and 2,4-dinitrophenol present at initial concentrations ranging from 50 to 180 mg/L. Analysis of the SBR-associated microbial biome revealed very diversified bacterial populations. Bacteria phylogenetically related to Candidate division TM7 appeared to be significant during SBR operation but did not yield sludge bulking. Based on aromatic ring-cleaving dioxygenase activities, putative degradative pathways for each of the tested NACs are discussed.

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