Biodegradation of multiple nitrosamines by the *Bacillus* species LT1C in drinking water biofilters

Wanfeng Wang, Yanling Guo, Yao Huang, Chunyou Zhu, Jing Fan and Feng Pan

**ABSTRACT**

To better clarify the biodegradation mechanism of nitrosamines at low concentrations and characterize the nitrosamine-reducing strains isolated from biological activated carbon (BAC) filters in drinking water treatment plants. The nitrosamine-reducing bacterial culture *Bacillus* species LT1C was cultivated, isolated and purified from a pilot filter system using BAC, and was then applied to evaluate the biodegradable capacity for nitrosamines. Five nitrosamines (N-nitrosomethylamine, N-nitrosodi-n-propylamine; *N*-nitrosopyrrolidine and *N*-nitrosodi-n-butylamine, *N*-nitrosopiperidine) were biodegraded in mineral salts medium with removal ratios which ranged from 23.6 to 51.2%. The removal ratios achieved by the isolate were in accordance with the structure, Log*K*<sub>ow</sub> and Henry’s constant of the nitrosamines examined. The findings of this study demonstrate increased potential for the establishment of nitrosamine control strategies in the water supply industry.

**Key words** | biodegradation, biological activated carbon, isolation, nitrosamines, nitrosamine-reducing bacteria

**INTRODUCTION**

Occurrence of nitrosamines in water supplies is an emerging issue of public health significance because many nitrosamines are potent carcinogens, teratogens and mutagens (*Mitch et al. 2003*). The US Environmental Protection Agency has expanded potential regulatory monitoring to include *N*-nitrosodimethylamine (NDMA) and the nitrosamines *N*-nitrosodiethylamine (NDEA), *N*-nitrosomethylamine (NMEA), *N*-nitrosodi-n-propylamine (NDPA), *N*-nitrosopyrrolidine (Npyr), *N*-nitrosobutylamine (NDBA), and *N*-nitrosodiphenylamine (NDPhA) in the Unregulated Contaminant Monitoring Regulation Rule 2 (USEPA 2006) and Contaminant Candidate List 5 (USEPA 2009). Emerging regulatory and notification levels for NDMA, NDEA, and NDPA have been set at or below 10 ng/L (*CADPH 2011*). However, many nitrosamines have been detected in excess of this threshold in both source water and finished water.

In an effort to reduce nitrosamines, reverse osmosis (RO) (*Plumlee et al. 2008*), ultraviolet and advanced oxidation processes (AOP, i.e., UV/H<sub>2</sub>O<sub>2</sub>, O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>) (*Jobb et al. 1994; Liang 2002*) have been used, however, only a partial reduction of nitrosamines can be expected using these processes under practical operating conditions, and application of these processes are limited due to their costliness and complexity (*Lee et al. 2005; Lee et al. 2007*). Furthermore, many nitrosamines generated early in drinking water treatment processes because pre-oxidation processes are used widely (such as pre-chlorination, pre-chloramination and pre-ozonation), which leads to the formation of nitrosamines before filtration and disinfection processes. Biological treatment, although slow under environmental conditions, is believed to be an important attenuation mechanism for removal of these nitrosamines in biofilters (biological activated carbon (BAC)), due to its many advantages, such as high removal efficiencies and capacities for carbonaceous organic substances, total nitrogen, ammonia and suspended solids. In addition, the extension of AC regeneration period could be obtained, and the risk of chemical treatments could also be avoided in drinking water treatment (*Wang...*).
et al. 2006; Zhou et al. 2009; Patterson et al. 2012). For the practical operation of drinking water treatment plants (DWTP), screening and isolation of microbial strains capable of reducing nitrosamines are urgently required.

Nowadays, it has been reported that nitrosamines could be reduced by anaerobic or aerobic bacteria. However, our understanding of the microbial nitrosamine degradation is largely limited to NDMA. Fournier et al. (2009) and Hatzinger et al. (2011) reported that the propanotroph Rhodococcus ruber ENV425 could biodegrade NDMA under an aerobic environment. Ralstonia pickettii PKO129 (Sharp et al. 2008) and Pseudomonas mendocina KR1 (Fournier et al. 2006) also have the capacity for reducing NDMA. These studies only focused on NDMA at high concentrations (μmol/L-level or mmol/L-level). Homme & Sharp (2013) clarified that the bacterial strain Rhodococcus jostii RHA1 could biotransform NDMA, NDEA, NDPA, Npyr, and possibly N-nitrosomorpholine (NMor) under high concentration (20–2,000 μg/L). However, in our previous survey (Wang et al. 2014), the dominant nitrosamines in source water and finished water were NDMA (6.4–46.9 ng/L), NDEA (1.9–16.3 ng/L) and NDBA (1.0–19.9 ng/L), which are usually present at much lower concentrations (<100 ng/L), and the degradation rate and attenuation mechanism of nitrosamines at these concentrations is unknown. Therefore, further studies on the biodegradation of nitrosamines at relatively low concentrations are of practical significance.

The aim of the present study was: (1) to track the possibility of biological nitrosamine reduction in the biofilters exposed to nitrosamines; (2) to isolate the nitrosamine-reducing strain from BAC samples; and (3) to characterize the biodegradation of multiple nitrosamines at relatively low concentrations. It is expected that the present study would provide useful information for the establishment of nitrosamine control strategies in the water supply industry.

**MATERIALS AND METHODS**

**Chemicals and preparation of nitrosamine-containing water**

Standard solutions of 1,000 μg/mL NDMA, NDEA, NMor, NDBA, NDPA, NMEA, Npyr, N-nitrosopiperidine (NPip), and NDPhA were purchased from Supelco (USA), and the other chemicals used in this study were in HPLC grade. Filtered water samples from DWTP containing approximately 15 ng/L NDMA, 3.0 mg/L dissolved oxygen, 0.58 mg/L ammonia (NH₃-N), and 0.6 mg/L dissolved organic carbon were used as the influent for the bench-scale experiment, and spiked with a mixture of nitrosamines (nine nitrosamines at a concentration of 1,000 ng/L each).

**Experimental apparatus setup**

The BAC samples used for the experiment were obtained from one DWTP during stable operation. Coal active carbon was used in BAC. The drinking water treatment processes of DWTP include pre-chlorination, coagulation and sedimentation, ozonation, BAC filter, and disinfection (chlorine). Two glass columns (inner diameter = 4.0 cm) with a working volume of 450 mL were filled with the obtained BAC samples, and one of them was used as a control by sterilizing the BAC before filling (Figure 1). The filtered water was circulated using peristaltic pumps.
Isolation and identification of nitrosamine-reducing bacterium

The BAC samples were collected from the bench-scale BAC filter after cultivating with the backwashed water for 60 days. A total of 10 g BAC was poured into a glass triangular flask containing 50 mL of phosphate buffer (pH 7.3) and stirred with glass beads (diameter = 4.0 mm) for 4 h. Then, a 1.0 mL suspension was put into another flask containing 50 mL of tryptone soya broth (TSB, Oxoid Ltd, England) liquid medium and the nine nitrosamines (1.0 μg/L for each), and then agitated on a rotary shaker at 150 r/min. After the culture was grown to the late logarithmic phase (optical density at 600 nm [OD600] of ~1.9), 100 μL cells were incubated on nitrosamine TSB plate for 3 days at 25 °C. Finally, three types of bacteria obtained were isolated from the TSB plates. To identify the purification, each bacterium was cultivated again in TSB liquid medium and then grown on TSB plates.

The nitrosamine-reducing behavior of the isolates were evaluated using the liquid mineral salts (MS) medium (4.25 g/L K2HPO4·3H2O, 1.0 g/L NaH2PO4·H2O, 0.20 g/L MgSO4·7H2O, 0.012 g/L FeSO4·7H2O, 0.005 g/L MnSO4·H2O, 0.003 g/L ZnSO4·7H2O, and 0.001 g/L CoSO4·7H2O, 2 g/L NH4Cl, and 0.054 g/L C6H6O6; pH 7.3) (Plumlee & Reinhard 2007). The 500-mL autoclaved amberized vial containing liquid MS medium was added nine nitrosamines (each at a final concentration of 200 ng/L) and 5 mL of purified bacterium (after incubation in liquid TSB containing nitrosamines at 25 °C), capped with sealing film in bioclean bench and then incubated at 25 °C in the dark. A total of 500 mL of the MS liquid medium containing residual nitrosamines after biodegradation by isolate was immediately filtered by glass fibre pads (GF/F, 0.7 μm, Whatman), and then enriched by solid phase extraction column (Resprep EPA Method 521 cartridges, 2 g /6 mL).

Micrographs of the isolate were taken using a scanning electron microscope (S-3000N, Hitachi, Japan). For bacteria identification, the genomic DNA of the purified strain was extracted. Primer pairs of 27F (5'-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGGTCACCTTGTTACGACTT-3’) were used to amplify the 16S rRNA genes. Polymerase chain reaction (PCR) was performed in 25 μL mixture containing 2.5 μL of 10× Fast-Pfu Buffer, 0.5 μL of dNTPs (10 mmol/L), 0.5 μL of each primer (5 μmol/L), 0.25 μL of Taq polymerase (5 U/μL), 1 μL template DNA and 19.75 μL DD water. The following conditions were used for PCR: 5 min of initial denaturation at 95 °C and 30 cycles of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C, followed by extension for 10 min at 72 °C and a hold at 10 °C. PCR products were purified using the Axy-Prep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and then were sent to Shanghai Majorbio Bio-pharm Technology Company for sequencing. The nucleotide sequence was analyzed by conducting a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS AND DISCUSSION

Screening of nitrosamine-reducing bacterium

Nitrosamine removal performance of the BAC filters (Figure 1) was investigated. The changes of nitrosamine concentrations in BAC filters after 5 days of cultivation showed that six nitrosamines were degraded, including NDMA, NMEA, NDPA, Npyr, NPip and NDBA (data not shown), indicated that some nitrosamine-reducing bacteria were probable in BAC filters. After 60 days cultivation, the BAC samples were collected, and screened for nitrosamine-degrading microorganisms. Bacterial growth was confirmed on TSB agar plates at 25 C, and three different bacterial strains were obtained. All the isolates formed clear zone on TSB agar plates around their colonies. These clear zone-forming colonies were then transferred onto nitrosamine-agar plates. All the isolates also formed a clear zone...
on nitrosamine plates. With the preliminary degradation of nitrosamines by the three isolates, the nitrosamine-reducing strain (deposited in China General Microbiological Culture Collection Center, CGMCC, No. 10067), *Bacillus* species LT1C, an effective multiple nitrosamines degrader, identified as *Bacillus*, this species is the first reported nitrosamine-reducing bacterium. Strain LT1C was a rod-shaped, spore-forming and non-unsheathed bacterium (Figure 2), and which belongs to the Gram-positive bacterium. The lengths range from 1.5 to 3.0 μm. Sequence analysis of the 1,167 bases of the 16S rRNA gene of strain *Bacillus* sp. LT1C also showed 100% identity with those of the *Bacillus* using the 16S rDNA sequence database (GenBank accession number GU458266.1).

**Identification of the role of nitrosamines in biodegradation by the *Bacillus* sp. LT1C**

Figure 3 describes the biodegradation of the nitrosamines examined as a carbon source (no glucose) or nitrogen source (no ammonia chloride) in MS medium after 5 days of bioattenuation. In comparison with nitrosamines...
as nitrogen source, degradation ratios of *Bacillus* sp. LT1C with nitrosamines as the carbon source were significantly higher, with increased removal ratios of 41.4% (NMEA), 15.0% (NDPA), 14.6% (NDBA), 8.8% (Npyr), and 11.8% (NPip). Sharp *et al.* (2007, 2011) and Fournier *et al.* (2009) reported that three strains (*Rhodococcus jostii* RHA1, *Rhodococcus* species RR1 and *R. ruber* ENV425) could biotransform NDMA when grown on pyruvate, soy broth, lysogeny broth (LB), or propane. A significant fraction of the carbon in the NDMA molecule was released as CO$_2$ by strain *R. ruber* ENV425, although growth on NDMA could not be confirmed. Furthermore, the rates of transformation by the RHA1 strain were enhanced by approximately 300–500-fold after growth on propane and when using a higher concentration of NDMA (mg/L level); RHA1 also biotransforms multiple nitrosamines after growth on media containing propane as the carbon source (Homme & Sharp 2013). Strains from other genera that biodegrade NDMA, including *P. mendocina* KR1, *Methyllosinus trichosporium* OB3b, *Mycobacterium vaccae* JOB-5 and *R. pickettii* PKO129, have also been shown to remove NDMA when grown on toluene or methane as the sole carbon and energy source (Sharp *et al.* 2005; Fournier *et al.* 2006). These reports proved that nitrosamines were used as the nitrogen source, and other cometabolic compounds (i.e. propane, methane or toluene) as carbon source degraded by different nitrosamine-reducing bacteria. However, the results in this study showed that strain LT1C utilized nitrosamine as the carbon source, but not as a nitrogen source, although the transformation rates of nitrosamines were very slow. The biotransformation mechanism of nitrosamines used as carbon source is unknown, and this needs further study.

**Biodegradation of nitrosamines by the isolated bacterium**

The nitrosamine-degrading ability of the isolate was characterized after incubation for 10 days in MS medium at 25 C. The degradation of NMEA, NDPA, Npyr, NPip and NDBA (each 200 ng/L) by the *Bacillus* sp. LT1C was higher when compared with that exhibited by the controls over a period of 10 days in the MS medium (Figure 4). The removal ratios ranged from 23.6 to 51.2%. Among the five nitrosamines, the 10 day removal ratio of NMEA by the nitrosamine-reducing strain was the highest (from 200 to 97.6 ng/L), followed by NDPA (from 200 to 120.5 ng/L) and NDBA (from 200 to 125.9 ng/L). The removal ratios of Npyr and NPip were less than 25%. The five nitrosamines could be arranged in the following order based on their decreasing removal ratios: NMEA (51.6%) > NDPA (40.0%) > NDBA (37.1%) > Npyr (24.9%) > NPip (23.6%).

In previous studies, many isolates belonging to different genera have shown the biodegradation capacities for NDMA at high concentrations, and propane monooxygenases, soluble methane monooxygenases and toluene monoxygenases enhanced the biodegradation of NDMA as the nitrogen source (Sharp *et al.* 2005, 2011; Fournier *et al.* 2006, 2009; Tezel *et al.* 2011). Multiple nitrosamines under high concentrations have also been biodegraded by an isolate reported by Homme & Sharp (2013), growth of cells on media with added propane as the sole carbon source greatly enhanced degradation rates. However, no study has focused on the biodegradation of multiple nitrosamines at low concentrations. The low reaction rates observed in this study indicated a biotransformation mechanism which uses nitrosamines as the sole carbon source only at low concentrations. LT1C is likely to degrade nitrosamine-derived carbon to CO$_2$, although the transformation rates of nitrosamines were very slow. In addition, there are other organics in BAC filters, even with toxic compounds; cell toxicity and the competitive interactions might have greater effects on the biotransformation mechanism at low concentrations of nitrosamines (Hatzinger *et al.* 2011), which requires further study.

With regard to the structure of the nitrosamines, the removal ratios of linear nitrosamines (NMEA, NDPA and NDBA) (Figures 4(a)–4(c)) were higher than those of cyclic nitrosamines (Npyr and NPip) (Figures 4(d) and 4(e)), and which were lower with the MW increase of the nitrosamines (Table 1). These results are consistent with those reported in previous studies (Drewes *et al.* 2006; Homme & Sharp 2013). Among the five nitrosamines examined, our data show that NDBA also degrades faster than the cyclic Npyr and NPip, although the MW of NDBA is big, this result is in contrast with that observed by Tezel *et al.* (2011), which observed that the removal rates by a mixed methanogenic culture in
the order of fastest to slowest were NDMA, NMEA, Npyr at concentrations of 16 μmol/L but NDEA, NDPA and NDBA were not degraded. Furthermore, the LogKow and Henry's constant for the five nitrosamines examined are consistent with the removal ratios (Table 1). Among the linear nitrosamines, the LogKow (−0.15, 1.35 and 2.63) and Henry's constant (1.44 × 10⁻⁶, 3.46 × 10⁻⁶ and 9.96 × 10⁻⁶ at 25 °C, atm m³/mol) for NMEA, NDPA, and NDBA are consistent with the removal ratios. Similarly, the LogKow (0.23 and 0.74) and Henry's constant (1.99 × 10⁻⁷ and 2.81 × 10⁻⁶ at 25 °C, atm m³/mol) for the cyclic Npyr and NPip are in accordance with the removal ratios. The results are consistent with those reported in a previous study (Homme & Sharp 2013). In other words, biodegradation of nitrosamines is complicated under multiple conditions and further studies are required for a better understanding of this process.

Figure 4 | Variation in the nitrosamines presented in MS medium of the strain Bacillus sp. LT1C. The initial concentration of each nitrosamine was 200 ng/L (NMEA, NDPA, NDBA, Npyr and NPip).
CONCLUSIONS

The bench-scale BAC filter after cultivating with the nitrosamine-containing water showed a biological nitrosamine reduction capacity. This study is the first to contribute to the knowledge on the nitrosamine-reducing bacterium, Bacillus sp. LT1C, which could effectively degrade NMEA, NDPA, Npyr, NPip and NDBA as the carbon source. The removal ratios achieved by the isolate were in accordance with the structure, LogK_{ow}, and Henry’s constant of the nitrosamines examined. The findings of this study provide new insights into the construction of a BAC filter to remove nitrosamines from drinking water.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 51208184), the Education Department of Henan Science and Technology Fund Project (13A610585), and the Technology Department of Henan Science and Technology Fund Project (No. 142102210456).

REFERENCES

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Table 1 | The molecular structure and properties of the five nitrosamines

<table>
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<tr>
<th>Nitrosamines</th>
<th>Structure</th>
<th>Formula</th>
<th>MW (g/mol)</th>
<th>LogK_{ow}</th>
<th>Henry’s constant (atm m^3/mol, at 25°C)</th>
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<td>114.1</td>
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<td>2.81 × 10^{-6}</td>
</tr>
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</table>

*aFrom the Hazardous Substances Data Bank and references cited therein.
*bFrom Fujioka et al. (2012).


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First received 23 February 2015; accepted in revised form 27 April 2015. Available online 14 May 2015