Nitrogen removal efficiency and microbial community analysis of ANAMMOX biofilter at ambient temperature
Zeng Taotao, Li Dong, Zeng Huiping, Xie Shuibo, Qiu Wenxin, Liu Yingjiu and Zhang Jie

ABSTRACT
An upflow anaerobic biofilter (AF) was developed to investigate anaerobic ammonium-oxidizing (ANAMMOX) efficiency in treating low-strength wastewater at ambient temperature (15.3–23.2 °C). Denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization were used to investigate treatment effects on the microbial community. Stepwise decreases in influent ammonia concentration could help ANAMMOX bacteria selectively acclimate to low-ammonia conditions. With an influent ammonia concentration of 46.5 mg/L, the AF reactor obtained an average nitrogen removal rate of 2.26 kg/(m³ day), and a removal efficiency of 75.9%. polymerase chain reaction-DGGE results showed that microbial diversity in the low matrix was greater than in the high matrix. Microbial community structures changed when the influent ammonia concentration decreased. The genus of functional ANAMMOX bacteria was Candidatus Kuenenia stuttgartiensis, which remained stationary across study phases. Visual observation revealed that the relative proportions of ANAMMOX bacteria decreased from 41.6 to 36.3% across three study phases. The AF bioreactor successfully maintained high activity due to the ANAMMOX bacteria adaptation to low temperature and substrate conditions.

Key words | ambient temperature, ANAMMOX, microbial community, nitrogen removal

INTRODUCTION
Nitrogen, a major nutrient, must be removed during wastewater treatment to avoid eutrophication and toxicity to aquatic life. Compared with conventional nitrification/denitrification processes, an anaerobic ammonium-oxidizing (ANAMMOX) process is a promising new and more sustainable process for removing nitrogen. In anoxic conditions, ANAMMOX bacteria convert ammonium and nitrite directly to dinitrogen gas (N2). This saves aeration energy, reduces biomass production, and decreases organic matter consumption (Kartal et al. 2010).

Based on the ANAMMOX process, several innovative processes, such as Single reactor High activity Ammonia Removal Over Nitrite (SHARON)–ANAMMOX (partial nitrification–ANAMMOX) and completely autotrophic nitrogen removal over nitrite (CANON), have been developed in wastewater treatment experiments (Vázquez-Padín et al. 2009; Shen et al. 2012). Limited by stringent operating conditions and slow bacterial growth rates, most ANAMMOX processes have been applied with high-strength wastewaters (> 500 mg/L NH4+-N) at mesophilic temperatures (50–40 °C), including digester effluents and anaerobically treated industrial effluents (Hendrickx et al. 2012). Lowering the temperature of warm ANAMMOX reactors results in an immediate large decrease in specific activity (Isaka et al. 2008).

Recently, several ANAMMOX reactors have been built for wastewater treatment with low-ammonia concentrations at a lower temperature (20–25 °C). For example, a laboratory-scale rotating biological contactor was developed to investigate ANAMMOX performance treating sewage-like nitrogen influent concentrations (29–66 mg N/L) at 25 °C (De Clippeleir et al. 2011). This investigation achieved nitrogen removal rates of 0.33–0.38 g N/(L day) in a short hydraulic retention time (HRT) of 1.2 h. A gaslift reactor with ANAMMOX biomass was operated at 20 °C and fed with synthetic...
wastewater (69 ± 5 mg (NH₄⁺ + NO₂⁻)/L). The reactor’s nitrogen removal rate was 0.26 g N/(L·day) at an HRT of 5.3 h. The influent was maintained at a constant temperature in these studies, requiring additional energy for heating. Natural sewage has a lower and variable temperature (~15–25 ºC outside tropical and sub-tropical regions). As such, there is considerable interest in developing an ANAMMOX process for low-strength sewage treatment at ambient temperatures.

Given this background, this study used an anaerobic biofilter (AF) to investigate ANAMMOX efficiency for low-strength wastewater treatment at an ambient temperature (15.3–23.2 ºC). Microbial compositions and functional populations were monitored using denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH).

**MATERIALS AND METHODS**

**Setup and operation of ANAMMOX biofilters**

An upflow AF was used as an ANAMMOX reactor, with a volume of 45 L and an 18.5 cm internal diameter. Table 1 presents the characteristics of the volcanic stone used as carrier. The reactor was inoculated with mature ANAMMOX sludge and fed with synthetic wastewater carrier. The reactor was inoculated with mature ANAMMOX sludge and fed with synthetic wastewater carrier. The reactor was inoculated with mature ANAMMOX sludge and fed with synthetic wastewater carrier. The reactor was inoculated with mature ANAMMOX sludge and fed with synthetic wastewater carrier. The reactor was inoculated with mature ANAMMOX sludge and fed with synthetic wastewater carrier.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Particle size (mm)</th>
<th>Specific surface area (m²/kg)</th>
<th>Bulk density (kg/m³)</th>
<th>Actual density (kg/m³)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volcanic stone</td>
<td>4–6</td>
<td>11.3</td>
<td>0.82 × 10³</td>
<td>1.6 × 10³</td>
<td>62.5</td>
</tr>
</tbody>
</table>

Table 1 | Characteristics of volcanic stone used in the anaerobic biofilter

**Table 2 | The main run status and the index of influent water characteristics**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Days</th>
<th>Temperature ( ºC)</th>
<th>HRT (h)</th>
<th>NH₄⁻-N (mg/L)</th>
<th>NO₂⁻-N (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I</td>
<td>1–30</td>
<td>15.3</td>
<td>3.2</td>
<td>191.1</td>
<td>249.5</td>
</tr>
<tr>
<td>Phase II</td>
<td>30–80</td>
<td>15.8</td>
<td>3.2–1.8</td>
<td>191.1–78.7</td>
<td>102.6–225.1</td>
</tr>
<tr>
<td>Phase III</td>
<td>80–122</td>
<td>20.3</td>
<td>1.8</td>
<td>78.7</td>
<td>105.9</td>
</tr>
<tr>
<td>Phase IV</td>
<td>122–170</td>
<td>23.2</td>
<td>1.1</td>
<td>46.5</td>
<td>61.4</td>
</tr>
</tbody>
</table>

Chemical oxygen demand, NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N levels were measured using *Standard Methods* (APHA 1998). Temperature, pH, and dissolved oxygen (DO) were detected using a WTW pH/DO meter (WTW Multi 340i, Weilheim, Germany). The nitrogen removal efficiency was calculated as (Nₙin − Nₙout), where Nₙin and Nₙout were the nitrogen concentrations (mg/L) of the influents and effluents, respectively. The nitrogen removal rate (kg N m⁻³ day⁻¹) was calculated as the daily nitrogen load removed (daily flow rate × (Nₙin − Nₙout)) per unit volume of reactor. Biofilm samples were collected during Phases I, III, and IV for molecular microbial population analysis.

**DNA extraction and PCR-DGGE**

Wet biomass samples of 200 mg each were collected from the ANAMMOX biofilter and rinsed twice with sodium phosphate buffer (PBS, 0.1 M, pH 8.0). Total community genomic DNA was extracted with DNA extraction liquid (100 mM Tris–HCl, 100 mM EDTA, 1.5 M NaCl, 100 mM Na₃PO₃, 1% CTAB, pH 8.0) and SDS (100 g L⁻¹) methods described in a previous report (Liu et al. 2003). Bacterial universal primers GC-338F/518R were used to directly amplify partial 16S rRNA gene fragments from total DNA (Chen et al. 2008). Polymerase chain reaction (PCR) products were loaded onto 8% polyacrylamide with a linear denaturing gradient ranging from 30 to 60% (100% denaturing gradient contains 7 M urea and 40% formamide). DGGE was performed with the D-code System (Bio-Rad, Hercules, CA, USA) using the manufacturer’s instructions. Electrophoresis was run at a constant...
Table 3 | The 16S rRNA-targeted oligonucleotide probes used in this study

<table>
<thead>
<tr>
<th>Probes</th>
<th>Sequence (5'→3')</th>
<th>Specificity</th>
<th>Fluorescent labeled</th>
<th>Formamide (%)</th>
<th>NaCl (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>GCTGCCCTCCGTAGGAGT</td>
<td>Eubacteria</td>
<td>FITC</td>
<td>20</td>
<td>225</td>
</tr>
<tr>
<td>EUB338-II</td>
<td>GCAGCCACCGTAGGAGT</td>
<td>Eubacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EUB338-III</td>
<td>GCTGCCACCGTAGGAGT</td>
<td>ANAMMOX organisms</td>
<td>AMCA</td>
<td>15</td>
<td>338</td>
</tr>
<tr>
<td>AMX368</td>
<td>CTTTCGGGCAATCGGA</td>
<td>ANAMMOX organisms</td>
<td>AMCA</td>
<td>15</td>
<td>338</td>
</tr>
</tbody>
</table>

Figure 1 | Nitrogen removal performance of ANAMMOX biofilter (a) and the corresponding temperature and pH variations (b).
voltage of 120 V for 5 h at 60 °C. Gels were subsequently silver stained using a procedure from Bassam & Gresshoff (2007), and pictures were taken using a Gel Doc XR system (Bio-Rad, Hercules, CA, USA).

Cloning and sequencing analysis

Obvious bands created by DGGE were cut and placed into 1.5 mL Eppendorf tubes, and incubated with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) at 4 °C for 24 h. The DNA bands were used as templates, and PCR was performed with primers 338F/518R without GC clamps. PCR products were purified using an Agarose Gel DNA Purification Kit (TIANGEN, Beijing, China), ligated into the pMD19-T vector (TaKaRa Biotechnology Co., Ltd, Dalian, China) and then transformed into competent *Escherichia coli* DH5α (TaKaRa Biotechnology Co., Ltd, Dalian, China). Positive colony PCR products were sent to Sangon Company (Shanghai, China) for sequencing. The basic local alignment search tool was used to compare the obtained sequences with available sequences in a nucleotide collection database.

Fluorescence in situ hybridization analysis

Biomass FISH assays were conducted to detect ANAMMOX bacteria. Biofilm samples were fixed in 4% freshly prepared paraformaldehyde solution for 2 h at 4 °C. Then, samples were rinsed twice with phosphate-buffered saline (PBS). In situ hybridizations were performed in a hybridization incubator using Amann et al.’s procedure (Amann et al. 1995). Table 3 lists probe sequences, as well as specific probes and the hybridization conditions. The AMX368 probe was used to investigate the presence of ANAMMOX bacteria (Schmid et al. 2003). The EUB338, EUB338II, and EUB338III probes were used together in an equimolar mixture to detect all bacteria (Liu et al. 2012a). All 5’-fluorescein tagged probes were purchased from Sangon Company (Shanghai, China). FISH images were collected and recorded using an acquisition system (camera: DP72 and software: cell-Sens Dimension) coupled to an Olympus BX-51 fluorescence microscope (Olympus, Tokyo, Japan).

RESULTS AND DISCUSSION

ANAMMOX performance of biofilter

In a previous study, an ANAMMOX biofilter was run with an influent ammonia concentration of 200 mg/L and a temperature of 16 °C. The average nitrogen removal rate was 1.6 kg/(m³ day), with a nitrogen removal efficiency of 60% (Zeng et al. 2012). In this study, stepwise decreases of influent ammonia concentrations were introduced to investigate ANAMMOX processes in low-ammonia conditions. Influent ammonia concentrations and HRT were varied over the 170-day study period, as shown in Table 2. Figure 1(a) shows the nitrogen removal rate and removal efficiency with continuous operation of the biofilter.

Phase I (0–30 days): during this phase, the influent ammonia concentration was 191 mg/L, with a low flow rate to prevent ANAMMOX bacteria loss. The average nitrogen removal efficiency of the biofilter was 42.7% with a temperature of 15.3 °C and HRT of 3.2 h. The total nitrogen removal rate was gradually increased from an initial 1.49 kg/(m³ day) to 2.44 kg/(m³ day) on Day 20. When this
change was made, the biofilter restarted successfully and exhibited better ANAMMOX performance.

Phase II (30–80 days): as a drastic change in operating conditions potentially destabilized the biological system (Yang et al. 2011), in this phase, the influent ammonia concentration was gradually decreased from 191.1 to 78.7 mg/L. For first 10 days, the lower ammonia concentration temporarily affected the nitrogen removal rate level. However, average the nitrogen removal efficiency and nitrogen removal rate were not affected since the biofilter quickly adapted to the low substrate concentration condition. The average total nitrogen load rate (NLR) was 3.44 kg/(m$^3$ day) when HRT was shortened from 3.2 to 1.8 h. The nitrogen removal rate was 1.86 kg/(m$^3$ day) and the average total nitrogen removal efficiency was 54.1% with a temperature of 15.8 °C, higher than Phase I.

Phase III (80–122 days): in this phase, the influent ammonia concentration was 78.7 mg/L and HRT was 1.8 h with a constant NLR of 3.17 kg/(m$^3$ day). Meanwhile, the operating temperature was progressively increased from 15.8 to 20.3 °C. ANAMMOX treatment performance was not affected at low substrate concentrations, where the nitrogen removal rate was 2.36 kg/(m$^3$ day) and total nitrogen removal efficiency was 74.5%. Results indicated that ANAMMOX bacteria gradually adapted to the new operating conditions.

Phase IV (122–170 days): in municipal sewage, the N-concentration typically ranges from 20 to 85 mg N/L. To achieve sufficiently high nitrogen loading rates, short HRTs would have to be applied, impacting sludge retention (Hendrickx et al. 2012). In this phase, HRT decreased from 1.8 to 1.1 h, as the influent ammonia concentration decreased from 78.7 to 46.5 mg/L (Table 2). This contributed to a constant NLR of 2.98 kg/(m$^3$ day). Stable performance was still obtained with an effluent average nitrogen removal rate of 2.26 kg/(m$^3$ day) and total nitrogen removal efficiency of 75.9%. The ANAMMOX reactor’s nitrogen removal capacity was satisfactory at ambient temperatures by shortening HRT without enhancing the influent total nitrogen concentration.

Changes in pH (pH$_{effluent}$–pH$_{influent}$) and temperature were also assessed each day, as illustrated in Figure 1(b), together with the nitrogen removal rate. The average temperatures of Phase I, Phase II, Phase III, and Phase IV were 15.7, 17.0, 22.6, and 23.2 °C, respectively. The average changes in pH during Phase I, Phase II, Phase III, and Phase IV were 0.89, 0.49, 0.80, and 0.62, respectively. Corresponding average nitrogen removal rates were 1.77, 1.86, 2.36, and 2.26 kg/(m$^3$ day), respectively, with increases from 1.77 kg/(m$^3$ day) to 2.36 kg/(m$^3$ day) over the previous three phases. In Phase IV, the nitrogen removal rate decreased to 2.26 kg/(m$^3$ day), due to influent substrate decline. During the 170 experiment days, effluent pH was significantly higher than influent pH, due to the ANAMMOX process’s acidity consumption (Tao et al. 2012).

In this study, the ammonia concentration in Phase IV was consistent with the ammonia level in residential sewage, with

Table 4 | Bacterial 16S rRNA sequences of anaerobic ammonium-oxidizing biofilter

<table>
<thead>
<tr>
<th>Band</th>
<th>Closest match (GenBank accession numbers)</th>
<th>Similarity (%)</th>
<th>Phase I</th>
<th>Phase III</th>
<th>Phase IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Oxalicibacterium</em> sp. (GU295961.1)</td>
<td>93</td>
<td>Y*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Igneavibacterium album</em> (AB478415.1)</td>
<td>84</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Bacterium rJ15</em> (AB021333.1)</td>
<td>88</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>Candidatus Kuenenia stuttgartiensis</em> (AF375995)</td>
<td>95</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>5</td>
<td><em>Alpha proteobacterium HIN4</em> (AB599867.1)</td>
<td>88</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>Kuenenia stuttgartiensis</em> (CT573071.1)</td>
<td>91</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td><em>Candidatus Kuenenia stuttgartiensis</em> (AF375995)</td>
<td>93</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>Clostridium</em> sp. DY192 (HQ696463.1)</td>
<td>98</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td><em>Bacterium w5ch14</em> (DQ416664.1)</td>
<td>89</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Comamonas nitrativora</em> (NR_025376.1)</td>
<td>98</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td><em>Hippea maritima</em> (AB072402.1)</td>
<td>95</td>
<td>Y</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>12</td>
<td><em>Thioprofundum lithothrophica</em> (AB468957.1)</td>
<td>91</td>
<td>Y</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>13</td>
<td><em>Sulfur-oxidizing bacterium</em> (AB266389.1)</td>
<td>91</td>
<td>Y</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>14</td>
<td><em>Rhodopseudomonas palustris</em> (D12700.1)</td>
<td>93</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

*Y: represents yes, which means the genus existed in this phase.
less nitrite but similar amounts of organic substances. To provide enough nitrite for ANAMMOX bacteria growth, partial nitrification should be carried out before the ANAMMOX process; or, partial nitrification could be coupled with ANAMMOX, such as CANON or OLAND processes (Molinuevo et al. 2009). Previous work suggested that ANAMMOX bacteria could use some C sources, such as formic acid, acetate, and propionate. Real sewage contains complex organic matter, inhibiting ANAMMOX activity. Therefore, influent organic matter should be treated before bringing ANAMMOX processes into real environments.

In a different study, Dosta et al. (2008) operated a sequencing batch reactor (SBR) at different temperatures (from 30 to 15°C) to assess ANAMMOX activities. That study achieved a nitrogen removal rate of 0.29 kg N/(m³ day) with an HRT of 24 h at a temperature of 18°C (Dosta et al. 2008). In this study, a nitrogen removal rate of 2.26 kg/(m³ day) was obtained with an HRT of 1.1 h, demonstrating the high ANAMMOX activity of the AF for nitrogen removal. This indicated that nitrogen could be removed effectively from low-strength wastewater using ANAMMOX bacteria at ambient temperature (15.3–23.2°C).

**Microbial community of ANAMMOX biofilter**

Total DNA with a length of 23 kDa was extracted, and PCR products of 16s rDNA V3 region were applied for DGGE analysis. DGGE profiles of 16s rDNA V3 region (Figure 2(a)) and the sketch map (Figure 2(b)) express microbial community abundance. There were 6, 8 and 8

![Phylogenetic tree of total bacteria based on 16S rDNA sequences.](https://iwaponline.com/wst/article-pdf/71/5/725/469398/wst071050725.pdf)
bacterial DGGE bands in Phase I, Phase III, and Phase IV, respectively, indicating that microbial diversity in the low matrix was greater than in the high matrix. Compared with Phase I, the similarities of Phases III and IV were 54.8% and 37.5%, respectively. The divergence revealed that microbial community structures changed when the influent ammonia concentration decreased.

Fourteen distinct bands (1–14, Figure 2(a)) were carefully excised from the ANAMMOX DGGE gel for sequencing. The nucleotide sequences were compared to available sequences in the GenBank database by BLASTN; Table 4 shows the results. Figure 3 presents the phylogenetic tree of total bacteria based on 16S rDNA sequences; it reveals that bands 4, 6 and 7 were similar to Candidatus Kuenenia stuttgartiensis with sequence homologies from 91 to 95%. Candidatus Kuenenia stuttgartiensis was the functional ANAMMOX bacteria, remaining stationary in all high and low matrix phases and contributing to biofilter nitrogen removal. Rhodopseudomonas palustris was a constant strain in all phases.

Alpha proteobacterium was found in Phases I and III, but died out in Phase IV. Hippea maritima and Thioprofundum lithotrophicum were found in Phases III and IV, indicating survivability in both high and low substrate matrix conditions. Oxalicipatrum sp., Irenabilisgracterium album and Bacterium r15 were found only in low-ammonia Phase IV.

Phase I, Phase III, and Phase IV temperatures were 15.7, 22.6, and 23.2 °C, respectively, and temperature affected the microbial community. Oxalicipatrum sp., Irenabilisgracterium album and Bacterium r15 appeared during the 23.2 °C phase, indicating that they could not adapt to low-temperature conditions. The functional bacteria Candidatus Kuenenia stuttgartiensis could adapt to temperatures from 15.7 to 23.2 °C, corresponding to the increased nitrogen removal capacity shown in Figure 1. Clostridium sp. DY192, Bacterium w5cb14, Comamonas nitrativorans, and Sulfur-oxidizing bacterium survived in the 22.6 °C phase only, indicating that they were interim bacteria. Rhodopseudomonas palustris was found in all phases, adapting to temperature fluctuation.

Sequencing results illustrated that influent substrates and temperature had combined effects on microbial composition. Most of the bacteria were anaerobic autotrophic microorganisms, corresponding to the inorganic synthetic influent and anaerobic environment of the biofilter. The functional bacteria Candidatus Kuenenia stuttgartiensis could survive well in low-temperature and low-substrate conditions, playing key roles in biofilter nitrogen removal.

**FISH analysis of ANAMMOX biofilm**

Positive biomass hybridization with the specific Amx368 probe confirmed the presence of ANAMMOX bacteria in the AF bioreactor after cultivation (Figure 4). By counting FISH-stained cells with an Amx368 probe and Eubmix probes, ANAMMOX bacteria abundance was estimated as shown in Figure 5. Visual observations of microbial composition confirmed that ANAMMOX bacteria were located in the middle of the biofilm, whereas a fraction of eubacteria was located outside the biofilm. On average, ANAMMOX bacteria accounted for 41.6%, 37.9%, and 36.3% of eubacteria in Phases I, III, and IV, respectively.
Compared with Phase I, the proportion of ANAMMOX bacteria reduced as influent substrate decreased in Phases III and IV. However, the nitrogen removal efficiency and the nitrogen removal rate (NRR) of the biofilter increased (Figure 1), illustrating that ANAMMOX bacterial activity was enhanced to compensate for population reductions.

CONCLUSIONS

This study’s results indicate that nitrogen can be removed effectively from low-strength wastewater with ANAMMOX activities at ambient temperature (15.3–23.2 °C). The nitrogen removal rate and removal efficiency of the AF reactor in low ammonia concentrations were 2.26 kg/(m³ day) and 75.9%, respectively. PCR-DGGE results showed that microbial community structures changed when the influent ammonia concentration decreased. However, the genus of functional ANAMMOX bacteria was stationary across the four study phases. Visual observation revealed that the relative proportions of ANAMMOX bacteria accounted for 41.6%, 37.9%, and 36.3% in Periods I, III, and IV, respectively. ANAMMOX bacteria (Candidatus Kuenenia stuttgartiensis) adapted effectively to variable temperature and substrate concentrations, and maintained high activity with low matrix influent. This study confirmed that ANAMMOX processes can be effective for treating low-strength wastewater at ambient temperature. To apply this work to real application, influent organic matter should be treated properly, which is the key issue for future study.

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