Abstract: Uranium and nitrate are common groundwater pollutants near in situ leach uranium mines. However, we still lack techniques that can simultaneously immobilize uranium and reduce nitrate using a single bacterial species. In this study, the potential of simultaneous uranium immobilization and nitrate reduction by a single AFODN (anaerobic Fe(II) oxidizing denitrifier), *Clostridium* sp. PXL2, was investigated. *Clostridium* sp. PXL2 showed tolerance to U(VI) concentrations varying from 4.2 µM to 42 µM. The U(VI) immobilization and nitrate reduction rates in groundwater samples inoculated with this bacterium reached up to 75.1% and 55.7%, respectively, under neutral conditions. Exposure to oxidation conditions led to further U(VI) removal but did not show any noticeable effect on nitrate reduction. The U(VI) immobilization rate reached up to 85% with an increased Fe(II) initial concentration, but this inhibited nitrate reduction. SEM (scanning electron microscopy) coupled with EDS (energy dispersive spectroscopy) showed that the U(VI) immobilization was mainly due to sorption to amorphous ferric oxides. U(VI) and nitrate bioremediation by AFODNs, including *Clostridium* sp. PXL2, may provide a promising method for the treatment of uranium- and nitrate-contaminated groundwater after the in situ leach mining of uranium.

Keywords: *Clostridium* sp. PXL2; immobilization; reduction

1. Introduction

Uranium pollution has become a serious environmental concern because of extensive uranium mining, leaching, and the poor processing of by-products generated during mining activities [1]. ISL (in situ uranium leaching) techniques were developed in the USA and have also been broadly used in Eastern Europe, including the former Soviet Union, and some parts of Asia. However, the leaching solutions from ISL mines contain high levels of uranium and nitrate, which contaminate nearby aquifers and cause serious pollution to the groundwater. The uranium and nitrate concentrations in leaching solutions at some typical ISL mining and milling sites can range from 1 mM to 210 mM [2,3]. Long-term exposure to uranium-polluted drinking water can cause serious health problems, including renal function damage or kidney failure [4,5]. The permissible value for uranium in drinking water is set as 2.0 µg L\(^{-1}\) by the World Health Organization [6] and is 30 µg L\(^{-1}\) in the USA [7]. Nitrate is another common pollutant produced by uranium leaching and processing due to the use of nitric acid or ammonia [8,9]. Groundwater nitrate levels have been observed to reach up to 130 mM in Oak Ridge, TN [8], and 19.7 mM in mill tailing sites near Tuba City, AZ (USA) [10]. The main health problem associated with nitrate is blue baby syndrome, which is caused by the reaction of nitrate with hemoglobin [11]. The guideline value for nitrate in groundwater is set as 10 mg L\(^{-1}\) in many countries [12].
Table 1. Leaching solution quality in some in situ leaching mines (mg L\(^{-1}\)).

<table>
<thead>
<tr>
<th></th>
<th>Czech Rep.</th>
<th>Uzbekistan</th>
<th>Yining, China</th>
</tr>
</thead>
<tbody>
<tr>
<td>nitrate</td>
<td>200–1400</td>
<td>750</td>
<td>-</td>
</tr>
<tr>
<td>U(VI)</td>
<td>20–500</td>
<td>86</td>
<td>75</td>
</tr>
</tbody>
</table>

Data derived from [13,14].

In recent decades, a number of biotechnologies, including biomimelarization [15–17], bioreduction [18–21], biosorption [22–25], and bioaccumulation [26], have been proposed in order to immobilize uranium in contaminated groundwater. Among the uranium bioremediation techniques available, uranium bioreduction has been studied extensively, where U(VI) is reduced to U(IV) by a variety of bacteria. In situ demonstrations of uranium bioreduction have been conducted successfully at the US DOE Rifle site, Colorado [27], and at the US DOE Oak Ridge site, Tennessee [28,29]. However, the stability of reduced U(IV) depends on protecting it from re-oxidation by nitrate [30,31] and O\(_2\) [28]. Even certain intermediates of denitrification products such as nitrite and nitric oxide have been shown to cause the re-oxidation of U(IV) [32]. Therefore, the coexistence of nitrate with uranium poses a challenge when using this technique as a viable uranium bioremediation strategy for polluted groundwater [31,32]. Generally, uranium biomimelarization has only been studied at the laboratory scale due to the high cost of organic phosphates and other toxic effects on cell metabolism [9,33].

There is, therefore, an urgent need to find cost-effective biological techniques that can remediate groundwater contaminated with uranium and nitrate. Although uranium and nitrate are frequently detected together in groundwater near uranium mines and milling sites, especially at in situ leaching uranium sites, currently we still lack biotechnologies that can simultaneously remove uranium and nitrate using a single species of bacteria. Bacterial species known as AFODNs have been reported to oxidize Fe(II) to Fe(III) under anoxic conditions [34,35]. AFODNs can simultaneously reduce nitrate to denitrification intermediates such as nitric oxide and nitrogen. They also produce ferric oxides that have a great adsorption capacity for U(VI) [36,37]. These anaerobes may therefore provide a novel solution to concurrently remediate uranium and nitrate contaminated groundwater arising from in situ uranium mining, milling, and leaching sites.

The purpose of this study was to simulate polluted groundwater after the in situ leach mining of uranium and to investigate the potential of the AFODN bacterium, *Clostridium* sp. PXL2, for the simultaneous removal of uranium and nitrate. The effects of environmental factors such as pH, oxidation, and Fe(II) on uranium immobilization and nitrate reduction by this anaerobe were assessed. SEM-EDS analysis was applied to characterize the adsorption of uranium by the biogenic iron oxides.

### 2. Materials and Methods

#### 2.1. Sample Collection and Processing

Groundwater samples were collected from a uranium rich coal mining site in Xinjiang, China. The groundwater was pumped up by an electric well water pump from a depth of about 150 m and collected in 100 L plastic containers, which were completely filled and capped tightly in order to avoid further oxidation. Some basic physical and chemical parameters of the groundwater are listed in Table 2.

Table 2. Physical and chemical parameters of groundwater sample.

<table>
<thead>
<tr>
<th>pH</th>
<th>U(VI)</th>
<th>Conductivity</th>
<th>Cl(^-)</th>
<th>SO(_4^{2-})</th>
<th>Ca(^{2+})</th>
<th>K(^+)</th>
<th>Mg(^{2+})</th>
<th>Na(^+)</th>
<th>CO(_3^{2-})</th>
<th>HCO(_3^-)</th>
<th>Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.74</td>
<td>8.71</td>
<td>1238</td>
<td>113.1</td>
<td>357.9</td>
<td>175.6</td>
<td>3.4</td>
<td>36.6</td>
<td>116.0</td>
<td>55.9</td>
<td>49.5</td>
<td></td>
</tr>
</tbody>
</table>

The pH was measured using a pH meter (Mettler SevenEasy); conductivity was measured by a conductivity detector (DDS-307); Cl\(^-\) and SO\(_4^{2-}\) were measured by ion chromatography (ICS-5000 Dionex, Waltham, MA, USA); Ca\(^{2+}\), K\(^+\), Mg\(^{2+}\), and Na\(^+\) were measured by ICP-OES (735, Agilent, Santa Clara, CA, USA); HCO\(_3^-\) and CO\(_3^{2-}\) were measured by an automatic potentiometric titrator (G20, Mettler); U(VI) concentration was measured by ICP-MS (8800, Agilent, USA); nitrate was measured by the photometric method of Slanina et al. [36].
2.2. Culture of Clostridium sp. PXL2

The anaerobic bacterium was originally isolated from activated sludge collected from the sewage management station of Miqian in Urumqi, China, and identified as Clostridium sp. PXL2 [12]. The bacterium was incubated at 30 °C in a medium of the following composition (L⁻¹ deionized water): 0.28 g L⁻¹ NH₄Cl, 0.25 g L⁻¹ KH₂PO₄, 0.1 g L⁻¹ MgSO₄, 0.1 g L⁻¹ yeast extract, 0.5 g L⁻¹ KNO₃, 0.5 g L⁻¹ FeCl₂·4H₂O. The growing culture was used for further experiments after 1 week of incubation at 30 °C. The initial optical density (OD₆₀₀) of the experimental bacterium was adjusted to 0.2.

2.3. Groundwater Treatments

Groundwater was amended with 0.3 g L⁻¹ of yeast extract and then sterilized using 0.22 µm hydrophilic polyestersulfone membrane filters. The sterile groundwater was placed in an anaerobic chamber overnight in order to remove dissolved oxygen. The pH was adjusted with 0.1 M sodium hydroxide solution or 0.1 M hydrochloric acid. The groundwater was supplemented with filter-sterilized 1.0 g L⁻¹ U(VI) stock solution and 130 g L⁻¹ sodium nitrate solution to provide initial concentrations of 1.5 mg L⁻¹ U(VI) and 1050 mg L⁻¹ nitrate, respectively. Various Fe(II) concentrations were obtained using filter-sterilized 35.2 g L⁻¹ Fe(II) (FeCl₂·4H₂O) stock solution. All manipulations and experiments were conducted in 250 mL serum bottles in an anaerobic workstation at 30 °C. A 2% (v/v) Clostridium PXL2 culture was inoculated into the test samples after supplementing with the other chemicals. The Fe(II), U(VI), nitrate, and nitrite concentrations were measured every 24 h. All experiments were conducted in triplicate.

2.4. Measurement of Growth

Clostridium sp. PXL2 was inoculated in the above-mentioned selective media in 100 mL serum bottles containing various U(VI) concentrations (0, 4.2, 10.5, 21.0, 42.0 µM) and cultivated at 30 °C in the DG250 anaerobic workstation. Samples without U(VI) were used as controls. Using a UV2800 spectrophotometer (Unico, Shanghai, China), we measured the bacterial growth at 600 nm (OD₆₀₀) at exposure to U(VI) for 12, 24, 36, 48, 60, 72, and 84 h.

2.5. Analytical Methods

The concentrations of Fe(II), U(VI), nitrate, and nitrite were measured by spectrophotometric methods using a UV2800 spectrophotometer. The Fe(II) concentration was determined by a modified phenanthroline method at 510 nm [39]. Instead of using a 25% HCl solution, 40 mM of sulfamic acid was used, as this reacts quickly with nitrite and prevents Fe(II) oxidation by the nitrite at acidic pH values [40]. U(VI) concentration was monitored using an optimized photometric Arsenazo-III method at 651 nm [41]. Briefly, several milliliters of prefiltered (0.22 µm) groundwater samples were heated slowly to near dryness followed by the addition of 0.007% Arsenazo-III solution. A total of 0.007% Arsenazo-III solution was prepared by dissolving Arsenazo-III in 3 mol L⁻¹ of HClO₄ (w/v). The final volume was made up to 5.0 mL in a volumetric flask by using the same Arsenazo-III solution. The absorbance was measured at 651 nm against the reagent blank after thorough mixing. The nitrate concentration was measured at 220 and 275 nm. Certain amounts of prefiltered groundwater samples were added to 50 mL colorimetric tubes and diluted to 50 mL with distilled water followed by the addition of 1 mL of 1 mol L⁻¹ hydrochloric acid. An amount of 50 mL of distilled water plus 1 mL of hydrochloric acid was used as reference. A quartz cuvette of optical length 10 mm was used for the measurement [38]. The nitrite concentration was determined to be 540 nm. Certain amounts of prefiltered groundwater samples were added to 50 mL colorimetric tubes and diluted to 50 mL with distilled water. A total of 1 mL of chromogenic agent (N-(a-naphthyl)-ethylenediamine) was added and thoroughly mixed. Distilled water was used as a reference. The samples were measured after resting for 20 min [42].
Biogenic Fe(II) oxidation sediments and bacterial cells were examined by SEM-EDS. Sediment samples for SEM-EDS analysis were prepared as follows. First, the samples were centrifuged for 5 min at 8000 rpm and supernatants were abandoned. Pellets were washed with distilled water. This process was repeated 3 times. Afterwards, the washed pellets were frozen at $-80\, ^\circ\text{C}$ for 4 h before drying at $-60\, ^\circ\text{C}$ for 48 h. A scanning electron microscope (Zeiss Super 55VP, Oberkochen, Germany) was used for analyzing our samples. Then, we used an energy dispersive X-ray spectrometer (Bruker XFlash 5010, Karlsruhe, Germany) to confirm the elemental composition of the sediments.

2.6. Chemicals

U(VI) was applied in the form of analytical-grade uranyl nitrate hexahydrate $[\text{UO}_2(\text{NO}_3)_2\cdot6\text{H}_2\text{O}]$ (Hubei Chushengwei Chemistry Co., Ltd., Wuhan, China). Initially, a 1.0 g L$^{-1}$ stock U(VI) solution was prepared in deionized water and stored at 4 $^\circ$C. Other chemicals included FeCl$_2\cdot4\text{H}_2\text{O}$ (analytical grade, Beijing Kangpu Huiwei Technology Co., Ltd., Beijing, China) and sodium nitrate (analytical grade, Tianjin Yongsheng Chemical Co., Ltd., Tianjin, China).

2.7. Statistical Methods

The Mann–Whitney–Wilcoxon method was applied to determine whether there were significant differences between the subgroups. The t-test statistical method was applied to determine whether there were significant differences between the values of this study and mean (or average) values of other studies.

3. Results and Discussion

3.1. Growth in the Presence of U(VI)

Figure 1 shows the growth of Clostridium sp. PXL2 in the presence of various concentrations of U(VI). The cell growth in all U(VI) concentrations showed a trend of steady increase over the 96 h of incubation. Compared to the control, the cell growth was not significantly affected by U(VI) exposure to up to 42 $\mu$M ($p > 0.05$), implying that U(VI) has no significant toxic effects on this bacterium in an aquatic environment. It is also noticeable from Figure 1 that there was a slight but not significant increase ($p > 0.05$) in the cell growth with the increase in U(VI) concentrations. This was possibly due to the precipitation of U(VI) with phosphate ions which were released from NB media during the bacterial cell growth.

3.2. Influence of pH

Figure 2 shows the changes in Fe(II), U(VI), nitrate, and nitrite concentrations in the control and inoculated (pH 5.5 and pH 7.0) cultures over the 96 h of incubation under anoxic conditions. We observed a decline in the concentrations of Fe(II), U(VI), and nitrate, whereas the nitrite concentration increased (Figure 2a,b). After the 96 h of incubation, the Fe(II) concentrations in the inoculated groundwater samples at initial pH 5.5 and pH 7.0 were reduced by 27.2% and 65.4%, from an initial concentration of 2.35 mM and 1.30 mM, respectively (Figure 2a). The lower Fe(II) oxidation rate at pH 5.5 was possibly due to the fact that Fe(II) is more resistant to oxidation in acidic conditions than at neutrality. Moreover, neutral conditions are more favorable for the growth of Clostridium sp. PXL2 than alkaline or acidic conditions [12]. The U(VI) concentrations in the inoculated pH 5.5 and pH 7.0 samples dropped by 70.0% and 75.1%, respectively (Figure 2a). The estimated molar ratios of immobilized U(VI) and oxidized Fe(II) at pH 5.5 and pH 7.0 were 0.0067 and 0.0047, respectively. The higher effective U(VI) immobilization at pH 5.5 was due to aqueous U(VI) being strongly adsorbed to ferric oxides at low pH [43,44]. In higher pH environments (pH $\geq 7$), UO$_2^{2+}$ formed strong aqueous complexes with CO$_3^{2-}$, which greatly enhanced the solubility of U(VI) in carbonate-containing aquatic environments [45,46]. During the 96 h of incubation, the nitrate concentrations at pH 5.5 and pH 7.0 rapidly declined with 43.5% and 55.7% levels of removal, respectively (Figure 2b). The higher nitrate reduction at
pH 7.0 was due to the better bacterial growth in a neutral environment [12]. Concurrently, 4.32 mM and 5.72 mM of nitrite were produced at pH 5.5 and pH 7.0 after 96 h, which are lower values than the stoichiometric loss of 7.24 mM and 9.49 mM nitrate. This indicates that nitrite may be further reduced to nitrogen, ammonia, or NO [12]. Comparatively, only 11.9% of the Fe(II) and U(VI) and 8.0% of the nitrate were removed in the control sample at pH 7.0, while 1.7%, 4.7%, and 2.4% of the Fe(II), U(VI), and nitrate were lost from the control sample at pH 5.5, respectively. This confirmed that the nitrate reduction and U(VI) immobilization in the groundwater samples were mainly due to the activities of the *Clostridium* sp. PXL2.

Figure 1. Growth of *Clostridium* sp. PXL2 at various U(VI) concentrations. ■, control; ●, 4.2 µM; ▲, 10.5 µM; ▼, 21.0 µM; ★, 42.0 µM. Data are presented as means ± standard deviations (SD) of triplicate measurements.

Figure 2. The effects of pH on changes in Fe(II), U(VI), nitrate, and nitrite concentrations during the growth of *Clostridium* sp. PXL2. ■ and □, pH 5.5; ▼ and ▶, pH 5.5 (non-inoculated control ○); ● and ○, pH 7.0; ▲ and △, pH 7.0 (uninoculated control ▲). Filled symbols, Fe(II) and nitrate; open symbols, U(VI) and nitrite. (a), Fe(II) and U(VI); (b), nitrate and nitrite. Data shown are means ± SD (n = 3).
3.3. Influence of Oxidation

Figure 3 shows the changes in Fe(II), U(VI), nitrate, and nitrite concentrations in the groundwater samples over a 2 h oxidation period after 96 h of incubation. The Fe(II) concentrations in the inoculated pH 5.5 and pH 7.0 samples dropped rapidly by 33.6% and 9.2% compared to the 37.3% and 58.9% losses in the pH 5.5 and pH 7.0 control samples over 2 h of oxidation (Figure 3). This means that Fe(II) is much more vulnerable to oxidation by oxygen than to bacterial oxidation. The U(VI) concentrations simultaneously decreased by 30.0% and 9.8% in the pH 5.5 and pH 7.0 inoculated samples, while 42.3% and 60.9% were lost in the pH 5.5 and pH 7.0 control samples (Figure 3). This rapid and efficient immobilization of U(VI) in oxic conditions can be explained by the fact that abiotically produced Fe(III) oxides have a better binding capacity for U(VI) than biogenic Fe(III) oxides [47]. During the 2 h oxidation period, the nitrate concentrations in both the inoculated pH samples decreased slightly \((p > 0.05)\) while the nitrite concentrations slightly increased \((p > 0.05)\) (Figure 3), implying, therefore, that the facultative bacterium *Clostridium* sp. PXL2 was still active in oxic conditions.

![Figure 3](image_url)

**Figure 3.** Changes in concentrations of Fe(II), U(VI), nitrate, and nitrite after oxidation for 2 h following 96 h of anaerobic incubation. Data shown are means \(\pm SD (n = 3)\).

3.4. Effect of Fe(II)

Figure 4 shows the effects of Fe(II) on the changes in the Fe(II), U(VI), nitrate, and nitrite concentrations in the groundwater samples over 96 h of anoxic incubation. The initial Fe(II) concentrations of 1.2 mM and 4.0 mM in the groundwater samples decreased rapidly over the first 48 h before declining slowly to 0.4 mM and 2.5 mM after 96 h, with 66.7% and 37.5% levels of oxidation, respectively (Figure 4a). This low Fe(II) conversion to Fe(III) at a high initial Fe(II) concentration was due to the fact that the bacterial cells tended to settle at the bottom of the reaction vessel with the produced Fe(III) oxides, inhibiting the further oxidation of Fe(II) in the system. Furthermore, it is possible that Fe(III) oxides might have toxic effects on bacterial growth [48]. About 73.7% and 84.5% of the supplied U(VI) were immobilized from the initial concentrations of 6.34 \(\mu\text{M}\) and 6.39 \(\mu\text{M}\), respectively (Figure 4a). The molar ratios of U(VI) immobilization to Fe(II) oxidation were 0.0058 and 0.0036, indicating that the U(VI) immobilization was more efficient at lower Fe(II) concentrations. It is also noticeable from Figure 4a that the U(VI) concentration in the control sample without the Fe(II) addition was reduced slightly \((p > 0.05)\), probably due to biosorption to the bacterial cells. Bacterial cell walls contain ligands such as phosphate, carboxyl, hydroxyl, sulfhydryl, and amine groups, which are able to bind metal cations [9]. As can be seen from Figure 4b, the concentration of nitrate within the samples showed a rapid decrease during the initial 48 h but gradually subsided to under 44.3–58.1% reduction in the 96 h from an initial concentration of about 17 mM. It is also noticeable that the nitrate...
reduction (58.1%) was higher in the control sample than in the 1.2 mM Fe(II) (54.2%) and 4.0 mM Fe(II) (44.3%) samples. The higher nitrate reduction in the 1.2 mM Fe(II) sample than in the 4.0 mM Fe(II) sample further suggests that high Fe(II) levels inhibited the bacterial growth.

![Graph](image)

**Figure 4.** The effects of Fe(II) on changes in Fe(II), U(VI), nitrate, and nitrite concentrations during the growth of Clostridium sp. PXL2. ■ and □, 0 mM Fe(II) (control); ● and ○, 1.2 mM Fe(II); ▲ and △, 4.0 mM Fe(II); ▼ and ▽, 1.2 mM Fe(II) (uninoculated). Filled symbols, Fe(II) and nitrate; open symbols, U(VI) and nitrite. (a), Fe(II) and U(VI); (b), nitrate and nitrite. Data shown are means ± SD (n = 3).

3.5. **SEM-EDS Analysis**

Figure 5 shows *Clostridium* sp. PXL2 and sediments containing various concentrations of Fe(II), U(VI), and other elements. The *Clostridium* sp. PLX2 cells were approximately 1.8 μm in length. Amorphous iron oxides were observed on the cell surfaces and crystalline iron oxides were not observed. The formation of amorphous iron oxides by some other AFODN species has also been reported [49,50]. Figure 6 shows the elemental composition of the sediments around the bacteria cells. The following, including C, O, N, Fe, and a small of proportion of P, U, and S based on their weight, primarily formed the sediments’ composition. The U(VI) percentages were 0.17% and 0.14% for D1 and D2 spots, indicating that the U(VI) was mainly immobilized due to adsorption on iron oxides produced by *Clostridium* sp. PXL2. The slightly higher content of U(VI) in D1 could be attributed to biosorption by bacterial cells [9].

![SEM Image](image)

**Figure 5.** SEM image of sediment from the *Clostridium* sp. PXL2 cultures. D1 and D2 are typical spot locations for EDS analysis on and around the bacterial cells. Typical images are shown from one of triplicate examinations.
The composition of the sediments around the bacteria cells. The following, including C, O, N, Fe, and a small proportion of P, U, and S based on their weight, primarily formed the sediments' composition. The U(VI) percentages were 0.17% and 0.14% for D1 and D2 spots, indicating that the U(VI) was mainly immobilized due to adsorption on iron oxides produced by *Clostridium* sp. PXL2. The slightly higher content of U(VI) in D1 could be attributed to biosorption by bacterial cells.

**Figure 5.** SEM image of sediment from the *Clostridium* sp. PXL2 cultures. D1 and D2 are typical spot locations for EDS analysis on and around the bacterial cells. Typical images are shown from one of triplicate examinations.

**Figure 6.** EDS analysis of the *Clostridium* sp. PXL2 sediment (corresponding to spots D1 and D2 in Figure 5). Typical spectra are shown from one of triplicate determinations.

### 4. Conclusions

Our results demonstrated that the AFODN bacterium *Clostridium* sp. PXL2 could simultaneously remove U(VI) and nitrate from polluted groundwater. The removal efficiency of these two pollutants was affected by environmental factors such as pH, redox conditions, and initial Fe(II) concentration. SEM-EDS analysis showed that the U(VI) was immobilized mainly by sorption to poorly crystalline ferric oxides that were produced by *Clostridium* sp. PXL2. Therefore, nitrate-dependent Fe(II) oxidizing bacteria including *Clostridium* sp. PXL2 are effective microbes for the bioremediation of uranium- and nitrate-contaminated groundwater after the in situ leach mining of uranium.

**Author Contributions:** X.P., W.L. and R.W. conceived the ideas and designed this study; R.W., J.D. and J.F. interpreted the data of spatial distribution and structure characteristics and analysis; R.W. wrote the manuscript; X.P. reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** This study does not involve humans and animals.

**Informed Consent Statement:** This study does not involve any humans.

**Data Availability Statement:** This study did not report any data.

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**Conflicts of Interest:** The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.
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