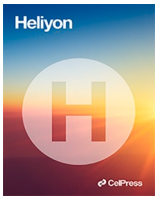
Heliyon 10 (2024) e27397

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Identification of groundwater microbes that decrease the concentration of the conservative tracer uranine

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ARTICLE INFO

Keywords:
Fluorescent dye
Groundwater tracer
Conservative tracer
Sodium fluorescein
Microbe

ABSTRACT

Fluorescent dyes are commonly used as conservative groundwater tracers to track the migration of water. Over- or underestimation of important parameters such as the water flow rate can occur if the concentration of a dye is changed by unexpected reactions. Because such errors may seriously affect the results of experiments, the reactions and processes that change fluorescent dye concentrations need to be understood. In this study, we focused on the widely used fluorescent dye uranine (UR) and aimed to identify microbes contributing to decreases in UR concentrations in groundwater. First, we identified the conditions (water temperature, pH, and salinity) under which significant decreases in UR concentrations occurred to show that the decrease in UR concentrations were caused by the effects of microbes in the groundwater. Next, we obtained information about the metabolism of organic matter by potential contributing microbes. These results were used to narrow down possible microbes that could decrease the UR concentration. Analysis of the microbial community in groundwater using 16S rRNA gene sequencing was then used to further identify contributing microbes. Finally, a verification experiment was conducted using a strain of one of the identified microbes (*Parapontixanthobacter aurantiacus*). Our results showed that conservation of the concentration of fluorescent dye solutions prepared with on-site groundwater was affected by several microbes with different metabolic characteristics, including *P. aurantiacus*. When fluorescent dye solutions prepared with on-site groundwater are used in field investigations or tracer tests, the pros and cons of using fluorescent dyes should be carefully evaluated because of the potential effects of microbes in the groundwater.

1. Introduction

Fluorescent dyes such as uranine (UR; sodium fluorescein) and eosin are widely used as conservative tracers to track water movement and contaminant transport. These dyes are conserved because they do not adsorb easily to rocks and are not readily degraded. Furthermore, they are simple to use with low background signals and detection limits, and have little impact on other analyses. They are also safe with low environmental impact and toxicity [1–4]. At the end of the nineteenth century, fluorescent dyes were applied to assess surface water flow to support growing demand for clean water for drinking and for use in industrial activities [2,5]. They have also been used to delineate catchment areas [6] and study groundwater trajectories and active conduit networks in karst aquifers and caves [7,8]. Furthermore, they have been used to evaluate pipe flow [9], investigate leaks from reservoirs [10], and

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https://doi.org/10.1016/j.heliyon.2024.e27397
Received 1 November 2023; Received in revised form 20 February 2024; Accepted 28 February 2024
Available online 6 March 2024

examine the behavior of contaminants (e.g., pesticides) in surface water [11,12]. Recently, fluorescent dyes have been used as tracers to distinguish between drilling fluid and on-site groundwater in deep subsurface borehole drilling [3,13–18], and as non-adsorbable tracers to compare with the behavior of adsorbable tracers in borehole-based solute transport tests [19,20].

If the concentration of a conservative tracer is changed by unexpected reactions, it can cause over- or underestimation of important parameters such as the water flow rate. Because such errors could seriously affect the results of experiments, the reactions and processes that change fluorescent dye concentrations need to be understood. The properties and limitations of fluorescent dyes and their use in groundwater studies have been reviewed [1,5,8,21], including their detection sensitivities, effects on water quality, photodegradation by sunlight, adsorption losses, environmental contamination risks, and toxicity. Nakata et al. [4] found that the pH, temperature, co-existence of natural organic matter, and filtration of fluorescent dye solutions greatly affected quantitative determination, and they proposed treatment methods to address each of these factors. However, in some cases, the concentrations of fluorescent dye solutions prepared with groundwater still decrease during storage [4,22]. After taking into consideration the effects of known mechanisms on the concentration, the remaining change in concentration has been attributed to microbes in groundwater [12,23–25]. The possibility of UR degradation by microbes in the natural environment has been mentioned in several studies [1,24,26]; however, to date, the impact of this has been considered negligible [27,28]. In a recent study we added four types of fluorescent dyes (UR, eosin, sodium naphthionate, and amino G-acid) to different samples of groundwater and evaluated changes in the dye concentrations over time [22]. In some of the samples, large decreases in the concentration were thought to be caused by microbial activity. Thus, strategies are needed to prevent decreases in the concentrations of fluorescent dyes by microbes. To develop these strategies, we need to identify possible contributing microbes and understand their characteristics.

In this study, we attempted to identify microbes that decreased UR concentrations in groundwater solutions. To achieve this, we investigated the following: (1) the solution conditions (water temperature, pH, or salinity) under which the microbes that decreased the UR concentrations were active; (2) the metabolism of organic matter by the active microbes; (3) changes in the microbial community constituents with and without addition of UR; and (4) the effect of a specific microbe (*Parapontixanthobacter aurantiacus*) on the UR concentration.

2. Material and methods

2.1. UR dye

Among the many fluorescent dyes commonly used as tracers to track the migration of water [1,29], we selected UR (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) because it is possible to visually confirm approximate concentration changes with this dye. Furthermore, in a previous study, UR showed the largest decreases in concentration among four fluorescent dyes commonly used as groundwater tracers [22]. The structure and fluorescence of UR are described elsewhere [4].

2.2. Groundwater

Groundwater was collected from a borehole drilled from the 350 m gallery (13-350-C01) at the Horonobe Underground Research Laboratory in Japan (45°02′43″N, 141°51′34″E). The borehole was divided into sections by packers. Nylon tubes were run from each section to the gallery to enable water sampling. The section with relatively high groundwater inflow was targeted for sampling, and groundwater was collected in autoclaved 2-L polypropylene bottles. The collected samples were taken back to the laboratory under cool and dark conditions and stored at 4 °C until the start of the experiment. Groundwater samples were collected in April, July, and December 2021 (Table S1). The groundwater had a temperature of 22.3 °C and neutral pH (7.2), and its salinity was lower than that of seawater (equivalent to 1% NaCl) [30].

2.3. Measurement of the UR concentration

The UR concentration was measured using a fluorescence spectrometer (FP-8300; JASCO, Tokyo, Japan). The analytical method is described in detail elsewhere [4,22]. Briefly, because the pH of the solution affects the analytical value of UR [1,3,4,31,32], the pH of the solution was adjusted to approximately 9 using 0.05 M borax (sodium tetraborate decahydrate in deionized water; FUJIFILM Wako Pure Chemical Corp.). Because microparticles in a sample can alter the fluorescence [4], the samples were filtered through a polytetrafluoroethylene syringe filter (pore size: 0.2 μm, diameter: 13 mm; Advantec, Tokyo Japan) before analysis. The filter material (hydrophilic polytetrafluoroethylene) was selected to prevent adsorption of UR, and 0.5 mL of the sample was washed through the filter and discarded before filtering the actual sample for measurement. UR standard solutions (0, 10, 50, 100, 200, and 400 ppb) for construction of a calibration curve were prepared by diluting a UR stock solution (1000 ppm in deionized water) with 0.05 M borax. The fluorescence intensities of the solutions were measured using a spectrofluorometer. For the samples from the experiments with unknown UR concentrations, we followed the same procedure of dilution with the borax solution and measurement of the fluorescence intensity using the spectrofluorometer. The UR concentrations were quantified by comparison with the calibration curve. Some of the fluorescence in a groundwater sample can be derived from natural organic matter and other sources in the groundwater. If this fluorescence occurs at wavelengths close to that for UR, it can increase the apparent UR concentration [4,33]. The filtered groundwater was also analyzed as described above to determine the background concentration to understand the effect of natural matter on UR concentrations. The experimental results are expressed as normalized concentrations (C/C_0) relative to the initial concentration after the background concentration was subtracted from the measured values.

2.4. Conservation experiments with the UR solution

2.4.1. Screening of solution conditions for microbial activity decreasing the UR concentration

To show that the decrease in UR concentrations was caused by the effects of microbes in the groundwater, the water temperature, pH, and salinity were selected as parameters that could affect the microbial activity. These parameters were examined using values that would be expected to decrease the microbial activity. Therefore, we included values that were very different from those commonly encountered in the subsurface environment. UR was added to the groundwater and its concentration over time was measured, while controlling the water temperature, pH, and salinity. Conditions that promoted decreases in the UR concentration were identified. We assumed that microbes that decreased the UR concentration were active under these conditions.

For each experiment, 10 mL of groundwater and UR stock solution (1000 ppm) were added to a 15-mL sterile polypropylene centrifuge tube to prepare a 2 ppm UR solution. The water temperature was investigated in two stages. In the first stage, the water temperatures investigated were 4 °C, 20 °C, 35 °C, 45 °C, 60 °C, and 80 °C. According to the results of the first stage, four more temperatures were investigated between that at which a large change in concentration was observed (35 °C) and that at which no decrease in concentration occurred (45 °C). The temperatures for the second stage were 37 °C, 40 °C, 42 °C, and 44 °C. To investigate the pH, the sample pH was adjusted to 6.0, 7.2 (in situ groundwater [30]), 9.0, or 12.0 using NaOH and HCl (FUJIFILM Wako Pure Chemical Corp.). To investigate the salinity, the sample NaCl mass fraction was adjusted to 1% (in situ groundwater with an electrical conductivity of 1940 mS/m [30]), 3%, 6%, or 10% (FUJIFILM Wako Pure Chemical Corp.). After adding the UR stock solution, the tubes were capped and the experiments were started. The samples for the water temperature experiments were stored in controlled thermostatic chambers set to the required temperatures. All other samples were stored at approximately 20 °C in an air-conditioned room. Samples were stored in the dark to prevent photodegradation. After a set time, the tubes were opened and 1 mL aliquots were collected for measuring the UR concentration.

2.4.2. Evaluation of organic matter metabolism

To identify microbes that could contribute to decreases in the UR concentration, we investigated the metabolism of organic matter by microbes in groundwater. Organic matter that could act as a carbon and energy source for the microbes was added to the UR solutions prepared with groundwater and changes in the concentration of UR were measured over time.

For these experiments, 10 mL samples of groundwater were added to a 15 mL sterile polypropylene centrifuge tubes with 0.02%, 0.2%, or 2% glucose, sodium acetate, or methanol (FUJIFILM Wako Pure Chemical Corp.). As a control, a sample was prepared with no added organic matter. Next, the UR stock solution was added at 2 ppm and the tubes were capped. All samples were stored in the dark at room temperature. After a set time, the tubes were opened and 1 mL aliquots were collected for analysis.

2.4.3. Comparison of microbial communities with and without addition of UR

The microbial communities in the groundwater with and without addition of UR were analyzed using 16S rRNA gene sequencing. We compared these communities to identify microbes that might decrease the UR concentration.

First, we aseptically filtered 2 L of groundwater using a 0.22 μm membrane filter (Merck, Darmstadt, Germany). Microbial cells in the groundwater were collected on the filter membrane. The filter was stored in a freezer until required for analysis. Another 2 L of groundwater was divided equally between two 2 L polypropylene bottles. We added the UR stock solution to one bottle to achieve a concentration of 2 ppm and left the other bottle with no added UR. A 1 mL aliquot was collected from each bottle for analysis. Then, the bottles were capped and stored in the dark for 6 days, at which point the sample with added UR had discolored. Another 1 mL aliquot was collected from each bottle after storage. For each bottle, the microbial cells were collected on the 0.22 μm filter membranes.

Bulk DNA was extracted from the microbial cells collected on the filter membranes using a PowerWater DNA Isolation kit (Qiagen, Hilden, Germany). The concentration of extracted DNA was determined using a Qubit 1.0 dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Libraries generated from extracted DNA were prepared by a two-step tailed polymerase chain reaction (PCR) [34] using 515f/806r primers specific for the V4 region (320 bp) of the 16S rRNA gene and analyzed using Miseq. A first PCR was performed using primers with overhang adapters from Illumina (San Diego, CA, USA). The reaction solution contained 12.5 μL of 2 × KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), 0.5 μL of primer (10 μM), 10.5 μL of double-distilled H_2O, and 1 μL of template DNA. The reaction conditions were 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. Amplicons were purified using AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA) after amplification was confirmed by electrophoresis on 1.5% agarose gel. A second PCR was performed to attach dual indices and Illumina sequencing adapters. The reaction conditions were 95 °C for 3 min, followed by eight cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. Purified second PCR amplicons were extracted with AMPure XP beads and quantified using a Qubit Fluorometer (Thermo Fisher Scientific-JP, Tokyo, Japan). They were then pooled in equimolar ratios for library construction, spiked with PhiX Control v3 kit (Illumina), loaded onto iSeq cartridges, and run on the iSeq 100 Sequencing System Guide (Illumina) using the next-generation sequencer iSeq 100 (Illumina) [35]. Paired-end reads were merged and processed using QIIME 1.91 [34]. Chimeric sequences were identified and removed using UCHIME [36]. Sequences were clustered into operational taxonomic units using UPARSE [37] with a 97% similarity cutoff. Taxonomy assignments were made using the USEARCH global alignment program [38] according to sequence similarity in RefSeq RDP 16S v3 and SILVA v132 (Version 1.10; [39]). Phylogenetic inference was performed using BaseSpace Sequence Hub (Illumina).

2.4.4. Culture of P. aurantiacus with UR solution

From the results in Sections 2.4.1–2.4.3, we identified *P. aurantiacus* as a potential contributor to the decreases in UR

concentrations. To study the effect of *P. aurantiacus*, we obtained a strain (JCM 19853) from the Japan Collection of Microorganisms and cultivated it in Marine Broth 2216 (Merck), which was dissolved in purified water and autoclaved [40]. The cell culture was grown for 3–5 days at 37 °C with shaking. After cultivation, cells were collected by centrifugation, washed with 1 × PBS buffer, and then suspended in PBS buffer at 1×10^8 cells/mL. Untreated or filter-sterilized groundwater (0.2-μm filter) (10 mL) and UR stock solution were added to a 15-mL sterile polypropylene centrifuge tubes to prepare 2 ppm UR solutions. The pH and NaCl mass fraction were set to 7.2% and 1%, respectively, to match the conditions for the in situ groundwater. The *P. aurantiacus* solution was added to each tube to give a density of 10^6 or 10^5 cells/mL. For both the untreated and filter-sterilized groundwater, two tubes were prepared with 10^6 cells/mL of *P. aurantiacus*. One of these tubes was used to study the effect of subsampling and the other was used to study the effect of no subsampling. The tubes were capped and incubated in the dark at room temperature. At set times, the caps of the subsampling tubes were opened and a 1 mL aliquot was collected from each tube for measuring the UR concentration. The tubes without subsampling were sampled for analysis once, after visually confirming the change in color of the solution.

3. Results

3.1. Solution conditions for microbial activity decreasing the UR concentration

Microbes are affected by environmental conditions, such as the water temperature, pH, and salinity. In this study, the changes in the UR concentration with water temperature, pH, and salinity (Fig. 1) were measured to narrow down potential microbes that could decrease the UR concentration according to the conditions required for them to thrive.

Marked decreases in the UR concentrations were observed with water temperatures of 20 °C and 35 °C, and the normalized concentrations reached almost zero within 3 days (Fig. 1a). A decrease in the concentration was also observed at 4 °C, but it took almost 1 week for the concentration to reach almost zero at this temperature. At temperatures of 45 °C and above, large (>20%) decreases in the concentration were not observed over the 2 weeks of the experiment. In the second stage of the temperature experiments (35°C-45 °C), the fastest decrease in the concentration was observed at 37 °C, followed by 40 °C (Fig. 1b). The concentration did not decrease at temperatures above 42 °C.

In the pH experiments, the largest decrease in the concentration was observed at pH 7.2, which is the pH of in situ groundwater [30]. The concentration approximately halved by the third day of the experiment, and within 1 week, the concentration was almost zero (Fig. 1c). At pH 6.0 and 9.0, the UR concentration decreased slowly. By contrast, at pH 12, large (>5%) decreases in the concentration were not observed over the 2 weeks of the experiment.

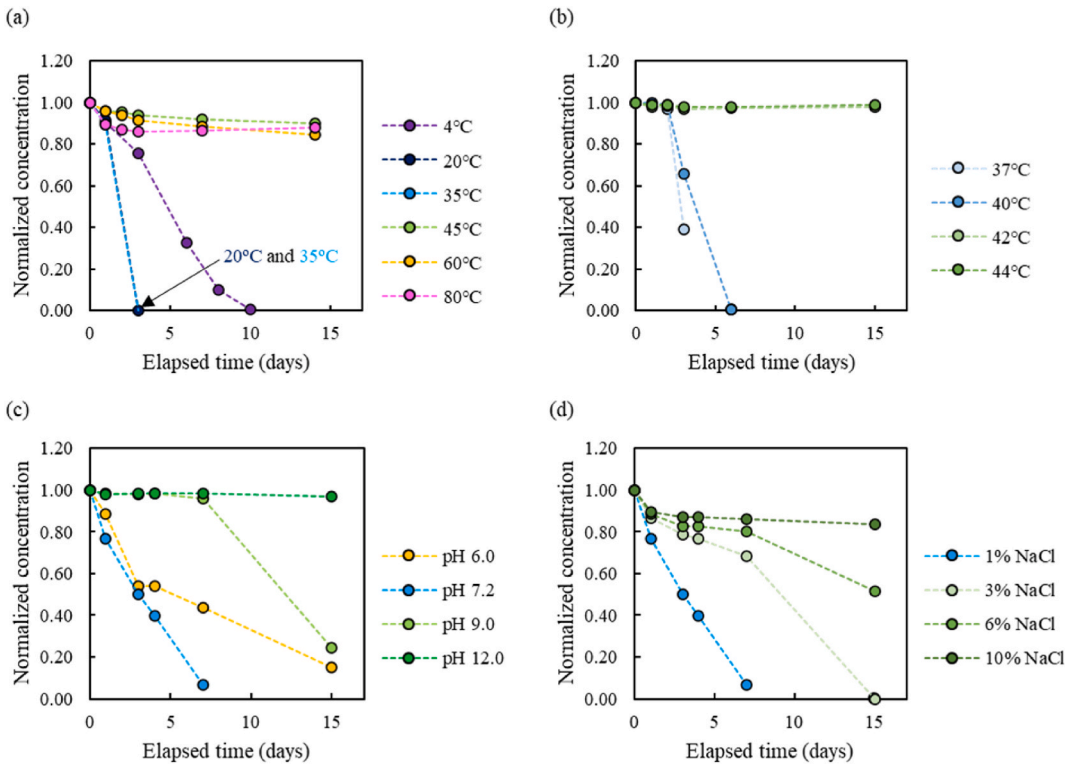

Fig. 1. Time course of uranine (UR) concentration changes under different conditions: (a) storage at temperatures ranging from 4 °C to 80 °C, (b) storage at temperatures ranging from 37 °C to 44 °C, (c) pH, and (d) salinity.

In the NaCl mass fraction experiments, the largest decrease in the UR concentration was observed with 1% NaCl, which was salinity equivalent to that of in situ groundwater [30] (Fig. 1d). The rate of the decrease in the UR concentration decreased with increasing salinity and the changes in the concentrations were minimal when the salinity was higher than that of seawater.

3.2. Metabolism of organic matter added to the UR solution

To clarify the involvement of microbes in decreasing the UR concentrations, we added organic matter to the UR solution prepared with groundwater. We hypothesized that if the decrease in UR concentration was caused by microbes, then the rate of the decrease would be affected by addition of organic matter, which would provide carbon and energy. The results and the microbial metabolic characteristics could then be used to further identify microbes that decreased the concentration. Glucose, sodium acetate, and methanol were added as organic matter at 0.02%, 0.2%, and 2%.

In the control, without added organic matter, the UR concentration decreased slowly and reached almost 50% of initial concentration after 2 weeks. When organic matter was added, the change in the UR concentration differed depending on the type of organic matter (Fig. 2). When glucose was added, the change in the UR concentration was consistent with 0.02%, 0.2%, and 2% glucose. Addition of acetate accelerated the decrease in the UR concentration compared with the control. When methanol was added at low concentrations (0.02% and 0.2%), the decrease in UR concentration accelerated, but addition of methanol at a high concentration (2%) inhibited the decrease in UR for the 2 weeks of the experiment.

3.3. Comparison of microbial communities with and without addition of UR

The microbial community constituents in the samples were compared after 6 days with and without addition of UR (Fig. S1). The microbial community constituents in the original groundwater were also evaluated for reference (Fig, S1). The background UR

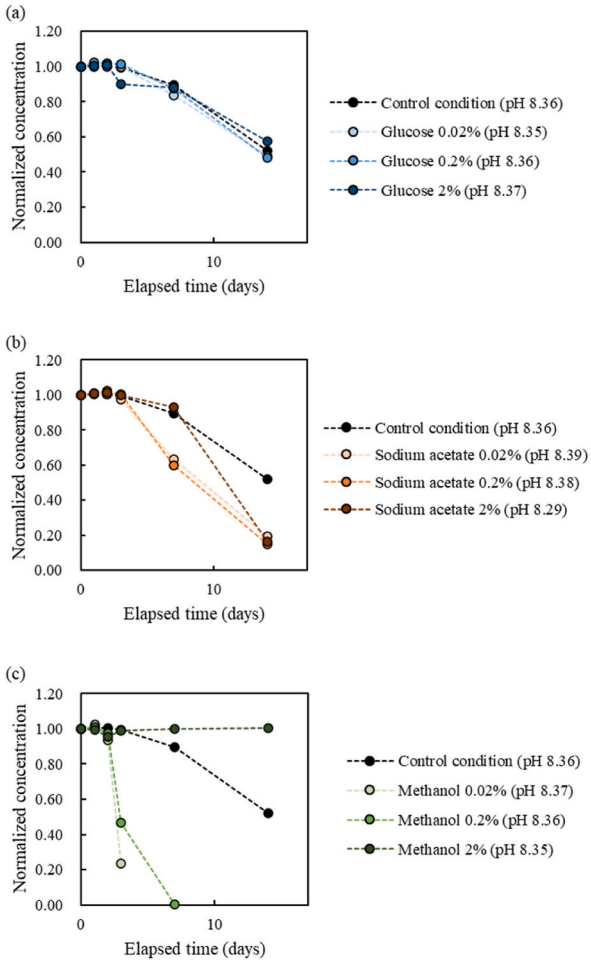

Fig. 2. Time course of uranine (UR) concentration changes with addition of organic matter: (a) glucose, (b) sodium acetate, and (c) methanol. The average pH of the solutions at the beginning of the experiment was 8.15. The pH values at the end of the experiment are shown in parentheses.

concentration in the examined groundwater was 0.00 ppm. For the sample with added UR, the UR concentration decreased from 2.07 ppm to 0.00 ppm over 6 days.

The microbial community in groundwater was dominated by the phyla *Proteobacteria* (classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*), *Bacteroidetes*, *Euryarchaeota*, and *Firmicutes*. These phyla accounted for more than 70% of the whole community. By the end of the experiments both with and without UR addition, *Proteobacteria* accounted for more than 90% of the community. Addition of UR resulted in an increase in the relative ratio of the class *Gammaproteobacteria* from 30.0% to 44.9%.

At the genus level, UR addition resulted in increases in the contents of *Silanimonas*, *Methylophaga*, and *Methylobacter*, which belong to the class *Gammaproteobacteria*, from 3.2% to 13.4%, 11.8%–18.2%, and 3.5%–7.2%, respectively, at the end of the experiment. At the same time, the contents of *Erythrobacter* and *Roseovarius*, which belong to the class *Alphaproteobacteria*, increased from 0.6% to 6.0% and 0.8%–2.9%, respectively. The sequences were similar to those of *Silanimonas mangrovi*, *Methylophaga alcalica*, *Methylobacter marinus*, *P. aurantiacus*, and *Roseovarius mucosus*.

3.4. Culture of *P. aurantiacus* with UR solution

To evaluate whether the microbes identified in Section 3.3 decreased the concentration of UR, a verification experiment was conducted using one strain. For a UR solution prepared using untreated groundwater, without addition of *P. aurantiacus*, the UR concentration decreased to 78% of the initial concentration on the day after the start of the incubation and 30% of the initial concentration on the second day (Fig. 3). When *P. aurantiacus* was added to the same UR solution, a higher density (10^6 vs. 10^5 cells/mL) of *P. aurantiacus* resulted in faster decrease in the UR concentration. For a UR solution prepared using filter-sterilized groundwater and without addition of *P. aurantiacus*, the UR concentration did not decrease over the 4 weeks of the experiment. Furthermore, no decreases in the UR concentration were observed in the subsampling experiments. However, in the experiments without subsampling, a change in the solution was visually confirmed on day 7 of the incubation. Furthermore, when we opened the tube and collected a sample on day 8, the UR concentration was zero.

4. Discussion

4.1. Solution conditions for microbial activity decreasing the UR concentration

Decreases in UR concentrations in groundwater have been observed in previous studies [4,22] and were observed in the present study (Fig. 1). In our results, the largest decreases in the UR concentration occurred when the groundwater had a temperature of approximately 37 °C, pH of 7.2, and NaCl mass fraction of 1%. We assumed that the microbes that decreased the UR concentration were active and thrived under these conditions. Therefore, mesophilic, neutrophilic, and halophilic or halotolerant microbes may decrease the UR concentration.

The temperature of the in situ groundwater was 22.3 °C [30], and this was within the temperature range at which UR concentration

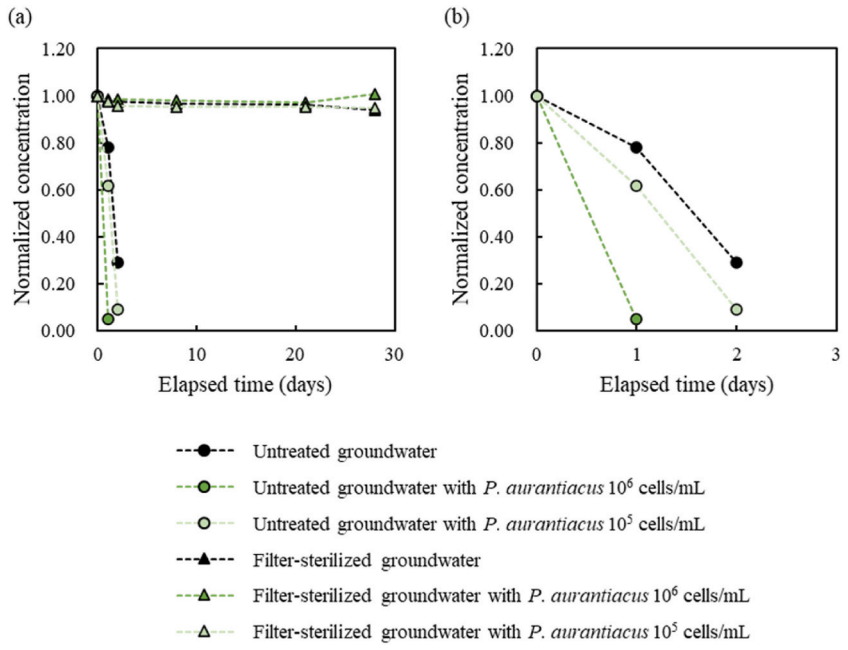

Fig. 3. (a) Time course of uranine (UR) concentration changes with addition of *Parapontixanthobacter aurantiacus* under subsampling conditions. (b) An enlargement of (a) up to the third day, showing only the results for untreated groundwater.

Table 1
Characteristics of microbes identified as contributing to decreases in the UR concentration.

Species		Silanimonas mangrovi (HE573746)	Methylophaga alcalica (AF384373)	Methylobacter marinus (AF304197)	Parapontixanthobacter aurantiacus (NR_146682.1)	Roseovarius mucosus (AJ534215)
Class		*Gammaproteobacteria*	*Gammaproteobacteria*	*Gammaproteobacteria*	*Alphaproteobacteria*	*Alphaproteobacteria*
Order		*Lysobacterales*	*Thiotrichales*	*Methylococcales*	*Sphingomonadales*	*Rhodobacterales*
Family		*Lysobacteraceae*	*Piscirickettsiaceae*	*Methylococcaceae*	*Erythrobacteraceae*	*Rhodobacteraceae*
Oxygen tolerance		Strictly aerobic	Strict aerobe	Strictly aerobic	Strictly aerobic	Aerobic
Gram stain		Negative	Negative	Negative	Negative	Negative
Growth temperature	Range	10–40	4–35	15–40	4–40	20–40
oC	Optimum	30–37	25–29	37	37	
Growth NaCl (w/v)	Range	0–8	< 10		0.5–7.0	0.3–10
%	Optimum	0–2	3–4	0.6	3	1–7
Grouwth pH	Range	6–12	7.0–11.5	5.5–9.0	6.0–10.0	6.0–8.8
	Optimum	7–8.5	9–9.5	7.0	9	
Carbon source	Glucose	+	−	−	−	−
	Acetate		−	−		+
	Methanol		+	+		−
Quinones		Q-8	Q-8	Q-8	Q-8, Q-9, Q-10	Q-10
Isolation		Mangrove sediment	Saline soda lake	Sea water	Deep sea sediment	Dinoflagellate
Reference		Srinivas et al. (2013)	Doronina et al. (2003)	Bowman et al. (1993)	Zhang et al. (2016)	Biebl et al. (2005)

decreases occurred. Therefore, decreases in the UR concentration could occur under the in situ environmental conditions. Solution conditions that promote decreases in UR concentrations are likely to be encountered during field investigations or tracer tests. Because the water quality characteristics of the tracer solution must be close to those of the on-site groundwater, fluorescent dye solutions are typically prepared using on-site groundwater. Therefore, there is an urgent need to evaluate the possibility that the UR concentration will decrease during use and storage of conservative tracer fluorescent dye solutions prepared using on-site groundwater.

In terms of the temperature, fluorescent dyes are usually stored in the dark at 4 °C [22,24]. This temperature is selected to suppress microbial activity, but not all microbial activity stops at 4 °C. Therefore, the concentration of the fluorescent dye will still decrease, albeit more slowly than at 20 °C or 35 °C.

No changes in the UR concentration were observed when temperatures were above 42 °C, or pH was over 12.0, or NaCl mass fraction was 10%. Furthermore, the decrease in the UR concentration was minimal when the solution was prepared using filter-sterilized (0.2 μm filter) groundwater (Fig. S2). This result suggested that microbes in groundwater decreased the UR concentration. Depending on the solution conditions, the effects of microbes in groundwater with similar microbial community constituents may be negligible in terms of their ability to decrease the UR concentration. The results of this study also showed that it may be possible to eliminate the effects of microbes on the UR concentration by controlling the solution conditions. However, this is an inference from the results of the present study in one location, and further research in the other locations is needed to generalize the results.

4.2. Metabolism of organic matter by microbes that decrease the UR concentration

Although addition of glucose did not affect the change in the UR concentration (Fig. 2), addition of acetate and low concentrations of methanol (0.02% and 0.2%) accelerated the decrease in the UR concentration compared with the control. These results are a strong indication that microbes contribute to the decreases in the UR concentration. Goldscheider et al. [8] mentioned that UR degrades in organic-rich environments. The input of organic matter may trigger the degradation of previously unreactive organic matter [41], and this has already been shown with contaminants [42]. Thus, the decrease in UR concentration may be caused by an increase in microbial activity resulting from the addition of organic matter. Microbes that show activity increases in the presence of acetate and low concentrations of methanol may promote decreases in the UR concentration. For instance, sulfate-reducing bacteria (SRB), which use hydrogen and formic acid as electron donors, thrive with acetate as a carbon source [43]. It is possible that these microbes may have an impact on UR concentrations. Obligate methylotrophic and aerobic bacteria, such as *Methylophaga* and *Methylobacter*, which were detected in the groundwater with added UR, use methanol to grow. Therefore, the increased activity of microbes such as SRB, or some methylotrophic bacteria, in groundwater with the addition of acetate or methanol to the UR solution may be responsible for the decrease in the UR concentration.

However, the decrease in the UR concentration was inhibited in the presence of high concentrations of methanol. There are two possible reasons for this. First, if the added organic matter is degraded by the same metabolic pathway as UR, it may act as an antagonist and slow the rate of the UR concentration decrease. Furthermore, if microbes in groundwater are degrading UR and using it

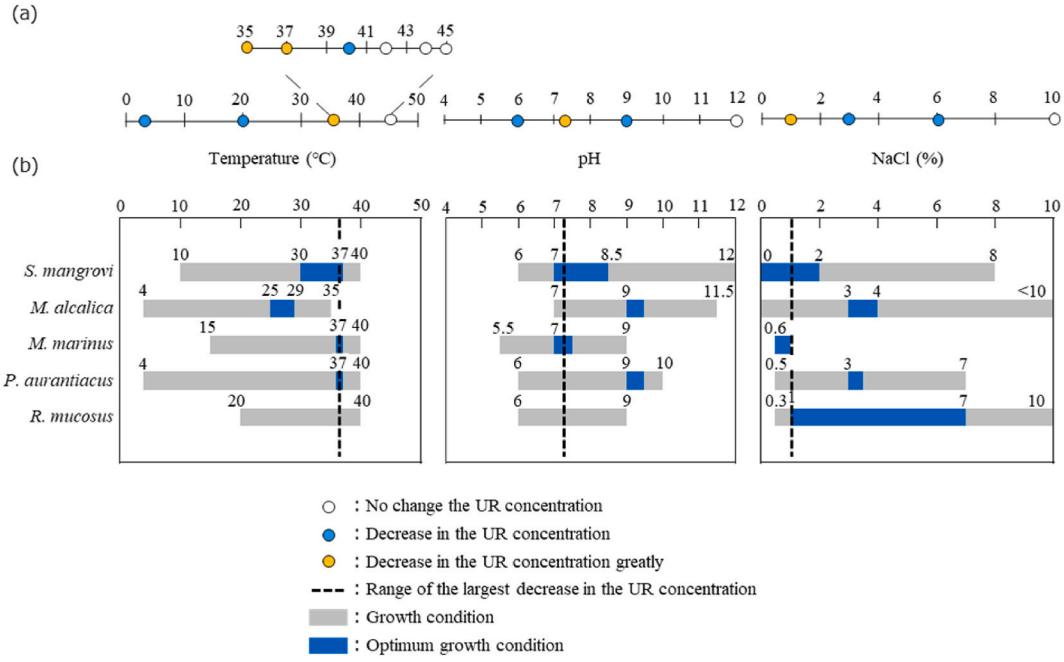

Fig. 4. Comparison of (a) solution conditions showing decreasing uranine (UR) concentration and (b) microbial growth conditions that contribute to the decreases in the UR concentrations in the examined groundwater.

as a carbon source, then the decrease in UR will be inhibited or reduced in the presence of more readily available organic matter. It is possible that methanol is a more accessible carbon source than UR. Second, methanol is an anti-bacterial agent at high concentrations [44], and can inhibit the activity of methylotrophs [45,46]. Thus, the addition of high concentrations of methanol could be effective for conservation of UR concentrations.

4.3. Microbe changes with addition of UR

The relative ratios of specific microbes that biodegrade UR may increase after the addition of UR. Therefore, the microbial community constituents may differ with and without UR. We found that the relative ratios of five microbes increased with addition of UR (Table 1). It is possible that these microbes biodegrade UR. These microbes were aerobic and either halophilic or halotolerant and had growth temperatures ranges of 4 °C–40 °C, pH ranges of 5.5–12.0, and salinity ranges of 0%–10% (w/v). Thus, if the UR solution meets these conditions, then UR may be biodegraded by these microbes.

Fig. 4 compares the solution conditions that resulted in large decreases in the UR concentration in the examined groundwater with microbial growth conditions. The temperature that caused the largest decrease in the UR concentration was 37 °C, which was consistent with the optimum growth temperatures for *S. mangrovi*, *M. marinus*, and *P. aurantiacus*, and within the growth temperature range for *R. mucosus*. The pH value that resulted in the largest decrease in the UR concentration was optimum for growth of *S. mangrovi* and *M. marinus*. For NaCl, the concentration that resulted in the largest decrease in the UR concentration was consistent with that for optimum growth of *S. mangrovi* and *R. mucosus*, and within the range for growth of the other species. Therefore, the solution conditions that resulted in large decreases in the UR concentration were consistent with growth conditions for microbes that could contribute to decreasing the UR concentration. This was especially true for *S. mangrovi* and *M. marinus*, and it was possible that the decreases in the UR concentration were caused by these microbes.

Five closely related microbes potentially contributed to the decreases in the UR concentration. These were *M. alcalica*, *M. marinus*, *P. aurantiacus*, and *R. mucosus*, which do not use glucose; *R. mucosus*, which uses acetate; and *M. alcalica* and *M. marinus*, which are obligate methylotrophic bacteria that use methanol (Table 1) [40,47–51]. Considering those microbial metabolic characteristics, microbes that do not use glucose and/or those that use acetate and/or low concentrations of methanol may contribute to the decreases in the UR concentration in the examined groundwater. The metabolic characteristics of *M. alcalica*, *M. marinus*, *P. aurantiacus*, and *R. mucosus*, which do not use glucose but do use acetate or methanol, are consistent with the experimental results for addition of organic matter. However, a similar experiment [52] conducted using groundwater sampled from different depths in the Horonobe area showed that the rate of decrease in the UR concentration slowed as the amount of glucose added increased. This suggests that microbes such as *S. mangrovi*, which uses glucose, may contribute to the decrease in the UR concentration. Therefore, the microbes identified as potentially contributing to the decreases in the UR concentration are consistent with the microbial metabolic characteristics inferred from the organic matter addition experiments. It is possible that all five species contributed to decreases in the UR concentration.

The microbial biomass, activity, and community constituents in groundwater collected from different depths in the Horonobe area reportedly show large differences [53] and the ecosystem hosts microbes from diverse lineages [54]. This suggests that differences in the microbial community of the examined groundwater may contribute to differences in the rate of decrease in the UR concentration and response to addition of organic matter.

4.4. Effect of P. aurantiacus on the UR concentration

When *P. aurantiacus* was added to a UR solution prepared with untreated groundwater, the UR concentration decreased more rapidly with a higher density of *P. aurantiacus* than with a lower density of *P. aurantiacus* (Fig. 3). This indicated that *P. aurantiacus* had a positive effect on the decrease in UR concentration. Addition of *P. aurantiacus* to filter-sterilized groundwater without subsampling also resulted in a decrease in the UR concentration. Therefore, *P. aurantiacus* directly contributed to the decrease in UR concentration.

The differences in the results with or without subsampling may be caused by oxygen conditions in the tubes. It is possible that some reactions under low-aerobic conditions contributed to the decrease in UR concentration, although, *P. aurantiacus* is a strictly aerobic bacterium [40]. Because some microbes are known to metabolize differently under aerobic and microaerobic conditions, or at low oxygen concentrations, it is possible that the difference in oxygen conditions contributed to the decrease in the UR concentration.

The present study is the first to reveal that a specific microbe, *P. aurantiacus*, decreases the UR concentration. *Parapontixanthobacter aurantiacus* has been isolated from deep-sea sediment in the west Pacific Ocean [40]. It is characterized as strictly aerobic. Growth occurs between 4 °C and 40 °C (optimum temperature: 37 °C), at pH 6–10 (optimum pH: 9) and in the presence of 0.5%–7% (w/v) NaCl (optimum mass fraction: 3%). Therefore, it is possible that *P. aurantiacus* could be present in groundwater when conducting tracer tests under these conditions, which could result in a decrease in the concentration of fluorescent dye. In fact, *P. aurantiacus* has been detected in groundwater in several locations, and it has been confirmed that it decreases the UR concentration [55]. Therefore, the use of UR should be avoided in groundwater where *P. aurantiacus* is potentially present or preliminary experiments should be conducted to evaluate UR degradation. We also identified four other microbes and the conditions (water temperature, pH, and electrical conductivity) under which they could decrease the UR concentration. If tests are conducted in groundwater under these conditions, the use of UR should be avoided, or preliminary experiments should be conducted.

Parapontixanthobacter aurantiacus belongs to the family *Erythrobacteraceae* [56], which includes bacteria that degrade aromatic compounds such as benzo[*a*]pyrene [57] and oil [58]. UR is an aromatic compound and could also be degraded by bacteria in this family. Groundwater that is rich in aromatic compounds may contain many microbes that use these substances as a source of nutrients, and these microbes could contribute to the degradation of UR. Bottrell et al. [59] highlighted that sulfide produced by SRB decreased

the UR fluorescence intensity in areas contaminated with unleaded petroleum fuel. Therefore, in addition to biodegradation of UR by microbes, it is possible that metabolites (e.g., sulfides) from microbial reactions will bind to UR and inhibit its fluorescence, which is equivalent to a decrease in UR concentration. Further experiments are required to determine whether microbes biodegrade UR [3, 24, 25 and references therein] or whether the metabolites inhibit its fluorescence [59,60].

4.5. Impact on water resource studies

Our results indicated that microbes in natural systems could affect the concentrations of fluorescent dyes. If UR is used and expected to act as a conservative tracer, large decreases in UR concentrations caused by microbes may result in misunderstanding of groundwater flow, and underestimation of flow rates and catchment areas of groundwater or surface water. In the following cases, the effects of microbes on the concentration of UR should be carefully considered.

4.5.1. Preparation of tracer solutions using natural water that contains microbes

In some experiments, such as tracer experiments, the composition of the tracer solution should be close to that of the natural system because the migration behavior of the tracer will be affected by the composition. Sometimes water obtained from the test site is used for preparation of tracer solutions. In such cases, microbes in the water could affect the concentrations of the fluorescent dyes. The use of simulated natural water prepared from tap water or pure water is one option to avoid the effects of microbes in groundwater.

4.5.2. Subsurface investigations

Photodegradation is known to dramatically affect the concentration of UR [17,24,26] and investigations of surface water are adversely affected by sunlight. Because UR is more rapidly photodegraded than degraded by microbes [27,28,61], the effects of microbes might be negligible for investigations or experiments conducted in surface water. By contrast, for subsurface investigations, degradation by microbes could be a major factor affecting the concentrations of fluorescent dyes.

4.5.3. Long (>1 day) experiments

In our experiments, the UR concentration decreased greatly a few days after starting the experiments. Thus, the effect of microbes on fluorescent dyes may be minimal for experiments that are shorter (<1 day). In longer experiments (several days to a week), the decrease in concentration for fluorescent dyes caused by microbes should receive attention.

In summary, if subsurface experiments are conducted, the tracer solution is prepared with groundwater, and the experiment is longer than 1 day, then the effects of microbes on the fluorescent dye concentration should be considered. In such cases, preliminary experiments should be performed to determine if the fluorescent dye act as a conservative tracer or not.

Decreases in the UR concentration can also occur during storage of samples collected for analysis. Therefore, it is recommended that samples collected for analysis are filtered and stored in a refrigerator to minimize microbial effects [22]. In future research, reasonable measures should be taken to ensure that the fluorescent dye behaves as a conservative tracer. Furthermore, the mechanisms by which microbes affect the concentrations of fluorescent dyes should also be elucidated. The information obtained in this study for conditions that accelerate UR degradation could be used to identify the mechanisms.

5. Conclusions

This study focused on the widely used fluorescent dye UR and tried to identify microbes that contributed to decreases in its concentration during storage. Experiments investigating the solution conditions and addition of organic matter strongly suggested the involvement of microbes. The largest decreases in the UR concentration occurred with a water temperature of approximately 37 °C, pH of 7.2, and NaCl mass fraction of 1%. The microbes that decreased the UR concentration likely did not metabolize glucose but did metabolize acetate or low concentrations of methanol. Microbial community analysis showed that the relative ratios of some bacteria increased with the addition of UR. The metabolic characterization and microbial community analysis results suggested that several microbes with different metabolic characteristics (e.g., use of acetate and methanol) contributed to the UR concentration decreases. Addition of one of these species, *P. aurantiacus*, to a UR solution decreased the UR concentration. Therefore, this microbe was identified as contributing to the decrease in UR concentration. The results of this study show that the concentrations of fluorescent dyes in natural systems are affected by microbes in groundwater. This could result in errors when using fluorescent dyes as conservative groundwater tracers. If subsurface investigations or experiments are conducted, the tracer solution is prepared with groundwater and the investigation or experiment takes more than 1 day, then the effects of microbes on the fluorescent dye concentration are likely to be significant. The use of UR should be avoided if the groundwater contains certain microbes, including *P. aurantiacus*, or if the groundwater conditions (water temperature, pH, and electrical conductivity) meet those required for microbial growth. Alternatively, preliminary experiments should be performed to determine whether the concentration of the fluorescent dye is conserved.

Funding sources

The study was performed under a contract with the Ministry of Economy, Trade and Industry (METI) as part of the R&D supporting program “Research and development on groundwater flow evaluation technology in bedrock” (financial year 2022) (grant number JPJ007597).

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Ayumi Sugiyama: Writing – original draft, Validation, Investigation. **Kotaro Nakata:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Shin-Ichi Hirano:** Writing – review & editing, Resources. **Takuma Hasegawa:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The study was performed under a contract with the Ministry of Economy, Trade and Industry (METI) as part of the R&D supporting program “Research and development on groundwater flow evaluation technology in bedrock” (financial year 2022) (grant number JPJ007597). The groundwater sampling in the Horonobe area was performed by Dr Kazuya Miyakawa (Horonobe Underground Research Center of the Japan Atomic Energy Agency). The authors thank Y. Matsumura, C. Nakayama, and R. Hagino for help with the laboratory work. We thank Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27397.

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