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Detection of human-associated bacteria in water from Akiyoshi-do Cave, Japan

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• Abstract
Akiyoshi-do Cave is one of the oldest and the largest show caves in Japan. Environmental alterations induced by tourism impacts have been suggested; however, only a few previous studies have investigated the impacts of tourism on the cave. In this study, enrichment culture procedures were applied to detect human-associated bacteria (HAB) including *Escherichia coli*, *Staphylococcus aureus*, and thermo-tolerant *Bacillus* spp. in cave water. Physical and bacterial parameters of water including total nitrogen, ammonia, phosphate, total number of bacteria, and total number of viable bacteria were collected as environmental factors. *Escherichia coli* was absent at all sites, but increased levels of total bacteria, viable bacteria, *S. aureus*, and thermo-tolerant *Bacillus* spp. were present at high-impact sites. Examination of the origin tracking of HAB suggested that cave tourists could be a source of HAB contamination, but other causes related to the surface land use could also contribute to HAB contamination.

• Practitioner points

- Detection of HAB was performed for the first time in Japanese show caves to consider tourism impacts.
- The greater number of HAB was detected from the tourist area than the nontourist area in the cave.
- It was suggested that the origin of HAB may not be limited to tourists, but may also include the surface.

• Key words
bacterial cultivation; cave water; environmental protection; human-associated bacteria; origin track; show cave; tourism impact

Introduction

Cave tourism, a form of geotourism, has recently gained popularity worldwide. In Japan, cave tourism began in the early 20th century when Western culture was introduced to Japan. Akiyoshi-do Cave is the first and most popular domestic show cave in Japan, with tourism beginning in 1904 and 500,000 visitors annually in recent years. Akiyoshi-do Cave was discovered by local people, but entrance was limited as the cave was seen as a holy place where the water god lives. Human-induced environmental changes were minimal or relevant until its establishment as a show cave, but none of the previous 104+ or not relevant studies conducted since the beginning of its use as a show cave have focused on tourism impacts. Generally, caves have a stable but fragile confined environment, which is highly susceptible to tourism-induced disturbances (Fernandez-Cortes et al., 2011). Tourism impacts on Akiyoshi-do Cave have become more noticeable, such that tourists have observed visible changes in the landscape, such as water pollution, lampenflora growth, decrease of cave animal species, and colonization by invasive species.

Previous tourism impact studies of other show caves demonstrated that trash, dust, and external soil left by cave visitors alter the quality of cave water and soil

(Chelius et al., 2009; Fernandez-Cortes et al., 2011). Cave microclimates are influenced by breathing and human movement, causing changes in carbon dioxide concentration, and air temperature (Hoyos, Soler, Cañaveras, Sánchez-Moral, & Sanz-Rubio, 1998). For Akiyoshi-do Cave specifically, cave water is especially important to protect, as it supports subterranean amphipods such as *Pseudocrangonyx akatsukai*, *Gammarus pulex*, *Gammarus nipponensis*, an isopod, and *Caecidotea akiyoshiensis*, and local people use cave water as drinking water and for Japanese sake production (Nakamura & Kuramoto, 1978; Uéno, 1927, 1933).

Although tourism impacts on the Akiyoshi-do Cave have not yet been investigated in depth, previous studies identified the cave's unique ecological structure, geomorphological features, and hydrological system, providing a baseline for future assessments. The value of the cave as a tourism resource and opportunity for teaching visitors about the natural environment are widely recognized, as the Akiyoshi-do Cave is included on lists of Special Natural Monuments, Quasi-National Parks, Ramsar sites, and Japanese Geoparks.

Tourism/human impact studies are essential to sustain the cave's long-term value. Among various methodologies to measure human impact, microorganisms have the most immediate and sensitive response, as demonstrated by prior studies in other cave systems. Ikner et al. (2006) revealed decreased isolated bacterial diversity as visitation frequency increased. Lavoie and Northup (2005) targeted *Escherichia coli*, *Staphylococcus aureus*, and thermo-tolerant *Bacillus* spp. as human-associated bacteria (HAB) and discovered increased HAB as visitation frequency increased. Mulec (2014) also quantified abundance of the HAB *E. coli* and *S. aureus*, and total/viable number of bacteria, confirming the same trend. Shapiro and Pringle (2010) qualified the decreased fungal diversity as visitation frequency increased. There is some overlap between the microbial diversity in the cave and the surface, but the microbial diversity is higher in the cave (Lavoie et al., 2017; Ortiz et al., 2013). Because microorganisms are adapted to the oligotrophic cave environment, their number and activity level is lower than on the surface (Rusterholtz & Mallory, 1994). Therefore, increasing inputs of organic matter that change the native microbial ecology are of significant concern for the cave environment (Ikner et al., 2006; Lavoie & Northup, 2005; Mulec, 2014).

Although cave-dwelling animals in the Akiyoshi-do Cave have been studied by multiple researchers for several decades (Kuramoto, 1961; Kuramoto & Matsumoto, 1967; Kuramoto, Nakamura, Shimoizumi, & Uchida, 1969; Kuramoto, Nakamura, & Uchida, 1985; ; Kuramoto, Nakamura, & Uchida, 1988; Kuramoto, Nakamura, Uchida, & Shimoizumi, 1973; Kuramoto, Nakamura, Uchida, & Shimoizumi, 1975; Nakamura & Kuramoto, 1973; Yaginuma, 1962; Yoon, Kuramoto, & Uchida, 1984a; Yoon, Kuramoto, & Uchida, 1984b; and others), only one study evaluated cave microbes. Hori, Ishida, and Shimano (2008) reported the cave-specific soil bacterial structure in the nontourist region of the Akiyoshi-do Cave, but no studies have compared the bacterial structure in the tourist versus nontourist regions. Although HAB examined in prior studies of other cave systems indicated tourism impacts, the origins of HAB were not evaluated in these studies. Therefore, the objectives of the present study were (a) detection of HAB and comparison between the tourist and nontourist regions of the cave, and (b) origin tracking of HAB.

Materials and methods

Study site

Akiyoshi-do Cave in Mine City of the Yamaguchi prefecture is located at 34.2°N, 131.3°E, which has a humid subtropical climate (Figure 1). Approximately 1 km in the total cave length of 10+ km is available for tourism, with three entrances, a tourist trail, cave lights, and voice guides. The average air temperature outside was 13.6°C, while the average annual precipitation was 1,994.7 mm, as recorded in observations from 1981 through 2010 (Japan Meteorological Agency, 2018). The average air temperature inside the cave is nearly stable at 17°C, and the relative humidity is 100% or slightly lower, with visible water vapor in certain areas. The temperature gap between inside and outside of the cave induces a chimney effect, in which the temperatures within the cave are higher than the outside in the winter (mean = 5.2°C), causing air movement from the lower entrance toward the higher entrance (Maeda, 1961). The air movement direction reverses in the summer when temperatures are lower in the cave.

Sampling site information

Study samples were collected, and in-situ environmental factors were measured in the Akiyoshi-do Cave in November 2016, and February, April, and November 2017. Two water samples were collected per site; one was for bacterial experiments, and the other was for physicochemical experiments. Figure 1 illustrates the map of the Akiyoshi-do Cave and the locations of sampling sites, while Figure 2 describes the positional relations of the sampling sites. Two sites in the Chimachida rimstone pools located adjacent to the tourist trail were selected as high-impact sites and named H1 and H2. There is not a great elevation between these pools and the tourist trail, so tourists have easy access, and can touch or step on the H1 and H2 rimstone pools. Another two pools located in the nontourist area, where no tourists can enter but researchers and cavers can, were selected as low-impact sites and named L1 and L2. In addition, three cave river water and inflow water sites, which are connected to high and low-impact sites, were selected to track the origins of HAB. The river water sites were named RW1, RW2, and RW3, from the upper to the lower stream. Another site with pumped-in river water inflows to H1 was named FL because the water is flowing into the pools. The other site of upwelling water, with inflow but no direct connection with H2, was named GW because the water is groundwater. All the sampling sites were selected where bat guano is absent to avoid the natural presence of *E. coli*. RW1-3, FL, and GW sites were surveyed a single time in November 2017.

Physical parameters of water

Water temperature, pH, and electrical conductivity were measured on-site using a HORIBA handy meter (model SSS054,

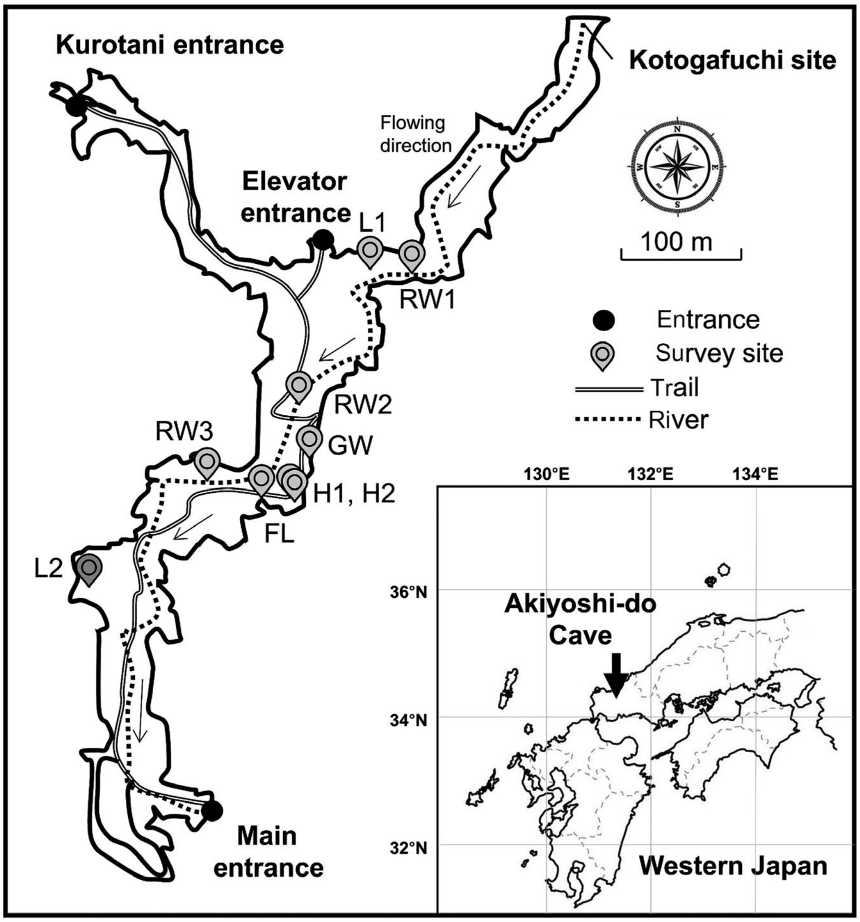

Figure 1. Location of the Akiyoshi-do Cave and survey sites. The cave river flows to the south. The map of Akiyoshi-do Cave was drawn based on karusuto.com (URL: https://akiyoshido.karusuto.com/html/guide/).

D-54). The quantity of dissolved oxygen was measured using another HORIBA handy meter (model SS 054, D-55s). Dissolved oxygen was not measured in February 2017 due to the equipment malfunction.

Alkalinity was measured by the acid–base titration method. Bicarbonate ion concentration was calculated from the alkalinity. Chemical oxygen demand was measured by the potassium permanganate titration method. Total nitrogen, total phosphorus, ammonia, and phosphate were measured by the spectrophotometric method. Ammonia and phosphate were not measured in November 2016.

Cation (Ca, Si, Na, Mg, and K) was measured by the inductively coupled plasma analysis. Anion (Cl, $NO_{3,}$ and SO_4) was measured by ion chromatography.

Bacterial counts

Total number of bacteria (TB) and total number of viable bacteria (TVB) were counted from each site to obtain biological environmental factors. Water samples for counting TB were collected by adding a 9 ml water sample to a sterilized tube, immediately packing the tube in a cooler box with ice and water (10°C), and adding 1 ml glutaraldehyde solution to the tube on the same day the field survey was conducted. Ethanol spraying and renewal of latex globes were enforced when sampling the water for bacterial experiments at different sites to prevent contamination. The glutaraldehyde solution was used as a fixation method for bacteria (Chao & Zhang, 2011). Water samples for quantification of total number of viable bacteria were collected by adding 10 ml sample water to a separate sterilized tube and immediately packing the tube in a cool box maintained under 10°C. In the laboratory, the water samples for total bacteria (2 ml) were filtered using a polycarbonate membrane filter with a pore diameter of 0.20 μm (Whatman), and DAPI (4′,6-diamidino-2-phenylindole) staining was used to count cell numbers under a fluorescent microscope (10 fields/sample). A total number of viable bacteria were counted by culturing samples on R2A medium (Daigo, Nippon Seiyaku) of $\times10^0$, $\times10^{-1}$, and $\times10^{-2}$ levels, in triplicate. Sample water was diluted to $\times10^{-s1}$ and inoculated onto plates in triplicate, which were incubated at 15°C for 10 days prior to counting the number of colony-forming units.

Human-associated bacteria

Escherichia coli is a Gram-negative coliform bacterium commonly found in the lower intestines of warm-blooded animals (Tenaillon, Skurnik, Picard, & Denamur, 2010). To detect *E. coli*, 0.1 ml of original sample water and 0.1 ml of diluted sample water were cultured with a bacteria spreader on sterilized Eosin Methylene Blue (EMB) agar media (Nissui Pharmaceutical Co., Ltd.) in triplicate and incubated at 37°C for 24 hr prior to counting the number of colony-forming units.

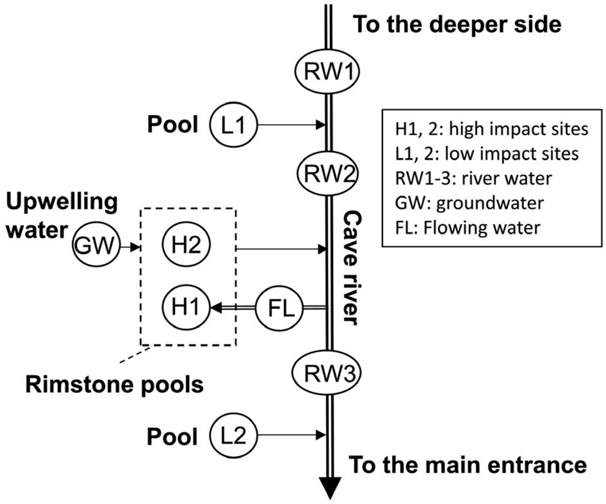

Figure 2. Positional relationships of the survey sites.

Staphylococcus aureus is a Gram-positive, normal flora of human skin and mucous membranes. To detect *S. aureus*, 0.1 ml nondiluted sample water and 0.1 ml diluted sample water were cultured with a bacteria spreader in triplicate on Mannitol Salt Agar media (Nissui Shiyaku) and incubated at 37.0°C for 36 hr prior to counting the number of colony-forming units.

Thermo-tolerant *Bacillus* spp. are found in soils heated by sunlight (Lavoie & Northup, 2005). To quantify thermo-tolerant *Bacillus* spp., approximately 5 ml sample water was heated in a 65°C water bath for 10 min to kill vegetative cells. The sample water was then diluted to $\times 10^{-1}$ using Nutrient Broth (Difco), cultured with a bacteria spreader on Nutrient Agar media (Difco) in triplicate, and incubated at 45°C for 72 hr prior to counting the number of colony-forming units.

Statistical analysis

F test and *t* test were performed with software R to clarify the differences between high and low-impact sites. Water temperature, pH, electrical conductivity, dissolved oxygen, alkalinity, chemical oxygen demand, total nitrogen, total phosphorus, ammonia, phosphate, cation (Ca, Na, Mg, K, Cl), anion (Cl, NO_3, SO_4), total bacteria, total number of viable bacteria, *S. aureus*, and thermo-tolerant *Bacillus* spp. were used in the analysis.

Table 1. Mean values of environmental factors

	HIGH-IMPACT SITES		LOW-IMPACT SITES						
	H1	H2	L1	L2	RW1[A]	RW2[A]	RW3[A]	FL[A]	GW[A]
T (℃)	14.6 ± 1.1	14.9 ± 0.6	14.3 ± 0.2	15.2 ± 0.3	15.2	15.2	15.9	15.4	15.4
pH	8.1 ± 0.2	7.9 ± 0.3	7.8 ± 0.4	8.1 ± 0.2	7.6	7.8	7.8	7.9	7.7
EC (mS/cm)	34.6 ± 6.5	32.6 ± 6.8	39.8 ± 20.0	27.5 ± 2.2	32.4	32.9	31.8	33.4	32
DO (mg/L)	7.1 ± 1.7[b]	6.3 ± 1.0[b]	7.4 ± 1.6[b]	7.5 ± 0.2[b]	9.6	9.2	9.8	10.0	9.9
ALK (meq/L)	2.8 ± 0.2	2.8 ± 0.3	2.6 ± 0.1	2.6 ± 0.9	3.0	3.0	3.0	3.0	3.0
BIC (mg/L)	172 ± 11	169 ± 17	155 ± 5	160 ± 50	180	180	183	183	183
COD (mg/L)	5.5[a]	6.4[a]	5.2[a]	11.2[a]	2.7	12.6	3.3	1.2	2.5
TN (mg/L)	1.9 ± 0.8	1.9 ± 0.4	1.2 ± 0.2	1.3 ± 0.4	1.7	1.9	2.1	1.7	1.8
TP (mg/L)	0.32 ± 0.19	0.30 ± 0.19	0.29 ± 0.33	0.29 ± 0.35	0.22	0.22	0.19	0.20	0.20
NH_4 (mg/L)	0.24 ± 0.11[c]	0.13 ± 0.05[c]	0.51 ± 0.41[c]	0.50 ± 0.26[c]	0.20	0.14	0.58	0.20	0.35
PO_4 (mg/L)	0.10 ± 0.04[c]	0.12 ± 0.03[c]	0.14 ± 0.05[c]	0.14 ± 0.01[c]	0.15	0.17	0.19	0.18	0.15
Ca (mg/L)	38.4 ± 23.3[b]	55.1 ± 6.0[b]	50.5 ± 15.7[b]	47.9 ± 10.5[b]	50.2	53.0	50.4	55.8	58.0
Si (mg/L)	1.6 ± 1.2[b]	2.3 ± 0.9[b]	1.6 ± 0.3[b]	1.9 ± 0.6[b]	1.8	1.7	1.7	1.9	2
Na (mg/L)	3.1 ± 1.9[b]	4.2 ± 0.6[b]	3.9 ± 0.7[b]	3.8 ± 0.3[b]	3.8	4.6	4	4	4
Mg (mg/L)	0.8 ± 0.5[b]	1.2 ± 0.3[b]	0.8 ± 0.2[b]	0.9 ± 0.2[b]	1.3	1.3	1.3	1.4	1.4
K (mg/L)	0.4 ± 0.3[b]	0.5 ± 0.1[b]	0.3 ± 0.2[b]	0.4 ± 0.1[b]	0.5	0.6	0.7	0.6	0.6
Cl (mg/L)	4.1 ± 3.0[b]	4.4 ± 4.0[b]	4.3 ± 3.0[b]	4.9 ± 2.8[b]	3.1	3.6	3.1	3.1	3.1
NO_3 (mg/L)	1.46 ± 0.68[b]	1.32 ± 0.25[b]	0.86 ± 0.97[b]	0.91 ± 0.82[b]	2.20	2.00	2.00	2.20	2.20
SO_4 (mg/L)	2.7 ± 2.8[b]	2.7 ± 3.0[b]	2.6 ± 3.0[b]	2.9 ± 6.5[b]	1.3	1.4	1.4	1.3	1.3

Note. H1 and H2 are high-impact water, L1 and L2 are low-impact water, and RW1-3 are river water. FL and GW are source of pumped-in river water and upwelling water, which flow into the high impact pools where H1 and H2 were collected.

Abbreviations. ALK: alkalinity; BIC: bicarbonate ion concentration; COD: chemical oxygen demand; EC: electric conductivity; T: water temperature; TN: total nitrogen; TP: total phosphorus.

[a]Values of a single survey in November 2017.

[b]Mean values of three surveys in November 2016, and April and November 2017.

[c]Mean values of three surveys in February, April, and November 2017.

Results

Environmental factors

Physicochemical environmental factors of the high- and low-impact sites and other sites are shown in Table 1. A *t* test was used to evaluate statistical significance between high- and low-impacts sites for all items. High-impact sites showed greater values than low-impact sites in total nitrogen, ammonia, and phosphate with the *p*-values of <0.05. Dissolved oxygen values showed greater values in high-impact sites than low-impact sites without the *p* value of <0.05.

Total bacteria was the highest at the high-impact sites, with 5.7×10^5 CFU/ml and 1.2×10^6 CFU/ml at H1 and H2, respectively (Figure 3). The high-impact sites showed greater values of total bacteria than low-impact sites, which were 1.8×10^5 CFU/ml and 9.9×10^4 CFU/ml for L1 and L2, respectively ($p < 0.05$). The total number of viable bacteria was highest at H2 with 2.3×10^4 CFU/ml (Figure 4). Low-impact sites had a lower total number of viable bacteria than the other sites including high-impact sites, except one of the three river water sites ($p < 0.05$).

Human-associated bacteria

Figure 5 summarizes the numbers of HAB. *E. coli* was not detected from any study samples. A *t* test was used to evaluate statistical significance between high- and low-impacts sites for *S. aureus* and thermo-tolerant *Bacillus* spp. *S. aureus* was detected at all sampling sites. H2 had the highest number of *S. aureus* (7.4×10^1 CFU/ml) among the all sites, followed by H1 (5.1×10^1 CFU/ml). The low-impact sites L1 and L2 had markedly less numbers of *S. aureus* than high-impact sites, with 2.1×10^1 CFU/ml at L1 and 2.5×10^1 CFU/ml at L2 ($p < 0.05$). RW2 had 2.0×10^1 CFU/ml *S. aureus*, which was higher than the other two river water survey sites. GW, the inflow to the rimstone pools sampled in H1 and H2, had 1.7×10^1 CFU/ml. Thermo-tolerant *Bacillus* spp. were detected at all sites except GW. H2 had the highest abundance of thermo-tolerant *Bacillus* spp. among all sample sites at 1.9×10^1 CFU/ml. The low-impact sites L1 and L2 both had markedly lower values than the high-impact sites at 0.1×10^0 CFU/ml and 1.5×10^0 CFU/ml, respectively ($p < 0.05$). RW3, which was the most downstream site of the cave river, had the second-highest value at 1.8×10^1 CFU/ml, and H1 had the third-highest value at 8.1×10^0 CFU/ml. RW1, RW2, and FL all had 1.7×10^0 CFU/ml.

DISCUSSION

Tourism impact on water quality

In the present study, we conducted water quality comparisons between high-impact and low-impact sites, and detected higher levels of total nitrogen, ammonia, phosphate, total bacteria, total number of viable bacteria, *S. aureus* and thermo-tolerant *Bacillus* spp., with statistically meaningful differences can be explained by the development of tourism in the cave. Akiyoshi-do Cave has been open to tourism for over 100 years and experienced steady growth until the late 20th century. The annual number of tourists rapidly increased in the high economic growth period from 1955 to 1975, with the greatest number of visitors in 1975, when the cave recorded 1,979,446 visits. The increased number of tourists is considered to be a direct cause of organic matter inputs into the H1 and H2 rimstone pools. Because these sites are located adjacent to the tourist trail without a great elevation difference, giving tourists easy access to the pools. There was no imminent reason to construct a fence for tourist safety, because the elevation difference between the pools and the trail is below 40 cm, and the pools are shallow. Tourists can touch or step on those pools. Therefore, cave tourists could contaminate high-impact sites with organic matter and HAB, increasing the nutrients inclusive of nitrogen and phosphorus. Consequently, the pools' microbial structures could be altered. Installation of fences between the tourist trail and the rimstone pools would be effective for decreasing the inputs of organic materials and foreign microorganisms. Cave managers also introduce foreign materials by cleaning the trail. They clean the trail using cave water and detergents several times in a year as a common duty and drain the wastewater into the rimstone pools. Although the managers had been educated about the cave before starting their jobs, they are not biospeleologists and do not have regular study meetings to gain knowledge from professionals or the latest studies. Cave researchers should take the initiative in the management of the cave to protect the water quality and cave microorganisms better.

The dissolved oxygen content of Akiyoshi Cave water was examined in the 1930s. At that time, the oxygen level was 9.5 mg/L at the Kotogafuchi site, which is the upper stream of RW1, and 10.7 mg/L at the Chimachida Rimstone Pools (H1 and H2) (Uéno, 1933). The present study identified that RW1 had a dissolved oxygen level of 9.6 mg/L, which was close to the prior value. The Kotogafuchi site and RW1 are both located in

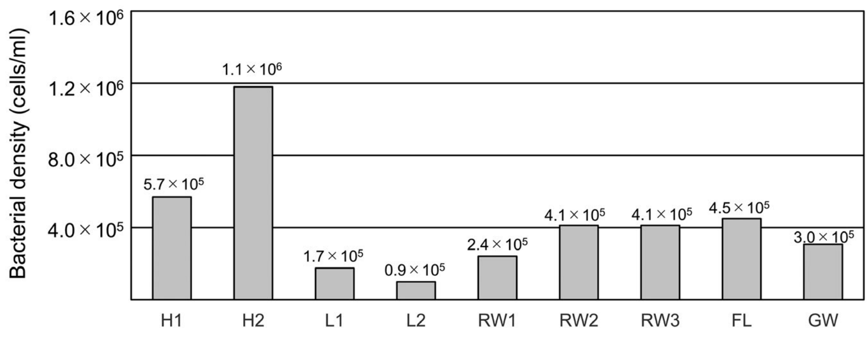

Figure 3. Summary of total number of bacteria (TB). Mean values of the results obtained from the two surveys performed in April and November 2017. A *t* test result showed a significant difference between high and low-impact sites, with a *p*-value <0.05.

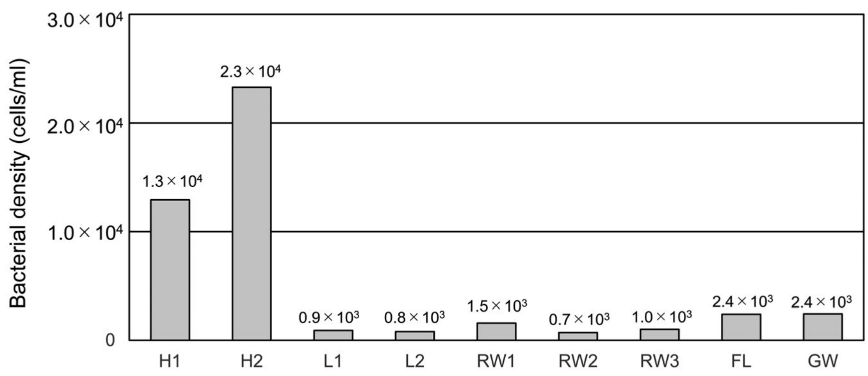

Figure 4. Summary of total number of viable bacteria (TVB). A *t* test suggested no significant difference between high and low-impact sites for TVB, as the *p*-value was not lower than 0.05.

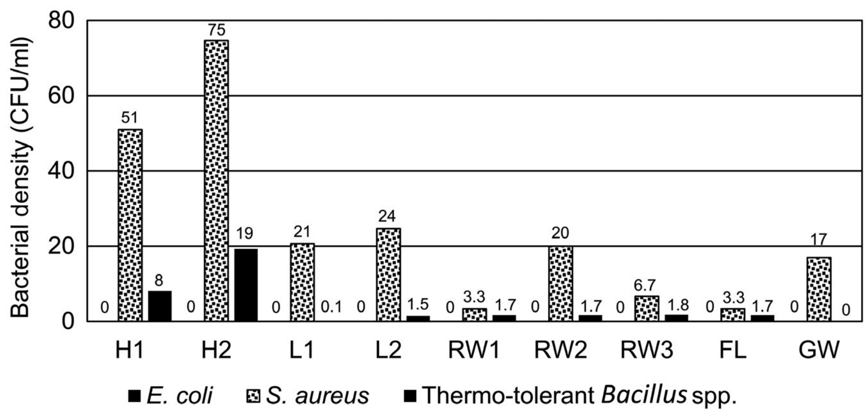

Figure 5. Summary of the number of human-associated bacteria. The values indicate average of 3 replicates.

the nontourist portion of the cave, so the dissolved oxygen did not change over time. However, for the sites in the tourist portion, H1 (7.4 ± 1.3 mg/L) and H2 (6.3 ± 1.0 mg/L) selected from the Chimachida Rimstone Pools had lower dissolved oxygen than the earlier study, which detected 10.7 mg/L (Uéno, 1933). Moreover, the dissolved oxygen of H1 and H2 was even lower than the FL (10.0 mg/L) and GW (9.9 mg/L), which are the inflow water into the Chimachida Rimstone Pools. FL and GW had similar oxygen levels to H1 and H2 in the 1930s, suggesting that inflow water quality has not been affected. Therefore, it is reasonable to presume that the changes in H1 and H2 are the direct result of cave tourist introduction of organisms and nutrients. Heterotrophic organisms are assumed to consume the oxygen in the high-impact pool and decrease dissolved oxygen.

The dissolved oxygen decreased from RW1 (9.6 mg/L) to RW2 (9.2 mg/L) as the water progressed downstream and increased to 10.0 mg/L at FL. Dissolved oxygen increases in environments where the water surface tension is broken, such as in waterfall and riffle areas. The river water flows over the rocks, which breaks the water's surface and increases oxygen levels. Consequently, FL water should have increased dissolved oxygen relative to RW1 and RW2. L2 had relatively low dissolved oxygen (7.5 ± 0.2 mg/L) but is defined as clean water, as it exceeds 7.0 mg/L, which satisfies the minimum value for aquatic life (Ministry of the Environment Government of Japan, 1971). However, when it is considered together with the chemical oxygen demand of L2 (11.2 mg/L), it can be presumed that reductive substances flowed in and bound to oxygen, decreasing dissolved oxygen. Further surveys are required to identify the reductive substances and their origins.

Bacteria as a function of human impact

Total bacteria, total viable bacteria, *S. aureus*, and thermo-tolerant *Bacillus* spp., total nitrogen, ammonia, and phosphate had greater values at high-impact sites than at low-impact sites with statistically meaningful differences. In the H1 and H2 pools, tourists touch or step in the water, as there is no restriction of entry into the pools. The environmental parameters suggest that tourists bring a variety of external materials such as skin oils, sweat, fibers, dust, lint, cells, and hair that could serve as nutrients to microbes (Ikner et al., 2006), accelerating eutrophication in tourist portions of the cave.

Bacterial parameters had sensitive responses to environmental contamination. H2 showed greater total bacteria, total number of viable bacteria and HAB values than H1, which could be caused by differences in water exchanging conditions and locations of the pools. The H1 pool directly receives river from a pump during business hours, and the inflow and the outflow of the pool are visible. In contrast, the H2 pool receives upwelling water from the cave wall nearby, but is apart from the upwelling spot, and does not have visible inflow and outflow. Therefore, water in the H2 pool is more stagnant than H1, which could partly explain increased HIB in H2. The elevation differences between the tourist trail and water surfaces of each site could also explain this difference. The elevation difference between the tourist trail and the H2 pool surface is approximately 15 cm, while the elevation difference between the tourist trail and the H1 pool surface is approximately 40 cm. The smaller elevation difference between the tourist trail and the H2 pool could increase tourist contact with this pool, therefore increasing contamination with HAB and nutrients.

Origin tracking of human-associated bacteria

Because either or both *S. aureus* or thermo-tolerant *Bacillus* spp. were detected at all sites, it is unlikely that the origin of HAB is limited to cave tourists. Low-impact sites had detectable HAB, although these sites are not open to tourists. However, these sites are accessible to a limited number of researchers and cavers, which could introduce minimal HAB. Bacterial contamination associated with human skin and hairs and belongings can occur unless they wear protective clothes. Contamination could also be introduced by the water from the surface which is impacted by human land use near the cave. Farms and tourist facilities were built after the development of Akiyoshi-do Cave. Changing surface land use impacts cave environments (Baker & Genty, 1998). A Shuho town-owned farm was present at the upper stream of the Akiyoshi-do Cave in the west from 1968 to 2014. An agricultural association-owned poultry farm has been present in the north since 1948. Both farms are located within the catchment area of the cave (Haikawa, 2006). The connection between the surface ground and the cave has been proven by prior researchers (Kawano & Fujii, 1985; Yoshimura & Inokura, 1992). Excrement from the farms is not treated, and likely flows into the cave, influencing water quality. Total nitrogen and total phosphorus may be increased due to contamination from this source since the 1940s, when the farms were opened. The farms may also be the source of HAB. However, no further studies have determined the type and amount of pollutants from the farms are introduced to the cave. The dye tracing method could be applied to answer this question in the future studies.

RW2 had a higher chemical oxygen demand value and the number of *S. aureus* than other river water sites, RW1 and RW3, could be due to inflow from the Yurei-daki upwelling spot located between RW1 and RW2. Yurei-daki usually provides water in a vertical opening just above RW2, with varying amounts according to precipitation. A sewer pipe is buried near Yurei-daki, which transports wastewater drained from hotels, restaurants, and other tourist facilities on the plateau. In the 1990s, the pipe ruptured, introducing wastewater via the Yurei-daki upwelling point. However, the pipe was repaired, such that wastewater contamination should not in theory be present. Lavoie and Northup (2005) reported that *S. aureus* decreased below the lower detection limit within 2–4 weeks in soil. However, relatively larger values of chemical oxygen demand and *S. aureus* in the RW2 site suggests that leakage may still affect this site. For example, if leaked wastewater was not removed completely which could continually provide nutrients to the microorganisms at this site. Although the broken pipe was repaired, soil purification to remove the pollutants has not been reported.

Need for monitoring

The indicators of water pollution in the present study indicated increased cave water eutrophication in the tourist portion of the Akiyoshi-do Cave. Only a few prior quantitative studies have investigated tourist impacts on cave water quality. (Nakamura & Kuramoto, 1978) suggested tourism impacts on cave water based on the observation results of the population density of amphipods in the Chimachida Rimstone Pools, where the high-impact sites of the present study were selected from. Tourism-induced water pollution of the rimstone pools was suggested in the 1970s, when a visible increase of wastewater residents such as *Tubifex tubifex* was observed (Nakamura & Kuramoto, 1978). The rimstone pools were originally inhabited by *P. akatsukai* and *G. nipponensis*, both stygobionts. Supplementation of pumped-in river water taken below the cave river surface began in 1971 in an attempt to purify the rimstone pools, which was successful in decreasing population densities of *T. tubifex* and troglophiles. This pump is still in operation today, and presumably the population density or habitat range of the amphipods is still affected, but the present status is unknown, as these populations have not been monitored. As the present study suggests, the Chimachida Rimstone Pools are subject to pollution with foreign materials introduced by tourists, as these pools are adjacent to the tourist trail. Previous studies described the Akiyoshi-do Cave's unique ecological system, but its microbial system, particularly in the cave's waters, has not been thoroughly evaluated. However, a prior microbial community analysis of the cave soil revealed the possibility of the existence of cave-specific species (Hori et al., 2008). Environmental monitoring such as carbon dioxide concentration in the air, water temperature, pH, dissolved oxygen, nutrients, and microbes should be monitored. Monitoring results will identify tourism impacts and allow preservation of the cave animals' populations and species diversity by developing appropriate conservational efforts based on monitoring data.

Conclusion

In summary, alterations in total nitrogen, ammonia, phosphate, total bacteria, total number of viable bacteria, *S. aureus*, and thermo-tolerant *Bacillus* spp. were identified in areas with greater visitation frequency in the Akiyoshi-do Cave. Cave tourists are potential influencers to alterations of the water quality of pools distributed in the tourist area since tourists have direct contact with pools via touch or step on the pools. It was assumed that pools in the high-impact areas' chemical and microbial status would have been similar to the low-impact sites where the tourism is absent. The development of tourism leading to the increase of annual visits could have increased foreign organic matter and microorganisms' inputs to the water, resulting in a greater number of HAB. However, human impacts on the cave could not be limited to cave tourists, as HAB were detected in low-impact sites restricted from tourism even though the detected number was significantly lower than high-impact sites. Excrement contamination from farms on the surface and residual wastewater from a previously broken sewer pipe could also influence the cave environment. These factors need to be assessed in detail in future studies. Among high-impact sites, we detected differences based on the location of the site relative to the tourist trail and water flow conditions between sites. However, the cave waters' microbial communities and the influence of tourist activities on these communities remain incompletely understood. Continued monitoring of the water quality in tourism and nontourism areas, as well as microbial community analysis to confirm bacterial diversity, may allow more definitive assertions regarding tourism impacts on microbial communities of the Akiyoshi-do Cave.

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