RESEARCH ARTICLE

Fungal Load of Groundwater Systems in Geographically Segregated Islands: A Step Forward in Fungal Control

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ABSTRACT

The fungal distribution, diversity, and load were analyzed in the geographically segregated island groundwater systems in Korea. A total of 79 fungal isolates were secured from seven islands and identified based on the internal transcribed spacer (ITS) sequences. They belonged to three phyla (Ascomycota, Basidiomycota, and Chlorophyta), five classes, sixteen orders, twenty-two families, and thirty-one genera. The dominant phylum was Ascomycota (91.1%), with most fungi belonging to the *Cladosporium* (21.5%), *Aspergillus* (15.2%), and *Stachybotrys* (8.9%) genera. *Cladosporium* showed higher dominance and diversity, being widely distributed throughout the geographically segregated groundwater systems. Based on the diversity indices, the genera richness (4.821) and diversity (2.550) were the highest in the groundwater system of the largest scale. As turbidity (0.064–0.462) increased, the overall fungal count increased and the residual chlorine (0.089–0.308) had low relevance compared with the total count and fungal diversity. *Cladosporium* showed normal mycelial growth in de-chlorinated sterilized samples. Overall, if turbidity increases under higher fungal diversity, bio-deterioration in groundwater-supplying facilities and public health problems could be intensified, regardless of chlorine treatment. In addition to fungal indicators and analyzing methods, physical hydrostatic treatment is necessary for monitoring and controlling fungal contamination.

1. Introduction

The use of natural water systems as drinking water sources requires systematic treatment to ensure water or food safety. Therefore, in various countries, water treatment and its internal and statistical quality control standards are designed and ruled by specific laws and regulations [1]. In the Republic of Korea, drinking water supply systems (DWSSs) are largely divided into three categories depending on the type of water source, i.e. 1) urban tap water purification and supply system, which uses rivers or lakes as raw water sources; 2) small water supply facilities, which use underground or spring water along the coastal region; and 3) reverse osmosis facilities, which treat deep marine or seawater or water derived from other sources.

Drinking water is essential for human survival, and systematic water purification and quality control are directly related to public health concerns. Owing to the diversification and complexity of the food supply chain [2–4], the microbial load of the drinking water used in manufacturing has had a greater impact on the microbiological safety of the final product. In particular, the introduction of fungi into human settlement environments results in the biodegradation of the diverse facilities and living environment of the final consumer [5–10]. It might also cause the bio-deterioration of water supply facilities. Furthermore, some fungal taxa have a high epidemiological correlation with damp building-related illnesses [11–13] and even cause respiratory allergies and asthma [14–16]. Thus, the importance of fungal management in drinking water is gradually increasing from a health perspective, and from other perspectives, such as bio-deterioration prevention.

Traditionally, food safety and water management have focused on monitoring and removing certain indicator bacteria. However, spore-forming fungi have a higher environmental resistance than bacteria [17,18], and therefore require additional treatment. However, very few studies on the systematic distribution, diversity, and load of fungi in large-scale public groundwater systems have been made. In addition, the mechanism by which fungi that are

naturally distributed in the groundwater system affect the human body and settlements, and what risks they pose in terms of food safety, have rarely been evaluated [19]. Therefore, this interdisciplinary study assessed a fungal cluster in islandic groundwater systems, among various water resources. The fungal state identified in the groundwater system might reflect the geological characteristics and ecological environment of the water resources.

First, water quality indicators (total fungal and bacterial count, residual chlorine level, and turbidity values) that affect the fungal and bacterial status of groundwater systems were analyzed. Second, to reveal the distribution of taxa and their ecological relationship with the groundwater characteristics, seven groundwater systems on large geographically segregated islands were evaluated using culture-dependent methods. Third, fungal diversity, richness, evenness, and dominance were also analyzed. The comprehensive state of groundwater fungi and water quality indicators (residual chlorine concentration, turbidity, and bacterial load) were analyzed to examine the outcomes of groundwater pollution due to management failure. Moreover, countermeasures for fungal control are suggested.

2. Materials and methods

2.1. Site description and geo-ecological segregation

Several islands are distributed on the coast of the Incheon metropolitan city, located on the west coast of the Korean Peninsula. Among them, the seven islands with the highest population were selected for this study (Tables 1 and 2). Sampling was performed at 236 points (public wells) distributed over the seven islands. Each island area (i.e. groundwater system) is located in the ocean and is geographically segregated from the mainland and from other islands (distance from the coast: Boreum–Seokmo islands, 7.5 km; Boreum–Gyodong islands, 8.2 km; Gyodong–Seokmo–Ganghwa islands, 2 km; Ganghwa–Buk islands, 5.8 km; and Buk–Jawol islands, 30.5 km) (Figure 1). Total groundwater supply systems (total facility capacity and the number of water supply tube wells) and the geographical scale (and extent area) of each island were evaluated (Table 1).

2.2. Sampling and pretreatment for microbial analysis

To measure the microbial (fungal and bacterial) load of the sub-surface drinking water and to isolate pure fungal colonies, the interior and exterior of the sampling target faucet were sufficiently sterilized using 75.0% ethanol and sterile gauze. The water was then discharged at full force for >5 min to remove all the substances from inside the drinking water faucet. To attain better accuracy, sampling was performed after discharging for a certain period to remove the stagnant water from inside the water pipe.

Sterile sampling bottles (2 L, World Medi Co., Ansan, Korea) were washed twice/thrice with the samples to be collected; then, the samples were collected in a sterile manner and stored at 4 °C in an ISO17025 lab facility for 4 h. After disinfecting the

Table 1. Groundwater system description on each island.

Groundwater system	Administrative district including the sampling area	Total facility capacity (m³/day)[a]	Number of water supply tube wells	Extent area of islands (km²)
Ganghwa island	Ganghwa-gun, Incheon, KR	7,681	129	302.6
Gyodong island	Gyodong-myun, Ganghwa-gun, Incheon, KR	1,210	33	46.89
Seokmo island	Samsan-myun, Ganghwa-gun, Incheon, KR	1,715	31	42.84
Boreum island	Seodo-myun, Ganghwa-gun, Incheon, KR	653	14	6.36
Buk island	Bukdo-myun, Ongjin-gun, Incheon, KR	200	11	17.64
Jawol island	Jawol-myun, Ongjin-gun, Incheon, KR	1,980	8	16.12
Daechung island	Daechung-ri, Daechung-myun, Ongjin-gun, Incheon, KR	1,150	10	12.63

[a]The maximum capacity of a water supply facility is to supply water for a day. Secondary processing of the data was obtained from the National Water Information System of the Korea Republic (https://www.waternow.go.kr/web/, accessed December 21, 2021).

Table 2. Water hygiene indicators and distribution of the fungal taxa in the groundwater systems of each island.

Groundwater system	Turbidity	Residual chlorine	Heterotrophic bacterial count	Total fungal count (average)	Total isolate number	Fungi				
						Phylum	Class	Order	Family	Genus
Ganghwa island	0.176	0.244	7	3,361 (26.25)	34	3	5	11	15	18
Gyodong island	0.075	0.308	0	72 (2.18)	2	1	2	2	2	2
Seokmo island	0.228	0.296	9	243 (7.83)	6	2	3	5	5	5
Boreum island	0.064	0.15	0	31 (2.21)	2	1	1	1	1	1
Buk island	0.139	0.18	0	28 (2.54)	17	1	3	6	8	8
Jawol island	0.154	0.089	0	66 (8.25)	4	1	3	3	3	3
Daechung island	0.462	0.18	0	168 (17)	14	2	4	6	7	8

Turbidity, residual chlorine, and total count of heterotrophic bacteria (average value per island = summary of value/number of water supply tube wells) results.

Figure 1. Images of the studied islands and total isolate numbers.
*The images cannot be enlarged because of geopolitical reasons (ceasefire state of the Korean Peninsula).

inlet of the sampling bottle with 75.0% ethanol, 500 mL of each sample was collected and filtered using a sterilized filter paper with a pore diameter of $0.45 \pm 0.02\,\mu$m (Advantec, Taipei, Taiwan). The filter paper was cut into small pieces using sterile scissors and immersed in tubes (SPL Life Sciences, Pocheon, Korea) containing 10 mL sterile phosphate-buffered saline (PBS). After vigorous shaking, the filter papers were discarded and centrifugation (Union 5KR, Hanil Co. Ltd., Daejeon, Korea) was performed at 4 °C and 8,000 rpm for 10 min. The supernatant was discarded, the pellets were diluted in 100 µL sterile PBS and inoculated onto sterilized bacterial (plate count agar [PCA]) and fungal (potato dextrose agar [PDA]) media.

2.3. Fungal and heterotrophic bacterial count

The heterotrophic bacterial count was analyzed based on the United States Environmental Protection Agency (EPA) method ES05702.1b and the Korean drinking water quality process test standard [20,21]. Pretreated samples (section 2.2) were serially diluted, and 100 µL of each sample was inoculated onto PCA (5.0 g of tryptone, 2.5 g of yeast extract, 1.0 g of glucose, and 15.0 g of agar per 1,000 mL of de-ionized water) and incubated at 35 °C ± 0.5 °C for 48 ± 2 h. The test was performed five times for each dilution rate, and culture plates with 30–300 colonies were selected to determine the fungal load (total fungal count). For fungal load (total fungal count), the sample was treated similarly as mentioned above: 100 µL of the

serially diluted specimen was inoculated onto sterilized PDA (pH 5.6; 100.0 g potato infusion, 10.0 g dextrose, and 10.0 g agar powder in 1 L deionized water), and then incubated at 18 °C and 25 °C, sequentially, for 30 days.

2.4. Isolation and pure culture of fungi

For the pure isolation of fungal colonies from each groundwater sample, pretreated samples (section 2.1) were inoculated onto PDA and cultured at 18 °C and 25 °C for 30 days. All fungal colonies were inoculated and incubated in 50% diluted PDA again, and then, the fungal isolates were finally isolated purely based on morphological characteristics. Glycerol stocks (15.0% sterilized glycerol) of the total fungal isolates or their spores were stored at −65 °C.

2.5. Genomic DNA extraction and polymerase chain reaction

All the 79 fungi isolates were inoculated onto potato dextrose broth (Difco, Detroit, MI, USA) and incubated at 25 °C for 7 days with shaking at 80 rpm using a rotary shaker (Lab companion SK-71, Jeiotec Co. Ltd., Korea). The filtered mycobionts were lyophilized for 2 days. The DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used to extract the genomic DNA from the lyophilized mycobionts; and primers targeting the universal sequences (internal transcribed spacer [ITS] 1, partial

sequence; 5.8S ribosomal RNA and ITS2, complete sequence; and 28S ribosomal RNA, partial sequence); ITS1 (5′–TCC GTA GGT GAA CCT GCG G–3′) and ITS4 (5′–TCC GCT TAT TGATAT GC–3′) were used for amplification [22]. The following polymerase chain reaction (PCR) amplification conditions were used: pre-denaturation (94 °C, 4 min), denaturation (94 °C, 1 min), annealing (55–58 °C, 1 min), and extension (72 °C, 2 min) for a total of 35 cycles, followed by a final extension (72 °C, 2 min) [22]. PCR products were confirmed using gel electrophoresis (1.5% agarose gel, stained with ethidium bromide), and the resulting band pattern was observed using a UV trans illuminator. An AccuPrep PCR and gel extraction kit (Bioneer, Daejeon, Korea) were used to purify the PCR products, and an ABI 3730XL DNA analyzer (Applied Biosystems, Waltham, MA, USA) was used for ITS sequencing.

2.6. Partial identification of the fungal isolates

A total of 79 fungal isolates were partially identified based on signature sequence similarity using the data collected from GenBank, NCBI. The sequences of ITS1 and 28S (partial sequences) and 5.8S and ITS2 (complete sequences) obtained from the fungal isolates were compared with the data deposited in GenBank using the BLASTn tool to determine their taxonomical identity. Furthermore, evolutionary analyses were performed using the molecular evolutionary genetics analysis version X (64-bit for Windows) [23,24] with sequence alignments prepared using Clustal W version 2.0.10 [25,26], for accurate partial identification. The evolutionary history was inferred using the minimum evolution method to assess the evolutionary relationships between taxa [27], in which the 'associated' taxa were those clustered together in a certain percentage of the replicate trees in the bootstrap test (1,000 replicates) [28]. Further, evolutionary distances were computed using the Tajima–Nei method [29] and expressed according to the number of base substitutions per site. The minimum evolution tree was explored using the close-neighbor-interchange algorithm [30] at a search level of 1. The neighbor-joining algorithm [31] was used to generate the initial phylogenetic tree. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The 79 fungal signature sequences obtained in this study were deposited in GenBank with assigned accession numbers (Table S1–S7).

2.7. Biodiversity assessment

The diversity indices of the fungal clusters were analyzed and compared between groundwater systems [32–34]. The diversity at the genus level was determined using the (1) Margalef richness [33,34], (2) the Menhinick [34,35], (3) the Shannon diversity (H′) [36], and (4) the Simpson's indexes (D) [34,37].

2.8. Residual chlorine and turbidity

Residual chlorine was measured using the method specified in the Standard Methods 4500-ClG: DPD Colorimetric Method [21,38] and in compliance with the field-testing principle [39]. For this purpose, a chlorine-free DPD chlorine reagent (Hach, Colorado, USA) was used [40]. For an accurate sampling, measurements were performed after groundwater discharge for a certain period to remove the stagnant water from inside the water pipe. Residual chlorine was repeatedly measured until the same value was obtained. For measuring the residual chlorine of the negative control (blank specimen), the glass cell used for measurement was repeatedly washed twice/thrice using the water sample, filled up, and then the measurement was performed using a residual chlorine meter (HACH, DR300-Cl$_2$) at 490–530 nm (light length: ≥ 1 cm).

Water turbidity was measured [21] using the nephelometric turbidity unit (NTU) method [41]. Samples collected at the site were stored at 4 °C and moved to an experimental facility within 4 h. Measurements were performed five times to obtain the median. Calibration was performed using standard solutions of 0.1, 20, 100, 1,000, and 4,000 NTU, to confirm linearity. The sample in the vial was sufficiently stabilized, for at least 1 min, to eliminate the air bubbles, and the measurements were then repeated.

3. Results and discussion

3.1. Taxa of groundwater-borne fungi

The methods used for analyzing the fungal clusters in a specific environment are classified as culture-dependent or -independent methods (e.g. next-generation sequencing (NGS)) [42]. In this study, a culture-dependent method was used. This is because, in environments where high-density nutrients or microorganisms exist, such as the gut of a host or soil terrain, the genetic elements rapidly degrade through enzymatic or oxidative reactions. Therefore, environmental-DNA (e-DNA) data are likely to reflect the present status of microbial clusters well. However, when subjected to natural water purification treatments, such as that used for groundwater, the concentration of microorganisms and their proliferation sources (organic/inorganic nutrients) are significantly scarce, and the factors

affecting the stability of nucleic acids (e.g. nucleases) are expected to be few. Therefore, the criteria for determining whether the DNA detected in the purified water of such groundwater is remnant DNA from the past or DNA that has been opportunistically introduced from other regions are ambiguous. In addition, as very few studies have focused on the culture-independent fungal cluster analysis of tap water, the strategy of first using a culture-based method and then employing NGS analysis—if necessary—appears to be appropriate.

In this study, 79 morphologically different fungal isolates were purely isolated (Table S1–S7); these belonged to 3 phyla (Ascomycota, Basidiomycota, and Chlorophyta), 5 classes, 16 orders, 22 families, and 31 genera (Figure 2). Ascomycota was the dominant (91.1%) phylum; Dothideomycetes (36.7%) and Sordariomycetes (32.9%), which belong to the Ascomycota phylum, were the dominant classes (Figure 2A). *Cladosporium* (21.5%), *Aspergillus* (15.3%), and *Stachybotrys* (8.9%) were the dominant genera (Figures 2 and 3).

3.2. Turbidity, residual chlorine, fungal and bacterial counts

In drinking water purification systems, residual chlorine refers to chlorine that is intentionally added for purifying the water before it reaches the final consumer (faucets) while maintaining bacteriostasis. Some vegetative forms of bacterial cells in supplied tap water may be successively controlled using residual chlorine [43]. Effective chlorine can be classified as free chlorine and residual chlorine [43]. Hydrochloric acid and its ions effectively maintain the chlorine concentration until the final consumer (faucets) [43]. Therefore, when supplied to the final consumer, the level of residual chlorine in water must be ≥ 0.1 mg/L (ppm) according to the law in the Republic of Korea [44]. The range of residual chlorine found in this study satisfies this level (Table 2); however, the algebraic proportional relationship between the distribution of residual chlorine and the count of fungi could not be confirmed in this study.
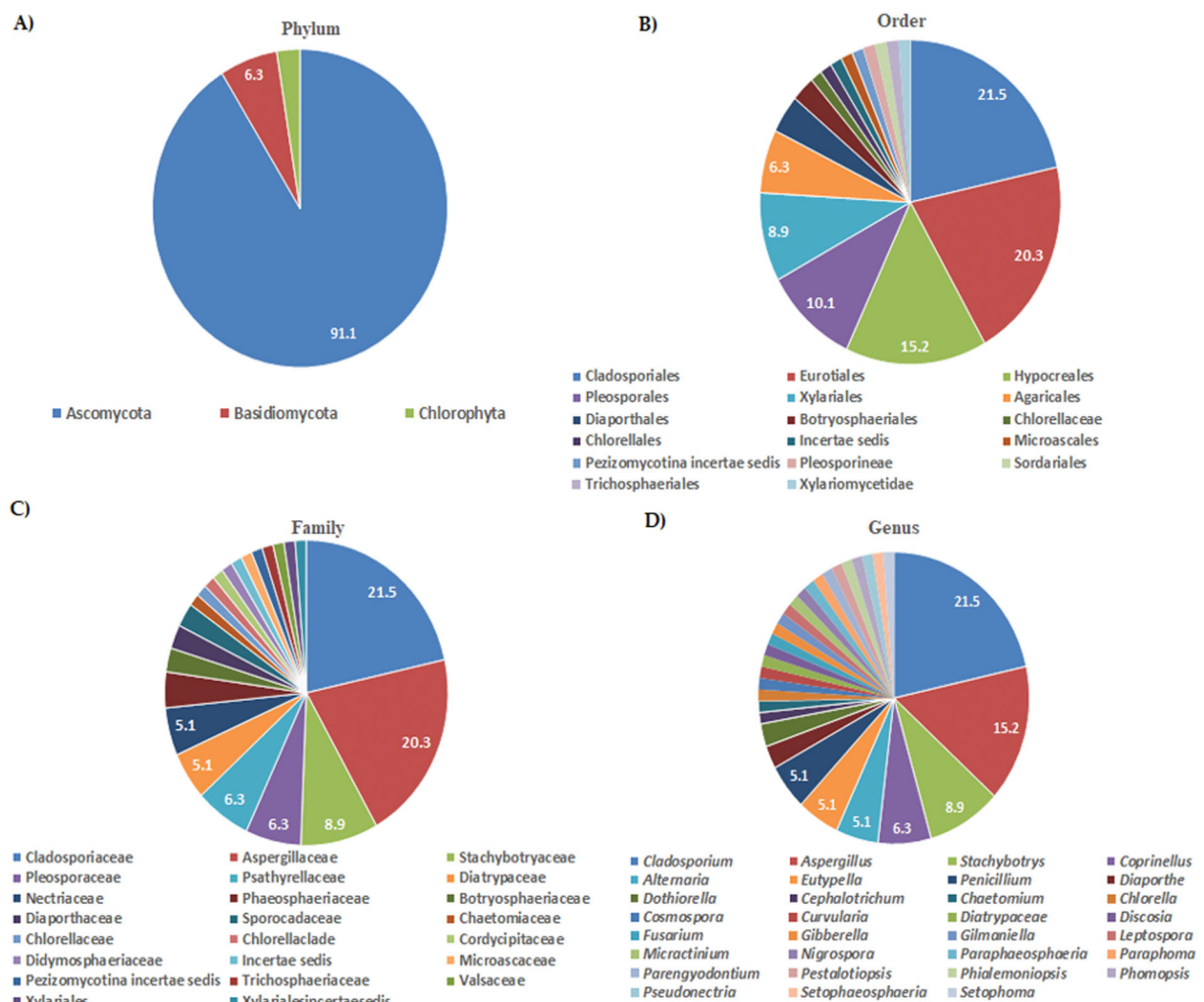
Figure 2. Fungal taxon ratio of the total groundwater-borne fungal isolates (unit: %).
Incertae sedis (Latin for 'of uncertain placement') is a term used for a taxonomic group whose broader relationships are unknown or undefined. Alternatively, such groups are frequently referred to as enigmatic taxa. For example, based on the '2021 work on the Outline of Fungi and fungus-like taxa [71]', there are 1,148 incertae sedis species belonging to the Ascomycota genus.

Figure 3. Richness, diversity, unevenness (A), and dominance ratio (B, unit: %) of groundwater-borne fungi.

As the turbidity of the water increases, the total fungal load increases. The water quality index can be evaluated based on th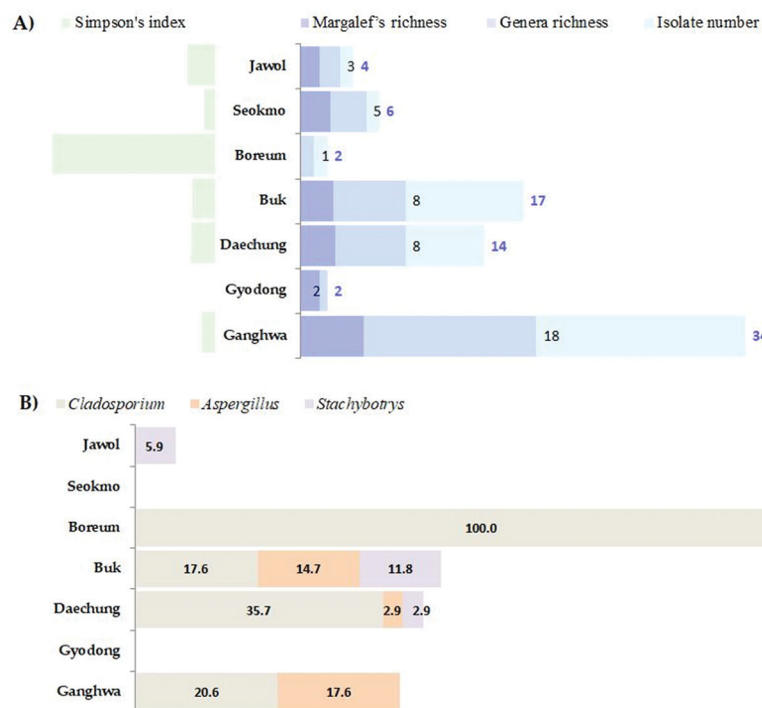e turbidity and the total heterotrophic bacterial count. As per article 22 of the enforcement regulation of the waterworks act of Korea, the turbidity value should be below 0.50 NTU and the bacterial count should be below 100 CFU/mL, when incubated in a PCA medium for 48 h [20]. In the present study, this total bacterial count criterium was met; however, not all samples met the turbidity criterion (Tables 2 and 3). Thus, along with monitoring the heterotrophic bacteria counts using the traditional PCA medium, additional analytical methods or intervention plans for monitoring and controlling fungi are needed. In addition, distinguishing bacteria and fungi using only the existing PCA medium is difficult; therefore, supplementary methods are needed to obtain more accurate heterotrophic bacterial counts. In this study, the total counts obtained using PCA and PDA medium showed a quantitative gap, suggesting the necessity of establishing standards for quantifying fungi using methods other than the conventional PCA method.

3.3. Distribution and dominance of fungi

In general, as the extent of the island area increased, the number of tube wells and the total facility capacity increased in proportion to each other (Table 1). Moreover, as the number of tube wells and total facility capacity increased, the fungi morphological diversity increased (Figure 2). The most widely distributed genus was *Cladosporium* (Figure 2D). This genus had the highest morphological diversity (Table 4) and dominance (Figure 3B) in most of the islands with a large total capacity of groundwater systems (Table 1) and large populations.

In general, *Cladosporium* spp. are highly cold-resistant microorganisms and can grow at $-10\,°C$ and $-3\,°C$ ($14\,°F$ and $27\,°F$) [45]; the physical environmental characteristics of groundwater systems with a low-temperature distribution may act as a major selective pressure for fungal clusters. In particular, *C. cladosporioides* grows well on wet building materials, paint, wallpaper, and textiles [15,46], as well as on paper, pulp, frescos, tiles, wet windowsills, and other indoor substrates, including salty and sugary foods according to previous studies [5–9,47]. Furthermore, diverse fungal isolates belonging to the *Cladosporium* genus (Table S1–S7) reported previously related to focal infection in humans (*C. anthropophilum*, *C. cladosporioides*, *C. colombiae*, *C. crousii*, *C. exasperatum*, *C. pseudocladosporioides*, *C. ramotenellum*, *C. tenuissimum*) [48]. Therefore, uncontrolled fungal contamination in DWSSs may lead to the deterioration and degradation (bio-corrosion) of the residential environment or pose potential public health problems. Bio-deterioration assays should be performed, and groundwater treatment or intervention technologies should be established.

Stachybotrys spp. were dominantly identified in the groundwater systems of three islands (Table 4), and all of them showed similarities to the species *S. chartarum* deposited in GenBank (Table S1–S7).

Table 3. Ranges of turbidity, residual chlorine, and fungal count in each groundwater system.

Drainage system	Value	Turbidity	Residual chlorine	Fungal load
Ganghwa	Ave.	0.176	0.244	26.3
	Max.	3.420	0.640	221.0
	Min.	0.058	0.120	0.0
	Total	–	–	3361.0
Gyodong	Ave.	0.075	0.308	3.0
	Max.	0.218	0.670	9.0
	Min.	0.060	0.120	0.0
	Total	–	–	72.0
Daechung	Ave.	0.462	0.182	16.8
	Max.	1.930	0.210	51.0
	Min.	0.063	0.160	0.0
	Total			168.0
Buk	Ave.	0.139	0.180	2.5
	Max.	0.600	0.210	9.0
	Min.	0.070	0.150	0.0
	Total			28.0
Boreum	Ave.	0.064	0.150	18.0
	Max.	–	–	–
	Min.	–	–	–
	Total	–	–	31.0
Seokmo	Ave.	0.228	0.296	9.3
	Max.	0.843	0.410	52.0
	Min.	0.062	0.120	0.0
	Total	–	–	243.0
Jawol	Ave.	0.129	0.179	8.3
	Max.	0.432	0.210	40.0
	Min.	0.064	0.150	1.0
	Total	–	–	66.0

Total: sum of total fungal count; Ave: average; Max: maximum; Min: minimum.

S. chartarum has a high epidemiological correlation with damp building-related illnesses according to previous studies [12,13,49,50]: *S. chartarum is* reported as causing inflammation and leads to upper and lower respiratory and neurological infections, and exhibit cytotoxic effects [51,52]. In certain epidemiological case studies, individuals frequenting school facilities that were exposed to water contaminated with this species developed immune response-related diseases. Some of them contracted the disease and a few died from pulmonary hemosiderosis, following exposure to unusually high levels of *S. chartarum* spores [10,53]. The present study reports a high prevalence of the *Stachybotrys* genus (Table 4, Figure 2D), implying that the abundance of this species cannot be efficiently controlled through residual chlorine disinfection of groundwater. In addition, increased water turbidity due to the lack of safety intervention or failure to ensure a proper water treatment is expected to lead to the proliferation of *Stachybotrys* in groundwater systems.

Generally, several species belonging to the *Aspergillus* genus have been reported as marine fungi [54–59]. This might indicate that the underground fungal community in the marine island area is affected by the surrounding marine terrains. Meanwhile, the existence of fungal isolates with higher similarity with the building material biodegradative *A. hiratsukae* (*Neosartorya hiratsukae*) [60–62] should be considered in subsequent studies. Ocean island areas exposed to marine environments

inevitably use groundwater or spring water as a major natural source of drinking water. Therefore, a strategy to keep these fungi under control is needed. *Cladosporium*, *Aspergillus*, or *Stachybotrys* spp. may be used as indicator strains or targets for future water hygiene control.

3.4. Fungal diversity and possible risks

In the Korean Peninsula, hygiene problems related to the use of groundwater as a drinking water source are becoming a concern due to several reasons. First, the implementation of a systematic water purification treatment is challenging because of the difficulty in managing the groundwater pipes. Second, the consumption of groundwater has a comprehensive effect on food safety, water hygiene, and human health. Therefore, if groundwater fungal contamination, which is reflected in higher fungal diversity and richness, is not properly controlled, it may lead to the explosive proliferation of specific fungal taxa.

If unlike the results of this study show, the abundance and diversity of fungi are very low, there is a low possibility that a certain organic/inorganic contamination level would induce the proliferation of certain groups of fungi. For the quantitative evaluation of the biodiversity at the genus level, the Margalef and Menhinick (richness of taxa), Simpson (evenness), and Shannon diversity indices were used. The richness refers to the number of species found in specific environmental conditions [33,35]. The evenness refers to the proportional equivalence of an emerging genus (or species) [34]; the Simpson index refers to the dominance of a particular genus (or species) [34]. Therefore, theoretically, the Simpson index decreases as the evenness of the constituent taxon increase. The stability of fungal clusters based on their diversity could be assessed using the Shannon index by comprehensively considering the richness and evenness indices [36]. Biological quantitative diversity evaluation based on these statistical values could be interpreted in various ways, depending on the situation. In this study, the bigger the size of the island (which is proportional to the number of residents and the size of the islandic DWSSs), the higher the morphological diversity of fungi and the genus richness values. In the case of Ganghwa Island, more than 75,000 residents and 40,000 floating populations were demographically distributed, showing the highest Margalef richness (4.821) and Menhinick (3.087) indices, with a relatively low (0.080) Simpson index, which indicates unevenness due to the dominance of specific genera (Table 5, Figure 3). Therefore, the groundwater

Table 4. Fungal genus distribution in the groundwater systems of each island.

Genera	Ganghwa	Gyodong	Daechung	Buk	Boreum	Seokmo	Jawol	Total
Alternaria	1		1	1			1	4
Aspergillus	6		1	5				12
Cephalotrichum						1		1
Chaetomium	1							1
Chlorella			1					1
Cladosporium	7		5	3	2			17
Coprinellus	3					2		5
Cosmospora	1							1
Curvularia	1							1
Diaporthe	1					1		2
Diatrypaceae insentia sedis	1							1
Discosia				1				1
Dothiorella		1		1				2
Eutypella	4							4
Fusarium		1						1
Gibberella	1							1
Gilmaniella							1	1
Leptospora	1							1
Micractinium	1							1
Nigrospora						1		1
Paraphaeosphaeria						1		1
Paraphoma	1							1
Parengyodontium	1							1
Penicillium	1		3					4
Pestalotiopsis	1							1
Phialemoniopsis				1				1
Phomopsis	1							1
Pseudonectria			1					1
Setophaeosphaeria				1				1
Setophoma			1					1
Stachybotrys			1	4			2	7

Table 5. Diversity, richness, and evenness of fungal clusters in each groundwater system.

Diversity	Ganghwa	Gyodong	Daechung	Buk	Boreum	Seokmo	Jawol	Total
Total number of isolates	34	2	14	17	2	6	4	79
Genus richness (S)	18	2	8	8	1	5	3	7
Margalef richness (Dmg)	4.821	1.443	2.652	2.471	0.000	2.232	1.443	1.373
Menhinick index (Dmn)	3.087	1.414	2.138	1.940	0.707	2.041	1.500	0.788
Shannon diversity index (H′)	2.550	0.693	1.829	1.840	0.000	1.561	1.040	2.862
Simpson index (D)	0.080	0.000	0.143	0.140	1.000	0.067	0.167	0.082

system on Ganghwa Island exhibited relatively stable fungal clusters.

In most island areas, the fungal richness and Simpson index increased with an increasing morphological diversity (Figure 3A), and the Simpson index was inversed to genus abundance (Figure 3A). In addition, the Shannon index was higher on islands with high species richness, and the Simpson index was close to zero (Table 5). An increased richness of fungi on larger and more populous islands may lead to selective fungal proliferation when loopholes occur in hygiene management, possibly leading to food safety problems and deteriorated water supply facilities in industries. In addition, as the average turbidity of the water system increases, the total fungal count increases, supporting this situation.

It is necessary to evaluate whether the fungal isolates secured in this study are only introduced opportunistically from the surrounding environment or can proliferate in the groundwater system. This can be confirmed by evaluating whether they proliferate without requiring any additional carbon or nitrogen sources in the actual sub-surface water sample. For this, 980 mL of a groundwater sample with high turbidity (turbidity, 0.316 NTU; nitrous nitrogen, 0.413 mg/L; undetected heterotrophic bacteria, total coliforms, *Escherichia coli*; ammonia nitrogen, 0.00 mg/L; and chromaticity, 0.0) was homogenized *via* sonication. Pure agar powder (15.0 g) was sterilized and added. The isolates belonging to *Cladosporium*, *Aspergillus*, and *Starchybotrys* were inoculated and incubated for 30 days (Section 2.2). Normal hyphal growth or pigment formation was observed. These results indicate the possibility of fungal deterioration (e.g. pigment formation) or proliferation under failed management conditions. In addition, the dominant species in this study were widely (commonly) detected in geographically segregated islands; this also might support the expected outcome, as these dominant species may be optimized for the groundwater conditions, and not just opportunistic inflow pathogens.

3.5. Direction of hygienic intervention

As the total fungal counts increase due to increased turbidity, the possibility of the bio-deterioration of water supply facilities, along with considerable hygiene problems, could increase, if hygiene management fails in the groundwater purification process under higher fungal diversity states. Therefore, additional intervention or control measures are needed when using water from groundwater treatment and supply systems. The existing processes used for controlling the microorganism counts in public water purification systems can be classified as chemical or physical methods. Based on previous studies, it cannot be asserted that chemical treatment is the best or sole treatment method [63–67].

Chlorine, which is conventionally used for the treatment of water systems, reacts characteristically with protein molecules, among various polymeric materials, leading to bacterial death and helping maintain the bacteriostatic effect until the consumer supply point (faucet of tap water). Chlorine treatment is normally used in the food manufacturing industry and in the drinking water purification process. In terms of food hygiene, the water remaining in the final food product or that used for processing or manufacturing is disinfected with residual chlorine in the 2 to 7 ppm (mg/L) range. A concentration of more than 200 ppm should be used for food or water processing environmental disinfection [68].

Meanwhile, in the case of the drinking water purification process, a concentration of 0.1 ppm or more should be maintained until the consumer supply point (faucet). In previous studies, it was reported that the chlorination effect varies depending on the state of fungal spores at an active chlorine concentration of 0.1–0.2% (1–2 ppm) [69]. Therefore, it is difficult to completely remove the fungal spores based on the usual residual chlorine levels found (legal criterion in the Republic of Korea: 0.1 ppm or more [1]), as was also verified in this study. However, if the chlorination level was increased, several disinfection by-products (trihalomethane, chloral-hydrate, dibromoacetonitrile, dichloroacetonitrile, trichloroacetonitrile, 1.2-dibromo-3-chloropropane, and haloacetic acid), whose presence can be attributed to an elevated density of chlorines, would threaten the safety of drinking water [70] or food products made from tap water. Therefore, physical methods are preferred for the successful control of fungi.

The same conclusion can be drawn when comparing the groundwater treatment process with the existing urban water treatment and supplying system. Considering that the general urban water purification process uses chlorine at the beginning of the water supply process, immediately after the tap water purification process, a certain bacteriostatic state is maintained due to the residual chlorine until the water reaches the consumer supply point (faucet). However, as groundwater is treated with chlorine when it reaches the consumers (faucet) without going through a separate sophisticated organic and inorganic purification process, it is questionable whether the residual chlorine may control the levels of the fungi that are already present in the groundwater system. Therefore, establishing a physical sterilization process that can remove fungi (or spores) in the final step of the groundwater supply process during the water purification treatment may be optimal.

4. Conclusion

We revealed that the total fungal load increases with increasing groundwater turbidity, with no clear relationship with the level of residual chlorine. Therefore, in a situation in which the diversity and richness of the fungal taxa constituting the fungal cluster are high, hygiene management failure or an inadequate purification process of the groundwater system on islands may lead to a selective and explosive increase in the total fungal load. In particular, *Cladosporium*, *Aspergillus*, and *Starchybotrys*, which have the potential to bio-deteriorate water supply facilities were distributed in a wide range of island groundwater systems, despite the geographical segregation of their native environment. These fungal taxa showed a higher dominance by adapting to the characteristics of groundwater and overcoming the selective pressure. Therefore, it is necessary to use them as indicator fungal strains for monitoring the groundwater systems and the effectiveness of the purification process. As chlorination is the final step of the groundwater purification process, there is a limit to the efficacy of disinfection for fungal spore control. Therefore, in addition to the traditional groundwater treatment procedures, physical purifying methods should be introduced or intensified to remove spores or reduce water turbidity.

Disclosure statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability statement

All data are available within the manuscript and its supplementary materials.

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