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# Temperature sensitivity of Antarctic soil-humic substance degradation by cold-adapted bacteria

**Dockyu Kim**,[1*] **Ha Ju Park**,[1] **Mincheol Kim**,[1] **Seulah Lee**,[1] **Soon Gyu Hong**,[1] **Eungbin Kim**[2] **and Hyoungseok Lee**[1]

[1]*Division of Life Sciences, Korea Polar Research Institute, Incheon, 21990, Republic of Korea.*

[2]*Department of Systems Biology, Yonsei University, Seoul, 03722, Republic of Korea.*

## Summary

**Heteropolymer humic substances (HS) are the largest constituents of soil organic matter and are key components that affect plant and microbial growth in maritime Antarctic tundra. We investigated HS decomposition in Antarctic tundra soils from distinct sites by incubating samples at 5°C or 8°C (within a natural soil thawing temperature range of −3.8°C to 9.6°C) for 90 days (average Antarctic summer period). This continuous 3-month artificial incubation maintained a higher total soil temperature than that in natural conditions. The long-term warming effects rapidly decreased HS content during the initial incubation, with no significant difference between 5°C and 8°C. In the presence of Antarctic tundra soil heterogeneity, the relative abundance of *Proteobacteria* (one of the major bacterial phyla in cold soil environments) increased during HS decomposition, which was more significant at 8°C than at 5°C. Contrasting this, the relative abundance of *Actinobacteria* (another major group) did not exhibit any significant variation. This microcosm study indicates that higher temperatures or prolonged thawing periods affect the relative abundance of cold-adapted bacterial communities, thereby promoting the rate of microbial HS decomposition. The resulting increase in HS-derived small metabolites will possibly accelerate warming-induced changes in the Antarctic tundra ecosystem.**

## Introduction

The maritime Antarctic region is extremely vulnerable to rapid environmental changes, including those caused by warming related to global climate change (Convey and Peck, 2019). This region has undergone rapid warming over the past several decades (Turner *et al*., 2019). Recent studies in the Antarctic have reported altered sexual reproduction and spatial distribution patterns of moss and lichen communities (Casanova-Katny *et al*., 2016; Kim *et al*., 2016) and expansion of Antarctic hair grass populations (Hill *et al*., 2011). Temperature is one of the most important factors controlling soil microbial community activity; therefore, rising temperatures may have already altered microbial responses such as growth rate and degradative activity. Although soil microbes are important decomposers involved in nutrient cycling, the impact of the current rapid warming on the maritime Antarctic microbial community has not been studied (Royles *et al*., 2013). Higher temperatures have been shown to directly increase experimentally measured colony-forming units of bacteria and fungi isolated from maritime Antarctic soils (Yergeau *et al*., 2007). Higher temperatures have also been proposed to facilitate fungal colonization in soil (Newsham *et al*., 2016). In contrast, another study reported that bacterial communities respond negatively to soil temperature increases (up to 2.4°C), with bacterial biomass reductions of 41%–46% (Dennis *et al*., 2013). Therefore, warming may be a main driver of changes in microbial community size and structure, which are likely to affect the degradation of soil organic matter (SOM) across a range of maritime Antarctic terrestrial ecosystems.

Heteropolymer humic substances (HS), primarily humic acids (HA) and fulvic acids (FA), are organic compounds formed by the spontaneous condensation of biomolecules derived from decaying plants and other organisms. HS are ubiquitous throughout the environment and are the largest constituent (60%–80%) of SOM (Gramss *et al*., 1999). HS have two main functions in soil ecosystems: carbon storage and supply. The detailed structures of HS depend on the plant and soil sources, and specific formation conditions. The average structural properties of HS are similar, including aromatic compounds with hydroxyl (—OH), carbonyl (C=O) and carboxylic (—COOH) groups, which increase the cation exchange capacity in soils. HS and HS-derived small compounds (primarily produced by microbial decomposition) enhance or

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regulate the growth of plants and microbes through continuous interactions in soils (Lipczynska-Kochany, 2018; Garcia *et al*., 2019); however, HS are relatively resistant to microbial degradation because of their large and complex structure (Grinhut *et al*., 2007). Accordingly, large quantities of HS are presumably stored in the maritime Antarctic region owing to low microbial degradative activities. Current evidence suggests that cold-adapted bacteria are directly involved in *in situ* biodegradation in cold environments. A detailed understanding of their temperature sensitivity and adaptation is crucial for estimating HS degradation and predicting future ecological effects of increased temperatures. Microbial degradative activities and community composition shifts have been reported (Park *et al*., 2015; Kim *et al*., 2018); however, few studies have investigated the ecological impact of increasing temperatures on soil bacteria and HS degradation in the maritime Antarctic region.

Despite their structural complexities, HS are thought to be inherently sensitive to higher temperatures, as their activation energies for decomposition are higher (Lehmann and Kleber, 2015). HS in polar tundra soils are composed of low lignin content materials derived from grasses, mosses and lichens (Abakumov and Alekseev, 2018). Therefore, the intrinsic conformational flexibility of HS from polar tundra region is an emerging topic related to global warming. Maritime Antarctic ecosystems are sensitive to warming due to their geographical location (Kim *et al*., 2007; Convey and Peck, 2019). A slight increase in soil temperature could enhance soil bacterial metabolic activities, which would subsequently drive a higher HS degradation rate and release more HS-derived small metabolites. These metabolites will be available as nutrient sources for surrounding microbes and plants, considerably affecting the maritime Antarctic tundra ecosystem. These considerations led us to undertake microcosm studies using Antarctic soils to evaluate ecological changes associated with microbial HS degradation under increased soil temperature and/or during prolonged periods of soil thawing in polar tundra regions.

## Results

### *HS degradation during microcosm incubation*

The initial HS contents of seven Antarctic soils were determined to be within a range of $109.9 \pm 3.8$ to $175.6 \pm 0.9$ mg $g^{-1}$ of dried soil, whereas that of the control soil (SNU1-1) was $41.1 \pm 1.9$ mg $g^{-1}$ dried soil. When compared with an average value of 157.4 mg $g^{-1}$ dried soil obtained from five other Antarctic soils, KS3-1A and KS3-1C soils contained approximately 27% lower (109.9 and 119.6 mg $g^{-1}$ dried soil respectively) HS contents than the average value, indicating that the initial HS contents and structures are variable among nearby soils, due to Antarctic tundra heterogeneity and dynamics. Generally, the initial HS contents rapidly decreased at both 5°C and 8°C incubation temperatures for up to 30 days ($T = 30$), and then the HS decomposition rates remained slow until $T = 90$. The differences in HS contents at 5°C and 8°C appeared to be insignificant. In contrast, the HS content in the control temperate zone soil did not decrease at either temperatures (Fig. 2A and B). The percentage change in HS content after $T = 90$ incubation clearly showed that HS in Antarctic tundra soils tended to sensitively respond to changes in soil temperatures, which may be attributed to higher microbial decomposition rates at higher temperatures (Fig. 2C).

### *Structural characterization of HA by solid-state $^{13}$C-NMR*

HA were partially purified from HS extracts of the control soil (SNU1-1) and randomly selected sample soils (KS1-6C, KS2-1 and KS3-1C) at $T = 0$. Solid-state $^{13}$C-NMR spectra of Antarctic HA and commercial humic acid (CHA) were compared to obtain information regarding the structural characteristics of HA (Fig. 3). Molecular fragments of structural compounds were identified according to the chemical shift values: 0-45–C, H-substituted aliphatic carbons; 45-60–methoxyl group and O, N-substituted aliphatic carbons; 60-110–aliphatic carbons doubly substituted by heteroatoms, secondary alcohols, and other oxygen-bound carbon atoms; 110-160–C, H, O, N-substituted aromatic carbons; 160-185–carboxyl groups; 185-200–carbonyl groups. The percentage of carbon atoms in the respective HA was determined using solid-state $^{13}$C-NMR section integrals (Table 1). Functional groups that fall within the range of 110–160 and 160–185 ppm were grouped into the term aromatic fragments, as the carboxyl groups in the HS structure are generally attached to aromatic rings and participate in constituting complex cyclic fragments linking aromatic rings together. HA from SNU1-1 and KS2-1 exhibited the lowest contents of aromatic fragments at 23% and 25% respectively. Considering that SNU1-1 was collected from the temperate zone and had a relatively low HS content, it can be assumed that the HA had already been subjected to some level of microbial degradation at $T = 0$, thereby representing a low percentage of aromatic fragments. KS2-1 HA exhibited the lowest content of aromatic fragments among the three maritime Antarctic tundra soils, although the other two also exhibited relatively low contents of aromatic fragments (26% and 29%) compared to CHA (41%). This result suggests that the HA in Antarctic tundra soils have low contents of aromatic functionalities and are presumably highly degradable, and KS2-1 HA are anticipated to be more prone to microbial degradation than the other two.

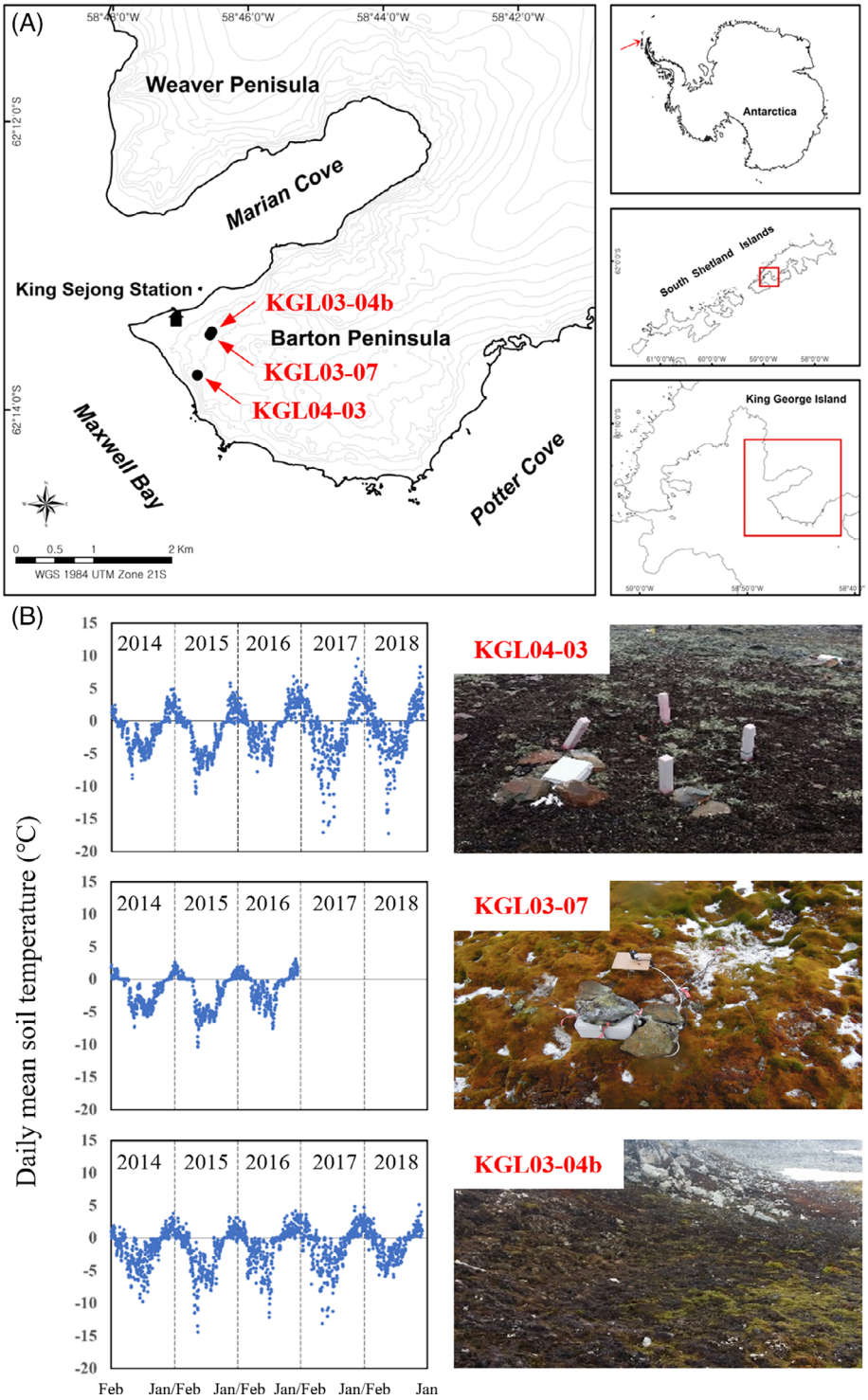


**Fig. 1.** Experimental site on Barton Peninsula on King George Island, Antarctic Peninsula.
A. Location of the humic substances (HS)-rich soil sampling sites.
B. Photos and time-series data of soil temperatures at the HS-rich soil sampling sites.

*Changes in HA content and structure in KS2-1 soil*

Although the difference in the decrease in HS content was insignificant between 5°C and 8°C, KS2-1 soil showed the highest decomposition rate (a decrease to ~51% at 8°C) among the seven Antarctic soils. Prior to this research, HS from KS1-3 soil (another sample from site KGL04-03) were characterized to be composed of HA and FA in a ratio of 90%:10% (unpublished experimental data). HA were partially purified from the HS extract of KS2-1 at 30-day intervals and then measured

by direct weighing. The HA content consistently decreased at 5°C and 8°C until $T = 90$ compared with that of the control soil frozen at −20°C ($T = 0$), and there was a transient and significant difference in the HA content at 8°C. The initial HA content (100%, 282.0 ± 8.9 mg $g^{-1}$ of dried soil) decreased to 67.5 ± 5.3% and 57.1 ± 2.0% at $T = 60$ in soils incubated at 5°C and 8°C respectively (Fig. 4A). Gel permeation chromatography (GPC) confirmed that the HA content at $T = 90$ was lower at 8°C than at 5°C. A higher molecular weight peak (8976 mRIU at 20 min) decreased to 7489 and 6101 mRIU in soils incubated at 5°C and 8°C respectively (Fig. 4B).

We examined the structural changes in HA by assigning major peaks in the Fourier-transform infrared (FTIR) spectra of HA at $T = 90$: peak 1, R-OH and R-NH; peaks 2–3, C=O of R-COO-R′ and R-CO-R′ groups (Fig. 4C). Peak 3 intensity significantly increased at 5°C compared to the control soil frozen at −20°C. This further increased at 8°C, with peak 3/peak 2 intensity ratios of 0.84, 1.03 and 1.61 at −20°C, 5°C and 8°C respectively. A new peak, i.e. peak 4, was detected in the HA spectra from both KS2-1 microcosm soils incubated at 5°C and 8°C. The appearance and increase of these functional group peaks in the HA FTIR spectra indicate that increased soil temperature enhanced microbial HA degradation, which subsequently increased HA functional group exposure to surrounding (micro)organisms.

### *Changes in bacterial community structure*

Bacterial 16S rRNA gene sequences of the seven Antarctic soils at $T = 0$ were grouped primarily into nine phyla: *Actinobacteria* (22.9% on average), *Proteobacteria* (18.7%), *Chloroflexi* (17.2%), *Acidobacteria* (14.3%), candidate division AD3 (6.1%), *Cyanobacteria* (5.6%), *Gemmatimonadetes* (4.2%), *Bacteroidetes* (3.1%) and *Verrucomicrobia* (2.8%) (Fig. 5). *Conexibacter* (*Actinobacteria* phylum) was the most dominant genus (5.5% on average) in all Antarctic soils at $T = 0$. *Aciditerrimonas* (*Actinobacteria*) (2.8%), *Edaphobacter*

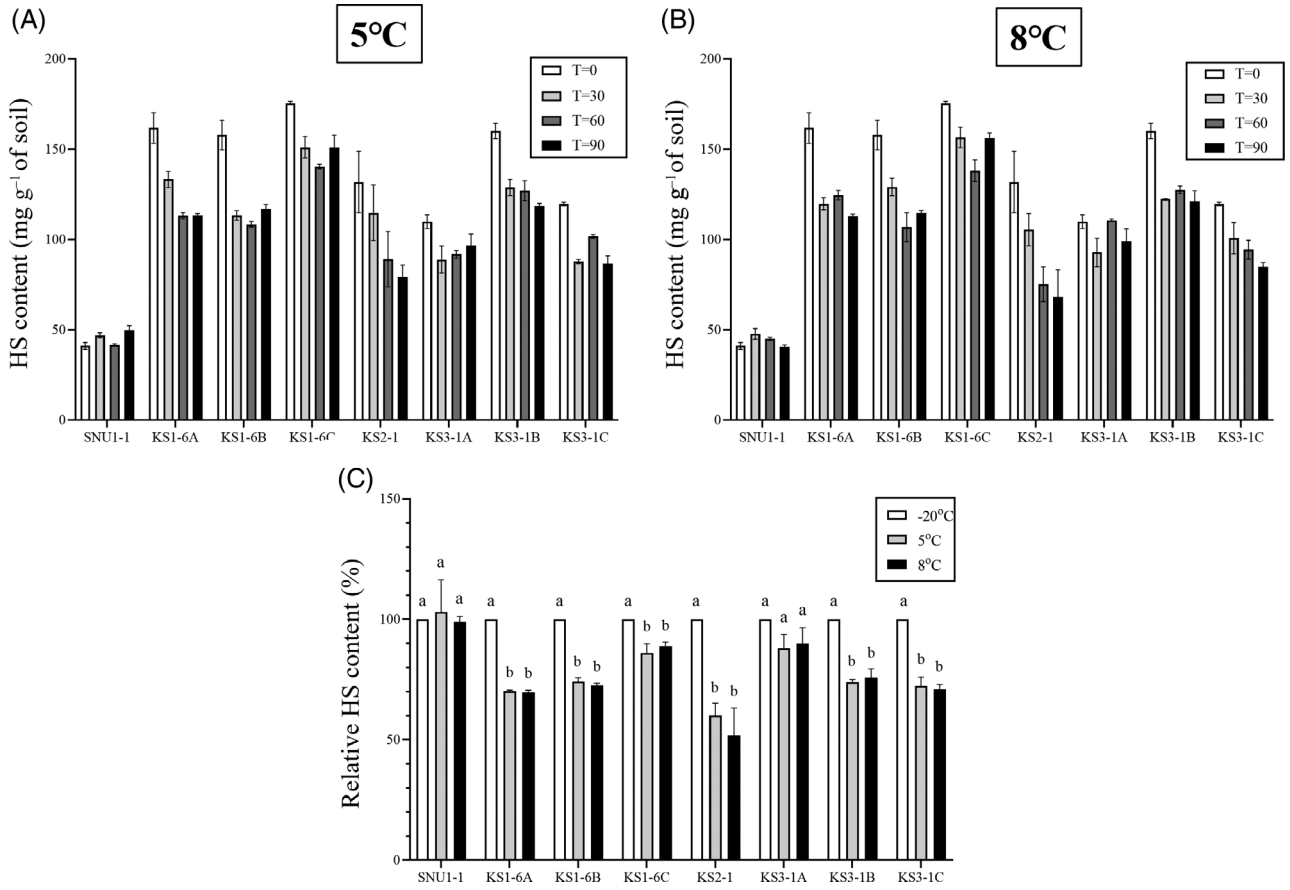


**Fig. 2.** Humic substances (HS) content changes in soil samples during the microcosm experiments.
A, B. Bar charts show data (mean ± standard deviation, $n = 3$) from one temperate zone soil (SNU1-1, control) and seven maritime Antarctic tundra soils (KS1-6, KS2-1 and KS3-1), which were incubated at 5°C or 8°C for 90 days ($T = 90$). (C) Bar chart presents percentage change in HS contents calculated from the original data (A, B) at −20°C, 5°C and 8°C after $T = 90$. Different lowercase letters indicate significant differences computed with analysis of variance (ANOVA) followed by Tukey's HSD test, $p < 0.05$ ($n = 3$).

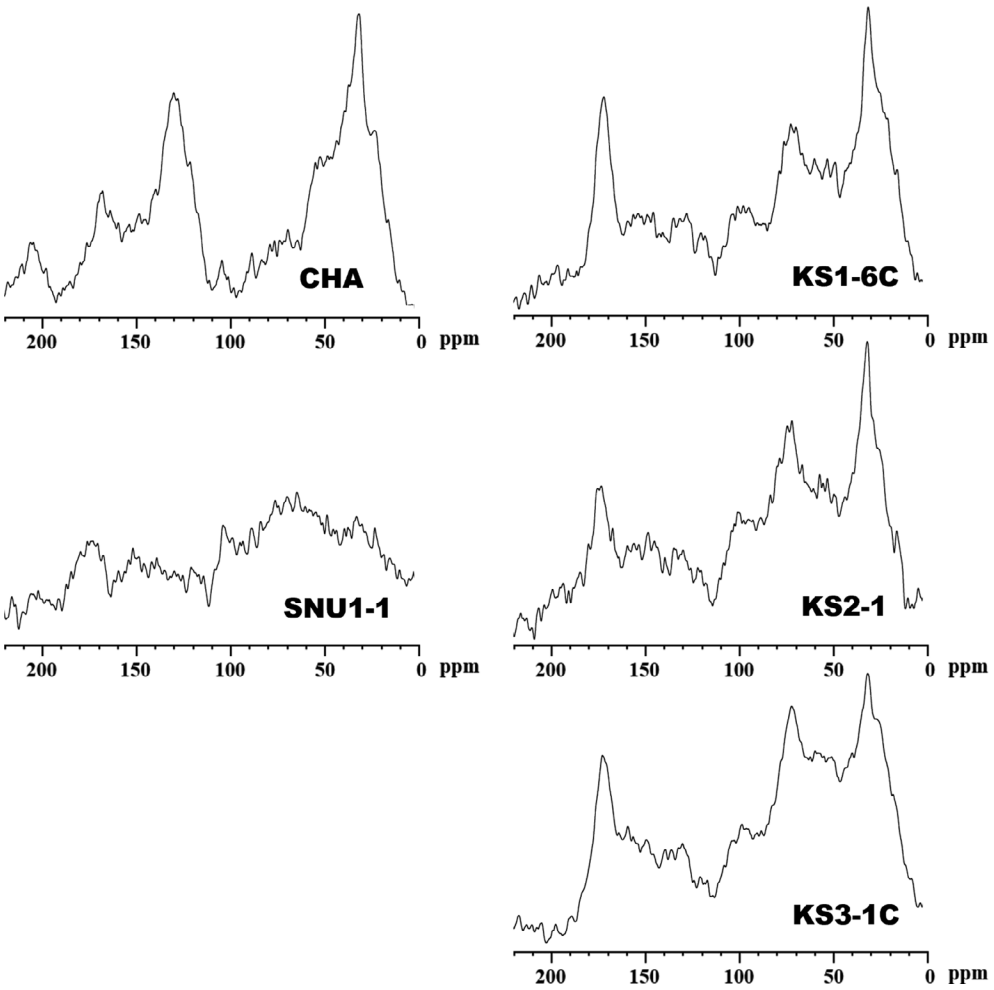


**Fig. 3.** $^{13}C$ (CP/MAS) NMR spectra of humic acids (HA) isolated from control soil SNU1-1 and Antarctic tundra soils (KS1-6C, KS2-1 and KS3-1C) before microcosm incubation ($T = 0$). Commercial humic acid (CHA, Sigma-Aldrich) was included for a comparison between HA from temperate and Antarctic tundra regions.

(*Acidobacteria*) (2.3%), *Solibacter* (*Acidobacteria*) (1.7%) and *Pseudolabrys* (*Proteobacteria*) (1.5%) were also consistently detected at high abundance. The control soil (SNU1-1) consisted of three major phyla at $T = 0$: *Actinobacteria* (41.3%), *Proteobacteria* (34.7%) and *Acidobacteria* (8.2%). Among various phyla, AD3 lineages displayed large variations in relative abundances across Antarctic soils (0.8%–13.0%) at $T = 0$ and were overrepresented in samples KS3-1A (8.5%) and KS3-1C (13.0%) compared with other soils. The KS3-1A and KS3-1C samples also contained 27% lower HS content at $T = 0$ than the average from other Antarctic soils. Thus, future work should investigate the relationship between HS content and AD3 functions.

Bacterial community compositions at the phylum level varied among $T = 90$ samples at both temperatures. Significant changes were detected in *Proteobacteria* responses after excluding samples that displayed relatively lower reductions in HS content during the incubation (i.e. KS3-1A and KS3-1C). The relative abundance of *Proteobacteria* significantly increased after incubation at 8°C ($18.8 \pm 5.0\%$ at $T = 0$ and $26.6 \pm 3.9\%$ at $T = 90$; *t*-test, $p < 0.05$). It also increased at 5°C ($21.9 \pm 4.2\%$ at $T = 90$), but the difference was not statistically significant ($p > 0.05$). The most dominant phylum, *Actinobacteria*, which degrades recalcitrant soil polymers such as lignin and HS, responded negatively (KS1-6A, KS1-6C and KS2-1) or unstably (KS1-6B, KS3-1B and KS3-1C) at both 5°C and 8°C, contrary to our expectations.

## Discussion

The Antarctic tundra has short and cool summers, with long and freezing winters. Microbial growth and decomposition are slow, resulting in a high accumulation of soil organic carbon (SOC) (Lee *et al*., 2013; Park *et al*., 2015).

Most ecosystem models predict that warming will stimulate microbial decomposition rates (Wang *et al*., 2017). It is crucial to identify changes in microbial metabolism in response to increase in soil temperatures to accurately predict the effects of warming on the maritime Antarctic ecosystem. During our microcosm incubations, the overall

**Table 1.** Percentage of humic acid carbon atoms based on solid-state $^{13}$C-NMR section integrals.

| | Chemical shift (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| Sample | 0–45 | 45–60 | 60–110 | 110–160 | 160–185 | 185–200 | Aromatic fragments (110–185) |
| SNU1-1 | 25 | 14 | 38 | 13 | 10 | 1 | 23 |
| KS1-6C | 34 | 10 | 26 | 16 | 13 | 1 | 29 |
| KS2-1 | 30 | 11 | 32 | 14 | 11 | 2 | 25 |
| KS3-1C | 32 | 12 | 30 | 15 | 11 | 1 | 26 |
| CHA[a] | 33 | 10 | 14 | 31 | 10 | 2 | 41 |

[a]CHA was purchased from Sigma-Aldrich (Cat. No. 53680).

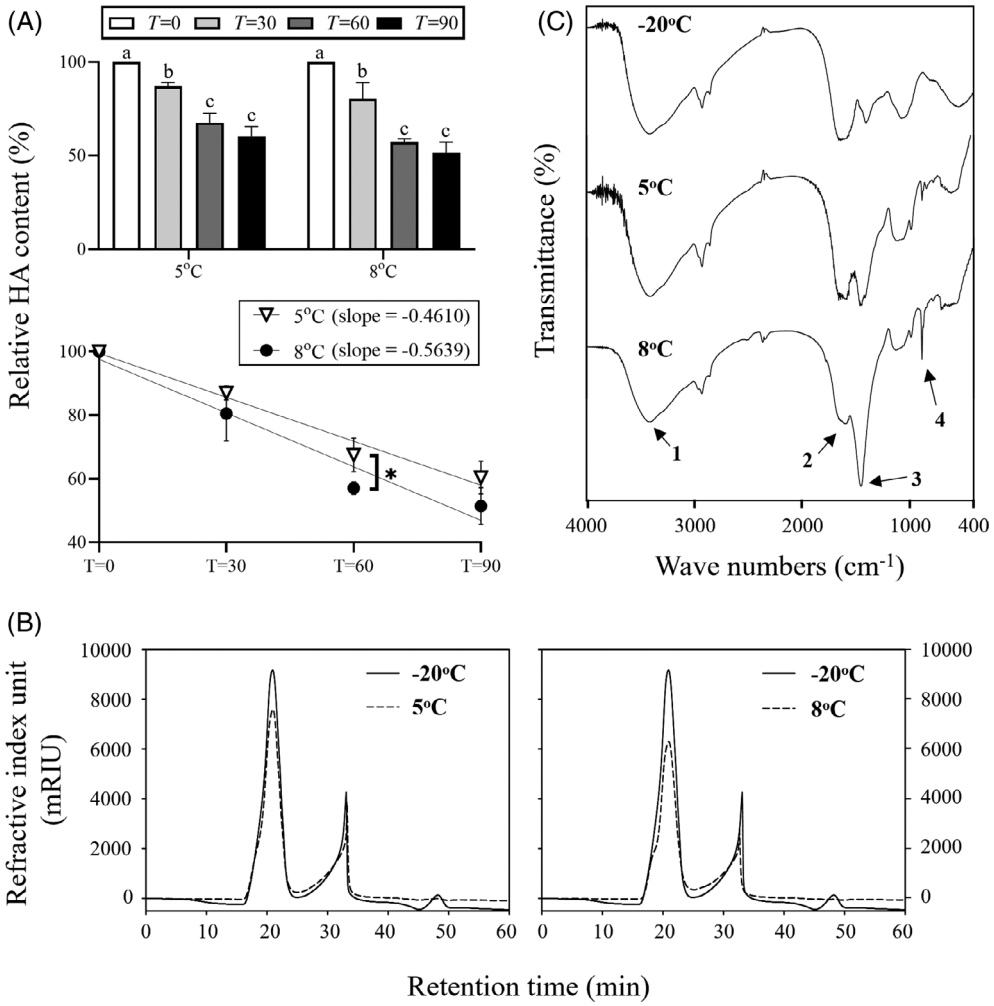


**Fig. 4.** Changes in humic acids (HA) content and structure were determined by direct weighing (A), gel permeation chromatography (B) and Fourier transform infrared spectroscopy (C) during the microcosm experiment of KS2-1 soil at 5°C and 8°C for $T = 90$. In (A), the difference in HA degradation rates between 5°C and 8°C (upper bar chart) was expressed as the linear regression slope over time from $T = 0$ until $T = 90$ (lower regression chart). Different lowercase letters indicate significant differences computed with analysis of variance (ANOVA) followed by Tukey's HSD test, $p < 0.05$ ($n = 3$). Asterisk indicates significant difference ($p < 0.05$) between at 5°C and 8°C at $T = 60$ based on an unpaired *t*-test. In (C), FTIR major peaks were assigned as follows: peak 1, R-OH and R-NH; peak 2, C=O of R-COO-R′; peak 3, C=O of R-CO-R′; peak 4, C—O of polysaccharides and polysaccharide-like substances.

HS contents of six Antarctic soil samples significantly decreased at both 5°C and 8°C for up to 30 days, with no significant difference between the two temperatures. Further obvious reductions in HS contents were not observed after 30 days until 90 days at both 5°C and 8°C (Fig. 2A and B). Interestingly, KS2-1 soil displayed the highest decrease in HS content after 90 days among the Antarctic soils (Fig. 2C). When HA (a major component of HS) were purified from HS of KS2-1 and characterized, the content decrease and structural change were more distinct at 8°C than at 5°C (Fig. 4). This difference among the soil samples might be due to different soil properties (e.g. total organic carbon and pH) or HS sources (e.g. mosses and lichens). Differences in HS physicochemical properties could affect microbial decomposition rates, which is an important aspect to be examined in future studies.

Solid-state $^{13}$C-NMR spectra of HA purified from SNU1-1, KS1-6C, KS2-1 and KS3-1C soils were analysed and compared with those of CHA (Fig. 3). Excluding the control soil SNU1-1, HA in KS2-1 soil displayed the lowest content of aromatic fragments, which directly corresponds with low humification (Table 1). The CHA structure is similar to that of lignin (an aromatic heterogeneous natural polymer in plants) and displayed the highest content of aromatic fragments. Considering the relatively low percentage of aromatic fragments of all three Antarctic HA (25%–29%), we estimate that polar region soils generally exhibit low humification levels because of the short vegetation season and low levels of plant materials with high lignin contents, with mosses, lichens and algae constituting the primary plant life in maritime Antarctic tundra (Polyakov and Abakumov, 2019). Thus, with higher aliphatic fragment contents than aromatic fragment contents, the HA from three Antarctic tundra soils are predicted to be highly degradable by microbes at low temperatures, especially HA from KS2-1 soil that contained the lowest percentage of aromatic fragments.

We examined sample KS2-1 as a representative maritime Antarctic tundra soil. The HA content steadily decreased at the two incubation temperatures (5°C and 8°C) to approximately 87% and 80% respectively, after 30 days, and then decreased to 60% and 51% after 90 days respectively, compared with the frozen control (100%). These results indicate that soil microbial degradative activity was higher at 8°C than at 5°C. This sharp decrease in HA content was maintained until 60 days when the difference between 5°C and 8°C was the most significant, and then the rate of HA degradation slowed at both temperatures (Fig. 4A). SOM containing HA is biologically degraded in three stages in a temperate climate: stage 1 (1–2 years) is the rapid decomposition of the labile SOM portion, stage 2 (10–100 years) is a slower degradation of the intermediate SOM portion and stage 3 (100–1000 years) is a very slow and complete decay of the remaining SOM (Lipczynska-Kochany, 2018). It is possible that the slow decline in HA degradation rate after 60 days (especially at 8°C) reflected stage 2 activity, and the labile HS was more quickly degraded at 8°C than at 5°C within 30 days.

If climate change increases the soil temperature or extends the soil thawing period in Antarctic tundra, then indigenous cold-adapted soil microorganisms will play a more crucial role in degrading SOC, such as HS. In our microcosm experiments, the relative abundance of *Proteobacteria* tended to increase according to increased HS decomposition at increased incubation temperatures from −20°C (assumed freezing temperature during winter) to 5°C or 8°C (assumed melting temperature during

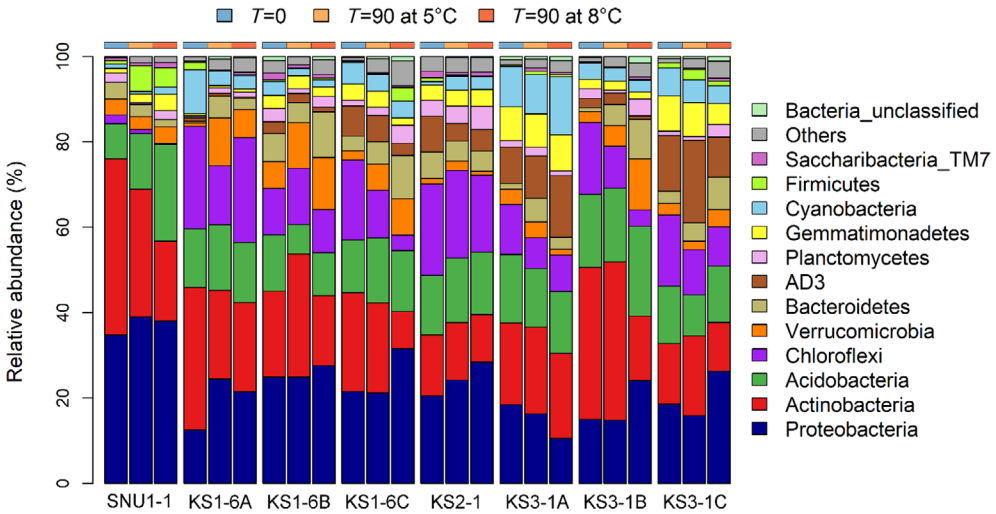


**Fig. 5.** Distribution of bacterial phyla across different soils under different incubation temperatures (5°C and 8°C). Bacterial phyla with less than 1% relative abundance are grouped into the 'Others' category after summing their relative abundance values.

summer) for 90 days. This trend was more evident at 8°C. Lately, members of the phylum *Proteobacteria* have been characterized to be abundant in organic matter-rich soils as the main HS degraders (Kim *et al*., 2020; Kulikova and Perminova, 2021; Park *et al*., 2021), with *Gammaproteobacteria* and *Betaproteobacteria* being the most prevalent classes from that phylum. Enrichment culturing of Alaska tundra soils at 5°C for 99-days in mineral medium containing HS stimulated the growth of *Proteobacteria*, with *Betaproteobacteria* being highly enriched, which indicated their involvement in HS decomposition at low temperatures (Park *et al*., 2015). In contrast, the relative abundance of *Actinobacteria* tended to decrease as that of *Proteobacteria* increased. Diverse extracellular laccase and peroxidase enzymes from white-rot fungi and soil *Actinobacteria* can oxidize and depolymerize lignin and, possibly, HS (Wang *et al*., 2016; Kim *et al*., 2019). This suggests that the degradative activity of polymer-degrading *Actinobacteria* increases to some extent at 5°C and further at 8°C, providing more bioavailable carbon sources to the environment. These sources could serve as substrates for microbial growth (in this case, *Proteobacteria*). We have previously reported a similar result from microcosm studies of subarctic Alaskan tundra soil (Park *et al*., 2015). *Proteobacteria* and archaeal *Euryarchaeota* largely increased in abundance when the initial HA content decreased to 48% after 99 days of incubation at 5°C, indicating their involvement in HA partial degradation and complete mineralization respectively. However, a systematic review for the interactions between HS-degrading bacteria and other heterotrophic microorganisms that utilize HS-derived organic compounds as a carbon and energy source is still missing. Any bacterial groups with both the capabilities of initial HS decomposition (depolymerization) and subsequent degradation of HS-derived organic compounds could take a competitive advantage over other surrounding microbes including *Actinobacteria* members.

Microbes in polar tundra soils are adapted to survive at extremely low temperatures, with cold-adaptation strategies, including the expression of cold-active enzymes and optimization of metabolic traits related to energy generation (Tribelli and Lopez, 2018). Their responses to the increase in total soil temperature (the sum of soil temperature for the duration of soil melting) may differ according to metabolic status and environmental conditions. Our results indicate that bacteria in maritime Antarctic tundra soils display higher HS decomposition rates with warming temperatures, generating higher accumulation of HS-derived small-molecule carbon sources for microbial and plant growth in the ecosystem. HS are widely recognized as an effective growth biostimulant and have a broad range of useful functions for plants (Shah *et al*., 2018). The expansion of Antarctic hair grass populations in maritime Antarctic regions (Hill *et al*., 2011) may have been possibly accelerated by climate-induced increases in HS-derived carbon sources. Some rare and latent soil microorganisms can be revitalized and proliferate as a result of increased nutrient supply. Some of these currently rare microorganisms could be pathogenic to plants or animals, and the potential emergence and dispersion of pathogens in polar tundra regions is an emerging field related to climate warming and permafrost thawing (Mogrovejo *et al*., 2020). The novel results of our study contribute to the understanding of how global warming in polar regions impacts microbial communities and their metabolic functions that regulate the carbon cycle.

## Experimental procedures

### *Site description and soil sampling*

Three sites with high HS contents were selected for sampling maritime Antarctic tundra soils. The sites were located near King Sejong Station, Korean Antarctic Research Station, on the Barton Peninsula of King George Island (Fig. 1A): Site KGL04-03 (KS1; 62°13′47″ S, 58°46′54″ W); Site KGL03-07 (KS2; 62°13′31″ S, 58°46′42″ W); and Site KGL03-04b (KS3; 62°13′29″ S, 58°46′40″ W). We selected one to three nearby locations for collecting soil samples during the following periods: KS1-6A, KS1-6B and KS1-6C from Site KGL04-03 in January 2018; KS2-1 from Site KGL03-07 in January 2014; and KS3-1A, KS3-1B and KS3-1C from Site KGL03-04b in January 2018. Each sample was collected from a depth of 0–10 cm, homogenized and stored at −20°C. As a control, a temperate zone soil (SNU1-1) sample was collected from a forest region (SNU1) in Seoul National University, Korea, in February 2014.

The climate of the Barton Peninsula is mild by Antarctic standards, with an average annual temperature of $-1.8 \pm 2.8$°C (1988–2015 unpublished meteorological data from the station) and 89% relative humidity (Kim *et al*., 2007). Most of the ice-free areas of the peninsula are covered by well-developed moss and lichen communities. The soils are generally poor in organic materials and available nutrients. Soil temperatures tended to increase annually during the summer seasons in some snow-melted areas. The average soil temperature at a depth of 5 cm at Site KGL04-03 ranged from −17.2 to 4.1°C (average −2.9°C) during 5 years (2014–2018, unpublished data) of Antarctic winters (April to November). During the thawing period (December to March), the temperature ranged from −3.8 to 9.6°C (average 2.1°C) (Fig. 1B).

### *Microcosm incubation*

The frozen soil samples were slowly thawed in a refrigerator (1.5°C) for 2 days. Then, a small fraction (120 g) of

each sample was incubated in a plant culture jar (72 × 72 × 100 mm) at 5°C or 8°C for up to 90 days. Each jar was wrapped in a plastic bag to maintain the water content of the initial soil sample. The soil in each jar was mixed with a spatula for aeration every 2 weeks. At 30-day intervals, a portion (12 g) of the soil was removed for analysis of the HS content and bacterial community.

### *Extraction and quantification of HS*

Soils were completely dried at 60°C for 5 days and then passed through a 1-mm pore sieve to remove debris. One gram of dried soil was mixed with 25 ml of 0.5 N NaOH, incubated at room temperature with continuous shaking overnight, and then centrifuged twice at 6000*g* for 10 min at 25°C. The supernatant was diluted 100-fold in 0.5 N NaOH, filtered through a 0.2-μm pore hydrophilic membrane (Advantec 13HP020AN Syringe Filters), and absorbance was measured at 350 nm. HS extraction was independently performed three times for each soil sample. The concentration of HS (HA + FA) was calculated using a standard curve of the measured absorbance at a concentration range of commercial HA (Sigma-Aldrich Cat. No. 53680) dissolved in 0.5 N NaOH (Badis *et al*., 2009).

### *Separation and structural analysis of HA*

HS were extracted from the dried soil sample using 0.5 N NaOH, and the HS extract was centrifuged twice (6000*g*, 10 min, 25°C) to produce HA-enriched supernatant as described above. The supernatant was acidified to pH 2.0, using 5.0 N HCl. The solution was continuously shaken at room temperature overnight, and then the insoluble HA fraction was separated from the soluble fraction containing FA and other small compounds by centrifugation at 6000*g* for 10 min at 25°C. Finally, the pelleted HA paste was freeze-dried, powdered and weighed.

We examined changes in the high molecular mass distribution and HA content using GPC. Powdered HA was resuspended in 0.1 N NaOH (10 mg $ml^{-1}$), and the resulting solution was filtered through a 0.2-μm membrane. Subsequently, 10 μl of filtrate was loaded onto an Ultrahydrogel-500 column (7.8 mm ID × 300 mm, Waters) linked to a Shodex OHpak SB-804 HQ column (8.0 mm ID × 300 mm, Showa Denko America) attached to a Hewlett Packard 1100 HPLC apparatus. The flow rate of the mobile phase (degassed water) was 0.5 ml $min^{-1}$, and HA content was examined with a refractive index detector. Structural changes in HA were examined using FTIR spectroscopy analysis. HA (5 mg) was mixed with 245 mg potassium bromide (KBr), and the mixture was pressed at 250 atm. The spectrum of HA in the KBr disc was recorded on a Nicolet 6700 FT-IR spectrophotometer (Thermo Fisher Scientific). The one-dimensional solid-state cross-polarization magic angle spinning $^{13}C$ nuclear magnetic resonance ($^{13}C$-CP/MAS NMR) spectrum of powdered HA was measured using a Bruker Avance 500 MHz system.

### *DNA extraction, PCR amplification and sequencing*

Soil metagenomic DNA was extracted from 0.3 to 0.4 g of soil using a FastDNA Spin Kit for Soil (MP Biomedicals). Bacterial 16S rRNA gene fragments were amplified by targeting the V1–V3 region (27F/519R for pyrosequencing) in Site KGL03-07 soil DNA or the V3–V4 region (341F/805R for MiSeq) in Site KGL04-03, KGL03-04b and SNU1 soil DNAs. The amplicons were sequenced using 454 GS FLX Titanium (454 Life Sciences, Roche Applied Science) for the 16S V1–V3 region or Illumina MiSeq Sequencing for the 16S V3–V4 region.

### *Bioinformatics analysis*

Primer and adapter sequences were trimmed, and pyrosequencing and MiSeq reads were processed using the DADA2 method (Callahan *et al*., 2016) to infer amplicon sequence variants (ASVs), which can resolve sequences down to the level of single-nucleotide differences. The resultant ASVs were taxonomically assigned to genus-level phylotypes against the EzBioCloud database (May 2018) for bacteria using the naïve Bayes classifier with a confidence threshold of 80% in mothur v.1.43.0 (Schloss *et al*., 2009). Sequencing results generated by two different platforms are not directly comparable; therefore, we used relative abundance values at higher taxonomic levels (class or phylum) for cross-sample comparisons. Raw sequence data were submitted to the NCBI Sequence Read Archive database under the BioProject accession number PRJNA604663.

### *Statistical analysis*

The experimental HS or HA extraction data were analysed using the GraphPad Prism ver. 9.1.0 software. Data from three technical replicates were expressed as the mean ± standard deviation. Analysis of variance (one-way ANOVA) was undertaken, followed by the multiple mean comparison test ($p < 0.05$, Tukey's honestly significant difference).

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