To Sporulate or not to Sporulate: Prevalence of Endospore-Forming Firmicutes along a Thermal Gradient in Alexandros Crater (Nisyros, Greece)

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"Extreme" is in the eyes of the beholder"
Lynn J. Rothschild & Rocco L. Mancinelli (2001)

"Nothing in Biology Makes Sense Except in the Light of Evolution"
Theodosius Dobzhansky (1973)

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To Sporulate or not to Sporulate: Prevalence of Endospore-Forming Firmicutes along a Thermal Gradient in Alexandros Crater (Nisyros, Greece)

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Abstract

Volcanos are extreme environments with elevated temperatures, ranging from 45°C to 120°C, low pH (between 1.5 and 2.5), high pressure and high concentration of heavy metals. Fumaroles, the vents from which escape hot gases, present high temperatures around 100°C and a very acidic pH around 2. Although these environments are extreme, evidence from literature supports the fact that microbes thrive in those environments. In order to thrive in volcanic environments and to withstand unfavorable conditions, microorganisms have developed adaptations and survival strategies. One of these strategies is endospore formation. Endospore formation is the production of a dormant structure called an endospore. Thanks to their structure, endospores are highly resistant to heat, cold, desiccation, UV radiation and chemicals. The aim of this study was to assess the role of endospore formation as a survival strategy along a temperature gradient in volcanic fumaroles. We hypothesize that endospore-forming Firmicutes (EFF) are more prevalent, the more extreme the site is. From this hypothesis, we formulated two research questions: (1) Is there a prevalence gradient of EFF along a thermal gradient from a fumarole? (2) Are EFF dominant in volcanic fields compared to « non-extreme » sites? In order to address these questions soil was sampled along two thermal gradients and a macro-scale gradient from the crater towards vegetation. The total bacterial community was assessed by qPCR of the 16S rRNA gene, EFF by qPCR of the *spo0A* gene, and EFF spores by dipicolinic acid (DPA) quantification. The results showed that a thermal gradient resulted in an abundance gradient of EFF: the relative proportion of EFF spores compared to the total community decreased with distance from the fumaroles. Conversely, bacterial abundance increased while temperature decreased. The same results were obtained for the macro-scale gradient towards vegetation. These results suggest that, because of their selective advantage under harsh environmental conditions, EFF are more prevalent in volcanic environments than in non-extreme ones. This study is the first to give an insight into the role of endospore-formation as a survival strategy in volcanic habitats.

Keywords: Extreme environments | Volcanos | Thermal gradient | Fumaroles | Endospore-forming Firmicutes | Endospores

Introduction

Extreme environments are considered as “extreme” from an anthropocentric perspective (Stetter, 1999). Volcanic habitats are such environments as they present high temperatures, up to 120°C, an acidic pH around 1.5-2 and high pressure (Stetter, 2001, Pirajno & van Kranendonk, 2005). Various gases such as CO₂, CH₄, H₂S or H₂ are produced and sulfates, nitrates, chlorides, ammonium and heavy metals are often present (Stetter, 1999, Teschner *et al.*, 2007). According to past studies, these conditions resemble the ones of the environment in which life is supposed to have appeared on Earth (Nisbet & Sleep, 2001).

Although conditions are extreme, these ecosystems are found to host microbial life (Stetter, 1999, Rothschild & Mancinelli, 2001). It is known that the geological setting along with the geochemical and physical characteristics of these environments select microbial communities that live there (Inskeep *et al.*, 2013, Klatt *et al.*, 2013, Meyer-Dombard & Amend, 2014). A number of studies have described the diversity of extremophilic microorganisms in volcanic habitats (Forget *et al.*, 2010, Wall *et al.*, 2015, Gagliano *et al.*, 2016). These studies showed that fumaroles (Wall *et al.*, 2015), geothermal soil (Gagliano *et al.*, 2016) and hydrothermal sediments of submarine volcanoes exhibit high microbial diversity.

In order to survive in extreme environments such as volcanic fields, microorganisms have developed diverse survival strategies (Roszak & Colwell, 1987, Mirouze & Dubnau, 2013) such as biofilm formation (Kreft, 2004), morphological plasticity (Justice *et al.*, 2008) or post-transcriptional modifications (Zhao *et al.*, 2002). Another of these strategies that can ensure survival under extreme environmental conditions is endospore formation in Endospore-forming Firmicutes (EFF) (Onyenwoke *et al.*, 2004). Endospore formation is the formation of a dormant structure called endospore (Driks, 2002, Errington, 2003). Sporulation begins with the detection of unfavorable environmental conditions by the vegetative cell through a two-component system (Fabret *et al.*, 1999, Vishnoi *et al.*, 2013). The cell commits to sporulation only if the concentration of phosphorylated Spo0A, the transcriptional regulator of sporulation, exceeds a certain threshold (Stages 0 and I) (Errington, 2003). This transcriptional regulator in turn activates several key sporulation genes. The next step is an asymmetrical cell division (Stage II) with the formation of a prespore, followed by its engulfment in the mother cell (Stage III)

(Errington, 2003). The cortex is then synthesized around the prespore (Stage IV) (Errington, 2003). Afterwards, the coat of the spore is formed (Errington, 2003). Finally, the spore matures and the mother cell lyses, leading to the release of a free spore (Stages VI and VII) (Errington, 2003). Dipicolinic acid (DPA), a small molecule that protects spore DNA from damage (Setlow, 2007), is synthesized along with the cortex formation (Errington, 2003, Setlow, 2007). Thanks to their structure, endospores are highly resistant to heat, UV radiation, chemicals and other stressors (Errington, 2003). Besides these survival strategies, extremophilic microorganisms such as thermophiles and acidophiles have evolved physiological adaptations to thrive in these environments. Thermophiles (between 45°C and 80°C) and hyperthermophiles (> 80°C) can, for example, increase the amount of saturated phospholipids to cope with the increase fluidity of the cell membrane at high temperatures (Madigan & Mairs, 1997, Stetter, 1999, Rothschild & Mancinelli, 2001). Acidophiles (pH < 5), as far as they are concerned, transport actively protons outside the cell by membrane-associated antiporters in order to maintain a neutral pH in their cytoplasm (Madigan & Mairs, 1997, Rothschild & Mancinelli, 2001).

Thermal gradients in geothermal sites are thought to create habitat gradients because of the different temperature fitness of the taxa present in the communities (Miller *et al.*, 2009, Weltzer & Miller, 2013). Although there are several studies on the structure of microbial communities along thermal gradients (Norris *et al.*, 2002, Miller *et al.*, 2009, Cuevas *et al.*, 2014), none of them studied endospore formation as a survival strategy. In this study, we assess the role of endospore formation as a survival strategy along thermal gradients in a volcanic setting and compare the abundance of EFF in volcanic environments to non-extreme sites. We hypothesize that EFF are more prevalent, the more extreme the site is. Indeed, as endospore formation is an energetically costly procedure, the cell enters sporulation only as a last resort solution (Errington, 2003). Therefore, endospore formation could be a disadvantage in stable environments compared to other bacterial taxa due to the absence of selective pressure for survival and the cost of sporulation (Abecasis *et al.*, 2013). However, it becomes an advantage under harsh and changing environmental conditions (Abecasis *et al.*, 2013). To test our hypothesis, we quantified the total community by 16S rRNA quantitative PCR (qPCR) and EFF by qPCR of *spo0A* gene and by DPA quantification of soil samples coming from two thermal gradients and a macro-scale gradient from the crater towards vegetation. In addition,

a culture-dependent approach was also used to isolate extremophilic endospore-forming Firmicutes from the samples along the thermal gradients. Our results indicate that a thermal gradient resulted in an abundance gradient of EFF spores and that spores are more abundant inside the crater than outside.

Materials and Methods

Site description & sampling

Sampling on Nisyros island in Alexandros crater was held in August 2015. Nisyros island is a calc-alkaline stratovolcano which belongs to the South Aegean active volcanic arc along with Methana, Milos and Santorini (Fig. 1) (Hunziker & Marini, 2005). The geological setting of Nisyros volcano result from the gradual evolution of calc-alkaline basalt into andesite, dacite and finally rhyolite (Vougioukalakis, 1998). The volcanic activity on Nisyros is expressed by five different surface manifestations: (i) thermal springs along the coasts, (ii) fumarolic fields in the south of the Lakki plain, (iii) ten hydrothermal craters, (iv) hot grounds and (v) mudspots (Kanellopoulos & Xirokostas, 2016). Alexandros is a small crater, about 70 m of diameter (Vougioukalakis, 1998), located at the north-west of the hydrothermal area, at the south-west of the caldera (Fig. 2A). Intense fumarolic activity is observed at the borders of the crater. Real-time sulfur crystal formation is observed at the fumaroles. A mat was present on the crater's surface among the fumaroles. This mat was gray, light yellow, orange or black at the surface and whitish beneath. Two fumaroles were selected (Fumarole 1, N 36°34.924' W 27°9.840' and fumarole 2, N 36°34.923' W 27°9.854') (Fig. 2B-2C) and temperature along the gradients were measured for 5 days. pH was also measured in the laboratory. At each point of the gradients (5 for fumarole 1 and 6 for fumarole 2), two bags of approx. 150 g of surface soil (max. depth 10 cm) were sampled. The soil samples were preserved at 4°C until transportation to the laboratory and then stored at 4°C and -20°C. Steam of both fumaroles was collected with a device of our own design (Fig. 2D, description in Supp. material). 2.85L and 2.8L of steam condensate were collected from fumarole one and fumarole two, respectively, and were filtered through a 0.22 µm pore size nitrocellulose membrane (Merck Millipore, Darmstadt, Germany). Chemical analyses (Sulfide; Sulfate; Phosphorus, reactive; Iron, ferrous; Iron, total; Zinc; Copper; Manganese) were performed on the steam condensate filtrates using a DR/890 Colorimeter (HACH LANGE GmbH, Düsseldorf, Germany), following the manufacturer's instructions. A third, macro-scale, thermal gradient was also sampled (Fig. 2E). Two bags of approx. 150 g of surface soil of a crater point (N 36°34.941' W 27°09.844', 0 m), two intermediate points (N 36°34.897' W 27°09.816', 45.48 m; N 36°34.895' W 27°09.825', 52.54 m)

and one point where the vegetation first appeared (N 36°34.838' W 27°09.863', 145.40 m) were collected and stored at 4°C and -20°C. Samples stored at 4°C were used for enrichment cultures (see Supplementary materials).

Figure 1 – Position of Nisyros island in the Southern Aegean volcanic arc. (A) Volcanic arc of Southern Aegean. (B) Nisyros-Kos system. (C) Nisyros island. (D) Hydrothermal craters area in the south of the caldera. Modified from Hunziker & Marini (2005).

DNA extraction

DNA from volcanic soil was extracted using a modified indirect extraction method described in Wunderlin *et al.* (2013), after optimization (see Supplementary material). Biomass was first separated from a total of 40 g of soil, as previously described (Wunderlin *et al.*, 2013), and then collected by filtration through a 0.45 µm pore size nitrocellulose membrane (Sartorius Stedim Biotech GmbH, Goettingen, Germany).

DNA was extracted using a modified protocol from MP FastDNA[®] SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) (Wunderlin *et al.*, 2013). Two sequential bead-beating steps were performed in order to lyse easy- and hard-to-lyse cells and endospores. Finally, the two extracts were pooled and DNA was precipitated and re-suspended in sterile water, as previously described (Sambrook & Russel, 2001). DNA yield was then measured with a Qubit[®] 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) using the Quant-iT dsDNA HS assay kit, following the manufacturer's instructions.

Quantification of the total bacterial community

Total bacterial community was quantified by real-time quantitative PCR of the V3 hypervariable region of the 16S rRNA gene using the primers 338f (5'-ACTCCTACGGGAGGCAGCAG-3') and 520r (5'-ATTACCGCGGCTGCTGG-3') (Ovreås *et al.*, 1997). The qPCR mix contained 5 µL Rotor-Gene[™] SYBR[®] Green PCR kit (Qiagen GmbH, Hilden, Germany), 0.3 µM of each primer and 2 µL of DNA template. A final volume of 10 µL was reached with PCR-grade water. The qPCR

Figure 2 – Sampling in Alexandros crater. (A) Alexandros crater. (B) Thermal gradient from fumarole 1. (C) Thermal gradient from fumarole 2. (D) In-house device to collect steam condensate. (E) Macro-scale thermal gradient towards vegetation (Modified from Hunziker & Marini (2005)).

war run with a Rotor-Gene™ 6000 instrument (Qiagen) with the following program: initial denaturation step at 95°C for 15 min, followed by 45 cycles composed of a denaturation at 95°C for 10 s, annealing at 55°C for 15 s, and elongation at 72°C for 20 s. The standard curve consisted of 10-fold dilutions (10^8 to 10^2 copies/ μ L) of a plasmid containing the V3 region of a *Bacillus subtilis* clone. All samples and standards were measured in triplicate.

Quantification of the endospore-forming Firmicutes

The endospore-forming Firmicutes part of the community was quantified by qPCR of *spo0A* gene using the primers spo0A655f (5'-GGHGTGCCNGCNCATATHAA-3') and spo0A834r (5'-CCAHGCGACTTCWATNGCRT-3') (Bueche *et al.*, 2013). The qPCR mix contained 5 μ L Rotor-Gene™ SYBR® Green PCR kit (Qiagen GmbH, Hilden, Germany), 1 μ M and 0.45 μ M of each primer and 2 μ L of DNA template. A final volume of 10 μ L was reached with PCR-grade water. The program was the following: initial denaturation of 95°C for 10 min, the 45 following cycles consisted of denaturation for 95°C for 15 s, annealing at 54°C for 15 s, and elongation at 68°C for 15 s. The standard curve consisted of 10-fold dilutions (10^8 to 10^2 copies/ μ L) of a plasmid containing the *spo0A* gene of a *Bacillus subtilis* clone. All samples and standards were measured in triplicate.

Quantification of endospores by DPA assay

Firmicute endospores were quantified in the volcanic soil by measuring their DPA content (Brandes Ammann *et al.*, 2011, Bueche & Junier, 2016), following optimization (see Supplementary material). Before autoclaving, the freeze-dried sediments were mixed with 2.7 mL of sodium acetate buffer (0.2 M, pH 5) and 300 μ L of aluminium chloride (AlCl_3 , 1.5 M). Fluorescence was measured within a Perkin-Elmer LS50B fluorometer. The excitation wavelength was set at 272 nm with a slit width of 2.5 nm. Emission was measured at 545 nm (slit width 2.5 nm). The device was set in the phosphorescence mode (equivalent to time-resolved fluorescence). The delay between emission and measurement was set at 50 μ s. Measurements were performed every 20 ms. The integration of signal was performed over a duration of 1.2 ms. Values recovered for each measurement corresponded to the mean of the relative fluorescence unit (RFU) values given by the instrument within the 30 s following sample introduction in the device. Finally, to transform RFU units into DPA concentrations,

a 10-point standard curve was established using increasing concentrations of DPA from 0.5 μM up to 10 μM .

Results

Thermal gradients and steam condensate analyses

In this study, two thermal gradients were selected for which temperature and pH were measured. For both gradients, temperature decreased with increasing distance from the fumaroles. Temperature gradient from fumarole one was steeper at the beginning, whereas that from fumarole two was more gradual. Temperature ranged between 41.28 and 99.60°C. However, pH was unstable and no gradients were observed (Fig. 3, Table S3). pH ranged between 1.6 and 4.

In addition, steam condensate from both fumaroles was collected in order to perform chemical analysis (Table 1). Specific metals were detected in the steam (Fe^{2+} , Fe_{tot} , Zn^{2+} , Cu^{2+} , Mn^{2+}), as well as sulfide, sulfate, and phosphate. Steam condensate of fumarole two presented a higher concentration of iron (0.53 mg/L) and zinc (0.26 mg/L) compared to that of fumarole one (0.18 mg/L Fe and 0.17 mg/L Zn). Same concentrations of

Figure 3 – Temperature and pH measurements along the two fumarole gradients. (A) Thermal gradient from fumarole one. (B) Thermal gradient from fumarole two. Thermal gradient from fumarole one is steeper than that of fumarole two. However, no pH gradient was observed for any of the two fumaroles.

copper and manganese were measured for both fumaroles (around 0.02 mg/L Cu and 0.022 mg/L Mn). 0.2 mg/L and 0.04 mg/L sulfide were measured for fumarole one and two, respectively. Sulfate concentrations was 2 mg/L for fumarole one and 5 mg/L for fumarole two. Finally, steam condensate of fumarole one presented a concentration of phosphate of 0.13 mg/L while that of fumarole two was 0.33 mg/L.

Table 1 – Chemical analyses of the steam condensates from the two fumaroles. Evian mineral water composition has been added for comparison (Evian, 2014). ND = non-detected.

Parameters	Test	Fumarole 1 [mg/L]	Fumarole 2 [mg/L]	Evian water [mg/L]
S^{2-}	Sulfide	0.2	0.04	/
SO_4^{2-}	Sulfate	2	5	13
PO_4^{3-}	Phosphorous, reactive	0.13	0.33	/
Fe^{2+}	Iron, Ferrous	0.16	0.45	ND
Fe_{tot}	Iron, Total	0.18	0.53	ND
Zn^{2+}	Zinc	0.17	0.26	ND
Cu^{2+}	Copper	0.02	0.01	ND
Mn^{2+}	Manganese	0.022	0.022	0.001

Total bacterial community

Total bacterial community was quantified in the samples collected along the two thermal gradients and along the macro-scale gradient from the crater towards the vegetation (Table S2). Due to low DNA yields (< 1 ng/ μ L), only the results from the second thermal gradient and the macro-scale gradient could be obtained. Bacterial abundance increases while temperature decreases along the thermal gradient (Fig. 4A). The same tendency is observed for the macro-scale gradient (Fig. 5A).

Relative abundance of Endospore-forming Firmicutes

Relative abundance of EFF was calculated as the ratio of log-transformed endospores count (DPA) per gram of sediment under total bacterial abundance ($1/C_t$) (Table S4). A temperature gradient resulted in an abundance gradient of EFF spores. Indeed, the relative abundance of EFF spores increased with temperature increase (Fig. 4B). Furthermore, spores were found to be more abundant inside the crater than outside in the macro-scale gradient (Fig. 5B).

Figure 4 – Total bacterial abundance and relative abundance of spores along the thermal gradient from fumarole 2. (A) Total bacterial abundance (1/Ct). Bacterial abundance increases with temperature decrease. (B) Relative abundance of spores. Relative abundance of spores decreases with increasing temperature.

Figure 5 – Total bacterial abundance and relative abundance of spores along the macro-scale gradient from the crater towards vegetation. (A) Total bacterial abundance ($1/C_t$). Bacterial abundance increases while going towards vegetation (B) Relative abundance of spores. Relative abundance of spores is higher inside the crater than outside.

Discussion

Nisyros volcanic activity is expressed by thermal springs, fumarolic fields, hot grounds and mudspots (Kanellopoulos & Xirokostas, 2016). Fumaroles were chosen because of their ubiquity in volcanic environments (Ellis *et al.*, 2008) and their poorly studied microbial diversity (Wall *et al.*, 2015). Moreover, it could be the source of microorganisms that would be “seeded” in nearby sites through airborne dispersal in fumarolic steam (Ellis *et al.*, 2008, Bizzoco & Kelley, 2013). In Alexandros crater, high fumarolic activity is observed. Two fumaroles were selected based on temperature gradient measurements along horizontal transects and in a way to avoid the thermal influence of other close-by fumaroles. As expected, and in accordance with previous literature (Sievert *et al.*, 1999, Sievert *et al.*, 2000, Norris *et al.*, 2002, Cuecas *et al.*, 2014), temperature decreases by distance from the heat source, in this case, the fumaroles. Thermal gradient one is quite steep in the first 45 cm, with a decrease in temperature from 100°C to 50°C, and afterwards temperature decreases slowly until reaching 40°C 110 cm from the fumarole. Thermal gradient two present a more gradual decrease in temperature from 100°C to approximately 45°C within two meters. To our knowledge, other studies on terrestrial thermal gradients show temperature gradients along larger transects than the ones we studied here (from 90°C to 40°C within 100 m in a hot spring in Cuecas *et al.* (2014) and from 65°C to 40°C within 5 m in a geothermal soil in Norris *et al.* (2002)). However, studies on submarine thermal gradients seem to be more within the range of those in Alexandros crater. Indeed, studies of Sievert *et al.* (1999) and Sievert *et al.* (2000) showed temperature gradients from 70°C to 45° within 140 cm and from 90°C to 45° within approximately 2 m, respectively. Due to geothermal activity, hot gases and steam coming from the geothermal reservoir escape the fumarole (Vougioukalakis, 1998). Fumarolic steam of Nisyros volcano is mainly composed of water (H₂O), carbon dioxide (CO₂), hydrogen sulfide (H₂S), dihydrogen (H₂), dinitrogen (N₂), methane (CH₄) and carbon monoxide (CO) (Chiodini, 2005, Teschner *et al.*, 2007). The real time formation of elemental sulfur on the fumaroles and the extremely low pH of the soil (between 1.5 and 3) are due to the presence of hydrogen sulfide in the steam (Marini *et al.*, 2002). Indeed, the hydrogen sulfide gas oxidizes to form elemental sulfur and reacts with water to produce sulfuric acid (Pirajno & van Kranendonk, 2005, Kanellopoulos & Xirokostas, 2016).

Moreover, chemical analyses on steam condensates revealed the presence of metals, such as copper, manganese, zinc and iron. These results are consistent with previous literature showing that metallic trace elements are found in fumarolic condensates (Gemmell, 1987). The presence of metals can be explained by the circulation of hot infiltrated water into the geothermal system. Indeed, in both submarine (hydrothermal vent) and terrestrial (fumaroles) geothermal environments, the infiltrated water is heated by the magmatic chamber and enriched with magmatic-derived elements, such as metals, through leaching of the crust by the hot hydrothermal fluids when ascending back to the surface (Baross & Hoffman, 1985, Pirajno & van Kranendonk, 2005). The presence of manganese, zinc and iron in fumarolic steam is consistent with the presence of manganese, zinc and iron, in mineral or native form, in Nisyros volcanic rocks (Hunziker & Marini, 2005).

The chemical composition of the steam and the soil samples along with the physical characteristics of the sites would influence the microbial communities living there. Total bacteria quantification by quantitative PCR (qPCR) of the 16S rRNA gene revealed that bacterial abundance increases with temperature decrease along the thermal gradient. These results are consistent with previous literature on microbial communities along temperature gradients (Sievert *et al.*, 2000, Miller *et al.*, 2009, Everroad *et al.*, 2012, Cuecas *et al.*, 2014, Sharp *et al.*, 2014). Similar results were found for the macro-scale gradient from the crater towards the vegetation. Temperature exerts a strong selective pressure on microbial communities. Indeed, bacteria are distributed along the thermal gradient depending on the optimal growth temperature of the taxa present in the communities (Stetter, 1999, Weltzer & Miller, 2013, Sharp *et al.*, 2014). While temperature decreases with distance from the fumarole, bacterial abundance increases because of the less extreme temperature conditions prevailing there. In the case of the macro-scale gradient, the closer to the vegetation, the less influence the crater has on the communities and thus the higher the bacterial abundance. Because of the extreme conditions prevailing in volcanic environments, DNA extraction is quite challenging (Herrera & Cockell, 2007). In addition to being relatively low, the biomass could be not well separated from the soil particles due to hydrothermal alteration of the rocks and minerals present in the soil. Indeed, rocks and minerals undergo hydrothermal alteration when in contact with acidic hot water or steam, producing a white material rich in clay minerals and silica

(Pirajno & van Kranendonk, 2005, Kanellopoulos & Xirokostas, 2016). It is known that cells or DNA can bind on clay minerals (Henneberger *et al.*, 2006, Herrera & Cockell, 2007). Moreover, interaction between cells or DNA and clay tend to increase at acidic pH (Henneberger *et al.*, 2006). These types of interaction could thus lead to an incomplete release of cells during the homogenization step. Problems of DNA binding on clay can also occur during the DNA extraction. Addition of skimmed milk led to improved DNA yields by competing with DNA for adsorption on soil particles, in a previous study (Takada-Hoshino & Matsumoto, 2004, Herrera & Cockell, 2007). In addition to that, as the soil samples were rich in clay, it was difficult to separate the cells from the very fine particles because of a density similar to that of bacteria and spores. When collecting bacterial biomass, a large amount of fine particles was also retained on the filter. Only results from the second thermal gradient and from the macro-scale gradient towards the vegetation could be obtained, due to the low DNA yields of the extraction (< 1 ng/ μ L) and low biomass of the samples, despite the use of an indirect extraction method. In order to improve DNA yields, separation of bacterial cells from soil matrix should be further optimized specifically for volcanic samples and samples should be first filtered through cell strainers with 40 μ m nylon mesh before collecting biomass on 0.45 or 0.22 μ m nitrocellulose membranes in order to remove as much particles as possible (Lever *et al.*, 2015).

Originally, the EFF fraction of the community would be quantified using the *spo0A* genetic marker. *Spo0A* is a gene that encodes a transcriptional factor responsible for the initiation of sporulation in EFF (Abecasis *et al.*, 2013), thus it is EFF specific. As *spo0A* is present in one single copy in the genome of EFF (Abecasis *et al.*, 2013), it is difficult to target when biomass is low, compared to the 16S rRNA gene which is present in an average copy number per cell of 7 in *Firmicutes* and 4 in all bacteria (Lee *et al.*, 2009). *Spo0A* gene was undetectable by qPCR, thus abundance of spores was assessed via DPA quantification. Relative abundance of spores was calculated along the thermal gradient and the macro-scale gradient from the crater towards the vegetation. For the thermal gradient, relative abundance of spores decreased with decreasing temperature. Similarly, relative abundance of spores decreased along the macro-scale gradient from the crater towards vegetation. A marine thermal gradient in the natural spring of Kanava on the island of Milos showed similar results (LAMUN, unpublished data,

Fig. S8). As already mentioned, temperature has a strong influence on the composition of the communities along a thermal gradient in springs in Sharp *et al.* (2014) and in a geothermal soil in Norris *et al.* (2002). Indeed, the thermal gradient creates habitat and niche gradient that select the bacteria that are adapted to the specific conditions existing there (Weltzer & Miller, 2013). Thus, temperature along the gradient favors bacteria based on their optimal growth temperature: thermophiles from 45°C to 80°C and hyperthermophiles above 80°C (Madigan & Marrs, 1997). The higher abundance of EFF in higher temperature sites could be linked to the resistance properties of endospores (Setlow, 2014). During the sporulation pathway, the addition of each component of the endospore will confer resistance to different extreme conditions: the spore coat gives resistance to UV radiation and chemicals (Riesenman & Nicholson, 2000) and the cortex is responsible for heat resistance (Atrih & Foster, 2001). Finally, low water content, DPA, and small acid-soluble proteins present in the spore core protect the DNA from damage. (Setlow, 2007, Setlow, 2014). All these components give resistance to wet and dry heat, desiccation, UV and γ -radiation and chemicals (Setlow, 2007, Setlow, 2014). Moreover, the dynamic state of the mature endospore contributes to its resistance to desiccation by controlling the water flux inside and outside the spore (Driks, 2003). It is known that spores of thermophiles have higher heat resistance than spores of mesophiles (Setlow, 2014). This is probably due to the fact that thermophiles have proteins that are more thermostable than those of bacteria that grow at lower temperature (Setlow, 2014). Moreover, it seems that sporulation temperature influences wet heat resistance (Setlow, 2014). Spores produced at higher temperature have a higher wet heat resistance than spores produced at lower temperature (Setlow, 2014). This is because the spore core water content is lower in spores produced at higher temperature (Setlow, 2014). Moreover, spores are more abundant in higher temperature locations probably due to the strong selective pressure of temperature on microbial communities. Indeed, the strong selective pressure for survival of temperature along the thermal gradient, along with pH, desiccation and other chemical factors, may have favored endosporulation at higher temperature sites (Abecasis *et al.*, 2013). With temperature decrease along the thermal gradient, the selective pressure becomes weaker, thus leading to a lower number of spores.

As endospore-formation is a complex and highly costly procedure (Errington, 2003), it should be favored and prevalent in extreme environments (Abecasis *et al.*, 2013). Indeed, it is a last resort solution, only when other strategies, such as biofilm formation or chemotaxis for example, fail (Mirouze & Dubnau, 2013). It has been shown that only the co-existence of multiple limiting factors increases the relative abundance of EFF in natural springs (Filippidou *et al.*, *In preparation*). The results obtained concerning the macro-scale gradient from the crater towards the vegetation suggest the same tendency. There is co-occurrence of multiple limiting factors in Alexandros crater: temperature, pH and desiccation. Extremity decreases from the crater towards vegetation and this creates a gradient of abundance of EFF.

Methods need to be further optimized, as these environments are relatively low in biomass. A tailored DNA extraction optimized for volcanic samples could be designed (Lever *et al.*, 2015), in order to yield more DNA for studying the community composition of the samples. A chemical characterization of the samples is needed in order to, on the one hand, understand whether there is a particular community assembly under particular chemical composition of the samples, and on the other hand, to design adequate enrichment media. Our *in situ* observations need to be verified *in vitro* in microcosm experiments using an artificial community with selected strains isolated from the environment. Finally, the role of fumaroles in shaping bacterial communities in volcanic environments needs to be further investigated. There is evidence of airborne dispersal of microorganisms in geothermal environments (Bonheyo *et al.*, 2005, Ellis *et al.*, 2008, Portillo & Gonzalez, 2008, Bizzoco & Kelley, 2013, Herbold *et al.*, 2014). However, the role of fumaroles as collectors (Wall *et al.*, 2015) or producers (Bonheyo *et al.*, 2005) in volcanic environment needs to be determined. We suggest that bacteria, and specifically endospores owing to their high dispersal capabilities (Nicholson *et al.*, 2000), are dispersed by the fumarole steam and that the local environmental conditions (e.g. temperature) will select the taxa in the communities, with a higher prevalence of EFF in more extreme sites, but this needs to be further analyzed.

We have shown that endospore-formation is an advantage in volcanic habitats compared to non-extreme sites (i.e. vegetation sample along the macro-scale gradient). Temperature seems to influence the relative abundance of EFF along a thermal gradient, in comparison to pH for

which no pattern was observed. However, no other parameters, such as the chemical composition of the samples, have been taken into account. The relative proportion of EFF compared to the total community decreases with temperature decrease. Moreover, the results showed that bacterial abundance increases with temperature decrease along thermal gradients and while departing from the crater towards the vegetation along a macro-scale gradient. The hypothesis by which a higher abundance of EFF is expected in more extreme sites is thus confirmed.

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Supplementary material

Description of the steam condensate collecting device

Steam of fumarole one and two was collected by condensation using a device of our own design. A large glass funnel held by a clamp and metal stand was placed upside down approximately 30 cm above the opening of the fumaroles. The funnel was oriented in a way to collect as much steam as possible and to allow steam condensate to flow down in second smaller funnel and then in a plastic Nalgene collection bottle. The tip of the big funnel was covered with aluminium foil to avoid escape of the evaporates and maximize collection of the steam. Approximately 500 mL of steam condensate was collected every 12h and then filtered.

Enrichment cultures and isolation of extremophilic endospore-forming bacteria

Enrichment cultures were attempted in various media in order to enrich and isolate thermo-acidophilic sulfo-oxidizing endospore-forming bacteria from samples along the thermal gradients. Indeed, geothermal habitats are often rich in reduced sulfur compounds such as hydrogen sulfide or elemental sulfur (Aragno, 1992), and Alexandros crater was found to be rich in sulfur deposits due to real-time formation of elemental sulfur.

A number of studies succeed in isolating *Sulfobacillus* sp., which are sulfur-, iron-, and mineral-sulphide-oxidizing moderately thermophilic and acidophilic endospore-forming bacteria (Norris *et al.*, 1996, Atkinson *et al.*, 2000, Johnson *et al.*, 2003, Melamud *et al.*, 2003, Bogdanova *et al.*, 2006, Jiang *et al.*, 2009), as well as *Alicyclobacillus* sp., which are iron- and sulfur-oxidizing thermo-acidophilic endospore-forming bacteria (Albuquerque *et al.*, 2000, Jiang *et al.*, 2009), from geothermal habitats. The aim of this culture-dependent part is to isolate such strains. Cultures were also attempted on the macro-scale gradient samples.

Enrichment and isolation of *Sulfobacillus* strains

A first attempt to isolate *Sulfobacillus* sp. was performed with a mineral salt medium described in Norris *et al.* (1996). For heterotrophic growth, the medium was supplemented with 0.025% w/v yeast extract and the pH was adjusted to 2 with H₂SO₄ 10M. For autotrophic growth, the medium was supplemented with 0.5% w/v elemental sulfur. pH was adjusted to 3 with H₂SO₄ 10M. Elemental sulfur was sterilized separately under UV during three cycles of 15 min.

Additionally, enrichment culture in NB (Nutrient Broth, Biolife, Milan, Italy) were also done to cultivate heterotrophic thermo-acidophilic bacteria. 1 g of soil samples and 1/6th steam condensate membrane was added aseptically in 50 mL screw-cap pyrex bottles containing 10 mL of medium. Incubation temperatures and pH conditions for each culture are presented in Table S1. Enrichment cultures were incubated under agitation (180 rpm) during about two weeks. Growth was checked by light microscopy. In case of growth, 100 µL of 10-fold suspension-dilutions of the culture were plated onto solid medium and the plate was incubated during 2-3 days for isolating colonies. In order to specifically isolate endospore-forming strains, a 1-mL aliquot of culture was pasteurized at 90°C during 10 min (Bonjour *et al.*, 1988) and 100 µL of 10-fold suspensions-dilutions were plated onto solid medium.

Table S1 – Enrichment culture to isolate *Sulfobacillus* strains and heterotrophic thermo-acidophilic bacteria. Incubation temperatures and pH are mentioned for each medium and for each samples. SH: *Sulfobacillus* heterotrophic medium; SA: *Sulfobacillus* autotrophic medium; NB: Nutrient Broth.

Samples ID	SH		SA		NB	
	T (°C)	pH	T (°C)	pH	T (°C)	pH
A10	50	2	50	3	90	4
A11	50	2	50	3	80	2.5
A12	50	2	50	3	50	2.5
A13	50	2	50	3	45	1.8
A14	50	2	50	3	50	2.5
A15	50	2	50	3	40	1.8
A20	50	2	50	3	90	4
A21	50	2	50	3	90	2.5
A22	50	2	50	3	70	1.8
A23	50	2	50	3	70	1.8
A24	50	2	50	3	60	1.8
A25	50	2	50	3	60	2.5
A26	50	2	50	3	45	1.8

For solid medium, a double strength gellan gum solution with 10 g/L gellan gum (Phytigel, Sigma, St. Louis, USA) and a double strength medium were prepared and autoclaved separately (Lindström & Sehlén, 1989). After autoclaving, the double strength Phytigel solution was poured into the double strength medium (Lindström & Sehlén, 1989). Solid medium to isolate *Sulfobacillus* strains was supplemented with elemental sulfur and yeast extract. pH was

adjusted to 2.5 and plates were incubated at 50°C. Solid NB medium with 10 g/L gellan gum was used to isolate heterotrophic thermo-acidophilic bacteria.

After more than two weeks of incubation, no growth was observed in none of the enrichment cultures.

Enrichment and isolation of sulfo-oxidizing strains of different sulfur sources

A second attempt was performed in order to isolate sulfo-oxidizing thermo-acidophilic bacteria in a mixotrophic mineral medium (modified DSMZ medium 1190) containing different sulfur sources: elemental sulfur, pyrite and sodium thiosulfate. The modified mixotrophic mineral medium DSMZ 1190 contained (for 1 L): 20 mL of 50x heterotrophic basal salts solution; 1 mL trace elements solution and 0.025% w/v TSB. MilliQ water was added to 1 L. The following modifications of the original medium recipe were applied: the 50x heterotrophic basal salts solution was supplemented with 0.5 g $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ in order to have a final concentration of 10 mg/L, as described in Norris *et al.* (1996). $\text{CrCl}_3 \cdot 6 \text{H}_2\text{O}$ and NH_4VO_3 replaced $\text{Cr}_2(\text{SO}_4)_3 \cdot 15 \text{H}_2\text{O}$ and NaVO_3 , respectively, in the trace element solution. Ferrous sulfate solution was replaced by one of the three following sulfur sources: 0.5% w/v elemental sulfur (Marsh & Norris, 1983), 0.2% w/v pyrite (FeS_2) (Marsh & Norris, 1983) or 10 mM final concentration of sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) (Norris *et al.*, 1986). Elemental sulfur and pyrite were UV-sterilized. A medium designed specifically for *Sulfobacillus* strains (medium DSMZ 665) was also used. All the media were adjusted to pH 2. In addition to that, enrichment cultures to isolate heterotrophic bacteria in TSB (Tryptic Soy Broth, Biolife, Milan, Italy) supplemented with soil extract were launched. TSB/Soil extract medium was prepared according to Michel Aragno's recipe (adapted from Pochon & Tardieux (1962)). For the soil extract, 400 g of soil were mixed by stirring in 1 L MilliQ water during 30 min. This soil suspension was then sterilized by autoclaving during 1h at 121°C. A first filtration through gaze to remove the biggest particles was effectuated, followed by a filtration through filter paper. Finally, the clear soil extract was autoclaved during 15 min at 121°C. For 1 L of TSB/Soil extract medium, 200 mL of soil extract was added to 800 mL of TSB containing 30 g of TSB dehydrated medium (Biolife, Milan, Italy). Table S2 show all enrichment cultures with their temperature and pH conditions. 1 g of soil samples was inoculated into 10 mL of medium in 50 mL screw-cap pyrex

bottles. The cultures were incubated during approx. one month. Growth was checked by light microscopy.

Table S2 – Enrichment culture to isolate sulfur-oxidizing thermo-acidophilic bacteria using different sources of sulfur. Incubation temperatures and pH are mentioned for each medium and for each samples.

Samples ID	Sulfur		Pyrite		Thiosulfate		<i>Sulfobacillus</i>		TSB/Soil	
	T (°C)	pH	T (°C)	pH	T (°C)	pH	T (°C)	pH	T (°C)	pH
A11	80	2	80	2	80	2			80	2.5
A12	50	2	50	2	50	2	50	2	50	2.5
A13	45	2	45	2	45	2	50	2	45	1.8
A14	50	2	50	2	50	2	50	2	50	2.5
A15	40	2	40	2	40	2	50	2	40	1.8
A21	90	2	90	2	90	2			90	2.5
A22	70	2	70	2	70	2			70	1.8
A23	70	2	70	2	70	2			70	1.8
A24	60	2	60	2	60	2			60	1.8
A25	60	2	60	2	60	2			60	2.5
A26	45	2	45	2	45	2	50	2	45	1.8

Isolation was done on FeSo-phytagel solid medium (Johnson, 1995) for mixotrophic media containing different sources of sulfur and for *Sulfobacillus* medium (medium DSMZ 665), and on TSB/Soil extract-phytagel solid medium for heterotrophic bacteria. FeSo solid medium is used for the isolation of autotrophic and mixotrophic sulfo-oxidizers (Johnson, 1995).

As the previous attempt, no growth was observed in none of the enrichments. A couple of hypotheses can be formulated to explain why no growth was observed in none of the enrichment cultures to isolate *Sulfobacillus* sp., thermo-acidophilic sulfo-oxidizing or thermo-acidophilic heterotrophic bacteria. Firstly, culture conditions could be not optimal to observe growth of *Sulfobacillus* or mixotrophic thermo-acidophilic bacteria. A purely autotrophic medium without any addition of organic carbon and a purely heterotrophic medium were used to isolate *Sulfobacillus* strains. However, *Sulfobacillus* sp. requires yeast extract to grow autotrophically on sulfur, ferrous iron or sulfide minerals (Aragno, 1992, Norris *et al.*, 1996, Bogdanova *et al.*, 2006). Moreover, no CO₂ was added into the bottles to stimulate autotrophic growth. This can explain why no growth at all was observed on autotrophic cultures, despite the fact of using a mixotrophic solid medium. Indeed, carbon dioxide is present in large amount in geothermal habitats (Aragno, 1992). During incubation, cultures were agitated at 180 rpm. However, it

has been shown that thermophilic sulfo-oxidizing bacteria are microaerophilic, thus requiring low amount of oxygen (Bonjour & Aragno, 1986, Aragno, 1992). The enrichment cultures would therefore not be agitated. Indeed, oxygen is quite limiting in geothermal areas (Aragno, 1992). For the isolation of heterotrophic thermophilic bacteria, the fail of growth could be explained by the high concentration of organic carbon under the form of peptone or yeast extract in NB and TSB/soil extract media. Indeed, organic matter is generally present in low amount in geothermal sites (Aragno, 1992). Moreover, Bogdanova *et al.* (2002) showed that the high concentrations of organic matter in rich heterotrophic medium, such as *Bacillus acidocaldarius* medium, did not support growth of acidophilic chemolithotrophic bacteria such as *Sulfobacillus* strains. Further attempts should thus be performed with a medium with a low content of organic carbon. Finally, as observed with DNA extraction, biomass is relatively low in those habitats. Enrichment cultures should therefore be launched in higher culture volumes with a larger amount of inoculum.

Other enrichment cultures could be attempted in order to target other types of metabolisms and thus enrich other types of thermo-acidophilic endospore-forming bacteria such as hydrogen-oxidizers (Aragno, 1992, Aragno, 1992), carbon monoxide-oxidizers (Aragno, 1992) or anaerobic metabolisms, such as for example anaerobic photosynthesis by *Heliobacteria* (Kimble *et al.*, 1995).

Enrichment and isolation of acidophilic heterotrophic endospore-forming bacteria in the macro-scale gradient

A last attempt was performed on the vegetation sample of the macro-scale gradient in order to isolate *Bacillus acidocaldarius* like strains (Zentgraf *et al.*, 1992). *Bacillus acidocaldarius* medium from DSMZ (DSMZ 13) was used. pH was adjusted to 4. 1 g of the soil from the vegetation sample was inoculated into 10 mL of medium DSMZ 13. The culture was incubated at 30°C with agitation (180 rpm) for 2 to 3 days. 100 µL of the initial culture was used to inoculate 10 mL of another culture. After incubation at 30°C during 2 to 3 days, growth was observed and confirmed by light microscopy. A 1-mL aliquot of culture was pasteurized at 90°C during 10 min (Bonjour *et al.*, 1988) and 100 µL of 10-fold suspensions-dilutions were plated onto *Bacillus acidocaldarius* agar plates. Solid cultures were incubated at 30°C during

2 to 3 days. White colonies grew on the solid medium. Two different colony morphologies could be identified: circular colonies with entire margins and circular colonies with undulate margins. Phase-contrast microscopy confirmed that the isolated strains were endospore-forming (Fig. S1).

Further physiological tests are needed to characterize the isolated acidophilic mesophilic endospore-forming bacteria from the vegetation sample of the macro-scale gradient, as well as a molecular identification.

Figure S1 – Phase-contrast microscopy image of the isolated endospore-forming strains on *Bacillus acidocaldarius* agar plate (400×). About 10 μm long rod shaped bacteria are visible in black. Endospores appear bright and are oval. Their position in the mother cell is subterminal to terminal. Bar = 50 μm.

Optimization of the methods

DNA extraction

1) *Test 1*: Standard direct extraction

DNA was extracted from 0.5 g of soil using the MP FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), following the manufacturer's instructions. Quantification showed no or low DNA present (<0.50 ng/mL).

PCR amplification of the 16S rRNA gene was attempted using the primers GM3f and GM4r (Muyzer *et al.*, 1995). PCR reaction were performed in a final volume of 50 µL containing: 1 µL sample, 1× Reaction Buffer (TaKaRa Bio, Shiga, Japan), 1.25 U of Ex Taq Polymerase (TaKaRa), 1x BSA (New England Biolabs), 200 µM of each dNTP (TaKaRa) and 0.2 µM of each primer. Negative control (1 µL PCR-grade water) and positive control (2 ng *Anoxybacillus rupiensis* DNA template) were included. PCR reaction were run on an Arktik Thermo Cycler (Thermo Fisher Scientific, Vantaa, Finland) with the following program: initial denaturation step at 98°C for 5 min, followed by 35 cycles composed of a denaturation at 94°C for 30 s, annealing at 51°C for 45 s, and elongation at 72°C for 1 min; and a final elongation at 72°C for 5 min. In order to see whether there were PCR inhibitors, some samples were spiked with DNA from an EFF strain (NEU1298-*Anoxybacillus rupiensis*).

None of the samples were amplifiable (Fig. S2). However, the spiked samples were amplifiable. There are thus no PCR inhibitors in the samples. In conclusion for this test, as there is no detectable DNA after the direct extraction, a tailored indirect method with biomass extraction is needed.

Figure S2 – Gel electrophoresis image of the 16S rRNA gene PCR of the thermal gradient with direct extraction. 1% agarose gel run at 100 V for 30 min. (L): BenchTop 1kb DNA Ladder (Promega); Line 1: (-): negative control; (+): positive control (NEU1298); (1): A11 spiked; (2): A15 spiked; (3): A21 spiked; (4): A26 spiked; (5): A11; (6): A12; (7): A13; Line 2: (8): A14; (9): A15; (10): A21; (11): A22; (12): A23; (13): A24; (14): A25; (15): A26

2) Test 2: Microscopic observation of biomass extracted from 20 g of soil and stained with SYBR Green I

As previous tests with indirect extraction of biomass from 1, 2 and 5 g of soil did not yield DNA (data not shown), a test with biomass extraction from 20 g was performed on a sample of thermal gradient from fumarole 1. 4-fold 8 g of soil sample were homogenized with 25 mL of extraction solutions (1% sodium hexametaphosphate) by vortexing at maximum speed for 2 min. Heaviest particles were then removed by centrifugation at 20 x g for 1 min, and the supernatant was collected. The cell separation step was then repeated. The supernatant was then filtered through black 0.22 μm pore size nitrocellulose filters (GTBP02500, Millipore,

Billerica, MA, USA). A quarter of the filter was placed on a microscopy slide with 50% glycerol and 50% Tris-EDTA and stained for 15 min with a SYBR Green I 20× solution. The slide was finally observed at a magnification of 400× and 1000× with an epifluorescence microscope using filter cube L5 (Leica DMR, Leica Microsystems, Wetzlar, Germany) (Sauvain *et al.*, 2013).

As there were a lot of sediment in the supernatant, the filter has clogged and only approx. 20 mL were filtered. Despite that, some rod-shaped bacteria were visible (Fig. S3) at 400 and 1000 magnification, along with a lot of larger particles (probably organic matter (Morono *et al.*, 2009)). We have thus decided to extract the biomass from 20 g of soil, to filter through 0.45 µm pore size nitrocellulose filters and to extract DNA.

Figure S3 – Microscopy images with SYBR® Green I staining. Arrows show bacterial cells. Other visible particles are probably organic matter (Morono *et al.*, 2009). (A) 400× magnification. (B) 1000× magnification. Bar = 10 µm

3) Test 3: Indirect DNA extraction from 20 g of soil

20 g of soil sample A23 were homogenized with 1% sodium hexametaphosphate in order to separate the biomass. Then, the supernatant containing the biomass was filtered through 0.45 µm pore size nitrocellulose filters and DNA was extracted from the filters with the MP FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) as described in Wunderlin *et al.* (2013). DNA was finally precipitated and its concentration measured with Qubit® 2.0 Fluorometer. The DNA yield after precipitation was 0.06 ng/µL.

The 16S rRNA gene was amplified using the same program as the first test. PCR amplification of the *spo0A* gene was attempted with the degenerate primers spo0A166f (5'-GATATHATYATGCCDCATYT-3') and spo0A748r (5'-GCNACCATHGCRATRAAYTC-3')

(Wunderlin *et al.*, 2013). PCR reaction were performed with 1 µL sample, 1× Reaction Buffer (TaKaRa Bio, Shiga, Japan), 1.25 U of Ex Taq Polymerase (TaKaRa), 1x BSA (New England Biolabs), 200 µM of each dNTP

(TaKaRa) and 1 µM of each primer in a total reaction volume of 50 µL, completed with PCR-grade water. Negative control (1 µL PCR-grade water) and positive control (2 ng *Anoxybacillus rupiensis* DNA template) were included. PCR reaction were run on an Arktik Thermo Cycler (Thermo Fisher Scientific, Vantaa, Finland) with the following program: initial denaturation

Figure S4 – Gel electrophoresis gel image of the 16S rRNA and *spo0A* genes PCR from 20 g. 1% agarose gel run at 100 V for 30 min. (A) 16S rRNA gene PCR. The PCR product is around 1500 bp. (B) *spo0A* gene PCR. The PCR product should be around 602 bp, but no amplification is observed. (L): BenchTop 1kb DNA Ladder (Promega); (-): negative control; (+): positive control (NEU1298); (A23): sample A23.

step at 98°C for 5 min, followed by 40 cycles composed of a denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 1 min; and a final elongation at 72°C for 5 min.

The 16S rRNA gene amplified, but not the *spo0A* one (Fig. S4). After these results with 20 g, the DNA of all the samples would be extracted from 40 g of soil.

Quantification of DPA

1) Test 1: of increasing soil sediments mass

A first test of DPA quantification was performed on the second thermal gradient with increasing sediment mass of 150 mg, 300 mg, 600 mg and 1.2 g without freeze-drying with addition of 300 µL of AlCl₃ 0.5 M solution as described in Bueche & Junier (2016). Sodium acetate buffer with addition of AlCl₃ 0.5 M was taken as negative control.

No DPA was detected, as all the values from the samples were under the value of the negative control (Fig. S5). This is probably due to the low biomass content of the samples. Further tests will be done on the sediments from the filters.

Figure S5 – DPA concentration for the second thermal gradient from increasing sediment mass. The value of the negative control is above the values of the samples; the measurements cannot thus be taken into account.

2) Test 2: of 150 mg of sediments from filters

A second test was performed as described for the previous one but with 150 mg of freeze-dried sediments from the filters (coming from the biomass separation step from 40 g of soil).

Once again, low DPA was detected (Fig. S6). DPA in samples A13 and A26 was high because of the turbidity of the lysate after autoclave. As phosphate ions are known to drastically reduce the fluorescence of the DPA-Terbium complex and that AlCl_3 reduces phosphate interference (Fell Jr *et al.*, 2001), a test with *Bacillus subtilis* spores' suspensions in sodium hexametaphosphate with addition of AlCl_3 0.5 M, 1 M or 1.5 M was performed.

Figure S6 – DPA quantification for both gradients from the sediments from the filters. 150 mg of sediment from the filters (coming from the biomass separation step from 40 g of soil) were used for the quantification. The values of the samples are very low. Samples A13 and A26 were turbid, which can explain that the values are higher than the other samples. The negative control was sodium acetate buffer with addition of AlCl_3 0.5M. T-tests between the samples and the negative control have been performed. $P < 0.05^*$, $P < 0.01^{**}$, $P < 0.001^{***}$.

3) Test 3: of *Bacillus subtilis* spores' suspensions in saline water or in 1% sodium hexametaphosphate with 0.5, 1 or 1.5 M AlCl_3

Spores of *Bacillus subtilis* (strain NEU1) were collected from cultures on NA (Nutrient Agar) supplemented with 10 mg/L MnSO_4 in order to produce spores. Then, spores were suspended

in 1 mL physiological water (0.9% NaCl) for the positive control and with 4 times 1mL 1% sodium hexametaphosphate. Suspension of *Serratia marcescens* (strain NEU1302) in 1 mL 1% sodium hexametaphosphate was used as negative control. The suspensions were then freeze-dried. The lyophilisates were processed as sediment samples. 1.5 M AlCl_3 was added for *S. marcescens* suspension and no, 0.5M, 1M or 1.5M AlCl_3 was added in *B. subtilis* spores' suspensions. No AlCl_3 was added for the positive control. The measurements were performed as described in Bueche & Junier (2016).

The concentration of AlCl_3 had an effect on DPA concentration (Fig. S7). DPA concentration increased with increasing AlCl_3 concentrations. No DPA was detected in *S. marcescens*. The positive control was turbid, maybe because not all spores were destroyed during autoclaving. This could explain why the measured DPA concentration is so low.

Following these results, all the samples would be processed with addition of AlCl_3 1.5 M.

Figure S7 – DPA quantification tests on *B. subtilis* suspensions with increasing AlCl_3 concentrations. Increasing amounts of AlCl_3 permit to reduce the interference of phosphate ions on the detection of DPA. Indeed, the highest DPA concentrations is measured with 1.5M AlCl_3 . *B. subtilis* in physiological water is used as a positive control, while *S. marcescens* in sodium hexametaphosphate supplemented with 1.5M AlCl_3 as a negative control. A concentration of 1.5M of AlCl_3 is thus used for further endospore quantification of the samples.

Kanava, Milos (GR)

Figure S8 – Relative abundance of EFF along a marine thermal gradient in the natural spring of Kanava (Milos, GR). The relative abundance of EFF increases with increasing temperature. 82% of the community is endospore-formers at around 100°C (bubbling water spot). It decreases to 0.4% at a lower temperature and finally reach 0.07% in the sand sample (ambient temperature).

Table S3 – Temperature and pH measurements for the two gradients.

Sample ID	Distance from fumarole [cm]	T (°C)	pH
Fumarole 1 thermal gradient			
A10	0	99.60	4
A11	30	84.00	2.32
A12	45	50.70	2.50
A13	60	47.25	1.85
A14	80	48.90	2.21
A15	110	41.28	1.93
Fumarole 2 thermal gradient			
A20	0	97.65	4
A21	45	90.68	2.87
A22	60	74.43	1.88
A23	80	72.43	1.60
A24	120	62.95	1.99
A25	160	57.70	2.50
A26	200	47.15	1.80

Table S4 – Summary of total bacterial abundance, DPA concentration, endospores count and relative abundance of EFF for the thermal gradient from fumarole 2 and the macro-scale gradient from crater towards vegetation. The values in the table are mean values $\pm$ SE (standard error), except for the ratio.

Samples	Total bacteria [1/Ct]	DPA [μ M]	Endospores count	Log(Endospores count)	Ratio Log(Endospores)/ Total bacteria
A21	0.04216 $\pm$ 0.00013	1.57E-07 $\pm$ 1.76E-08	8.28E+06 $\pm$ 9.26E+05	6.92 $\pm$ 0.84	164.09
A22	0.04801 $\pm$ 0.00047	7.03E-07 $\pm$ 1.65E-08	3.70E+07 $\pm$ 8.68E+05	7.57 $\pm$ 0.88	157.65
A23	0.04948 $\pm$ 0.00014	6.07E-07 $\pm$ 6.60E-09	3.19E+07 $\pm$ 3.46E+05	7.50 $\pm$ 0.88	151.64
A24	0.04950 $\pm$ 0.00104	2.49E-07 $\pm$ 3.07E-08	1.31E+07 $\pm$ 1.62E+06	7.12 $\pm$ 0.85	143.74
A25	0.05020 $\pm$ 0.00008	2.55E-07 $\pm$ 3.22E-08	1.25E+07 $\pm$ 1.58E+06	7.10 $\pm$ 0.85	141.35
A26	0.04938 $\pm$ 0.00035	3.81E-07 $\pm$ 2.09E-08	2.48E+07 $\pm$ 1.36E+06	7.39 $\pm$ 0.87	149.75
Crater (0 m)	0.04548	1.38E-07 $\pm$ 1.30E-08	9.56E+06 $\pm$ 8.99E+05	6.98 $\pm$ 0.84	153.50
Inter. point 1 (45.48 m)	0.08008 $\pm$ 0.00031	2.24E-07 $\pm$ 2.76E-08	1.58E+07 $\pm$ 1.95E+06	7.20 $\pm$ 0.86	89.90
Inter. point 2 (52.54 m)	0.05450 $\pm$ 0.00074	7.52E-07 $\pm$ 1.04E-08	3.57E+07 $\pm$ 4.94E+05	7.55 $\pm$ 0.88	138.59
Vegetation (145.40 m)	0.22051 $\pm$ 0.00966	7.18E-07 $\pm$ 1.14E-08	1.52E+08 $\pm$ 2.40E+06	8.18 $\pm$ 0.91	37.10

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