Microbiome profiling in extremely acidic soils affected by hydrothermal fluids: the case of Solfatara Crater (Campi Flegrei, southern Italy)

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Abstract

An integrated geochemical and microbiological investigation of soils from the Solfatara Crater (Campi Flegrei, southern Italy) demonstrated that interstitial soil gases dominated by CO₂ and other typical hydrothermal gaseous species (e.g. H₂S, CH₄, ethane, benzene, alkenes and S-bearing organic compounds) influenced the composition of microbial communities. The relatively high concentrations of hydrothermal fluids permeating the soil produced acidic conditions and whitish deposits that characterize the Solfatara Crater floor. Archaea and Bacteria showed almost equal cell abundance (up to 3.2 x 10⁹ and 4.2 x 10⁹ cell/g, respectively) with relatively low levels of biodiversity and equitability in sites characterized by elevated temperatures (up to 70 °C), very low pH values (up to 2.2) and reducing conditions. In these sites, high-throughput sequencing showed the marked selection of microorganisms, mainly affiliated with the genera Thermoplasma, Ferrophilus and Acidithiobacillus. A relatively high biodiversity and concomitant distinctive structure of the microbial community were observed in soils poorly affected by fumarolic emissions that were toxic and rich in organic matter.

Introduction

During the last few decades, studies in hydrothermal, geothermal and anthropogenic high-temperature ecosystems have discovered previously unknown diversity of thermophilic microorganisms (Huber et al. 2000; Menzel et al. 2015; Colman et al. 2018). Genomic studies of thermophilic microbes inhabiting these extreme environments have predicted numerous microbial metabolic pathways directly involved in key global processes such as nutrient (e.g., C, N, S, Fe) and trace element (e.g., As, Sb, Hg) cycling and gas exchange (CO₂ and CH₄) (Inskoep et al. 2013). Solfaric fields, characterized by low pH and high temperature, represent one of the most extreme environments widespread on Earth (e.g. Krisuvik, Hveragerthi, Kerlingarfjöll - Iceland, Yellowstone National Park - USA, White Island - New Zealand, Kamchatka peninsula - Russia, Hokkaido - Japan, and Campi Flegrei - Italy).

Solfatara Crater is a relatively young tuff cone (ca. 4,000 yr BP) located in the town of Pozzuoli, a few km west of Naples (southern Italy). It represents the most prominent surface hydrothermal manifestation in the Campi Flegrei caldera. It is characterized by the occurrence of a relatively strong emission of hydrothermal fluids, mainly consisting of water vapor and CO₂ (Chiodini et al. 1998; Chiodini et al. 2001; Caliro et al. 2007; Chioldini et al. 2010; Tassi et al. 2015a) and significant amounts of CH₄, H₂S, CH₃ and gaseous elemental mercury (GEM) in both fumarolic fluids and soils (Tassi et al. 2013, 2015a, 2016; Sicola et al. 2015). Steam condensation at shallow depths produces significant thermal anomalies and hot soils in the Solfatara Crater, which was known to the Romans as the Forum Vulcani (in Latin: home of the god of fire), as in Strabone’s “Strabonis geographicae”. Hydrothermally altered soils carpet the crater floor that includes native sulfur, sulfate and sulfide encrustations, alunite and amorphous silica. Anomalous enrichments in deep-originated elements, e.g. K, Cs, Sb, Cl, F and Sr, were also recognized (Allard et al. 1991; Bruno et al. 2007; Sgavetti et al. 2009; Piochi et al. 2015; Mayer et al. 2016). The Solfatara soils exhibit a typical whitish color induced by intense hydrothermal alteration of the volcanic rocks under acidic conditions (Turney et al. 2008; Piochi et al. 2015). The altered deposits progressively turn grayish approaching the central part of the Solfatara Crater, named Fangia (an Italian term to define a muddy zone), which hosts bubbling acidic mud pools that form through interactions of rainwater, steam condensation and hydrothermal gases. This area, as well as the north- and south-eastern fumarolic fields, shows the highest soil temperatures (Chiodini et al. 2001). In contrast, Mediterranean maquis, with holms and oaks, strawberry-trees and pines covers the north-western...
portion of the crater with no significant heat and gas flux anomalies (e.g. Tassi et al. 2013, 2016). Recent monitoring of the Solfatara Crater within the Campi Flegrei caldera has shown increasing seismic and deformation activity and significant chemical variations of the fumarolic gas discharges (Chiordini et al. 2016, 2017; Kilburn et al. 2017).

The chemical characterization of Solfatara volcano and the entire Campi Flegrei has been thoroughly documented (e.g. Tassi et al. 2013; Chiordini et al. 2015a; Cardellini et al. 2017; Montanaro et al. 2017; Venturi et al. 2017a), but little is known about the microorganisms living in these extreme environments. A previous low-throughput molecular analysis of the bacterial composition of soil and crust samples collected near the fumarolic vents and the acidic mud pools in the center of Solfatara Crater identified the presence of bacteria mainly affiliated with Proteobacteria, Acidobacteria, and Actinobacteria (Glamoclija et al. 2004a). The analysis revealed the occurrence of thermophilic or thermostolerant iron and hydrogen oxidizers or methane/methanol consumers, although many phylotypes were too distantly related to cultivated species to infer potential metabolic properties (Glamoclija et al. 2004a). To understand the adaptive strategies and the ecological role of the microbial communities in these acidic and high-temperature environments, it is important to identify those metabolic features that allow them to thrive in these extreme conditions.

Our study focused on profiling at high-resolution both the archaeal and bacterial components of high-temperature and extremely acidic soils in Solfatara Crater by combining results of Next Generation Sequencing with single-cell quantification assays. The study plan also included the integration of geochemical and microbiological data to develop an overview of soil community composition in relation to different degree of enrichments in deep-origin gases and elements.

Materials and methods

Study site: Solfatara Crater

Solfatara Crater (Fig. 1) hosts one of the largest fumarolic discharges worldwide (mostly represented by Bocca Grande and Bocca Nuova fumaroles), whose associated thermal energy flux was estimated to be ~100 MW (Chiordini et al. 2001). Most thermal energy is released by widespread diffuse degassing fed by a 1.5-2 km deep column of an ascending mixture of (i) magmatic gases (about 26 %) released by the degassing of a magma body at ~5 km depth (Gottsmann et al. 2006) and (ii) hydrothermal fluids (about 74 %) originated by the boiling of overlying aquifer(s) (e.g. Chiordini et al. 2001; Caliro et al. 2007; Chiordini et al. 2015b; Cardellini et al. 2017). Whilst fumarolic activity is mainly restricted to the south- and north-eastern sectors of the crater, diffuse degassing is widespread and anomalous soil CO2 fluxes nearly affect the entire bottom of the crater. According to Cardellini et al. (2017), a total soil CO2 output varying from 1116±138 to 1524±6164 tons/day was recorded in 2012-2013, which is comparable to the CO2 flux associated with a "medium-large" volcanic plume from persistently degassing volcanoes such as Soufrière Hills or Mount Erebus (Burton et al. 2013; Cardellini et al. 2017).

In January 2016, five soil sampling sites at Solfatara Crater were selected together with two acidic mud pools in the Fangaia area for biogeochemical investigations (Fig. 1). The selection of the sites for collecting soil and mud samples was based on field measurements of temperature and diffuse CO2 flux. Soil samples were collected at 10 cm and 30 cm depth for subsequent analyses. The soil samples strongly affected by fumarolic gas emissions (S1, S2, S3) were characterized by a whitish coverage typically produced by sulfuric acid. Sampling sites S4 and S5 were collected from hydrothermalized terrains far from fumarolic emission and covered by typical Mediterranean vegetation. Muds S6 and S7 were sampled from the acidic Fangaia mud pools.

Sampling methods and field measurements

At each site, diffuse CO2 (ΦCO2) and CH4 (ΦCH4) fluxes from the soil were measured according to (i) the "accumulation chamber (AC) method" (Chiordini et al. 1998) and (ii) the "static closed-chamber (SCC) method" (Tassi et al. 2013), respectively. Equipment and procedures are described in Tassi et al. (2015a). Interstitial soil gases were collected from sites S1 to S5 by inserting a 1 m long stainless-steel tube down to the sampling depth (10 and 30 cm at each site). At 10 and 30 cm the soil temperature was measured with a Thermocouple Sensor K 3K350. The soil gases were stored into two 12 cm³ Labco Exetainer® glass vials, equipped with a porous membrane, for subsequent chemical and isotopic analysis. The sampling procedure is described in Tassi et al. (2015b). Soil samples for chemical and microbiological analyses were collected at 10 and 30 cm depth using sterilized spoons. Aliquots for microbiological analyses were immediately fixed with formaldehyde solution (final concentration 1%) and stored at 4 °C for total cell counts and fluorescence in situ hybridization. Aliquots of 15 mL for DNA extraction were placed in dry ice immediately after collection and then stored in laboratory at -20 °C until further processing. Soil samples for chemical analyses were stored and sealed in plastic bags.
Bubbling gases from sites S6 and S7, i.e. the Fangaia mud pools, were conveyed to a pre-evacuated 60 mL glass flask, equipped with a Thorion® valve and containing 20 mL of 4 M NaOH solution (Giggenbach, 1975), using an inverted funnel positioned above the bubbles. A glass vial for the analysis of VOCs was also collected. Water samples from the mud pools were filtered at 0.45 µm and collected in 125 mL polyethylene bottles. Temperature, pH and Eh of pool waters were measured in situ. Mud samples for chemical and microbiological analyses were collected from the pools by plastic bags and sterilized tubes, respectively. Mud aliquots for microbiological and chemical analyses were preserved as described for soil samples.

Chemical and isotopic analyses of gas samples

Inorganic gases both in the headspace of the bubbling pool sampling flasks (N₂, O₂, H₂, Ar, CO) and in the glass vials (CO₂, H₂S, N₂, O₂, Ar) were analyzed using a Shimadzu 15A gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a 10 m long 5A Molecular Sieve column or, for CO₂ and H₂S analysis in the glass vials, a 3 m long column packed with Porapak Q 80/100 mesh. Light hydrocarbons (C₁-C₄) were analyzed using a Shimadzu 14A gas chromatograph equipped with a Flame Ionization Detector (FID) and a 10 m long stainless-steel column packed with Chromosorb PAW 80/100 mesh coated with 23 % SP 1700. CO₂ and H₂S dissolved in the alkaline solution of the flasks were analyzed as CO₂⁻ by automatic titration (AT) with 0.5 M HCl solution (Metrohm 794 Basic Titirno) and SO₄²⁻ by ion chromatography (Metrohm 761 Compact IC) after oxidation with H₂O₂ (Montegrossi et al. 2001; Vaselli et al. 2006). Volatile Organic Compounds (VOCs) were analyzed, after Solid Phase Micro Extraction (SPME; Arthur and Pawliszin, 1990), by using a Thermo Trace GC Ultra gas chromatograph coupled to a Thermo DSQ Quadrupole Mass Spectrometer. The SPME and analytical procedures are described in Tassi et al. (2015a), together with instrumental specifications. Analytical errors for AT, GC and IC were <5%.

The ¹³C/¹²C ratios in CO₂ (expressed as δ¹³C-CO₂ % V-PDB) were determined by using a Finnigan Delta Plus mass spectrometer after a two-step extraction and purification procedures of the gas mixtures by using liquid N₂ and a solid-liquid mixture of liquid N₂ and trichloroethylene (Evans et al. 1998; Vaselli et al. 2006). Internal (Carrara and San Vincenzo marbles) and international (NBS18 and NBS19) standards were used for estimation of external precision. The analytical uncertainty and the reproducibility were ±0.05‰ and ±0.1‰, respectively.

Chemical analysis of soil, mud and water samples

Soil and mud samples were dried at 40 °C for 48 hours, sieved at 2 mm and ground with a planetary agate ball mill until an impalpable powder was obtained. Loss On Ignition (L.O.I.), i.e. the total volatile content, was determined by gravimetry. The powdered sample was dried at 110 °C for 8 hours, weighted (dry weight) and heated at 950 °C for 2 hours using a CEM microwave oven. The L.O.I. values were expressed as percentage by weight. The bulk chemical composition of soil samples was analyzed by X-ray fluorescence (XRF) on pressed powder pellets, using a Rigaku II wavelength dispersive spectrometer equipped with a Rh anode at CRIST (Center of Structural Crystallography – University of Florence). The composition of the main and trace elements was acquired using a ZSX Rigaku Software Package version 3.50 and determined with the SQX semi-quantitative element analysis routine. The Fundamental Parameter (FP) software was used to allow elemental quantification of the samples without standards.

The pH of the soils was potentiometrically measured in the supernatant suspension of 1:2.5 soil:KCl (1M) solution mixture (ISRIC 1995; Peech 1965). For each sample, 10 g of soil were amended with 25 mL of KCl 1 M solution. Before measuring the pH, the obtained mixture was shacked for two hours and then centrifuged at 5000 rpm for 5 min.

Soluble elements were determined through H₂O extraction by adding 100 mL of MilliQ water to 5 g of soil and mud samples and mechanically stirring overnight for 8h. The final solution, obtained by filtering (0.45 µm) the leachate, was acidified by adding 1 mL of Suprapur® HNO₃ and analyzed by ICP-OES using a PerkinElmer Optima 8000 (PerkinElmer, Waltham, MA, USA) for Mn, Fe, Co, Ni, Cu, Zn, As, Sb, Al, Hg, W, Si, Pb, P and Cd. The analytical error was <10 %.

Water samples from the Fangaia pools were analyzed by ion-chromatography using an 861 Advanced Compact IC-Metrohm for cations (Na, K, Mg, Ca, Li) and a 761 Compact IC-Metrohm for anions (F, Cl, Br, SO₄, NO₃). The analytical error was <3 %.

Total prokaryotic abundance, CARD-FISH and NGS

The total prokaryotic abundance was estimated by DAPI staining, and Bacteria and Archaea abundances by Catalyzed Reported Deposition - Fluorescence in situ Hybridization (CARD-FISH) following the protocol optimized by Fazi et al. (2007, 2013). Specific rRNA-target Horseradish peroxidase (HRP) labeled
Bacterial/Archaeal 16S rRNA gene sequencing libraries were prepared by the Marine Biological Laboratory, Woods Hole, MA, in the framework of Census of Deep Life Sequencing Opportunities 2016. The v4v5 regions of the 16S rRNA gene were amplified separately for bacteria and archaea. Bacterial 16S rRNA gene was amplified in triplicate using primers 518F (5′ - CCACGACGCTGGAAN -3′) and a combination of three reverse primer variants 926R (5′-CCGCTAATTCNCTTTRAGT CCGTCAATTTCTTTTGGAGT CCGTCAATTCCTTTTGGANT -3′) as previously described (Huse et al. 2014a). The archaeal v4v5 16S rRNA gene was amplified in triplicate using a combination of five forward primer variants (527F; GCCTAAAGCATTACGTAGC, GCCTAAARCCTGCYCGTAGC, GTCTAAACGGCTCGTAGC, GCCTAAAGNCTGCYCGTAGC, GTCTAAACGGCCYCGTAGC) and a single reverse primer (958R; CCGCCGTGANTCCATT) (Topçuoğlu et al. 2016). The amplification products were visualized on a Caliper High Sens assay (Perkin Elmer, Waltham MA), quantified by PicoGreen assay (Life Technologies, Grand Island NY) and pooled in equimolar concentrations. The pool was sequenced as paired-end 250 cycle reads on the Illumina Miseq (Illumina, San Diego CA). The sample datasets were demultiplexed using a combination of Miseq Reporter software and custom python scripts. Data processing and diversity analyses were performed at the MBL. Specifically, paired reads were merged to produce high-quality full-length v4v5 sequences and the taxonomic affiliation of each unique sequence was assigned using GAST (Huse et al. 2008). GAST assigns taxonomy based on the results of VSEARCH (Rognes et al., 2016). Alignments must cover 80% of the read length and 66% of the matched sequences must share the same taxonomic string. If the reference taxonomies are discordant, the assignment is moved up a taxonomic level until 2/3 consensus is reached. Sequences that shared the same taxonomic assignment were grouped into OTUs. The results were uploaded to the VAMPS web site (Visualization and Analysis of Microbial Population Structures, http://vamps.mbl.edu; Huse et al. 2014b). Downstream analyses used operational taxonomic units (OTUs) that were comprised of tags having the same taxonomic assignment.

Statistical analysis

The non-parametric Mann-Whitney U test was applied to verify the statistical differences between strongly and not-strongly affected samples by hydrothermal fluids for all physical, chemical and microbial parameters (Clarke et al. 1993). The chemical variables were incorporated into a Non-metric MultiDimensional Scaling ordination plot (NMDS) in order to graphically synthesize the Euclidean dissimilarity between soil samples. Chemical and microbial data were then projected onto the NMDS ordination using a vector-fitting procedure, in which the length of the arrow is proportional to the correlation between NMDS-axes and each variable. This method allowed determining the variation pattern of each projected variable discriminating the samples (Foulquier et al. 2013; Amalfitano et al. 2014). Chemical data were log-transformed, whereas values of microbial groups revealed by NGS (clusters > 1% of total OTUs were considered) were normalized by log(X+1). Shannon and Simpson indices for each sampling site were also generated using PAST software (PALAEONTOLOGICAL STATISTICS, ver. 2.17) (Hammer et al. 2001).

Results

Physicochemical characteristics of the sampling sites

The variation patterns of major physicochemical parameters differentiated samples in transition from the area not affected by hydrothermal fluids (S4, S5) relative to the most affected one (S1, S2, S3 and Fangaia mud pools), as revealed by NMDS analysis (Fig. 2).

Sites S1, S2 and S3 were characterized by temperature-depth gradients ≥1.1 °C/cm, with soil temperatures at 10 cm depth ranging from 26.3 (S3) to 42.9 °C (S2) and from 50.6 (S3) to 60.1 °C at 30 cm depth (S2). The pH values ranged from 1.3 to 1.7 at S1 and S2, whereas they were slightly higher (from 2.0 to 2.2) at S3. The ΦCO2 and ΦCH4 values ranged from 557 to 3,350 and from 0.0021 to 0.059 g m⁻² day⁻¹, respectively, in agreement with those previously measured at Solfatara Crater (Cardellini et al. 2003; Tassi et al. 2013). The chemical composition of the interstitial soil gases was
dominated by CO$_2$ (943 mmol/mol), with relevant concentrations of H$_2$S and CH$_4$ (up to 1.9 and 0.091 mmol/mol) and low O$_2$ contents (from 0.32 to 6.1 mmol/mol at 30 cm depth). The δ$^{13}$C-CO$_2$ values ranged from -1.86 to -1.1 % vs. V-PDB and increased passing from 30 cm to 10 cm depth (Table 1). The organic gases mostly consisted of alkanes (up to 73 % ΣVOCs) and aromatics (up to 27 % ΣVOCs), whilst cyclic, O-substituted and S-bearing and alkene compounds were ≤4.5, ≤3.2, ≤1.1 and ≤0.30 % ΣVOCs, respectively (Table 2).

Soils at S1, S2 and S3 were characterized by L.O.I. values ranging from 4.8 up to 9.9 wt% and relatively high concentrations of SiO$_2$ (up to 91 wt%), followed by Al$_2$O$_3$ (up to 4.5 wt%), S (up to 2.8 wt%), K$_2$O (up to 2.7 wt%) and TiO$_2$ (up to 1.35 wt%). Fe$_2$O$_3$, CaO, Na$_2$O, Ba, P$_2$O$_5$ were detected at concentrations ranging from 0.10 to 0.48 wt%, whilst Sr, Cl, MgO, Nb, MnO, Pb, Rb, Zr and Cu were ≤0.06 wt%. Cr, As, Y and Hg were only occasionally detected (Table 3). Al, Fe and Si were the most abundant metals measured in the leachates (Table 4). Cu, Mn, Zn, P, As and Pb were present at concentrations ranging from 1.1 to 7.2 mg/L, whilst the contents of W, Ni, Co, Cd and Sb were ≤0.73 mg/L (Table 4).

Relatively highly acidic pH (<2.1) and high temperatures (between 40 and 70 °C) were measured for the S6 and S7 muds. The chemical composition of the bubbling gas from Fangia mud pool was similar to that of soil gases from sites S1, S2 and S3, i.e. largely dominated by CO$_2$ (987 mmol/mol) and H$_2$S (1.88 mmol/mol) (Table 1). Significant H$_2$ and CH$_4$ contents (1.85 and 0.25 mmol/mol, respectively), markedly higher than those recorded in soil gases, were measured. Carbon monoxide was also found at detectable amount (0.015 mmol/mol), whilst O$_2$ content was 0.011 mmol/mol. The organic gas fraction was largely dominated by ethane and benzene (Table 2). The chemical composition of the waters was characterized by high concentrations of SO$_4^{2-}$ (~1.800 mg/L) and pH values of 1.62-1.75 (Table 5). Mud samples showed relatively high L.O.I. values (Table 1). The leachates had relatively high concentrations of Al, Fe and Si, as well as of As, Cu and P (Table 4). The concentrations of Mn, Zn, W, Ni, Co, Sb, Pb and Cd were ≤0.52 mg/L.

Sites S4 and S5 displayed soil temperature gradients ≤0.1 °C/cm and temperatures ≤17.4 °C, whilst the pH values ranged from 2.8 to 3.6 (Table 1). The ΦCO$_2$ and ΦCH$_4$ values were ≤161 and ≤0.00046 g m$^{-2}$ day$^{-1}$, respectively. Interstitial soil gases were enriched in atmospheric components, i.e. N$_2$, O$_2$ and Ar. The CO$_2$ contents largely differed among these sites, being ≥330 mmol/mol at S4 and ≤30 mmol/mol at S5. Similarly, H$_2$S and CH$_4$ concentrations of S4 were higher than those of S5 (Table 1). The δ$^{13}$C-CO$_2$ values at S4 were 0.6 and -0.07 % vs. V-PDB at 10 and 30 cm depth, respectively. VOCs were below detection limit at S5, whereas at S4 they were dominated by alkanes (up to 61 % ΣVOCs) and aromatics (up to 24 % ΣVOCs) (Table 2). Whilst relative abundances of alkenes (up to 0.20 % ΣVOCs) and cyclic compounds (up to 5.01 % ΣVOCs) were similar to those of S1, S2 and S3, S-bearing species exhibited lower concentrations (<0.84 % ΣVOCs) and those of O-substituted species were higher (up to 23 % ΣVOCs).

Soils were characterized by L.O.I. values ranging from 5.8 to 15 wt% (Table 1) and dominated by SiO$_2$ (up to 86 wt%), followed by Al$_2$O$_3$ (up to 5.2 wt%) and K$_2$O (up to 2.7 wt%). TiO$_2$ and S were up to 0.95 and 1.2 wt%, whereas Fe$_2$O$_3$, CaO, Na$_2$O, MgO, P$_2$O$_5$, Cl and MnO were less than 1.9, 0.72, 0.38, 0.22, 0.21, 0.12 and 0.02 wt%, respectively (Table 3). Ba, Zr, Sr, Pb, Nb, Rb and Cu were present at concentrations ranging from 0.004 to 0.13 wt%, whereas those of Cr and As were below the instrumental detection limit. Ni, Zn and Br were only detected in soils from S5. The chemical composition of leachates showed relatively high concentrations of Al, Fe and Si (Table 4).

### Microbial community composition

Overall, CARD-FISH analysis showed that approximately 74.3 % of total DAPI-stained cells represented Bacteria and Archaea. On average, 36.6 % were Archaea and 37.7 % belonged to Bacteria domain. Abundance of Archaea varied between 6.1 x 10$^6$ and 3.2 x 10$^7$ cell/g and bacterial cell abundance ranged from 9.4 x 10$^8$ – 4.2 x 10$^7$ cell/g (Fig. 3). A total of 1,902,856 reads that mapped to Archaea resolved into 43 OTUs. The archaeal 16S rRNA gene fragments resolved into 2 main phyla: (i) *Europaarchaeota* dominated the S1, S2, S3, S6, and S7 samples (on average 96.5 % of total OTUs); (ii) *Thaumarchaeota* dominated S4 and S5 samples (on average 89.7 % of total OTUs). *Crenarchaeota* phylum represented low-abundance taxa only in S2 and S3 deep samples (Fig. 4).

*Europaarchaeota* phylum was largely represented by the genera *Acidiplasma* (up to 75.8 % in S6), *Ferroplasma* (more than 90 % in S1-10, and S7 samples) and *Thermoplasma* (on average 91.5 % of total OTUs in S3 sample). On average, more than 30 % of total OTUs affiliated with the *Thaumarchaeota* terrestrial group in S4 and S5 samples, a large number of OTUs that affiliated with this phylum (up to 96.7 % of total OTUs in S4-30) could not be identified at a more specific level and
were described as ‘other Thaumarchaeota’. Crenarchaeota were represented by members affiliated with the Sulfolobaceae family, mainly belonging to Acidianus brierleyi species.

A total of 2,122,238 reads were generated using the bacterial v4-v5 primers and they resolved into 921 OTUs. Proteobacteria were the most abundant phylum at S1, S2, S3, S6, and S7 (on average 77.5 % of total OTUs), with Gammaproteobacteria belonging to Acidithiobacillus genus ranging from 29.5 % to 87.8 % of total OTUs. Alpha-, Beta- and Deltaproteobacteria were mainly retrieved from S2-30 and S3, with relative abundances ranging between 2.5 - 17.2 %, 0.1- 2.2 % and 0.4 - 2.3 %, respectively.

Firmicutes retrieved in sites S1, S2, S3, and S7 (range 5.8 - 14.5 % of total OTUs) were mainly affiliated with the Sulfolobus (S1, S2, and S3) (range 1.2 - 10.3 %) and Bacillus (S2-30) (9.1 %). Actinobacteria (average 7.1 %) were predominantly represented by the Acidimicrobiales order and Mycobacterium genus. OTUs affiliated to Thermotogae phylum were retrieved at relatively low abundances in all samples (< 0.1 %) with the exception of sample S6 where they represented 40.9 % of total OTUs. Members of Nitrospirae phylum that primarily affiliated with Leptospirillum genus, represented 5.6 % and 1.9 % of total OTUs in S3 -10 and S3 - 30 samples, respectively (Fig. 5).

Remarkably, sites S4 and S5 harbored completely different bacterial community compositions. The Shannon index showed the highest values in these samples (range between 1.9 and 3.7) and the lowest ones in all other samples (on average 1.2). Similar trends were also found using the Simpson index showing values on average equal to 0.4 in all samples. In S4 and S5 samples the main phyla retrieved were Acidobacteria (36.7 % of total OTUs), Chloroflexi (25.7 %), Proteobacteria (14.1 %), Actinobacteria (5.7 %), Firmicutes (4.1 %), Bacteroidetes (2.9 %), Planctomycetes (2.7 %), and Verrucomicrobia (1.1 %). Between 4 % and 12 % of bacterial OTUs were not identified at the phylum level in S4 sample by GAST (see ‘other bacteria’ in Fig. 5). The majority of these ‘bacterial’ OTUs (up to 99%) showed high sequence identity to Nitrososphaerae and “Candidatus Parvarchaeum” when the sequences were queried against the full NCBI nucleotide database (Altschul et al. 1990, 1997) and the RDP classifier database (Wang et al. 2007). Acidobacteria were affiliated with Acidobacteriales order and Bryobacter genus, although a large amount (up to 39.3 % in S4-10) was not identified at genus level. Chloroflexi were mainly affiliated with Ktedonobacterales order (up to 53 % in S4-30), mostly belonging to Ktedonobacteraceae and Thermosporotrichaceae families. Proteobacteria showed the highest percentage in S5 (up to 25 %), represented by Alpha- (Rhodospirillales), Betaproteobacteria (Neisseriales) and Gammaproteobacteria (Haemophilus genus). OTUs affiliated to Streptococcus genus, belonging to Firmicutes phylum, represented more than 11 % of total OTUs in S5-10 (Fig. 5).

Discussion

Geochemical features

The selected sites showed a wide range of environmental conditions, in terms of temperature, pH, and supply of hydrothermal fluids. The latter can roughly be estimated from the measurements of CO₂ diffuse fluxes from the Solfatara Crater floor. Among the measuring sites, the highest ΦCO₂ values were measured at S1, S2 and S3, where temperatures ranged from mesophilic (at 10 cm depth) to thermophilic (at 30 cm depth) values. Similarly, the ΦCH₄ values were up to 2 orders of magnitude higher than those measured in sites S4 and S5, suggesting a strong contribution of hydrothermally-derived fluids. Accordingly, the chemical composition of interstitial soil gases collected at both 10 and 30 cm depth in sites S1, S2 and S3 approached that of the fumarolic gas emissions, being dominated by CO₂ and characterized by relatively high contents of H₂S and CH₄, as well as ethane, benzene, alkenes and S-bearing compounds. The isotopic composition of CO₂ in the interstitial soil gases from S1, S2 and S3 (from -1.86 to -1.1 ‰ vs. V-PDB) was consistent with that reported for the fumarolic emissions from Solfatara (i.e. from -2 to -0.95 ‰ vs. V-PDB; Chiodini et al. 2008; Vaselli et al. 2011; Tassi et al. 2015a), confirming a dominating hydrothermal fluid input. The strong uprising of hydrothermal fluids was responsible for the peculiar acidic conditions (pH ≤2.2) measured in these sites, since H₂S is rapidly oxidized, either abiotically or biotically, under aerobic conditions to form sulfuric acid that affects minerals producing whitish deposits, as those recognized in the Solfatara crater floor and at S1, S2 and S3 (Fig. 1). When compared to the parent material (Turney et al. 2008 and references therein), the analyzed soil samples were enriched in immobile elements, such as Ti and Si (Fig. 6a), and depleted in mobile species, i.e. Na, Ca, K and Mg (Fig. 6a), as well as Mn and Cl (Fig. 6b), testifying intense alteration processes. The depletion in Al and Fe observed in the soil samples with respect to volcanic rocks was related to the acidic conditions, which increase the mobility of these metals during leaching processes. The relatively high S concentrations (up to 2.79 wt%), e.g. those measured in S1, S2 and S3, confirmed the strong interaction with hydrothermal H₂S-rich fluids. Relatively high concentrations of Al, Fe, Cu, Mn, Zn, P, Pb, W, Cd, Co and Ni were measured in the leachates from S1, S2 and

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S3 soil samples (Table 4), suggesting that these metals were available for uptake due to the low pH conditions. Similarly, the S6 and S7 samples were characterized by a relatively high mobility of metals such as Fe, Al, Cu, P, Sb, Co, Ni and, particularly, As, which reached concentrations ~3 mg/L in the leachates. The occurrence of such peculiar high concentrations of metals in the Fangaia muds was likely caused by an enhanced hydrothermal fluid contribution. Waters collected from S6 and S7 displayed temperatures in the thermophilic range and a typical sulfate-acidic composition, which was likely produced by the condensation of hydrothermal fluids into near-surface groundwater. The relatively high CH4 and H2 concentrations in the bubbling gas from Fangaia mud pools with respect to those measured in the fumarolic vents may be related to a higher contribution of reduced hydrothermal fluids. The markedly high concentrations of ethane and benzene in the Fangaia gases support this hypothesis.

In contrast, S5 was characterized by N2-dominated interstitial soil gas compositions and relatively high O2 contents (≥138 mmol/mol), corresponding to the lowest ΦCO2 and ΦCH4 values, pointing to a low supply of hydrothermal fluids. This was confirmed by both psychrophilic soil temperatures and relatively high pH values. The 10 and 30 cm samples from S5 showed relatively high concentrations of metals with respect to those recorded in other soil samples but they were depleted relative to the parent volcanic rocks. This implies a less intense hydrothermal alteration process with respect to the other sampling sites. The higher pH values produced the observed lower mobility of metals, except for P, the latter being enriched in S5 at 10 cm depth with respect to the P content measured in the parent material. Such enrichment likely reflects the decomposition of organic matter. Accordingly, S5 was located in a portion of the crater covered by luxuriant vegetation, i.e. a typical mesophilic Mediterranean forest (Fig. 1). A circumscribed vegetation cover was also present at S4, which was totally enclosed among hydrothermalized terrains (Fig. 1). Here, CO2 was the most abundant gas species at 30 cm depth, where increased concentrations of H2S, CH4, ethane, benzene, alkenes and S-bearing organic compounds from the hydrothermal source were also measured.

Hints of possible biochemical processes affecting C-bearing species in soil gases

The correlation among (i) CO2, CH4 and H2S contents, (ii) high temperatures, (iii) acidic conditions, and (iv) CO2 and CH4 fluxes clearly indicates a hydrothermal origin for both CO2 and CH4 in the interstitial soil gases from the analyzed sites throughout the Solfatara Crater. A dominant deep CO2-rich source was confirmed by the carbon isotopic composition in S1 soil gases, which resembled that of the fumarolic emissions. Moving from S1 to S4, the isotopic composition of CO2 progressively diverged from that of the fumarolic discharges, increasing up to 0.6 ‰ vs. V-PDB in S4 at 10 cm depth. Despite the progressive depletion in CO2 and enrichment in atmospheric gases (N2, O2, Ar) suggested the occurrence of a certain air dilution, a mixing of hydrothermally-derived CO2 with air (δ13C-CO2 value around -8 ‰ vs. V-PDB; Scripps CO2 Program) would lead to a progressive decrease in δ13C-CO2 values. Hence, a simple dilution processes cannot explain the observed geochemical features. Hence, fractionation processes able to modify the isotopic signature of hydrothermal CO2 were likely occurring. The increase of the δ13C-CO2 values from 30 cm to 10 cm depth at S1, S2, S3 and S4 is consistent with the occurrence of isotopic fractionation processes affecting the deep-sourced CO2 in the shallowest portions of the system. According to Federico et al. (2010), the carbon isotopic composition of interstitial soil gases is expected to be controlled by fractionation effects during the CO2 uprising through the soil. However, no clear relationship between δ13C-CO2 variations from 30 to 10 cm depth and ΦCO2 values were observed (Table 1). On the other hand, the relative enrichment in 13CO2 observed in the soil gases at decreasing CO2 contents could be related to biochemical processes. Autotrophic CO2 fixation preferentially consumes the isotopically light CO2, causing a 13C enrichment in residual soil CO2 compatible with the observed increasing trend in δ13C-CO2 values at decreasing CO2 contents in soil gases (Table 1).

The CO2/CH4 ratio in the soil gases increased from S1 to S4, suggesting that deep-sourced CH4 was consumed more efficiently than CO2 at decreasing hydrothermal fluid input. Although CH4 can be considered almost chemically inert, the presence of specific inorganic catalysts (minerals, metals) or the enzymatic activity of methanotrophs may allow either abiotic or biotic oxidation of CH4, respectively. The increase of the CO2/CH4 ratio from 30 cm to 10 cm depth at S1 to S4 suggests a significant consumption of deep-sourced CH4 at shallow depths in the soil. Particularly favorable conditions for the development of CH4 consumption processes seem to characterize S4 and S2 at 10 cm depth. In contrast, the peculiarly low CO2/CH4 ratio at S5 indicates prevailing CO2 consumption processes over CH4 oxidation. The CO2 content in the S5 soil declined by 50% between 30 cm and 10 cm depths.

As observed for CO2 and CH4, evidences of shallow secondary processes affecting the hydrothermal fluids during their migration through the soil towards the atmosphere can also be recognized among VOCs. Hydrocarbons (alkanes, aromatics, alkenes) and S-bearing compounds were detected at higher concentrations in soil gases from S1, S2 and S3 with respect to S4 (Table 2). Their contents in soil gases decreased from 30 cm to 10 cm depth at each sampling site, pointing to a deep origin of these compounds. Accordingly, alkanes, aromatics, alkenes and S-bearing species were recognized as the major organic constituents in fumarolic
gases from Solfatara Crater (Tassi et al. 2015a). Similarly, cyclics showed higher amounts in soil gases from (i) S1, S2 and S3 with respect to S4, and (ii) at 30 cm depth relative to 10 cm depth in each site. The production of cyclics is thought to occur at relatively great depth through incomplete aromatization of alkanes at temperature <150 °C (Tassi et al. 2012, 2015a; Venturi et al. 2017b). The O-bearing compounds (e.g. aldehydes, ketones, carboxylic acids) showed an opposite trend, since increasing concentrations passing from S1, S2 and S3 to S4 and from 30 cm to 10 cm depth were measured. This was likely due to increasing oxidizing conditions related to the availability of free O₂, being the O-bearing compounds typical by-products of metabolic pathways of microorganisms. For instance, ketones and aldehydes may result from the consumption of alkanes under aerobic conditions (e.g. Fritzche and Hofrichter, 2008; Rojo, 2009). At S1, S2 and S3 the C₁₀/C₆ alkane ratio in soil gases decreased approaching the surface, suggesting the occurrence of efficient consumption of long-chain alkanes at shallow depth. Differently, C₁₀ alkane were enriched in the S4 soil gases, whose C₁₀/C₆ alkane ratio increased approaching the soil-air interface, pointing to a shallow source of long-chain saturated hydrocarbons. The latter might be represented by the degradation of organic matter since, differently with respect to what observed at S1, S2 and S3, a vegetation cover was present at site S4.

**Microbiological features**

Due to its extreme and harsh conditions, some investigators regard Solfatara as a plausible terrestrial analogue for Martian hydrothermal systems where physicochemical conditions might be comparable to those of the Archaean Earth (e.g. Glamocilja et al. 2004a, 2004b, 2004c; Sgavetti et al. 2009). Previous microbiological studies (Huber et al. 2000) recognized the Solfatara Crater, together with Yellowstone National Park (U.S.A.), as model systems for studying thermophilic and hyper-thermophilic microorganisms in hydrothermal environments. Hot springs, mud holes and solfataric fields are natural biotopes of thermophilic and hyperthermophilic microorganisms due to their extreme chemical-physical characteristics. Other than Solfatara and Yellowstone, the most studied hydrothermal environments worldwide are those located in Kamchatka peninsula (Russia), the Taupō Volcanic Zone (New Zealand) and Krisuvik, Hveragerði and Kerlingarfjöll (Iceland) (Huber et al. 2000). A comparison of the biodiversity and community composition in high-temperature, acidic, sulfur-rich sites outlined common microbiological features driven by extreme environmental characteristics. In particular, members belonging to *Sulfolobus, Acidithiobacillus, Hydrogenobaculum* were frequently recovered (Kivist et al. 2007; Mathur et al. 2007; Inskipp et al. 2010, 2013; Lin et al. 2015; Colman et al. 2018). At lower extent, members of *Sulfurospirillum, Nanoarchaeum* (Inskipp et al. 2010, 2013; Hou et al. 2013; Sahn et al. 2013; Menzel et al. 2015), and *Acidianus* genera were also observed (Stout et al. 2009; Menzel et al. 2015).

This study provides the first detailed description of microbial communities from the Solfatara soils and sediments using high-throughput sequencing and in-situ quantification of Archaea and Bacteria. Overall, CARD-FISH analysis showed a nearly equal occurrence of archaeal and bacterial cells in all screened samples with cell densities (up to 3.2 x 10⁴ and 4.2 x 10⁴ cell/g for Archaea and Bacteria, respectively - Fig. 3) in the range of those observed in previous studies in analogous environments (Huber et al. 2000; Inskipp et al. 2010). The sequencing data analysis clearly showed the strong influence of the Solfatara physicochemical characteristics on the structure and composition of the mixed microbial communities. A marked distinction in the microbial composition from the different communities between sites highly affected by hydrothermal fluids (S1-S2-S3-S6-S7) and sites characterized by lower temperature and higher O₂ concentration (S4-S5) (Fig. 7) was recorded. Low levels of archaeal and bacterial biodiversity and equitability were found in S1, S2, S3, S6 and S7, typically characterized by elevated temperatures and very low pH values. Higher bacterial biodiversity and concomitant distinctive structure of the archaeal community were observed in S4 and S5 characterized by lower temperature (<17.4 °C) and slightly higher pH values (pH= 2.8 - 3.6). Consistent with the extremely acidic conditions and the high concentrations of metal observed in S1-S2-S3-S6-S7, most of the OTUs retrieved in Archaea domain were affiliated with acidophilic members of family *Ferroplasmaceae*. *Ferroplasma* were found in environments characterized by high temperature (> 50 °C), suggesting the possibility of the existence of thermophilic members of this genus (Uribeta et al., 2012; Menzel et al., 2015). At the species level, the analysis revealed the occurrence of “*Ferroplasma acidarmanus*”, a facultative anaerobe previously observed in acidophilic hot environments with the temperature of approx. 40°C (Edwards 2000; Aliaga-Golsman et al. 2015). This microorganism is able to grow at pH ranging between 0 and 1.5 either chemoorganotrophically or chemomixotrophically on Fe(II) and a variety of carbon sources (yeast extract, casaminoacids, and sugars) (Dopson et al. 2004). It can also proliferate, although at low growth rate, by coupling oxidation of organic carbon sources to Fe(III) reduction (Dopson et al. 2004).

The prevalence of *Acidiplasma* spp., facultatively anaerobic, acidophilic and thermophilic archaea able to oxidize and reduce iron, was specifically found in S6, which is characterized by the highest temperature registered among the screened sampling sites (70 °C) (Golyshina et al., 2009). As often observed in acidic
solfataric environments (Segerer et al. 1986; Benson et al. 2011), the acidic hot and oxygen-exposed surface of terrestrial fields typically harbor aerobic or facultative aerobic archaiae such as *Acidimicrobium" found in S2 and S3 samples at lower abundance relative to *Ferroplasma*. The occurrence of Archaea capable of oxidizing sulfur and inorganic sulfur compounds is in line with the biotic H₂S oxidation observed in S2 and S3.

Furthermore, in metal-rich samples (S1-S2-S3-S6-S7) with particular high As concentrations, the analysis revealed the occurrence of microorganisms potentially involved in arsenic detoxification and metabolic reactions, such as members of genera *Acidiplasma*, *Ferroplasma* and *Thermoplasma* (Baker-Austin et al. 2007; Volant et al. 2012; Yelton et al. 2013). Arsenic is one of the most prominent heavy metals in high temperature environments reflecting the occurrence of thermophilic As-transforming microorganisms (Huber et al. 2000; Hug et al. 2014; Wheaton et al. 2015). The possible involvement of hyperthermophiles in the As-cycle from these environments is consistent with the previously reported occurrence of arsenate-reducing prokaryotes in Pisciarelli Solfatara and the isolation of *Pyrobaculum arsenaticum* (Huber et al. 2000). Members of *Thermoplasmata* class could also be involved in anaerobic methane oxidation, as frequently observed in sediments (Inagaki et al. 2006; Roalkvam et al. 2011; Avrahamov et al. 2014).

Completely different archaean communities were observed in S4 and S5 (Fig. 7). These sites were characterized by both relatively higher pH values, lower temperatures, lower H₂S and higher O₂ and N₂ concentrations relative to those sites strongly affected by the presence of hydrothermal fluids. The large majority of OTUs was affiliated with *Thaumarchaeota*, a deep-branching phylum within Archaea domain. Members of this phylum represent the most abundant Archaea on Earth and include all known autotrophic archaean ammonia oxidizers as well as environmental sequences representing microorganisms with unknown metabolism (Stuegmeier, Alves and Schleper 2014). However, comprehensive information about energy metabolisms of *Thaumarchaeota* is still lacking. Thus, the involvement of members of this phylum in methane oxidation cannot be excluded. It is well known, that ammonia monooxygenase and methane monooxygenase enzymes are evolutionarily linked (Holmes et al. 1995), leading to functional similarities enabling both methanotrophy and ammonia oxidation (O’Neill and Wilkinson 1977; Jones and Morita 1983; Zheng et al. 2014).

Only a few OTUs affiliated with archaean groups that typically include methanogens (e.g. orders *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales*) were detected by the sequencing analysis thus likely indicating a marginal contribution of methanogenesis to the overall metabolic traits of the microbial communities living in this environment.

Among bacteria, most of the OTUs retrieved from S1, S2, S3, S6 and S7 samples belonged to *Acidithiobacillus* genus, which includes acidophilic aerobic bacteria able to autotrophically grow on iron and/or sulfur compounds (Dopson and Johnson 2012). In addition, some strains of *Acidithiobacillus* genus are able to grow under anaerobic conditions via Fe³⁺ respiration, using elemental sulfur, tetrathionate or hydrogen as electron donor (Hedrich and Johnson 2013). Members of this genus were previously observed at Campi Flegrei and in other acidophilic hot hydrothermal springs, rivers and pools worldwide (Sahm et al. 2013; Wemheuer et al. 2013; Uribea et al. 2012; Menzel et al. 2015). It is well known that *Acidithiobacillus* spp. also plays a key role in biofilm formation and sulfur oxidation in extremely acidic environments (Jones et al. 2012; Quatrini and Jonson 2018). Together with *Acidithiobacillus*, the occurrence of sequences affiliated with *Sulfobacillus* and *Alicyclobacillus* genera, previously observed in the Pozzuoli area and in other hydrothermal vents (Menzel et al. 2015; Guo et al. 2016), indicates the presence of a microbial community involved in the biotic H₂S oxidation observed in sites S1, S2 and S3. The analysis also revealed the marginal occurrence of OTUs affiliated to *Leptospirillum* genus (Rawlings et al., 1999; Hippe 2000) and *Acidimicrobiales* order (Stackebrandt et al., 1997), typically found in acidic metal contaminated environments and capable of Fe³⁺-oxidation (Christel et al. 2017; Clum et al., 2009). Furthermore, the sequencing analysis highlighted the presence of thermophilic methanol consumers belonging to genus *Methylobacterium* in S2 at 30 cm depth. OTUs affiliated with the phylum *Thermotogae* were abundant in S6, which had the highest temperature and L.O.I. values, in agreement with the results of studies carried out in sites having similar temperature and pH conditions (Sahm et al. 2013; Wemheuer et al. 2013; Menzel et al. 2015; Li et al. 2015). As observed for Archaea, the bacterial communities in S4 and S5 exhibited a diversity that was remarkably higher than that found in the other sites. Most OTUs retrieved in these samples was affiliated with the phylum *Acidobacteria* but they exhibited low relationships to described species. Members of the phylum *Acidobacteria* represent one of the most abundant bacterial groups in soil environments (Jones et al. 2009; Foese et al. 2014), but their ecological roles are poorly understood, likely due to a paucity of cultured representatives (Kielak et al. 2016). *Acidobacteria* were profusely found in a previous clonal analysis performed on samples taken from Solfatara (Glammocemica et al. 2004a) and in various environments including shallow- and deep-sea vents (Sievert et al. 2000; Lopez-Garcia et al. 2003) and in soil after a geothermal heating event at Yellowstone National Park (Norriss et al. 2002),
Verrucomicrobial methanotrophs were not detected in this study, although novel species of *Methylacidibacterium* were recently discovered in the Solfatara Crater (van Teeseling et al. 2014). However, OTUs generically affiliated to the phylum *Verrucomicrobia* or specifically to genus *Chondriobacter* were retrieved in our samples suggesting the possible presence of new methanotrophic *Verrucomicrobia*. Within *Chloroflexi*, it is worth noting the occurrence of OTUs, mainly not identified at genus level, belonging to the class *Ktedonobacteria*. Members of this class were previously isolated from geothermal soils (Yabe et al. 2010, 2011, 2017). Remarkably, the occurrence of OTUs not identified at genus level revealed a new microbial diversity within *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia* and *Thaumarchaeota* phyla in such an extremely acidic environment, which deserves further investigation.

The combination of high-throughput sequencing with single-cells quantification assays provided a deep insight on biodiversity in Solfatara Crater, highlighting some differences with previously well-studied, high-enthalpy geothermal systems e.g. lack of evidence of members of *Aquificae* phylum typically reported in hyperthermophilic, alkaline systems such as Yellowstone National Park (Takacs-Vesbach et al. 2013). A wide distribution of different *Aquificales* lineages as a function of the geochemical conditions was reported for Yellowstone National Park, with the dominance of *Hydrogenobaculum* spp. specifically associated with the acidic, sulfidic sites (Takacs-Vesbach et al. 2013).

In this study, the integrated geochemical and microbiological characterization highlighted how the microbial communities are shaped by deep-originated gases and elements. Fumarolic gas emissions (rich in CO$_2$, CH$_4$, H$_2$S), high temperature and acidity select microbial communities with high metabolic versatility as a strategy to deal with extreme reaction environments.

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**References**


Figure 1 Map of Solfatara Crater in Campi Flegrei caldera (Italy) with the location of sampling sites (1-7) and Bocca Grande and Bocca Nuova fumaroles.
Figure 2 NMDS ordination plot, based on Euclidean distance matrix of log-transformed data, showing the variation patterns of chemical variables. The vector length is proportional to the correlation between the NMDS axes and each chemical variable. The stress value (i.e., <0.2) suggests for an accurate representation of the dissimilarity among areas affected or not affected by hydrothermal fluids.
Figure 3 Bacteria and Archaea abundance estimated by CARD-FISH and expressed as number of cells per gram of Dry Weight (DW). Cells were hybridized by the specific probes EUB338 I-III and ARCH915 for Bacteria and Archaea respectively.
Figure 4 Relative abundance (% of total OTUs) of OTUs belonging to the domain of Archaea estimated by NGS. Crenarchaeota (red); Euryarchaeota (green); Thaumarchaeota (yellow).
Figure 5 Relative abundance (% of total OTUs) of OTUs belonging to the domain of Bacteria estimated by NGS. Clusters making up less than 1% of total composition and bacteria not identified at phylum level were classified as ‘other bacteria’.
Figure 6 (a) Primitive mantle (PM)-normalized element patterns for soil samples collected at 10 cm (up triangle) and 30 cm (down triangle) depth from sites S1 (red color), S2 (orange color), S3 (green color), S4 (cyan color) and S5 (blue color). Normalization values are from Palme and O’Neill (2004). The PM-normalized concentrations of parent materials, i.e. Solfatara and Astroni trachytes and Agnano Monte Spina phono-trachytes (according to Turney et al., 2008 and references therein), are also reported (gray shaded area). (b) Enrichment factors (EF) for selected elements relative to parent materials in soil samples from sites S1 (red color), S2 (orange color), S3 (green color), S4 (cyan color) and S5 (blue color) collected at 10 cm (first bar) and 30 cm (second bar) depth. EF values were calculated for each element as follows: $EF_i = \frac{[C_i/C_{TiO_2}]_{\text{sample}}}{[C_i/C_{TiO_2}]_{\text{parent}}}$, where $[C_i/C_{TiO_2}]_{\text{sample}}$ and $[C_i/C_{TiO_2}]_{\text{parent}}$ are the concentrations ratios of an element $i$ normalized to TiO$_2$ in the sample and in the parent material, respectively.
Figure 7 The typifying microbial composition revealed by NGS (clusters > 1% of total OTUs were considered) projected onto the NMDS ordination synthesizing the chemical dissimilarity between areas affected or not affected by hydrothermal fluids (see Figure 2). The vector length is proportional to the correlation between the NMDS axes and each microbial variable, upon normalization by log(X+1). Bacteria: Aci, Acidobacteria; Act, Actinobacteria; Bac, Bacteroidetes; Chl, Chloroflexi; Fir, Firmicutes; Fus, Fusobacteria; Nit, Nitrospirae; Pla, Planctomycetes; Alpha-, Beta-, Gamma-, Deltaproteobacteria; The, Thermotogae; unk, bacteria unknown. Archaea (in red): Cren, Crenarchaeota; Eury, Euryarchaeota; Thau, Thaumarchaeota.
Table 1. Main physicochemical parameters (T, pH, CO$_2$ and CH$_4$ soil fluxes, and chemical and isotopic composition of interstitial soil gases and bubbling gases from Solfatara Crater. Loss on ignition (L.O.I.) values (in wt%) for soil and mud samples are also reported; n.a.: not analyzed.

<p>| Sample ID | Sampling Site | East UTM | North UTM | ϕCO$_2$ (g m$^{-2}$ day$^{-1}$) | ϕCH$_4$ (g m$^{-2}$ day$^{-1}$) | depth cm | T °C | pH | wt% CO$_2$ | wt% CH$_4$ | wt% H$_2$ | wt% S | wt% CH$_4$ | wt% N$_2$ | wt% O$_2$ | wt% Ar | wt% H$_2$ | wt% CO | δ$^{13}$C - CO$_2$ |
|-----------|---------------|----------|-----------|-------------------------------|-------------------------------|----------|------|----|------------|------------|----------|------|-----------|----------|----------|-------|--------|-------|----------------|-----------|
| S1_1      | S1            | 427588   | 4519913   | 3.35                          | 0.05                          | 10       | 37.2 | 1.3 | 7.3        | 887        | 1.6      | 0.064 | 109       | 1.4       | b.d.l.   | b.d.l. | -1.42 |
| S1_3      | S1            | 427539   | 4519923   | 0.00                          | 21                            | 30       | 57.6 | 1.4 | 6.2        | 943        | 1.9      | 0.091 | 54        | 0.32      | b.d.l.   | b.d.l. | -1.69 |
| S2_1      | S2            | 427352   | 4519984   | 0.00                          | 25                            | 10       | 42.9 | 1.5 | 9.9        | 887        | 1.3      | 0.021 | 107       | 3.3       | b.d.l.   | b.d.l. | -1.1  |
| S2_3      | S2            | 427352   | 4519984   | 0.00                          | 25                            | 30       | 60.1 | 1.7 | 5.5        | 900        | 1.9      | 0.051 | 96        | 0.7       | b.d.l.   | b.d.l. | -1.26 |
| S3_1      | S3            | 427352   | 4519984   | 0.00                          | 25                            | 10       | 26.3 | 2.2 | 6.4        | 804        | 1.2      | 0.047 | 147       | 4.6       | b.d.l.   | b.d.l. | -1.25 |
| S3_3      | S3            | 427352   | 4519984   | 0.00                          | 25                            | 30       | 50.6 | 2.0 | 4.8        | 888        | 1.6      | 0.056 | 103       | 6.1       | b.d.l.   | b.d.l. | -1.86 |
| S4_1      | S4            | 427571   | 4520048   | 0.00                          | 046                           | 10       | 10.5 | 2.8 | 6.5        | 330        | 0.31     | 0.0078 | 591       | 7.7       | b.d.l.   | b.d.l. | +0.6  |
| S4_3      | S4            | 427571   | 4520048   | 0.00                          | 046                           | 30       | 12.4 | 3.4 | 5.8        | 590        | 0.56     | 0.015  | 390       | 4.8       | b.d.l.   | b.d.l. | -0.07 |
| S5_1      | S5            | 427388   | 4520113   | 0.00                          | 021                           | 10       | 14.4 | 3.4 | 15         | 15         | 0.05     | 0.0033 | 733       | 89        | b.d.l.   | b.d.l. | b.d.l. |
| S5_3      | S5            | 427388   | 4520113   | 0.00                          | 021                           | 30       | 17.4 | 3.6 | 8.6        | 80        | 0.06     | 0.0039 | 741       | 91        | b.d.l.   | b.d.l. | b.d.l. |
| S6 Fangua | S6            | 427411   | 4519902   | -                             | -                             | 10       | 14.4 | 3.4 | 15         | 15         | 0.05     | 0.0033 | 733       | 89        | b.d.l.   | b.d.l. | b.d.l. |
| S7 Fangua | S7            | 427380   | 4519910   | -                             | -                             | 10       | 14.4 | 3.4 | 15         | 15         | 0.05     | 0.0033 | 733       | 89        | b.d.l.   | b.d.l. | b.d.l. |</p>
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<td>150</td>
<td>7.3</td>
<td>b.d.l.</td>
<td>1.6</td>
<td>2.8</td>
<td>4.1</td>
<td>5.5</td>
<td>4.7</td>
</tr>
<tr>
<td>S2_30</td>
<td>250</td>
<td>110</td>
<td>80</td>
<td>50</td>
<td>160</td>
<td>9.0</td>
<td>b.d.l.</td>
<td>2.1</td>
<td>3.6</td>
<td>2.6</td>
<td>3.1</td>
<td>3.5</td>
</tr>
<tr>
<td>S3_10</td>
<td>250</td>
<td>120</td>
<td>60</td>
<td>80</td>
<td>150</td>
<td>9.0</td>
<td>b.d.l.</td>
<td>1.4</td>
<td>4.8</td>
<td>7.4</td>
<td>9.1</td>
<td>6.9</td>
</tr>
<tr>
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<td>290</td>
<td>150</td>
<td>90</td>
<td>110</td>
<td>180</td>
<td>12</td>
<td>b.d.l.</td>
<td>1.7</td>
<td>6.9</td>
<td>3.7</td>
<td>4.4</td>
<td>3.1</td>
</tr>
<tr>
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<td>50</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>30</td>
<td>2.0</td>
<td>b.d.l.</td>
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<td>0.7</td>
<td>9.5</td>
<td>16</td>
<td>7.8</td>
</tr>
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<td>110</td>
<td>30</td>
<td>20</td>
<td>20</td>
<td>70</td>
<td>4.0</td>
<td>b.d.l.</td>
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<td>2.2</td>
<td>7.8</td>
<td>11</td>
<td>6.1</td>
</tr>
<tr>
<td>S6</td>
<td>1900</td>
<td>12</td>
<td>11</td>
<td>6.1</td>
<td>850</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>S7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Bulk chemical composition (expressed in wt%) resulted from XRF analysis on soil samples; b.d.l.: below detection limit; n.a.: not analyzed.

| Sample ID | Na₂O | MgO | Al₂O₃ | SiO₂ | P₂O₅ | K₂O | CaO | TiO₂ | MnO | Fe₂O₃ | S | Cl | Cu | Rb | Sr | Zr | Nb | Ba | Pb | Cr | As | Y | Hg | Ni | Zn | Br |
|-----------|------|-----|-------|------|------|-----|-----|------|-----|-------|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| S1_10     | 0.19 | 0.040 | 4.5 | 81 | 0.099 | 2.7 | 0.24 | 0.99 | b.d.l. | 0.48 | 2.6 | 0.020 | 0.004 | 0.016 | 0.057 | 0.046 | 0.008 | 0.13 | 0.009 | 0.009 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. |
| S1_30     | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| S2_10     | 0.046 | 0.016 | 1.6 | 83 | 0.081 | 0.53 | 0.071 | 1.1 | b.d.l. | 0.19 | 2.8 | 0.022 | 0.004 | 0.004 | 0.011 | 0.054 | 0.009 | 0.14 | 0.013 | 0.013 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. |
| S2_30     | 0.064 | 0.021 | 1.4 | 89 | 0.090 | 0.44 | 0.080 | 1.2 | b.d.l. | 0.19 | 1.5 | 0.050 | 0.006 | 0.004 | 0.010 | 0.062 | 0.011 | 0.15 | 0.008 | b.d.l. | 0.012 | 0.000 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. |
| S3_10     | 0.065 | 0.024 | 3.2 | 86 | 0.100 | 0.98 | 0.070 | 1.1 | b.d.l. | 0.18 | 1.3 | 0.040 | b.d.l. | 0.004 | 0.012 | 0.063 | 0.015 | 0.12 | 0.015 | b.d.l. | b.d.l. | b.d.l. | 0.006 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. |
| S3_30     | 0.028 | 0.022 | 1.1 | 91 | 0.10 | 0.17 | 0.070 | 1.4 | 0.009 | 0.11 | 0.65 | 0.030 | b.d.l. | 0.003 | 0.006 | 0.098 | 0.017 | 0.12 | 0.018 | b.d.l. | b.d.l. | b.d.l. | 0.009 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. |
| S4_10     | 0.12 | 0.050 | 3.2 | 85 | 0.12 | 1.4 | 0.23 | 0.49 | 0.010 | 0.06 | 0.89 | 0.12 | 0.005 | 0.006 | 0.019 | 0.048 | 0.008 | 0.12 | 0.018 | b.d.l. | b.d.l. | 0.000 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. |
| S4_30     | 0.12 | 0.050 | 3.5 | 86 | 0.099 | 1.5 | 0.15 | 0.62 | b.d.l. | 0.45 | 1.2 | 0.039 | 0.004 | 0.007 | 0.019 | 0.052 | 0.009 | 0.11 | 0.012 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. |
| S5_10     | 0.38 | 0.22 | 5.2 | 72 | 0.21 | 2.1 | 0.72 | 0.80 | 0.020 | 1.9 | 1.0 | 0.080 | 0.005 | 0.010 | 0.026 | 0.059 | 0.009 | 0.13 | 0.023 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | 0.008 | 0.015 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. |
| S5_30     | 0.25 | 0.11 | 3.9 | 82 | 0.12 | 1.9 | 0.43 | 0.95 | 0.010 | 0.04 | 0.79 | 0.059 | 0.004 | 0.009 | 0.023 | 0.058 | 0.011 | 0.18 | 0.020 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | 0.003 | 0.004 | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. | b.d.l. |
| S6        | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| S7        | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
Table 4. Chemical composition resulted from ICP-OES analysis of solutions obtained after leaching tests in MilliQ on soil and mud samples; b.d.l.: below detection limit.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Mn (mg/L)</th>
<th>Fe (mg/L)</th>
<th>Co (mg/L)</th>
<th>Ni (mg/L)</th>
<th>Cu (mg/L)</th>
<th>Zn (mg/L)</th>
<th>As (mg/L)</th>
<th>Sb (mg/L)</th>
<th>Al (mg/L)</th>
<th>Hg (mg/L)</th>
<th>W (mg/L)</th>
<th>Si (mg/L)</th>
<th>Pb (mg/L)</th>
<th>P (mg/L)</th>
<th>Cd (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1_10</td>
<td>3.7</td>
<td>488</td>
<td>0.17</td>
<td>0.11</td>
<td>2.4</td>
<td>1.2</td>
<td>0.17</td>
<td>b.d.l.</td>
<td>1110</td>
<td>b.d.l.</td>
<td>0.73</td>
<td>47</td>
<td>1.09</td>
<td>1.6</td>
<td>0.03</td>
</tr>
<tr>
<td>S1_30</td>
<td>2.8</td>
<td>229</td>
<td>0.16</td>
<td>0.18</td>
<td>5.0</td>
<td>1.9</td>
<td>0.15</td>
<td>b.d.l.</td>
<td>873</td>
<td>b.d.l.</td>
<td>0.59</td>
<td>93</td>
<td>0.15</td>
<td>0.83</td>
<td>0.11</td>
</tr>
<tr>
<td>S2_10</td>
<td>0.62</td>
<td>13</td>
<td>0.03</td>
<td>0.04</td>
<td>0.59</td>
<td>0.48</td>
<td>0.55</td>
<td>0.04</td>
<td>278</td>
<td>b.d.l.</td>
<td>0.54</td>
<td>24</td>
<td>0.06</td>
<td>0.75</td>
<td>0.03</td>
</tr>
<tr>
<td>S2_30</td>
<td>1.0</td>
<td>59</td>
<td>0.11</td>
<td>0.15</td>
<td>7.2</td>
<td>0.9</td>
<td>1.4</td>
<td>0.04</td>
<td>273</td>
<td>b.d.l.</td>
<td>0.48</td>
<td>24</td>
<td>0.61</td>
<td>0.51</td>
<td>0.05</td>
</tr>
<tr>
<td>S3_10</td>
<td>0.67</td>
<td>8.7</td>
<td>0.04</td>
<td>0.04</td>
<td>0.17</td>
<td>0.41</td>
<td>0.35</td>
<td>0.04</td>
<td>107</td>
<td>b.d.l.</td>
<td>0.44</td>
<td>79</td>
<td>b.d.l.</td>
<td>0.63</td>
<td>0.03</td>
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<tr>
<td>S3_30</td>
<td>1.0</td>
<td>10</td>
<td>0.03</td>
<td>0.05</td>
<td>0.94</td>
<td>0.69</td>
<td>0.55</td>
<td>0.07</td>
<td>157</td>
<td>b.d.l.</td>
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<td>71</td>
<td>b.d.l.</td>
<td>0.48</td>
<td>0.04</td>
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<tr>
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<td>0.82</td>
<td>29</td>
<td>0.04</td>
<td>0.04</td>
<td>0.21</td>
<td>0.46</td>
<td>0.21</td>
<td>0.03</td>
<td>15</td>
<td>b.d.l.</td>
<td>0.40</td>
<td>96</td>
<td>0.01</td>
<td>0.42</td>
<td>0.04</td>
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<td>0.04</td>
<td>0.18</td>
<td>0.34</td>
<td>0.17</td>
<td>0.04</td>
<td>24</td>
<td>b.d.l.</td>
<td>0.39</td>
<td>91</td>
<td>b.d.l.</td>
<td>0.27</td>
<td>0.03</td>
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<td>5.9</td>
<td>0.02</td>
<td>0.04</td>
<td>0.29</td>
<td>0.48</td>
<td>0.27</td>
<td>0.06</td>
<td>23</td>
<td>b.d.l.</td>
<td>0.40</td>
<td>125</td>
<td>0.08</td>
<td>1.4</td>
<td>0.03</td>
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<tr>
<td>S5_30</td>
<td>1.1</td>
<td>4.0</td>
<td>0.02</td>
<td>0.04</td>
<td>0.28</td>
<td>0.64</td>
<td>0.48</td>
<td>0.08</td>
<td>10</td>
<td>b.d.l.</td>
<td>0.40</td>
<td>114</td>
<td>b.d.l.</td>
<td>0.66</td>
<td>0.07</td>
</tr>
<tr>
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<td>133</td>
<td>0.27</td>
<td>0.28</td>
<td>1.8</td>
<td>0.51</td>
<td>3.7</td>
<td>0.22</td>
<td>139</td>
<td>b.d.l.</td>
<td>0.41</td>
<td>28</td>
<td>0.13</td>
<td>1.2</td>
<td>0.04</td>
</tr>
<tr>
<td>S7</td>
<td>0.52</td>
<td>53</td>
<td>0.02</td>
<td>0.20</td>
<td>0.22</td>
<td>0.37</td>
<td>2.9</td>
<td>0.03</td>
<td>133</td>
<td>b.d.l.</td>
<td>0.41</td>
<td>24</td>
<td>0.14</td>
<td>1.6</td>
<td>0.03</td>
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</table>

Table 5. Main physicochemical parameters (Eh and pH) and chemical composition of waters from Fangaia mud pools; b.d.l.: below detection limit.

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<th>Sample ID</th>
<th>pH</th>
<th>Eh (mV)</th>
<th>F⁻ (mg/L)</th>
<th>Cl⁻ (mg/L)</th>
<th>Br⁻ (mg/L)</th>
<th>NO₃⁻ (mg/L)</th>
<th>SO₄²⁻ (mg/L)</th>
<th>Ca²⁺ (mg/L)</th>
<th>Mg²⁺ (mg/L)</th>
<th>Na⁺ (mg/L)</th>
<th>K⁺ (mg/L)</th>
<th>NH₄⁺ (mg/L)</th>
<th>Li⁺ (mg/L)</th>
</tr>
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<tbody>
<tr>
<td>S6</td>
<td>1.62</td>
<td>447</td>
<td>5.30</td>
<td>101</td>
<td>b.d.l.</td>
<td>4.36</td>
<td>1810</td>
<td>20</td>
<td>2.45</td>
<td>80</td>
<td>47</td>
<td>b.d.l.</td>
<td>b.d.l.</td>
</tr>
<tr>
<td>S7</td>
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<td>388</td>
<td>2.77</td>
<td>95</td>
<td>b.d.l.</td>
<td>0.78</td>
<td>1800</td>
<td>11</td>
<td>b.d.l.</td>
<td>5.31</td>
<td>14</td>
<td>b.d.l.</td>
<td>1.33</td>
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