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Microbial community differentiation between active and inactive sulfide chimneys of the Kolumbo submarine volcano, Hellenic Volcanic Arc

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Abstract Over the last decades, there has been growing interest about the ecological role of hydrothermal sulfide chimneys, their microbial diversity and associated biotechnological potential. Here, we performed dual-index Illumina sequencing of bacterial and archaeal communities on active and inactive sulfide chimneys collected from the Kolumbo hydrothermal field, situated on a geodynamic convergent setting. A total of 15,701 OTUs (operational taxonomic units) were assigned to 56 bacterial and 3 archaeal phyla, 133 bacterial and 16 archaeal classes. Active chimney communities were dominated by OTUs related to thermophilic members of Epsilonproteobacteria, Aquificae and Deltaproteobacteria. Inactive chimney communities were dominated by an OTU closely related to the archaeon *Nitrosopumilus* sp., and by members of Gammaproteobacteria, Deltaproteobacteria, Planctomycetes and Bacteroidetes. These lineages are closely related to phylotypes typically involved in iron, sulfur, nitrogen, hydrogen and methane cycling. Overall, the inactive sulfide chimneys presented highly diverse and uniform microbial communities, in contrast to the active chimney communities, which were dominated by chemolithoautotrophic and thermophilic lineages. This study represents one of the most comprehensive investigations of microbial diversity in submarine chimneys and elucidates how the dissipation of hydrothermal activity affects the structure of microbial consortia in these extreme ecological niches.

Introduction

Submarine hydrothermal vent fields are ecosystems with global distribution across the seafloor and a considerable impact on ocean chemistry (Elderfield and Schultz 1996). Diverse chemoautotrophic microbial communities are hosted by hydrothermal vent deposits, such as vent chimneys, benefiting from the interaction of the acidic, metal-rich, chemically reduced hydrothermal fluids with the cold and oxygenated surrounding seawater. This interaction results in physicochemical gradients along the hydrothermal vent deposits, creating a variety of different microhabitats that can be colonized by different types of microbes (Mccollom and Shock 1997; Tivey 2004; Flores et al. 2012). Hydrothermal vents are distributed mainly in mid-ocean ridges, intra-oceanic volcanic arcs and in the back-arc basins of subduction systems (Takai et al. 2006; Orcutt et al. 2011).

The Kolumbo submarine volcano is situated in the north-eastern part of the Santorini Island, 7 km off the coast (Nomikou et al. 2012) and it is part of the Hellenic Volcanic Arc (HVA), South Aegean Sea. The HVA (Methana, Milos, Santorini, Nisyros) is separated from the Hellenic Sedimentary Arc (HSA) (Peloponnesus, Crete, Rhodes) by the Cretan

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basin, a 'back-arc' mollasic basin which lies behind the HSA but in front of the HVA (Kilias et al. 2013). Kolumbo is the largest cone of a series of at least 20 smaller volcanic cones along a NE–SW trending rift zone, within Anyhdros basin (Hübscher et al. 2015; Nomikou et al. 2016). Kolumbo has a 1500 m wide, oval-shaped crater at a depth of 505 m (Sigurdsson et al. 2006; Carey et al. 2011).

The Kolumbo volcano consists of active and inactive sulfide vent chimneys of spire or mound shape up to 4 m high, formed by the successive precipitation of sulfide and sulfate minerals (Kilias et al. 2013). Temperatures up to 220 °C have been recorded in fluids flowing out of the active vents (Sigurdsson et al. 2006; Carey et al. 2011). Kolumbo's crater floor is covered with a thick layer of sediment consisting of microbial mats and amorphous Fe-oxyhydroxide deposits. This layer surrounding the active vents exhibits sites with low-temperature (≤ 70 °C) diffuse venting (Kilias et al. 2013). The discharge of gaseous CO_2 (> 99%) is the most salient characteristic of active vent chimneys of Kolumbo (Rizzo et al. 2016). Besides pH reduction, the dissolution of venting CO_2 causes an increase of water density that leads to the accumulation of acidic seawater (as low as pH 5.0) near the crater floor (Carey et al. 2013).

Until now, several cultivation- and molecular-based studies have been performed to elucidate the diversity of microorganisms residing in hydrothermally active environments. Microbial communities inhabiting inactive sulfide hydrothermal deposits have also been studied to elucidate the changes occurring in microbial composition after the cessation of hydrothermal activity (Suzuki et al. 2004; Brazelton et al. 2010; Kato et al. 2010; Sylvan et al. 2012, 2013; Jaeschke et al. 2012). With the advent of high throughput sequencing, more light has been shed to the structure of the microbial communities present in active hydrothermal vent deposits and sulfides originating mainly from mid-ocean ridges (Flores et al. 2011; Jaeschke et al. 2012; Olins et al. 2013; Frank et al. 2013; Dahle et al. 2015; He and Zhang 2016) and back-arc basin (Flores et al. 2012).

A recent work compared the microbial communities inhabiting hydrothermally active and inactive sulfide deposits from several hydrothermal sites (Olins et al. 2013) and proposed that these communities are distinct and attributed the observed differences to the presence and composition of hydrothermal fluids (Toner et al. 2013). This study was limited by the employment of 16S rRNA gene clone libraries, which prevents the extensive coverage of microbial diversity; a limitation shared by other previous studies (Suzuki et al. 2004; Kato et al. 2010; Toner et al. 2013). Furthermore, those findings were based on a comparison of active and inactive chimneys from distantly located hydrothermal sites. Other active-vs-inactive comparisons of microbial diversity were affected by similar constraints (Kato et al. 2010; Sylvan et al. 2012, 2013).

In contrast to previous studies, our investigation examined microbial diversity in closely located active and inactive chimneys using high-throughput sequencing. By analyzing 16 samples using Illumina MiSeq sequencing, we conducted one of the most comprehensive characterizations of microbial communities inhabiting hydrothermal chimneys. A detailed comparison of the observed microbiomes provided insights in the succession of bacterial and archaeal groups from active to inactive chimneys and elucidated the microbial taxa dominating different points of a single sulfide chimney. The differences of the microbial community structures and the potential metabolisms associated with specific taxa allowed the examination of the potential biochemical capabilities and the ecological role of Bacteria and Archaea, following the cessation of hydrothermal activity (Fig. 1).

Materials and methods

Chimneys collection

The hydrothermal chimneys samples were collected from the Kolumbo submarine volcano during the expedition 2Biotech of the EU-FP7 Seabiotech project in September 2013, using the R/V Aegaeo of HCMR. Sampling was carried out using the Hellenic ROV Max Rover. In total, 6 sulfide chimneys were sampled named V16, V59, V64, V08, V65, and V67 (Fig. 2). During sampling the ROV-mounted CTD (SBE 39; Seabird Scientific) recorded the surrounding temperatures of the sulfide chimneys. Chimneys V16, V59 and V67 showed no signs of active venting (See inactive chimney V16 in Supplementary Video S1), whereas chimneys V64, V08 and V65 exhibited diffuse venting of fluids (See active chimney V08 in Supplementary Video S2). Two fragments were collected from chimney V16 and two fragments from chimney V59. For each of the remaining five chimneys, one fragment was collected. Upon return to the surface, all chimney fragments were transported onboard carefully. Samples were collected using sterile scalpels, placed into sterile Petri dishes, and then stored at − 20 °C until further analysis took place. In detail, sample V16_c was collected by scraping the surface of the first fragment of V16, while the second fragment resulted into four samples (Fig. 2a). Samples V16_c.out, V16_c.mid and V16_c.in were collected from three distinct layers (outer layer, middle layer, inside layer, respectively) on the surface of the first fragment and sample V16_c.Red was collected from material on the axial section of the second fragment (Fig. 2a). Samples V59_2a.Red was collected from the surface and sample V59_2a.As from the axial section of the first fragment of V59 (Fig. 2b). Distinct white material on the surface of the second fragment of V59 resulted in sample V59_b, the rest of the surface in sample V59_a and material from the axial section of the fragment
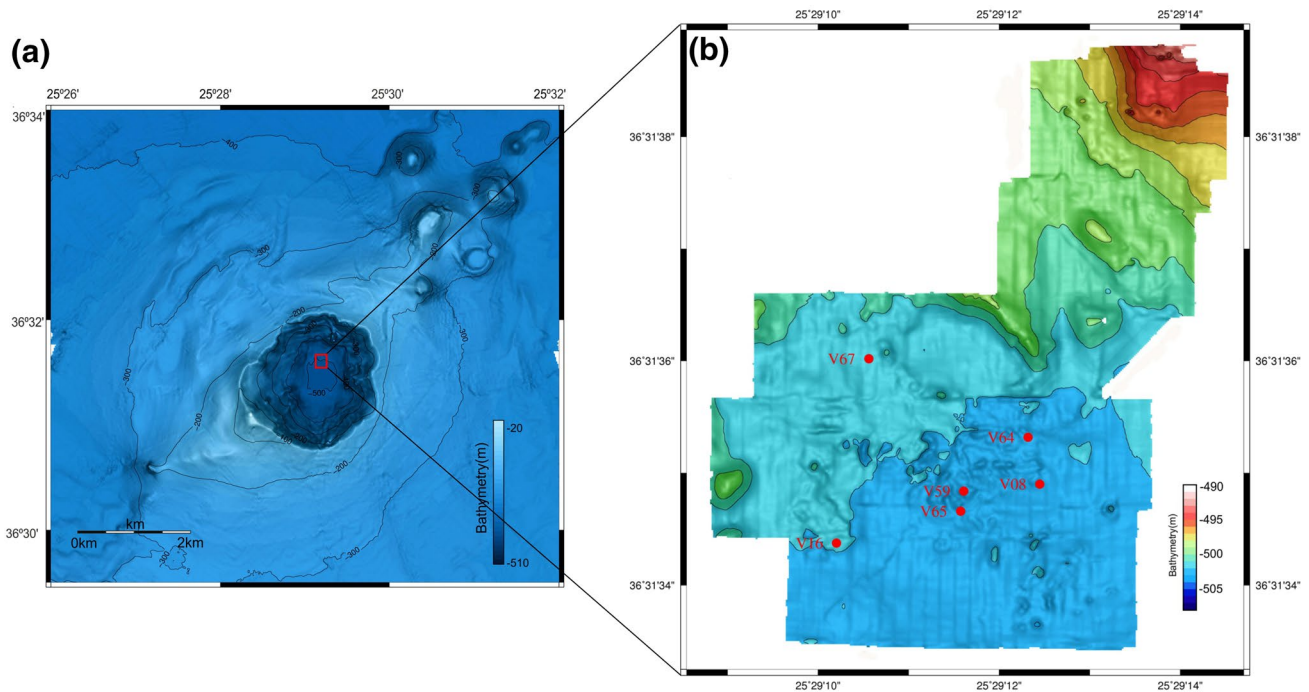
Fig. 1 Bathymetric maps for Kolumbo volcano and hydrothermal vents. **a** Bathymetry of the Kolumbo volcano (modified from Rizzo et al. 2016, license: https://creativecommons.org/licenses/by/4.0/). **b** Bathymetric map of the Kolumbo hydrothermal field (modified from Kilias et al., 2013, license: https://creativecommons.org/licenses/by-nc-sa/3.0/). Kolumbo is located in the northern part of the crater (red rectangular in **a**). The red dots represent the location of hydrothermal chimneys: V59, V16, V67, V64, V08 and V65

resulted in V59_c (Fig. 2b). The fragment of V64 gave two samples: V64_a, from the axial section of the fragment and V64_b from the surface of the fragment (Fig. 2c). V08_a was collected from the white–grey material on the surface of the V08 fragment and V08_b from the red–orange material of the fragment's surface (Fig. 2d). Samples V67 (Fig. 2e) and V65 (Fig. 2f) were collected from the uniform surfaces of the respective V65 and V67 fragments.

Six subsamples for metal analysis were collected, due to the small amount of material of the collected samples. Subsamples were stored in sterile plastic bags at − 20 °C until further analysis. For the methods of the analyses see the Supplementary Material. The results of the elemental analysis are detailed in the Supplementary Table S1.

Nucleic acid extraction and 16S rRNA gene library construction

Approximately, 1 g of material from each sample was used to extract the total microbial community DNA by employing the MoBio UltraClean Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following a slightly modified protocol of the manufacturer. More specifically, the bead-beating step was performed in a tissue lyser for at least 30 min (frequency at 30 l/s; TissueLyser II, Qiagen). DNA concentrations were quantified using the NanoDrop

ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, USA) and the V4 region of the 16S rRNA gene was amplified using the universal primers 515f (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806r (5′-GGA CTA CHV GGG TWT CTA AT-3′;). In brief, the 515f/806r PCR primers with 8-base barcodes on the forward and reverse primers were used in a PCR reaction with the KAPA HiFi HotStart DNA polymerase (1 U μL^{-1}) (KAPA Biosystems) under the following conditions: 95 °C for 2 min (initial denaturation), followed by 32 cycles of 98 °C for 20 s (denaturation), 61 °C for 10 s (primer annealing), and 72 °C for 15 s (extension), with a final extension at 72 °C for 5 min. Positive and negative control samples were used during the PCR reactions, to ensure the validity of the PCR products. It should be mentioned that despite the inclusion of positive and negative PCR controls, the possibility of contaminants presence in the analysed samples cannot be excluded (Tanner et al. 1998; Salter et al. 2014). Part of the PCR products and the controls (5 µL) were then run on a 1.5% agarose gel in order to check amplification and the relative intensity of bands. The remaining quantities of the products were purified with AMPure XP magnetic beads (Perkin-Elmer, UK). Quantification of the PCR products was performed with Quant-iT PicoGreen dsDNA Assay using a TECAN Infinite F200 Pro fluorescence microplate reader. After DNA quantitation, an equimolar pool of the samples was prepared. Final DNA

Fig. 2 Images of the active and inactive chimneys at the seafloor and of the chimney fragments onboard the R/V upon recovery. **a–c** Active chimney samples, **d–f** inactive chimney samples. Images of the chimneys at the seafloor are stills of videos taken during sampling (for **a** see Supplementary Video S1 and for **b** see Supplementary Video S2). Images of the chimney fragments were taken upon recovery. Red lines represent the chimney fragments collected with the ROV, and yellow lines represent the samples collected from the chimney fragments
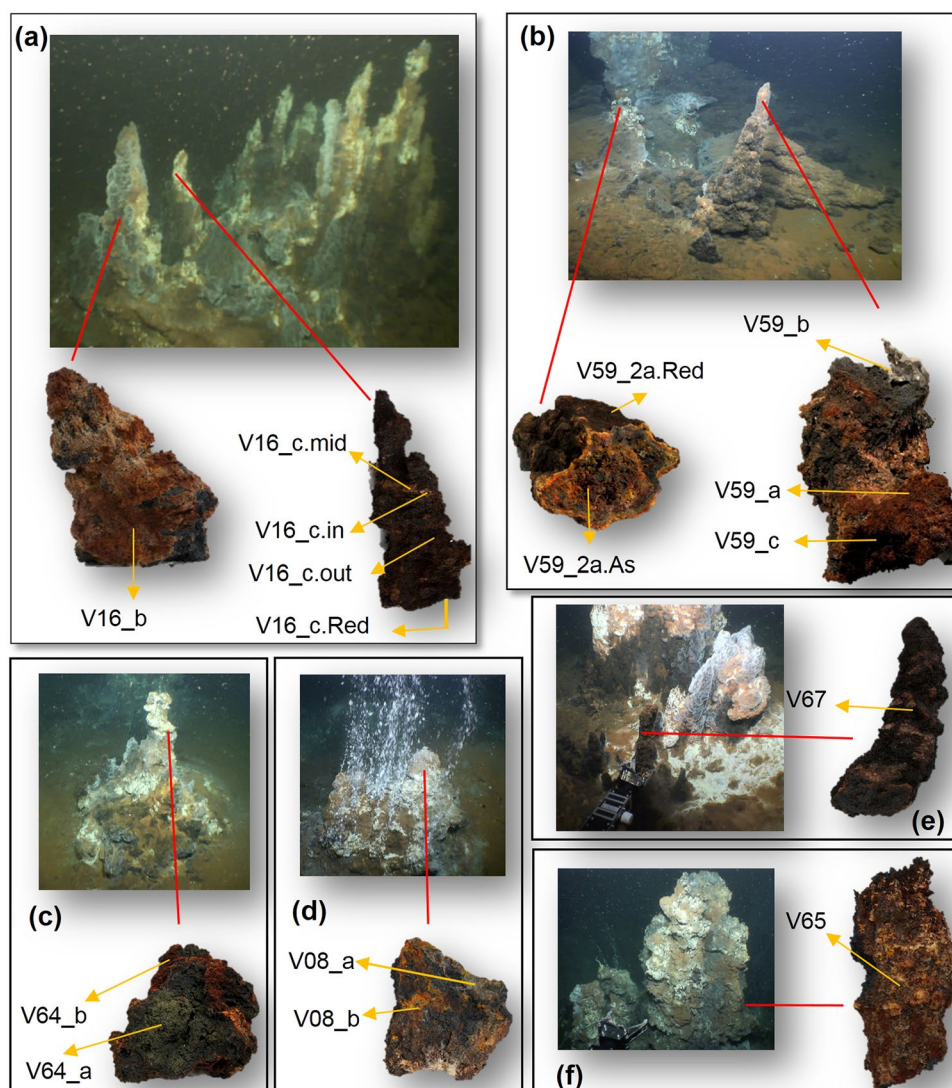
quantification was done by qPCR (KAPA Library Quantification Kit—Illumina/Universal). Finally, the sequencing was performed at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of HCMR in Crete using one run of an Illumina MiSeq with v3 chemistry for 2×280 cycles.

Sequence data analysis

Primers and adapters were removed from the raw demultiplexed sequences using cutadapt version 1.9.2.dev0 (Martin 2011), while further quality trimming was performed using Trimmomatic version 0.32 (Bolger et al. 2014). Sequence data curation and analysis were carried out using mothur 1.35.0 (Schloss et al. 2009) following the Illumina MiSeq Standard Operation Procedure (Kozich et al. 2013). Paired-end reads were joined into contigs and any remaining sequences with homopolymers longer than 8 bp and any ambiguous base calls were removed. Contigs

were then aligned to the mothur compatible Silva database from release v.119 (Quast et al. 2013). Sequences were further de-noised from PCR amplification and sequencing errors, by applying the pre-cluster algorithm with 2 mismatches (Schloss et al. 2011). The UCHIME algorithm was applied for detection and removal of chimeric sequences (Edgar et al. 2011). Trimmed and denoised sequences were classified using the greengenes reference taxonomy (Release August 2013) (DeSantis et al. 2006), the RDP 16S rRNA reference database (Cole et al. 2014) (version 14; data not shown) and the Silva reference database (data not shown). Greengenes reference taxonomy places the genera *Nitrosopumilus* and *Cenarchaeum* in the phylum Crenarchaeota, despite its reclassification in the Thaumarchaeota phylum. Due to this, the taxonomy of Thaumarchaeota members was edited manually. Sequences were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity, using the

average neighbor clustering algorithm. The raw tag data are available through NCBI's Sequence Read Archive under study accession number SRP075670.

OTU matrix visualizations and statistical analysis

Rarefaction curves and cluster dendrogram, using the Chao1 richness estimator and UPGMA algorithm, respectively, were calculated through mothur and visualized using R. Ecological indicators and richness estimators were calculated under R with the use of the vegan package 2.3-4 version (Oksanen et al. 2017). Heatmaps were created using the package pheatmapv.1.0.8 (Kolde 2015) with UPGMA as a clustering method. Bubble chart was created using the package ggplot2 (Wickham 2009).

Results

The temperature of the ambient water surrounding the sulfide chimneys varied between 15.8 and 16.2 °C (V16: 15.9 °C, V59: 16.2 °C, V64: 15.8, V08: 15.9 °C, V65: 15.8 °C, V67: 15.9 °C). An analysis for 53 major and trace elements was performed for 6 samples (Supplementary Table S1). The major elements in the samples were Fe, S, Pb, Na, As, Sb, Mn and Sr with average concentrations of 111,333 ppm Fe, 23,850 ppm S, 6043 ppm Pb, 4182 ppm Na, 2656 ppm As, 2616 ppm Sb, 2075 ppm Mn and 1828 ppm Sr samples (Supplementary Table S1).

Microbial diversity

Dual index Illumina MiSeq sequencing resulted in a total of 14,456,487 demultiplexed reads for all 16 samples. After sequence data analysis, a normalization of the number of sequences of each sample to the sample with the lowest number of reads (V64_a:175,524 reads) was performed, which resulted in 2,808,384 total reads (Supplementary Table S2). Indices were calculated to estimate biodiversity, including Shannon–Weaver (H), Simpson (D), inverse Simpson (iD), Fisher, Chao1 and ACE (Table 1). A rarefaction analysis was also performed to verify the accuracy of diversity indices (Supplementary Fig. S1). Sample V16_c. out collected from the inactive chimney V16, was the most diverse according to the H index (5.9), the Fisher index (850), the Chao1 (6973 ± 168) and ACE (6818 ± 45) estimators, and the second most diverse according to the D (0.98) and iD (52) indices (Table 1). In contrast, samples V08_b and V08_a from the active chimney V08 had the lowest microbial diversity, as revealed by the number of observed OTUs (V08_b:666 and V08_a:906), the H index (V08_b:2.2 and V08_a:2.8), the D index (V08_b:0.81 and V08_a:0.83), the iD index (V08_b:5.3 and V08_a:5.8), the Fisher index (V08_b:88 and V08_a:125), the Chao1 estimator (V08_b:1309 ± 111 and V08_a:1632 ± 124) and the ACE estimator (V08_b:1234 ± 21 and V08_a:1519 ± 21).

A total of 15,701 OTUs at 3% dissimilarity were obtained from all samples analyzed, which were phylogenetically assigned to 3 archaeal and 56 bacterial phyla (Fig. 3a; Supplementary Table S3), and in 17 archaeal and 219 bacterial families (Supplementary Table S4). Species richness showed

Table 1 Diversity statistics for all different chimney samples

	Obs. OTUs	Shannon–weaver (H)	Simpson (D)	Inverse Simpson (iD)	Fisher	Chao1	Chao1 sd	ACE	ACE sd
V59_a	3437	5.333	0.976	42.439	605.938	6364	220	6236	46
V59_b	1568	3.369	0.914	11.584	237.305	2365	92	2275	26
V59_c	3736	5.882	0.988	80.815	670.594	5193	116	5132	38
V59_2a.As	3688	5.502	0.965	28.742	660.120	6139	185	5805	42
V59_2a.Red	1916	4.784	0.975	39.848	300.737	3972	209	3805	37
V16_b	3819	5.615	0.978	46.160	688.789	6567	196	6516	47
V16_c.Red	2941	4.507	0.942	17.233	501.874	4177	113	3910	32
V16_c.out	4535	5.888	0.981	51.930	850.033	6973	168	6818	45
V16_c.mid	1285	4.916	0.976	41.136	187.837	2428	174	2100	25
V16_c.in	2220	4.961	0.980	50.370	358.281	4513	211	4480	41
V67	1889	4.340	0.958	23.557	295.720	2810	103	2661	27
V64_a	3450	5.018	0.936	15.555	608.719	6400	224	5942	45
V64_b	2383	4.144	0.879	8.263	389.897	3539	126	3144	29
V08_a	906	2.793	0.828	5.799	125.001	1632	124	1519	21
V08_b	666	2.213	0.812	5.332	87.593	1309	111	1234	21
V65	1113	3.222	0.890	9.108	158.801	1625	75	1564	21

Fig. 3 **a** Bacterial and archaeal distribution among the different samples of the assigned sequences at the phylum level. Proteobacterial distribution among the different samples of the assigned sequences at the class (**b**) and order (**c**) level. Black lines and brackets denote the samples that originate from active and inactive sulfide chimneys
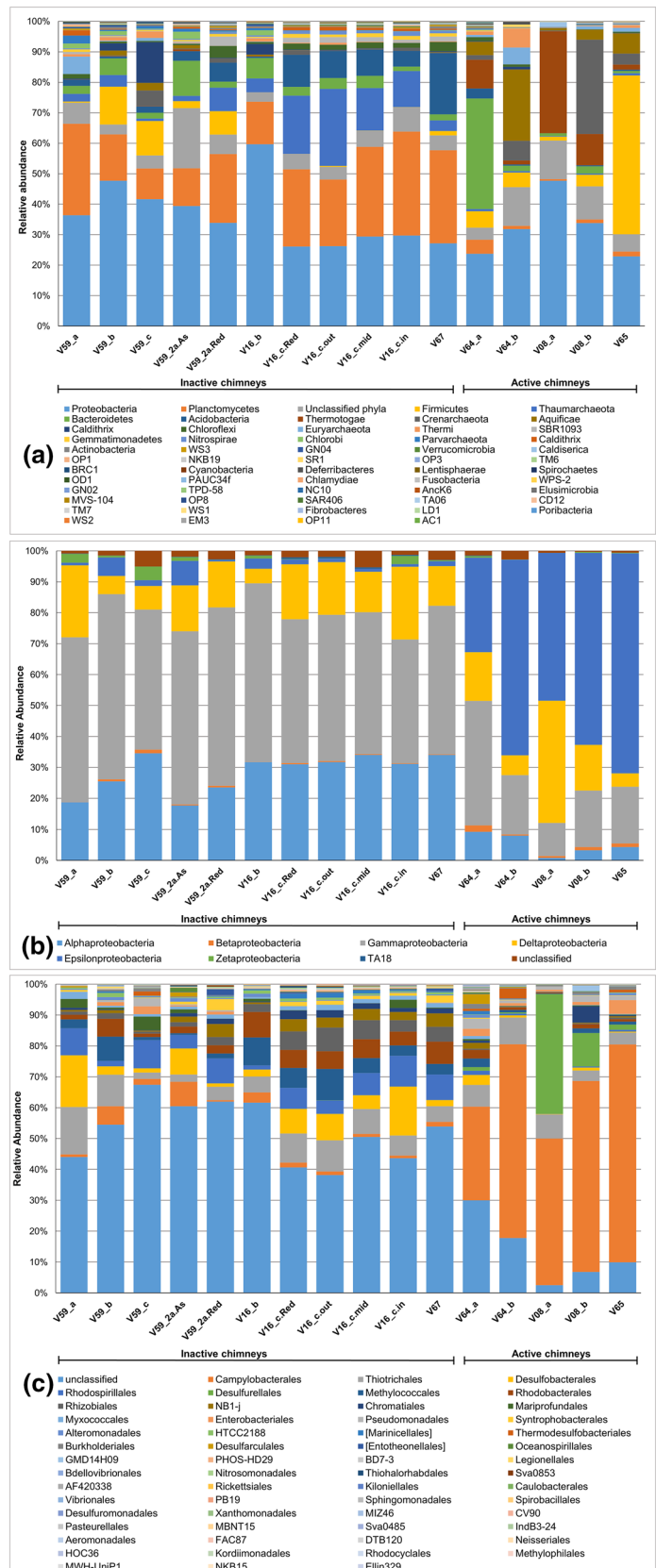
considerable variability between samples obtained from different chimneys, different points across the same chimney and particularly between active and inactive chimneys. Based on OTU numbers alone, samples collected from V16 inactive chimney (V16_c.out and V16_b) exhibited the highest species richness (4535 and 3819, respectively), followed by the samples from the inactive V59 chimney (V59_c: 3736 OTUs, V59_2a.As: 3688 OTUs).

Taxonomic groups and their distribution

Overall, Bacteria dominated over Archaea in terms of sequence abundance and accounted for 89% of all the observed sequences (Fig. 3a). The phylum Proteobacteria was the most abundant in all samples, making up 23–60% (with an average of 35%) of total microbial sequences (Fig. 3a). A particularly high contribution was also observed for another two phyla, namely Planctomycetes (0.53–34%, mean 16%) and the archaeal phyla Crenarchaeota (0.03–31%, mean 3.3%) and Thaumarchaeota (0.17–25%, mean 6.1%). Other phyla with intermediate abundances were Firmicutes (6.5%), Bacteroidetes (5.4%), Acidobacteria (4.5%), Thermotogae (3.6%), Aquificae (2.8%), Caldithrix (1.4%), Chloroflexi (1.3%) and Euryarchaeota (1.0%). Additional phyla with relatively low abundance (< 1%) were Thermi, Gemmatimonadetes, Nitrospirae, Chlorobi, Parvarchaeota, Caldithrix, Actinobacteria, Verrucomicrobia and Caldiserica among others. Some phyla were much more prominent in specific samples, like the phylum Acidobacteria in inactive chimney samples V16_c.Red, V16_c.out, V16_c.mid, V16_c.in and V67 (5.2–20%). With regard to active chimneys, the prevailing phyla were Firmicutes in sample V65 (52%), Bacteroidetes in sample V64_a (36%), Crenarchaeota in V08_b (31%), Aquificae (24%), Euryarchaeota (5.6%), Thermi (6.2%) in V64_b, and Thermotogae in samples V08_a (33%) and V08_b (10%) (Fig. 3a).

Members of all proteobacterial classes, such as Alpha-, Beta-, Gamma-, Delta-, Epsilon-, Zetaproteobacteria, and TA18, were present in all samples at varying abundances (Fig. 3b). Gamma- and Alphaproteobacteria sequences were predominant in the samples from inactive chimneys (51 and 29%, respectively), as opposed to the ones from the active chimneys (21 and 5.2%, respectively). In contrast, Epsilonproteobacteria presented an overwhelming abundance in the samples from the active chimneys (55%) and made only a minor contribution (2.4%) in those from inactive chimneys. A large portion of the proteobacterial sequences (averaging 40%) could not be assigned beyond the order level (Fig. 3c). The order Campylobacterales (19%) was the only order of Epsilonproteobacteria that was identified in the analyzed samples. On the other hand, 17 orders of Gammaproteobacteria were observed, with Thiotrichales (6.8%) being the most prevalent one. Among the identified orders

of Deltaproteobacteria, Desulfobacterales demonstrated the most consistent presence among the samples analyzed (0.1–17% with a mean of 4.8%), while the order of Desulfurellales was especially prevalent only in the active chimney samples V08_a (39%) and V08_b (11%). Strikingly, the extensive presence of Desulfurellales in those two samples was indeed observed due to the detection of a single unclassified OTU.

Dominant OTUs

Of the entire dataset, only 48 OTUs were shared between all 16 samples (of the total 15,701 OTUs and 36% of all sequences), while 125 OTUs were shared among 15 samples (of the total 15,701 OTUs and 61% of all sequences). Moreover, 995 OTUs were identified in the various samples with more than 200 sequence counts each and they collectively accounted for 6.3% of the total OTUs and for 94% of all sequences (Supplementary Table S5). The dataset from chimney samples were divided into two groups corresponding to active and inactive chimneys and further comparison was performed. A number of 2372 OTUs (15%) of the total 15,701 unique OTUs were present in both the active and inactive chimney groups, but these OTUs account for the 94% of the total reads. The active and inactive chimney groups shared 2033 unique OTUs (13% of the total OTUs), and 11,296 OTUs (72% of the total OTUs), respectively.

In order to discern any further differences of the most dominant OTUs among chimney samples, we performed clustered heatmap analysis using the abundances of the top 1000 most prevalent OTUs (Fig. 4). This analysis resulted in the hierarchical clustering of samples into three groups: one group representing active chimneys (AC), and two groups representing inactive chimneys (IN1, IN2). Interestingly, the IN1 group displays the highest numbers of OTUs, the AC group the lowest, while the IN2 group displays a more intermediate state.

The dominant OTUs were further scrutinized with regards to their abundance and the degree of hydrothermal activity in the originating chimneys (Fig. 5, Supplementary Table S5). OTUs closely related to *Nitrosopumilus* genera were present in all samples, but they were more prevalent in the samples from inactive chimneys and especially in chimney V16 (Fig. 5). For example, the OTU assigned to *Nitrosopumilus pIVWA5* was also present in all samples, but it was more prevalent in samples from inactive chimneys (on average 1601 and 106 sequences were detected in inactive and active chimney samples, respectively) (Fig. 5). A similar pattern was evident for additional OTUs belonging to other genera, such as *Planctomyces* sp. *1* (average: 5638 in inactive, 153 in active) and *Planctomyces* sp. *2* (average: 2229 in inactive, 51 in active), unclassified *Acidobacteria 2* (average: 2256 in inactive, 74 in active), and *Acidobacteria*
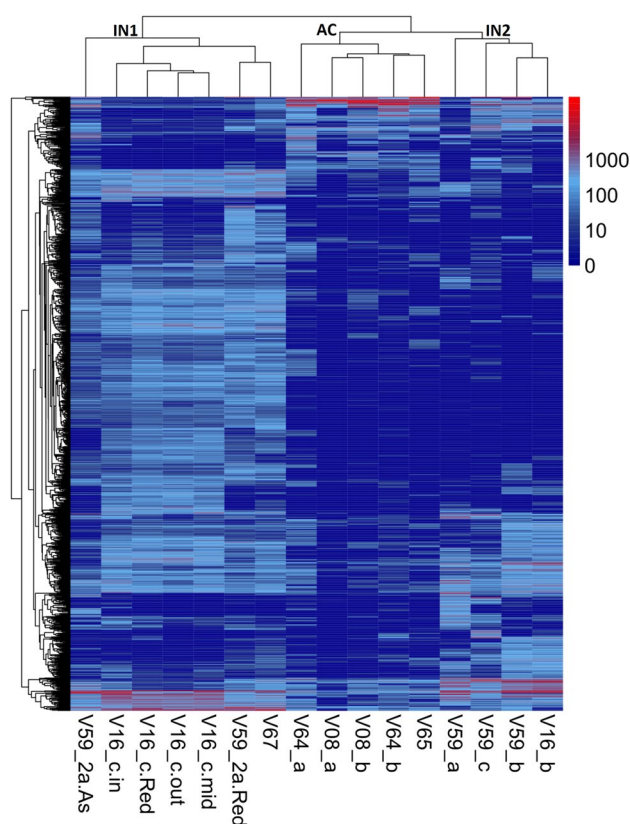
Fig. 4 Occurrence of the 1000 most abundant OTUs derived from the reads in chimney samples. Heatmap denoting relative abundance of the OTUs at 3% cutoff level. Hierarchical clustering of OTUs and samples was performed with UPGMA. Color intensity signifies abundance ranging from dark blue (low abundance) to red (high abundance). The samples are clustered into three groups: IN1, IN2 (samples from inactive chimneys) and AC (samples from active chimneys)

3 (average: 2307 in inactive; 107 in active), *Alphaproteobacterium 1* (average: 2653 in inactive; 357 in active) and *Gammaproteobacterium 2* (average: 3088 in inactive; 156 in active) and 3 (average: 1464 in inactive; 374 in active) (Fig. 5). On the other hand, several OTUs were found to prevail in the samples from active chimneys, such as *Aeropyrum* sp. (average: 86 in inactive; 3483 in active), *Sulfurimonas* sp. *1* (average: 153 in inactive; 6959 in active), *unclassified Bacterium* 1 (average: 34 in inactive; 12,240 in active), *unclassified Campylobacterales* (average: 223 in inactive; 18,352 in active), *unclassified Thermotogae* (average: 172 in inactive; 19,693 in active), *unclassified Desulfurobacteriaceae* (average: 39 in inactive; 4512 in active) (Fig. 5). There were also OTUs with high abundance in samples from both active and inactive vents such as: *Anaerococcus* sp. (average: 4988 in inactive; 8214 in active) and *Oceanithermus* sp. (average: 1464 in inactive; 374 in active) (Fig. 5). Moreover, while there were OTUs present in all or most of the samples, some OTUs prevailed in just one sample, e.g., *Streptococcus infantis* in sample V65 (62,172 sequences),

unclassified Desulfurellaceae in V08_a (32,508 sequences), *Desulfurococcaceae* in V08_b (48,126 sequences), *unclassified Hydrogenothermaceae* (34,003 sequences) and *unclassified Thermococcaceae* in V64_b (9138 sequences) (Fig. 5).

Discussion

In the present study we investigated the prokaryotic diversity of the active and inactive sulfide chimneys of the Kolumbo submarine volcano. This field is of particular interest due to its special convergent setting where volcanism and hydrothermal activity occur through thinned continental crust and the concurrent presence of both active and inactive chimneys in close proximity in the same hydrothermal field (Nomikou et al. 2012; Kilias et al. 2013). We applied high-throughput sequencing in a series of sulfide minerals collected from both active and inactive chimneys, as well as from different points along the length and depth of those chimneys. We further investigated the differences occurring in the structure of microbial communities as a result of the different hydrothermal activity and/or physicochemical conditions encountered within localized microhabitats of chimneys.

The ambient water temperatures of the sulfide chimneys (15.8–16.2 °C) were similar with the previously recorded temperatures of the microbial mats surrounding the Kolumbo (16.2–17 °C) hydrothermal vent field (Kilias et al. 2013). Similarly, the bulk elemental compositions of the samples were comparable to the compositions of other sulfide chimneys of the same area (Kilias et al. 2013).

Previous studies on hydrothermal chimneys have used high-throughput amplicon pyrosequencing to describe the diversity and structure of microbial communities (Brazelton and Baross 2010; Flores et al. 2011, 2012; Sylvan et al. 2012; Jaeschke et al. 2012; Kilias et al. 2013; Olins et al. 2013; Dahle et al. 2015; He and Zhang 2016). These studies produced sequence datasets varying between 5485 (Jaeschke et al. 2012) and 206,647 (Sylvan et al. 2012) 16S rRNA gene reads, while the previous study of the Kolumbo hydrothermal field reported 11,566 16S rRNA gene sequences (Kilias et al. 2013). Illumina sequencing platforms are able to produce large numbers of longer DNA reads, thus enabling a more accurate discrimination of archaeal and bacterial communities (Caporaso et al. 2012). Recently, Illumina amplicon sequencing was employed in order to characterize deep-sea microbial communities, such as those in seafloor basalt rocks (Lee et al. 2015) and sediments of inactive hydrothermal vent (Zhang et al. 2013), but this method has not been applied in active and inactive hydrothermal chimneys. To our knowledge, the present study provides the most extensive dataset (14,456,487 reads) generated by deep sequencing for hydrothermal chimneys.

Microbial diversity differences in active and inactive chimneys

The microbial communities that were hosted on the sampled active chimneys had Chao1 values of 1309–6400 (Table 1; Supplementary Table S6). The Chao1 values observed in the active chimneys of Kolumbo were higher than those reported in previous studies (Flores et al. 2011; Sylvan et al. 2012; Olins et al. 2013; Frank et al. 2013), but they were lower than the maximum values from active sulfide sample from the East Pacific Rise (EPR; Chao1: 4311–10821) and the South Atlantic Ridge (SAR, Chao1: 3953–12426) (He and Zhang 2016). Similarly, the microbial communities hosted on inactive deposits had 2365–6973 Chao1 values (Table 1). These were much higher compared to all other deep sequencing studies undertaken in inactive deposits, such as the inactive carbonate chimneys of Lost City Hydrothermal Field (Chao1 for Archaea: 129–599 and for Bacteria: 217–995) (Brazelton et al. 2010) and the inactive sulfide chimneys of EPR (Chao1: 1147–1651) (Sylvan et al. 2012). Before considering the implications of the differences in the biodiversity index values, a portion of these differences can be attributed to the variety of the analytical methods followed by each study. Supplementary Table S6 summarizes some of the key differences of the analytical methods used in the studies, i.e., sequencing technology, 16S rRNA gene target hypervariable region, performance of preclustering and cut off levels.

The most diverse sample analyzed from Kolumbo chimneys was V16_c.out, while the least diverse were V08_b and V08_a, as indicated by Chao1 estimator (Table 1). These findings were further confirmed by the ACE estimator and Simpson indices, which provided similar results about the microbial richness of the specific samples (Table 1). Interestingly, the sample with the highest diversity V16_c.out, also exhibited the lowest content in major and minor elements such as Fe, S, As, Zn, Cu, Tl, Hg, Cd and Ag, while its content in Mn, Ca and P was the highest among the samples (Supplementary Table S1). Regarding the rest of the samples, different estimates were obtained using Chao1/ACE estimators and Simpson indices. These differences can be explained considering that Chao1 and ACE indices take into account singletons (i.e. OTUs represented by only a single sequence), while Simpson index is a richness estimator that is less affected by singletons. The high diversity estimates obtained by species richness indices were also confirmed by rarefaction curves (Supplementary Fig. S1), which despite the deep sequencing effort, did not reach a plateau for most of the samples suggesting that a portion of microbial diversity remained undetected. These diversity analyses also suggest that inactive sulfide chimneys can host highly diverse microbial communities, contrary to the suggestion of Sylvan et al. (2012) who claimed that inactive sulfides represent low

diversity habitats. Indeed, the inactive chimneys from the Kolumbo volcano seem to host more diverse microbial communities than the active chimneys of the same area, as well as other active sulfides from EPR and SAR (He and Zhang 2016). Moreover, the Kolumbo inactive chimneys displayed higher numbers of OTUs, in comparison to those reported in 2013 for other inactive sulfides and microbial mats of the Kolumbo volcano (2221–3881 OTUs) (Kilias et al. 2013). At the same time, the microbial communities from the inactive chimneys from Kolumbo displayed higher diversity than those previously reported for hydrothermal fluids of EPR (2224 observed OTUs) (Campbell et al. 2013), Guaymas Basin (732 observed OTUs) (Campbell et al. 2013) and Mid-Cayman Rise (~ 600 observed archaeal OTUs, ~ 6000 observed bacterial OTUs) (Reveillaud et al. 2015).

The cessation of hydrothermal activity affects microbial succession and biochemical activity

As a first attempt to discern the differences between the microbiomes of active and inactive vents, hierarchical sample clustering was applied using the abundances of the 1000 most prevalent OTUs in our sequence dataset (Fig. 4). A cluster of seven samples originating from inactive chimneys (IN1) was distinctly separated from the cluster representing the five samples collected from the active chimneys (AC). Though an additional cluster was observed for inactive chimneys which comprised four samples (IN2), this smaller cluster showed a higher similarity with the group of active chimneys than the rest of the inactive chimneys. It is difficult to provide a definite explanation for this finding, but it is tempting to speculate that the four samples of IN2 represent inactive chimneys that were influenced by hydrothermal activity over the recent past and represent the transitional phase of microbial community transformation after the cessation of the hydrothermal activity.

To further evaluate the differences of the microbial composition between active and inactive chimneys, we examined the microbial richness and membership in greater detail. The composition of bacterial and archaeal communities has been suggested to be altered considerably once the hydrothermal condition venting ceases and the system is shifted from a reduced state to a more oxidized condition (Toner et al. 2013). This concept has been supported by several previous studies (Suzuki et al. 2004; Kato et al. 2010; Sylvan et al. 2012), which revealed particularly low percentages of Epsilonproteobacteria and Aquificae on inactive chimneys, as compared to active ones. Similar findings were obtained from the analysis of Kolumbo chimney samples. This characteristic pattern shared between the sulfide chimneys of the Kolumbo volcano and other inactive sulfides worldwide (Suzuki et al. 2004; Kato et al. 2010; Sylvan et al. 2012), reinforces the suggestion by Toner et al. (2013), that
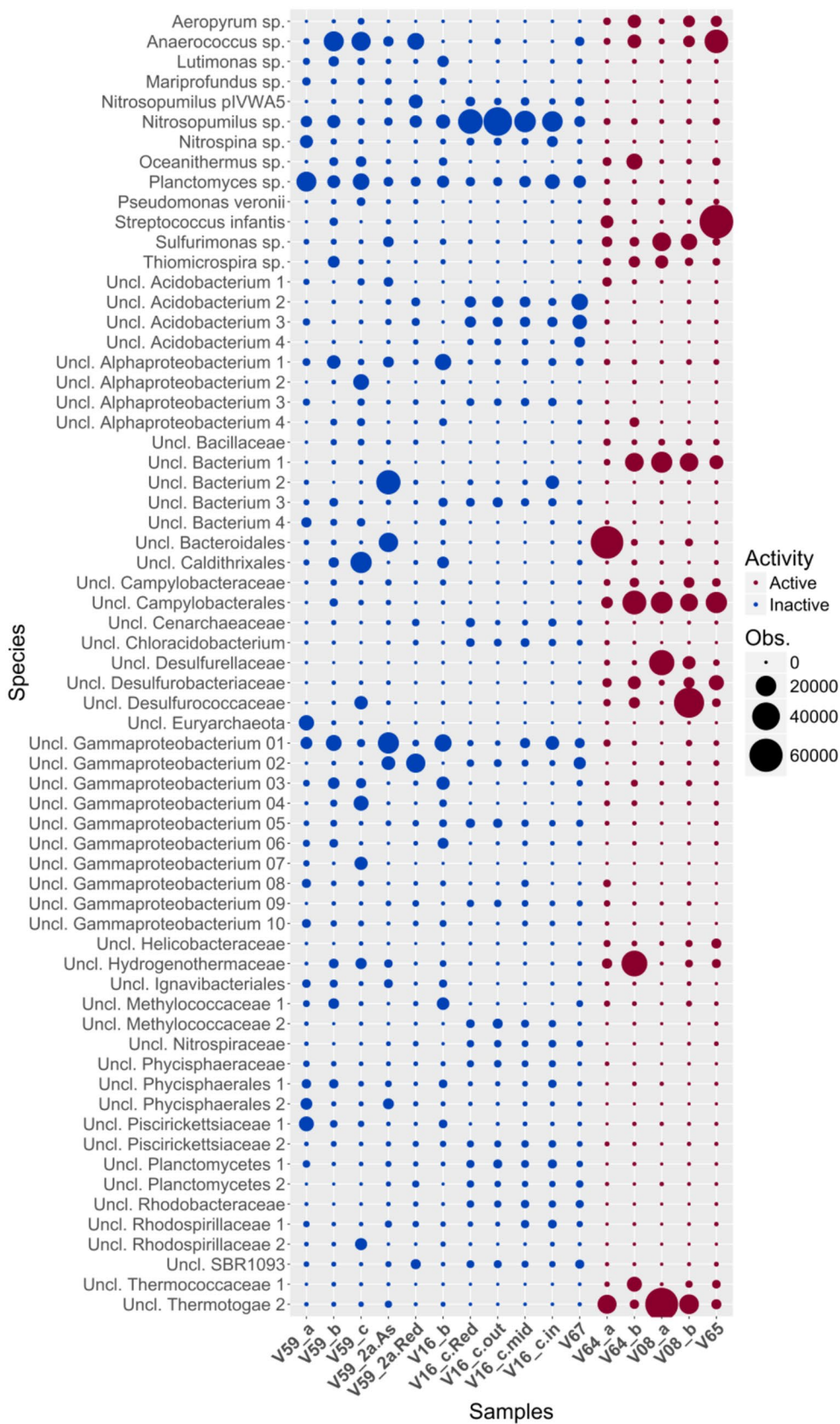
◀**Fig. 5** Comparison of the abundance of the 56 most abundant OTUs among different active and inactive chimney samples. Proportions of the reads in each OTU is indicated by size of circles, as shown in legend. Color indicates the origin of samples: red from an active chimney, blue from an inactive chimney. OTUs that could not be assigned to a species, they were assigned to the next available taxonomic level as unclassified (Uncl.)

common microbial biomes are the result of common physicochemical conditions rather than of similar geographic origin.

Prior studies on inactive sulfide chimneys of the Okinawa Trough, the Central Indian Ridge and the East Pacific Rise identified bacterial sequences that were primarily related to Gammaproteobacteria, Alphaproteobacteria, Deltaproteobacteria, Bacteroidetes and Planctomycetes (Suzuki et al. 2004; Kato et al. 2010; Sylvan et al. 2012). These results are in close agreement with the findings of the present study. In particular, Gammaproteobacteria were found to be the most prevalent group in inactive chimneys of Kolumbo, primarily due to the high abundance of ten unclassified Gammaproteobacteria OTUs and two OTUs of the family Piscirickettsiaceae (Fig. 5, Table 2). Gammaproteobacteria were also found to be prevalent in inactive sulfides in a previous study of the Kolumbo hydrothermal field (Kilias et al. 2013). Members of Gammaproteobacteria have been shown to be effective iron oxidizers (Hedrich et al. 2011), while members from the Piscirickettsiaceae family isolated from hydrothermal environments have been previously implicated in sulfur oxidation and carbon fixation (Brazelton and Baross 2010). Furthermore, the high abundance of Alphaproteobacteria in inactive vents is largely due to the detection of large number of sequences closely related to Rhodobacteraceae family (Fig. 5, Table 2). A Rhodobacteraceae strain recently isolated from a hydrothermal vent of the Southwest Indian Ridge has been shown to oxidize sulfur-containing compounds and to use H_2 as an electron donor (Jiang et al. 2014). The cosmopolitan marine phylum Plactomycetes had a strong presence in inactive sulfide chimneys of the Kolumbo volcano, mainly due to the occurrence of two unclassified Planctomyces OTUs (Fig. 5). A *Planctomyces* species previously isolated from hydrothermal environments was considered to be involved in sulfur reduction (Elshahed et al. 2007). Moreover, the Bacteroidetes phylum has been shown to be abundant in inactive sulfides (Sylvan et al. 2012), while the VC21 group of the Bacteroidetes phylum has been shown to be abundant in mats from the Kolumbo volcano and the Santorini submarine caldera of the HVA (Oulas et al. 2016). Members of the Acidobacteria phylum were found to be among the major components of the rare biosphere of the inactive sulfides in EPR (Sylvan et al. 2012). In the present study, three unclassified Acidobacteria OTUs (Fig. 5) were found to be abundant in the inactive chimneys of Kolumbo. On the other hand, the

most abundant OTU in all inactive chimney samples was a sequence related to the ammonia oxidizing Thaumarchaeon *Nitrosopumilus* sp. (Figure 5, Table 2), confirming previous findings in other chimneys of the same submarine volcano (Kilias et al. 2013).

In contrast to inactive chimneys, the bacterial phyla of active chimneys presented distinct differences. In particular, the most dominant OTUs in all investigated active chimneys of Kolumbo were members of the class of Epsilonproteobacteria. These included two unclassified members of Campylobacterales and one member of the genus *Sulfurimonas* (Fig. 5, Table 2). The denitrifying sulfur oxidizing *Sulfurimonas* has been previously found in other active hydrothermal fields (Kato et al. 2012), whereas members of Campylobacterales have been also isolated from other hydrothermal vents (Mid-Atlantic Ridge and EPR) and have been found to be nitrate reducing chemolithotrophs (Vetriani et al. 2014). Additional bacterial groups were present in all samples of active chimneys, but their abundances fluctuated between samples. Members of Thermotogae, a bacterial phylum with thermophilic and mesophilic anaerobic representatives, dominated in two samples from the V08 chimney (Fig. 5, Table 2). The phylum Deinococcus-Thermus had a strong presence (Table 2) only in the sample collected from the inside part of chimney V64 (Fig. 5). This was mainly due to the dominance of OTUs closely related to the thermophilic chemolithoheterotrophic bacterium *Oceanithermus*, which has been previously isolated from a deep sea hydrothermal vent (Miroshnichenko et al. 2003). An OTU closely related to the Aquificae family of Desulfurobacteraceae dominated all samples retrieved from active chimneys, while another Aquificae OTU closely related to the Hydrogenothermaceae family was dominant in the inside part of chimney V64 (Fig. 5, Table 2). Aquificae have been reported to include thermophilic or hyperthermophilic hydrogen and/or sulfur oxidizing members (Hügler et al. 2007).

Besides the phyla showing a clear preference in active or inactive sulfide chimneys of Kolumbo, some other phyla existed in both of them. Albeit a small part of the microbial communities in Kolumbo chimneys, Zetaproteobacteria were ubiquitously present in all the samples analyzed (Fig. 5, Table 2) and the same was evident for Crenarchaeota (including Thaumarchaeota). *Nitrosopumilus* was the dominant thaumarchaeon in inactive chimneys of Kolumbo, whereas the most prevalent crenarchaeon OTU identified in active chimneys were closely related to the thermophilic sulfur-respiring Desulfurococcaceae family and *Aeropyrum* sp. which is a hyperthermophilic archaeon. The Deltaproteobacteria phylum was also present in all samples, but it exhibited distinct representatives in the active and inactive chimneys (Fig. 5, Table 2). An OTU closely related to Desulfurellaceae was prevalent in the active chimneys (Fig. 5, Table 2). This thermophilic family is involved in sulfate

Table 2 Potential ecological roles associated with certain taxa in both the active and inactive chimneys of the Kolumbo submarine volcano

Potential ecological role	Taxa	Active chimneys			Inactive chimneys				References
		No. of sequences	% active chimney sequences	% all sequences	No. of sequences	% inactive chimney sequences	% all sequences		
Ammonia oxidation	Thaumarchaeota:Nitrosopumilus	4218	0.5	0.2	166475	8.6	5.9	6	Kilias et al. (2013)
Hydrogen oxidation	Aquificae:Desulfurobacteraceae	25628	2.9	0.9	434	>0.1	>0.1	38	Hügler et al. (2007)
	Aquificae:Hydrogenothermaceae	40931	4.7	1.5	9850	0.5	0.4	38	HÜgler et al. (2007)
Iron oxidation	Zetaproteobacteria	543	0.1	>0.1	9010	0.5	0.3	30	Hedrich et al. (2011)
Denitrification	Epsilonproteobacteria:Sulfurimonas	44818	5.1	1.6	11336	0.6	0.4	35	Kato et al. (2012)
Nitrate reduction	Epsilonproteobacteria:Campylobacterales	152569	17.4	5.4	19010	1.0	0.7	36	Vetriani et al. (2014)
	Bacteroidetes	2297	0.3	0.1	10841	0.6	0.4	17	Sylvan et al. (2012)
Nitrification	Betaproteobacteria:Nitrosomonadales:	48	>0.1	>0.1	924	>0.1	>0.1	52	Schmidt (2002)
Nitrite oxidation	Deltaproteobacteria:Nitrospina	800	0.1	0.0	33641	1.7	1.2	53	Spieck and Bock (2005)
	Nitrospira	655	0.1	0.0	13245	0.7	0.5	53	Spieck and Bock (2005)
Nitrogen fixation	Alphaproteobacteria:Rhizobiales	741	0.1	0.0	21596	1.1	0.8	54	Black et al. (2012)
Sulfate reduction	Deltaproteobacteria:Desulfobacterales	2570	0.3	0.1	41994	2.2	1.5	55	Pereira et al. (2011)
	Deltaproteobacteria:Desulfovibrionales	177	>0.1	>0.1	0	0.0	0.0	55	Pereira et al. (2011)
	Deltaproteobacteria:Desulfuromonadales	150	>0.1	>0.1	46	>0.1	>0.1	55	Pereira et al. (2011)
	Deltaproteobacteria:Syntrophobacterales	358	>0.1	>0.1	6640	0.3	0.2	55	Pereira et al. (2011)
	Deltaproteobacteria:Desulfurellaceae	40314	4.6	1.4	45	>0.1	>0.1	39	Bonch-Osmolovskaya et al. (1990)
	Deltaproteobacteria: Thermodesulfobacteriales	2474	0.3	0.1	1145	0.1	>0.1	55	Pereira et al. (2011)
Sulfur oxidation	Alphaproteobacteria:Rhodobacteraceae	3027	0.3	0.1	32095	1.7	1.1	32	Jiang et al. (2014)
	Gammaproteobacteria:Chromatiales	3689	0.4	0.1	7438	0.4	0.3	4	Orcutt et al. (2011)
	Gammaproteobacteria:Thiotrichales	17816	2.0	0.6	48454	2.5	1.7	4	Orcutt et al. (2011)
	Epsilonproteobacteria:	153537	17.5	5.5	19039	1.0	0.7	4	Orcutt et al. 2011
	Gammaproteobacteria:Piscirickettsiaceae	16579	1.9	0.6	40398	2.1	1.4	31	Brazelton et al. (2010)
	Epsilonproteobacteria:Sulfurimonas	44818	5.1	1.6	11336	0.6	0.4	35	Kato et al. (2012)

Table 2 (continued)

Potential ecological role	Taxa	Active chimneys			Inactive chimneys				References
		No. of sequences	% active chimney sequences	% all sequences	No. of sequences	% inactive chimney sequences	% all sequences		
Sulfur reduction	Aquificae:Desulfurobacteraceae	25628	2.9	0.9	434	>0.1	>0.1	38	Hügler et al. (2007)
	Aquificae:Hydrogenothermaceae	40931	4.7	1.5	9850	0.5	0.4	38	Hügler et al. (2007)
Sulfur respiration	Crenarchaeota:Desulfurococcaceae	74077	8.4	2.6	8347	0.4	0.3	56	Macur et al. (2013)
Methane oxidation	Gammaproteobacteria:Methylococcaceae	2327	0.3	0.1	23955	1.2	0.9	57	Lesniewski et al. (2012)
Thermophiles and hyperthermophiles	Thermotogae	98559	11.2	3.5	23957	0.1	0.1	4	Orcutt et al. (2011)
	Deinococcus-Thermus:Thermales	14927	1.7	0.5	23956	0.4	0.3	4	Orcutt et al. (2011)
	Total	748647	85.3	26.7	585207	28.3	19.5		

reduction (Bonch-Osmolovskaya et al. 1990) and isolates from hydrothermal vents have been shown to be thermoacidophilic and involved in metal cycling (Flores et al. 2012).

The present analyses of bacterial and archaeal diversity revealed some interesting trends in the succession of microbial communities taking place in the sulfide chimneys of Kolumbo submarine volcano after the termination of their hydrothermal activity. Table 2 provides an overview of the dissimilarities between active and inactive chimneys on the basis of their microbial communities and provides valuable insights about the potential geochemical role of the changing microbial groups. According to this comparison, bacterial taxa on active (venting) chimneys, such as Epsilonproteobacteria and Aquificae are deemed to be replaced by Gammaproteobacteria, Alphaproteobacteria, Planctomycetes and Bacteroidetes on non-venting chimneys. The case of Deltaproteobacteria, which were abundant in all chimneys, presents a shift from the Desulfurellaceae family in active chimneys to the Desulfobacterales order in inactive chimneys (Table 2).

On the basis of the extensive literature reviewed in present study, it was possible to hypothesize the potential ecological role that the main microbial groups play in geochemical cycling. (Table 2). In the active Kolumbo chimneys, over 85% of the identified sequences corresponded to bacteria and archaea with potential participation in sulfide oxidation/reduction, as well as to (hyper)thermophilic species capable of functioning at the elevated temperature of venting hydrothermal fluids. In contrast, the corresponding contribution of the specific microbial groups in inactive chimneys was only 28%. We can infer that, during active venting, microbes proliferate due to the chemical energy and carbon input provided by the mineral-rich hydrothermal fluids, resulting in microbial communities dominated by chemolithoautotrophs that participate in the cycling of S, N, Fe and H_2. When the venting ceased the energy is provided by the sulfide structure of the chimneys, allowing bacteria and archaeal to occupy every microniche of the sulfide structure and resulting in higher diversity.

In conclusion, we described the microbial communities of the active and inactive hydrothermal sulfides. Many of the bacteria and archaea present in these communities are possible to be involved in reduction–oxidation reaction of Fe, S and N, which are known to be present in high concentrations. In the case of active sulfides, carbon fixation is likely to occur due to the high availability of CO_2 in the diffuse flowing hydrothermal fluids, and the strong presence of thermophilic and hyperthermophilic communities in the inner part of the chimneys. This is evidenced by the dominance of Epsilonproteobacteria, Aquificae and Deltaproteobacteria. These chemolithotrophs appear to colonize the internal and external microniches of the sulfides and utilize the fluids and precipitates of the hydrothermal venting. Despite any apparent differences among individual active chimneys these were generally found to host microbial communities of limited diversity. The highly diverse communities of inactive sulfides are dominated by the archaeon *Nitrosopumilus* sp. and bacteria of the classes Gamma-, Alpha-, and Deltaproteobacteria, and the phyla Planctomycetes, Bacteroidetes and Acidobacteria. These communities were found to be more evenly distributed among the different chimneys and utilize the mineralogy of sulfides chemolithotrophically in order to maintain an unambiguous presence in this peculiar microbial ecosystem.

Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest exists.

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