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Review

Phylogenetic Diversity of Archaea in Shallow Hydrothermal Vents of Eolian Islands, Italy

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Received: 16 July 2019; Accepted: 3 September 2019; Published: 5 September 2019

Abstract: Shallow hydrothermal systems (SHS) around the Eolian Islands (Italy), related to both active and extinct volcanism, are characterized by high temperatures, high concentrations of CO_2 and H_2S, and low pH, prohibitive for the majority of eukaryotes which are less tolerant to the extreme conditions than prokaryotes. Archaea and bacteria are the key elements for the functioning of these ecosystems, as they are involved in the transformation of inorganic compounds released from the vent emissions and are at the basis of the hydrothermal system food web. New extremophilic archaea (thermophilic, hyperthermophilic, acidophilic, alkalophilic, etc.) have been isolated from vents of Vulcano Island, exhibiting interesting features potentially valuable in biotechnology. Metagenomic analyses, which mainly involved molecular studies of the 16S rRNA gene, provided different insights into microbial composition associated with Eolian SHS. Archaeal community composition at Eolian vent sites results greatly affected by the geochemistry of the studied vents, principally by hypersaline conditions and declining temperatures. Archaeal community in sediments was mostly composed by hyperthermophilic members of Crenarchaeota (class Thermoprotei) and Euryarchaeota (Thermococci and Methanococci) at the highest temperature condition. Mesophilic Euryarchaeota (Halobacteria, Methanomicrobia, and Methanobacteria) increased with decreasing temperatures. Eolian SHS harbor a high diversity of largely unknown archaea, and the studied vents may be an important source of new isolates potentially useful for biotechnological purposes.

Keywords: Archaea; archaeal diversity; PCR/DGGE; Illumina sequencing; shallow hydrothermal systems

1. Introduction

Marine hydrothermal systems (shallow and deep-sea vents) are associated with areas of active submarine volcanism and occur at mid-oceans ridges, inland arcs, and submerged volcanoes at both shallow and deep-sea locations, which are currently defined by a cut off at a depth of 200 m [1], that coincides with the limit of the photic zone. The hydrothermal conditions considerably influence the chemical and biological properties of the surrounding environments, which result prohibitive for most organisms. In contrast, hydrothermal conditions have a low impact on prokaryotes (bacteria and archaea), which are able to tolerate both the strong physical and chemical gradients and the so-called extreme conditions. Archaea and bacteria exert a pivotal role in the functioning of the hydrothermal ecosystems, since they are involved in the transformation of inorganic compounds released from vent emissions into biomass and therefore, they are at the basis of the hydrothermal system food web [2,3]. Since many archaea were firstly isolated from thermal springs or deep-sea hydrothermal vents, they have been considered as organisms restricted to extreme environments, although they are now known to be widespread throughout the oceans, where they constitute a relevant fraction of the microbial community [4–7]. Despite the success in culturing heat-loving archaea and bacteria from hydrothermal vents, where the fluid temperature can reach very high temperatures, most of the microbial diversity is still known only as gene sequences. The limited number of archaeal isolates greatly limits our knowledge of their metabolic pathways, and therefore we cannot fully interpret their biochemistry and physiology, and consequently, their impact on biogeochemical cycles, ecological significance, and their role within the ecosystems are until now largely unknown.

The Mediterranean Sea hosts several shallow hydrothermal systems (SHS), of which those located on the Eolian Islands (Italy) [8–15] and the Aegean Volcanic Arc (Greece) [16–18] have been studied for over more than a decade. Shallow hydrothermal systems sustain a more complex microbial community than deep-sea vents, since both sunlight and hydrothermal energy support both photosynthetic and chemosynthetic organisms [19–22]. Although their ubiquitous distribution in tectonically active coastal zones, microbial communities associated with SHS have been less investigated than deep-sea vents.

The SHS off the Eolian Islands (Italy), characterized by high temperatures, high concentrations of CO_2, and low pH, provide excellent natural fields to investigate how microorganisms respond to potential global changes, and to study the effects of increasing temperature and acidification in the oceans. Such ecosystems represent valuable sources of so far unknown types of extremophiles potentially useful for biotechnological purposes.

Here, we compare the phylogenetic diversity of archaeal groups in shallow hydrothermal vents of Eolian Islands using recent results and insights from metagenomic approaches. The incredible uncultivated diversity in Eolian SHS suggests there is much more to be done.

1.1. Diversity of Archaea as Currently Known

The archaea were phylogenetically distinguished from the bacteria in 1977, when Carl Woese and Fox [23] revolutionized phylogeny by comparing conserved small subunit (16S and 18S) ribosomal RNA (rRNA) sequences. Successively, archaea were divided into two groups with the taxonomic rank of kingdoms: the Crenarchaeota, all hyperthermophilic, and the Euryarchaeota, containing species with a variety of phenotypes (hyperthermophilic, mesophilic, methanogenic, and halophilic) (Woese et al. 1990) [24]. There are many debates for taxonomy of archaea, since several possible novel phyla have been also proposed only based on phylogenetic analyses in metagenomic or single cell genomic studies. More recently, phylogenetic analyses have supported a clade uniting the Thaumarchaeota, Aigarchaeota, Crenarchaeota, and Korarchaeota which has been informally named the “TACK” archaea [25] or “Proteoarchaeota” [26]. A novel candidate archaeal phylum, Lokiarchaeota, was described as related to the TACK superphylum, representing the nearest relative of eukaryotes in phylogenomic analyses [27]. Other superphyla of Euryarchaeota actually include DPANN (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota, Nanohaloarchaeota, Woesearchaeota, Pacearchaeota, and potentially Altiarchaea) and the more recently described Asgards (in Norse mythology, one of the Nine Worlds, home to the Æsir gods) consisting of Lokiarchaeota, Thorarchaeota, Odinarchaeota, and Heimdallarchaeota [28–30].

Members of Crenarchaeota phylum were initially believed to be mostly thermophilic, sulphur-metabolizing archaea, but their detection in different environments of oceans indicated that this domain is more widely distributed than previously assessed [31]. Crenarchaeota are generally the most abundant component of archaea populations in oxygenated deep waters [32,33] and surface sediments, surpassing the Euryarchaeota abundances of ca. fivefold [34,35]. Thaumarchaeota, embracing mesophilic Crenarchaeota [36], with the genera *Cenarchaeum* and *Nitrosopumilus*, are numerically dominant in deep marine waters and in non-diffuse flow hydrothermal vents than in normal deep and surface marine waters [37–39]. The Korarchaeota were detected in hydrothermal springs by using culture-independent molecular techniques [40] and the first genome of *Candidatus* Korarchaeum cryptofilum was physically isolated from an enrichment culture inoculated with sediments from Obsidian Pool, Yellowstone [41].

The Euryarchaeota phylum comprises methanogens (e.g., genera *Methanococcus* and *Methanothermus*), methanotrophs, extreme halophiles (e.g., genera *Halococcus* and *Halobacterium*), and extreme thermophiles, such as *Archaeoglobus* and *Thermococcus*. The Nanoarchaeaota have been recognized as a group in Euryarchaeota [42], represented by single co-cultured and characterized isolates *Nanoarchaeum equitans*, an ultra-small ectosymbiont residing on the marine hyperthermophilic crenarchaeote *Ignicoccus hospitalis* [43], *Nanopusillis acidilobi*, hosted by *Acidilobus* from a terrestrial geothermal environment [44], and the more recently described *Nanoclepta minutus*, hosted by *Zestosphaera tikiterensis* from a New Zealand hot spring [45].

1.2. Characteristics of Thermo- and Hyperthermophilic Cultivated Archaea

In the respect of temperature conditions, several archaeal genera are thermophilic (with optimal growth temperature >60 °C), and most of them also hyperthermophilic (with optimal growth at >80 °C) (Table 1) [46–54], while only two orders of bacteria, Thermotogales and Aquificales, are known to grow optimally above 80 °C.

Table 1. Some characteristics of archaea that grow above 70 °C. The genera, optimal growth temperatures, type of metabolism (A: autotrophic, H: heterotrophic, FA facultatively autotrophic), and electron acceptors are provided for each listed order.

Phylum	Class	Order	Genus	T opt (°C)	Metabolism	Electron Acceptors	Reference
Crenarchaeota	**Thermoprotei**	**Acidilobales**	*Acidilobus, Caldisphaera*	70–85	H	S°, O_2, Fe(III)	[46]
		Desulfurococcales	*Aeropyrum, Desulfurococcus, Hyperthermus, Ignicoccus, Ignisphaera, Pyrodictium, Pyrolobus, Staphylothermus, Stetteria, Sulfophobococcus, Thermodiscus, Thermogladius, Thermosphaera*	85–106	A, H, FA	H+, S°, $S_2O_3^{2-}$, SO_3^{2-}, Fe(III), NO_3^-, O^2	[47]
		Thermoproteales	*Caldivirga, Pyrobaculum, Thermocladium, Thermofilum, Thermoproteus, Vulcanisaeta*	75–100	H, FA	O2, S°, SO_4^{2-} $S_2O_3^{2-}$, NO_3^-, Fe(III), AsO_4^{3-}, SeO_4^{2-}	[48,49]
		Sulfolobales	*Acidianus, Metallosphaera, Stygiolobus, Sulfolobus, Sulfurisphaera*	70–85	A, FA	S°, O_2, Fe(III)	[50,51]
Euryarchaeota	**Archaeoglobi**	**Archaeoglobales**	*Archaeoglobus, Ferroglobus, Geoglobus*	70–88	A, H, FA	SO_4^2, $S_2O_3^{2-}$, NO_3, Fe(III)	[52]
	Methanobacteria	**Methanobacteriales**	*Methanothermus*	83–88	A	CO_2	[53]
	Methanococci	**Methanococcales**	*Methanocaldococcus, Methanotorris*	80–88	A	CO_2	
	Methanopyri	**Methanopyrales**	*Methanopyrus*	95–100	H	CO_2	
	Thermococci	**Thermococcales**	*Palaeococcus, Pyrococcus, Thermococcus*	80–100	H	H^+, S°	[54]

An extremely piezophilic strain of *Pyrococcus yayanosii* was isolated from the deep hydrothermal field of Ashadze [55]. The highest temperature at which *Methanopyrus kandleri* can grow under concomitant elevated pressure is 122 °C [56].

2. Eolian Shallow Hydrothermal Systems

The Eolian Archipelago (Tyrrhenian Sea, Italy), located at 25 km north of Sicily, consisting of seven main islands (Figure 1), hosts numerous SHS related to both active and extinct volcanism, at a depth allowing investigations by scuba divers.

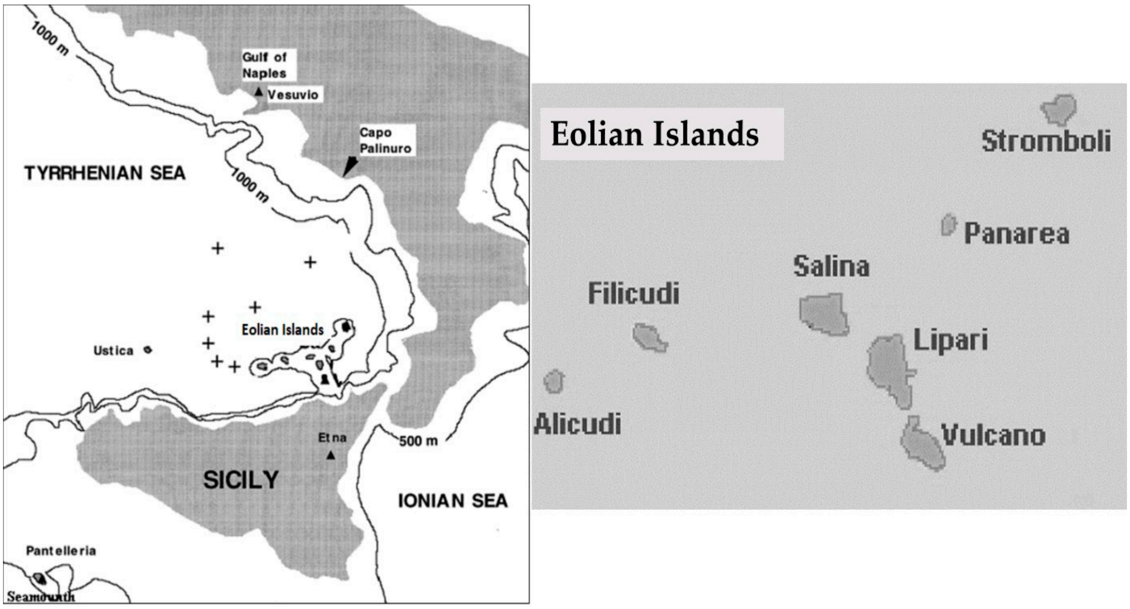

Figure 1. Hydrothermal areas in the Tyrrhenian Sea and the Eolian Islands (Italy).

At present, significant volcanic manifestations exist on only two of the Eolian Islands: Vulcano with fumarolic activity at the crater of La Fossa, and Stromboli with permanent explosive (strombolian) activity at the homonymous crater.

Submarine hydrothermal vents, common to all Eolian Islands, release both thermal waters and gases whose temperatures, detected at the emission points, are in the range of 27–130 °C. Elemental sulfur is a common mineral found in sediments around the vents. Fluids diffusely escape from open fractures of the rocks or diffuse through the sand from the sea-floor. The hydrothermal fluids might emit significant amounts of heavy metals that can precipitate around the vents. On the basis of the geochemistry of the emitted fluids, Italiano [57] proposed the existence of geothermal systems beneath all the Eolian Islands, and estimated the deep temperature to be in the range of 150–350 °C. The fluid composition is similar to those of deep-sea hydrothermal vents, and gases consist mostly of CO_2 plus variable concentrations of the reactive gases H_2S, O_2, CH_4, CO, and H_2, as well as inert gases (N_2, Ar, He). The pH values range between 1.9 and 5.7 [58–60].

2.1. Vulcano Island SHS

Levante Harbor of Vulcano Island is a shallow bay (Figure 2) where venting and diffuse emissions of hydrothermal fluids are easily visible.

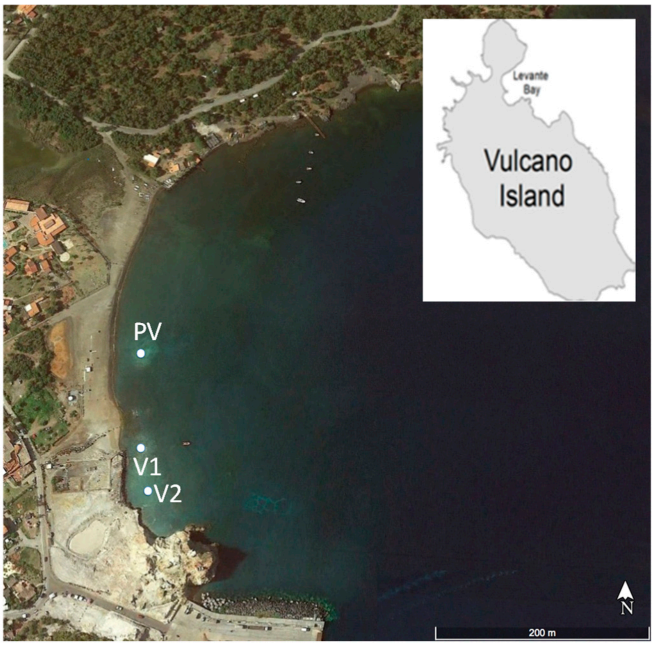

Figure 2. Levante Harbor at Vulcano Island and sampling site locations: V1, V2, and PV.

Characteristics of sampling sites are reported in Table 2.

Table 2. Main features of the fluids vented at Levante Harbor, Vulcano Island.

	Physical and Chemical Composition of Fluid							
Site and Sample	**Depth (m)**	**T (°C)**	**pH**	**Conductivity (mS/cm)**	**CO$_2$ (%)**	**O$_2$ (%)**	**H$_2$S (%)**	**Reference**
Levante Harbor	6	32–38	4.4–6.3	47.9	nd	bdl-5.7	bdl-8.4	[8]
V1	2	35	6.1	47.9	1010	0.6	1.8	[61]
V2	0.8	60	5.8	48.8	992	0.2	5.1	
PV	1	60	6.4	39.1	nd	6.5	nd	[62]
Reference Site	Sea level	15	8.1	54.0	0.24	4.8	bdl	[9]

bdl: below the detection limit. nd: not determined.

The studied vents of Vulcano Island showed values really variable along the sampling sites. In particular pH showed an increasing range from value of 5.8 (site V2) to value of 8.1 at the reference site, not influenced by the vent area.

2.2. Panarea Island SHS

The submarine hydrothermal system off Panarea Island hosts the most active vents of the whole Mediterranean area and has been studied since the mid 1980s. The interest in the area around the Panarea Island increased since November 2002 when a great exhalative event occurred. The volcanic structure has evolved in recent geological times following different stages of activity: First, the central apparatus developed with the Island of Panarea, then the volcanic structure enlarged to the East by a fault system NE-SW oriented (Figure 3).

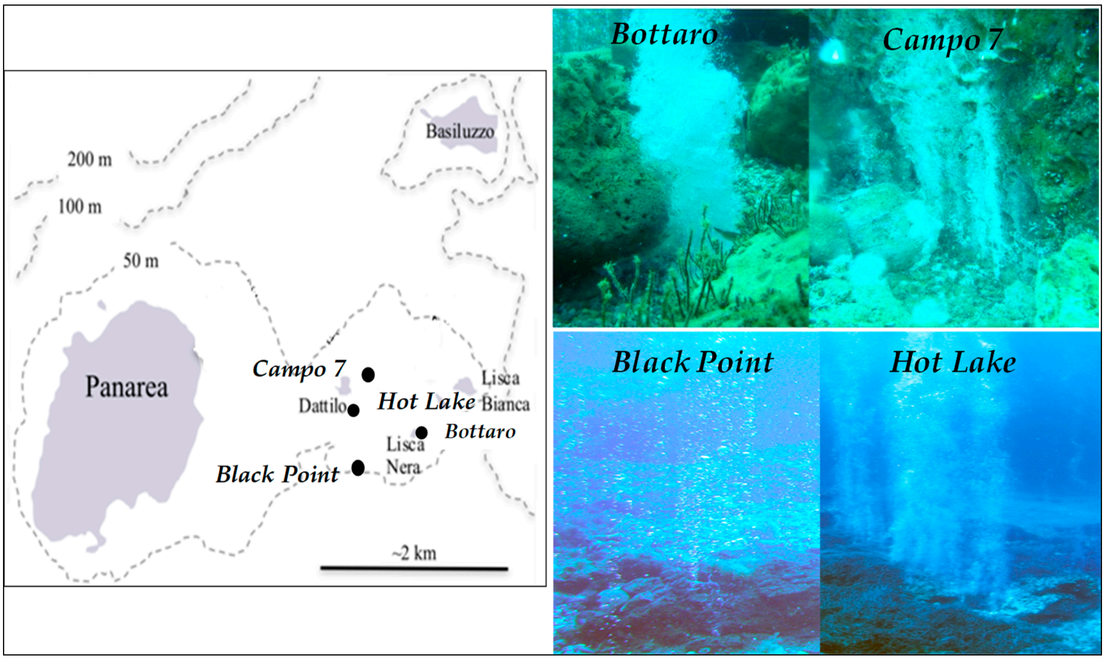

Figure 3. Map of Panarea Island and surrounding islets. Sampling sites' location (closed circles) and vents emissions at Bottaro, Campo 7, Black Point, and Hot Lake.

The exhalative area, which is recognized as the remnants of a crater rim, is delimited by a group of islets (Dattilo, Bottaro, Lisca Bianca, and Lisca Nera) located 2.5 km east of the main island. The vents occur at shallow depths although hydrothermal deposits and chimneys can be found in deep waters (>400 m; Italiano, personal communication).

The Black Point site, characterized by the presence of black sulfide and manganese incrustations, is a submarine crater in the seafloor located at a depth of 23 m, and it extends about 25 m in the north-south and about 20 m in the east-west directions [14,21,60]. When compared to other investigated shallow vents in the same area of Panarea, fluids from Black Point vents are characterized by the highest temperatures (120 and 130 °C) and the lowest pH (2.4 and 3.3) (Table 3).

Table 3. Analytical data of hydrothermal fluids emitted from vents of Panarea Island and collected sample type (F: fluid and S: sediment).

		Physical and Chemical Properties of Fluid				**Gas Concentration in Vol%**			
Site	**Sample Type**	**Depth (m)**	**T (°C)**	**pH**	**Conductivity (mS/cm)**	**CH_4**	**He**	**N_2**	**Reference**
Bottaro	Bottaro1 (F, S)	8	52	5.3	51.3	5.1×10^{-4}	6.0×10^{-4}	0.29	[12]
Campo 7	Campo 7 (F, S)	21	65	4.9	44.7	bdl	7.0×10^{-4}	bdl	
Black Point	BP120 (S)	23	120	3.3	63.7	1.0×10^{-4}	bdl	0.01	Unpublished data
Black Point	BP130 (F, S)	23	130	3.3	46.2	6.0×10^{-2}	1.0×10^{-3}	0.37	[21]
Black Point Vent 1	BP74 (F, S)	23	74	3.3	66	9.0×10^{-2}	1.1×10^{-2}	0.44	[14]
Black Point Vent 2	BP27 (F, S)	23	27	2.4	55	1.7×10^{-1}	9.6×10^{-3}	0.85	
Hot Lake	HL94 (S)	~20	94	4.7	94	5.0×10^{-4}	nd	0.5	[15]
Hot Lake	HL28 (S)	~20	28.5	4.5	98	5.0×10^{-4}	nd	0.5	

bdl: below the detection limit. nd: not determined.

Gases are mainly composed of CO_2. Concentrations of CH_4 and He from Black Point were higher than those reported from the exhalative area at Bottaro and Campo 7 vents. Among sites of the submarine hydrothermal system of Panarea, higher redox values (up to about + 300 mV) were recorded at Black Point [59], indicating the presence of only partial reducing conditions.

Hot Lake is a recently described shallow brine-pool located north-west of the Lisca Bianca and Bottaro islets, off Panarea Island, where emitted fluids (pH 4.6) are highly saline and rich in CO_2 and H_2S [15,59]. The Hot Lake pond is an oval-shaped depression in the seafloor at depth of 20–23 m [59].

3. Archaea in Eolian SHS

3.1. Archaeal Isolates

The thermal springs in the Levante harbor of Vulcano Island host dozens of aerobic and anaerobic, thermophilic, and hyperthermophilic microorganisms belonging to bacteria and archaea domains [13]. Thermophilic and hyperthermophilic archaea were isolated from the thermal springs of Vulcano Island, some of them are of interest in pure and applied research (Table 4).

Table 4. Thermophilic and hyperthermophilic archaea isolated from thermal springs of Vulcano Island.

Phylum	Class	Species	Reference
Crenarchaeota	Thermoprotei	*Acidianus brierleyi*	[63]
		Acidianus infernus	[64]
		Pyrodictium occultum	[65]
		Staphylothermus marinus	[66]
		Thermodiscus maritimus	[67]
Euryarchaeota	Archaeoglobi	*Archeoglobus fulgidus*	[68]
		Ferroglobus placidus	[69]
	Methanococci	*Methanococcus aeolicus* PL1/5H	[70]
	Thermococci	*Palaeococcus helgesonii*	[71]
		Pyrococcus furiosus	[72]
		Pyrococcus woesei	[73]
		Thermococcus acidaminovorans	[74]
		Thermococcus alcaliphilus	[75]
		Thermococcus celer	[76]
	Thermoplasmata	*Thermoplasma volcanium*	[77]

The first isolated organism at temperatures above 103 °C was the sulfur-reducing archaeon *Pyrodictium occultum* [65] with optimal growth temperature of 105 °C. The furious fireball, *Pyrococcus furiosus* (optimal growth temperature of 100 °C) [72], is the source of a very thermostable, commercially available DNA polymerase. *Archaeoglobus fulgidus* still holds the record as the highest temperature marine sulfate reducer, able to oxidize H_2 [68], and *Ferroglobus placidus* (65–95 °C) couples nitrate reduction with iron oxidation [69].

Several Eolian isolates were closely related to those isolated from deep-sea locations. Among them, *Methanococcus aeolicus* PL-15/H, a CO_2-reducing methanogen isolated from Eolian marine sediments [70], producing three different restriction enzymes, was closely related to two strains from sediments taken from the Nankai Trough near the coast of Japan. The new species *Palaeococcus helgesonii*, one of the rare oxygen tolerant hyperthermophiles, was isolated from a geothermal well of Vulcano Island [71]. This species belongs to the same genus first isolated from a deep-sea hydrothermal vent chimney in Japan [78]. All these species make the Levante harbor of Vulcano Island the "type locality" for cultivated hyperthermophiles.

From Panarea submarine vents (20 m depth, fluid temperature 80 °C), Amend and colleagues (personal communication) isolated *Thermococcus barossii, T. celer*, *T. peptonophilus*, *T. profundus*, and *T. stetteri*, and two strains closely related to *Thermococcus* retrieved in Loihi Seamount (Hawaii).

Due to the frequent isolation of archaea from Eolian shallow hydrothermal vents, it was assumed that archaea could dominate prokaryotic communities of these sites.

3.2. Archaeal Community Composition by Culture-Independent Approach

With advances in culture-independent approaches, which mainly involve techniques based on 16S rRNA genes, an unpredictable, high diversity of microbial community was observed. Archaeal communities at shallow hydrothermal vents are generally found to be less abundant and diverse than the coexisting bacterial communities [17,79]. The location and nature of substrata could greatly influence the composition of the archaeal community. As demonstrated by culture-independent 16S rRNA gene surveys, thermophiles and hyperthermophiles appear segregated into specific vents, to higher temperature sediments, or internal chimney habitats [80,81]. In spite of the great variety of archaea previously isolated by several authors from Vulcano Island, a quite different picture was revealed when molecular methods were used. Samples from hydrothermal seeps of Vulcano Island, investigated by fluorescent in situ hybridization (FISH) using new probes, reported that the diversity of thermophiles at Vulcano was far greater than that represented by cultivated strains [82]. Sequences of DNA extracted from water and sediment samples, and from a geothermal well (Pozzo Istmo), detected a dozen of crenarchaeal, euryarchaeal, and korarchaeal lineages belonging to phylogenetic groups that have no cultured representatives at all [83]. However, one of the sequences from Pozzo Istmo was nearly identical (99% similarity) to *Palaeococcus helgesonii*, the euryarchaeon previously isolated from this well [71].

The diversity of both bacteria and archaea thriving at Eolian SHS has been investigated by a fingerprinting method, the denaturing gradient gel electrophoresis (DGGE) [12,13,21]. This method is considered one of the most reliable tools for screening and analyzing the microbial community of complex ecosystems. Since this approach allows for the detection of microorganisms only if their proportion is greater than 1% of the total community, the microbial community diversity is expressed in terms of dominant phylotypes [84]. Fragments resolved by PCR/DGGE of 16S rRNA from bacteria and archaea from sediment samples collected at two Vulcano vents (VS1 and VS2), characterized by different temperatures (35 and 60 °C, respectively), indicated that the richness of archaea was lower than that of bacteria [21]. The dominant phylotypes retrieved at these vents were referred to uncultivated clones of Euryarchaeota, with the only exception of one sequence affiliated with *Natronorubrum thiooxidans* (Halobacteriaceae), a hyperhalophilic archaeon currently detected only in other extreme habitats [85].

More recently, investigations were carried out along a pH gradient at increasing distance from the primary vent (PV) in the Levante Harbor [62]. DGGE results showed that archaeal richness and diversity increased the narrowing PV site. Archaeal sequences were affiliated with Euryarchaeota and Crenarchaeota and all phylotypes were related to archaea retrieved from extreme environments, most of which were characterized by high temperatures. Sequences were affiliated with members assigned to hyperthermophilic Euryarcheota belonging to the genus *Thermococcus* (Thermococci) and to the thermophilic genus *Methanobrevibacter* (Methanobacteria). Norteworthy, more sequences were retrieved within Crenarcheota and related to different members of the class Thermoprotei, with the hyperthermophilic genera *Desulfurococcus, Vulcanisaeta* and *Aeropyrum*. Hyperthermophilic Thermococci (*Ferroglobus, Palaeococcus, Pyrococcus* and *Thermococcus*) and Thermoprotei (*Staphylothermus*) were demonstrated using DGGE in sediments from two extremely hot (100°C) shallow hydrothermal vents of Vulcano Island by Antranikian et al. [86]. Moreover, as revealed using enrichment cultures, the high dominance of *Thermococcus* and *Palaeococcus* in the two vents was mainly dependent on the carbon source available, rather than the site temperature [86].

The DGGE technique, applied in samples collected from three different vents off Panarea Island (Bottaro1, Campo 7, and Black Point), showed that archaeal populations from the vents were more homogenously distributed in sediments than in fluids, due to the fact that changes in chemical properties and gas composition in fluids were more evident. Phylogenetic analysis of archaeal DGGE 16S rRNA gene sequences revealed that most of sequences were referred to Euryarchaeota, followed by Crenarchaeota. Almost all sequences were affiliated with uncultured clones of archaea mainly retrieved from hot springs and hydrothermal vents of different geographical zones (Table 5)

Table 5. Archaeal distribution arranged by samples collected from Bottaro, Campo 7, and Black Point vent sites, at Panarea system and sample type (F: fluid and S: sediment), as elucidated by PCR/DGGE profiles.

Sample	Band	Sample Type	BLASTn Result	Percentage of Identity	Phylogenetic Affiliation	Found in Environment
Bottaro 1 $^{(a)}$	3	F	Uncult. archaeon clone 20c-54	94	-	Sediments of the Aegean Sea
	4	F	Uncult. clone Y5x	96	Crenarchaeota	Basaltic flanks
	5	F	Uncult. archaeon clone VulcPIw.66	96	-	Geothermal well
	6	F	Uncult. clone a87R72	96	Euryarchaeota	Basaltic flanks
	7	F	Uncult. clone D_A04	96	Crenarchaeota	Hydrothermal Vent, New Zealand
	8	F	*Paleococcus helgesonii* DSM 15127	96	Euryarchaeota	Geothermal well
	9	S	Uncult. *Ferroglobus* sp. clone IAN1-2	87	Euryarchaeota	Deep hydrothermal fluids
Campo 7 $^{(a)}$	13	F	Uncult. clone LDS17	95	Euryarchaeota	Dagow Lake
	14	S	Uncult. clone G37A	91	Euryarchaeota	Salt crust
Black Point 130 $^{(b)}$	1	F, S	Uncult. archaeon clone pCIRA-E	95	-	Deep-sea hydrothermal field, Japan
	2	F, S	Uncult. archeon clone VulcPIw.164	94	-	Geothermal well on Vulcano Island, Italy
	3	F, S	Uncult. clone ESYB61	86	Euryarchaeota	Estuarine sediment at the mouth of Orikasa River
	4	F, S	Uncult. archaeon clone CaR3s.09	92	-	Coastal Arctic ecosystem
	5	F, S	Uncult. clone PNG_TB_4A2.5H2_A3	90	Crenarchaeota	Arsenic-rich sediment shallow vent, Papua Guinea
	6	F	*Paleococcus helgesonii* DSM 15127	85	Euryarchaeota	Geothermal well on Vulcano Island, Italy

a: Maugeri et al. [12]. b: Maugeri et al. [21].

Some sequences were only moderately related (<95% similarity) to database entries and therefore they could represent new archaeal taxa. All retrieved uncultered clones were related to Archaea detected at extreme environments, most of which were characterized by high temperature. Only two sequences were related to *Palaeococcus helgesonii*, the euryarchaeon isolated from a Vulcano Island well [68], demonstrating that Panarea and Vulcano hydrothermal systems may host similar members of archaeal populations. Sequences related to an uncultured *Ferroglobus* sp. (Archaeoglobi), reported from deep-sea fluids [87], were also retrieved.

3.3. Data from Next Generation Sequencing Technologies

To analyze the real microbial diversity, powerful sequencing technologies have recently been applied to marine samples from various deep-sea hydrothermal vents [39,88–90] and in shallow-water hydrothermal vents [14,17,91–93].

High-throughput sequencing techniques represent powerful tools for studing microbial diversity, since the increased numbers (millions) of operational taxonomic units (OTUs) offer the opportunity of revealing simultaneously a large number of individuals and their taxonomic affiliation [14,94]. To gain a better understanding of microbial diversity associated with Panarea vents, we applied Illumina high-throughput amplicon sequencing of the V3 region of the 16S rRNA gene for bacteria and archaea to samples collected from Black Point [14,95] and Hot Lake sites [15]. This tool enabled us to detect and enumerate also microorganisms occurring at very low relative abundance (lower than 0.01%). As resulted by the analysis of high-quality reads, archaea represented a minor component of the prokaryotic community at Black Point (from 0.03% to 1.6% of high-quality reads), and Hot Lake (from 0.05% to 3.5%) sites, at both low- and high-temperature conditions.

In fluids emitted at Black Point sites, Euryarchaeota dominated the archaeal community at high (74 °C, BPF74) and low (27 °C, BPF27) temperatures, Halobacteria were prevalent (Figure 4) and were affiliated with several genera (*Haloarcula, Halobacterium, Halobiforma, Halomicrobium, Haloplanus, Halorubrum, and Natronomonas*) (Table 6).

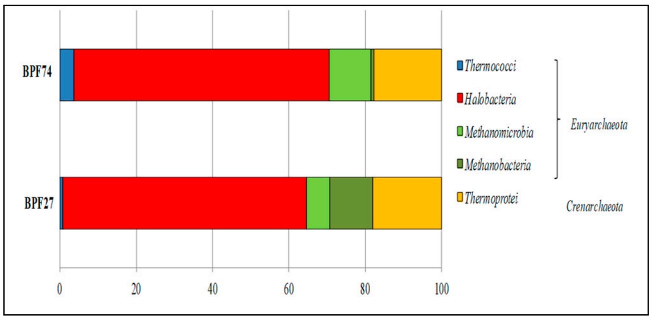

Figure 4. Archaeal community composition in fluids collected from Black Point site at high (BPF74) and low temperature (BPF27).

Sequences referred to class Methanobacteria, with the genus *Methanobrevibacter* were more abundant in the low-temperature fluid (BPF27), while those affiliated class Methanomicrobia, with the genus *Methanosarcina*, were prevalent in the high-temperature fluid (BPF74) (Table 6). Sequences affiliated with the Thermococci class, felting into the genus *Palaeococcus*, were more abundant in the high-than in the low-temperature fluid. All crenarchaeotal sequences were related to hyperthermophilic members of the Thermoprotei class. Among them, the most abundant genus was *Staphylothermus* (Table 6) of which the type strain *Staphylothermus marinus*, a strictly anaerobic, heterotrophic, and S°-dependent archaeon was isolated from the heated submarine sediments at Vulcano Island [66]. Very few sequences were referred to the genus *Thermocladium*, firstly isolated from solfataric muds in Japan, and reported as inhabitant of acidic, extremely thermophilic (i.e., 65–80 °C), terrestrial hot spring area [96].

A high number of archaeal sequences from sediments collected from BP and HL remained unclassified at the phylum and the class level. Archaeal community composition in the sediment samples from BP and those from HL vents greatly differed, determining distinct communities at the different temperature conditions (Figure 5).

Members of Euryarchaeota dominated the archaeal community associated with sediments at Hot Lake, while Crenarchaeota (Thermoprotei class) prevailed at BP120. The archaeal community composition from the two sites was distinct, since some groups retrieved at BP were not found at HL and *vice versa* (Table 6).

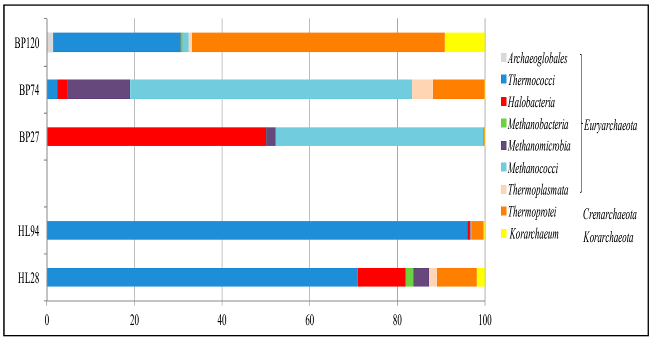

Figure 5. Relative abundances of the archaeal phyla and classes retrieved at different temperature conditions from sediment samples, at Black Point (BP120, BP74, and BP27) and Hot Lake (HL94 and HL28) sites.

Sequences referred to *Methanococcus* and *Methanothermococcus* (Methanococci) were found only in sediments from Black Point, while those referred to *Halococcus*, *Methanosphaera*, and *Methanohalophilus* were only retrieved from Hot Lake, whereas *Methanobrevibacter* (Methanobacteria) were in both sites. Among Methanomicrobia class, the genus *Methanococcoides* was present in samples (BP74, BP120, and HL94) with higher temperatures. Sample BP120 was the richest in hyperthermophilic genera within Thermococci (*Palaeococcus*, *Pyrococcus*, and *Thermococcus*) and Thermoprotei (*Ignicoccus*, *Ignisphaera*, *Staphylothermus*, *Thermodiscus*, *Thermocladium*, *Thermofilum*, and *Vulcanisaeta*). The single genus of Korarchaeota, related to "*Candidatus* Korarchaeum", was retrieved in samples from both Black Point (BPF74 and BP120) and Hot Lake (HL94 and HL28) sites.

Differences in archaeal communities from Black Point and Hot Lake sites emerged more clearly when abundances of archaeal classes were analyzed together with some physical and chemical properties (Table 3) (Figure 6).

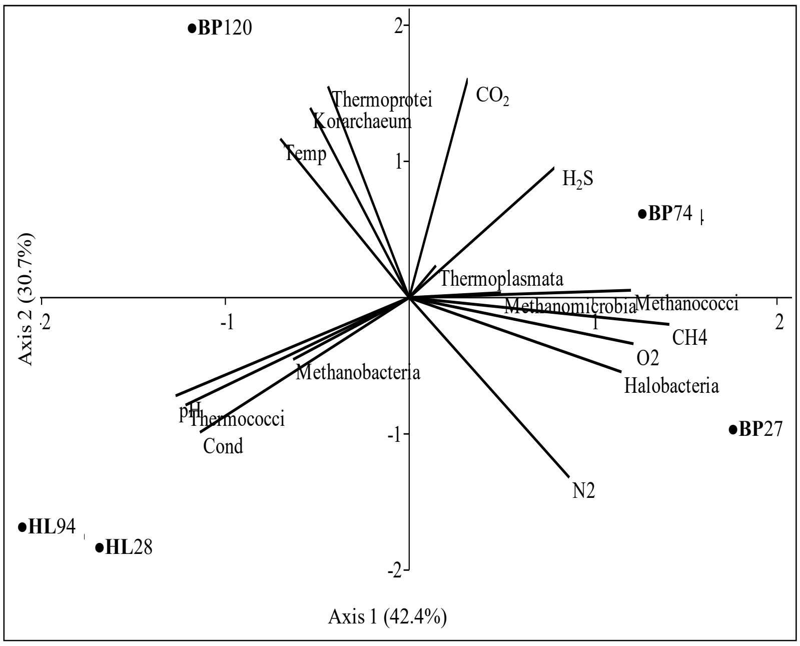

Figure 6. Principal component analysis based on the relative abundances of archaeal classes Thermococci, Halobacteria, Methanobacteria, Methanococci, Methanomicrobia, Thermoplasmata, Thermoprotei, and Korarchaeum, detected in sediments and fluids, and pH, conductivity (Cond), temperature (Temp), O_2, N_2, CH_4, CO_2, H_2S from BP (BP27, BP74, and BP120) and HL (HL28, HL94) sites.

Table 6. Archaeal class, order, and genus distribution in fluids and sediments from Black Point (BP120, BP74, and BP27) and Hot Lake (HL94 and HL28) sites, as elucidated by Illumina sequencing technology.

Phylum	Class	Order	Genus	Black Point					Hot Lake	
				Fluid		Sediment			Sediment	
				BPF74 (a)	BPF27 (a)	BP 120	BP 74 (a)	BP 27 (a)	HL 94 (b)	HL 28 (b)
Euryarchaeota	Archaeoglobi	Archaeoglobales	*Archaeoglobus*			*				
	Halobacteria	Halobacteriales	*Haloarcula*		*					
			Halobacterium	*	*					
			Halobiforma	*	*					
			Halococcus						*	
			Halomicrobium	*	*					
			Haloplanus		*					
			Halorubrum	*	*					
			Natronomonas	*	*					
	Methanobacteria	Methanobacteriales	*Methanobrevibacter*	*	*	*				*
			Methanosphaera							*
	Methanococci	Methanococcales	*Methanococcus*				*	*		
			Methanothermococcus			*	*	*		
	Methanomicrobia	Methanosarcinales	*Methanococcoides*			*	*		*	
			Methanohalophilus							*
			Metanosarcina	*	*					
	Thermoplasmata	Thermoplasmatales	*Thermogymnomonas*				*		*	*
	Thermococci	Thermococcales	*Paleococcus*	*	*	*	*		*	*
			Pyrococcus			*				
			Thermococcus			*			*	*
Crenarchaeota	Thermoprotei	Desulfurococcales	*Ignicoccus*			*				
			Ignisphaera			*				
			Staphylothermus	*	*	*				
			Thermodiscus			*	*	*	*	
		Thermoproteales	*Thermocladium*	*	*	*				
			Thermofilum			*				
			Vulcanisaeta			*				
Korarchaeota			*Candidatus* Korarchaeum	*		*			*	*

a: Lentini et al. [14]. b: Gugliandolo et al. [15].

As expected, Halobacteria and Methanococci greatly depended on O_2 and CH_4, respectively, whereas Thermoprotei (Crenarchaeota) and Korarchaeum by temperature. Halobacteria were exclusive in fluids, whereas Methanococci in sediments at BP sites, even if each sample was related to different members at the genus level (Table 6).

Archaeal communities in samples from BP were distinguished from each other. The hottest sample (BP120) was associated with Thermoprotei, and Korarchaeum abundances. This finding distinguished the sample from those previously analyzed from Eolian SHS, but makes it similar to deep-sea hydrothermal locations [97]. Sample BP74 was linked with CO_2, H_2S, and Thermoplasmata, mainly represented by the moderately thermophilic *Thermogymnomonas* genus. Members of methanogens, strictly linked to CH_4, distinguished BP74 communities from those of the BP27 sample, since abundances of Methanococci were linked to BP74, whereas those of mesophilic Methanomicrobia to BP27.

Despite the great difference in temperature, samples from HL sites grouped together, characterized by higher salinity and pH values (4.5–4.7) and less CO_2 content than BP sites. HL28 and HL94 were strictly associated with Thermococci (mainly represented by *Palaeococcus* and *Thermococcus*) whereas Methanobacteria (with genera *Methanobrevibacter* and *Methanosphaera*) were distinctive of the coldest site HL28 (Table 6).

3.4. Comparison with Archaeal Communities from Other Hydrothermal Vents

After the definition of the Vulcano Island shallow hydrothermal system as the site with the most isolated hyperthemophiles, more insights on the archaeal community composition of Eolian SHS have been obtained by applying molecular culture-independent techniques. As resolved by Illumina sequencing in Vulcano and Panarea sites, the retrieved archaeal communities appeared remarkably diverse, especially when observed at class level, from each to other, and also from those of different SHS, examined by next generation methods (Table 7).

Archaeal communities of extremely hot hydrothermal vents, with temperatures above 100 °C, are composed by well-known hyperthermophyles. Archaeal communities from two shallow vents at Vulcano were dominated by members of class Thermoprotei and Thermococci [80], with the highly abundant genera *Staphylothermus*, *Thermococcus, Aeropyrum*, and *Pyrodictium*, most of them already isolated from the same area in the past (Table 4).

Differences in samples are also quite distinct among samples from Black Point sites, mainly referred to the occurrence of Crenarchaeota (Thermoprotei) and hyperthermophilic members of Euryarchaeota (Archaeoglobi and Thermococci) at the hottest BP120 site. Although Crenarchaeota are reported as more abundant than Euryarchaeota in cold deep-sea sites [98], and at seamounts in the Tyrrhenian Sea [99], they were generally less abundant than Euryarchaeota at different SHS [16]. Thermoprotei from BP120 were referred to several genera, among them *Thermodiscus* was the most commonly retrieved genus in samples from both Black Point and Hot Lake sites. Euryarchaeotal Archaeoglobi, mainly referred to the genus *Ferroglobus*, and Thermocococci, with *Palaeococcus* and *Thermococcus* genera, were also reported as the dominant archaeal groups at the RP site of Palaeochori Bay (Milos Island, Greece) [17]. Moreover, also the genus *Pyrococcus* (Thermocococci) was only retrieved at the hottest site BP120. Our results suggest that the differences observed in the major archaeal populations at Black Point and Palaeochori Bay sites are mainly due to the different temperatures and pH values, rather than the salinity conditions. The moderately acidophilic and thermophilic, euryarchaeotal genus *Thermogymnomonas* (Thermoplasmata) was commonly retrieved from samples at Black Point and Hot Lake sites, but not in the RP site of Palaeochori Bay (Milos Island), where indeed it was dominant at the less saline (W) site. The presence at Black Point sites of several strictly anaerobic thermophilic archaeal groups, such as Methanococci (including the genera *Methanococcus* and *Methanothermococcus*), Thermococci, and Archaeoglobi indicates that these vents are seeded by subseafloor communities.

Hyperthermophilic members of Thermococci (*Palaeococcus* and *Thermococcus*) were also predominant at sites from the Hot Lake thermal brine pool, under high-temperature conditions (HL94). Thermococci have been also reported as dominant in deep-sea high-temperature locations, where they act as prominent degraders of organic matter within marine hot-water ecosystems [78]. The genus *Palaeococcus* was retrieved as the most abundant genus in all the examined sites of Panarea Island. The isolation and occurrence of sequences referred to *Paleococcus hegelsonii* indicate that the respiration and/or S°- fermentation of complex organic compounds may represent the metabolic key pathways at Eolian Island vents, as well as in other hydrothermal systems [12,21,71,83]. The decreasing temperature registered at Hot Lake site was accompanied by a concomitant increasing of diversity (Shannon and evenness indices, Table 7) and a marked shift in the archaeal community composition [15], since the majority of hyperthermophilic members retrieved at high temperature (HL94) was successively substituted by more specific mesophilic archaea of the classes Halobacteria, Methanomicrobia, and Methanobacteria [15].

The occurrence of sequences affiliated to Halobacteria, as demonstrated by different molecular sequencing techniques, confirms that they represent dominant archaeal populations at Panarea system, as well as in the shallow system Kueishan Island, Taiwan [100].

As recently evaluated by 16S rRNA gene clone library sequencing, archaeal communities in microbial mats collected from low-temperature, hydrothermal fields of Basiluzzo Islet (Eolian Islands) at different depths (from 26 to 211 m), were dominated by Thaumarcheota and Woesearchaeota [92].

These results suggest that other than temperature, depth, and salinity, different geochemical factors are essential to constraining the archaeal community associated with Eolian hydrothermal sites.

The single genus of the Korarchaeota phylum, related to "Candidatus Korarchaeum" has been commonly detected in different terrestrial and marine hydrothermal habitats, including vents of Vulcano hydrothermal system [83], sites of Palaeochori Bay (Milos Island) [17], and at hot springs of Yellowstone Park [83,97], indicating a broad diffusion in different hydrothermal systems.

Table 7. Archaeal major components retrieved by next generation sequencing at Vulcano, Black Point, and Hot Lake sites in comparison with those from other shallow vents.

Site and Sample	Depth (m)	Temp (°C)	pH	Salinity (Cl^- mM)	OTUs	*H′*	*J*	Major Components	Reference
Vulcano (Italy)	-	-	-	-	-	-	-	-	-
I	0.7	100	5.9	-	-	-	-	Crenarchaeota (Thermoprotei), Euryarchaeota (Thermococci)	[86]
II	0.7	100	5.9	-	-	-	-	Crenarchaeota (Thermoprotei), Euryarchaeota (Thermococci)	-
Hot Lake, Panarea Is (Italy)	-	-	-	-	-	-	-	-	-
HL94	~20	94	4.7	1292	454	3.27	0.53	Euryarcheota (Thermococci)	[15]
HL28	~20	28.5	4.5	1200	129	4.08	0.84	Euryarcheota (Thermococci and Halobacteria)	
Black Point, Panarea Is (Italy)	-	-	-	-	-	-	-	-	-
BP74	23	74	3.3	547.4	-	-	-	Euryarcheota (Methanococci)	[14]
BP27	23	27	2.4	583.8	-	-	-	Euryarcheota (Methanococci and Halobacteria)	
BP120	23	120	3.3	793.8	-	-	-	Crenarchaeota (Thermoprotei), Euryarcheota (Archaeoglobi and Thermococci)	This study
Palaeochori, Milos Is (Greece)	-	-	-	-	-	-	-	-	-
RP [(a)]	4.5	79	5.3	745	9–14	1.9–2.3	0.1–0.2	Euryarcheota (Archaeoglobi and Thermococci)	[17]
Kueishan Is, Taiwan (China)	-	-	-	-	-	-	-	-	-
W	21	49.5	4.7	971	436	5.74	-	Euryarchaeota (Halobacteria)	[100]

a: surface sediments (0–4.5 cm).

4. Conclusions

The Eolian SHS sites, characterized by elevated temperatures and salinity, high concentrations of CO_2 and low pH, constitute excellent natural fields to investigate how microorganisms respond to potential global changes, and to study the effects of the acidification and increasing temperature in the oceans.

With the advent of modern molecular techniques, the diversity of archaea has been recognized, but relatively little is already known about the ecological role of archaea at the Eolian SHS. Archaea represented a minor component of prokaryotic community at the examined samples from Vulcano [86] and Panarea sites [12,14,15,92], and as resulted by phylogenetic analysis Eolian shallow vents harbor a high diversity of largely unknown archaea. Although many members of archaea have not been cultivated yet, their phylogenetic diversity suggests a greater physiological diversity than previously documented. Such ecosystems therefore represent valuable sources of so-far unknown types of archaea and should be studied further, also including innovative cultivation based approaches and molecular tools targeting functional genes to deeply understand their ecological role in these environments.

Archaea communities residing in hydro-thermal fluids are quite different from those present in sediments primarily due to the fact that fluids are strongly influenced by the mixing with overlying seawater, determining steep geochemical gradients that influence the structure and composition of the resident archaeal communities in each site. The detection in high-temperature samples of members assigned to hyperthermophilic Crenarchaeota (Thermoprotei) and Euryarchaeota (Archaeoglobi and Thermococci) makes Eolian SHS similar to those retrieved from different geographical areas, i.e., from Milos Island. However, at the hot and acidic vents of Black Point sites the presence of several strictly anaerobic thermophilic archaeal groups, such as Thermococci, Archaeoglobi, and Methanococci indicates that these vents are similar to those of deep-sea hydrothermal systems.

Temperature values greatly differed in the different sampling occasions at Panarea sites, and the decrease in temperature in the same site indicates that the supply of geothermal heat to the vents becomes exhausted. The considerable loss of the majority of hyperthermophilic representatives in low-temperature samples was accompanied by an increase in the abundance of more specific mesophilic archaea of the classes Halobacteria, Methanobacteria, and Methanomicrobia. In addition to temperature, other geochemical factors are essential to constraining the archaeal community associated with hydrothermal sites.

Our findings extend our view of archaeal diversity and of their phylogenetic composition in shallow hydrothermal environments. These results may be used as baseline information to follow the microbial community responses to potential global changes and to anthropogenic impacts.

Author Contributions: Conceptualization and data curation, C.G. and T.L.M.; Writing—original draft preparation, C.G. and T.L.M.; Writing—review and editing, C.G. and T.L.M.

Funding: This research received no external funding.

Acknowledgments: We are indebted to Jörg Overmann at the Leibniz-Institute DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig GmbH, Germany) and Valeria Lentini for their valuable scientific and technical support.

Conflicts of Interest: The authors declare no conflict of interest.

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