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ORIGINAL PAPER

# Bacterial and archaeal populations at two shallow hydrothermal vents off Panarea Island (Eolian Islands, Italy)

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**Abstract** The aim of this study was to investigate the microbial community thriving at two shallow hydrothermal vents off Panarea Island (Italy). Physico-chemical characteristics of thermal waters were examined in order to establish the effect of the vents on biodiversity of both Bacteria and Archaea. Water and adjacent sediment samples were collected at different times from two vents, characterised by different depth and temperature, and analysed to evaluate total microbial abundances, sulphur-oxidising and thermophilic aerobic bacteria. Total microbial abundances were on average of the order of $10^5$ cells $ml^{-1}$, expressed as picoplanktonic size fraction. Picophytoplanktonic cells accounted for 0.77–3.83% of the total picoplanktonic cells. The contribution of bacterial and archaeal taxa to prokaryotic community diversity was investigated by PCR–DGGE fingerprinting method. The number of bands derived from bacterial DNA was highest in the DGGE profiles of water sample from the warmest and deepest site (site 2). In contrast, archaeal richness was highest in the water of the coldest and shallowest site (site 1). Sulphur-oxidising bacteria were detected by both culture-dependent and -independent methods. The primary production at the shallow hydrothermal system of Panarea is supported by a complex microbial community composed by phototrophs and chemolithotrophs.

**Keywords** Archaea · Bacteria · Prokaryote community · PCR–DGGE · Shallow hydrothermal vents

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## Introduction

Submarine hydrothermal vents are inhabited by a variety of microorganisms that tolerate environmental extremes and could have some yet undescribed industrial potential.

Shallow hydrothermal systems in the Mediterranean Sea are located in tectonically active areas that are present both in the Eastern (in the Aegean Sea, close to Milos Island, Greece) and in the Western (in the Tyrrhenian Sea, close to the Eolian Islands, Italy). Despite the accessibility of shallow hydrothermal vents, several aspects concerning microbial ecology of these ecosystems have been much less investigated than those of deep-sea vents. The shallow marine hydrothermal vents of the Eolian Islands provide easily accessible sampling locations for studying microorganisms inhabiting extreme marine ecosystems. The thermal springs in the Baia di Levante of Vulcano Island (Eolian Islands) host dozens of aerobic and anaerobic, thermophilic, and hyperthermophilic microorganisms belonging to Bacteria and Archaea domains (Stetter et al. 1983; Fiala and Stetter 1986; Huber et al. 1986; Stetter 1988; Huber and Stetter 1989; Hafenbradl et al. 1996; Caccamo et al. 2000; Maugeri et al. 2001, 2002; Amend et al. 2003a; Gugliandolo et al. 2003).

The most active Eolian submarine hydrothermal system is located off the coast of Panarea Island, where several gaseous emissions spread over the sea bottom up to a depth of 150 m

(Italiano and Nuccio 1991). The island of Panarea consists of a volcanic structure that 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. A group of islets (Dattilo, Bottaro, Lisca Bianca and Lisca Nera) located on the East of the main island, are recognised as the remnants of a crater rim. The interest toward the area around the group of islets increased since November 2002 when a great exhalative event occurred.

The studies on the microbial community thriving in hydrothermal vents of Panarea are very limited and mainly regarded microorganisms studied by cultivation-based techniques. A mesophilic chemoautotrophic sulphur-oxidising bacterium, resembling to *Thiobacillus* sp., has been isolated from Panarea fluids (Gugliandolo et al. 1999). From Panarea submarine vents (20 m depth) Amend et al. (personal communication) isolated *Thermococcus stetteri*, *T. peptonophilus*, *T. celer*, *T. profundus* and *T. barossii* and two strains closely related to the thermococci retrieved in Loihi Seamount (Hawaii).

Microorganisms inhabiting shallow hydrothermal vents possess nutritional requirements and overall metabolic pathways ideally suited to such ecosystem that represents a clear example of the close connection between geosphere and biosphere. It has been recognised that only a small fraction of microorganisms occurring in their natural habitat is revealed by using culture-dependent techniques. Isolation efforts of Bacteria and Archaea from thermal vents were often unsuccessful mostly due to the difficulties in reproducing the complex geochemical composition of the environment in growth media. This results in a limited knowledge of the real microbial diversity (Muyzer 1999). Culture-independent methods are at present considered the best tool to reflect the greater part of microbial community composition because of the existence of high numbers of as-yet-uncultured microorganisms. Molecular methods based on 16S rDNA have widely been used to reveal intrinsic genetic diversity. PCR–DGGE fingerprinting method was first used to obtain insights into the bacterial community present at a marine shallow water hydrothermal vent near Milos (Aegean Sea) by Sievert et al. (1999, 2000). Microbial community analysis based on fluorescent in situ hybridisation targeting thermophilic Bacteria and Archaea was reported for Vulcano Island (Rusch and Amend 2004). Using the Terminal-restriction fragment length polymorphism fingerprinting technique, high bacterial diversity was observed at shallow hydrothermal vents of Panarea related with the highly variable vent emissions, which could favour the coexistence of several prokaryotic species (Manini et al. 2008).

The aim of this study was to examine at different times the microbial community thriving at two shallow hydrothermal vents characterised by different depth and temperature. Water and sediment samples were collected close to the fluid emissions to evaluate the cellular abundances by microscopic and cultural methods, with particular attention paid to sulphur-oxidising bacteria and thermophilic aerobic bacteria. A PCR–DGGE fingerprinting method was applied to samples from the two vents to obtain information about the occurrence of the dominant bacterial and archaeal populations.

## Materials and methods

### Study sites and sampling

Water and sediment samples were collected from two vents (site 1 and site 2) in the immediate vicinity of the emission (<5 cm). Site 1 (Lat. 38° 38′ 31″N-Long. 15° 06′ 597″E) (8 m depth) was located close to the Bottaro Islet, and site 2 (Campo 7) (Lat. 38° 37′ 59″N-Long. 15° 06′ 360″E) (21.3 m depth) was between Dattilo and Lisca Bianca Islets (Fig. 1). Samples were taken at site 1 in June 2005, September 2005 and July 2006 and at site 2, in June 2005 and July 2006.

Water samples were collected by SCUBA diving using sterile bottles from the emission of the vents. Surface sediment samples were collected using sterile polycarbonate tubes. Temperature was recorded in situ. Reference seawater sample was collected from a site that was 12 m deep and not influenced by the hydrothermal area.

The bubbling gases were collected at the sea bottom by means of an inverted stainless-steel funnel that drives the gases to a two-way sampling bottle filled by seawater to avoid any atmospheric contamination. The gas samples were taken by water displacement. Chemical analyses of He, $O_2$, $N_2$, $CH_4$ and $CO_2$ were carried out using a Perkin Elmer 8500 gas-chromatograph equipped with a 4-m Carbosieve 5A column and double detector (Flame Ionisation Detector with methanizer and Hot Wire Detector). The

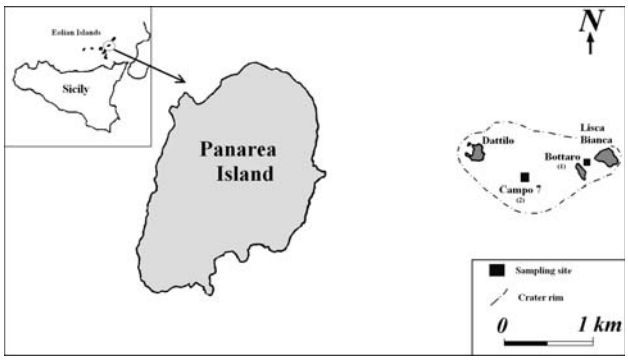


**Fig. 1** Sampling sites' location (site 1 and site 2) off the Island of Panarea, Italy

detection limits were 5 ppmv for He, $O_2$, and $N_2$, 2 ppmv for $H_2$ and 0.1 ppmv for $CH_4$ and $CO_2$. Analytical errors were ±5% for He, and ±3% for the other gases.

The analysis of the dissolved gases from the reference site (namely air-saturated seawater) was carried out according to Sugisaki and Taki (1987) and Capasso and Inguaggiato (1998).

The $H_2S$ content was evaluated on board by using reactive tubes (Dräger) calibrated for both high and low sensitivities in the range 0.01–10 vol%. The pH and $HCO_3^-$ values of water samples were determined on board immediately after sampling. The $Na^+$ and $Cl^-$ concentrations were determined in the laboratory by ion chromatography (Dionex model 2001SP) with reproducibility within ±2%.

## Microscopic counts

The microbial community was enumerated by direct count of picoplankton (cells ranging from 0.2 to 2 μm in diameter) (total counts, TC). Water samples were fixed with formalin (2%, v/v); prefiltered through 2 μm pore size polycarbonate membrane filters, and filtered through 0.2 μm polycarbonate (black) filters to obtain the picoplankton size fraction according to the procedure proposed by Maugeri et al. (1990). Sediment samples were placed on ice and sonicated to dislodge cells from particles. Cell counts were obtained using dilutions of their suspension filtered through membrane Nuclepore filters.

Picoplankton counts and counts of the cells associated with sediment samples were obtained by DAPI (4′6-diamidino-2-phenylindole) fluorochrome staining onto 0.2 μm black Nuclepore filters using epifluorescence microscopy (Olympus BX-60 M) at 1,000× magnification. The microscope was equipped with a halogen (HG 100) light. A G 330-385 exciter filter, an FT 400 chromatic beam splitter and an LP 420 barrier filter were used for picoplanktonic direct counts.

To estimate the total number of picophytoplankton (PP), water samples were prefiltered and fixed as described earlier, and observed microscopically according to the procedure described by Maugeri et al. (1990). Blue light excitation, a G 470-490 exciter filter, an FT 510 chromatic beam splitter, and an LP 520-barrier filter were used to determine picophytoplanktonic density.

The viability of cells was assessed with nucleic acid staining dyes (LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit; Molecular Probes, Eugene, Oregon). This two-colour fluorescence assay determines viability based on cell membrane integrity. Membranes of all cells stained with SYTO® 9 have a green fluorescence. Propidium iodide stain penetrates cells with damaged membranes and generates a red fluorescence. Therefore, living cells (LC) with intact membranes stain green, while dead cells are red.

## Cultural methods

### *Aerobic thermophilic bacteria*

To enrich aerobic thermophilic bacteria, water samples (100 ml filtered through 0.2 μm membrane filters) and sediment (1 g) were inoculated in Bacto Marine Broth 2216 (Difco) and incubated at 60, 70 and 80°C (for 3 days in aerobic conditions). Sub-cultures were made on the same medium supplemented with agar (2%, w/v). All colonies obtained on plates were picked and purified by streaking onto Bacto Marine Agar 2216 (Difco) at least three times. Phenotypic studies on thermophilic isolates were performed as suggested by Maugeri et al. (2001).

### *Sulphur-oxidising bacteria (SOB)*

Sulphur-oxidising bacteria were enumerated by the most probable-number (MPN) method in a mineral medium with 40 mM thiosulphate (Tuttle and Jannasch 1972) as sole electron donor (pH 6.8). MPN dilution series of vent water and sediment samples were performed in triplicate and incubated at room temperature and at in situ temperature of both sites until a colour change of the pH indicator (methyl red) and a visual increase in turbidity was observed. The numbers of SOB were determined by using the MPN index of the American Public Health Association (1969).

## Molecular methods

### *Molecular assessment of taxonomic affiliation*

For the DNA extraction from heterotrophic thermophilic isolates, a DNeasy tissue kit (Qiagen, GmbH) was used according to the manufacturer's directions. Amplified ribosomal DNA restriction analysis (ARDRA) was done with two enzymes *Alu* I (Boehringer Mannheim) and *Taq* I (Fermentas Life Sciences). The digestion of PCR product was conducted according to Caccamo et al. (2001).

Reference strains used in this study included five *Geobacillus* species (*G. thermodenitrificans* DSM 465$^T$; *G. kaustophilus* DSM 7263$^T$; *G. thermocatenulatus* DSM 730$^T$; *G. vulcani* DSM 13174 $^T$; *G. thermoleovorans* DSM 5366$^T$) and two *Bacillus* species (*B. caldotenax* DSM 406; *B. licheniformis* lab strain B3-15).

### *Polymerase chain reaction (PCR)*

DNA was extracted from 1 l (filtered through 0.2 μm membrane filters) and 1 g of sediment using a FastDNA SPIN Kit for Soil (BIO 101 System, Q-Biogene) according to the manufacturer's directions and used as template in the PCR assays.

The primers used for amplification of the region V3 of 16S rDNA of Bacteria were 357F-GCclamp (*Escherichia coli* positions 338–357: 5′-ACT CCT ACG GGA GGC AGC AG-3′) (Lane 1991) and 518R (*E. coli* positions 534–518: 5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer et al. 1993). The primers used for amplification of the region V9 of 16S rDNA of Bacteria were 1070F (*E. coli* positions 1,055–1,070: 5′-ATG GCT GTC GTC AGC T-3′) and 1406R- GCclamp (*E. coli* positions 1,392–1,406: 5′-ACG GGC GGT GTG AC-3′) (Amann et al. 1995). Amplification of Archaea 16S rDNA sequences was carried out using primer ARC344F with GC-clamp (5′-ACG GGG YGC AGC AGG CGC GA-3′) (Raskin et al. 1994) and ARC915R (5′-GTG CTC CCC CGC CAA TTC CT-3′) (Stahl and Amann 1991).

PCR conditions for members of Bacteria were carried out as described by Muyzer et al. (1993) and Ferris et al. (1996) to amplify V3 and V9 bacterial regions of the 16S rDNA, respectively.

PCR conditions for archaeal 16S rDNA genes were as follows: initial step of 3 min at 98°C, annealing for 90 s at 55°C and primer extension for 2 min at 72°C. After 40th cycle, the extension step was prolonged for 5 min to complete synthesis of all strands.

### *Denaturing gradient gel electrophoresis analysis*

Denaturing gradient gel electrophoresis of Bacteria was carried out in a DCode System (Bio-Rad Laboratories, Inc.) with slight modifications to a previously described method (Muyzer et al. 1996). Briefly, bacterial PCR products (25.0–45.0 µl) were resolved in 1-mm-thick 10.8% (w/v) polyacrylamide gels, in 1 × TAE electrophoresis buffer (40 mM Tris–HCl pH 8.3, 20 mM acetic acid, 1 mM EDTA) using denaturing gradients ranging from 35 to 70% (where 100% denaturant contains 7 M urea and 40% formamide).

Archaeal PCR products were separated using 6.5% polyacrylamide gels with a denaturant gradient between 40 and 55% (Casamayor et al. 2000).

After electrophoresis at a constant voltage of 70 V, for 16 h at 60°C, the gels were stained in SYBR Green I for 45 min and photographed with a transilluminator (Polaroid Gelcam, Cambridge, MA). All bands in DGGE gels were excised and the small blocks of acrylamide were placed in sterile TE buffer at +4°C overnight to allow diffusion of DNA out of the gel fragments. A suitable volume of the eluate was used as template DNA in a reamplification PCR using the primers and reaction conditions described earlier. Following amplification, the PCR products were re-run on DGGE gels to confirm their positions relative to the bands from which they were excised. Before being sequenced, PCR products were purified with the QIAquick Spin PCR purification kit (Qiagen GmbH, Germany) as specified by the manufacturer.

### *Sequencing of DGGE bands*

Sequencing was performed with primers SteF 1Tex (5′-GCG TTC TTC ATC GTT GCG AG-3′) (Van der Gucht et al. 2001) and PRABA1406R for Bacteria and PARCH915R for Archaea using an automated sequencer CEQ 8000Genetic Analysis System. A nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) was performed in order to obtain information on the phylogenetically closest relative (Altschul et al. 1997). Sequences assigned to Archaea and selected reference sequences were used for phylogenetic tree construction generated by the neighbour-joining (NJ) using the Kimura-2 parameters algorithm. Distance matrix trees were generated by the NJ method with the Felsenstein correction as implemented in the PAUP 4.0B software (Sinauer, Sunderland, MA, USA). The NJ calculation was subjected to bootstrap analysis (1,000 replicates). The partial 16S rRNA gene sequences were deposited in the GenBank database.

## Results

### Analysis of thermal waters

Temperature, pH, conductivity and composition of dissolved gases of hydrothermal fluids from the two vents in comparison with the reference seawater are given in Table 1. The pH and conductivity of thermal fluids were lower than those of the reference site. Thermal fluids appeared less saline and contained more dissolved $CO_2$ than the control water.

The chemical composition of samples from thermal sites showed a slight depletion in $Na^+$ and $Cl^-$ as compared to reference seawater. Dissolved gases showed the typical characters of the geothermal fluids. All the sampled gases showed negligible $O_2$ content and $N_2$ concentrations well below those of the atmosphere. The $CH_4$ content varied greatly in site 1, and helium was in the range of few parts per million by volume.

### Direct counts

Direct counts of total picoplanktonic cells (TC) varied between $4.59 \times 10^5$ ml$^{-1}$ (site 2, June) and $3.62 \times 10^6$ ml$^{-1}$ (site 1, July) (Fig. 2). The densities of autofluorescent PP cells varied from $1.30 \times 10^4$ cells ml$^{-1}$ (site 1, June) and $3.20 \times 10^4$ cells ml$^{-1}$ (site 2, July) in water samples.

**Table 1** Physical and chemical characteristics of thermal waters from the two sites (1 and 2) off Panarea Island in comparison with seawater of reference site

| Physical and chemical properties of the water | | | | | | | | | Gas composition (%)[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Site | Date | Depth (m) | T°C | pH | Conductivity (mS/cm) | $Na^+$ (mg $l^{-1}$) | $Cl^-$ (mg $l^{-1}$) | $HCO_3^-$ (mg $l^{-1}$) | $CH_4$ | He | $N_2$ | $CO_2$ | $O_2$ | $H_2S$ | $H_2$ |
| Bottaro (1) | June 2005 | 8.0 | 52 | 5.28 | 51.30 | 22.71 | 17.96 | 0.12 | $5.1 \times 10^{-4}$ | $6.0 \times 10^{-4}$ | 0.29 | 98.15 | bdl | 1.4 | $4.0 \times 10^{-4}$ |
| | Sept 2005 | 8.0 | 53 | 5.30 | | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| | July 2006 | 8.0 | 55 | 5.42 | 42.90 | 22.30 | 18.4 | 0.08 | $6.0 \times 10^{-4}$ | $8.0 \times 10^{-4}$ | 0.28 | 95.9 | bdl | bdl | $1.5 \times 10^{-3}$ |
| Campo 7 (2) | June 2005 | 21.3 | 65 | 4.91 | 44.70 | 21.59 | 16.51 | 0.05 | bdl | $7.0 \times 10^{-4}$ | bdl | 98.5 | bdl | 1.8 | bdl |
| | July 2006 | 21.3 | 60 | 4.92 | 49.20 | 21.60 | 17.43 | 0.051 | $2.0 \times 10^{-2}$ | $8.0 \times 10^{-4}$ | 0.3 | 96.4 | 0.03 | bdl | $5.0 \times 10^{-4}$ |
| Reference site | | 12.0 | 15 | 8.10 | 54.00 | 24.01 | 18.7 | 0.04 | bdl | $2.8 \times 10^{-4}$ | 65.6 | 1.64 | 32.8 | bdl | bdl |

*nd* not determined, *bdl* below the detection limits (see text)

[a] The gas composition at the reference site refers to the composition of the dissolved gases in an ASSW (air-saturated seawater)

Total cells in sediment samples ranged from $2.90 \times 10^6$ $g^{-1}$ (site 1, September) to $1.50 \times 10^7$ $g^{-1}$ (site 1, June) (Fig. 3).

The percentage of LC on TC ranged from 51.9 (site 2, July) to 97.3 (site 1, September) in water and from 62.1 (site 1, June) to 94.0 (site 2, June) in sediment samples.

Total picoplanktonic cells of reference site were $2.0 \times 10^5$ $ml^{-1}$. Autofluorescent PP cells of reference site accounted for $3.0 \times 10^4$ $ml^{-1}$. The percentage of LC at this site was 35.0% for water and 65.0% for sediment.

## Cultural methods

### *Aerobic thermophilic bacteria*

A total of 53 strains were isolated from MB enrichment cultures incubated at 60, 70 and 80°C. Twenty strains from site 1 and 16 from site 2 with growth range of 37–70°C and optimum of ≥55°C were considered thermophilic strains and further characterised. All these strains belonged to *Firmicutes*; 13 of them had optimal growth at 70°C, 19 at 60°C and 4 at 55°C. Most of the isolates were able to grow with pH range of 5.5–9 and 0–3% of NaCl.

### *Suphur-oxidising bacteria (SOB)*

Counts of aerobic SOB, obtained after incubation at room temperature, in water samples varied from < 3 (site 2, July) to $2.1 \times 10^2$ MPN 100 $ml^{-1}$ (site 1, June), and from < 1 (site 1, September) to $2.1 \times 10^5$ 100 MPN $g^{-1}$ (site 2, July). Cultures incubated at in situ temperature were negative. SOB were undetected in the reference site.

## Molecular methods

### *Molecular assessment of thermophilic isolates*

Results obtained by restriction analysis of 16S rRNA allowed affiliation of isolates to the genera *Geobacillus* and *Bacillus*. Three strains showed an ARDRA profile similar to *G. thermodenitrificans* DSM 465$^T$, 13 strains were similar to *G. kaustophilus* DSM 7263$^T$, 12 strains were similar to *G. thermocatenulatus* DSM 730$^T$ and six isolates were similar to *B. caldotenax* DSM 406. The remaining 19 isolates did not match any ARDRA profile of the reference strains.

### *Denaturing gradient gel electrophoresis analysis*

The genetic diversity of microbial community at the two vents, examined by amplification of the V3 and V9 regions of 16S rDNA of thermal water and sediment samples, is

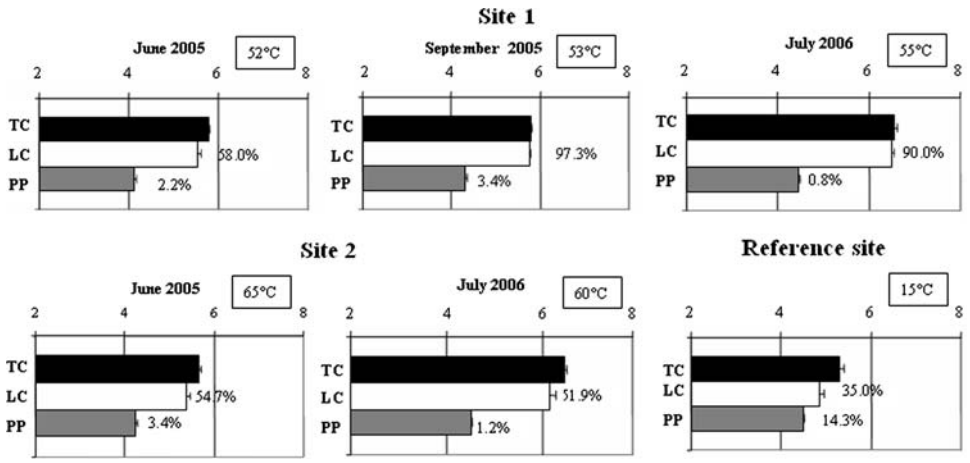


**Fig. 2** Direct counts of total picoplankton (TC) (*filled square*), living cells (LC) (*open square*) and picophytoplankton (PP) (*grey filled box*) (expressed as log of number of cells $ml^{-1}$) in water samples from site 1, site 2, and reference site. The *error bars* indicate the 95% confidence intervals. *Numbers* shown next to the living cells and *picophytoplankton bars* indicate their relative percentage to total picoplankton

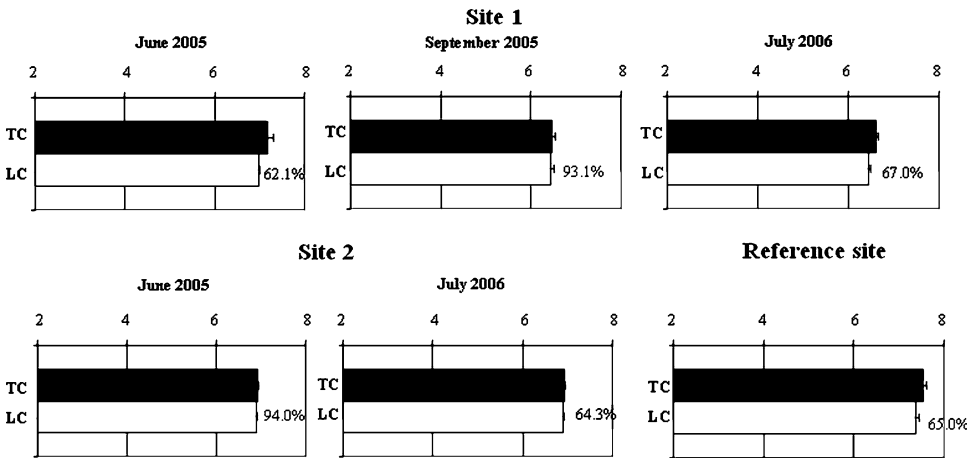


**Fig. 3** Direct counts of total picoplankton (TC) (*filled square*), living cells (LC) (*open square*) (expressed as log of number of cells $g^{-1}$) in sediment samples from site 1, site 2 and reference site. The *error bars* indicate the 95% confidence intervals. *Numbers* shown next to the living cells bar indicate their percentage to total picoplankton

shown in Figs. 4 and 5. The number of resolved bands is indicated at the bottom of each profile.

Amplification of the V3 region from site 1 in June resulted in a larger number of bands than those obtained from the V9 region. The largest number of bands was observed in water sample collected from site 1 in September (14), while among sediments the sample collected in June produced the highest number of bands (5) (Fig. 4).

At site 2 (Fig. 5) the number of bands of the V9 region was higher than that obtained from site 1 (Fig. 4).

DGGE fragments amplified with the primers specific for Archaea are shown in Fig. 6. The number of bands was higher in water samples (12 and 4 from site 1 and 2, respectively) than in sediment samples. The pattern of profiles appeared different for each sample, except for a few bands that are at the same position. The largest number of bands was observed at site 1 for water sample.

*Phylogenetic analysis*

Phylogenetic analysis of the predominant bacterial populations at the two vent sites was determined by sequencing of all bands excised from denaturing gradient gels. About 77% of the excised bands from DGGE of Bacteria were sufficiently pure to yield a sequenceable product. Fragments that did not yield sufficient PCR product or further sequencing failed because multiple PCR fragment migrating at the same position, are not reported.

Blast results on bacterial 16S rRNA gene sequences extracted from excised DGGE bands from site 1 are reported in Table 2. The major part of sequences of the dominant bacterial populations related them to representatives of Proteobacteria. Most of these sequences derived from water of site 1 were similar to uncultured bacterial clones detected in deep-sea hydrothermal systems or in other marine environments. The sequences from bands 1V3, 2V3, 3V3, 4V3, 6V3, 7V3, 9V9, 11V9, 12V9 and 13V9 showed similarity with those of the class Epsilonproteobacteria retrieved from marine environments, mainly from deep-sea hydrothermal systems. Sequence of band 7V3 was almost identical (97%) to the sequence of *Thiomicrospira denitrificans* (now renamed *Sulfurimonas denitrificans*, Takai et al. 2006).

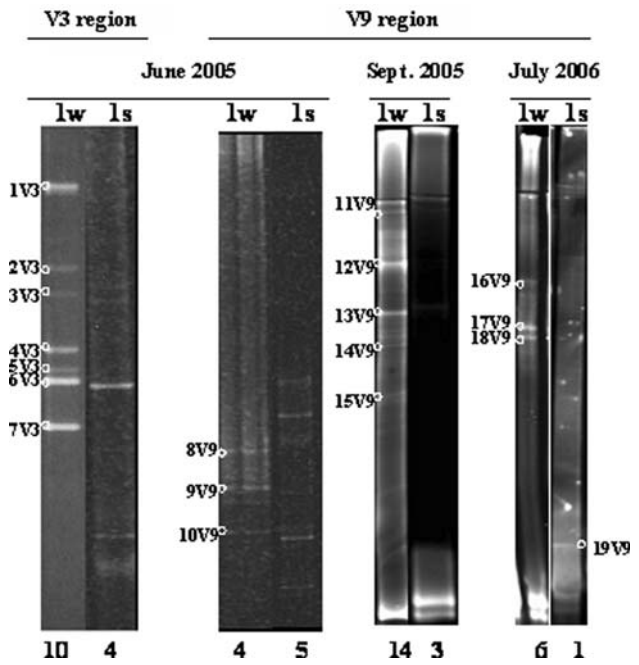


**Fig. 4** Denaturing gradient gel electrophoresis profiles of Bacteria 16S rDNA V3 and V9 regions PCR products of samples (*w*: water and *s*: sediment) collected from site 1 in June 2005, September 2005 and July 2006

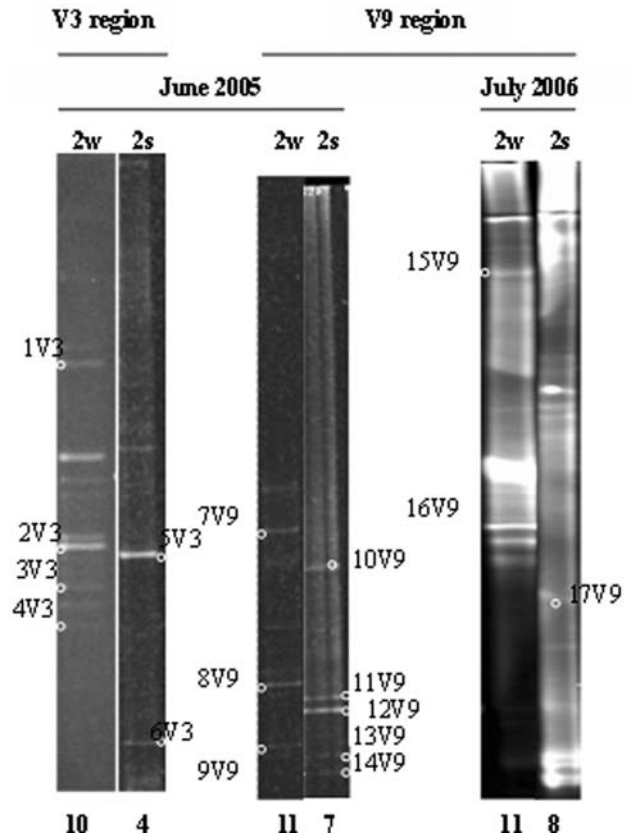


**Fig. 5** Denaturing gradient gel electrophoresis profiles of Bacteria 16S rDNA V3 and V9 regions PCR products of samples (*w*: water and *s*: sediment) collected from site 2 in June 2005 and July 2006

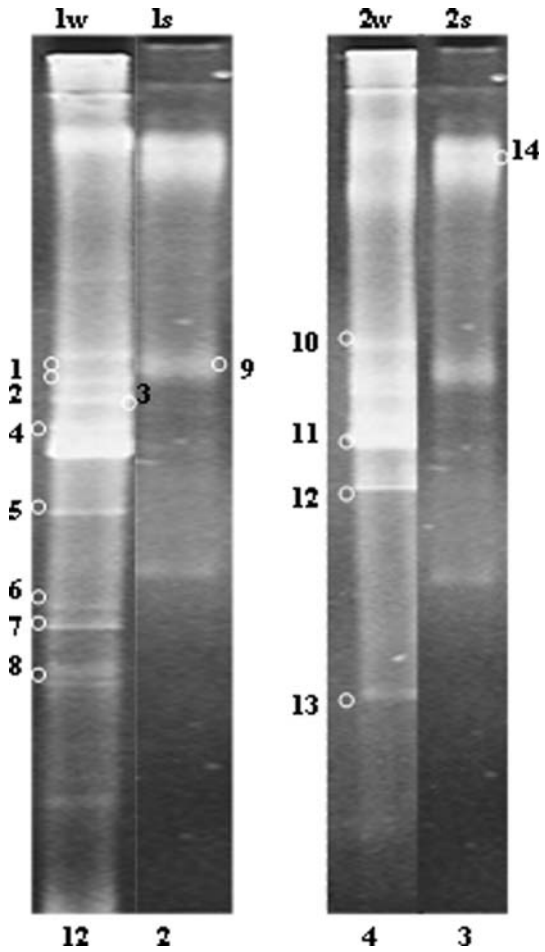


**Fig. 6** Denaturing gradient gel electrophoresis of 16S rDNA fragments of Archaea obtained after PCR amplification on samples (*w*: water and *s*: sediment) collected from sites 1 and 2 in June 2005

Sequences 8V9 and 14V9 obtained from water were closely (96%) and moderately (92%) similar to members of Bacteria, while that of 19V9 band was distant (62%) to any other deposited bacterial sequences.

Fragment 10V9 showed 100% similarity with *Pseudomonas* sp. As-33 (Gammaproteobacteria) isolated from Adriatic Sea. Fragment 5V3 showed 92% similarity with an uncultured bacterium of the order *Thiotrichales* (Garrity et al. 2005).

Sequences of 17V9 and 18V9, retrieved from water collected in July, were similar to *Chlorobi*, phototrophic, sulphur-oxidising bacteria.

Most of sequences derived from site 2 were representative of Proteobacteria (Table 3). Three bands (3V3, 14V9 and 16V9) were affiliated with the class Alphaproteobacteria, but only the fragment 14V9 shared a significant similarity (97%) to *Caulobacter* sp., retrieved from marine environment. The sequence of 17V9 band, affiliated with the class Deltaproteobacteria, did not show any significant similarity to cultivated or uncultivated bacteria. Both

**Table 2** Blast results on bacterial 16S rRNA sequences derived from excised DGGE bands from samples collected from site 1

| Band | BLASTn result | Percentage of identity | Phylogenetic affiliation | Found in environment (References) | Accession no. |
|---|---|---|---|---|---|
| 1V3 | Uncult. clone ATOS Iris 7 Rainbow 98 | 97 | ε-Proteobacteria | Deep hydrothermal vent, Mid Atlantic Ridge (Unpub.) | FJ234151 |
| 2V3 | Uncult. *Arcobacter* sp. clone IG-1-19 | 97 | ε-Proteobacteria | Sea water of the Ishigaki—jima Island (Unpub.) | FJ234152 |
| 3V3 | Uncult. clone MS12-2-B02 | 94 | ε-Proteobacteria | Hydrothermal vent Kermdec Arc, New Zealand (Unpub.) | FJ234153 |
| 4V3 | Uncult. clone T6-Ph07-962 | 96 | ε-Proteobacteria | Deep hydrothermal vent (Alain et al. 2004) | FJ234154 |
| 5V3 | Uncult. *Thiotrichales* clone SIMO-493 | 92 | γ-Proteobacteria | Salt marsh (Unpub.) | FJ234163 |
| 6V3 | Uncult. clone GoM HDB-15 | 95 | ε-Proteobacteria | Gas hydrates (Mills et al. 2005) | FJ234155 |
| 7V3 | *Sulfurimonas denitrificans* DSM 1251 | 97 | ε-Proteobacteria | Hydrothermal vent system (Sievert et al. 2008) | FJ234156 |
| 8V9 | Uncult. clone F5396_454_400bp_684B | 96 | Bacteria | Environmental samples (Unpub.) | FJ234170 |
| 9V9 | *Sulfuricurvum kujiense* | 97 | ε-Proteobacteria | Underground crude oil (Kodama and Watanabe 2003) | FJ234157 |
| 10V9 | *Pseudomonas* sp. As-33 | 100 | γ-Proteobacteria | Adriatic Sea (Unpub.) | |
| 11V9 | Uncult. clone PS-B28 | 90 | ε-Proteobacteria | Active deep-sea vent chimney (Page et al. 2004) | FJ234158 |
| 12V9 | *Sulfurimonas paralvinellae* DSM 17229 | 90 | ε-Proteobacteria | Deep-sea hydrothermal vent (Takai et al. 2006) | FJ234159 |
| 13V9 | Uncult. clone VC1.2-cl32 | 82 | ε-Proteobacteria | Deep hydrothermal vent (Corre et al. 2001) | FJ234160 |
| 14V9 | Uncult. clone JH-FQ-33-4 | 92 | Bacteria | Agricultural soil (Unpub.) | |
| 17V9 | *Chlorobium vibrioforme* subsp. *Thiosulfatophilum* strain NCIB 8346 | 97 | Chlorobi | Environmental samples (Alexander et al. 2002) | FJ234171 |
| 18V9 | *Chlorobium ferrooxidans* DSM 13031 | 92 | Chlorobi | Sediments (Heising et al. 1999) | FJ234172 |
| 19V9 | Uncult. 106 clone I3 K-0150 | 62 | Bacteria | Mediterranean Sea (Zaballos et al. 2006) | |

**Table 3** Blast results on bacterial 16S rRNA sequences derived from excised DGGE bands from site 2

| Band | BLASTn result | Percentage of identity | Phylogenetic affiliation | Found in environment (References) | Accession no. |
|---|---|---|---|---|---|
| 2V3 | Uncult. isolate DGGE band 115-28-D3 | 89 | Bacteria | Environment samples (Miller et al. 2007) | |
| 3V3 | Uncult. clone MB13H04 | 87 | α-Proteobacteria | Coastal marine bacterioplankton (Suzuki et al. 2001) | |
| 5V3 | Uncult. clone PYK04_18B_6 | 95 | ε-Proteobacteria | Black smoker (Unpub.) | FJ234161 |
| 6V3 | Unidentif. clone 47_L_Rhizo_L1_T7S | 87 | Bacteria | Soil (Kielak et al. 2008) | FJ234166 |
| 10V9 | *Sulfurimonas denitrificans* DSM 1251 | 97 | ε-Proteobacteria | Hydrothermal vent system (Sievert et al. 2008) | FJ234162 |
| 11V9 | Bacterium RRP-E6 | 95 | Bacteria | Italian rice field soil (Graff and Stubner 2003) | FJ234167 |
| 14V9 | *Caulobacter* sp. Tibet-S913 | 97 | α-Proteobacteria | Permafrost region (Zhang et al. 2007) | FJ234164 |
| 15V9 | Bacterium K7 | 97 | Bacteria | Hawaiian hotspot (Unpub.) | FJ234168 |
| 16V9 | *Rhodomicrobium* sp. ShRmc01 | 75 | α-Proteobacteria | Lake Shira (Unpub.) | FJ234165 |
| 17V9 | Uncult. Desulfobacteraceae clone cLaki-JM30 | 72 | δ-Proteobacteria | Lake Kinneret (Unpub.) | FJ234169 |

**Table 4** Blast results on archaeal 16S rRNA sequences derived from excised DGGE bands from sites 1 and 2

| Site | Band | BLASTn result | Percentage of identity | Phylogenetic affiliation | Found in environment (References) | Accession no. |
|---|---|---|---|---|---|---|
| 1 | 3 | Uncult. archaeon clone 20c-54 | 94 | | Sediments of the Aegean Sea (Unpub.) | FJ234173 |
| | 4 | Uncult. clone Y5x | 96 | Crenarchaeota | Basaltic flanks (Ehrhardt et al. 2007) | FJ234174 |
| | 5 | Uncult. archaeon clone VulcPIw.66 | 96 | | Geothermal well (Rogers and Amend 2005) | FJ234175 |
| | 6 | Uncult. clone a87R72 | 96 | Euryarchaeota | Basaltic flanks (Ehrhardt et al. 2007) | FJ234176 |
| | 7 | Uncult. clone D_A04 | 96 | Crenarchaeota | Hydrothermal Vent, New Zealand (Unpub.) | FJ234177 |
| | 8 | *Paleococcus helgesonii* DSM 15127 | 96 | Euryarchaeota | Geothermal well (Amend et al. 2003b) | FJ234178 |
| | 9 | Uncult. *Ferroglobus* sp. clone IAN1-2 | 87 | Euryarchaeota | Deep hydrothermal fluids (Nakagawa et al. 2005) | FJ234179 |
| **2** | 13 | Uncult. clone LDS17 | 95 | Euryarchaeota | Dagow Lake (Glissman et al. 2004) | FJ234180 |
| | 14 | Uncult. clone G37A | 91 | Euryarchaeota | Salt crust (Unpub.) | |

sequences of 5V3 and 10V9 bands were similar to those of Epsilonproteobacteria. The sequence of band 10V9, identical to that of 7V3 from site 1, was affiliated with *S. denitrificans* DSM 1251.

Sequences 2V3, 6V3, 11V9 and 15V9 were affiliated with uncultured clones of Bacteria.

Sequencing of PCR-DGGE fragments of archaeal 16S rRNA gene showed the presence of Crenarchaeota (2/9) and Euryarchaeota (5/9), mostly related to uncultured organisms (Table 4). A phylogenetic tree of the obtained archaeal sequences was constructed to see their phylogenetic position (Fig. 7). Only the sequence of fragment 8, collected from water of site 1, showed similarity (96%) with *Paleococcus helgesonii*, a species of Euryarchaea recently isolated from a geothermal well on Vulcano Island (Amend et al. 2003b). All retrieved clone types were related to Archaea detected at extreme environments, most of which were characterised by warm temperature. Three phylotypes (3, 5 and 8) were closely related to archaeal clones detected in Mediterranean area. Four sequences (4, 6, 7 and 9) were similar to uncultured clones described in deep-sea vent systems. One fragment (13) was affiliated with an uncultured methanogenic euryarchaeon from Dagow Lake (Glissman et al. 2004).

### *Nucleotide sequence accession numbers*

The 16S rRNA gene sequences of the phylotypes reported here have been submitted to GenBank under accession numbers FJ234151 through FJ234180.

## Discussion

A series of geomicrobiological surveys were carried out to elucidate the microbial abundances and the composition of microbial community in the shallow submarine hydrothermal system off Panarea Island. In this report, we identify bacterial and archaeal components inhabiting the shallow hydrothermal water and sediment at two different vent sites. Direct counts, cultivation-dependent and molecular techniques were combined to analyse the population size, the percentage of LC, the occurrence of characteristic bacterial populations and the microbial phylogenetic diversity.

The submarine hydrothermal vents of Panarea are composed of thermal waters of marine origin, modified by high-temperature water–rock interactions (Italiano and Nuccio 1991; Caracausi et al. 2005; Capaccioni et al. 2007), and by $CO_2$ as the dominant gas (Italiano and Nuccio 1991; Caracausi et al. 2005). Gases containing magmatic-type helium reveal a direct contribution of a magmatic source, either related to a cooling magma body or to a deeper connection with an active magmatic source, and both are able to provide the thermal energy feeding the local geothermal reservoir (Italiano and Nuccio 1991; Caracausi et al. 2005). The detection of $O_2$ in the sampled gases highlights that atmospheric contamination occurs at shallow levels as oxygen from air-saturated seawater (ASSW, Table 1) is normally carried by marine water circulating in the bottom sediments. Caracausi et al. (2005) observed that samples from different venting sites shift from the ASSW line towards low or undetectable oxygen contents, highlighting the fact that atmospheric $O_2$ is consumed by chemical reactions that probably involve the oxidation of sulphides.

Almost all the gases vented from the submarine hydrothermal system off the island of Panarea are marked by very low $H_2$ concentrations suggesting disequilibrium conditions of the gas mixtures to be referred to $H_2$ loss during the ascent of the gas towards the surface (Caracausi

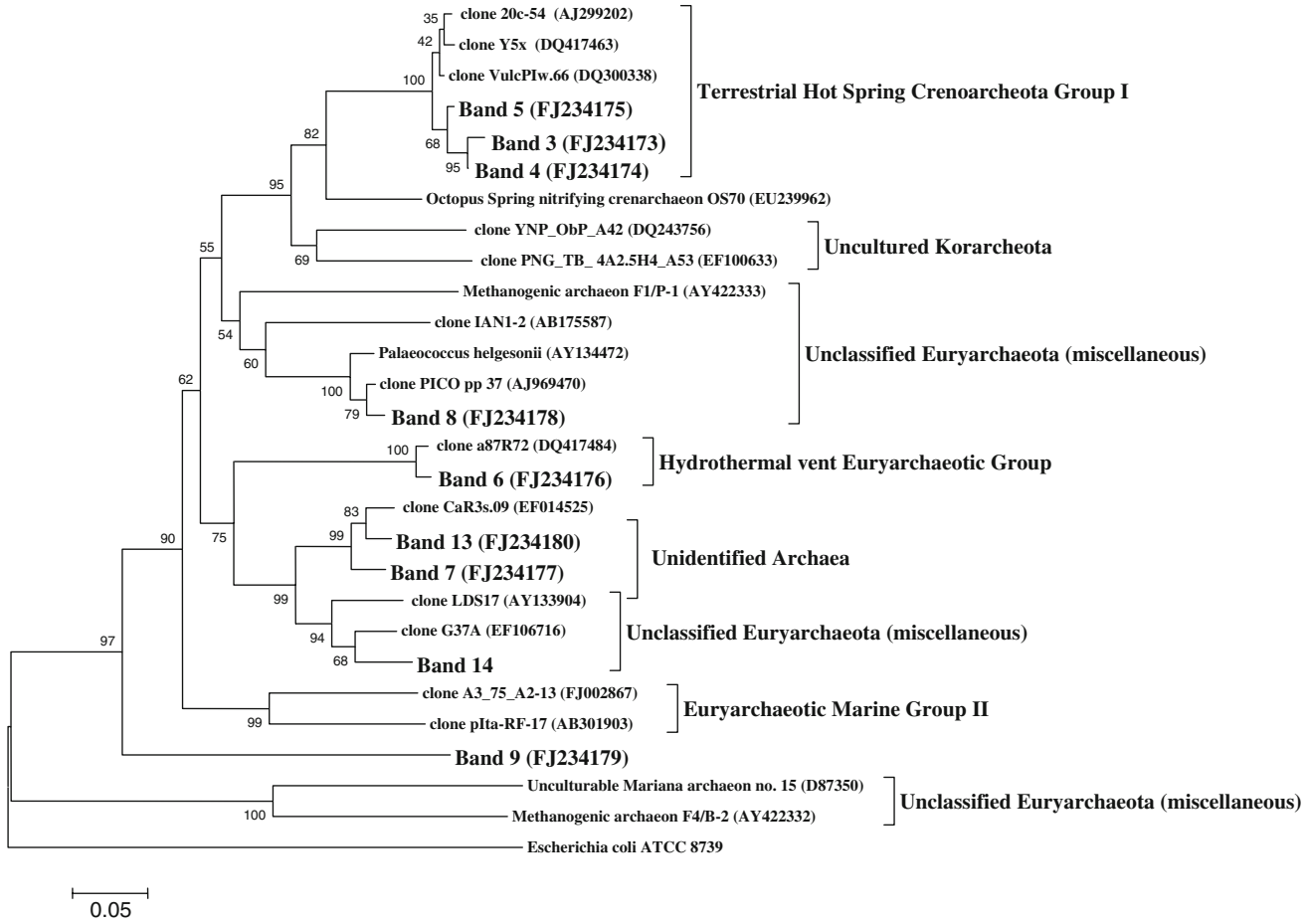


**Fig. 7** Phylogenetic tree of bands excised from the DGGE gels shown in Fig. 6. Neighbour-joining tree showing phylogenetic relationships among 16S rRNA genes of DGGE bands compared to those of representative archaeal species and environmental clones. Sequences of DGGE bands were added to the tree using DNA distance programme with Kimura-2 parameter. Bootstrap tree were constructed (Remark 4) using 1,000 replicates

et al. 2005). Following the geochemical model proposed by Caracausi et al. (2005) to explain $H_2$ loss due to gas–water interactions and low-temperature re-equilibration of the gas phase in submarine hydrothermal systems, we argue that even though chemical reactions and bacterial activity may account for the observed low hydrogen concentrations, hydrogen contents higher by one-two orders of magnitude can be considered for the deep located thermal fluids.

Vent water composition at the investigated vents showed large variations mainly due to the mixing of hydrothermal reservoir fluids: vapour condensate and seawater altered by interactions of fluid-sediment-bacteria in different proportions. The studied vents of Panarea showed values of pH, conductivity and oxygen content lower than those observed at the near island of Vulcano. On the other hand, the $Na^+$, $Cl^-$, $CO_2$, $CH_4$ and $H_2S$ content at Panarea was higher than that observed at Vulcano (Gugliandolo et al. 1999). The chemical characters of shallow submarine thermal waters were similar to those reported for the hydrothermal solutions from deep oceanic vents, showing that similar processes occur at hydrothermal vents whatever was their location at depth.

These large variations have little influence on the total prokaryotic counts. At our study sites, cell densities, on the average order of $10^5$ ml$^{-1}$ and $10^6$ g$^{-1}$, compared well to those previously counted at same shallow hydrothermal system of Panarea (Gugliandolo et al. 1999; Manini et al. 2008) and of Vulcano (Gugliandolo and Maugeri 1998; Rusch et al. 2005). In our study the abundance of phototrophic microorganisms (PP), evaluated by microscopic method, accounted for 0.77–3.83% of the total counts and appeared lower than that observed at the reference site.

In the cultivation assays, several thermophilic, thermoresistant and spore-forming strains were isolated and identified as *Bacillus* and *Geobacillus* spp., which are known to thrive in similar habitats (Maugeri et al. 2001). The growth of the resident heterotrophs can be supported by both autochthonous and allochthonous organic matter. Amino acids and sugar have been recently demonstrated in

the near-shallow hydrothermal vent system of Vulcano Island (Svensson et al. 2004; Skoog et al. 2007).

Larger numbers of SOB were obtained in the sediment of the warmest site 2. Reduced sulphur compounds ($H_2S$, $S_2O_3^{-2}$, S, etc.) occurring in the fluids may serve as electron donors for sulphur oxidisers, both in oxic and anoxic conditions.

Although the total cell abundances and their viability did not appear to correlate with temperature, the relative abundance of aerobic bacteria, such as thermophilic heterotrophic and sulphur-oxidising bacteria, noted as part of the microbial community structure, seems to be related to the complex geochemical regime.

Until today, the number of investigations on the microbial community diversity at shallow submarine hydrothermal systems is scarce. DGGE method is now considered the most reliable tool to analyse the microbial community diversity expressed as dominant phylotypes. It is undeniably a valuable approach for screening and analysing microbial community of complex ecosystems. The number and intensity of DGGE bands provide valuable information about variations in highly abundant operative taxonomic units and subsequent sequencing of the major bands allows the identification of the dominant populations. The DGGE profiles obtained by amplification of 16S rRNA regions demonstrated that resident microbial community at Panarea vents is composed by few populations of Bacteria and Archaea only which, however, are more diverse than those demonstrated for the reference site (data not shown).

The DGGE patterns also showed that bacterial and archaeal populations of the two vents were more homogenously distributed in sediment than in water, where changes in chemical properties and gas composition were more evident. Bacterial richness, expressed as the number of bands in each profile, was higher in water than in sediment samples of both sites. Relative bacterial richness was highest in the water sample of the warmest and deepest site (site 2). In contrast to Bacteria, archaeal richness was highest in water of the coldest and shallowest site (site 1).

Phylogenetic analysis of Bacteria showed that the major part of the phylotypes, detected by DGGE analysis, from the two sites was identified as members of Proteobacteria. Since one-third of the sequences had no close relatives (similarity $< 90\%$) in the database, we argue that novel bacterial species inhabit the Panarea hydrothermal system.

Comparison between the sites with respect to the dominant bacterial populations showed a different pattern. Chlorobi, Epsilon-and Gamma-proteobacteria were more frequently detected from site 1, Alfa- and Epsilon-proteobacteria were more frequently detected from site 2. These differences could be due to the different location of the two sites. Site 2 was an isolated vent deeper, warmer and with a slightly acidic pH than site 1 (a site selected from a wide venting area). The bacterial phylotypes retrieved from site 1 showed a different temporal pattern: while Chlorobi dominated in July, Epsilonproteobacteria were dominant in June and September. At site 2, Alfaproteobacteria were present in all sampling times.

Although most of the retrieved sequences are similar to uncultured Bacteria, some of them are phylogenetically associated with environmental clones obtained from deep-sea hydrothermal vents. Five phylotypes were closely related to sulphur-oxidising bacteria affiliated with different subdivisions of Proteobacteria, suggesting a high level of diversity among sulphur oxidisers in these vents. Two sequences were related to *S. denitrificans* and *S. paralvinellae*, autotrophic bacteria isolated from shallow (Hirayama et al. 2007) and deep-sea hydrothermal vents (Takai et al. 2006). Also a sequence related to *Arcobacter* sp., a sulphide-oxidiser epsilonproteobacterium, was recovered. The versatile Epsilonproteobacteria are considered key players in sulphidic habitats distributed in modern and ancient systems all over the world (Campbell et al. 2006). Mesophilic sulphur-oxidising bacteria then represent a specialised community, common in hydrothermal vents, involved in the chemosynthetic primary production and in the turnover of the reduced sulphur compounds. Therefore, differently to other shallow habitats, chemolithoautotrophic sulphur-oxidising bacteria appear to dominate the composition of microbial autotrophic community at the two vents off Panarea.

Other than chemical energy sources, microbial primary production at shallow hydrothermal vents is supported by sunlight. Dominant phototrophic bacteria in water from site 1 (the shallowest vent) were represented by *Chlorobi* (green sulphur anoxigenic phototrophs), while at site 2 (the deepest and warmest vent) they were represented by *Rhodomicrobium* spp. (purple non sulphur anoxigenic phototrophs). Therefore, the different vent conditions (mainly the depth) can exert a selective pressure on the more adapted type of phototrophs.

There are few comparable studies regarding the microbial community in shallow hydrothermal vents. Sievert et al. (1999) demonstrated that there were more heterotrophs than autotrophs in the sediment community of the shallow system of Milos, despite the fact that high numbers of chemolithoautotrophic sulphur-oxidising bacteria were cultivated from the same hydrothermal sediments. Our results agree with those of Hirayama et al. (2007), who demonstrated that, in accord with geochemical characteristics, the chemolithoautotrophs and methanotrophs, including members of the Epsilonproteobacteria and unique members of the Gammaproteobacteria,

predominated in the fluid and in the microbial mat associated with the main vent site in the Taketomi system (Japan).

Phylogenetic analysis of archaeal DGGE 16S rRNA gene sequences revealed that almost all of them were affiliated with uncultured clones of Archaea retrieved mainly from hot springs and hydrothermal vents of different geographical zones. Some sequences were only moderately related to database entries and could, therefore, represent new archaeal taxa (Fig. 7). A sequence related to uncultured methanogenic Archaea from site 2, and a sequence similar to an uncultured *Ferroglobus* sp., reported from deep-sea fluids (Nakagawa et al. 2005) were detected in site 1. These findings make our shallow hydrothermal vents similar to extreme environments, where anaerobic microorganisms constituted a significant component of the microbial community. Furthermore, two sequences recovered from site 1 were similar to those detected in a geothermal well in the near Vulcano Island (Amend et al. 2003b), demonstrating that Panarea and Vulcano hydrothermal systems may host similar members of archaeal populations.

The microbial diversity depicted by DGGE analysis is probably lower than that actually present as only 28% of bands obtained for Bacteria and 42.8% for Archaea were sequenced. Since DGGE technique detects only dominant populations higher than 1% (Muyzer et al. 1993), we can assume that an unknown fraction of the microbial populations remained undetected. Among these populations are members of thermophilic bacilli, which, even though frequently isolated by cultural methods, were not retrieved by DGGE analysis of environmental samples. Concomitant DGGE analysis (data not shown) carried out after inoculating water and sediment samples from site 1 and site 2 in MB enrichment (incubated at 80°C), showed sequences assigned to thermophilic bacilli. It suggests that the occurrence of thermophilic bacilli is demonstrated only after their enrichment in appropriate laboratory conditions.

Variations in fluid composition, linked to temporal and spatial variations, seem to influence not only the microbial structure and composition, but also the metabolic strategies of the resident microbial community. Both bacterial and archaeal specialised populations, mainly involved in the oxidative phase of sulphur cycle, were dominant components of the microbial community at Panarea shallow marine vents.

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