

Prokaryote Diversity and Virus Abundance in Shallow Hydrothermal Vents of the Mediterranean Sea (Panarea Island) and the Pacific Ocean (North Sulawesi-Indonesia)

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Abstract Despite their ubiquitous distribution in tectonically active coastal zones, shallow water hydrothermal vents have been less investigated than deep-sea vents. In the present study, we investigated the role of viral control and fluid emissions on prokaryote abundance, diversity, and community structure (total Archaea, total Bacteria, and sulphate-reducing bacteria) in waters and sediments surrounding the caldera of four different shallow-water

hydrothermal vents (three located in the Mediterranean Sea and one in the Pacific Ocean). All vents, independent of their location, generally displayed a significant decrease of benthic prokaryote abundance, as well as its viable fraction, with increasing distance from the vent. Prokaryote assemblages were always dominated by Bacteria. Benthic Archaea accounted for 23–33% of total prokaryote abundance in the Mediterranean Sea and from 13 to 29% in the Pacific Ocean, whereas in the water column they accounted for 25–38%. The highest benthic bacterial ribotype richness was observed in close proximity of the vents (i.e., at 10-cm distance from the emissions), indicating that vent fluids might influence bacterial diversity in surrounding sediments. Virioplankton and viriobenthos abundances were low compared to other marine systems, suggesting that temperature and physical-chemical conditions might influence viral survival in these vent systems. We thus hypothesize that the high bacterial diversity observed in close proximity of the vents is related with the highly variable vent emissions, which could favor the coexistence of several prokaryotic species.

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Introduction

Hydrothermal vent areas are extreme environments that support life forms adapted to highly unstable physicochemical conditions [12, 26, 33]. Deep-sea hydrothermal vents are known to be characterized by high pressure, wide temperature ranges, and the presence of variable composition of fluids and gases, which are hostile for the life of most eukaryotes [10, 11, 25, 56]. These ecosystems offer a variety of habitats and microniches, which can potentially be inhabited by metabolically diverse microorganisms [2,

25, 26]. Previous studies revealed that hydrothermal vents release high concentrations of iron and zinc sulphides, which are important substrates for microbes [33, 45]. Food webs associated with hydrothermal vents are complex and, although primary production of the hydrothermal-vent environment is chemosynthetic, much of the microbial biomass in these extreme environments is accounted by heterotrophic prokaryotes, which use organic matter released from animals and other microbes as a food source [24, 25, 50–52]. Hydrothermal plumes can influence the growth, diversity, and distribution of allochthonous heterotrophic and autochthonous autotrophic prokaryotes even in areas relatively distant from the active seepage fields [15, 25, 32].

Viruses are recognized as an abundant and dynamic component of surface and deep waters [for reviews, see 19, 59, 60, 63], but information on the interactions between prokaryotes and viruses in vent systems are very limited. Available information for other aquatic systems indicates that viral lysis of their hosts, releasing dissolved organic matter (DOM), can shortcut the microbial loop, reducing bacterial production and the energy transfer to higher trophic levels [8, 19, 35]. If this applies also to hydrothermal vents, virus-induced prokaryote mortality could influence prokaryote diversity and, potentially, carbon cycling. The only studies carried out on virus dynamics in a deep-sea hydrothermal vent suggested that virus-mediated mortality of prokaryotes is significant and may reduce energy flow to higher trophic levels [40, 64].

It is surprising to note that shallow-water hydrothermal vents, despite their abundance and ubiquitous distribution in tectonically active coastal zone, have been much less investigated than deep-sea hydrothermal vents. As a consequence, several important aspects of the research on shallow vent microbial ecology, such as prokaryote activity and diversity, viral distribution, and virus/prokaryotes interactions, are still poorly studied [2, 10, 11, 20, 21, 48, 49, 54]. Studies on these aspects can be crucial for understanding the overall functioning of hydrothermal shallow vent ecosystems [22].

In this study, we investigated the role of biotic (viral control) and abiotic (fluid emissions) factors on prokaryote abundance, diversity, and community structure in waters and sediments surrounding the caldera of shallow-water hydrothermal vents. To do this, we selected three vents in the Mediterranean Sea and one in the Pacific Ocean (North Sulawesi, Indonesia) and utilized epifluorescence microscopy and molecular techniques (including Terminal-Restriction Fragment Length Polymorphism [T-RFLP] and CAtalyzed Reporter Deposition [CARD]-Fluorescence *in Situ* Hybridization [FISH]) to: (1) determine the abundance, distribution, and diversity of prokaryotes in seawater and sediments collected along a gradient of increasing distance from the vent, and (2) to provide new insights on the viral

distribution in relation to fluid emissions, prokaryote diversity, and community composition.

Materials and Methods

Study Area

The first investigated area is part of the Aeolian Volcanic Archipelago in the Southern Tyrrhenian Sea (Mediterranean Sea). The 110KY BP volcano is characterized by an emerged portion comprising the Panarea Islands. The area is known for the presence of gas emissions from the sea bottom, entering in an explosive phase early November 2002, when several new vents appeared. Vent emissions reached the water surface from up to 20 m depth [1, 4, 5]. Gas composition (98% CO₂, H₂S, and other gases) was determined using gas chromatography according to Chiodini et al. [5]. The high-pressure gas created sinkholes and the collapse of the seafloor, including a large crater (20 × 10 m) close to the Bottaro Islet (site EC1; 15°06'34.61"E and 38°31'14.14"N; fluid temperature 30°C, gas flux intensity 241 L min⁻¹ m⁻²), another wide exhaling area (site EC2; 15°06'35.66"E and 38°38'23.29"N; fluid temperature 90°C; gas flux intensity 67 L min⁻¹ m⁻²) and a single emission point defined "black smoker" because of black and sulphur-rich gas emissions flowing in a small plume from the vent (site BP; 15°06'18.27"E and 38°38'14.15"N; fluid temperature 110°C, gas flux intensity 380–550 L min⁻¹ m⁻²) [57]. The vent EC1 was located on a plateau of about 14 m, partially filled to a depth of 7–8 m. This site was characterized by gravel sediment, covered by a layer of a few centimeters of snow-like, bright yellow colloidal material (here called "precipitate") containing sulphur salts. Because of the presence of hard substrate around EC1 caldera, only samples of bottom seawater and precipitate were collected. The vent EC2 ("Sink-Hole") was located on a small depression with a cavern at about 16 m depth and was characterized by sandy sediments interspersed with pebbles. The intensity of fluid emission was lower compared to the EC1 site, but spread out over a larger area. At this site, bottom seawater, precipitate, and sediment samples were collected. The BP vent was located at about 23 m depth, on a circular depression covered by boulders and sandy sediments. No precipitate was present, so that only seawater and sediment samples were collected at this site.

The second investigated area is part of the Indonesian archipelago (Pacific Ocean) and is characterized by the presence of 129 volcanoes, which are responsible for gas emissions from the sea bottom in several coastal areas. The investigated area was located in the Strait of Lembeh, between Sulawesi and the Moluccas Sea, in the Northern

region of the Sulawesi Island (site SC1; 123°48'19.45"E and 1°20'22.18"N; 3 m depth; fluid temperature 90°C, gas flux intensity 809 L min⁻¹ m⁻²).

Sampling

Sampling was carried out in spring–summer 2003. The three vents in the Mediterranean Sea are hereafter reported as EC1, EC2 ("Sink-Hole"), and BP, whereas the vent in the Pacific Ocean is reported as SC1. Sampling strategy was designed to investigate the influence of the vent system on the surrounding sediments [48]. Sediment sampling was carried out by SCUBA divers wearing specific protective gloves, using sterile polycarbonate tubes (Ø 5.5 cm), along a gradient of increasing distance from the seepage (i.e., inside the vent, at 10, 100, and 200 cm). Three independent replicates were collected according to a randomized stratified sampling strategy [3]. Prokaryotic and viral analyses were carried out contextually in the same samples, which were collected synoptically. Immediately after sediment collection, tubes were carried onboard and processed in sterile conditions to avoid contamination. Precipitate samples were collected around the vent field in EC1, EC2, and SC1 using sterile propylene test tubes (50 mL capacity).

Water samples were collected by SCUBA diving using sterile bottles from the emission of the three Mediterranean vents (EC1, EC2, and BP). Moreover, water samples were collected by using a Rosette sampler at three control sites located at a distance of 1000–2000 m from the calderas (St. 01 at 36 m, St. 02 at 75 m, St.10 at 23 m). Conductivity, temperature, and depth (CTD) casts (SBE911) were conducted at vent and control sites, to measure the following environmental variables: temperature, oxygen, pH, and salinity.

Vent fluid temperatures were measured by means of a glass thermometer by SCUBA divers. The gas flux was estimated by measuring the time necessary to completely remove water from an upside-down tank connected to the gas vent by a funnel. Because of the shallowness (3 m), CTD deployments were not carried out in Indonesia (vent SC1). In this vent, water samples were not collected because of logistical and technical problems. At this vent, temperature and pH were measured using a portable probe mod. HD 8705 (Delta Ohm, Italy), whereas salinity was measured using a portable refractometer (OxyGuard, Italy).

To provide unbiased estimates induced by the use of preservative, sediment samples for viriobenthos analyses were stored into sterile Falcon test tubes (50 mL) and immediately frozen at −20°C (without any fixation) until processing [23, 40, 58]. For virioplankton analyses, samples (1 mL) were filtered on board through a 0.02-μm pore size Anodisc filters (AIO₃, Whatman) and immediately

frozen at −20°C [40] until processing in the laboratory. For prokaryote counts in both sediments and water column, samples were fixed with 2% formalin as previously described and stored at 4°C until processing [34, 44].

For T-RFLP analyses of prokaryotic diversity, samples of sediment and precipitate were immediately placed into sterile 50-mL Falcon tubes and stored at −20°C until processing. Water samples were filtered onto 0.2-μm polycarbonate filters (diameter 47 mm; Millipore), placed into sterile petri dishes and stored at −20°C.

Total and Viable Prokaryote Abundances

Total and viable prokaryotic counts were performed using a staining/de-staining procedure [30]. This technique is based on the presence of a DNA organized in a visible nucleoid region [66] that can be stained with a DNA-specific fluorochrome, such as DAPI or Acridine Orange. The procedure involves a washing step of cells with isopropanol, after staining with DAPI or Acridine Orange. The isopropanol washing removes the nonspecific staining of cellular components of dead cells that do not contain a significant amount of DNA, leaving stained only those prokaryotes with an identifiable nucleoid. These cells are defined as nucleoid-containing cells (NuCC) compared to those devoid of nucleic acid. Samples were preincubated for 1 h with Acridine Orange (0.025% final concentration) to improve the staining efficiency [27]. Sediment samples were treated with ultrasound (three times for 1 min; Branson sonifier 2200, 60 W), which enabled the detachment of the prokaryotes from the sediment matrix and then incubated again for 2 h in the dark with acridine orange (0.025% final concentration) and Triton X-100 (0.1% [v/v]). Sediment samples were then diluted 100–500 times using Milli-Q and filtered onto 0.2 μm pore size Nucleopore black polycarbonate filters under low vacuum (<100 mmHg). Filters were washed with 10 mL of 2-propanol for 10 min before being mounted on microscopic slides. Filters were analyzed as described by Fry [17] using epifluorescence microscopy (Zeiss Axioskop 2, ×1000). After NuCC counting, according to the de-staining/staining procedure (0), filters were mounted again on the filtration apparatus, restained with Acridine Orange, washed once with 5 mL of sterile water and then mounted on microscopic slides for total prokaryotic count. For each filter, at least 20 microscope fields, corresponding to at least 400 cells, were counted to achieve a proper statistical significance [28]. Values of NuCC and total prokaryote abundances in seawater samples were normalized to milliliters of seawater. In both sediment and precipitate samples, these abundances were normalized to gram of sediment and precipitate dry weight after desiccation (60°C, 24 h).

Virus Abundances

Direct counts of virus-like particles in water samples were carried out using the method described by Noble and Fuhrman [39], with few modifications. Filters were stained with 20 μL of SYBR Green I (stock solution diluted 1:20 with virus-free Milli-Q water), incubated in the dark for 20 min and then mounted on glass slides with 20 μL of a solution of 50% phosphate buffer (6.7 mM, pH 7.8) and 50% glycerol containing 0.5% ascorbic acid.

Direct counts of virus-like particles in sediment samples were carried out according to Danovaro et al. [13, 14]. Viruses were detached from sediment samples using Tetrasodium pyrophosphate (5 mM final concentration; stock solution 500 mM, pH 9.5; SIGMA) and ultrasound treatment (three times for 1 min each; Branson sonifier 2200, 60 W) and then centrifuged for 60 s (800 $\times g$, ALC PK 120 centrifuge). To eliminate uncertainties in virus counting caused by extracellular DNA interference, subsamples diluted 100 times with Milli-Q sterile water were added with DNase I from bovine pancreas (10 U mL^{-1} final concentration) and incubated for 15 min at room temperature [13]. After incubation, samples were filtered on 0.02- μm pore-size filters (Anodisc; diameter 25 mm; Al_2O_3) and then processed as described for water samples.

Viral counts were then obtained by epifluorescence microscopy (Zeiss Axioskop 2, magnification $\times 1000$), by examining at least 10 fields per slide, to count at least 400 viral particles per filter. Virus counts were normalized to sediment dry weight after desiccation (60°C, 24 h).

Prokaryote Community Structure

We used fluorescence *in situ* hybridization with rRNA-targeted oligonucleotide probes (FISH) and signal amplification (CATalyzed Reporter Deposition, CARD) for both seawater and sediment samples. This method has been shown to allow a better visualization of benthic prokaryotic cells compared to conventional FISH, as a result of enhanced fluorescence intensities and signal-to-background ratios [41]. We used the following oligonucleotide probes: EUB338 for total Bacteria; SRB385 for sulphate-reducing bacteria; ARCH915 for Archaea; NON338 as a negative control (all probes from [41, 47]). Samples were prepared for *in situ* hybridization as described by Pernthaler et al. [41]. For bacterial cell wall permeabilization, filters were incubated in a lysozyme solution (10 mg mL^{-1}) at 37°C for 1 h. For specific Archaeal permeabilization, incubations were carried out with proteinase K solution (0.15 $\mu\text{g mL}^{-1}$ in 2.5 U mg^{-1}), as previously described [53]. Four microliters of probe (100 pmol μL^{-1}), each labeled with the enzyme horseradish peroxidase (HRP), were added to filter sections with 400 μL of hybridization buffer (0.9 M NaCl, 20 mM

Tris-HCl pH 7.5, 10% dextran sulphate, 0.02% sodium dodecyl sulfate (SDS), 55% formamide, 1% blocking reagent) and incubated for 2 h at 37°C. Filter sections were removed from the hybridization mixture and incubated in prewarmed washing buffer (3 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 20 mM Tris-HCl pH 7.5, 0.01% SDS) for 10 min at 37°C. To equilibrate, the probe-delivered HRP sections were placed in a phosphate-buffered saline (PBS) solution amended with 0.05% of Triton X-100 for 15 min at room temperature. Filter sections were transferred to a substrate mix containing 1 part of tyramide-Cy3 and 10 parts of amplification buffer (PerkinElmer) and incubated for 10 min at room temperature in the dark. Filter sections were then washed in PBS and Triton (0.05%). To decrease background fluorescence, sections were subsequently washed in Milli-Q water and 96% ethanol for 1 min. Microscopic evaluation was obtained by epifluorescence on a Zeiss Axioskop 2 microscope with an appropriate filter set for Cy3 fluorescence. The percentage of total Bacteria, determined using the probe EUB338, allowed estimating the abundance of all other Bacteria by difference with the abundance of SRB.

Bacterial Diversity

We used the Terminal-Restriction Fragment Length Polymorphism fingerprinting technique (T-RFLP) to obtain estimates of bacterial richness, diversity indices, and community composition in the investigated areas. Genomic DNA was extracted from seawater, precipitate, and sediment samples by *in situ* lysis, a technique previously utilized for investigating benthic prokaryote diversity [31]. For each sediment and precipitate sample, ca. 5 g of material were placed into a 50-mL Falcon test tube and the DNA was extracted according to the Zhou et al. [65] procedure. From each seawater sample, an aliquot of 50 mL was filtered onto 0.2- μm polycarbonate filters (Millipore). DNA was extracted from these latter filters according to the procedure of Fuhrman et al. [18]. DNA extracted was purified using a Wizard purification column (Promega, Madison, WI) and DNA concentrations were determined spectrofluorimetrically using SYBR Green I [9]. The fluorescence of each sample was converted into DNA concentrations using standard solutions of *Escherichia coli* DNA (Sigma). Bacterial 16S rDNA was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'; [29]) and 1492R (5'-GGTACCTTGTTACGACTT-3'; [29]). These primers were selected for assessing the overall bacterial diversity. Primer 27F was fluorescently labeled at the 5' end with the phosphoramidite dye 6-FAM (MWGspa BIOTECH). Polymerase chain reactions (PCR) were performed in a final volume of 50 μL in a thermocycler (Biometra), using the MasterTaq® kit (Eppendorf), which

reduces the effects of PCR-inhibiting contaminants. DNA was added in a volume of 1 μ L, containing about 1–5 ng of template. For both 27F-1492R and 385F-907R primers, we used 30 PCR cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, preceded by 3 min of denaturation at 94°C and followed by a final extension of 10 min at 72°C. For each PCR, negative controls containing the PCR mixture except DNA template were run, together with positive controls containing genomic DNA of *E. coli*. PCR products were run on agarose-TBE gel (1%) containing ethidium bromide for DNA staining and visualization. This also allowed excluding the presence of undesired or nonspecific PCR products. For each sample, two independent 50- μ L PCR were performed, and the amplified products were pooled together to reduce the occurrence of PCR biases. The products were again purified using a Wizard PCR clean-up system (Promega, Madison, WI), to remove unincorporated nucleotides and labeled primers. DNA was eluted in 50 μ L of water supplied with the clean-up system and then about 50 ng of purified 16 S rDNA amplicons (measured spectro-fluorimetrically after staining with SYBR Green I) were digested in duplicate in a 20- μ L reaction volume, containing 10 U of the enzyme Rsa I (Promega, Madison, WI.) at 37°C for 3 h. Restriction digestions were stopped by incubating at 65°C for 20 min, and samples were then stored for 2 days at –20°C until analysis. One microliter of each digest was mixed with 14 μ L of internal size standard (GS1000-ROX; Applied Biosystems, Foster City, CA) in deionized formamide, and then denatured at 94°C for 2 min and immediately chilled in ice. Fragments were analyzed in GeneScan mode in a ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using 47 cm $\times$ 50 μ m capillaries, POP-4 polymer (PerkinElmer), 40 s injection time, 15 kV injection voltage, 15 kV run voltage, and 60°C capillary temperature. Terminal restriction fragment sizes

between 47 and 946 bp were determined using Genescan analytical software 2.02 (ABI). For analysis and interpretation of T-RFLP profiles, Genescan numerical outputs from each electropherogram were extracted and transferred to Microsoft Excel. A standardization of the DNA quantity between all the replicate and samples profiles analyzed was adopted, as described by Dunbar et al. [16]. After standardization, peaks less than 1.5 bp apart from a larger peak were classified as “shoulders” and thus eliminated. As a subsequent step, peaks not present in the two replicates (irreproducible peaks) were considered as PCR artifacts and thus removed. Only terminal restriction fragments whose peak heights accounted for at least 0.17% of the total integrated height were included. This cut-off was specifically calculated on the basis of: (1) sensitivity of the technique, (2) length of the amplified region, and (3) size standard utilized [31]. Results are reported as operational taxonomic unit (OTU) or ribotype number (or richness).

Ecological Indexes and Statistical Analyses

The Shannon-Wiener index (H' , calculated on ln bases) and the Pielou index (J' , evenness) were obtained from T-RFLP profiles using the bacterial universal primers [43]. In all calculations we assumed that the number of TRFs represents the species number and that the peak height represents the relative abundance of each component. All diversity indexes and the similarity in community composition among samples (measured by means of a cluster analysis based on Bray-Curtis similarity) were calculated using the PRIMER program (Plymouth Marine Laboratory; [6]). Differences in environmental and microbial variables (e.g., total and viable prokaryotic abundance, viral abundance, prokaryote biodiversity) between the two areas were analyzed using one-way analysis of variance (ANOVA),

Table 1 Temperature, oxygen concentration, pH and salinity 1 in surface and bottom waters of the Mediterranean and Pacific vents

	Site	Sampling depth	Temperature °C	Oxygen mL L ⁻¹	pH	Salinity
Mediterranean Sea	St. 01 Control	Surface	28.5	3.9	7.9	38.1
		Bottom	19.6	5.1	7.9	37.6
	St. 02 Control	Surface	28.8	3.9	7.8	38.2
		Bottom	14.3	4.3	7.7	38.1
	St. 10 Control	Surface	28.1	3.9	8.0	38.1
		Bottom	16.8	5.0	7.9	37.6
	EC 1	Surface	28.5	4.3	6.4	38.0
		Vent	28.9	3.7	6.7	38.1
	EC 2	Surface	28.8	3.9	7.9	38.0
		Vent	27.8	3.9	7.6	38.0
	BP	Surface	28.7	3.9	7.9	38.1
		Vent	21.2	4.6	7.5	37.6
Pacific Ocean	SC1	Surface	30.2	–	6.0	34.5
		Vent	74.1	–	6.0	34.0

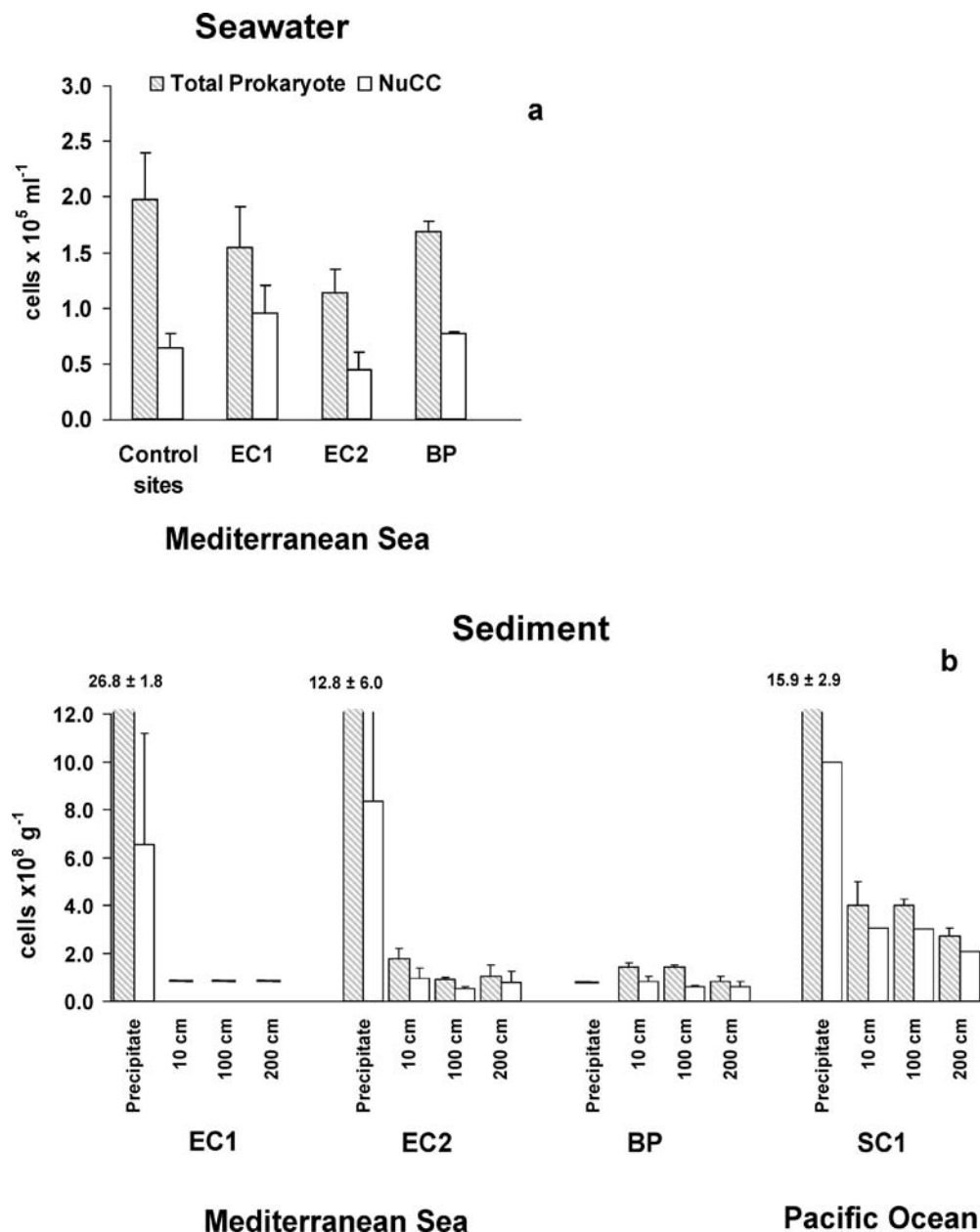


Figure 1 Total prokaryotic abundance and nucleoid-containing cells (NuCC) in (a) seawater (in Control, EC1, EC2, and BP sites) and (b) sediment samples (in EC1, EC2, and BP sites in Mediterranean Sea and in SC1 in the Pacific Ocean). Sediment samples were collected at

10, 100, and 200 cm from the caldera. Sediments were not collected at site EC1 (Mediterranean Sea) because of the hard substrate composition. Mean values are reported for the Control sites in seawater samples. Standard deviations ($n=3$) are reported. — not available

followed by the post hoc Newman–Keuls test (NK) with significant differences set at $p \text{ level} \leq 0.05$.

Results

Environmental Parameters

In the Mediterranean sea, the highest fluid temperature was observed at site BP (110°C), whereas the lowest tempera-

ture was observed at EC1 (30°C). Fluid temperature in the Pacific Ocean was ca. 90°C (site SC1). At this vent, the temperature in the surrounding sediments was ca. 74°C (at 10-cm distance from the caldera), 55°C at 100 cm and 30°C at 200 cm from the vent.

Temperature and pH values in surface and bottom waters in the Mediterranean Sea and the Pacific Ocean are reported in Table 1. Temperature in surface waters did not show differences among Mediterranean sites (on average 28.6°C), whereas bottom temperatures were higher in the vent sites

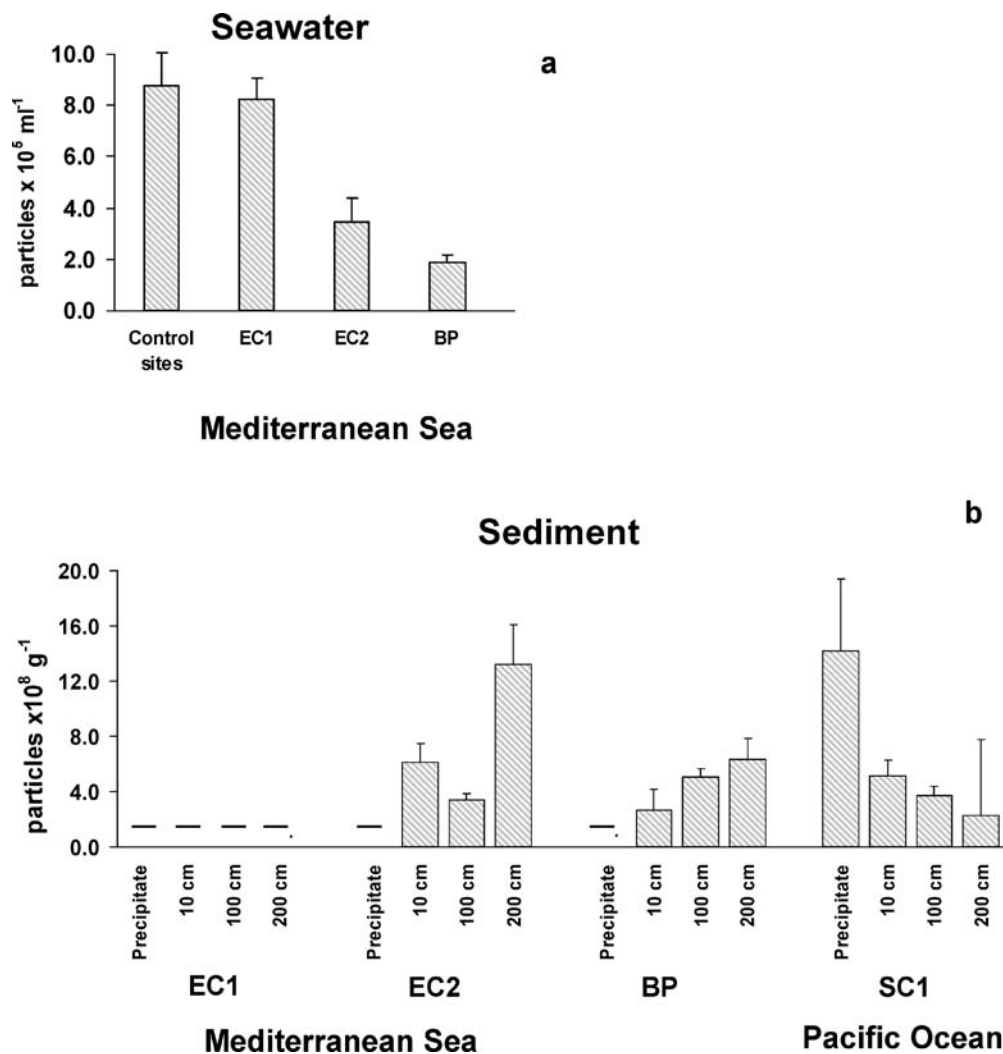


Figure 2 Viral abundance in (a) seawater (in Control, EC1, EC2, and BP sites) and (b) sediment samples (in EC1, EC2, and BP sites in the Mediterranean Sea and in SC1 in Pacific Ocean). Sediment samples were collected at 10, 100, and 200 cm from the caldera. Sediments

were not collected at site EC1 (Mediterranean Sea), because of the hard substrate composition. Mean values are reported for the Control sites in seawater samples. Standard deviations ($n=3$) are reported. — not available

than at the control sites (mean values: 26 vs 17°C, respectively). At all sites, pH values decreased when approaching the vent field, and in SC1 and EC1 the strong fluid emissions determined a low pH (ca 6.02 and 6.7, respectively). Salinity did not display differences among sites nor between surface and bottom waters (on average 38.1 vs 37.8, respectively). Oxygen content at the three Mediterranean vents was rather constant, with values in bottom waters ranging from 3.74 to 4.62 mL L⁻¹ (vs 4.28–5.06 mL L⁻¹ in controls). No values are available for the SC1 vent. The three sites also displayed differences in the dissolved nutrient concentrations (e.g., SO_4^{2-} , NO_3^- , NO_2^- , and SiO_2), with clear differences comparing surface and bottom waters. In EC1 and BP, bottom waters displayed a significantly higher content of nitrite and silicate than EC2 (on average 5.55, 7.73, and 0.98 $\mu\text{mol L}^{-1}$ nitrite in EC1,

BP, and EC2, respectively), whereas sulphates were higher at the EC2 site.

Prokaryote Abundance and Viable Fraction

The abundance of prokaryotes in Mediterranean seawater samples ranged from 1.1 ± 0.2 to $2.0 \pm 0.4 \times 10^5$ cells mL⁻¹ (Fig. 1a). No significant differences in prokaryote abundance were observed among vent sites or between vent and control sites. Benthic prokaryote abundance in both Mediterranean and Pacific vents was higher in the precipitate (range 12.8 ± 6.0 – $26.7 \pm 1.8 \times 10^8$ cells g⁻¹) than in other sediment samples (range 0.83 ± 0.1 – $4.01 \pm 0.9 \times 10^8$ cells g⁻¹), with a decreasing pattern from the vent center to 200-cm distance (ANOVA, $p < 0.01$; Fig. 1b). The contribution of the viable fraction to the total prokaryote

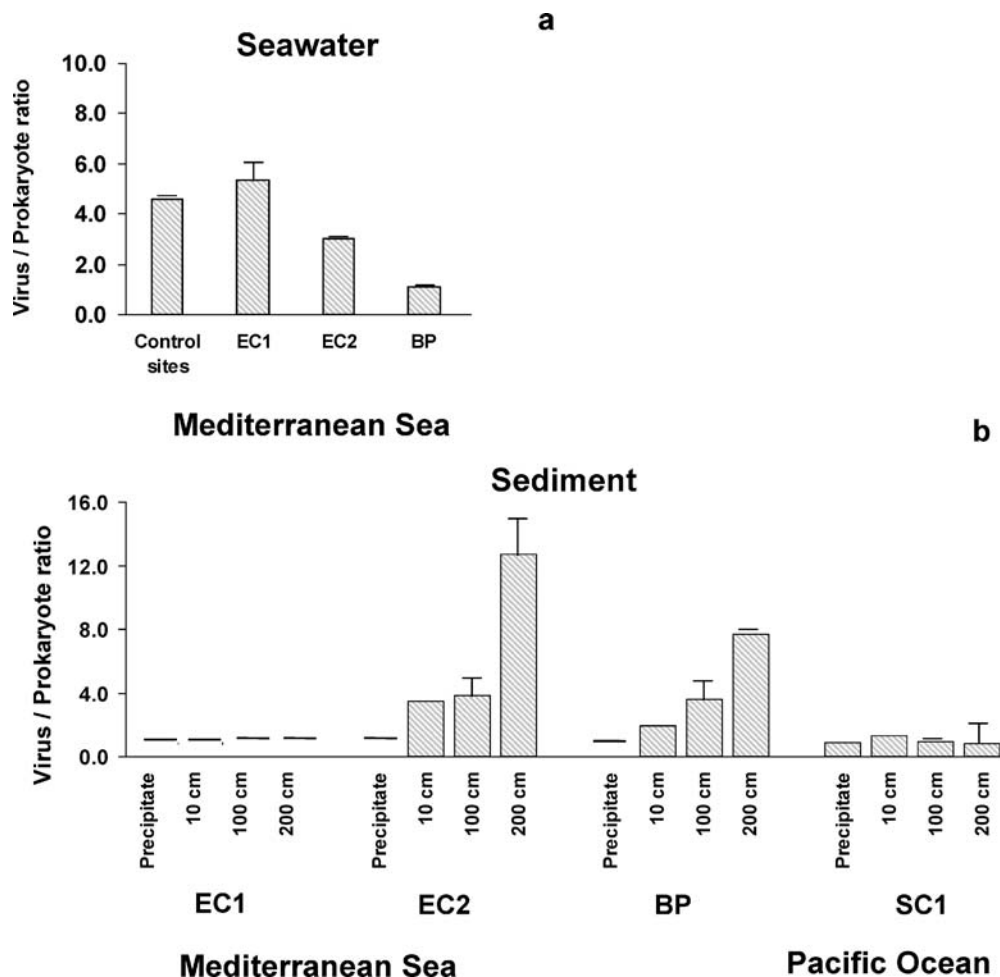


Figure 3 Virus to prokaryote abundance ratio in (a) seawater (in Control, EC1, EC2, and BP) and (b) sediment samples (in EC1, EC2, and BP sites in Mediterranean Sea and in SC1 in Pacific Ocean). — not available

abundance in both seawater and sediments did not display significant differences among Mediterranean vents (range 33–42% in seawater samples, 13–42% in the precipitate, 41–74% in the sediments). In the Pacific vent, the number of viable cells was significantly higher in the precipitate ($10.0 \pm 0.2 \times 10^8$ cells g^{-1}) than in all other stations (ANOVA, $p < 0.01$; Fig. 1b). The contribution to the total prokaryote abundance was 63% in precipitate, 76% at 10 and 100 cm, and 77% at 200 cm, with no significant differences observed.

Viral Abundance

Spatial patterns of viral abundance and virus to prokaryote ratios (VPR) are shown in Figs. 2 and 3, respectively. Viral abundance was, on average, ca five times higher than bacterial abundance, both in seawater and sediment samples. Virioplankton abundance ranged from 1.9 ± 0.3 to $8.8 \pm 1.3 \times 10^5$ viruses mL^{-1} (at BP and control sites, respectively). In seawater samples collected in the Mediterranean Sea, the highest viral abundance was observed in control sites and in the vent EC1 (NK test $p <$

0.001, Fig. 2a); a similar trend was also observed for VPR (4.6 and 5.4, respectively; Fig. 3a).

In the Mediterranean and Pacific vents, viriobenthos abundance ranged from 2.7 ± 1.5 to $5.2 \pm 1.1 \times 10^8$ viruses g^{-1} , respectively (at site BP 10 cm and SC1, respectively). In Mediterranean sediments, viral abundance displayed a significant increase with increasing distance from the vent (NK test, $p < 0.001$). In the Pacific vent, virus abundance was highest in the precipitate and decreased with increasing distance from the vent. Benthic VPR ranged from 0.8 to 12.7 (at site SC1 and EC2, respectively), with highest values at 200-cm distance from the vents (Fig. 3b).

Prokaryote Community Composition

The fraction of prokaryotes detected using CARD-FISH (calculated by summing the cells visualized using the probes EUB338 and ARCH915) in water and sediment samples ranged from 65 to 97% of the total prokaryote abundance in the Mediterranean samples and from 50 to 80% in the Pacific samples (Fig. 4a,b).

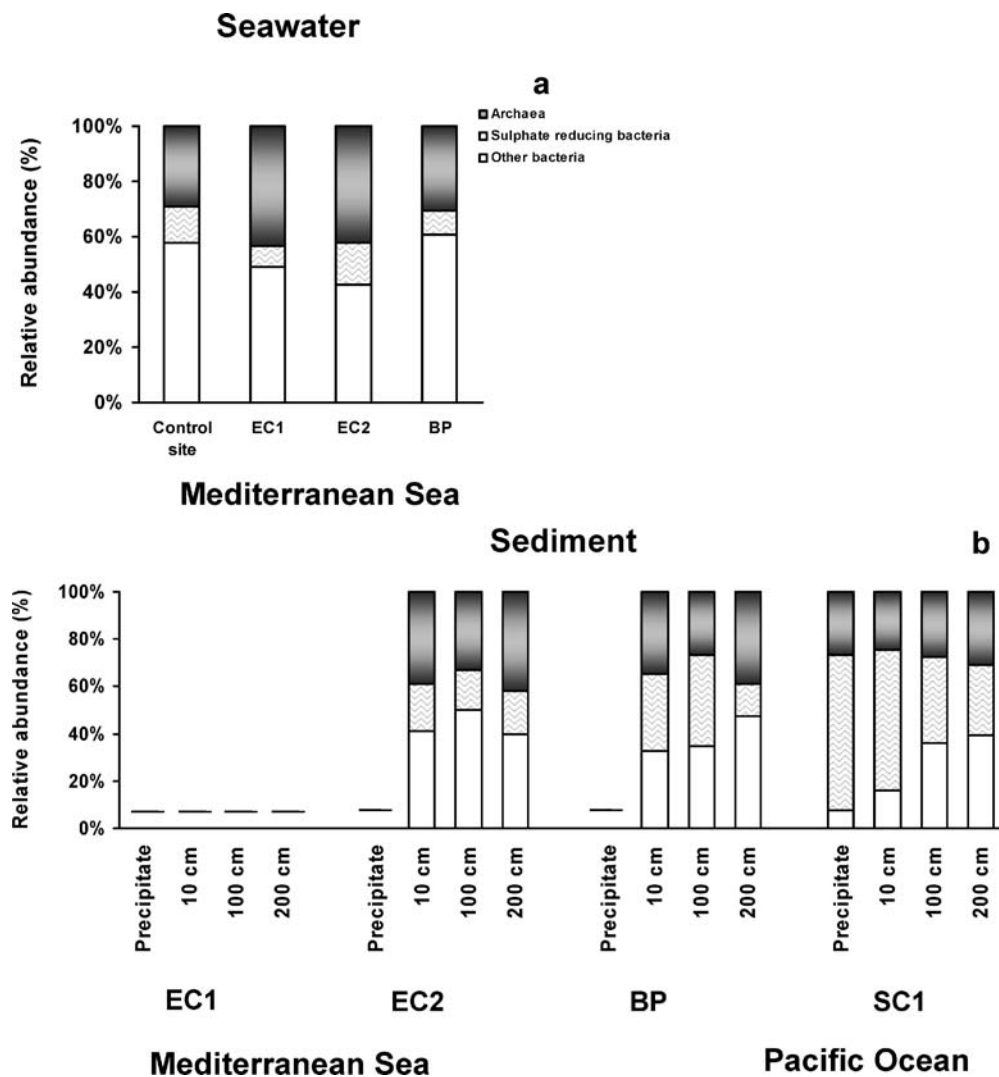


Figure 4 Relative abundance of sulphate-reducing bacteria (SRB), other Bacteria and Archaea in seawater and sediment samples in the investigated vents. — result not available. Mean values are reported for the Control sites in seawater samples

In seawater samples, the highest percentage of Bacteria (ca 60%) was observed in BP vent and in the control sites ($1.0 \pm 0.5 \times 10^5$ cell mL⁻¹ vs $0.9 \pm 0.1 \times 10^5$ cell mL⁻¹; NK test, $p < 0.05$). No significant differences were observed in terms of SRB contribution to Bacteria (range 7–11%) among sites (ANOVA, ns). The percentage of Archaea (on average 38%; Fig. 4) was highest at vent EC1 ($5.9 \pm 0.9 \times 10^4$ cell mL⁻¹) and varied within a narrow range in all other sites (range 25–28%).

In sediment samples, the percentage of the Bacteria ranged from ca. 36 to 65%, with higher values at BP vent in Mediterranean Sea (on average, $7.8 \pm 4.9 \times 10^7$ cell g⁻¹). The relative importance of sulphate-reducing bacteria (SRB) was significantly higher than in the water column, ranging from 11 to 34% in the Mediterranean sea and from 23 to 39% in the Pacific Ocean (NK test, $p < 0.001$). The percentage of Archaea did not display significant differences among all vents. In the Pacific vent, the abundance of

Archaea was significantly higher (NK test, $p < 0.001$) at 100-cm and 200-cm distance (29% and 24%, respectively) than in the precipitate and at 10-cm distance (13–15%).

Bacterial Diversity

Bacterial diversity, expressed as total ribotypes or OTU richness, ranged from 7 to 15 in seawater samples and from 35 to 53 in precipitate and sediment samples (Fig. 5a,b). In seawater samples, bacterial richness was highest at vents EC1 and BP. In these samples, some ribotypes, such as the 127 bp and 139 bp in EC1 and the 417 bp and 475 bp in BP, accounted together for ca. 30% of the total peak height in the T-RFLP profile (data not shown). In the sediment, bacterial richness was highest around the vent and decreased with increasing distance from the caldera. In seawater samples, the Shannon index (Table 2) was highest at vents EC1 and BP

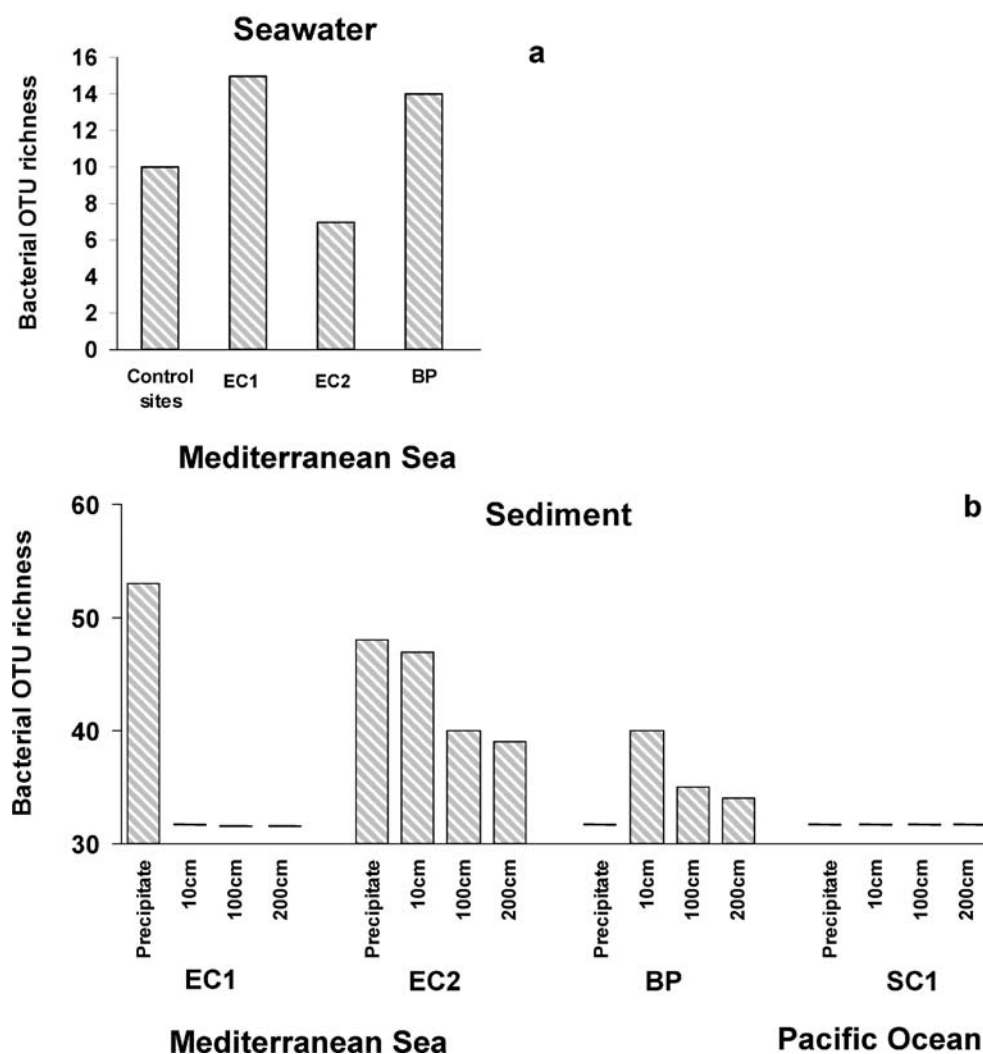


Figure 5 Bacterial diversity (as ribotype richness) in (a) seawater and (b) sediment samples. Mean values are reported for the control sites in seawater samples. — result not available

(ANOVA, $p < 0.01$). Conversely, values of the Pielou index were significantly lower at vents EC1 and BP than at EC2 and control sites (ANOVA, $p < 0.01$). In sediment samples, the highest values of the Shannon index were found in sediments close to the vent ($p < 0.01$). The Shannon index was always significantly higher in sediment and precipitate samples than in water samples (ANOVA, $p < 0.01$). No data are available for the Pacific Ocean.

Discussion

Deep-sea hydrothermal vents have been intensively investigated in the last 10 years [7, 25, 36–38], but information available on prokaryote and virus assemblages from shallow water vent areas is very limited [48, 49]. In this study, we investigated, for the first time, virus distribution

and prokaryote abundance, diversity, and community structure in four coastal vents characterized by different geographical locations, settings, and intensities of fluid emissions.

Several methods are available for estimating the active fraction of marine prokaryotes [30, 34 and references therein]. The method utilized in this study is based on the identification of cells containing a visible nucleoid region (NuCC) and as such does not provide information on the metabolic state of the cells [27]. Nonetheless, the identification of a nucleoid region remains useful to discriminate viable from nonviable cells. Results reported in this article indicate that samples around the vents in both Mediterranean and Pacific vents hosted a high number of prokaryotes, the majority of which (up to 70%) was accounted by NuCC. In particular, in the Pacific vent, this fraction decreased with increasing distance from the gas emission,

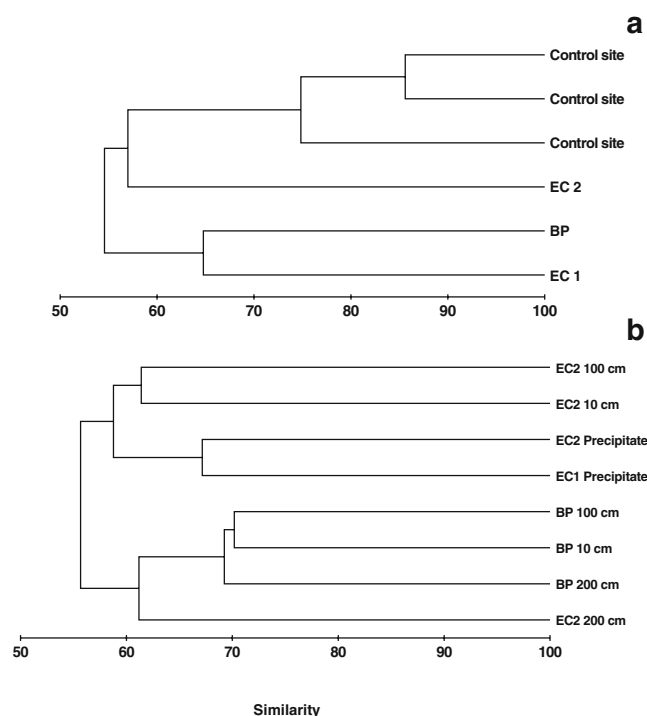
Table 2 Bacterial diversity (Shannon-Wiener index, H') and ribotype evenness (Pielou index, J) applied on the number of OTU) in seawater and sediment samples of the Mediterranean and Pacific vents

Station	Seawater	Precipitate						Sediment					
		H'	std	J	std	H'	std	J	std	H'	std	J	std
Mediterranean Sea	Control Sites	1.90	0.05	0.90	0.01	—	—	—	—	—	—	—	—
	EC 1	2.30	0.12	0.87	0.01	3.48	0.17	—	—	*	—	**	—
	EC 2	1.86	0.06	0.96	0.02	3.27	0.21	0.88	0.03	3.24	0.13	0.87	0.03
	BP	2.21	0.09	0.86	0.03	*	0.05	3.32	0.08	2.77	0.19	0.78	0.04
	SC1	*	—	*	—	*	—	3.22	0.05	*	—	*	—
Pacific Ocean	Control Sites	1.90	0.05	0.90	0.01	—	—	—	—	—	—	—	—
	EC 1	2.30	0.12	0.87	0.01	3.48	0.17	—	—	*	—	**	—
	EC 2	1.86	0.06	0.96	0.02	3.27	0.21	0.88	0.03	3.24	0.13	0.87	0.03
	BP	2.21	0.09	0.86	0.03	*	0.05	3.32	0.08	2.77	0.19	0.78	0.04
	SC1	*	—	*	—	*	—	3.22	0.05	*	—	*	—

Mean values are reported for the Control sites in seawater samples.

std: standard deviation

*Result not available

**Figure 6** Cluster analysis of T-RFLP patterns of bacterial community composition for (a) seawater and (b) sediment samples

suggesting that the hydrothermal activity might have an impact on the abundance and viability of microbial communities. These results also indicate that shallow vents, as previously reported for the deep-sea hydrothermal vents [61], are able to support the growth of viable prokaryotic assemblages.

An alternative estimate of the viable fraction of marine prokaryotes can be obtained using universal 16 S rRNA probes [27]. In the present study, the use of EUB338 and ARCH915 probes allowed to provide estimates of the percentage of viable cells. In 10 of the 14 investigated samples, the percentage of nucleoid containing cells (63–77%) was lower than the percentage obtained using the rRNA probes. If we assume that rRNA FISH probing works for all cells, the fraction of living cells, especially in proximity of the calderas, can be close to 90%.

In seawater and sediment samples of all investigated vents, prokaryote community was dominated by Bacteria, but Archaea represented an important fraction of total prokaryote abundance (on average 18% and 27%, in Pacific and Mediterranean vents, respectively). Previous findings based on rRNA blotting technique suggested that Bacteria accounted on an average for 95% of the prokaryotic assemblage at a shallow vent located in the Eastern Mediterranean (Aegean Sea) [49]. However, the amount of rRNA extracted in previous studies was too low to make reasonable quantifications of the Archaea [49], and the use of such different methodology could hamper a proper

comparison with our results. At the same time, our findings are consistent with those based on the FISH technique, which reported a high fraction of Archaea (on average 35% of the total prokaryote abundance) in surface sediments around the hydrothermal vent of Volcano Island (Italy) [46]. All these results suggest that the abundance of Archaea in shallow water vents can be higher than previously thought [49].

We cannot exclude that the use of Arch915 overestimated the abundance of Archaea, as recent studies reported that this probe can also target some Bacteria, such as Cytophaga-like bacteria [42]. However, Rusch et al. [46], using several independent probes that targeted different Archaeal groups, did not report evidence of overestimates caused by the use of Arch915, which remains one of the best tools for comparing our results with those available in literature.

In seawater, within the Bacteria domain, the fraction of sulphate-reducing bacteria (SRB) was relatively constant. In the BP and SC1 vent sediments, the SRB fraction decreased moving away from the caldera, whereas the relative importance of “other bacteria” and Archaea displayed an opposite spatial pattern. These patterns, although not observed in all vents investigated, suggest that the SRB fraction might increase its relevance in vents characterized by higher fluid emissions. However, data reported in this article are too limited to draw general conclusions from and the effect of vent emissions on the quantitative abundance of SRB requires further investigations.

In seawater, precipitate, and sediment samples, with the exception of a single seawater sample (i.e., EC2), total bacterial ribotype richness and diversity were highest near the vent emissions and decreased significantly with increasing distance from the vent, highlighting a positive effect of the fluid emissions on prokaryote diversity. In seawater samples of the EC1 and BP vents, the species evenness (Pielou index, J) was significantly lower than in the control sites (ANOVA, $P < 0.05$). This indicates a shift in the distribution of abundances among ribotypes, which resulted in the increased dominance of few ribotypes. A more detailed analysis of the prokaryote community composition in seawater samples collected from the vent fluids revealed also the presence of ribotypes not present in any other sample (data not shown). As evident from the cluster analysis (Fig. 6a), which illustrates the similarity in community composition between the different samples, bacterioplankton communities at the EC1 and BP vents were highly different from those of the control sites. Analysis of community composition in sediment and precipitate samples (Fig. 6b) indicated a high similarity in bacterial community composition among precipitates collected in different vents (e.g., EC1 and EC2), suggesting the occurrence of similar ribotypes within these peculiar matrices, independent of their geographical location. Ben-

thic bacterial similarity decreased at increasing distance from the vent at both EC2 and BP vents. These results are consistent with the analysis of the community composition reported for other shallow water vents [48, 49], thus suggesting that the vents might influence benthic prokaryote community composition.

Viral ecology in hydrothermal vents has been almost completely neglected so far. To our knowledge, the only available information deals with sparse investigations carried out on plume waters of deep-sea hydrothermal vents located in the Pacific Ocean [25, 40, 64]. The presence of high numbers of viable prokaryotes in the investigated vent emissions could potentially favor the development of a large abundance of viruses. It is known that marine viruses have the potential to control prokaryote abundance, and recent ecological models suggest that viruses might also increase prokaryote diversity by “killing the winner” [55, 58]. In our study, at all Mediterranean sites, both virioplankton and viriobenthos abundances significantly increased with increasing distance from the vent emission. Also, the virus to prokaryote abundance ratio, utilized as a proxy of potential viral infection [14, 62], was lowest in vent samples and increased significantly in control samples (ANOVA, $p < 0.01$). These trends were primarily influenced by the highest viral abundance and the lowest prokaryotic abundance in the sites closed to the vents. Although an opposite pattern of viral abundance was observed in the Pacific vent, the virus-to-prokaryote-abundance ratio in the Pacific samples was always very low and, indeed, lower than in the Mediterranean vents. In addition, virioplankton abundances were lower than those typically observed in coastal waters [35, 63]. These results allowed us to hypothesize that viruses can be less tolerant than prokaryotes to the high temperatures and fluid emissions and consequently have a low impact on prokaryote abundance and biodiversity. At the same time, we cannot exclude that a low viral abundance is related with lysogenic rather than lytic infections, as lysogeny could be an important survival mechanism in harsh environments [59]. Further studies are therefore needed to investigate the potential impact of lysogeny on prokaryote abundance and diversity in shallow hydrothermal vents. Moreover, although the dynamics of vent associated organisms are related with geothermal activity, we cannot exclude that viral abundance can change with time.

Overall, the results of the present study open new perspectives for the investigation of these poorly studied systems.

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