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Ferrous iron- and ammonium-rich diffuse vents support habitat-specific communities in a shallow hydrothermal field off the Basiluzzo Islet (Aeolian Volcanic Archipelago).

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25 microbial community, chemosynthesis

ABSTRACT

Ammonium- and Fe(II)-rich fluid flows, known from deep-sea hydrothermal systems, have
30 been extensively studied in the last decades and are considered as sites with high
microbial diversity and activity. Their shallow-submarine counterparts, despite their easier
accessibility, have so far been under-investigated, and as a consequence, much less is
known about microbial communities inhabiting these ecosystems. A field of shallow
expulsion of hydrothermal fluids has been discovered at depths of 170-400 meters off the
35 base of the Basiluzzo Islet (Aeolian Volcanic Archipelago, Southern Tyrrhenian Sea). This
area consists predominantly of both actively diffusing and inactive 1-3 metres-high
structures in the form of vertical pinnacles, steeples and mounds covered by a thick
orange to brown crust deposits hosting rich benthic fauna. Integrated morphological,
mineralogical and geochemical analyses revealed that, above all, these crusts are formed
40 by ferrihydrite-type Fe^{3+} oxyhydroxides. Two cruises in 2013 allowed us to monitor and
sampled this novel ecosystem, certainly interesting in terms of shallow water iron-rich site.
The main objective of this work was to characterize the composition of extant communities
of iron-microbial mats in relation to the environmental setting and the observed patterns of
macrofaunal colonization. We demonstrated that iron-rich deposits contain complex and
45 stratified microbial communities with a high proportion of prokaryotes akin to ammonium-
and iron-oxidizing chemoautotrophs, belonging to *Thaumarchaeota*, *Nitrospira* and
Zetaproteobacteria. Colonizers of iron-rich mounds, while composed of the common
macrobenthic grazers, predators, filter-feeders and tube-dwellers with no representatives
of vent endemic fauna, differed from the surrounding populations. Thus, it is very likely that
50 reduced electron donors (Fe^{2+} and NH_4^+) are important energy sources in supporting
primary production in microbial mats, which form a habitat-specific trophic base of the
whole Basiluzzo hydrothermal ecosystem, including macrobenthic fauna.

INTRODUCTION

55 During the past decades, the influence of environmental conditions in shaping composition and structures of microbial communities have been extensively studied. However, the question to what extent the energy availability determines the structure of autochthonous microbial communities has received less attention. On the land, sea surface and in shallow waters, solar power is the main energy source favoring light-
60 scavenging and CO₂-fixing photoautotrophs to become the primary producers. In contrast, there are ecosystems fuelled by chemical energy derived from inorganic redox reactions between electron donors (H₂, NH₄⁺, CO, CH₄, Fe²⁺, HS⁻ [or other reduced sulfur intermediates]) and acceptors (O₂, NO₃⁻, Fe³⁺, CO₂, SO₄²⁻ [or other oxidized sulfur intermediates]). Chemolithotrophic organisms are capable of fixing inorganic carbon using
65 all possible pairings of these compounds, and thus sustaining chemosynthetically-driven ecosystems (Berg *et al.*, 2010; Hügler & Sievert, 2011; Smedile *et al.*, 2014). It is well known that chemosynthetic ecosystems are widely distributed on our planet, spanning from terrestrial acid mines and hot springs to deep subsurface, sub-seafloor aquifers, cold seeps, deep-sea anoxic lakes and submarine hydrothermal systems. The last of these
70 habitats represents in many ways an excellent natural laboratory for the exploration of how energy landscapes shape communities of organisms thriving in these otherwise extreme environments. The best examples are deep-sea hydrothermal vent fields, initially found in the late 1970s (Corliss *et al.*, 1979). Currently, over 200 such hydrothermal fields have been found in various areas, mainly on mid-ocean ridges, arc volcanoes, back-arc basins
75 and hot-spot volcanoes (<http://www.interridge.org/irvents/>). In contrast to the deep-sea vents, situated in the deep and dark part of the ocean, the shallow ones are exposed to solar irradiation. Light availability permits an existence of complex ecosystems based on primary production activity of both photo- and chemolithoautotrophs and thus, shallow

vents represent unique intermediate sites, which can serve as suitable models for various studies including climate change, ocean warming and ocean acidification (Tarasov, 2006; Hirayama *et al.*, 2007; Manini *et al.*, 2008; Gugliandolo *et al.*, 2015). Nevertheless, it is surprising to note that despite their much easier accessibility and presumably ubiquitous distribution all over the world, shallow vents have been currently under-investigated, compared to their deep-sea counterparts.

The hydrogen and sulfide-diffusing submarine hydrothermal fields are typically inhabited by microorganisms capable of using these compounds as electron donors. This is consistent with the high energetic potential of hydrogen- and HS⁻-oxidation reactions under physicochemical conditions in these habitats. Another type of chemosynthetic microbial ecosystems occurs in ferrous iron-rich submarine vents and is based on Fe(II)-oxidation (Emerson and Moyer, 2002; Emerson *et al.*, 2007; Kato *et al.*, 2009a,b; Edwards *et al.*, 2011; Kato *et al.*, 2012). Before these studies, the biologically-mediated oxidation of ferrous to ferric iron in the ocean was greatly underestimated. This was mainly due to the fact that this conversion proceeds abiotically extremely rapidly in typically well-oxygenated and slightly alkaline marine environments. As calculated elsewhere (Hedrich *et al.*, 2011), at circumneutral pH values, the spontaneous (abiotic) rate of Fe²⁺ oxidation in an oxygen-saturated solution containing 100 mg L⁻¹ ferrous iron is 8.4 mg min⁻¹, while at pH 2.0 the corresponding rate is 10¹⁰ times slower (8.4 × 10⁻⁷ μg min⁻¹). Oxidation rates of the same hypothetical solution (pH 7.0, 100 mg L⁻¹ ferrous iron) are much slower under micro-aerophilic conditions. For example, at 6% of oxygen saturation (which corresponds to 0.5 mg L⁻¹) this process is almost 20-fold slower (0.47 mg min⁻¹) (Johnson *et al.*, 2012). Thus, exploitation of ferrous iron as an important energetic resource is much more efficient in acidic and oxygen-depleted environments. However, even under such favorable conditions, this low energy-yielding oxidative process can be used by autotrophic

prokaryotes only when ferrous iron is present in a great excess. It has been estimated, that
105 to fix one mole of CO₂, the chemolithoautotrophic acidophile *Acidithiobacillus ferrooxidans*
oxidizes about 70 moles of ferrous iron (Kelly, 1978). At circumneutral pH values, the
ferrous carbonate/ferric hydroxide couple redox potential is sufficiently low to allow other
compounds alternative to oxygen, such as nitrate or nitrite, to be used as electron
acceptors (Hedrich *et al.*, 2011). Additionally, iron oxidation at pH ~ 7.0 can also be
110 coupled to photosynthesis by anoxygenic phototrophic bacteria (Widdel *et al.*, 1993). This
implies that despite obvious energetic constraints at circumneutral pH, the dissimilatory
Fe(II) oxidation can be mediated in either micro-aerobic (oxygen-depleted) or anoxic
environments. Abiotic conversion of Fe(II) into Fe(III) is slow under these conditions, so
prokaryotes, thriving in such environmental niches, have opportunity to use this low
115 potential electron donor as an energy source. Indeed, marine iron(II)-oxidizing prokaryotes
significantly contribute to the global iron and carbon cycling in the ocean and especially in
submarine hydrothermal ecosystems, where a flux of Fe²⁺ was estimated at 3 × 10¹¹ mol
per year (Kato *et al.*, 2012).

Hosting numerous active vents and other hydrothermal formations, such as black
120 smokers (Manini *et al.*, 2008) and hot brine pools (Gugliandolo *et al.*, 2015), the shallow
hydrothermal vents located close to Panarea Island (Aeolian Volcanic Archipelago,
Southern Tyrrhenian Sea) is likely the most diverse active submarine hydrothermal system
within the Mediterranean Sea. Despite the fact that this area has been extensively studied
since the mid-1990s (Dando *et al.*, 1999, 2000; Sedwick and Stüben, 1996; Stüben *et al.*,
125 1996; Maugeri *et al.*, 2009), it remains not fully explored. One such novel finding occurred
while studying the high-gradient eastern slope of Basiluzzo Islet (NE of Panarea Island).
The vast area of hydrothermal massive Fe-oxyhydroxide deposits were found (Gamberi *et al.*
et al., 1997, 1998; Marani *et al.*, 1997). This area, located at depths ranging between 170

and 400 m, houses numerous, up to 2-3 m in height, diffuse ferruginous seeping structures
130 (DFS) in the form of vertical pinnacles, mounds and flanges, usually top-tapered. These
structures were very friable, composed of unconsolidated sedimentary material. DFS were
lacking an axial conduit, spares and beehive structure, that typify actively venting
chimneys (Tivey, 2007). Additionally, no bubbling or shimmering water was visible. Further
extensive geological studies suggested the ferruginous hydrothermal seeping origin of
135 these formations (Savelli *et al.*, 1999; Petersen *et al.*, 2011).

In this study, for the first time we investigated the prokaryotic and macrobenthic
communities inhabiting the Basilluzzo DFS structures, which were sampled in the twilight
zone at the depths of 175-220 m. We characterized the community structure of microbial
mats recovered from a few centimeters-thick orange to brown smooth sediments
140 consisting of Fe-encrusted and amorphous Fe-oxyhydroxide deposits. This material was
collected from two different iron-oxyhydroxide structures, situated at the depth of 175 m
and 211 m. Additionally, white-greyish suspended filamentous microbial streamers, grown
at diffuse CO₂-venting site (DCS) exterior of DFS site, were taken for comparative
biodiversity analysis. Coupling with geochemical factors we summarized the variation of
145 microbial communities and through comparisons we aimed at identifying some of the key
factors and processes that shape the microbial communities of iron (II) and ammonium-
rich diffuse flow areas at the base of the Basiluzzo Islet.

MATERIALS AND METHODS

150 **Bathymetric surveys and sediment sampling**

During two research cruises conducted in June and November 2013, the volcanic complex
was mapped using a EM2040 multibeam echosounder (Kongsberg Maritime AS, Norway),
including backscatter analysis, to provide detailed seabed information and to assist the

scenario of the remotely operated vehicle (ROV) surveys. High-resolution on-board on-line
155 magnetometry and gravimetry were also used to locate hydrothermally altered sediments
and rocks. The high-resolution multi-beam bathymetry revealed interesting geomorphic
features, including possible chimney fields, on the edges of slope failures and settled on
areas of relative lower positive magnetic anomaly, indicating possible shallow depth level
of hydrothermal alteration. Finally, conductivity, temperature, and depth (CTD) casts were
160 conducted at DFS and control site, to evaluate possible plumes and to measure the
following environmental variables: temperature, oxygen, pH, and salinity. A fine scale ROV
survey, covering the area mapped by the multibeam, was performed to classify and
characterize the hydrothermal structures. For these purposes, the *Pollux* ROV was
equipped with a high resolution camera (Nikon D80), full HD video camera (Sony HDR-
165 CX560V) to collect imagery of the seabed. The USBL (Ultra Short BaseLine) underwater
acoustic positioning system was applied to accurately record the ROV's position relative to
the multibeam map. The main hydrothermal structures were identified and mapped.
Recorded and processed images were further analyzed to identify the associated
macrobenthic assemblages.

170 Two pinnacle-like structures, DFS1 and DFS2, covered at the top by a thick iron microbial
mat (Fig. 1), were collected using *ad hoc* designed sampler equipped with 5 l plastic
scuffle box. The sampler chamber was flushed between each sampling of different mats.
Once retrieved, the Fe-rich mat material was sub-sampled for chemical, mineralogical and
biological analyses. One sample was taken from surface of a small (1.5 m high) diffuse
175 ferruginous structure (DFS1), located at a water depth of 175 m (38°40.331'N;
15°07.828'E). Two others samples, DFS2 (superficial part of iron microbial mat) and DFS3
(inner part of iron-rich deposits) were collected at the depth of 211 m from a 2 m high
vertical pinnacle possessing characteristic lateral flange (Fig. 1) (38°40.315'N;
15°07.846'E). Voltammetric measurements of oxygen and pH were performed immediately

180 after recovery of samples. pH was registered in pore water, after removal superficial parts
of collected material. For comparative reasons, we also collected the white-greyish
suspended filamentous microbial biofilms (streamers), observed at diffuse CO₂-venting
site DCS (38°38.232'N; 15°06.293'E). This was done at a depth of 26 m by SCUBA diving.
The DCS exhalative field is located between Bottaro and Dattilo islets (Fig. 1 and Fig. S1)
185 and characterized by occurrence of a vigorous gas and fluid emissions (Price *et al*, 2015).
Aliquots of the mat samples were collected in cryotubes for microscopic examination and
in 50 ml plastic vials for molecular analysis. Additionally, Fe-rich mat sub-samples (0.5 g
each) were directly inoculated into 120-ml serum bottles previously filled with minimal
medium for cultivation of iron-oxidizing chemolithotrophs (Emerson & Floyd, 2005). The
190 rest of samples were stored in sterile plastic bags at -20 °C for future analyses.

Scanning electron microscopy

Scanning electron microscopy – energy dispersive spectrometry (SEM-EDS)
measurements were collected by an environmental scanning electron microscope ESEM-
195 FEI Inspect-S coupled with a spectrometer Oxford INCA PentaFETx3 EDS, an Si (Li)
detector equipped by a ultra-thin window ATW2, by using a resolution of 137 eV at 5.9
keV. The spectral data were acquired under ESEM conditions at working distance of 10
mm with an acceleration voltage of 20 kV, counting times of 60 s, count for second
approximately 3000 cps with dead time below 30%. The results were processed by INCA
200 software Energy. This software uses the XPP matrix correction scheme developed by
Pouchou & Pichoir (1990).

X-ray powder diffraction

The X-ray powder diffraction analyses (XRPD) were performed using a Bruker D8
205 ADVANCE diffractometer with Cu K-alpha radiation on a Bragg-Brentano theta-theta
goniometer, equipped with a SiLi solid-state detector, Sol-X. Acquisition conditions are 40
kV and 40 mA. Scans were obtained typically from 2° to 80° 2θ, with step size of 0.02° 2θ,
with a count time of 1 second. Raw diffraction scans were stripped of ka2 component,
background corrected with a digital filter (or Fourier filter). Observed peak positions were
210 matched against the ICDD JCPDS database.

X-ray fluorescence spectrometry

The elemental composition was performed by X-ray fluorescence (XRF) spectrometry to
determine the bulk-sediment chemistry of the rocks in terms of major, minor and trace
215 elements by the method WDXRF with Bruker model S8 Tiger setup. The excitation source
was a tube of Rh at 4 kW. To avoid the detector saturation, power and current intensity
were changed according to the analyzed element and its quantity. The concentrations of
the major and minor elements have been calculated through the use of the software
package GEO-QUANT M. For the calculation of the trace elements, however, the software
220 GEO-QUANT T, a simple solution for the determination of these elements in geological
materials, was used. The latter is a pre-calibrated and standardized method by the
manufacturer, installed in the instrument present in the laboratory. This method was
validated using two standard samples GBW07103 and GBW07406.

225 DNA extraction, 16S rDNA PCR and clone library analysis and sequencing

Before proceeding with DNA isolation, the DFS and DCS samples were centrifuged (2500
rpm, 5 min) to eliminate excessive seawater. DNA was further extracted from
approximately 10 g of material using the UltraClean Mega Soil DNA Kit (Mo BIO, Carlsbad,

CA, USA), following the manufacturer's instructions. The quantity and purity of the nucleic acids were estimated using the NanoDrop® ND-1000 Spectrophotometer (Wilmington, DE, USA), the quality was evaluated by electrophoresis on agarose gel 0.8%. Following a decimal dilution of the purified DNA extracts, eubacterial 16S rRNA gene sequences were amplified by PCR using the universal bacterial primers Eub-8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and Eub-1492R (5'-GTT TAC CTT GTT ACG ACT T-3'). Archaeal 16S rRNA genes were amplified using specific primers archaea: Arch20F (5'-TTC CGG TTG ATC CYG CC RG-3') and Arch915R (5'-GTG CTC CCC CGC CAA TTC CT-3'). PCR was performed with a MasterCycler 5331 Gradient PCR (Eppendorf) using the following conditions: 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C; with a final extension of 10 min at 72°C. Amplicons were visualized on agarose gel (1%), specific bands were cut off and purified with the Wizard SV Gel and PCR Clean-up System kit (Promega, Madison, WI, USA). The purified products were cloned and sequenced as described in La Cono *et al.* (2013).

Quantitative real-time PCR (qPCR) was performed in triplicates using an ABI Prism 7300 real-time PCR system (Applied Biosystems) in a total volume of 25 µL, contained 12.5 µL of SYBR green mater mix, 200 nM of each primers, and 50 ng of template DNA. Volume of each reaction was adjusted to 25 µL by adding DNase-free ultrapure water. The primers and annealing conditions used through this analysis are listed somewhere (Swan *et al.*, 2010). Published assays for the quantification of the 16S rRNA gene copy numbers of Archaea (Takai and Horikoshi, 2000) and Bacteria (Nadkarni *et al.*, 2002) were applied. 16S rRNA gene copy numbers were converted to cell numbers using conversion factors of 1.5 for Archaea and 4.1 for Bacteria, as previously done (Schippers *et al.*, 2005).

Sequence analysis and phylogenetic tree construction

To check sequences for possible chimeric origin Pintail software (Ashelford *et al.*, 2005) was used. 16S rRNA gene sequences were identified with BLAST (Altschul *et al.*, 1997) aligned using the SILVA alignment tool and manually checked with ARB (Pruesse *et al.*, 2007). The phylogenetic tree based on distance analysis for 16S rRNA genes was generated using the neighbour-joining algorithm and Jukes-Cantor distance matrix of ARB program package. To assign a confidence level to the trees topology, 1000 bootstrap re-samplings were performed. As described elsewhere (La Cono *et al.*, 2013), cut-off of 97% of sequence identity was used for statistical analyses in each 16S rRNA gene library to define phylotypes or operational taxonomic units (OTUs) by means of software program Dotur (Schloss and Handelsman, 2005). PAST software version 2.17c (<http://folk.uio.no/ohammer/past>; Hammer *et al.*, 2001) was used to calculate diversity indices: rarefaction analysis, taxa, total clones, singletons, Shannon dominance, equitability, Simpson and chao2. Coverage values given as $C=1-(n_1 / N)$, where n_1 is the number of clones which occurred only once in the library of N clones (Good, 1953), were calculated to determine how efficient clone libraries described the complexity of original bacterial community. Principal Component Analysis (PCA), and Hierarchical Cluster Analysis (HCA), obtained by Primer 6 ecological software package developed by the Plymouth Marine Laboratory were used to analyze similarity between microbial populations obtained from the different sampling stations. PCA was applied on relative abundance matrix of the all prokaryotic OTUs, detected at different sampling points, HCA was obtained using Bray-Curtis similarity resemblance applied on the same matrix used for PCA.

Enumeration of macrobenthic colonists

The macrobenthic colonizers of DFS hydrothermal structures were collected during ROV surveys, fixed with ethanol (90% vol / vol), sorted and identified via microscopy.

280 Additionally, the active CO₂-seeping sites together with adjacent habitats with no apparent hydrothermal expulsion (black sulfide deposits and flat dark brown Fe-rich crusts) were sampled in nearby area (Fig. S1). To access the potential differences in the macrobenthic assemblages, a multivariate analysis of variance (PERMANOVA) was performed on species abundance. Data were transformed to presence/absence and analyzed on the
285 basis of a Sorensen similarity index using 4999 permutations. Pairwise comparisons were computed when significant differences ($p < 0.05$) among factor levels were detected. Additionally, non parametric multidimensional scaling (nMDS) and hierarchical cluster analysis were used to test the similarity of species composition of macrobenthic communities, associated with the different habitats.

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Nucleotide sequence accession numbers

The sequences representing the OTUs reported in this paper have been submitted and are available through GenBank under accession numbers KX524526-KX524601 for bacterial and KX524602-KX524673 for archaeal clones, respectively.

295

Results and Discussion

Geological setting of the Basiluzzo islet and features of the investigated hydrothermal field

The islet of Basiluzzo belongs to the Panarea volcanic complex (Aeolian Island
300 Archipelago, Tyrrhenian Sea, Central Mediterranean), which also includes Panarea Island and the surroundings islets (Fig. 1 and Fig. S1). The eruptive history is divided into six successive eruptive epochs with periods of volcanic activity separated by quiescence stages, the last of which contribute to the emplacement of the Basiluzzo endogenous dome (54 ± 8 ka) (Lucchi *et al.* 2003; Favalli *et al.*, 2005). Panarea and Basiluzzo islet, are
305 surrounded by an abrasion platform, similar in size to that of the other Aeolian Islands. The

large plateau is flanked by heavily dissected, steep slopes well evidenced by multibeam image shown in Figure 1. Gas emissions and hot springs making up the seafloor through cracks and fissures are currently detected in the area and documented by the submarine monitoring systems of the National Institute of Geophysics and Volcanology (INGV, Italy) (Favalli *et al.*, 2005; <http://www.ingv.it/panarea/panarea.html>). Data relative to fluid and gas chemical compositions connected with the activity of the involved hydrothermal system are available in databases of INGV monitoring systems (monsoon.pa.ingv.it) and in recent manuscript of Price *et al.* (2015).

The samples analyzed through this study were recovered from a zone of maximum density of ferruginous deposits, located in the North-Easternmost area of Basiluzzo islet, on the slope of the escarpment, at about 130 and 400 meters below sea level (mbsl) (Fig. 1). Several interesting sites were identified in the investigated area by ROV surveys during two research cruises conducted in June and November 2013. Analysis of these images allowed us to describe the various structural elements associated with the extensive hydrothermal field: brown and reddish mats and incrustations; whitish crust depositions; relict chimneys; field of vertical pinnacles (less than 3 m high) and mounds covered by thick bacterial mats (171 - 200 mbsl for BA11_RV220 and >200 mbsl for CC_P210 transects). Microbial mats were highly localized around vent orifices and were rust colored (Fig. 1). Despite the evident lack of visible gas and fluid emissions, the investigated ferruginous depositions were defined as diffusive ferruginous seeping structures (DFS). Two of such structures, named DFS1 and DFS2, located at different depths and characterized by similar morphological features, have been sampled. The maximum temperature, recorded by the temperature sensor directly above these structures, ranged from 18.3 to 21.8 °C. Considering that the ambient seawater temperature was significantly lower (13.8 to 14.1 °C), both DFS structures were ranked as hydrothermally active.

Collected by ROV material was treated as described in the “Materials and Methods” section with caution to avoid possible cross-contamination.

Structural, mineralogical and geochemical characterization of the DFS samples

335 The DFS fragments were analyzed through SEM-EDS to define micro-texture and mineral compositions. Additionally, XRPD and XRF analyses were respectively applied to identify mineral phases and to constrain the bulk chemistry in terms of major and trace elements. DFS samples showed reddish-orange to brown homogeneous appearance with porous-clotted texture and were very fragile (Fig. S2), whereas massive consistent iron-rich crusts
340 show red-orange to red-brown-grey coloration (Fig. 1). XRPD pattern and spot SEM-EDS analyses, demonstrate the dominance of amorphous ferric Fe-(hydrated)-oxyhydroxide deposits for both analyzed DFS1 and DFS2 samples (Fig. 2). Major and trace elements were analyzed by XRF and compared with sulfide- and Fe-(hydrated)-oxyhydroxides-rich mounds of Kolumbo vent complexes (Santorini, Greece) (Table 1). XRF bulk chemistry
345 data of Basiluzzo DFS samples suggested that they have a very similar major element geochemistry for Na, Cl, Si, Ca, K, Fe, and Mg, occurring mainly as halite, ferrihydrite/goethite and silicates indicating a common source in the reaction zone. Similarly to the distribution of major components, the trace element composition of DFS samples is rather uniform, with the exception of some transition metals V, Ni, Cu, Zn, Mo
350 and As exhibiting slightly variable values. No sulfide components such as pyrite, chalcopyrite or related phases were found in the original mineralogy composition data. Notable concentration of phosphorus ($16.43 \pm 1.57 \text{ g kg}^{-1}$), and Cr ($1.22 \pm 0.06 \text{ g kg}^{-1}$) was detected in DFS samples. In contrast to Kolumbo sulfide-sulfate-rich samples and to the majority of other seafloor sulfide deposits, DFS samples from Basiluzzo hydrothermal site
355 were significantly depleted in total sulfur ($0.18 \pm 0.04 \text{ g kg}^{-1}$), reflecting the notable lack of

sulfide- and sulfate-rich minerals. Combined Zn + Cu + Pb for DFS samples was less than 0.1 wt%, which is far lower compared to the most seafloor hydrothermal deposits (Hannington *et al.*, 2011). Such reduced quantity of these metals is typically detected in low-temperature hydrothermal iron-silica-rich vent fields (Li *et al.*, 2012; Kiliyas *et al.*, 2013).

360 Thus, as suggested for other submarine sulfide-depleted ferric iron-rich deposits (Boyd & Scott, 2001), mixing of oxygenated seawater and anoxic ferrous iron-rich hydrothermal flows results in the massive abiotic precipitation of amorphous iron-oxyhydroxides at the Basiluzzo vent site. This continuous and focused oxyhydroxide seafloor deposition formed slowly growing mounds and pinnacles, which we observed at
365 the Basiluzzo DFS area. Apparently, their slightly acidic and low-oxygenated environment hampers rapid abiotic oxidation of ferrous iron making Fe²⁺ a plausible energy source for iron-oxidizing prokaryotes. As revealed by electron microscopy of the DFS samples, amorphous iron-oxyhydroxides were coated by straight and branching filaments that formed complex networks (Fig. 2). As suggested elsewhere (Boyd & Scott, 2001), such
370 filamentous textures may have formed as a result of the interaction between the iron-oxidizing microorganisms and the initially precipitated ferrosic hydroxide that provided a source of ferrous iron needed for their growth. Additionally to filamentous textures, some observed patterns were remarkably similar to encrusted microbial structures like straight sticks, rods, cocci (Fig. 2), typically described from various hydrothermal vents (Edwards
375 *et al.*, 2003, 2011; Toner *et al.*, 2013; Peng *et al.*, 2010; Li *et al.*, 2012; Kiliyas *et al.*, 2013).

DFS prokaryotic communities are habitat-specific

The microbial community structures in Basiluzzo submarine hydrothermal samples (DFS1-3 and DCS) were investigated using culture-independent molecular methods based on

380 16S rRNA gene clone library construction and sequencing. After denoising and chimera removal, 265 bacterial and 288 archaeal clones were obtained by Sanger sequencing for all eight subsamples. The analyses of clone libraries confirmed the presence of highly diverse and specialized microbial communities that are spatially associated with different layers of iron-oxyhydroxide deposits of the Basiluzzo DFS mounds. Based on sequence
385 similarity (operational taxonomic units [OTUs] were defined at a 97% sequence identity threshold), a total of 75 bacterial and 60 archaeal OTUs were discerned in the samples. High coverage values (over 80%) of the bacterial and archaeal libraries were attained (Table S1), indicating that the majority of dominant prokaryotic members in all subsamples were recovered. The rarefaction curves for the bacterial and archaeal libraries did not
390 reach the plateau stage (Fig. S3) and Chao-1 richness estimates were greater (up to 2.8-fold) than the number of observed operational taxonomic units (OTUs) at a 97% sequence identity level, both indicating the presence of more rare prokaryotic species in the subsamples. As reflected by the high values of Simpson, Shannon and Chao-1 indices, the highest and lowest grades of diversity were correspondingly observed in archaeal
395 libraries DCSA and DFS1A. We found that bacterial communities exhibited more homogenous structure without an evident dominance of a singular OTUs (Table S1). To compare microbial community compositions between the iron-rich mat samples (DFS) and diffuse CO₂- and sulfide seeping sediments (DCS), we performed principal coordinate analysis (PCA). The PCA showed that the CO₂-seepage DCS prokaryotic community was
400 clearly distant from those of iron-rich DFS (Fig. 3), which was consistent with the geochemical differences between these hydrothermal sites (Price *et al.*, 2015). Remarkably, the prokaryotic community of oxygen-depleted DFS3 sample (internal part of iron-rich deposits core) differed from those of superficial DSF1 and DFS2 samples. This difference could reflect the influence of oxidizing/reducing conditions on structure of
405 microbial communities and their stratification.

Structure of eubacterial communities in the DFS and DCS samples

Bacterial 16S rRNA genes detected in the DFS and DCS samples were affiliated with 17 high-rank taxonomic groups: *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, *Zetaproteobacteria*,
410 *Planctomycetes*, *Acidobacteria*, *Deferribacteres*, *Chlorobi*, *Chloroflexi*, *Nitrospirae*,
Bacteroidetes, *Lentisphaerae*, *Armatimonadetes*, and TA06, WS3 and OP8 candidate divisions. Phylogenetic tree and detection frequencies of these major phyla and proteobacterial classes are shown in Fig. 4 and Fig. S4 and S5, respectively. Only representatives of *Gamma-*, *Deltaproteobacteria* and *Planctomycetes* were found in all
415 four eubacterial libraries. In accordance with the geochemical data, the lack of reduced sulfur intermediates in DFS samples significantly affected the occurrence of sulfur-oxidizing members of the *Epsilonproteobacteria*, typically predominant in the sulfide-rich hydrothermal ecosystems (Kato *et al.*, 2010, 2015; Sylvan *et al.*, 2012, 2013; Giovanelli *et al.*, 2013; Price *et al.*, 2015). Indeed, none of these organisms were found to be present
420 within all three DFS1-3 libraries. Noteworthy, the *Epsilonproteobacteria*-related clones were also lacking in DCS streamers library, indirectly indicating that this CO₂-venting site was likely impoverished by H₂S at the time of sampling, as was described by Capaccioni *et al.* (2007).

With regard to the Basiluzzo DFS mounds, they were covered by thick Fe-
425 oxyhydroxide-rich microbial mats, and in which we expected to find the signatures of iron-oxidizing microorganisms. These organisms are likely to play a pivotal role in the productivity of this ecosystem. As mentioned above, bacteria capable of the dissimilatory oxidation of iron at circum-neutral pH can be subdivided into three main physiological groups (Hedrich *et al.*, 2011): (i) aerobic/microaerophilic iron oxidizers; (ii) anaerobic
430 photosynthetic iron oxidizers and (iii) anaerobic/nitrate-dependent iron oxidizers. Within the

first group, most species identified so far in ferrous iron-rich submarine environments fall into class *Zetaproteobacteria*. The phylotypes belonging to this group were among the most abundant bacteria (up to 20% of the total clone numbers) in the DFS1 and DSF2 libraries. These neutrophilic Fe(II)-oxidizing chemoautotrophic microaerophiles are frequently detected in Fe-rich microbial mats at various deep-sea hydrothermal sites (Emerson *et al.*, 2007; Hodges & Olson, 2009; Sudek *et al.*, 2009; Forget *et al.*, 2010; Fleming *et al.*, 2013). However, to our knowledge, this study is the first direct indication of their presence in shallow hydrothermal fields within the Mediterranean Sea. Neither the inner core of iron-rich microbial mat (DSF3 library) nor the streamers from the CO₂-venting site (DCS library) contained *Zetaproteobacteria*. This observation could be explained either by anoxic conditions in the DFS3 sample or by lack of a suitable electron donor (Fe²⁺) at the CO₂-venting site DCS. We are aware that autotrophic growth by iron oxidation and biogenic Fe(III)-(hydrated)-oxyhydroxide formation is difficult to prove in iron-rich microbial mats, unless microorganisms are “caught in action” of oxidizing ferrous iron and fixing inorganic carbon into cellular biomass (Toner *et al.*, 2013). Consequently, we can only hypothesize that the *Rhodospirillales*-related clones detected only in superficial layer of mats (up to 15.4% of all analyzed DFS2 clones) are physiologically similar to known phototrophic iron-oxidizing purple bacteria of the class *Alphaproteobacteria* representing the second group of iron oxidizers. Thus, we do not exclude that together with *Zetaproteobacteria* they are involved in biogenic formation of iron oxyhydroxide.

Similarly to other iron-rich microbial mats and low-temperature hydrothermal iron-rich precipitates (Edwards *et al.*, 2003, 2011; Toner *et al.*, 2013; Li *et al.*, 2012), the most dominant phyla observed in DFS1 and DSF2 libraries (34% and 40%, respectively) were not related to obligate iron-oxidizing bacterial groups and were closely affiliated to various uncultured *Planctomycetes* lineages (Fig. 4 and Fig. S4). DFS3 library also contains

Planctomycetes-related clones (12%), 86% of which belong to uncultured bacteria, recovered from low-temperature diffuse vents at the South West Indian Ridge (GenBank accession number JN860338; Fig. S4). Although we are aware about restriction of physiological interpretation of 16S rRNA genes, it is noteworthy, that some representatives
460 of the phylum *Planctomycetes* are capable of chemoautotrophic growth fuelled by nitrate-dependent oxidation of ferrous iron and by anaerobic oxidation of ammonium with nitrite as electron acceptor (Kartal *et al.*, 2007; Oshiki *et al.*, 2013). To verify the potential availability of these sources of energy, we analyzed the presence of dissolved inorganic nitrogen (DIN) intermediates NH_4^+ , NO_2^- and NO_3^- in the pore water of the subsurface iron mat
465 sample DFS3. Seawater profiles collected at the depth of 100 meters (70-110 meters above DFS1 and DFS2) from the Basiluzzo hydrothermal field exhibited the DIN values, comparable to that of oligotrophic seawater ($50 - 900 \text{ nmol L}^{-1}$ for NO_3^- , $20-100 \text{ nmol L}^{-1}$ for NO_2^- and $60-300 \text{ nmol L}^{-1}$ for NH_4^+). The nitrite values in the pore water of DFS3 were within the seawater concentration range, while for nitrate and, especially, for ammonium
470 we recorded a sharp increase in their concentrations ($3.6 \mu\text{mol L}^{-1}$ for NO_3^- and $70.7 \pm 1.4 \mu\text{mol L}^{-1}$ for NH_4^+). Such 250-1000 fold increase in ammonium vs common seawater values indicated an injection of significant quantities of this important hydrothermal compound. As revealed by voltammetric measurements, the internal space of the iron-rich microbial mat DFS3 was significantly depleted in oxygen ($< 5 \mu\text{mol L}^{-1}$), thus representing
475 a set of suitable environmental conditions for the anaerobic processes.

Inter-comparison of the nitrogenous species profiles does not excluded an eventual upward gradual aerobic nitrification of NH_4^+ to NO_2^- and finally to NO_3^- . This process, termed nitrification, involves two separate steps: the initial oxidation of ammonia to nitrite followed by the oxidation of nitrite to nitrate. Typically, it requires the activity of two distinct
480 groups of organisms: ammonia-oxidizing microorganisms (AOMs; including bacteria and

archaea) and nitrite-oxidizing bacteria (NOB). The representatives of both groups were found on the surface of iron-rich DFS1 and DFS2 microbial mats. Besides ammonium-oxidizing archaea (see below), more than 16% of DFS1 clones were distantly related to marine nitrifying bacteria *Nitrosococcus* and *Nitrospira* (Fig S4 and S5).

485 It is generally accepted that community structures correspond well with their habitat types and the differences in the habitat conditions impose the changes in the abundance of phylotypes (Kato *et al.*, 2012, 2015; Toner *et al.*, 2013). As we mentioned above, according to the PCA, the abundant phylotypes in the DFS3 library were different from those of superficial layers of DFS1 and DFS2 of microbial iron hydroxide mats (Fig. 3).
490 Due to the reduced conditions in the anoxic inner space of iron oxyhydroxide mounds, the DFS3 bacterial community mainly consisted of organisms from three “anaerobic” taxa: *Deltaproteobacteria*, *Deferribacteres* and candidate phylum OP8. Their members, combined, accounted for two-third (23%, 23% and 20%, respectively) of all bacterial clones analyzed. As observed in Fig. S5, most of DFS3 *Deltaproteobacteria* clones (53.5%)
495 belonged to SEEP-SRB1 group. This group of sulfate-reducing bacteria was initially described as a bacterial partner of anaerobic methanotrophs of the ANME-2 clade (Schreiber *et al.*, 2010). Recently, SEEP-SRB1 members were found in deep-sea brine lakes of Red Sea and in the low temperature sulfide- and Fe-rich hydrothermal environments at the South West Indian Ridge (GenBank accession number JN860335).
500 The second most abundant group of DFS3 *Deltaproteobacteria* clones (20%) belonged to the family *Geobacteraceae*, a group comprising mesophilic strictly anaerobic chemolitho- or chemo-organotrophs, capable of utilizing Fe³⁺ as an electron acceptor (Röling, 2014). Noteworthy, the *Deltaproteobacteria* was also observed in both DFS1 and DFS2 libraries, however, this group was represented almost exclusively by mesophilic heterotrophic
505 aerobic myxobacteria of the family *Sorangineae* (Fig. S5). Members of *Deferribacteres*,

with nearly a quarter of all the DFS3 clones analyzed, represent a typical component of deep ocean and sub-seafloor bacterial communities (Garrity & Holt, 2001; Orcutt *et al.*, 2011). Some *Deferribacteres* members are able to live under highly reduced conditions in hydrothermal vent chimneys, enriched by both sulfide and iron, while displaying versatility for energy and utilizing either various organic compounds or H₂ as electron donors and S⁰, Mn(IV), nitrate or Fe(III), as electron acceptors (Alauzet & Jumas-Bilak, 2014; Takaki *et al.*, 2010). Elevated presence of the latter two interspecies in DFS3 could support activity and, thus eventual importance of *Deferribacteres* in nitrogen and iron cycling at the iron- and ammonium-rich Basiluzzo vents. As indicated above, the physiology of the recovered phlotypes cannot be determined from their phylogeny, but only inferred from the closest cultured species. Recent studies suggested a prevalent anaerobic/facultative mode of mesophilic heterotrophic metabolism within the *Aminicenantes* (candidate phylum OP8) (Rinke *et al.*, 2013; Farag *et al.*, 2015). Following these findings, a high abundance of the *Aminicenantes*, detected only in oxygen-depleted samples DFS3 (20%) and DCS (27%), corroborate with their environmental settings.

Structure of archaeal communities in the DFS and DCS samples

In contrast to some Fe-rich hydrothermal sediments of submarine volcanoes and ocean crusts, where repeated PCR attempts with universal archaeal primers yielded no product (Forget *et al.*, 2010), the archaeal 16S rRNA genes were detected in all the Basiluzzo samples analyzed. Moreover, the relative archaeal abundance as a function of total amount of 16S rRNA genes was estimated by qPCR. Archaeal abundance varied from 40.5% to 26.6% in DFS samples and encountered for one fifth (18.8%) of total recovered 16S rRNA genes in DCS sample (Fig. 4 and Table S2).

530 Phylogenetic analysis of archaeal clone libraries revealed that they were affiliated to
7 major phyla. Among all 52 detected OTUs, 34 OTUs were attributed to the Marine Group
II (MGII), the Marine Group III (MGIII), the Marine Benthic Group E (MBGE), The Deep-
Sea Hydrothermal Vent Euryarchaeotal Group 6 (*Woesearchaeota*) and the Marine
Hydrothermal Vent Group (MHVG). 16 OTUs were classified in the Marine Group I (MGI)
535 of *Thaumarchaeota*, while remaining two OTUs were affiliated to Miscellaneous
Crenarchaeotal Group 3 (C3). Phylogenetic tree and relative frequencies of these major
phyla and detected OTUs are shown in Fig. 4 and Fig. S6. The most abundant cluster of
observed species was present in all four samples and its members belonged to the MGI
group of phylum *Thaumarchaeota*. Basiluzzo OTUs were closely related to clones
540 previously retrieved from deep-sea environments including sediments, mud volcanoes,
submarine brine lakes / seawater interfaces, hydrothermal vents and Fe-rich mats. Of note
is that all currently cultivated representative members of the MGI are either aerobic or
microaerophilic chemolithoautotrophic ammonium-oxidising organisms (Stieglmeier *et al.*,
2014). In accordance with these metabolic constraints, thaumarchaeal assemblages
545 varied significantly among the analyzed Basiluzzo samples. Their fraction significantly
declined in presumptive anaerobic libraries (15% in DCS, 25% in DFS3) compared to their
obvious dominance in oxygenated samples (84 and 94% in DFS1 and DFS2 libraries,
respectively). This observation, taken together with the detection of a quantity of
Planctomycetes-related organisms in DFS1 and DFS2, strongly suggests that besides the
550 oxidation of Fe²⁺ and biogenic formation of Fe-oxyhydroxide, ammonium oxidation is likely
the second pivotal autotrophic process supporting the functioning of the Basilluzzo vent
ecosystem. Different heterotrophic micro- and macro-organisms, associated with DFS
primary producers, likely form unique metabolic network and contribute to the carbon, iron
and nitrogen cycling at whole Basiluzzo hydrothermal area.

555 According to the phylogenetic analysis, members of *Woesearchaeota* (formerly
named Deep-Sea Hydrothermal Vent Euryarchaeotal Group 6; Castelle *et al.* 2015) and
MHVG were the second and the third dominant groups, observed both in iron-rich
microbial mats and in CO₂-sulfide streamers. The MHVG Archaea were originally detected
at hydrothermal vent sites near Japan (clone pMC2A15; Takai and Horikoshi, 1999) and
560 were defined as a phylogenetic lineage after finding closely related phylotypes in cold
sediments in the Okhotsk Sea (Inagaki *et al.*, 2003). Since this genomically unexplored
lineage has so far only been detected in a few studies, its biogeographical distribution,
habitat and metabolic preferences remain poorly understood (Teske and Sørensen, 2008;
Spang *et al.*, 2013). Opposite to the MGI group of *Thaumarchaeota*, fraction of
565 *Woesearchaeota* significantly increased in presumptive anaerobic libraries (from 12% in
DFS2 to 54-59% of all DFS3 and DCS archaeal clones). As the MHVG, *Woesearchaeota*
was originally defined by Takai & Horikoshi (1999) as a hydrothermal vent lineage,
although currently most sequences available in SILVA database are widely distributed in
saline aquatic environments both marine or inland waters, mainly in microbial mats,
570 sediments, plankton and hydrothermal vents, followed by hot springs and freshwater, and
very few in soils (Ortiz-Alvarez and Casamayor, 2016). Single-cell genome re-construction
of *Woesearchaeota* members (Castelle *et al.*, 2015) showed their small genomes sizes
and limited metabolic capabilities. They lack important metabolic pathways, suggesting
that these archaea may have a symbiotic or parasitic lifestyle. In this study, we observed
575 relative abundances of *Woesearchaeota* positively correlated with bacterial phylogenetic
diversity. Thus, a larger than expected range of distinct archaeal populations is present in
Fe(II)- and ammonium-rich diffuse vents of Basiluzzo hydrothermal field. Which are the
mechanisms of interaction between archaea and the remaining planktonic organisms is
something that remains to be disclosed still.

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Habitat-specific macrobenthic DFS communities

Evidence that the Basiluzzo hydrothermal ecosystem represents a site of high biodiversity was confirmed by analysis of macrobenthic communities, associated with different types of active hydrothermal flows (CO₂ and sulfide emissions, diffuse ferrous vents) and with the sites of past hydrothermal evidences (inactive Fe-rich crusts and sulfide deposits). Although all sites were inhabited by common subset of grazers, filter-feeders and tube-dweller organisms, they were dominated by different species (Table S3). These benthic assemblages are characterized by exceptional population density, which is likely supported by the increased availability of organic matter due to elevated microbial productivity. Pair-wise comparisons and nMDS biplot showed that macrobenthic communities, apart from the DFS-associated assemblages, showed at least 40% of similarity, while Fe-rich crust and sulfide deposits sites exhibit 50% of similarity (Fig. 5). Among a total of 13 macrofaunal species, living on the DFS surfaces (Table S4), the most commonly found organisms were the amphipods *Stenothoe elachista* followed by the caprellids *Phtisica marina*. Of all sites studied at the Basiluzzo seafloor, rare carnivorous sponges *Asbestopluma hypogea* (family *Cladorhizidae*) were found only in the DFS-associated assemblages. Noteworthy, cladorhizid sponges are frequently detected in close proximity to deep vent systems, where they occur in greater number and diversity (Vacelet, 2006; Erickson et al. 2009). Possessing the capability to capture small crustaceans for their nutrition, these demosponges obviously do not belong to true vent-associated fauna, which directly benefits from vent effluent. Noteworthy, they are known to carry prokaryotic endosymbionts, likely involved in chitin hydrolysis (Vacelet et al. 1996; Vacelet 2006; Dupont et al., 2014).

Alltogether, we can conclude that the DFS-associated assemblages, while differed significantly from the others assemblages observed in the study area, are composed of a

subset of the standard macrobenthic organisms (grazers, filter-feeders, tube-dwellers, predators, etc.) with no representatives of vent endemic fauna. According to Tarasov *et al.* (2005), typical hydrothermal vent communities dominated by vent endemic species are found only at depth > 200 mbsl. These communities are usually sustained by the organic matter derived mainly from the energy-rich chemosynthetic transformation of sulfide and hydrogen, driven by endosymbiotic prokaryotes. Iron and ammonium oxidation are low energy-gaining processes and cannot support this form of direct mutualistic relationships between prokaryotes and higher organisms. Nonetheless, the benthic assemblages, associated with iron- and ammonium-rich diffuse vents, are likely flourishing on increased availability of organic matter due to elevated microbial productivity.

CONCLUSION

We conclude that Basiluzzo's unique geodynamic setting is balanced by mixing of oxygenated seawater and anoxic ferrous iron-rich hydrothermal flows which causes massive precipitation of amorphous Fe-oxyhydroxides. Despite the fact that this process is mainly abiotic, the slightly acidic and oxygen-impoverished microenvironment of Basiluzzo's Fe-oxyhydroxides mounds hampers rapid abiotic oxidation of ferrous iron and makes Fe²⁺ as plausible energy source for iron-oxidizing prokaryotes. Additionally to Fe²⁺, we found that Basiluzzo hydrothermal diffuse flows are enriched by ammonium, which likely support metabolic activities of ammonium-oxidizing chemolithotrophs. Samples from two Fe-oxyhydroxide mounds DFS1 and DFS2 are characterized by similar environmental settings. They both host very similar microbial communities, dominated by various chemolithotrophic prokaryotes, highly specialized on iron (II) and ammonium oxidation. In contrast to the recent studies on microbial diversity in Fe-oxidizing marine samples

630 (Hodges & Olson, 2009; Forget *et al.*, 2010; Kiliyas *et al.*, 2013), the simultaneous presence of neutrophilic Fe(II)-oxidizing *Zetaproteobacteria*, ammonium-oxidizing *Thaumarchaeota* and *Planctomycetes* was observed in Basiluzzo's Fe-oxyhydroxide-rich mounds, while no representatives of *Epsilonproteobacteria* were detected. Internal, oxygen-depleted layer of Fe-oxyhydroxides mounds harbor a different microbial population, which is dominated by
635 *Deltaproteobacteria* *Defferibacteres* and *Woesearchaeota*. We propose their eventual implication in dissimilatory Fe³⁺ reduction and thus, in recovery of iron intermediates, required for a full microbially-mediated iron cycle. Low similarity of many DFS1-3 clones to other published sequences suggests that new species and perhaps new families thrive in low-temperature Fe-oxyhydroxide-rich microbial mats of Basiluzzo hydrothermal field,
640 which makes it distinct among seafloor hydrothermal systems known anywhere in the world and characterized so far.

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905 **Table 1** Short description and average content in ppm (mg kg⁻¹) of major ions in iron-rich vent samples from the Basiluzzo hydrothermal field

Sample	DFS1	DFS2	DFS3	Kolombo Vent Complex ^a
Depth, mbsl	175	211		504
Description	FeO(OH) diffuse chimney			S ²⁻ -FeO(OH) mounds
pH (pore water)	5.71	5.66	5.80	5.0
Conductivity, mS/cm	33.3	35.3	33.2	Not available
Total carbon	4610	4470	3950	Not available
Total nitrogen	950	1010	740	Not available
Total phosphorus	17520	13330	18440	Not available
Total sulphur	220	150	160	Not available
Fe	314500	337800	391500	242000
FeO(OH) equivalent	500370	537440	622900	385020
Si	23970	26470	24860	25100
Al	320	280	360	1170
As	520	540	420	2910
Br	100	100	60	Not available
Ca	2770	2510	2770	Not available
Cl	31530	24410	21270	Not available
Cr	1050	1150	1300	Not available
Cu	23.5	22.7	25.0	1510
K	1790	1350	1820	Not available
Mg	3690	2700	3600	Not available
Mn	170	180	170	Not available
Mo	173	160	195	Not available

Na	21780	15710	21880	Not available
Ni	20	18	22	Not available
Pb	39	54	42	2880
V	408	290	300	Not available
Zn	30	20	20	2630

^a Data correspond to the site NA014-027 Champagne active mound-1 of Kolombo hydrothermal field (Kilias *et al.*, 2013)

Figure Legends

Fig. 1 Location of the Island Panarea and the Basiluzzo submarine hydrothermal field. High-resolution swath bathymetry map of the Basiluzzo Fe-rich hydrothermal field depicting the location of diffuse ferrous-seeping structures evidenced by black dots. DFS1 and DFS2 hydrothermal structures were identified and photographed by ROV surveys, than sampled and analyzed in the present study.

Fig. 2 SEM-SE micrographs of hydrothermal DFS2 precipitates with fragile morphology: A) external surface of DFS2 structure; B) internal structure of DFS sample with overview of amorphous ferrihydrite-type (characterized by XRPD) phase morphologies, including clustered microspheres and globular aggregates of various sizes (1-10 μm) grouped in straight, curved and branching filaments, which form intertwined structures. XRPD pattern of DFS2 precipitates showing peaks (in red) of Fe-oxyhydroxides (C).

Fig. 3 Comparison of Basiluzzo microbial community compositions by PCA. Each axis indicates the first and second principal coordinates (PC1 and PC2). The percentages in the axis labels represent the percentages of variation explained by the principal coordinates. Colors of the lines and figures indicate level of cluster similarity (%) as shown in the box.

Fig. 4 Overview of prokaryotic diversity and stratification of phylogenetic groups recovered from the different structures of the Basiluzzo Fe-rich hydrothermal field. Stratification and relative abundance of each phylogenetic group is shown as the percentage of all clones sequenced in appropriate libraries. The clones recovered from the DSF1, the DSF2, the DSF3 and the DCS are shown in yellow, red, green and black squares, respectively. Scale bar corresponds to 6% estimated difference in nucleotide sequence positions. The qPCR data of the relative abundance of Archaea are outlined in the small nested box.

Fig. 5 nMDS bi-plot depicting the similarity in the composition of macrobenthic assemblages associated with the different categories of hydrothermal activity identified in the study area of Basiluzzo Islet. Colors of the lines and figures indicate level of cluster similarity (%) as shown in the box.

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