Microbial biofilms associated with fluid chemistry and megafaunal colonization at post-eruptive deep-sea hydrothermal vents

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Abstract

At deep-sea hydrothermal vents, reduced, super-heated hydrothermal fluids mix with cold, oxygenated seawater. This creates temperature and chemical gradients that support chemosynthetic primary production and a biomass-rich community of invertebrates. In late 2005/early 2006 an eruption occurred on the East Pacific Rise at 9° 50'N, 104° 17'W. Direct observations of the post-eruptive diffuse-flow vents indicated that the earliest colonizers were microbial biofilms. Two cruises in 2006 and 2007 allowed us to monitor and sample the early steps of ecosystem recovery. The main objective of this work was to characterize the composition of microbial biofilms in relation to the temperature and chemistry of the hydrothermal fluids and the observed patterns of megafaunal colonization. The area selected for this study had local seafloor habitats of active diffuse flow (in-flow) interrupted by adjacent habitats with no apparent expulsion of hydrothermal fluids (no-flow). The in-flow habitats were characterized by higher temperatures (1.6 - 25.2 °C), and H₂S concentrations (up to 67.3 μM) than the no-flow habitats, and the microbial biofilms were dominated by chemosynthetic *Epsilonproteobacteria*. The no-flow habitats had much lower temperatures (1.2 - 5.2 °C), and H₂S concentrations (0.3 - 2.9 μM), and *Gammaproteobacteria* dominated the biofilms. Siboglinid tubeworms colonized only in-flow habitats, while they were absent at the no-flow areas, suggesting a correlation between siboglinid tubeworm colonization, active hydrothermal flow, and the composition of chemosynthetic microbial biofilms.

1. Introduction

Studies of diffuse-flow hydrothermal vents at 9° 50'N on the East Pacific Rise (hereafter referred to as EPR) following seafloor volcanic eruptions (in 1991 and 2006) have provided information about processes controlling the initial colonization by microbial biofilms and subsequent succession of vent metazoans (Shank et al., 1998; Govenar, 2012; Sievert and Vetriani, 2012). While, in general, metazoan colonization correlates with temperature and hydrothermal fluid chemistry (Shank et al., 1998; Luther et al., 2001; Luther et al., 2012), biological interactions may also play a role in colonization patterns (e.g. Mullineaux et al. 2003; Govenar et al., 2012). Many vent invertebrates rely on chemosynthetic microorganisms that convert the energy of the reduced chemical species dissolved in the hydrothermal fluids into biochemical energy (ATP) to fix carbon dioxide. Also, in other marine habitats, microbial cues of metazoan settlement are critically important. Dispersive larvae respond not only to the texture and composition of microorganisms in biofilms, but also to chemicals produced by microbial consortia or individual microbial species (reviewed by Underwood and Keough, 2001, Hadfield, 2011). In addition, metamorphosis from the free-swimming larval stage to the sessile adult stage can be initiated by a different composition of the biofilms (Tebben et al., 2011, Yang et al., 2013).

Hence, chemosynthetic microorganisms may provide useful cues that indicate habitat quality for metazoan colonizers. Indeed, biologists have long hypothesized that microbial biofilms at deep-sea hydrothermal vents may interact with vent larvae to induce settlement and metamorphosis (Van Dover, 2000; Adams et al., 2012). However, this hypothesis remains largely untested.

Direct observation of the colonization of diffuse-flow vents following a volcanic eruption on the EPR has shown that microbial biofilms are the pioneer colonizers of newly-formed vents (Lutz et al., 2001; Sievert and Vetriani, 2012). Further, small subunit rRNA gene surveys of vent microbial communities indicated that *Epsilonproteobacteria* were the dominant microorganisms associated with

the colonization of substrates, as well as the most abundant primary producers at diffuse-flow hydrothermal vents (Lopez-Garcia et al., 2003; Alain et al., 2004; Campbell et al., 2006; Sievert and Vetriani, 2012).

In December 2003, we initiated colonization studies at diffuse-flow vents located on the EPR to
80 examine the correlation between microbial and megafaunal colonizers. In late 2005/early 2006, a volcanic seafloor eruption buried many of the pre-existing benthic communities in fresh lava (Cowen et al., 2007). The eruption also created more than 20 nascent vent habitats, establishing a time-zero for subsequent biological colonization and community development. While previous studies have documented post-eruptive changes in the chemistry of diffuse-vent fluids (Shank et al , 1998; Von
85 Damm and Lilley, 2004), an integrated investigation that simultaneously examined the structure of early microbial colonists, megafaunal colonization and fluid chemistry in diffuse-flow vents had not been conducted.

Here we conducted time-series *in situ* experiments to examine patterns of microbial community structure and composition, fluid temperature and chemistry and megafaunal colonization at various
90 post-eruptive diffuse-flow vents on the EPR. The goal of this study was to examine for the first time co-located linkages between hydrothermal fluid chemistry, microbial biofilms and megafaunal colonization. Specifically, amongst newly-formed, post-eruptive vents, we aimed to: 1) characterize the composition of microbial colonizers; 2) correlate patterns of microbial colonization in response to different geochemical regimes; and 3) examine patterns of megafaunal colonization. Our findings
95 revealed marked differences in the composition of microbial biofilms and vent-endemic megafaunal colonization at hydrothermally active (in-flow) and inactive (no-flow) areas.

2.0 Materials and Methods

2.1 Site description and sampling strategy

100 To investigate patterns of microbial and megafaunal colonization at post-eruptive hydrothermal

vents in relationship to different thermal and geochemical regimes, we designed and deployed experimental colonization substrates that allow the coordination and integration of these data. The substrates, hereafter referred to as TAMS (Temporal Autonomous Multi-disciplinary Substrates), were designed to allow hydrothermal fluids to pass through the colonization surface. Each surface was constructed from a 4" x 4" polyethylene frame holding two layers of 1 mm wide polyester mesh to facilitate permeability and one 100 μ m layer in the middle to provide substrate for microbial colonization. Each TAMS was equipped with a VEMCO temperature data logger for continuous monitoring of the temperature throughout the deployment.

TAMS were deployed on the EPR at either visible diffuse-flow (position designated as in-flow) or onto basalt where there was no visible diffuse-flow or evidence of exposure to hydrothermal fluids (position designated as no-flow). For the purpose of the present study, eight TAMS were deployed in in-flow and four in no-flow positions (Table 1). Nearly all of the TAMS were deployed ~6 months after the eruption for 196-202 days, at a site named "Tamtown" (Marker 8/11, 9°50.09'N 104°17.46'W, 2503 m). In this study, we also included two TAMS that were deployed ~12 months after the eruption for 49 days, at a site named "Marker 19" (9°47.44'N 104°16.97'W, 2510 m). We found no substantive differences in the fluid chemistry, microbial composition, or megafaunal colonization between duration length, deployment time, or sites, so the analyses presented here focus on the differences between in-flow and no-flow habitats.

The sites were established during Alvin dives 4203, 4207 and 4282. TAMS were transported to the seafloor in filtered seawater to prevent contamination by surface-water microbial and metazoan populations and were recovered in individual temperature-insulated containers (bioboxes). Upon recovery, TAMS were immediately transferred into the shipboard 4°C cold room. TAMS were divided into quadrants (I, II, III, IV), inspected and documented using digital imaging. The TAMS were disassembled in either cold sterile seawater or preservative (depending on the method of recovery), and both sides of the mesh were carefully examined for megafaunal colonists, which were documented

using high-quality digital imaging at 10x magnification, physically removed from the mesh, measured (for body length), and frozen individually. After removal of all megafaunal colonists, the mesh was stored for microbial diversity analyses.

130 2.2 *In situ* fluid chemistry

Fluid chemistry measurements were conducted by voltammetry using both submersible-mounted and autonomous solid-state microelectrodes (Luther et al., 2008). Measurements of sulfide ($\text{H}_2\text{S}/\text{HS}^-$), oxygen (O_2) and temperature ($^{\circ}\text{C}$) on each TAMS were taken at deployment and recovery using microelectrodes operated by the *DSV Alvin* manipulator as previously described (Luther et al., 2008).

135 Detection limits (dl) are $0.2\ \mu\text{M}$ for H_2S and $3\ \mu\text{M}$ for O_2 . TAMS-associated VEMCO data loggers recorded *in situ* temperatures every 15 seconds.

Fluid chemistry measurements were performed using an *in-situ* electrochemical analyzer (AIS-ISEATM-III) operated manually by an operator within the *DSV Alvin*. Temperature and cyclic voltammetry (CV) electrochemical scans (7-10) were collected at the same point using a wand, which
140 encased 4 Au/Hg micro-electrodes (Luther et al., 2008) and a thermocouple. VEMCO temperature loggers were used instead of thermocouples as they are smaller, allowing for easier positioning of the electrodes. For a detailed description of the operation of the ISEA, see Luther et al. (2001, 2008) and Moore et al. (2009).

Gold amalgam micro-electrodes, as described in Brendel and Luther (1995) and Luther et al. (2008)
145 were used for all experiments. Electrodes were sanded, polished and then plated with Hg by reducing Hg(II) from a $0.1\ \text{N}\ \text{Hg}(\text{NO}_3)_2$ in a $0.05\ \text{N}\ \text{HNO}_3$ solution for 4 minutes at a potential of $-0.1\ \text{V}$, while purging with Ar. The mercury/gold amalgam interface was conditioned using a 90 s $-9\ \text{V}$ polarization procedure in a $1\ \text{N}\ \text{NaOH}$ solution. The solid state Ag/AgCl reference and Pt counter electrodes were made in the same fashion as the working electrodes, using a $500\ \mu\text{m}$ or greater diameter Ag or Pt wire
150 that extends from the tip of the electrode by at least 20 mm. A grounding wire was connected to the Al

shield braid of the reference electrode wire, which was used by the electrochemical analyzer to minimize electrical noise. Electrodes were standardized for O₂ and H₂S prior to deployment and checked after deployment as described in Luther et al. (2008). CV was used for all in situ measurements. CV scans were initially conditioned for 5 seconds at -0.9 V in order to remove any H₂S that may have deposited on the Hg-amalgam surface while it was inactive. CV experiments were then run by conditioning at -0.1 V for 2 s, then scanning from -0.1 V to -1.8 V to -0.1 V at 2 V s⁻¹ on a 100 nA or 1 μA current scale, taking ~10 s per voltammogram (electrode response time at this scan rate is ~2 s).

2.3 Enumeration of megafaunal colonists

Identification and enumeration of TAMS megafaunal colonists were conducted via microscopy. Siboglinid tubeworms too small for unambiguous morphological classification were identified using Restriction Fragment Length Polymorphism (RFLP) analyses and DNA sequencing of *mtCOI* as previously described in Shank et al. (1999) and Adams et al. (2010).

2.4 Genomic DNA extraction and PCR amplification

TAMS-colonizing biofilms were chosen for microbial diversity analyses based on the three criteria indicated in section 3.3. Subsections for DNA extraction were randomly selected from each of the chosen TAMS (marked with * in Table 1) and stored at -80 °C until processed. Total genomic DNA was extracted from subsections (1 x 1 cm) cut from each of the quadrants using the UltraClean Soil DNA extraction kit (MoBio, CA) according to the manufacturer's protocol. The composition of the microbial community on mesh subsections of different sizes (one 3 x 1 cm, one 1 x 1 cm and four 1 x 1 cm) was compared in a pilot experiment to assess possible biases associated with the mesh size. The test did not reveal statistically significant differences (data not shown), and 1 x 1 cm squares were used for the study. DNA purity was checked using a 1% agarose gel. The bacterial 16S rRNA gene was

amplified by polymerase chain reaction (PCR) using bacterial universal primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1517R (5'-ACGGCTACCTTGTTACGACTT-3').

2.5 Denaturing gradient gel electrophoresis (DGGE)

180 A preliminary assessment of the diversity of 15 biofilm samples developed on 12 TAMS was carried out by Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the bacterial 16S rRNA gene. The full-length 16S rRNA gene was obtained as described above, gel-purified and used as a template for nested PCR to amplify the V3 region using the primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') with a 5' GC clamp (5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGG-3') and reverse primer 519R (5'-GWATTACCGCGGCKGCTG-3'). DGGE analysis was performed as previously described (Giovannelli et al., 2013). Briefly, an aliquot of each PCR reaction (18 µl) was applied onto a 6% (wt/vol) polyacrylamide gel in 1x TAE (40 mM Tris, 20 mM acetate, 1 mM EDTA) with denaturant gradient from 40 to 60% (where 100% denaturant contains 7 M urea and 40% formamide).

190 Electrophoresis was performed at a constant voltage of 57 V for 16 h at 60 °C with a 10 min initial run at 200 V. After electrophoresis, gels were incubated for 15 min in ethidium bromide (0.5 mg/L), rinsed for 10 min in distilled water and photographed with a UV Foto Analyst imaging system (Fotodyne, Inc., Hartland, WI).

2.6 Construction of clonal libraries, RFLP analysis and sequencing

Libraries of 16S rRNA genes were constructed from six samples selected based on their microbial community DGGE profile, availability of temperature and chemistry data (Table 2), and biogeochemical regime and positioning (in-flow and no-flow). In brief, 16S rRNA amplicons were excised from agarose gels, purified and cloned into the pCR2-TOPO vector using the TOPO-TA

200 Cloning Kit (Invitrogen, Inc., Carlsbad, California) following the manufacturer's instructions. Clones

were grown in Luria Bertani media containing 100 µg/ml ampicillin overnight at 37°C. Recombinant plasmids were extracted using the QIAprep spin miniprep kit (Qiagen) as described in the manufacturer's instructions. A total of 500 randomly selected colonies were analyzed from the six libraries. 16S rRNA gene fragments were digested for 2 h at 37°C on a thermocycler using the tandem tetrameric restriction endonucleases *Hae*III and *Msp*I (Promega). A 3% (w/v) Metaphor agarose gel containing ethidium bromide was prepared using manufacturer's specifications (Lonza). The restriction products were run for 1.5 h at 75 V and 4°C on the Metaphor gel and visualized under UV light. Clones were grouped into operational taxonomic units (OTUs) based on their RFLP profiles, and sequences were obtained from representative clones of each OTU utilizing an automated DNA sequencer (3100 Avant Genetic Analyzer; Applied Biosystems).

2.7 Phylogenetic and statistical analyses

Sequences obtained from the six libraries were manually checked for quality and were aligned using ClustalW (Larkin et al., 2007) and SeaView (Galtier et al., 1996). The software Bellerophon (Huber et al., 2004) was used to identify chimeric sequences, which were removed from the dataset. Neighbor-Joining trees were constructed using the Jukes-Cantor correction, and tree topologies were tested using 500 bootstraps replications. To increase readability, each taxonomic cluster was collapsed and a separate tree was plotted using FigTree software v. 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Sequences of cultured bacteria with highest identity to the dataset were included as references in the alignment. Affiliation of the clones to a specific group was obtained by integrating blastn and EzTaxon results with phylogenetic analyses (Chun et al., 2007).

Statistical analyses were performed with the R-software (R-Cran project, <http://cran.r-project.org/>). DGGE profiles were analyzed with ImageJ (Schneider et al., 2012) recording the position of each DGGE band. The resulting presence/absence matrix was fed to R for the determination of the distance matrix based on Jaccard dissimilarity and used for cluster analysis with the average linkage method in

Vegan (Oksanen et al., 2012). Differences among in-flow and no-flow habitats were investigated using the two-tailed student t-test. The OTU table was imported into R to carry out cluster analysis using the Jaccard dissimilarity and the average linkage method in Vegan (Oksanen et al., 2012).

The sequences from this study are available through GenBank under accession numbers KP896578

230 - KP896688.

3. Results

3.1 Fluid temperature and chemistry

Temperature, oxygen and sulfide concentration were measured *in situ* on TAMS during deployment
235 and recovery using the solid-state microelectrodes and the temperature sensor. Additionally,
temperatures were recorded using VEMCO data loggers directly above the mesh of each TAMS for the
entire duration of the deployment. The results of the *in situ* measurements are reported in Table 2. The
temperatures measured with the manipulator-held sensor during the deployment and recovery of the
TAMS were within the range of those measured by the VEMCO data loggers, but never reached the
240 highest or lowest values measured by the VEMCO data loggers. The difference in recorded
temperatures may be due to the long-term spatial and temporal variability of fluid venting or to the
different positioning of the VEMCO data loggers (directly attached to the TAMS frame) and the
temperature sensor held by the *Alvin* manipulator directly above (>1 cm) the mesh. Only the
temperatures recorded by the VEMCO data loggers are reported in Table 2, and these were used for the
245 analyses. The maximum temperatures recorded by loggers ranged from 3.1 to 25.2°C, and the
minimum temperatures fluctuated around ambient seawater temperatures (1.2 to 2.6°C). The average,
maximum, and minimum recorded temperatures were significantly different between in-flow and no-
flow positioned TAMS (t-test, $p < 0.001$).

At the vents investigated in this study, *in situ* sulfide concentrations ranged between 0.3 and 67.3
250 μM , although some measurements were below detection limits. The maximum and average sulfide

concentration was statistically different among in-flow and no-flow habitats (t-test, $p < 0.001$), while the minimum recorded value did not vary significantly.

Oxygen concentrations ranged between 9.8 and 93.0 μM , with an average value of 43.7 μM for the in-flow TAMS and 61.8 μM for the TAMS positioned in no-flow areas. In several in-flow TAMS, the minimum oxygen concentrations were below the detection limit (3 μM). In contrast to sulfide, the maximum oxygen concentration did not vary significantly among in-flow and no-flow TAMS colonizers, while minimum and average values were statistically different (t-test, $p < 0.001$).

Average measured temperatures were strongly correlated with sulfide concentrations (Pearson product-moment correlation coefficient, $r = 0.867$, $p < 0.001$) and were inversely correlated with oxygen concentrations ($r = -0.57$, $p < 0.001$). As expected, oxygen and sulfide concentrations were inversely correlated ($r = -0.50$, $p < 0.001$).

3.2 Composition of TAMS-associated megafaunal species

Of all TAMS deployed at the seafloor, siboglinid tubeworms were found only in the in-flow TAMS. *Tevnia jerichonana* was identified on all in-flow TAMS and was the most abundant siboglinid tubeworm (Table 3). Among the other siboglinid tubeworms, as many as two individuals of *Riftia pachyptila* were identified on three in-flow TAMS, and one individual of *Oasisia alvinae* on TAMS 12.

In contrast, one larva of the mussel *Bathymodiolus thermophilus* was identified on both in-flow and no-flow TAMS.

3.3 DGGE-based analysis of TAMS-associated microbial biofilms

To obtain a general overview of the composition of the selected microbial biofilms, DGGE analysis was performed on several TAMS-associated biofilms (Fig. 1). To this end, biofilms were chosen based on the following criteria (Table 1): 1) presence or absence of foundation species (tubeworms); 2) in-flow or no-flow location; and 3) the chemical and temperature regimes during the deployment.

Overall, cluster analysis of the banding patterns of each samples revealed a shift in community between in-flow and no-flow TAMS. The only exception was TAMS 13, which clustered among the no-flow TAMS. In general, the cluster analysis revealed that different quadrants selected from the same TAMS clustered close to each other.

3.4 Diversity of TAMS-associated microbial biofilms

To obtain a detailed assessment of the composition of the different microbial biofilms, samples were selected based on their unique DGGE patterns, and 16S rRNA clone libraries were constructed. Two biofilms (TAMS 4 and TAMS 29) were chosen as representative no-flow habitats, while TAMS 20, TAMS 9, TAMS 12 and TAMS 16 were chosen as in-flow representatives due to their unique DGGE band profiles and their range of sulfide concentrations.

A total of 323 16S rRNA clones were screened. Following RFLP analysis, sequencing and chimera removal, the remaining 272 clones represented 112 unique sequences. *Proteobacteria*-related sequences dominated the libraries, accounting on average for 95.39% of the clones without significant differences at the phylum level between in-flow and no-flow samples. Within the *Proteobacteria*, sequences related to the *Epsilonproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria* and *Deltaproteobacteria* class were identified (Fig. 2).

Epsilonproteobacteria related sequences were ubiquitous in all investigated libraries, representing on average 90.6% of the sequences retrieved for in-flow positioned TAMS and 7.7% for the no-flow. The sequences were exclusively related to the *Campylobacterales* order (Fig. 2), and were prevalently represented by sequences related to members of the *Helicobacteraceae* family. Phylogenetic analysis of the *Epsilonproteobacteria*-related sequences revealed the presence of four distinct clusters (Fig. 3).

Cluster I (Fig. 3) is represented by sequences related to clones present in the database retrieved in different hydrothermal vent areas worldwide, including Lau Basin, the EPR and the Kermadec Arc (New Zealand). Some of the retrieved clones are related to hydrothermal vent gastropod epibionts or to

sequences associated with the tubes of siboglinid worms (Suzuki et al., 2005; Zbinden et al., 2014; clones Tams 16_6, Tams16_7, Tams9_33 and Tams12_9, respectively). The closest cultured relatives to this cluster are members of the *Campylobacter* and *Sulfurospirillum* genera, with sequence similarities between 88-90%.

305 The sequences in Clusters II, III and IV (Fig. 3) were related to members of the epsilonproteobacterial genus *Sulfurovum*, with similarities ranging from 88% to 98.3% (clone Tams29_1 and Tams20_20, respectively). Cluster II is composed of sequences related to environmental clones identified in hydrothermal vent areas with similarities between 92 and 95.5% to *Sulfurovum lithotrophicum* (AB091292, Inagaki, 2004). Cluster III is the largest cluster identified in the study (Fig. 310 3). Several clones in this cluster are related to sequences previously identified during survey of epibionts or ectosymbionts of macrofauna associated with hydrothermal-influenced environments (Watsuji et al., 2009; Ruehland and Dubilier, 2010; Zbinden et al., 2014). Cluster III also includes clone Tams16_5, which represents the largest OTU within the epsilonproteobacterial-related sequences (12 identical clones) with an average similarity of 94.4% to *S. lithotrophicum*. Cluster IV is entirely 315 comprised of OTUs related to environmental clones obtained from the East Pacific Rise hydrothermal vent segment (Lopez-Garcia et al., 2003; Sylvan et al., 2012), with the exception of clone Tams20_13 and Tams12_5, which are related to sequences from an Indian Ocean deep-sea hydrothermal vent (Goffredi et al., 2004) and the Okinawa Trough cold seeps (Watsuji et al., 2014), respectively. All the epsilonproteobacterial-related OTUs retrieved from no-flow positioned TAMS were related to the 320 genus *Sulfurovum* (Fig. 3). OTUs Tams29_6, Tams 4_18 (positioned within cluster III) and OTU Tams29_1 were related to environmental sequences retrieved from geothermally influenced environments, while OTUs Tams29_7 and Tams 4_10 positioned near Cluster IV were related to sequences from low-temperature methane seeps.

OTUs related to the *Gammaproteobacteria* class were identified in all no-flow positioned TAMS 325 and only in the in-flow TAMS 9 (Fig. 4). On average, *Gammaproteobacteria* represented 84.7% of the

retrieved sequences in no-flow TAMS, while only 8.5% in the in-flow TAMS 9. Most of the gammaproteobacterial sequences were related to the family *Thiotrichales*: the three most abundant OTUs (Tams29_2, Tams4_2 and Tams4_6), representing 73% of the *Gammaproteobacteria*-related sequences, were related to *Cocleimonas flavas*, a sulfur-oxidizing bacterium isolated from a sand snail (AB495251, Tanaka et al., 2011) and to *Leucotrix mucor* (Harold and Stanier, 1955), a colorless, filamentous, sulfur-oxidizing, sessile bacterium (Fig. 4; sequence similarity 89.6 - 92.6%). Sequences related to the class *Alphaproteobacteria* were only identified in no-flow positioned TAMS (Fig. 4), constituting only 0.74% of the total sequences, and clustered within the *Rhodobacterales* family (Fig. 4).

Deltaproteobacteria-related sequences were predominantly retrieved from in-flow positioned TAMS (Fig. 5). The sequences were related to the orders *Myxococcales* and *Desulfobacterales* and are primarily related to environmental clones obtained from deep-sea hydrothermally influenced sediments. The closest sequences in the database related to the *Deltaproteobacteria* include clones identified as epibionts of the deep-sea hydrothermal shrimp *Rimicaris exoculata* (97.3% similarity, Hügler et al., 2011), environmental clones from hypersaline microbial mats (89.20%, Harris et al., 2013) and from oceanic dead zones (Walsh et al., 2009). All these clones were classified as members of the *Desulfobulbaceae* family (Fig. 5).

Unidentified bacterial sequences accounted on average for 3.75% of the libraries and did not differ between in-flow and no-flow samples at the phylum level, while sequences related to the *Bacteroidetes* constituted the remaining 0.97% and were significantly more abundant in in-flow derived libraries (1.08 vs 0.74 %). All *Bacteroidetes*-related sequences were affiliated with the order *Flavobacteria* (Fig. 2). Phylogenetic analyses revealed no distinct clustering of the *Flavobacteria*-related sequences between in-flow and no-flow derived samples (Fig. 6). All sequences have environmental clones obtained from deep-sea hydrothermal vents as closest relatives in the database (Sylvan et al., 2012; Zwirgmaier et al., 2014), with the exception of clone Tams20_9, which was distantly related to a

Cythophaga sp. Sva1038 clone obtained from deep-sea cold sediments in the Svaldbarg (89.6% similarity; Fig. 6; Ravensschlag et al., 1999).

4. Discussion

Microbial biofilms are important for marine invertebrate recruitment in a variety of habitats. While early evidence suggested that it was the texture or the composition of micoorganisms that increased habitat suitability for settling larvae, additional work has demonstrated the importance of chemical signals produced by microbial consortia or individual species of bacteria that indicate a particular area as suitable for colonization (reviewed by Underwood and Keough, 2001, Hadfield, 2011).

Furthermore, different groups of bacteria can play an important role of triggering metamorphosis from free-swimming larval forms to adult sessile forms (*e.g.*, Tebben et al., 2011, Yang et al., 2013)..

In this study we conducted co-registered measurements of the composition of microbial biofilm communities, associated megafaunal colonization and fluid chemistry at post-eruptive deep-sea hydrothermal vents located on the EPR at 9° 50' N. Identification and enumeration of megafaunal colonists on TAMS revealed that siboglinid tubeworms, which are foundation species at deep-sea vents, were found only in the in-flow TAMS. *Tevnia jerichonana* was the most abundant siboglinid tubeworm (93 individuals), followed by *Riftia pachyptila* (five individuals) and *Oasisia alvinae* (one individual) along with unidentified siboglinid (Table 3). The prevalence of *T. jerichonana* at the early stages of colonization of post eruptive vents is consistent with previous observations: this polychaete was identified as the dominant colonist at diffuse-flow vents within one year of the 1991 eruption on the EPR (Shank et al., 1998). Furthermore, Shank et al. (1998) hypothesized that, since the early colonization of *T. jerichonana* coincided with high levels of hydrogen sulfide, *Tevnia* larvae might be more resistant to sulfide than *Riftia* larvae. However, Nees et al. (2009) tested this hypothesis following the 2005/2006 eruption on the EPR, by carrying out *in situ* electrochemical measurements and showed

375 that, although the median sulfide concentrations increased two- to four-fold from *Riftia*-dominated, pre-
eruptive vents in 2004-2005 to *Tevnia*-dominated, post-eruptive vents in 2006-2008, oxygen
concentrations also decreased to below detection over the same period. Therefore, based on the low
oxygen concentrations, Nees et al. (2009) hypothesized that *T. jerichonana* may also be adapted to
microaerobic conditions.

380 Our DGGE-based analysis of the TAMS-associated microbial biofilms showed that, with the
exception of TAMS 13 (in-flow), the in-flow biofilm communities were more similar to each other than
to the no-flow communities (Fig. 1). TAMS 13 was exposed to hydrothermal fluids whose temperature
was lower than that of the other in-flow habitats. The lower fluid temperature could, in part, explain the
similarity of the microbial community of TAMS 13 to that of the no-flow TAMS. The observation that
385 the temperature and sulfide concentrations at which the in-flow and no-flow TAMS were exposed were
significantly different (Table 2) implies that the vent thermal and redox regimes control the
composition of the biofilm communities. In line with the DGGE data, cluster analysis of the OTUs of
the microbial biofilms revealed that the in-flow biofilm communities were more similar to each other
than to the no-flow communities, and that *Epsilonproteobacteria* dominated the in-flow microbial
390 biofilms (Fig. 2).

Epsilonproteobacteria are very abundant at diffuse-flow hydrothermal vents, are discharged in
elevated numbers from cracks on the seafloor during eruptions and have been identified as early
colonizers of deep-sea vents (Moyer et al., 1995; Longnecker and Reysenbach, 2001; Huber et al.,
2002; Alain et al., 2004). Hence, the observed dominance of *Epsilonproteobacteria* in areas of active
395 hydrothermal venting is consistent with their adaptation to sulfidic habitats (Campbell et al., 2006). The
majority of the epsilonproteobacterial sequences retrieved from the in-flow TAMS were related to the
genus *Sulfurovum*, which appears to be ubiquitous in marine geothermal environments. The only two
formally described species within the genus *Sulfurovum*, *S. lithotrophicum* and *S. aggregans*, were
isolated from vents located in the Mid-Okinawa Trough and Mid-Indian Ridge, respectively (Inagaki,

400 2004; Mino et al., 2014). Recently, a new species, *S. riftiae*, has been isolated from the EPR (Vetriani and Giovannelli, personal communication). All *Sulfurovum* strains that have been cultured in the laboratory to date are chemolithoautotrophic mesophiles; however, while *S. lithotrophicum* and *S. riftiae* conserve energy by coupling the oxidation of reduced sulfur species to the reduction of nitrate, *S. aggregans* is a strict hydrogen oxidizer. The role of *Epsilonproteobacteria*-dominated
405 chemosynthetic biofilms in facilitating larval settlement at active vents has not been established, although in other marine habitats larvae have been shown to select settlement sites in response to microbial biofilms, which may provide cues about the quality of the environment (Hadfield and Paul, 2001). Considering that *Sulfurovum* spp. are chemosynthetic sulfide-oxidizers, it is possible that these bacteria could facilitate the settlement and development of siboglonid larvae by simultaneously
410 providing a source of fixed carbon and detoxifying sulfide to sulfate.

In contrast, *Gammaproteobacteria* related to the family *Thiotrichales*, which includes diverse bacteria often associated with geothermal and sulfur-rich habitats, dominated the no-flow TAMS (Fig. 2). The observed shift from *Epsilonproteobacteria*-dominated in-flow communities to a higher proportion of *Gammaproteobacteria* in the no-flow biofilms is in agreement with previous studies that
415 compared the microbial communities of active and inactive hydrothermal vents. These studies showed that the composition of the microbial communities changed dramatically between active and inactive vents, and that *Epsilonproteobacteria* were nearly absent at the inactive sites (Suzuki et al., 2005; Kato et al., 2010; Sylvan et al., 2012). Taken together, these findings suggest that *Epsilonproteobacteria* thrive in reducing habitats and that they rely on a continuous flux of reduced chemical species (*e.g.*,
420 hydrogen sulfide) used as energy sources for growth. Hence, *Epsilonproteobacteria* depend on active venting. The most abundant *gammaproteobacterial* OTUs detected in the no-flow TAMS were related to *Cocleimonas flavas* and *Leucothrix mucor*, two aerobic, sulfur-oxidizing bacteria of the family *Thiotrichales* (Harold and Stanier, 1955; Tanaka et al., 2011). *Leucothrix*-like filamentous bacteria were identified in an early morphological survey of natural sessile vent microbial communities that

425 colonized natural and artificial substrates in the vicinity of vents at the Galapagos Rift (Jannasch and
Wirsén, 1981). More recently, Guezennec et al. (1998) reported the presence of filamentous bacteria
similar to *Leucothrix* spp. on artificial substrates deployed at vents on the Mid-Atlantic Ridge. It is
possible that the obligate aerobic metabolism of *Leucothrix*-related filamentous bacteria prevented
them from colonizing TAMS deployed at the reducing in-flow vents, while these microorganisms
430 thrived on TAMS at the no-flow habitats, where oxygen is less limiting and sulfide is significantly
lower (Table 2). Further, *Leucothrix*-related phylotypes have been commonly detected as
ectosymbionts of non-sessile vent-endemic invertebrates, including the shrimp *Rimicaris exoculata*, the
crab *Kiwa hirsuta* and the gastropod *Crysomallon squamiferum*, as well as in shallow-water snails and
oligochaetes (Goffredi et al., 2004; Petersen et al., 2010; Ruehl and Dubilier, 2010). These studies
435 suggest that the association of *Leucothrix* spp. with motile hosts may be an adaptation to redox gradients
typically found in sulfidic habitats, allowing these bacteria to move between oxic-anoxic interfaces and
ensure access to oxygen (Polz et al., 2000).

At deep-sea hydrothermal vents, chemosynthetic biofilms mediate the transfer of geothermal
energy into biomass available for higher trophic levels. However, our current understanding of the role
440 of biofilms in facilitating larval settlement and of the successional transition from early microbial
colonists to invertebrate communities is extremely limited. While the data presented here provide a first
order correlation between microbial diversity, fluid chemistry and megafaunal colonization,
experiments aimed at identifying metabolites produced by natural biofilm communities and by
representative biofilm bacteria could help elucidate the molecular signals that influence larval
445 settlement and megafaunal colonization.

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Table 1. Summary of TAMS deployed during the study and presence/absence of colonization.

Quadrants (QI, QII, QIII, QIV) of TAMS colonized (+) or uncolonized (-) by siboglinid tubeworms.

470 TAMS quadrants selected for microbial diversity analyses (DGGE and/or clone libraries) are marked with a *.

Position	TAMS	QI	QII	QIII	QIV
in-flow	8	+	+	-	-*
in-flow	9	+	-*	+	+
in-flow	11	+	-*	0	+
in-flow	12	+	+	+	+
in-flow	13	-	+	-	-*
in-flow	16	+	+	+	+
in-flow	20	+	+	+	+
in-flow	21	+	+	+	+
no-flow	4	-	-	-	-*
no-flow	5	-*	-	-	-
no-flow	22	-	-	-*	-
no-flow	29	-	-*	-	-

475 **Table 2.** Summary of TAMS deployments for which temperature and fluid chemistry were measured.

Temperature was recorded using VEMCO probes over 221 hours on average (min 115, max 268 hours), while oxygen and sulfide concentrations were measured at deployment or recovery using the microelectrode manipulated by DSV *Alvin* or an autonomous data recorder. Values are reported as minimum – maximum (average standard deviation). bdl = below detection limit.

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Habitat	TAMS	Duration (days)	Recovery Dive	Temperature (°C)	Oxygen (µM)	Sulfide (µM)
in-flow	8	202	4299	1.9 – 22 (4.9±3.1)	52.1 – 93 (63.4±9.21)	bdl – 36.1 (21.1±9.4)
in-flow	9	202	4297	1.8 – 23.5 (4.9±2.9)	9.8 – 28.4 (20.6±5.57)	6.8 – 9.8 (8.2±1.3)
in-flow	12	197	4297	1.6 – 19.1 (5.1±2.3)	62.1 – 77.4 (69.2±5.2)	bdl – 3.4 (0.8±1.3)
in-flow	13	196	4298	1.6 – 13.1 (4.2±1.6)	NA	NA
in-flow	16	196	4297	1.8 – 24.2 (6.9±2.9)	bdl-35.8 (12.35±13.47)	2.7 – 18.5 (10.7±5.5)
in-flow	20	49	4304	2.6 – 25.2 (9.2±2.6)	bdl – 47.4 (21.5±17.7)	bdl – 67.3 (54.2±19.3)
no-flow	4	203	4300	1.8 – 5.2 (2.3±0.5)	43.6 – 65.7 (57.1±6.3)	0.3 – 1.1 (0.6±0.3)
no-flow	5	199	4300	1.2 – 3.1 (1.6±0.3)	38.4 – 57.2 (49.8±4.76)	0.7 – 1.4 (0.9±0.2)
no-flow	29	49	4304	1.6 – 4.5 (1.8±0.1)	51.5 – 92.9 (78.5±12.9)	2.1 – 2.9 (2.5±0.3)

Table 3. Presence of megafaunal colonists associated with microbial biofilms whose composition was assessed.

Species	TAMS					
	in-flow				no-flow	
	9	12	16	20	4	29
<i>Riftia pachyptila</i>	1	2	2	0	0	0
<i>Tevnia jerichonana</i>	15	19	50	9	0	0
<i>Oasisia alvinae</i>	0	1	0	0	0	0
unidentified vestimentiferans (total)	5	4	32	0	0	0
<i>Bathymodiolus thermophilus</i>	1	0	0	0	1	0

Figure Captions

Figure 1. Bacterial denaturing gradient gel electrophoresis, gel profile, and cluster analysis of the resulting diversity. Scale represents distance computed as Jaccard dissimilarity on the presence/absence matrix. TAMS number and quadrant are designated as T#Q#. No-flow TAMS are in boldface. QI-IV of TAMS 21 were pooled together.

Figure 2. Dendrogram showing the microbial community composition similarities obtained by sequencing the 16S rRNA of in-flow and no-flow (in boldface) TAMS. Scale represents distance computed as Jaccard dissimilarity on the abundance of each operational taxonomic unit (OTU) clustered at the order level. For each TAMS the microbial diversity at the order level is reported.

Figure 3. Phylogenetic tree showing the *Epsilonproteobacteria* 16S rRNA gene diversity. The tree was constructed using the neighbor-joining method from a similarity matrix based on the Jukes-Cantor distance model using 500 replicated bootstrap. Clones from no-flow TAMS are indicated in boldface.

Figure 4. Phylogenetic tree showing the *Alpha*- and *Gammaproteobacteria* 16S rRNA gene diversity. The tree was constructed using the neighbor-joining method from a similarity matrix based on the Jukes-Cantor distance model using 500 replicated bootstrap. Clones from no-flow TAMS are indicated in boldface.

Figure 5. Phylogenetic tree showing the *Deltaproteobacteria* 16S rRNA gene diversity. The tree was constructed using the neighbor-joining method from a similarity matrix based on the Jukes-Cantor distance model using 500 replicated bootstrap. Clones from no-flow TAMS are indicated in boldface.

Figure 6. Phylogenetic tree showing the *Bacteroidetes* 16S rRNA gene diversity. The tree was constructed using the neighbor-joining method from a similarity matrix based on the Jukes-Cantor distance model using 500 replicated bootstrap. Clones from no-flow TAMS are indicated in boldface.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6