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New insights into Siboglinidae microbiota - external tube contributes to an increment of the total microbial biomass

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Siboglinid worms were sampled from four mud volcanoes in the Gulf of Cádiz (El Cid MV, Bonjardim MV, Al Gacel MV and Anastasya MV). These invertebrates are characteristic to cold seeps and are known to host chemosynthetic endosymbionts in a dedicated trophosome organ. However, little is known about their tube as a potential niche for other chemosynthetic and non-chemosynthetic microorganisms. Analyses by scanning and transmission electron microscopy showed dense biofilms on the tube in Al Gacel MV and Anastasya MV specimens by prokaryotic cells. Methanotrophic bacteria were the most abundant forming these biofilms as further confirmed by 16S rRNA sequence analysis. Furthermore, elemental analyses with electron microscopy and EDX point to the progressive mineralization of the biofilm and the tube in absence of nutrients. Environmental bacterial and archaeal 16S rRNA sequence libraries revealed abundant microorganisms related to these siboglinid worms and variation in microbial communities among samples. We argue that these differences must be related to variance in seepage activity, as it is the main source of nutrients. Thus, the tube remarkably increases the microbial biomass related to the worms and needs to be incorporated as an important part of the worm's microbiota. Furthermore, empty tubes may still influence the composition of the active microbial community at those sites.

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New insights into Siboglinidae microbiota — external tube contributes to an increment of the total microbial biomass

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Abstract

Siboglinid worms were sampled from four mud volcanoes in the Gulf of Cádiz (El Cid MV, Bonjardim MV, Al Gacel MV, and Anastasya MV). These invertebrates are characteristic to cold seeps and are known to host chemosynthetic endosymbionts in a dedicated trophosome organ. However, little is known about their tube as a potential niche for other chemosynthetic and non-chemosynthetic microorganisms. Analyses by scanning and transmission electron microscopy showed dense biofilms on the tube in Al Gacel MV and Anastasya MV specimens by prokaryotic cells. Methanotrophic bacteria were the most abundant forming these biofilms as further confirmed by 16S rRNA sequence analysis. Furthermore, elemental analyses with electron microscopy and EDX point to the progressive mineralization of the biofilm and the tube in the absence of nutrients. Environmental bacterial and archaeal 16S rRNA sequence libraries revealed abundant microorganisms related to these siboglinid worms and variation in microbial communities among samples. We argue that these differences must be related to variance in seepage activity, as it is the main source of nutrients. Thus, the tube remarkably increases the microbial biomass related to the worms and needs to be incorporated as an important part of the worm's microbiota. Furthermore, empty tubes may still influence the composition of the active microbial community at those sites.

Introduction

Chemosynthetic fauna is widely distributed and often found in deep-sea areas of active fluid seepage where oxygen levels are normally low, such as in hydrothermal vents and cold seeps. However, they can also be found in other reduced environments, such as whale and wood falls (Stewart, Newton & Cavanaugh, 2005; Dubilier, Bergin & Lott, 2008; Lösekann et al., 2008; Roeselers & Newton. 2012; Levin et al., 2016). While the composition of the seepage fluids is variable, some bacteria and archaea have adapted to use some of the most abundant constituents as their energy and/or carbon source, i.e. methane and sulfur compounds. These chemosynthetic microorganisms produce organic compounds and act as primary producers supporting higher trophic levels at these habitats (Jannasch & Mottl, 1985; Jannasch, 1989).

Characteristic fauna found in these ecosystems include bivalves (within the Mytilidae, Vesicomyidae, Solemyidae, Thyasiridae and Lucinidae families; Duperron et al., 2007; 2013; Roeselers & Newton, 2012; Raggi et al., 2013), tubeworms (within the Alvinellidae and Siboglinidae families; Schulze & Halanych, 2003; Lösekann et al., 2008; Raggi et al., 2013), and protozoans like ciliates (Ott, Bright & Bulgheresi, 2004; Edgcomb et al., 2011), that are symbiotic with these chemolitoautotrophic bacteria. These bacteria provide their hosts with rich source of nutrients in a high methane and sulfur environment where they are protected inside the hosts.

Tube fossils of siboglinid worms from vent sites are dated from the Silurian period, ca. 430 Ma ago (Little et al., 1998; Hilário et al., 2011; Georgieva et al., 2015). Taxonomic groups of the Siboglinidae family are described as a fundamental part of the core chemosynthetic community in reduced environments (Hilário et al., 2011). Siboglinids are normally found in the oxic/anoxic interface, as the symbiotic microorganisms require oxygen as the electron acceptor to oxidize methane or sulfide. For instance, seep siboglinids are normally found with the anterior part of their chitin tube (Blackwell, Parker & Rudall, 1965) in contact with the water column, from where they acquire the oxygen, while the posterior part is inside the reduced sediment, from where they collect the nutrients for their endosymbionts (Dubilier, Bergin & Lott, 2008).

Adult siboglinids lack of gut and rely on their endosymbiotic bacteria for nutrition, which are located in bacteriocytes inside the highly vascularized trophosome organ (Bright & Giere, 2005; Southward, Schulze & Gardiner, 2005). Thiotrophic Gammaproteobacteria are the most common microorganisms found in siboglinid trophosomes (Petersen & Dubilier, 2009). However, methanotrophic symbionts in siboglinid species from methane vents have also been reported, i.e. *Siboglinum poseidoni* (Schmaljohann & Flügel, 1987; Rodrigues, Hilário & Cunha, 2013) and *Sclerolinum contortum* (Pimenov et al., 2000). To date, all methanotrophic symbionts identified are related to type I methanotrophs from the Gammaproteobacteria, while type II methanotrophs from the Alphaproteobacteria have not been found as symbionts in any marine invertebrate (Petersen & Dubilier, 2009).

While most studies are focused on the interaction between siboglinids and their endosymbionts, few studies have reported the presence of microorganisms colonizing the tube or considered these tubes as potential niches for other chemosynthetic and non-chemosynthetic microorganisms. Microbial communities have not only been described on the outside of the tubes of *Riftia pachyptila* (López-García, Gaill & Moreira, 2002) and *Lamellibrachia* sp. (Duperron et al., 2009); bacteria have also been found in the internal face of the tube (Duperron et al., 2009). Furthermore, Georgieva et al. in 2015 found bacterial biofilms inside the tube of *Alvinella* sp. worms (Alvinellidae family), acting as one more concentric layer of the multiple layers that constitute the tube of the worms. These extraneous microbial inner cores were proposed to be formed due to the colonization of the surface of the tube followed by its normal progressive mineralization.

In the present study, we elucidate the tube of siboglinid worms as a potential niche for microorganisms. This implies an increase in the microbiota of the worms as well as in the total microbial biomass of cold seep ecosystems. For this purpose, we examined different specimens of small siboglinids recovered from four mud volcanoes in the Gulf of Cádiz, i. e. El Cid MV, Bonjardim MV, Al Gacel MV and Anastasya MV. We used transmission electron microscopy (TEM) and scanning electron microscopy coupled to EDX (SEM-EDX) for the characterization of the tube and tissue of these worms, as well as Illumina next generation sequencing for the amplification of environmental 16s rRNA genes of the prokaryotes present in the specimens. Based on our findings, we attempt to characterize the endosymbionts of the sampled specimens, as well as to characterize the diversity of microbiota of the worms. Moreover, we consider the importance of the tube as a part of the total biomass of the siboglinids' microbiota, and how this microbial community may vary depending on the availability of nutrients.

Materials & Methods

Specimen collection

Field experiments were approved by the Spanish Ministry of Science, Innovation and Universities (project SUBVENT CGL2012-39524-C02 and project EXPLOSEA CTM2016-75947) and the Irish Marine Institute (project Deep-Links: Ecosystem services of deep-sea biotopes CE15012). To study the microbiota of small siboglinid worms, specimens were recovered from different mud volcanoes in the Gulf of Cádiz. El Cid MV, Bonjardim MV and Al Gacel MV were sampled during the 2014 Subvent-2 cruise (R/V Sarmiento de Gamboa), while the Anastasya MV was sampled during the 2015 Deep-Links cruise (R/V Celtic Explorer) (**Fig. 1**). From each mud volcano between 5 and 10 worms were fixed for transmission and scanning electron microscopy (TEM & SEM, respectively), and between 10 and 15 worms were stored in ethanol or kept at −80 °C for staining technics and DNA analysis.

Transmission electron microscopy (TEM)

Specimens from Al Galcel MV and Anastasya MV were fixed in 2.5% (w/v) glutaraldehyde. After washing several times with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), a dehydration series was performed (15%, 30%, 50%, 70%, 95% and 100% aqueous ethanol solution), followed by embedding the samples with Medium LR white resin (Plano, Wetzlar, Germany). Polymerization of the resin was at 60 °C during 24 h. A milling tool (TM 60, Fa. Reichert and Jung, Vienna, Austria) was used to make a truncated pyramid on the gelatin capsules. Furthermore, an ultramicrotome (Ultracut E, Reichert & Jung, Vienna, Austria) and glass knives were used for obtaining ultrathin sections of the sample. Ultrathin sections were 80 nm in thickness, mounted on 300 mesh specimen Grids (Plano), further stained with 4% (w/v) uranyl acetate (positive stain). The sections were inspected in a Jeol EM 1011 transmission electron microscope (Jeol, Eching, Germany).

Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) analysis

Specimens from El Cid MV, Al Gacel MV and Anastasya MV were fixed in 2.5 % (w/v) glutaraldehyde. After washing several times with phosphate-buffered saline, a dehydration series was performed (15%, 30%, 50%, 70, 80%, 90%, 95% and 100% aqueous solution), followed by hexamethyldisilazane (HMDS; Sigma-Aldrich, Germany) in order to avoid drying artefacts. Samples were mounted on SEM sample holders and sputtered with Au–Pd (13.9 nm for 120 s). They were further visualized in a SEM LEO 1530 Gemini (Zeiss, Oberkochen, Germany) combined with an INCA X-ACT EDX.

Fluorescent staining of chitin tubes

Specimens recovered from the Al Gacel MV were stained with calcofluor white (Sigma-Aldrich, Germany) to identify the chitin tube. Previous staining of the samples, they were fixed on a slide and embedded in paraffin followed by a graded ethanol series (100%, 90%, 70% and 50%). Afterwards, one drop of staining and one drop of KOH 10% were placed onto the slide with the sample. The samples were examined under UV filters with different excitation ranges (i. e. 365 nm, 395 – 440 nm and 450 – 490 nm) of a Zeiss Axioplan microscope (Oberkochen, Germany).

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DNA extraction and amplification of bacterial and archaeal 16S rRNA genes

Specimens from El Cid MV, Bonjardim MV, Al Gacel MV and Anastasya MV were used for this analysis. About 1 g of sample was first mashed with mortar and liquid nitrogen to fine powder. Total DNA was isolated with Power Soil DNA Extraction Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Bacterial amplicons of the V3 – V4 region were generated with the primer set S-D-Bact-0341-b-S-17 primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (see Klindworth et al., 2013). Likewise, archaeal amplicons of the V3 – V4 region were generated with the primer set Arch514Fa forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTGBCAGCCGCCGCGGTAA-3') and the reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCCGCCAATTYCTTTAAG-3') (Wemheuer et al., 2019). The PCR reaction mixture for bacterial DNA amplification, with a total volume of 50 µl, contained 1 U Phusion high fidelity DNA polymerase (Biozym Scientific, Oldendorf, Germany), 5% DMSO, 0.2 mM of each primer, 200 µM dNTP, 0.15 µl of 25 mM $MgCl_2$, and 25 ng of isolated DNA. Furthermore, PCR protocol for bacterial DNA amplification was: initial denaturation for 1 min at 98 °C, 25 cycles of 45 s at 98 °C, 45 s at 60 °C, and 30 s at 72 °C, and a final extension at 72 °C for 5 min.

The PCR reaction mixture for archaeal DNA amplification was similarly prepared but containing 1 µl of 25 mM $MgCl_2$ and 50 ng of isolated DNA. PCR protocol for archaeal DNA amplification was: initial denaturation for 1 min at 98 °C, 10 cycles of 45 s at 98 °C, 45 s at 63 °C, and 30 s at 72 °C, 15 cycles of 45 s at 98 °C, 45 s at 53 °C, and 30 s at 72 °C, and a final extension at 72 °C for 5 min. PCR products were then checked by agarose gel electrophoresis (1.3 % agarose, 100 bp ladder) and purified using the GeneRead Size Selection Kit (QIAGEN GmbH, Hilden, Germany).

Bioinformatic processing of amplicons

Paired-end sequencing of the amplicons and further processing of the sequence data were performed in the Göttingen Genomics Laboratory (Göttingen, Germany). Paired-end sequences were merged, and sequences containing unresolved bases and reads shorter than 305 base pairs (bp) were removed using PANDAseq v2.11(Masella et al., 2012) employing the PEAR algorithm v0.9.8 (Zhang et al., 2013). Non-clipped reverse and forward primer sequences were removed by employing cutadapt v1.15 (Martin, 2011). QIIME 1.9.1 was used to process the amplicon sequences (Caporaso et al., 2010). The sequences were dereplicated and checked for chimeric sequences (de novo). Sequenced were clustered at 97 % sequence identity to operation taxonomic units (OTUs). The taxonomic classification of the OTU sequences was performed with QIIME 1.9.1 against the SILVA database 132 employing the assignment method mothur

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(Yilmaz et al., 2014) Extrinsic domain OTUs, chloroplasts, and unclassified OTUs were removed from the dataset. Sample comparisons were performed at the same surveying effort, utilizing the lowest number of sequences by random subsampling (30,563 reads for bacteria, 4,080 reads for archaea). The paired-end reads of the 16S rRNA gene sequencing were deposited in the National Center for Biotechnology Information (NCBI) in the Sequence Read Archive SRR8944123 with the accession number PRJNA533037.

Results

Samples and in situ variables' measurement

Worm samples were recovered from different mud volcanoes at sites where reduced sediment was observed. Exact location of the samples, as well as physical and chemical measured variables at those sites were collected from the remoted operated vehicle (ROV) sensors (CH_4 and CTD) and are shown in **Table 1**. El Cid MV and Bonjardim MV specimens were sampled from grey mounds (**Fig. 1, B–C**). El Cid MV sample was taken with a push-core and worms were in the first 5 cm of sediment. In the case of the Bonjardim MV sample, the sediment was recovered with a suction sampler and emanated a strong hydrogen sulfide smell. Siboglinids recovered from the Al Gacel MV were located in a pockmark, beneath an AOM-derived carbonate and facing an active bubbling seepage (**Fig. 1, D**; Rincón-Tomás et al., 2019). Furthermore, Anastasya MV worms were obtained from a field of *Beggiatoa*-like biofilms (**Fig. 1, E**). All specimens were around 100 µm width and no longer than 15 cm. They had a light-brownish color due to their tubes. No morphological identification of the worms could be made, presuming they are *Siboglinum* sp. or *Sclerolinum* sp. worms due to their size and external appearance.

Endosymbionts imaging

Worm tissues were only observed in Al Gacel MV (see supplementary data **Figure S1**) and Anastasya MV samples (**Fig. 2**). The other samples were empty tubes. SEM micrographs from one specimen of Anastasya MV revealed the posterior region of the worm (**Fig. 2, A**) — segmented opisthosoma is observed— and the trophosome (**Fig. 2, B**). A hole in the trophosome exposed abundant bacteria inside (**Fig. 2, C–D**). These bacteria were cocci, ca. 0.5 µm of diameter and had inner-membranes like the ones expressed by methanotrophic bacteria (**Fig. 2, D**).

Structure and composition of the tubes

The fluorescent stain calcofluor white is an indicator for polysaccharides such as chitin that is part of the organic matrix of siboglinids' tubes. Sections of empty tubes from the Al Gacel MV expressed high fluorescence when observed under UV-light with different absorption band filters (**Fig. 3**). Furthermore, an external biofilm de-attached from the tube (probably due to handling of the sample) was slightly fluorescent (**Fig. 3, E**).

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SEM micrographs revealed transversal-segmented tubes which were covered by minerals (El Cid MV specimen, **Fig. 4, A**), a thick biofilm (Al Gacel MV specimen, **Fig. 4, B**) or putative remains of microbial extracellular polymeric substances or EPS (Anastasya MV specimen, **Fig. 4, C**). Disrupted tubes revealed their composition of multiple concentric layers 6 – 10 µm of thickness (**Fig. 4, D**). Some of the layers displayed a filamentous matrix, with attached globular particles of ca. 200 nm in diameter (**Fig. 4, E**). Layers consisting of these particles, show a silica signal in EDX analysis (see supplementary data **Figure S2**). Other layers contained significant amounts of iron, sulfur, and calcium, without notable differentiation between them. Detailed interpretation of EDX-analysis is discussed in supplementary data. Furthermore, bacteria were detected in the internal surface of the tube of the Al Gacel MV specimen (**Fig. 4, D**; **Fig. 5, G–H**). A model of the different layers observed in a tube in shown in **Fig. 4, F**.

Microbial biofilm of the tubes

TEM and SEM micrographs from the Al Gacel MV revealed a high microbial colonization of the outside surface of the tube (**Fig. 4, B; Fig. 5**). The biofilm was ca. 1 – 2 µm thick. Bacteria with intracytoplasmic membranes arranged as known for methanotrophic proteobacteria were the most abundant along the tube, forming densely packed bacteriocyte-like bodies (**Fig. 5, A–C**). Other microbial morphotypes were observed, i.e. prosthecate, rod shaped, helically shaped and filamentous bacteria (**Fig. 5, D–F**). Rod-shaped bacteria were also observed attached to the inside surface of the tube (**Fig. 5, G–H**) Furthermore, some microorganisms appeared to be actively penetrating the chitin tube (**Fig. 5, I**). Likewise, siboglinids from Anastasya MV under the TEM revealed a biofilm on the external tube-face. However, the biofilm appeared to be dead and in a degradation process, because cells appeared as “ghosts” (just cell walls, no cytosolic contents were visible; **Fig. 6**). Remains of EPS forming bacteriocyte-like bodies indicate abundance of methanotrophic-like bacteria, similar to the ones observed in Al Gacel MV specimens (**Fig. 6, A**). Remains of bacteria between single layers of the tube were also observed (**Fig. 6, B–D**).

Prokaryotic community composition

Bacterial and archaeal 16S rRNA gene libraries revealed relative abundances of taxa typically found thriving in the water column, such as Acidobacteria, Actinobacteria, Bateriodetes, Chloroflexi, Thermoplasmata, Woesearchaeia, and *Candidatus* Nitrosopumilus (**Fig. 7**). Sulfide-oxidizing bacteria are detected in all samples, with high representation in El Cid MV sample (**Fig. 7, C**), being mostly *Thiohalophilus* and bacteria from the Thiotrichaceae family (**Fig. 7, A**). *Sedimenticola* endosymbionts, which are also sulfide-oxidizing bacteria, are abundant in Al Gacel MV specimens, as well as Desulfobacterales sulfate-reducers. In fact, sulfate-reducers are highly representative (>15%) in Al Gacel MV and Anastasya MV samples, while in El Cid MV and Bonjardim MV represent 3% of the total relative abundance (**Fig. 7, C**). In Anastasya MV sample, Marine Methylotrophic group 2 (MMG-2) methanotrophic bacteria, and *Desulfobacter* sulfate-reducing bacteria are highly abundant (**Fig. 7, A**). Additionally, *Methylotenera* methylotrophic bacteria taxa (Kalyuzhnaya et al., 2006) are also representative in Al Gacel MV (7%). In Al Gacel MV and Anastasya MV up to 50 % of the bacteria are represented by methane-oxidizing, sulfide-oxidizing and sulfate-reducing bacteria (**Fig. 7, C**). Likewise, Chitinivibrionia (known chitin-degraders) were detected in all our samples, especially in Anastasya MV worms (**Fig. 7, A**).

The archaeal community profile was dominated by Woeserarchaeia (or DHVEG-6, Nanoarchaeota), followed by methane-oxidizing archaea (ANME-1 and ANME-2) as the second most abundant taxa — except in Anastasya MV, where methanogens are slightly more abundant (**Fig. 7, B & D**). ANME archaea are known to participate in the anaerobic oxidations of methane (AOM) together with sulfate-reducing bacteria (Boetius et al., 2000). Additionally, methanogenic archaea were homogeneous among the samples, except in the Al Gacel MV where they were almost absent (**Fig. 7, D**).

Discussion

Endosymbionts in Siboglinidae worms

Since the first time siboglinids were discovered in 1900s and described by Caullery in 1914 (Tobar-Hernández & Salazar-Vallejo, 2009), researchers have collected data on their life history characteristics and, in particular, adaptations allowing them to survive in reduced environments at high hydrogen sulfide concentrations and low oxygen (Petersen & Dubilier, 2009). To date, it has been established that these tube-dwelling annelids harbor chemolithoautotrophic endosymbionts in the super-vasculated trophosome (Bright & Giere, 2005; Southward, Schulze & Gardiner,2005). Those endosymbionts are facultative free-living bacteria which are acquired from the environment by the worms during their juvenile stage, at the same time as their guts are reduced (Cary et al., 1993; Di Meo et al., 2000). Once they become adults, they have established a permanent mutualistic microbe-animal symbiosis, with the host lacking gut and acquiring organic carbon solely from their endosymbionts (e. g. Nussbaumer, Fisher & Bright, 2006). This mechanism of obtaining endosymbionts horizontally from the environment has been described in other animals (Nussbaumer, Fisher & Bright, 2006 and references therein).

Siboglinidae worms mostly harbor thiotrophic bacteria in their trophosomes (Petersen & Dubilier, 2009; Hilário et al., 2011), and only some punctual specimens have been reported to harbor instead methanotrophic endosymbionts, i. e. *Siboglinum poseidoni* recovered from central Skagerrak (Schmaljohann & Flügel, 1987), and *Sclerolinum contortum* sampled at the Haakon Mosby MV (Pimenov et al., 2000) and the Gulf of Cádiz (Rodrigues, Hilário & Cunha, 2013). In the current study, endosymbionts from specimens collected in El Cid MV, Al Gacel MV and Anastasya MV were identified. Environmental bacterial 16s rRNA genes from El Cid MV sample presented an OTU with 99 % similarity to a thiotrophic endosymbiont of *Siboglinum* worms recovered from Gemini MV in the Gulf of Cádiz (OTU_0; see Rodrigues et al., 2011). Likewise, Al Gacel MV worms revealed high abundance of an OTU with 98 % similarity to *Sedimenticola* sp., a thiotrophic endosymbiont of *Sclerolinum contortum* (OTU_4; see Eichinger et al., 2014). Furthermore, we found evidence for methanotrophic bacteria inside of the trophosome (**Fig. 2, C–D**) of a small siboglinid from the Anastasya MV (attempted to be classified as *Siboglinum* sp., due to its lack of girdles between the trophosome and opisthosoma; **Fig. 2, A**; Southward, Schulze & Gardiner, 2005). Previous studies have reported *Siboglinum sp.* worms in this volcano (Rueda et al., 2012). The presence of methanotrophs inside the sampled individuals agrees with a previous report of *Siboglinum* sp. living in symbiosis with methanotrophic bacteria also in the Gulf of Cádiz, in this case in Captain Arutyunov MV (Rodrigues, Hilário & Cunha, 2013).

Environmental analysis of the bacterial 16s rRNA genes revealed that the most abundant methane-oxidizing bacteria in Anastasya MV specimens were related to Marine Methylotrophic Group 2 (MMG-2, Methylococcales; **Fig. 7, A**). MMG-2 bacteria have not previously been described as endosymbionts, but MMG-1 and MMG-3 (Ruff et al., 2013). However, since each new generation of siboglinids acquire their endosymbionts from the environment (Nussbaumer, Fisher & Bright, 2006), it is possible that MMG-2 bacteria have been adapted as endosymbionts in the *Siboglinum* sp. worms recovered from the Anastasya MV. Consequently, this study would be the first to attempt to include MMG-2 bacteria as potential endosymbionts of chemosynthetic invertebrates. This suggestion argues for the need to further study the role of MMG-2 bacteria related to siboglinids and other chemosynthetic invertebrates.

The tube as a new niche

To date, only few studies have reported microbial organisms related to siboglinid tubes (López-García, Gaill & Moreira, 2002; Duperron et al., 2009; Petersen et al., 2012). The tubes of all siboglinids have in common that they produce a chitinous matrix secreted by the worm that is incorporated in the tube (Blackwell, Parker & Rudall, 1965). Since they are in contact with water and reduced sediments, tubes are rich in minerals and other inorganic compounds which may vary depending on the environment (Duperron et al., 2014). Haas et al. in 2009, for instance, observed that the organic tubes were replaced by aragonite after the death of the worms, possibly due to the mineralization of bacterial communities colonizing the tube.

External and internal colonization of siboglinid tubes has previously been described (López-García, Gaill & Moreira, 2002; Duperron et al., 2009; Petersen et al., 2012) with high abundance of Epsilonproteobacteria (López-García, Gaill & Moreira, 2002; Georgieva et al., 2015). SEM and TEM micrographs showed highly colonized tubes in Al Gacel MV specimens (**Fig. 4, B**; **Fig. 5**). The biofilm was composed of mostly methanotrophic bacteria forming bacteriocyte-like bodies (**Fig. 5, A–C**), but also filamentous (**Fig. 5, D–E**), prosthecate- and spirillum-shaped (**Fig. 5, F**), and rod-shaped bacteria were observed (**Fig. 5, G–H**). Yet, bacteria penetrating the chitin tube were also detected (**Fig. 5, I**). Environmental 16s rRNA genes related to Al Gacel MV sample revealed the highly abundance of bacteria related to Methylococcales (mostly MMG-2), possibly forming the characteristic microbial biofilm. Rod-shaped bacteria could be related to *Methylotenera* sp. bacteria or sulfate-reducing Deltaproteobacteria (**Fig. 7, A**). Few reads were related to Hyphomonodaceae, prosthecate bacteria which could explain the morphotypes observed on the external biofilm of Al Gacel MV worm (**Fig. 5, F;** supplementary **Table S1**).

Furthermore, sequences related to Chitinivibrionia bacteria (chitin degraders) were also found in the sample, although only in minor amounts. The presence of these bacteria could explain the active penetration of the biofilm inside the tube (**Fig. 5, I**).
Al Gacel MV specimens represent a good example of how the tubes of siboglinids provide a viable niche for microorganisms. In fact, microbial biofilms are known to be ecosystems themselves, capable of self-regulation in which all microorganisms are linked and provide each other with stable sources of nutrients and protection (e. g. Davey & O'Toole, 2000). Those microorganisms increment the impact of siboglinid worms in the ecosystem, since they constitute part of the worms' microbiota. However, the stability of these biofilms may be disrupted if the worms die or the main source of nutrients decrease, i. e. seepage decreases. This would lead to the decay of this chemosynthetic-based biofilm. For instance, Anastasya MV specimens have remains of biofilm on the surface of their tubes (**Fig. 4, C; Fig. 6**). The decay of the microbial biofilm implies a decrease on the consumption of certain compounds present in the environment, such as methane and sulfur compounds. Thus, the rapid mineralization of the biofilm is expected.
High amounts of iron, calcium and sulfur compounds were detected in all the tubes with EDX-analysis (supplementary **Figure S2**), indicating the precipitation of minerals such as pyrite and aragonite (Peckmann, Little & Reitner, 2005; Haas et al., 2009; Georgieva et al., 2015). The tubes of El Cid MV specimens were externally covered by those minerals (**Fig. 4, A**).
Furthermore, the microbial mineralization is accompanied by the silicification of the chitin-tube (**Fig. 4, E;** Georgieva et al., 2015), which eventually mineralized due to the continuous exposure to the environment and more rapidly after the decay of a protective microbial biofilm. If the decay and consequent mineralization of the microbial biofilm occurs due to a decrease of nutrients — i. e. decrease of seepage activity — the tube of siboglinids would be re-colonized by new microorganisms once nutrients are available. Thus, a model has been proposed showing the composition of the different layers given in the tube of a siboglinid worm based on our results and other studies (**Fig, 4, F**; Peckmann, Little & Reitner, 2005; Haas et al., 2009; Georgieva et al., 2015). Additionally, the high presence of sequences related to chitin-degraders in Anastasya MV samples (**Fig. 7, A**) could explain the active participation of the biofilm on the decay of tubes once the worm dies. Consequently, it is important to consider the tube of siboglinids as an important niche, which increases the biomass and provides a large source of microorganisms which are part of the microbiota of the worms. Further studies focused on the life cycle of these biofilms, as well as their interaction and impact in the environment, are warranted.

The microbiota of small Siboglinidae worms

Siboglinidae worms do not only harbor microorganisms in their trophosome, but also on their tubes. Besides, rod-shaped bacteria were observed on Anastasya MV worm, as potential epibionts (supplementary **Figure S3**). The microorganisms associated with Siboglinidae worms conform the microbiota (or microflora) of these invertebrates. This microbiota is part of its host, and the metabolisms driven by these microorganisms contribute to the total ecological impact of the worm on the environment. Worm and microbiota constitute therefore a unique ecological unit, sometimes referred as holobiont (Margulis & Fester, 1991). Thus, in the same way the community of a mud volcano switches between chemosynthetic and non-chemosynthetic organisms depending on changes of the source of nutrients (i. e. seeped fluids *versus* organics from photic zone), we observed disparity in the microbiota of siboglinids sampled from different mud volcanoes and sites with different seepage activity (**Fig. 7**).

El Cid MV and Bonjardim MV specimens were recovered from sites where non-active emission of fluids was detected, and methane concentration was relatively low (70 – 90 nM and 50 – 65 nM, respectively; Sánchez-Guillamón et al., 2015) (**Fig. 8**). The site of El Cid MV from where worms were sampled, was surrounded by non-chemosynthetic fauna (shrimps, fish; **Fig. 8, A**), while Bonjardim MV sampling was performed in an area where patches of reduced sediment (biofilm-like) and dead bivalves were observed (**Fig. 8, B**). The sampled sediment with siboglinids from Bonjardim MV emanated a strong smell of hydrogen sulfide, potentially indicating the occurrence of anaerobic oxidation of methane (AOM) in the past. Likewise, the high relative abundance of sulfide oxidizers in El Cid MV samples may also indicate past AOM events (**Fig. 7, C**). In fact, DNA related to ANME in both inactive sites is detected (El Cid MV and Bonjardim MV; **Fig. 7, B**). Furthermore, sulfate-reducing bacteria are much less abundant in these samples when compared to known active sites (i. e. Al Gacel MV and Anastasya MV; **Fig. 7, A**).

On the other hand, the Al Gacel MV and Anastasya MV sampling sites showed bubbling of gas methane hydrates (**Fig. 8, C–D**) with methane concentrations as high as 191 nM at the time of *Sclerolinum* worm sampling (Sánchez-Guillamón et al., 2015). At both sites a thick biofilm covering the tube of mainly methanotrophic bacteria was detected — in Anastasya MV specimens only remains of the biofilm were observed — and environmental 16S rRNA genes revealed a higher presence of methane-oxidizing and sulfate-reducing microorganisms in these samples (**Fig. 7**). Since siboglinids normally colonize the oxic-anoxic interface in sites of fluids emission, they optimize at the same time the access to the seeped fluids for both aerobic methane-oxidizing bacteria and anaerobic sulfate-reducing bacteria, allowing them to co-exist in the same niche (the worm).

Interestingly, non-active (El Cid MV, Bonjardim MV) and active (Al Gacel MV, Anastasya MV) sites clear differ in the composition of their microbial communities. Therefore, this study represents a first insight into the microbiota of Siboglinidae tubeworms and demonstrates the high microbial diversity and variability among individuals located in nearby mud volcanoes. The seepage activity at these sites directly influence the composition of the microbial community (**Fig. 7**).

Conclusions

Small Siboglinidae worms recovered from four different mud volcanoes in the Gulf of Cádiz (El Cid MV, Bonjardim MV, Al Gacel MV and Anastasya MV) appeared to have a higher microbial biomass related to them than previously realized. In addition to the chemosynthetic endosymbionts harbored inside their trophosome, specimens from Al Gacel MV and Anastasya MV revealed that the tube was colonized by a thick microbial biofilm. We propose Marine Methylotrophic Group 2 as potential endosymbionts of Anastasya MV worms. Furthermore, the external biofilm of the tubes was mostly composed of bacteriocyte-like bodies of methanotrophic bacteria, but other morphotypes like filamentous, prosthecate, spirillum-like and rod-shaped bacteria were also observed. Comparison of environmental 16S rRNA gene libraries showed different microbial communities among samples, with marked differences between non-active and active sites. Since all sampled siboglinids had similar morphology, we assumed that these differences in the microbiota are due to changes in seepage activity at each sampling site, which ultimately influences the microbial community as seeped fluids are the main source of nutrients for microbial primary producers in these ecosystems.

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References

Blackwell J, Parker K, Rudall K. 1965. Chitin in pogonophore tubes. *Journal of the Marine Biological Association of the United Kingdom* 45, 659–661 DOI: 10.1017/S0025315400016489.

Boetius A, Ravenschlag K, Schubert CJ, Rickert D, Widdel F, Gieseke A, Amann R, Jørgensen BB, Witte U, Pfannkuche, O. 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* 407, 623–626, DOI: 10.1038/35036572.

Bright M, Giere O. 2005. Microbial symbiosis in Annelida. *Symbiosis* 38, 1–45.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, González Peña A, Goodrich JK, Gordon JI, Huttlex GA, Kellex ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature methods* 7(5), 335–336 DOI: 10.1038/nmeth.f.303.

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Cary SC, Warren W, Anderson E, Giovannoni S. 1993. Identification and localization of bacterial endosymbionts in hydrothermal vent taxa with symbiont-specific polymerase chain reaction amplification and in situ hybridization techniques. Molecular Marine Biology and Biotechnology 2, 51–62.

Davey ME, O'Tolle GA. 2000. Microbial biofilms: from Ecology to Molecular Genetics. *Microbiolology and Molecular Biology Reviews* 64(4), 847–867 DOI: 10.1128/MMBR.64.4.847-867.2000.

Di Meo CA, Wilbur AE, Holben WE, Feldman RA, Vrijenhoek RC, Cary SC. 2000. Genetic variation among endosymbionts of widely distributed vestimentiferan tubeworms. *Applied and Environmental Microbiology* 66, 651–658 DOI: 10.1128/AEM.66.2.651-658.2000.

Dubilier N, Bergin C, Lott C. 2008. Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology* 6(10), 725–740 DOI: 10.1038/nrmicro1992.

Duperron S, De Beer D, Zbinden M, Boetius A, Schipani V, Kahil N, Gaill F. 2009. Molecular characterization of bacteria associated with the trophosome and the tube of *Lamellibrachia* sp., a siboglinid annelid from cold seeps in the eastern Mediterranean. *FEMS Microbiology Ecology* 69(3), 395–409 DOI: 10.1111/j.1574-6941.2009.00724.x.

Duperron S, Gaudron SM, Lemaitre N, Bayon G. 2014. A microbiological and biogeochemical investigation of the cold seep tubeworm *Escarpia southwardae* (Annelida: Siboglinidae): Symbiosis and trace element composition of the tube. *Deep-Sea Research Part I: Oceanographic Research Papers* 90, 105–114 DOI: 10.1016/j.dsr.2014.05.006.

Duperron S, Gaudron SM, Rodrigues CF, Cunha MR, Decker C, Olu K. 2013. An overview of chemosynthetic symbioses in bivalves from the North Atlantic and Mediterranean Sea. *Biogeosciences* 10(5), 3241–3267 DOI: 10.5194/bg-10-3241-2013.

Duperron S, Sibuet M, MacGregor BJ, Kuypers MM, Fisher CR, Dubilier N. 2007. Diversity, relative abundance and metabolic potential of bacterial endosymbionts in three *Bathymodiolus* mussel species from cold seeps in the Gulf of Mexico. *Environmental Microbiolology* 9(6), 1423–1438 DOI: 10.1111/j.1462-2920.2007.01259.x.

Edgcomb VP, Leadbetter ER, Bourland W, Beaudoin D, Bernhard JM. 2011. Structured multiple endosymbiosis of bacteria and archaea in a ciliate from marine sulfidic sediments: a survival mechanism in low oxygen, sulfidic sediments? *Frontiers in Microbiology*, 2 (55), 1–16 DOI: 10.3389/fmicb.2011.00055.

Eichinger I, Schmitz-Esser S, Schmid M, Fisher CR., Bright M. 2014. Symbiont-driven sulfur crystal formation in a thiotrophic symbiosis from deep-sea hydrocarbon seeps. *Environmental Microbiology Reports* 6(4), 364–372 DOI: 10.1111/1758-2229.12149.

Georgieva MN, Little CTS, Ball AD, Glover AG. 2015. Mineralization of *Alvinella* polychaete tubes at hydrothermal vents. *Geobiology* 13(2), 152–169 DOI: 10.1111/gbi.12123.

Haas A, Little CTS, Sahling H, Bohrmann G, Himmler T, Peckmann J. 2009. Mineralization of vestimentiferan tubes at methane seeps on the Congo deep-sea fan. *Deep-Sea Research Part I: Oceanographic Reasearch Papers* 56, 283–293 DOI: 10.1016/j.dsr.2008.08.007.

Hilário A, Capa M, Dahlgren TG, Halanych KM, Little CTS, Thornhill DJ.,Verna C, Glover AG. 2011. New perspectives on the ecology and evolution of siboglinid tubeworms. *PLoS One* 6(2), e16309 DOI: 10.1371/journal.pone.0016309.

Jannasch HW. 1989. Chemosynthetically sustained ecosystems in the deep sea. In: Schlegel HG, Bowien B, eds. *Autotrophic bacteria*. Berlin, Heidelberg, New York, London, Paris, Tokyo: Springer Verlag, 147–166.

Jannasch HW, Mottl MJ. 1985. Geomicrobiology of deep-sea hydrothermal vents. *Science* 229, 717–725 DOI: 10.1126/science.229.4715.717.

Kalyuzhnaya MG, Bowerman S, Lara JC, Lidstrom ME, Chistoserdova L. 2006. *Methylotenera mobilis* gen. nov., sp. nov., an obligately methylamine-utilizing bacterium within the family Methylophilaceae. *International Journal of Systemic and Evolutionary Microbiology* 56(12), 2819–2823 DOI: 10.1099/ijs.0.64191-0.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, 41(1), e1 DOI: 10.1093/nar/gks808.

Levin LA, Baco AR, Bowden DA, Colaco A, Cordes EE, Cunha MR, Demopoulos AWJ, Gobin J, Grupe BM, Le J, Metaxas A, Netburn AN, Rouse GW, Thurber AR, Tunnicliffe , Van Dover CL, Vanreusel A, Watling L. 2016. Hydrothermal vents and methane seeps: rethinking the sphere of influence. *Frontiers in Marine. Sci*ence 3(72), 1–23, DOI: 10.3389/fmars.2016.00072.

Little, CTS, Herrington RJ, Maslennikov V, Zaykov V. 1998. The fossil record of hydrothermal vent communities. *Geological Society London Special Publications* 148(1), 259–270 DOI: 10.1144/GSL.SP.1998.148.01.14.

López-García P, Gaill F, Moreira D. 2002. Wide bacterial diversity associated with tubes of the vent worm *Riftia pachyptila*. Environmental Microbiology 4(4), 204–215 DOI: 10.1046/j.1462-2920.2002.00286.x.

Lösekann T, Robador A, Niemann H, Knittel K, Boetius A, Dubilier N. 2008. Endosymbioses between bacteria and deep-sea siboglinid tubeworms from an Arctic Cold Seep (Haakon

Mosby Mud Volcano, Barents Sea). *Environmental Microbiology* 10(12), 3237–3254 DOI: 10.1111/j.1462-2920.2008.01712.x.

Margulis L, Fester R. 1991. *Symbiosis as a source of evolutionary innovation*. MIT Press.

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. Journal* 17(1), 10–12 DOI: 10.14806/ej.17.1.200.

Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. 2012. PANDAseq: paired-end assembler for illumina sequences. *BMC bioinformatics* 13(31), 1–7 DOI: 10.1186/1471-2105-13-31.

Nussbaumer AD, Fisher CR, Bright M. 2006. Horizontal endosymbiont transmission in hydrothermal vent tubeworms. *Nature letters* 441, 345–348 DOI: 10.1038/nature04793.

Ott J, Bright M, Bulgheresi S. 2004. Marine microbial thiotrophic ectosymbioses. In: Gibson RN, Atkinson RJA, Gordon JDM, eds. *Oceanography and Marine Biology: An Annual Review*. Boca Raton, London, New York,Washington, D.C.: CRC Press, 95–118.

Peckmann J, Little CTS, Gill F, Reitner J. 2005. Worm tube fossils from the Hollard Mound hydrocarbon-seep deposit, Middle Devonian, Morocco: Palaeozoic seep-related vestimentiferans? *Palaeogeography Palaeoclimatology Palaeoecology* 227, 242–257 DOI: 10.1016/j.palaeo.2005.04.021.

Petersen JM, Dubilier N. 2009. Methanotrophic symbioses in marine invertebrates. *Environmental Microbiology Reports* 1(5), 319–335 DOI: 10.1111/j.1758-2229.2009.00081.x.

Petersen JM, Wentrup C, Verna C, Knitte, K, Dubilier N. 2012. Origins and evolutionary flexibility of chemosynthetic symbionts from deep-sea animals. *The Biological Bulletin* 223(1), 123–137 DOI: 10.1086/BBLv223n1p123.

Pimenov NV, Savvichev AS, Rusanov II, Lein AY, Ivanov MV. 2000. Microbiological processes of the carbon and sulfur cycles at cold methane seeps of the North Atlantic. *Microbiology* 69(6), 709–720 DOI: 10.1023/A:1026666527034.

Raggi L, Schubotz F, Hinrichs K-U, Dubilier N, Petersen JM. 2013. Bacterial symbionts of *Bathymodiolus* mussels and *Escarpia* tubeworms from Chapopote, an asphalt seep in the southern Gulf of Mexico. *Environmental Microbiology* 15(7), 1969–1987 DOI: 10.1111/1462-2920.12051.

Rincón-Tomás B, Duda J-P, Somoza L, González FJ, Schneider D, Medialdea T, Madureira P, Hoppert M, Reitner J. 2019. Cold-water corals and hydrocarbon-rich seepage in the Pompeia Province (Gulf of Cádiz) — living on the edge. *Biogeosciences* 16, 1607–1627 DOI: 10.5194/bg-16-1607-2019.

Rodrigues CF, Hilário A, Cunha MR, Weightman AJ, Webster G. 2011. Microbial diversity in Frenulata (Siboglinidae, Polychaeta) species from mud volcanoes in the Gulf of Cadiz (NE Atlantic). *Antonie Van Leeuwenhoek* 100(1), 83–98 DOI: 10.1007/s10482-011-9567-0.

Rodrigues CF, Hilário A, Cunha MR. 2013. Chemosymbiotic species from the Gulf of Cadiz (NE Atlantic): distribution, life styles and nutritional patterns. *Biogeosciences* 10, 2569–2581 DOI: 10.5194/bg-10-2569-2013.

Roeselers G, Newton IL. 2012. On the evolutionary ecology of symbioses between chemosynthetic bacteria and bivalves. *Applied Microbiology and Biotechnology* 94(1), 1–10 DOI: 10.1007/s00253-011-3819-9.

Rueda JL, González-García E, Marina P, Oporto T, Rittierott C, López-González N. 2012. Biodiversity and geodiversity in the mud volcano field of the Spanish margin (Gulf of Cádiz). In: Freitas MC, Cascalho J, Taborda R, Ribeiro M, Drago T, Andrade C, Fatela F, Cruces A, eds. *Actas do VII Simpósio sobre a Margem Ibérica Atlântica (MIA 2012), Extended abstracts*. Liboa: REPRO 2000, 137–141.

Ruff SE, Arnds J, Knittel K, Amann R, Wegener G, Ramette A, Boetius A. 2013. Microbial communities of deep-sea methane seeps at Hikurangi continental margin (New Zealand). *PLoS One* 8(9), e72627 DOI: 10.1371/journal.pone.0072627.

Sánchez-Guillamón O, García MC, Moya-Ruiz F, Vázquez JT, Palomino D, Fernández-Puga MC, Sierra A. 2015. A preliminary characterization of greenhouse gas (CH_4 and CO_2) emissions from Gulf of Cádiz mud volcanoes. In: Díaz-del-Río V, Bárcenas P, Fernández-Salas LM, López-González N, Palomino D, Rueda JL, Sánchez-Guillamón O, Vázquez JT, eds. *Volumen de Comunicaciones presentadas en el VIII Simposio sobre el Margen Ibérico Atlántico.* Málaga: Instituto Español de Oceanografía, 525–528.

Schmaljohann R, Flügel HJ. 1987. Methane-oxidizing bacteria in Pogonophora. *Sarsia* 72(1), 91–98 DOI: 10.1080/00364827.1987.10419707.

Schulze A, Halanych KM. 2003. Siboglinid evolution shaped by habitat preference and sulfide tolerance. *Hydrobiologia* 496(1-3), 199–205 DOI: 10.1023/A:1026192715095.

Southward EC, Schulze A, Gardiner SL. 2005. Pogonophora (Annelida): form and function. *Hydrobiologia* 535, 227–251 DOI: 10.1007/s10750-004-4401-6.

Stewart FJ, Newton IL. Cavanaugh CM. 2005. Chemosynthetic endosymbioses: adaptations to oxic–anoxic interfaces. *Trends in microbiology* 13(9), 439–448 DOI: 10.1016/j.tim.2005.07.007.

Tobar-Hernández MA, Salazar-Vallejo SI. 2009. Siboglinidae Caullery, 1914. In: León-González JA, Bastida-Zavala JR, Carrera-Parra LF, García-Garza ME, Peña-Rivera A, Salazar-Vallejo SI, Solís-Weiss V, eds. *Poliquetos (Annelida: Polychaeta)*. Monterrey: Universidad Autónoma de Nueva León, 555–567.

Wemheuer F, von Hoyningen-Huene AJE, Pohlner M, Degenhardt J, Engelen B, Daniel R, Wemheuer B. 2019. Primary Production in the Water Column as Major Structuring Element of the Biogeographical Distribution and Function of Archaea in Deep-Sea Sediments of the Central Pacific Ocean. *Archaea* 2019, 3717239 DOI: 10.1155/2019/3717239.

Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J, Ludwig W, Glockner FO. 2014. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acid Research* 42, 643–648 DOI: 10.1093/nar/gkt1209.

Zhang J, Kobert K, Flouri T, Stamatakis A. 2013. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* 30(5), 614–620 DOI: 10.1093/bioinformatics/btt593.

Table 1 (on next page)

Exact sampling sites and variables' measurement obtained from CH_4 and CTD sensors of the ROV

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1 **Table 1:**

2 **Exact sampling sites and variables' measurement obtained from CH_4 and CTD sensors of the ROV**

Mud Volcano	Coordinates	Depth (m)	T (°C)	O_2 (%)	CH_4	pH	ORP (mV)	Description
El Cid	35° 26.32' N -7° 29.03 W	1229	9.6	57	Yes	7.86	214	grey mound surrounded by non-chemosynthetic fauna
Bonjardim	35° 27.52' N -8° 59.99' W	3051	2.8	6.14	Yes	7.91	188	mud breccia with strong sulfidic smell and shells of dead bivalves
Al Gacel	35° 26.47' N -6° 58.27 W	791	10	54	Yes	7.88	149	bottom of AOM authigenic carbonate from pockmark with active bubbling
Anastasya	36º 31.32' N -7º 9.02 W	461	-	-	Yes	-	-	black mud underneath white sulfur-oxidizing bacterial mat with active bubbling

3

Figure 1

Location of the mud volcanoes sampled for this study in the Gulf of Cádiz and an overview of the sites where samples were recovered.

(A) General view of the Gulf of Cádiz. The mud volcanoes from where the samples were taken are pointed with an arrow. (B–E) ROV still frames from the different sampling sites. (B) El Cid MV. (C) Bonjardim MV. (D) Al Gacel MV. (E) Anastasya MV. Exact coordinates in **Table 1**.

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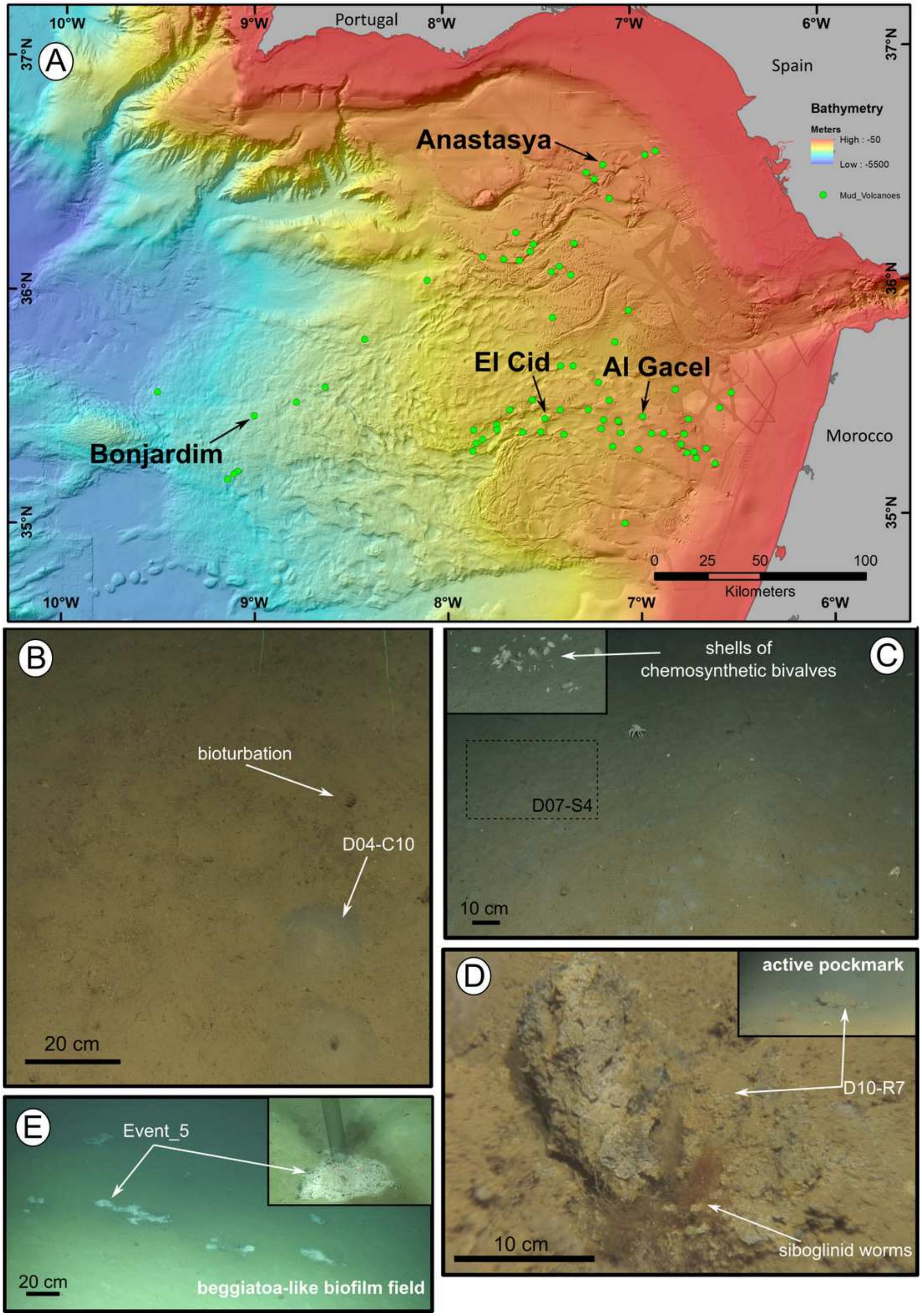


A
10°W
9°W
Portugal
8°W
7°W
6°W
37°N
36°N
35°N
Spain
Bathymetry
Meters
High : -50
Low : -5500
Mud_Volcanoes
Anastasya
El Cid
Al Gacel
Bonjardim
Morocco
0
25
50
100
Kilometers
B
bioturbation
D04-C10
20 cm
C
shells of
chemosynthetic bivalves
D07-S4
10 cm
D
active pockmark
D10-R7
10 cm
siboglinid worms
E
Event_5
20 cm
beggiatoa-like biofilm field

Figure 2

SEM micrographs of one specimen from Anastasya MV.

(A) General view of the sample. Posterior part of the worm is exposed and outside of the tube. (B) Trophosome. (C–D) Closer view to a hole in the trophosome where methanotrophic-like bacteria are observed.

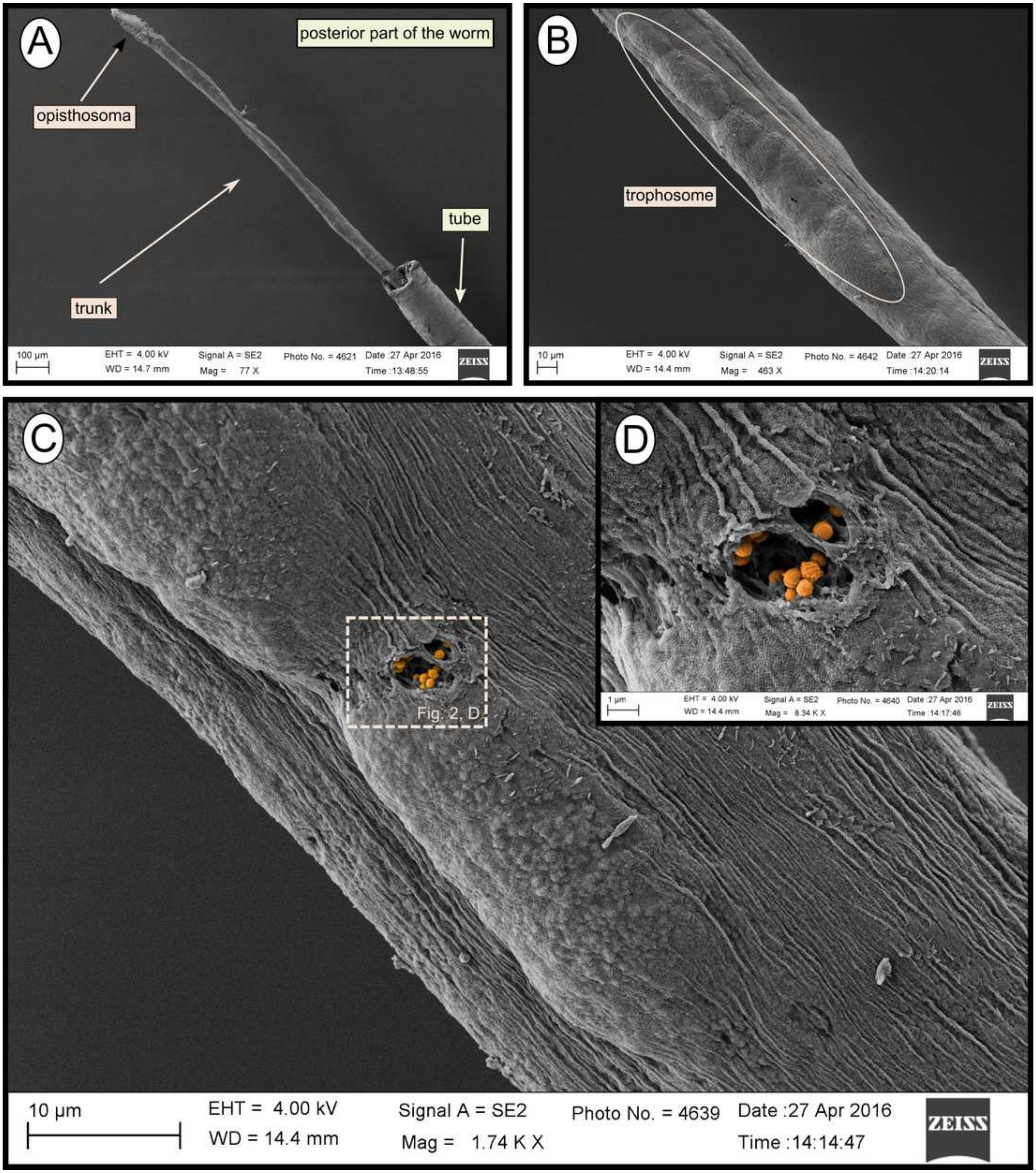

A
posterior part of the worm
opisthosoma
trunk
tube
B
trophosome
C
Fig. 2, D
D
10 µm
EHT = 4.00 kV
WD = 14.4 mm
Signal A = SE2
Mag = 1.74 K X
Photo No. = 4639
Date :27 Apr 2016
Time :14:14:47
ZEISS

Figure 3

Calcofluor white staining of empty tubes recovered from Al Gacel MV.

The fluorescence of the tube indicates the presence of chitin. (A–C) Fluorescence of the same tube section varies between filters. (D–E) Same section under normal light (D) and using Filter 09 (E). Notice the fluorescence of the tube and of the detached biofilm (marked with an arrow).

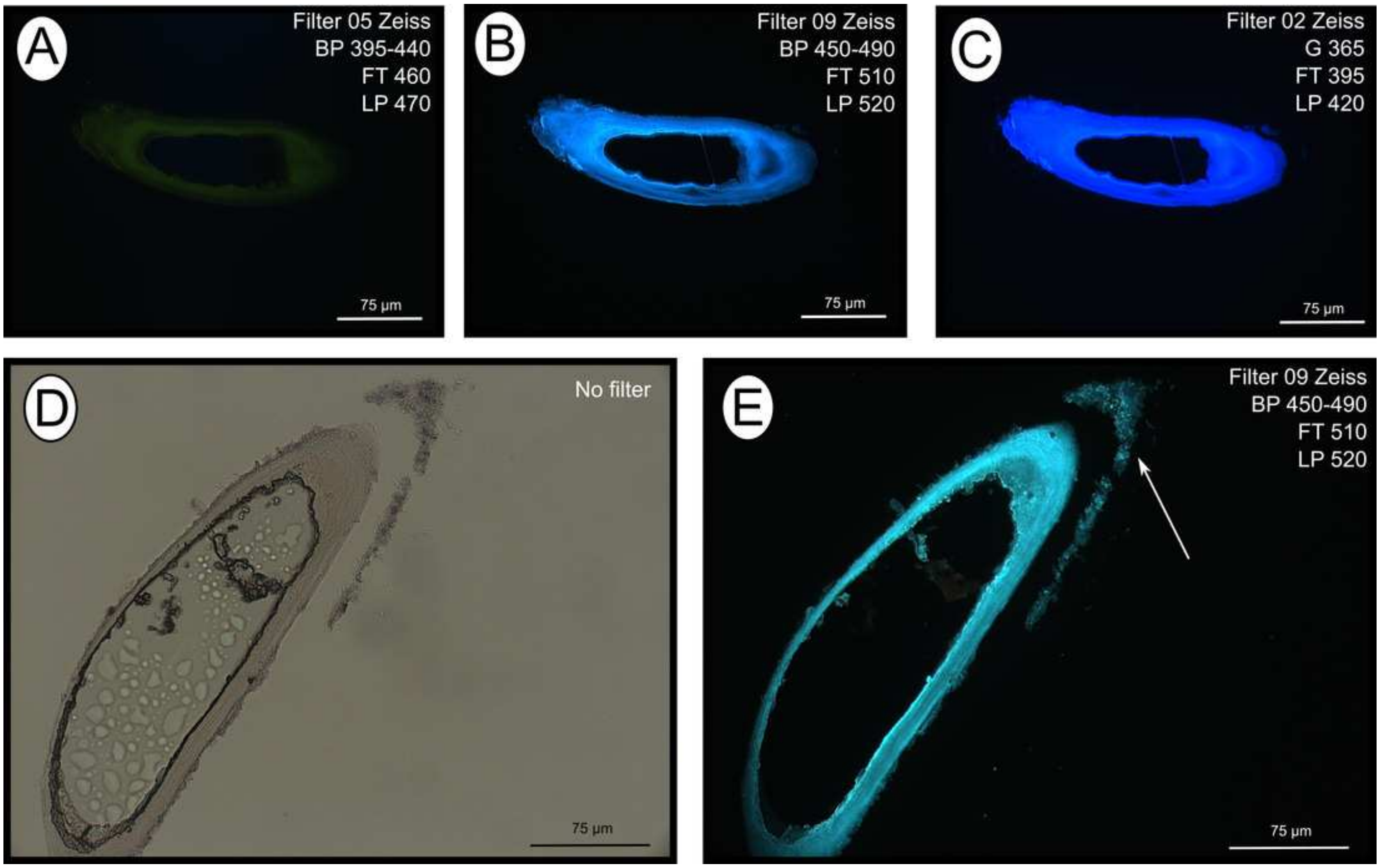
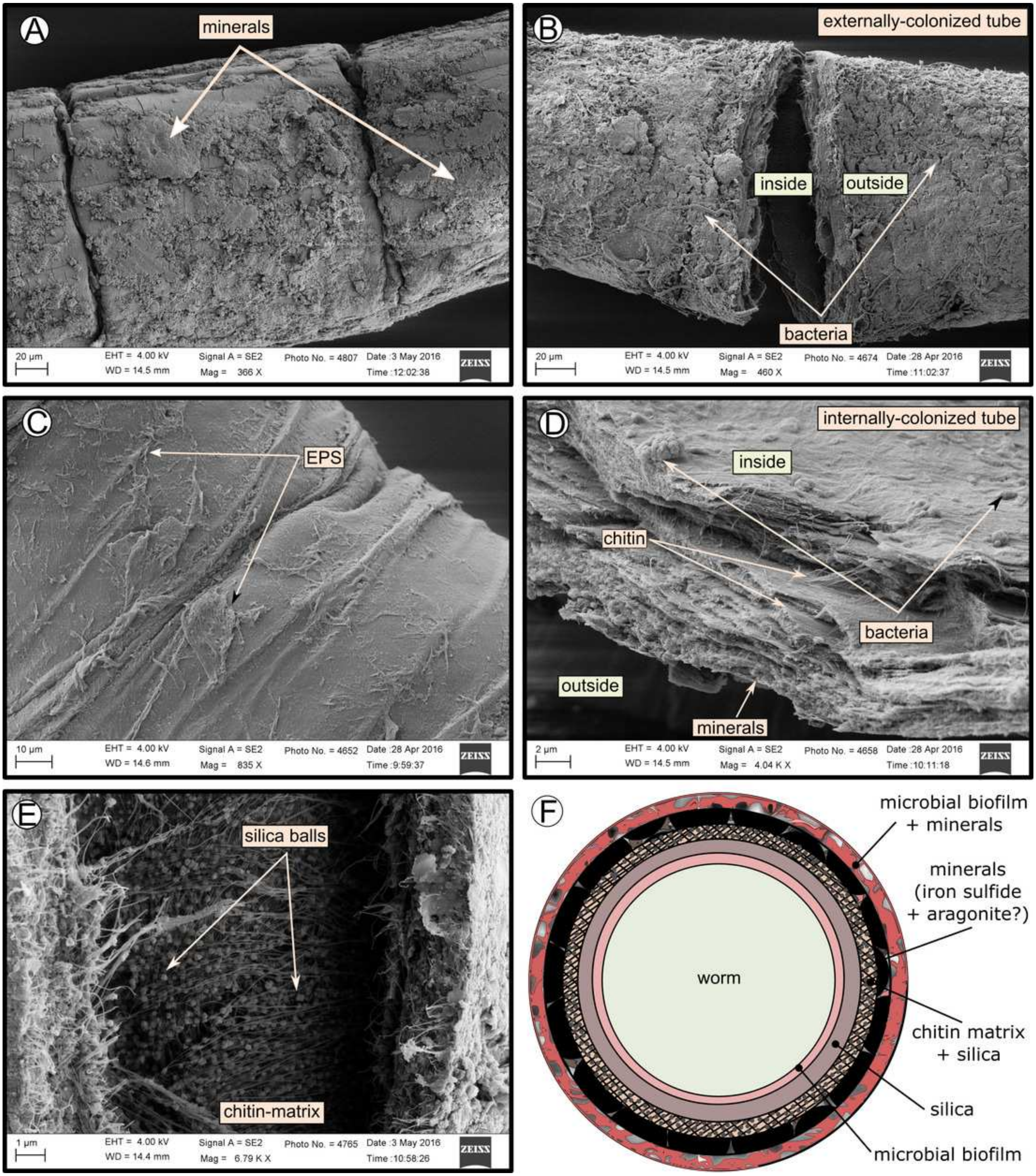


Figure 4

SEM micrographs of the tube of different specimens from different mud volcanoes and the expected display of their layers.

(A) El Cid MV specimen, with minerals on its external surface. (B) Al Gacel MV specimen, with a thick biofilm on its external surface. Microbial colonizers detailed in **Figure 5**. (C) Anastasya MV specimen with remains of EPS on its external surface. (D) Al Gacel MV specimen with bacteria on its internal surface. A multilayer organization of the tube can be observed, chitin layers and minerals can be differentiated. (E) Internal layer of chitin with rounded silica from El Cid MV specimen. (F) Model of what is expected to be the arranging of the tube, based on the SEM micrographs, EDX analysis, and references (Peckmann, Little & Reitner, 2005; Haas et al., 2009; Georgieva et al., 2015).



A
minerals
20 µm
EHT = 4.00 kV
WD = 14.5 mm
Signal A = SE2
Mag = 366 X
Photo No. = 4807
Date :3 May 2016
Time :12:02:38
B
externally-colonized tube
inside
outside
bacteria
20 µm
EHT = 4.00 kV
WD = 14.5 mm
Signal A = SE2
Mag = 460 X
Photo No. = 4674
Date :28 Apr 2016
Time :11:02:37
C
EPS
10 µm
EHT = 4.00 kV
WD = 14.6 mm
Signal A = SE2
Mag = 835 X
Photo No. = 4652
Date :28 Apr 2016
Time :9:59:37
D
internally-colonized tube
inside
chitin
bacteria
outside
minerals
2 µm
EHT = 4.00 kV
WD = 14.5 mm
Signal A = SE2
Mag = 4.04 K X
Photo No. = 4658
Date :28 Apr 2016
Time :10:11:18
E
silica balls
chitin-matrix
1 µm
EHT = 4.00 kV
WD = 14.4 mm
Signal A = SE2
Mag = 6.79 K X
Photo No. = 4765
Date :3 May 2016
Time :10:58:26
F
microbial biofilm + minerals
minerals (iron sulfide + aragonite?)
worm
chitin matrix + silica
silica
microbial biofilm

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Figure 5

SEM and TEM micrographs of colonized tubes from Al Gacel MV.

(A–C) Methanotrophic-like bacteria, organized in bacteriocyte-like bodies and expressing intracytoplasmatic membranes. (D–F) Different microbial morphotypes observed in the biofilm. (G–H) Rod-shaped bacteria colonizing the internal surface of the tube. (I-J) Bacterial biofilm penetrating the tube.

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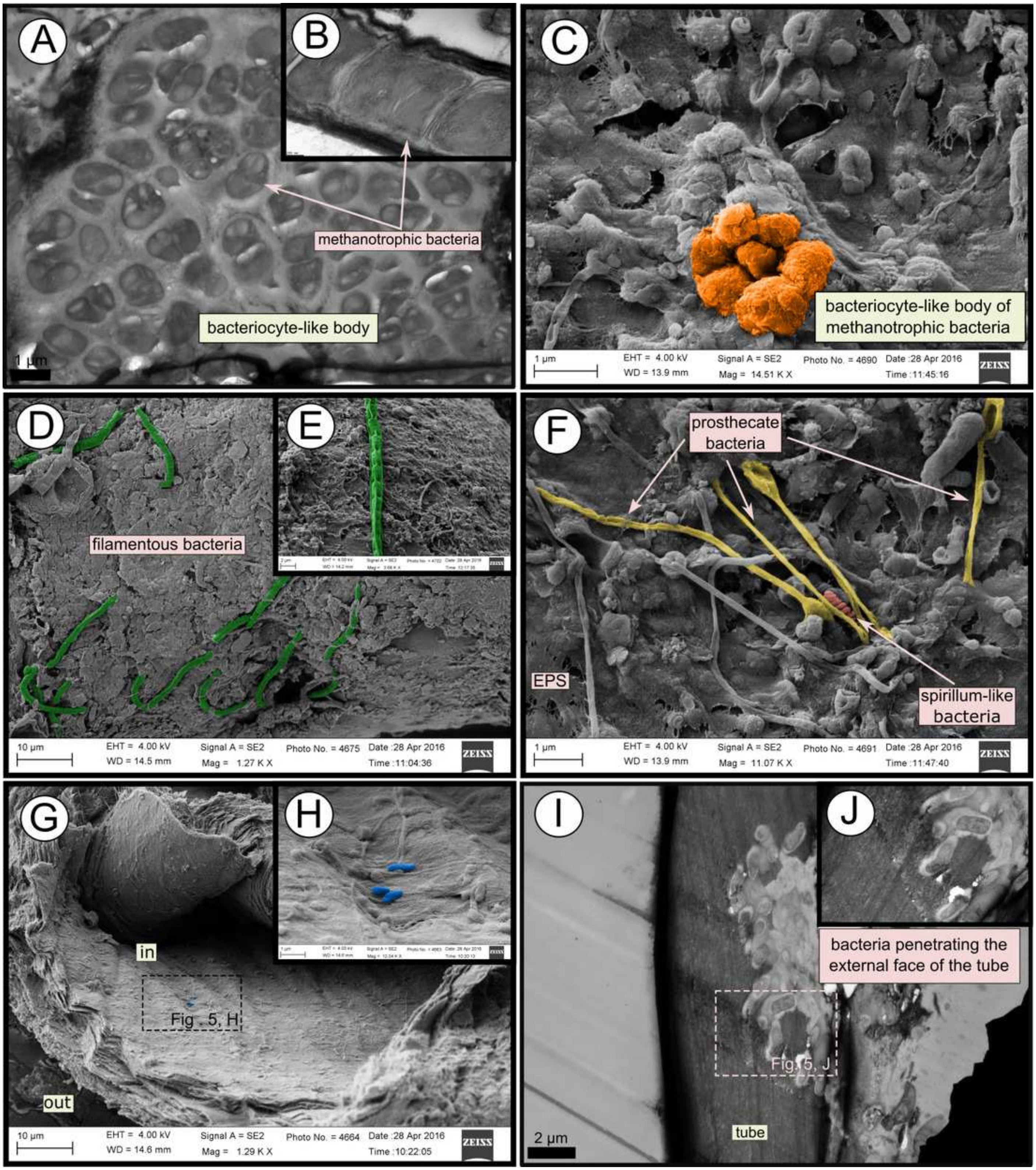
A
B
methanotrophic bacteria
bacteriocyte-like body
1 µm
C
bacteriocyte-like body of methanotrophic bacteria
D
E
filamentous bacteria
F
prosthecate bacteria
EPS
spirillum-like bacteria
G
H
in
Fig. 5, H
out
I
J
bacteria penetrating the external face of the tube
Fig. 5, J
tube
2 µm

Figure 6

TEM micrographs of remains of a microbial biofilm from tubes of Anastasya MV worms.

(A) Remains of methanotrophic-like bacteria are commonly observed. (B–D) Many bacteria appear to be embedded by the tube.

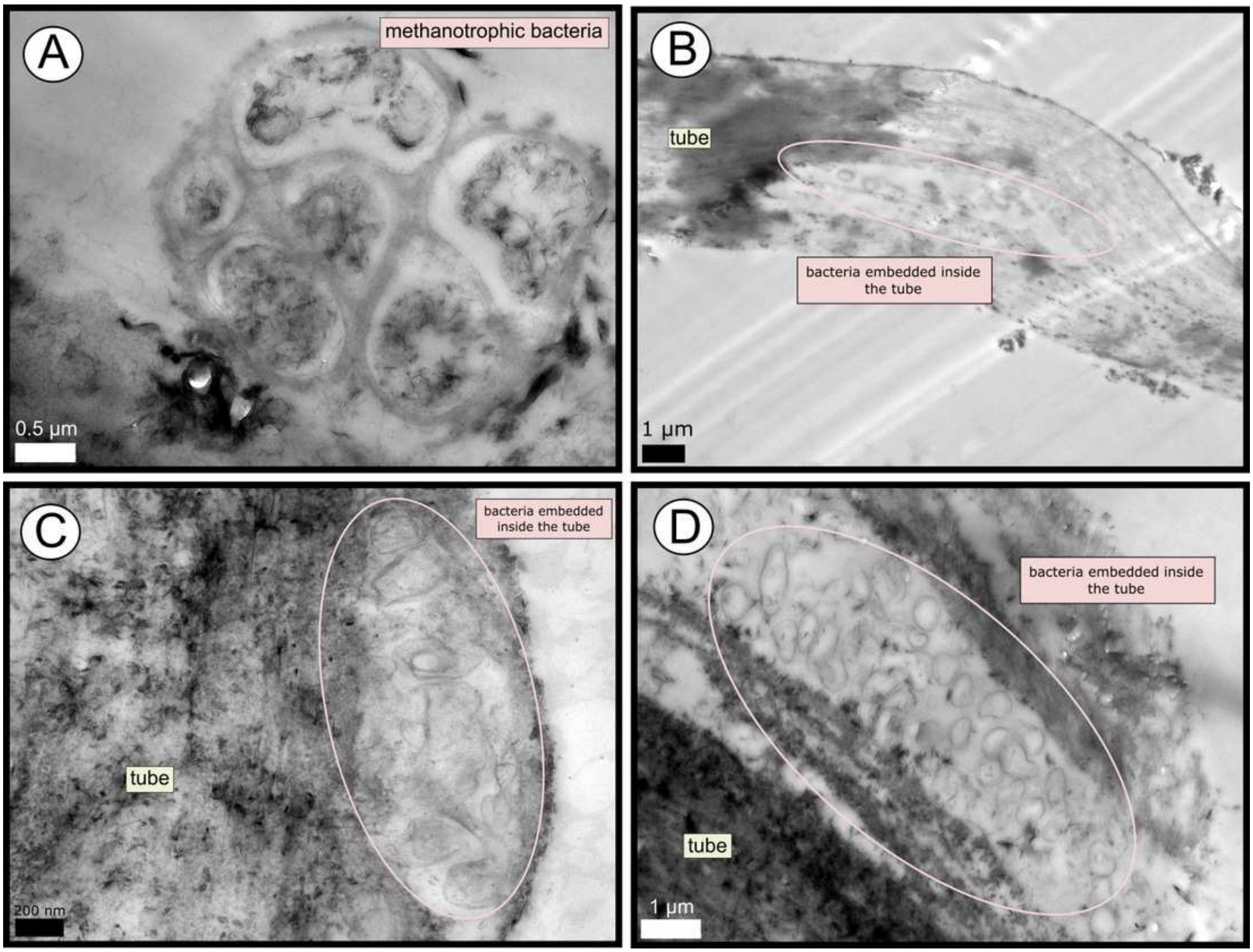

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Figure 7

Heatmap and grouped-bar charts of bacterial and archaeal relative abundances in each sample.

Microbial communities were grouped according to their metabolic preferences.

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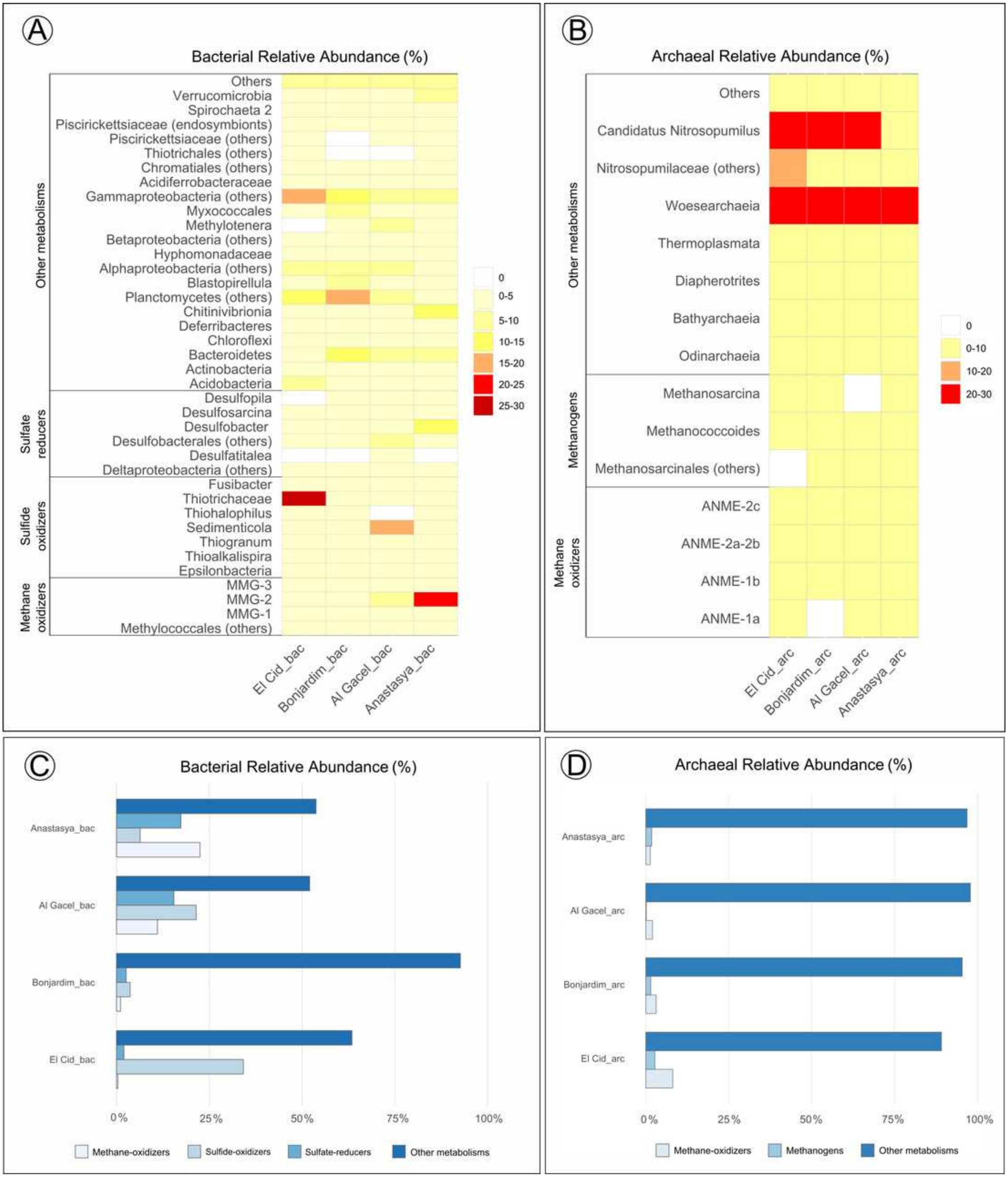
A
Bacterial Relative Abundance (%)
Other metabolisms
Sulfate reducers
Sulfide oxidizers
Methane oxidizers
Others
Verrucomicrobia
Spirochaeta 2
Piscirickettsiaceae (endosymbionts)
Piscirickettsiaceae (others)
Thiotrichales (others)
Chromatiales (others)
Acidiferrobacteraceae
Gammaproteobacteria (others)
Myxococcales
Methylotenera
Betaproteobacteria (others)
Hyphomonadaceae
Alphaproteobacteria (others)
Blastopirellula
Planctomycetes (others)
Chitinivibrionia
Deferribacteres
Chloroflexi
Bacteroidetes
Actinobacteria
Acidobacteria
Desulfopila
Desulfosarcina
Desulfobacter
Desulfobacterales (others)
Desulfatitalea
Deltaproteobacteria (others)
Fusibacter
Thiotrichaceae
Thiohalophilus
Sedimenticola
Thiogranum
Thioalkalispira
Epsilonbacteria
MMG-3
MMG-2
MMG-1
Methylococcales (others)
0
0-5
5-10
10-15
15-20
20-25
25-30
El Cid_bac
Bonjardim_bac
Al Gacel_bac
Anastasya_bac
B
Archaeal Relative Abundance (%)
Other metabolisms
Methanogens
Methane oxidizers
Others
Candidatus Nitrosopumilus
Nitrosopumilaceae (others)
Woesearchaeia
Thermoplasmata
Diapherotrites
Bathyarchaeia
Odinarchaeia
Methanosarcina
Methanococcoides
Methanosarcinales (others)
ANME-2c
ANME-2a-2b
ANME-1b
ANME-1a
0
0-10
10-20
20-30
El Cid_arc
Bonjardim_arc
Al Gacel_arc
Anastasya_arc
C
Bacterial Relative Abundance (%)
Anastasya_bac
Al Gacel_bac
Bonjardim_bac
El Cid_bac
0%
25%
50%
75%
100%
Methane-oxidizers
Sulfide-oxidizers
Sulfate-reducers
Other metabolisms
D
Archaeal Relative Abundance (%)
Anastasya_arc
Al Gacel_arc
Bonjardim_arc
El Cid_arc
0%
25%
50%
75%
100%
Methane-oxidizers
Methanogens
Other metabolisms

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Figure 8

Scheme of the conditions given in the different sampling sites.

Notice principal metabolism of siboglinids depending on seepage activity and presence or absence of other chemosynthetic organisms. Methane concentration values are given in Sánchez-Guillamón et al., 2015.

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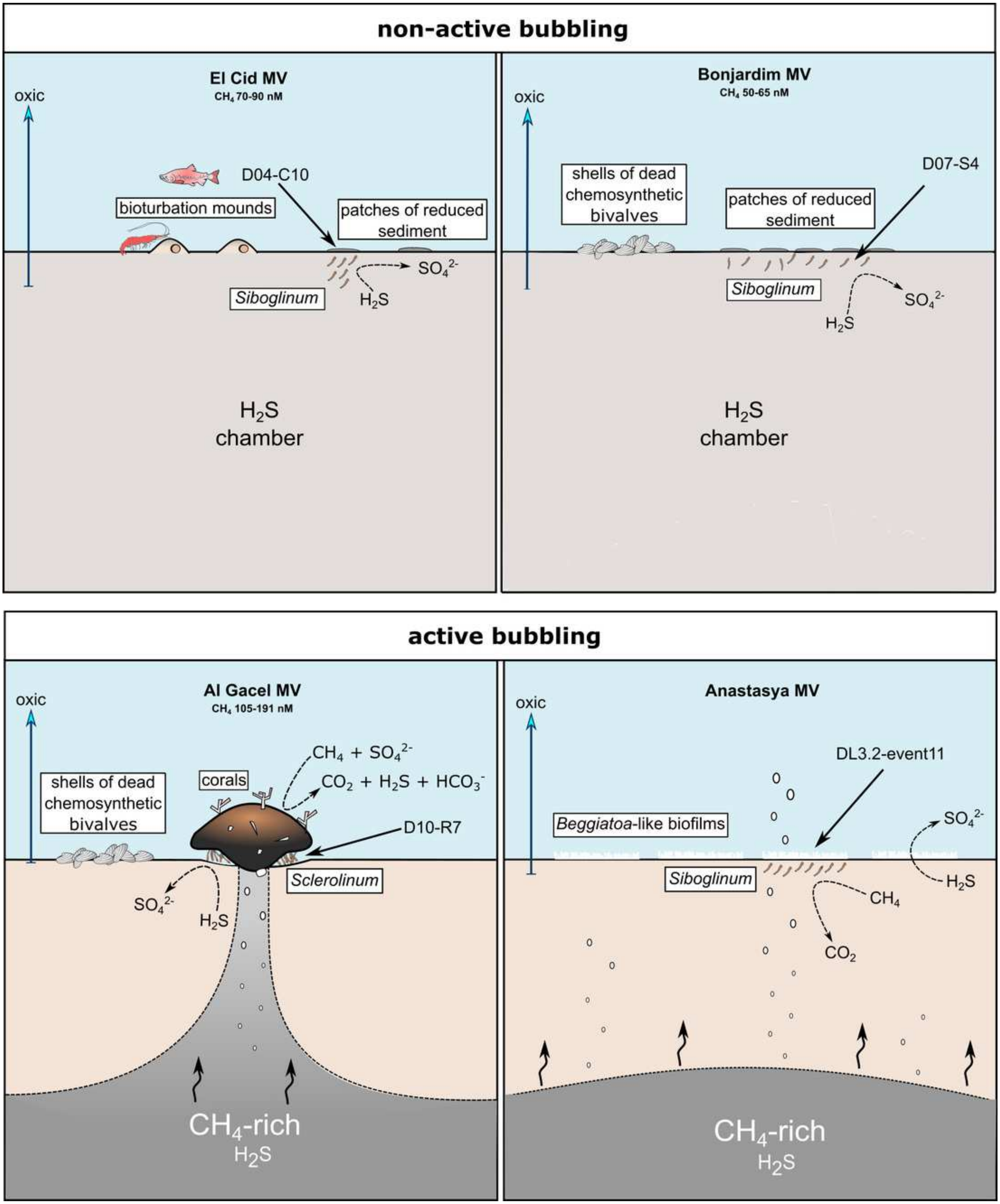
non-active bubbling
El Cid MV
CH4 70-90 nM
oxic
D04-C10
bioturbation mounds
patches of reduced sediment
Siboglinum
SO4 2-
H2S
H2S chamber
Bonjardim MV
CH4 50-65 nM
oxic
shells of dead chemosynthetic bivalves
D07-S4
patches of reduced sediment
Siboglinum
SO4 2-
H2S
H2S chamber
active bubbling
Al Gacel MV
CH4 105-191 nM
oxic
CH4 + SO4 2-
CO2 + H2S + HCO3 -
shells of dead chemosynthetic bivalves
corals
D10-R7
Sclerolinum
SO4 2-
H2S
CH4-rich
H2S
Anastasya MV
oxic
DL3.2-event11
Beggiatoa-like biofilms
SO4 2-
H2S
Siboglinum
CH4
CO2
CH4-rich
H2S