Cold-water corals and hydrocarbon-rich seepage in the Pompeia Province (Gulf of Cádiz) — living on the edge

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Abstract. Azooxanthellate cold-water corals (CWCs) have a global distribution and have commonly been found in areas of active fluid seepage. The relationship between the CWCs and these fluids, however, is not well understood. This study aims at unraveling the relationship between CWC development and hydrocarbon-rich seepage in the Pompeia Province (Gulf of Cádiz, Atlantic Ocean). This region comprises mud volcanoes, coral ridges, and fields of coral mounds, which are all affected by the tectonically driven seepage of hydrocarbon-rich fluids. The type of seepage such as focused, scattered, diffused or eruptive, however, is tightly controlled by a complex system of faults and diapirs. Early diagenetic carbonates from the currently active Al Gacel MV exhibit δ13C-signatures down to $-28.77 \%$ VPDB, indicating biologically derived methane as the main carbon source. The same samples contained $13C$-depleted lipid biomarkers diagnostic for archaea such as crocetane ($\delta^{13}C$ down to $-101.2 \%$ VPDB) and PMI ($\delta^{13}C$ down to $-102.9 \%$ VPDB), evidencing microbially mediated anaerobic oxidation of methane (AOM). This is further supported by next generation DNA sequencing data, demonstrating the presence of AOM related microorganisms (ANME archaea, sulfate-reducing bacteria) in the carbonate. Embedded corals in some of the carbonates and CWC fragments exhibit less negative $\delta^{13}C$ values ($-8.08$ to $-1.39\%$ VPDB), pointing against the use of methane as the carbon source. Likewise, the absence of DNA from methane- and sulfide-oxidizing microbes in a sampled coral does not support a chemoautotrophic lifestyle of these organisms. In the light of these findings, it appears that the CWCs benefit rather indirectly from hydrocarbon-rich seepage by using methane-derived authigenic carbonates as a substrate for colonization. At the same time, chemoautotrophic organisms at active sites prevent coral dissolution and necrosis by feeding on the seeping fluids (i.e., methane, sulfate, hydrogen sulfide), allowing cold-water corals to colonize carbonates currently affected by hydrocarbon-rich seepage.

1. Introduction

Cold-water corals (CWCs) are a widespread, non-phylogenetic group of cnidarians that include hard skeleton scleractinian corals, soft-tissue octocorals, gold corals, black corals and hydrocorals (Roberts et al., 2006; Roberts et al., 2009; Cordes et al., 2016). Typically, they thrive at low temperatures ($4 - 12 ^\circ C$) and occur in water depths of ca. $50 - 4000$ m. CWCs are azooxanthellate and solely rely on their nutrition as energy and carbon sources (Roberts et al., 2009). Some scleractinian corals (e.g. *Lophelia pertusa*, *Madrepora oculata*, *Dendrophyllia cornigera*, *Dendrophyllia alternata*, *Euchipsammia cornucopia*) are able to form colonies or even large carbonate
mounds (Rogers et al., 1999; Wienberg et al., 2009; Watling et al., 2011; Somoza et al., 2014). Large vertical mounds and elongated ridges formed by episodic growth of scleractinian corals (mainly *Lophelia pertusa*) are for instance widely distributed along the continental margins of the Atlantic Ocean (Roberts et al., 2009). These systems are of great ecological value since they offer sites for resting-, breeding-, and feeding for various invertebrates and fishes (Cordes et al., 2016 and references therein).

Several environmental forces influence the initial settling, growth, and decline of CWCs. These include, among others, an availability of suitable substrates for coral larvae settlement, low sedimentation rates, oceanographic boundary conditions (e.g. salinity, temperature and density of the ocean water) and a sufficient supply of nutrients through topographically controlled current systems (e.g. Mortensen et al., 2001; Roberts et al., 2003; Thiem et al., 2006; Dorschel et al., 2007; Dullo et al., 2008; Van Rooij et al., 2011; Hebbeln et al., 2016). Alternatively, the “hydraulic theory” supports that CWC ecosystems may be directly fueled by fluid seepage, providing a source of sulfur compounds, nitrogen compounds, P, CO$_2$ and/or hydrocarbons (Hovland, 1990; Hovland and Thomsen, 1997; Hovland et al., 1998; 2012). This relationship is supported by the common co-occurrence of CWC-mounds and hydrocarbon-rich seeps around the world, for example at the Hikurangi Margin in New Zealand (Liebetrau et al., 2010), the Brazil margin (e.g. Gomes-Sumida et al., 2004), the Darwin Mounds in northern Rockall Trough (Huvenne et al., 2009), the Kristin field on the Norwegian shelf (Hovland et al., 2012), the western Alborán Sea (Margreth et al., 2011), and the Gulf of Cádiz (e.g. Díaz-del-Río et al., 2003; Foubert et al., 2008). However, CWCs may also benefit rather indirectly from seepage. For instance, methane-derived authigenic carbonates (MDACs) formed through the microbially mediated anaerobic oxidation of methane (AOM; Suess & Whiticar, 1989; Hinrichs et al., 1999; Thiel et al., 1999; Boetius et al., 2000; Hinrichs & Boetius, 2002) potentially provide hard substrata for larval settlement (e.g. Díaz-del-Río et al., 2003; Goedert & Peckmann, 2005; Van Rooij et al., 2011; Magalhães et al. 2012; Le Bris et al., 2016; Rueda et al., 2016). In addition, larger hydrocarbon-rich seepage related structures such as mud volcanoes and carbonate mud mounds act as morphological barriers favoring turbulent water currents that deliver nutrients to the corals (Roberts et al., 2009; Wienberg et al., 2009; Margreth et al., 2011; Vandorpe et al., 2016).

In the Gulf of Cádiz, most CWC occurrences are “coral graveyards” with only a few living corals that are situated along the Iberian and Moroccan margins. These CWC systems are typically associated with diapiric ridges, steep fault-controlled escarpments, and mud volcanoes (MVs) such as the Faro MV, Hesperides MV, Mekness MV, and MVs in the Pen Duick Mud Volcano Province (Foubert et al., 2008; Wienberg et al., 2009). MVs (and other conspicuous morphological structures in this region such as pockmarks) are formed through tectonically induced fluid flow (Pinheiro et al., 2003; Somoza et al., 2003; Mediaide et al., 2009; León et al., 2010; 2012). This is because of the high regional tectonic activity and high fluid contents of sediments in this area (mainly CH$_4$, and, to a lesser extent, H$_2$S, CO$_2$, and N$_2$; Pinheiro et al., 2003; Hensen et al., 2007; González et al., 2012). However, the exact influence of fluid flow on CWC growth in this region remains elusive.

This study aims at elucidating the linkage between the present-day formation of MDACs and CWCs development along the Pompeia Province (Fig. 1), which encompasses mud volcanoes as the Al Gacel MV (León et al., 2012), diapiric coral ridges and mounds. We address this question by the combined analyses of high-resolution ROV underwater images, geophysical data (e.g. seabed topography, deep high-resolution multichannel seismic reflection data), and sample materials (petrographic features, $\delta^{13}$C- and $\delta^{18}$O-signatures of carbonates, lipid biomarkers and environmental 16s rDNA sequences of the prokaryotic microbial community). Based on our
findings, we propose an integrated model to explain the tempo-spatial and genetic relations between CWCs, chemosynthetic fauna and hydrocarbon-rich seepage in the study area.

2. Materials and Methods

This study is based on data and samples from the Pompeia Province that were collected during the Subvent-2 cruise in 2014 aboard the R/V Sarmiento de Gamboa (Fig. 1). In order to elucidate the tempo-spatial and genetic relations between CWCs, chemosynthetic fauna and hydrocarbon-rich seepage in this area, we explored geological features by means of underwater imaging and geophysical data. Based on these findings, we sampled different geological structures (mud volcanoes and coral ridges). All samples were taken with a ROV arm and immediately stored at room temperature (petrographic analysis), −20 degrees (stable isotopic analysis), and −80 degrees (environmental DNA analysis). Subsequently, we conducted detailed analyses on selected sample materials from sites that were characterized by different types of seepage during sampling. These include sites at the Al Gacel MV (D10-R3, D10-R7, D11-R8) and the Northern Pompeia Coral Ridge (D03-B1) (Fig. 1).

2.1. Geophysical survey

Seabed topography of the studied sites was mapped by using an Atlas Hydrosweep DS (15 kHz and 320 beams) multibeam echosounder (MBES). Simultaneously, ultra-high resolution sub-bottom profiles were acquired with an Atlas Parasound P-35 parametric chirp profiler (0.5 – 6 kHz). Deep high-resolution multichannel seismic reflection data was obtained using an array of 7 SERCEL gi-guns (system composed of 250 + 150 + 110 + 45 cubic inches) with a total of 860 cubic inches. The obtained data were recorded with an active streamer (SIG®16.3x40.175; 150 m length with 3 sections of 40 hydrophones each). The shot interval was 6 seconds and the recording length 5 seconds two-way travel time (TWT). Data processing (filtering and stacking) was performed on board with Hot Shots software.

2.2. Video survey and analysis

A remotely operated vehicle (ROV-6000 Luso) was used for photographic documentation (high definition digital camera, 1024x1024 pixel) and sampling. The ROV was further equipped with a STD/CTD-SD204 sensor (in-situ measurements of salinity, temperature, oxygen, conductivity, sound velocity and depth), HydroC™ sensors (in-situ measurements of CO₂ and CH₄) and Niskin bottles (CH₄ concentrations).

2.3. Petrographic analysis

General petrographic analysis was performed on thin sections (ca. 60 μm thickness) with a Zeiss SteREO Discovery.V8 stereomicroscope (transmitted- and reflected light) linked to an AxioCam MRc 5-megapixel camera. Additional detailed petrographic analysis of textural and mineralogical features was conducted on polished thin sections (ca. 30 μm thickness) using a DM2700P Leica Microscope coupled to a DFC550 digital camera. Carbonate textures have been classified following Dunham (1962) and Embry & Klovan (1971).

2.4. Stable isotopes (δ¹³C, δ¹⁸O) of carbonates

Stable carbon and oxygen isotope measurements were conducted on ca. 0.7 mg carbonate powder obtained with a high precision drill (φ 0.8 mm). The analyses were performed with a Thermo Scientific Kiel IV carbonate device
coupled to a Finnigan Delta Plus gas isotope mass spectrometer. Accuracy and reproducibility were checked through the replicate analysis of a standard (NBS19) and reproducibility was better than 0.1 ‰. Stable carbon and oxygen isotope values are expressed in the standard δ notation as per mill (‰) deviations relative to Vienna Pee Dee Belemnite (VPDB).

2.5. Lipid biomarker analysis

2.5.1. Sample preparation

All materials used were pre-combusted (500 °C for >3 h) and/or extensively rinsed with acetone prior to sample contact. A laboratory blank (pre-combusted sea sand) was prepared and analyzed in parallel to monitor laboratory contaminations. The preparation and extraction of lipid biomarkers was conducted in orientation to descriptions in Birgel et al. (2006). Briefly, the samples were first carefully crushed with a hammer and internal parts were powdered with a pebble mill (Retsch MM 301, Haan, Germany). Hydrochloric acid (HCl; 10 %) was slowly poured on the powdered samples which were covered with dichloromethane (DCM)-cleaned water. After 24 h of reaction, the residues (pH 3 – 5) were repeatedly washed with water and then lyophilized.

3 g of each residue was saponified with potassium hydroxide (KOH; 6 %) in methanol (MeOH). The residues were then extracted with methanol (40 mL, 2x) and, upon treatment with HCl (10 %) to pH 1, in DCM (40 mL, 2x) by using ultra-sonification. The combined supernatants were partitioned in DCM vs. water (3x). The total organic extracts (TOEs) were dried with sodium sulfate (NaSO₄) and evaporated with a gentle stream of N₂ to reduce loss of low-boiling compounds (cf. Ahmed and George, 2004).

Fifty percent of each TOE was separated over a silica gel column (0.7 g Merck silica gel 60 conditioned with n-hexane; 1.5 cm i.d., 8 cm length) into (a) hydrocarbon (6 mL n-hexane), (b) alcohol (7 mL DCM/acetone, 9:1, v:v) and (c) carboxylic acid fractions (DCM/MeOH, 3:1, v:v). Only the hydrocarbons were subjected to gas chromatography–mass spectrometry (GC-MS).

2.5.2. Gas chromatography–mass spectrometry (GC-MS)

Lipid biomarker analyses of the hydrocarbon fraction were performed with a Thermo Scientific Trace 1310 GC coupled to a Thermo Scientific Quantum XLS Ultra MS. The GC was equipped with a capillary column (Phenomenex Zebron ZB-5MS, 30 m length, 250 μm inner diameter, 0.25 μm film thickness). Fractions were injected into a splitless injector and transferred to the column at 300 °C. The carrier gas was He at a flow rate of 1.5 mL min⁻¹. The GC oven temperature was ramped from 80°C (1 min) to 310 °C at 5 °C min⁻¹ (held for 20 min). Electron ionization mass spectra were recorded in full scan mode at an electron energy of 70 eV with a mass range of m/z 50 – 600 and scan time of 0.42 s. Identification of individual compounds was based on comparison of mass spectra and GC retention times with published data and reference compounds.

2.5.3 Gas chromatography–combustion–isotope ratio mass spectrometer (GC-C-IRMS)

Compound specific δ¹³C analyses were conducted with a Trace GC coupled to a Delta Plus IRMS via a combustion-interface (all Thermo Scientific). The combustion reactor contained CuO, Ni and Pt and was operated at 940°C. The GC was equipped with two serially linked capillary columns (Agilent DB-5 and DB-1; each 30 m length, 250 μm inner diameter, 0.25 μm film thickness). Fractions were injected into a splitless injector and
transferred to the GC column at 290°C. The carrier gas was He at a flow rate of 20 ml min⁻¹. The temperature program was identical to the one used for GC-MS (see above). CO₂ with known δ¹³C value was used for internal calibration. Instrument precision was checked using a mixture of n-alkanes with known isotopic composition. Carbon isotope ratios are expressed as δ¹³C (‰) relative to VPDB.

2.6. Amplicon sequencing of 16S rRNA genes

2.6.1. DNA extraction and 16S rRNA gene amplification

Environmental DNA analyses of microbial communities were performed on a carbonate sample with embedded corals from the base of the Al Gacel MV (D10-R3), a carbonate sample from an active pockmark close to the summit of the Al Gacel MV (D10-R7), and a necrotic fragment of a living Madrepora oculata recovered from the Northern Pompeia Coral Ridge (D03-B1). About 1 – 4 g of sample material was first mashed with mortar and liquid nitrogen to fine powder. Three biological replicates were used per sample. Total DNA was isolated with a Power Soil DNA Extraction Kit (MO BIO Laboratories, Carlsbad, CA). All steps were performed according to the manufacturer’s instructions.

Bacterial amplicons of the V3 – V4 region were generated with the primer set MiSeq_Bacteria_V3_forward primer (5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’) and MiSeq_Bacteria_V4_reverse primer (5’-GTCTCGTGGGCTCGAGATGTGTATAAGAGACAGTACHVGGGTATCTAATCC-3’). Likewise, archaeal amplicons of the V3 – V4 region were generated with the primer set MiSeq_Archaea_V3_forward primer (5’-TCGTCGGCACGTCAGATGTGTATAAGAGACAGGGTGBCAGCCGGCCCGGTGTA-3’) and MiSeq_Archaea_V4_reverse primer (5’-GTCTCGTGGGCTCGAGATGTGTATAAGAGACAGTACHVGGGTATCTAATCC-3’). 50 µl of the PCR reaction mixture for bacterial DNA amplification, 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 MgCl₂, and 25 ng of isolated DNA. The PCR protocol for bacterial DNA amplification included (i) initial denaturation for 1 min at 98 °C, (ii) 25 cycles of 45 s at 98 °C, 45 s at 60 °C, and 30 s at 72 °C, and (iii) a final extension at 72 °C for 5 min. The PCR reaction mixture for archaeal DNA amplification was similarly prepared but contained instead 1 µl of 25 mM MgCl₂ and 50 ng of isolated DNA. The PCR protocol for archaeal DNA amplification included (i) initial denaturation for 1 min at 98 °C, (ii) 10 cycles of 45 s at 98 °C, 45 s at 63 °C, and 30 s at 72 °C, (iii) 15 cycles of 45 s at 98 °C, 45 s at 53 °C, and 30 s at 72 °C, and (iv) a final extension at 72 °C for 5 min.

PCR products were checked by agarose gel electrophoresis and purified using the GeneRead Size Selection Kit (QIAGEN GmbH, Hilden, Germany).

2.6.2. Data analysis and pipeline

Illumina PE sequencing of the amplicons and further process of the sequence data were performed in the Göttingen Genomics Laboratory (Göttingen, Germany). After Illumina MiSeq processing, sequences were analyzed as described in Egelkamp et al. (2017) with minor modifications. In brief, paired-end sequences were merged using PEAR v0.9.10 (Zhang et al., 2014), sequences with an average quality score below 20 and containing unresolved bases were removed with QIIME 1.9.1 (Caporaso et al., 2010). Non-clipped reverse and forward primer sequences were removed by employing cutadapt 1.15 (Martin, 2011). USEARCH version 9.2.64 was used following the
UNoise pipeline (Edgar, 2010). In detail, reads shorter than 380 bp were removed, dereplicated, and denoised with the UNoise2 algorithm of USEARCH resulting in amplicon sequence variants (ASVs) (Callahan et al., 2017). Additionally, chimeric sequences were removed using UCHIME2 in reference mode against the SILVA SSU database release 132 (Yilmaz et al., 2014). Merged paired-end reads were mapped to chimera-free ASVs and an abundance table was created using USEARCH. Taxonomic classification of ASVs was performed with BLAST against the SILVA database 132. Extrinsic domain ASVs, chloroplasts, and unclassified ASVs were removed from the dataset. Sample comparisons were performed at same surveying effort, utilizing the lowest number of sequences by random subsampling (20,290 reads for bacteria, 13,900 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 SRP156750.

3. Results

3.1. The Pompeia Province — geological settings

The Pompeia Province is situated in the Gulf of Cádiz offshore Morocco, within the so-called Middle Moroccan Field (Ivanov et al., 2000) at water-depths between 860 and 1000 m (Fig. 1). It compromises the active Al Gacel MV (Fig. 1, C), another mud volcano which is extinct (further referred as extinct MV) and two east-west elongated ridges (Northern Pompeia Coral Ridge and Southern Pompeia Coral Ridge). Scattered coral-mounds surround the ridges with a smooth relief (Fig. 1, B). CWCs were observed on seismic profiles resting on all these morphological features. Detailed geological profiles and 3D images of these features are shown in Figs. 2 and 3.

The Al Gacel MV is a cone-shape structure, 107 m high and 944 m wide, with its summit at 762 m depth and surrounded by a 11 m deep rimmed depression (León et al., 2012) (Fig. 1, C). It is directly adjacent to the Northern Pompeia Coral Ridge (Fig. 2, A–B), which extends ca. 4 km in westward direction (Fig. 2, A–B) and it is terminated by the Pompeia Escarpment (Fig. 1, B; Fig. 2, C). High resolution seismic profiles of the Pompeia Escarpment show CWC build-ups (R1 to R4) with steep lateral scarps of ca. 40 m height (Fig. 2, C). This MV is of sub-circular shape and exhibits a crater at its top (Fig. 2, A–B).

Ultra-high resolution sub-bottom seismic profile crossing the Pompeia Province from northwest (NW) to southeast (SE) (Fig. 3, A), shows (i) the Al Gacel MV surrounded by bottom-current deposits, (ii) an up to 130 m high CWC framework, growing on top the Southern Pompeia Coral Ridge, and (iii) semi-buried CWC mounds surrounding the ridge in areas of low relief. These CWC mounds locally form smooth, up to 25 – 30 m high top-rounded reliefs that are exposed, but then taper downward below the seafloor (applying sound speeds of 1750 m/s in recent sediments). Additionally, a multichannel seismic profile following the same track but with higher penetration below the seafloor (Fig. 3, B) shows high amplitude reflections inside the Al Gacel cone and enhanced reflections at the top of the diapirs (yellow dotted-line in Fig. 3, B), pointing to the occurrence of gas (hydrocarbon)-charged sediments. It furthermore exhibits breaks in seismic continuity and diapiric structures at different depths below the Southern Pompeia Coral Ridge and the Al Gacel MV, evidencing a fault system (Fig. 3, B). These tectonic structures may promote the development of overpressure areas (OP in Fig. 3, B) and consequent upward fluid flow to the surface.

3.2. ROV observation and measurements
Submersible ROV surveys at the Al Gacel MV (Fig. 1, C) revealed the presence of dispersed pockmark depressions at the eastern (Dive 10, 790 m) and northern flanks (Dive 11, 760 – 825 m depth). These sites are characterized by focused but low intensity seafloor bubbling (e.g. Fig. 4, B; Fig. 5, A). Analysis of water samples revealed CH₄-concentration up to 171 nM during Dive 10 and up to 192 nM during Dive 11 (Sánchez-Guillamón et al., 2015). Pockmarks were essentially formed by grey-olive mud breccia sediments and characterized by deposits of authigenic carbonates appearing in the center and edges, together with typical methane-seep related organisms (e.g. sulfide-oxidizing bacterial mats, chemosynthetic bivalves, siboglinid tubeworms) (Fig. 4, B–C; Fig. 5). Communities of non-chemosynthetic organisms (e.g. sponges, corals) were also found at pockmarks (Fig. 4, B–C; Fig. 5, C), but were more abundant in places where no seepage was detected (Fig. 4, A).

Observations with the submersible ROV at the Northern Pompeia Coral Ridge and the extinct MV (Dive 03) revealed widespread and abundant occurrences of dead scleractinian-corals (mainly Madrepora oculata and Lophelia pertusa) currently colonized by few living non-chemosynthetic organisms (e.g. Corallium tricolor, other octocorals, sea urchins) (Fig. 6, B–D). Locally, grey-black colored patches of sulfide-oxidizing bacterial mats surrounded by shells of chemosynthetic bivalves (Lucinoma asaphes and Thyssira vulcolutre) were detected (Fig. 6, A). CH₄-seepage appeared to be less than at the Al Gacel MV, with concentrations of 80 – 83 nM.

Water parameters display homogenous values between the four sampling sites (10 °C temperature, ca. 52 – 55 % dissolved oxygen, ca. 31 Kg/m³ density) (Table 1).

3.3. Petrography and stable isotopes signatures of carbonates (δ¹⁸O, δ¹³C)

Sample D10-R3 derives from a field of carbonates at the base of the Al Gacel MV which is inhabited by sponges and corals (Fig. 4, A). The sample is a framestone composed of deep water scleractinian corals (Madrepora and rare Lophelia) (Fig. 7, A–B). The corals are typically cemented by microbial automicrite (sensu Reitner et al. 1995) followed by multiple generations of aragonite. A matrix of dark allomicrite (sensu Reitner et al. 1995) with oxidized frambooidal pyrites and remains of planktonic foraminifera is restricted to few bioerosional cavities (ca. 5%) in the skeletons of dead corals (Fig. 8, A–B). δ¹³C signatures of the matrix and cements range from −26.68 to −18.38 ‰, while the embedded coral fragments exhibit δ¹³C values between −5.58 and −2.09 ‰ (Fig. 7, B; Table 2). The δ¹⁸O values generally range from +2.35 to +3.92 ‰ (Fig. 9; Table 2).

Sample D10-R7 was recovered from a pockmark on the eastern site of the Al Gacel MV that is influenced by active seepage (Fig. 3, C). It consists of black carbonate and exhibits a strong hydrogen sulfide (H₂S) odor (Fig. 5, B; Fig. 7, C–D). The top of this sample was inhabited by living octocorals (Fig. 5, C), while chemosymbiotic siboglinid worms were present on the lower surface (Fig. 5, D). The sample is characterized by a grey peloidal wackestone texture consisting of allogenicite with abundant planktonic foraminifers and few deep water miliolids. The sample furthermore exhibits some fractured areas which are partly filled by granular and small fibrous cement, probably consisting of Mg-calcite. Locally, light brownish crusts of microbial automicrite similar to ones in D10-R3 are present (see above). Frambooidal pyrite is abundant and often arranged in aggregates (Fig. 8, C–D). The carbonate exhibits δ¹³C values ranging from −28.77 to −21.13 ‰ and δ¹⁸O values from +2.37 to +3.15 ‰ (Fig. 9; Table 2).

Sample D11-R8 comes from an area with meter-sized carbonate blocks at the summit of the Al Gacel MV (Fig. 4, D) and is mainly colonized by sponges and serpulid worms (Fig. 7, E). The sample generally exhibits a light grey mud- to wackestone texture consisting of allogenicrite with few scleractinian-coral fragments and planktonic foraminifers (Fig. 7, E–F). The carbonate furthermore contains abundant quartz silt and, locally, pyrite.
enrichments. A further prominent feature are voids that are encircled by dark grey halos and exhibit brownish margins (due to enrichments of very small pyrite crystals and organic matter, respectively). $\delta^{13}$C signatures of the matrix and cements range from $-14.82$ to $-14.74\%$, while embedded coral fragments exhibit $\delta^{13}$C values of $-4.91$ to $-2.99\%$ (Fig. 7; Table 2). $\delta^{18}$O values generally range from $+1.49$ to $+5.60\%$ (Fig. 9; Table 2).

Sample D03-B1 is a necrotic fragment of a living scleractinian coral (*Madrepora oculata*) recovered from the Northern Pompeia Coral Ridge (Fig. 6; D; Fig. 7, G). The coral-carbonate exhibits $\delta^{13}$C values ranging from $-8.08$ to $-1.39\%$ and $\delta^{18}$O values from $-0.31$ to $+2.26\%$ (Fig. 9; Table 2).

### 3.4. Lipid biomarkers and compound specific carbon isotope signatures

The hydrocarbon fractions of the carbonate recovered from the active pockmark (D10-R7) mainly consist of the irregular, tail-to-tail linked acyclic isoprenoids 2,6,11,15-tetramethylhexadecane (C$_{26}$; crocetane), 2,6,10,15,19-pentamethylicosane (C$_{25}$; PMI), as well as of several unsaturated homologues of these compounds (Fig. 10). Additionally, it contains the regular, head-to-tail linked acyclic isoprenoid pristane (C$_{19}$).

The hydrocarbon fraction of the carbonate recovered from the summit of the Al Gacel MV (D11-R8) is dominated by n-alkanes with chain-lengths ranging from C$_{14}$ to C$_{33}$ (maxima at n-C$_{16}$ and, subordinated, at n-C$_{20}$ and n-C$_{31}$) (Fig. 10). The sample further contains pristane, crocetane, the head-to-tail linked acyclic isoprenoid phytane (C$_{20}$) and traces of PMI.

Crocetane and PMI exhibited strongly depleted $\delta^{13}$C values in the carbonate from the active pockmark (D10-R7) ($-101.2\%$ and $-102.9\%$, respectively), while they showed less depleted $\delta^{13}$C values in the carbonate from the summit of the volcano (D11-R8) ($-57.2\%$ and $-74.3\%$, respectively). $\delta^{13}$C values of n-alkanes in the carbonate D11-R8 (n-C$_{17}$-C$_{22}$) ranged between $-30.8\%$ and $-33.0\%$ (Table 3).

### 3.5. Prokaryotic community structure

Bacterial DNA (Fig. 11, A) from samples D10-R3 (authigenic carbonate, base of the Al Gacel MV) and D03-B1 (*Madrepora oculata* fragment, Northern Pompeia Coral Ridge) mainly derives from taxa that typically thrive in the water-column (e. g. Actinobacteria, Acidobacteria, Chloroflexi, Bacteroidetes, Woesiaceae, Dadabacteria, Kaiserbacteria, Poribacteria, Planctomycetes, Gemmatimonadetes). The sample D10-R3 furthermore contains bacterial DNA of the nitrite-oxidizing bacteria *Nitrospira sp.*, while the sample D03-B1 contains DNA of the bacterial taxa Verrucromicrobia, Enterobacteria, *Nitrosococcus*. Noteworthy, one amplicon sequence variant (ASV_189) with low number of clustered sequences has been found in D03-B1, identified as a methanotrophic symbiont of *Bathymodiolum maurtianicus* (see Rodrigues et al., 2013).

Up to 50% of bacterial DNA in sample D10-R7 (authigenic carbonate, top of the Al Gacel MV) derives from taxa that are commonly associated with fluid seepage and AOM, i.e. sulfide-oxidizing bacteria, sulfate-reducing bacteria (SRB) and methane-oxidizing bacteria. The most abundant are SRB taxa like SEEP-SRB1, SEEP-SRB2, *Desulfatiglans, Desulfofulvus* and *Desulfococcus*, which typically form consortia with ANME archaea.

Archaeal DNA (Fig. 11, B) from samples D10-R3 and D03-B1 mainly consist of *Cenarchaeum sp.*, which represents 70 – 90%. *Candidatus Nitrosopumilus* is the second most abundant in both samples, representing 5 – 20%. In contrast, around 90% of archaeal DNA in D10-R7 is related to ANME-1 and ANME-2 groups, in good concordance with the relative abundances of SRB DNA.

Details of the number of reads per taxa are shown in the supplementary data, Tables 1 and 2.
4. Discussion

4.1. Evidence of hydrocarbon-rich seepage affecting the Pompeia Province

Two-dimensional multichannel-seismic images show that the Pompeia Province is affected by fluid expulsion related to compressional diapirc ridges and thrust faults (Fig. 3, B), as it has been reported from other areas of the Gulf of Cádiz (Somoza et al., 2003; Van Rensbergen et al., 2005; Medialdea et al., 2009). There seem to be different types of fault-conduit systems that link the overpressure zones (OP) with the seafloor (Fig. 3, B), controlling both type and rate of seepage (e.g. eruptive, focused, diffused or dripping-like). Dripping-like refers to intermittent bubbling fluids. At the Al Gacel MV, conduits are for instance mainly linked to faults and a dense hydro-fracture network, allowing the migration of hydrocarbon-rich muds from the overpressure zone to the surface. During active episodes, eruptions lead to the formation of mud-breccia flows as observed in gravity cores (e.g. León et al., 2012). During rather dormant episodes, focused and dripping-like seepage predominates, forming pockmark features (Fig. 4, B).

Currently, the Al Gacel MV is affected by continuous and focused dripping-like seepages. These sites of active seepage are characterized by carbonates that are suspected to be methane-derived (e.g. sample D10-R7, Fig. 4, B–C). In-situ ROV-measurements and subsequent water sample analysis demonstrated high concentrations of CH₄ in fluids that were escaping upon removal of the carbonate D10-R7 from the active pockmark (171 nM; Fig. 5, A) (Sánchez-Guillamón et al., 2015). This association suggests a genetic relationship between hydrocarbon-rich seepage and the carbonate, as also evidenced by the low δ¹³C-values of the carbonates analyzed herein (down to ca. −30 ‰, Fig. 9; Table 2). Indeed, the grey peloidal texture of this sample resembles that of AOM-derived automicrites from the Black Sea that are related to micro-seepage of methane (cf. Reitner et al., 2005). The here observed isotopically depleted acyclic isoprenoids such as crocetane and PMI (δ¹³C values between ca. −103 and −57‰; Fig. 10; Table 3) are typical fingerprints of AOM-associated Archaea (Hinrichs et al., 1999; Thiel et al., 1999, 2001; Peckmann et al., 2001; Peckmann & Thiel, 2004), which is also in good accordance with the high abundance of DNA related to ANME. At the same time, abundant frambooidal pyrite in the carbonate (Fig. 8, C–D) and SRB-related DNA (Fig. 11) evidences microbial sulfate reduction in the environment. All these data clearly demonstrate that the carbonates have been formed via AOM, fueled by fluids from the underlying mud diapir.

Other carbonate samples from the Al Gacel MV (i.e. D10-R3 and D11-R8) probably have also been formed due to AOM as they are also isotopically depleted (δ¹³C values between ca. −25 and −15 ‰, Fig. 9, Table 2). However, no active gas bubbling was observed during sampling, even though both samples still contain open voids which could form pathways for a continuous migration of fluids. In fact, several characteristics of these voids (e.g. dark halos formed by pyrite, brownish margins due to organic matter enrichments) are very similar to those of methane-derived carbonate conduits (cf. Reitner et al., 2015). This could imply that the intensity of hydrocarbon-rich seepage and consequently AOM, may have fluctuated through time. The relatively low dominance of crocetane and PMI in the carbonate D11-R8 from the summit of Al Gacel MV (Fig. 10), as well as their moderately depleted δ¹³C values (−57.2 ‰ and −74.3 ‰, respectively; Table 3), could be due to mixing effects and thus be in good accordance with varying intensities of AOM in the environment. Also, the presence of only few AOM-related DNA sequences (Fig. 11) and partly oxidized pyrites in the carbonate D10-R3 from the base of the Al Gacel MV (Fig. 8, A–B) are well in line with this scenario.

There is no evidence for eruptive extrusions of muddy materials at the coral ridges. In the Southern Pompeia Coral Ridge (Fig. 3), diapirs appear to rather promote an upward migration of hydrocarbon-rich fluids in a divergent
way throughout a more extensive seafloor area. This results in a continuous and diffused seepage, which promotes
the occurrence of AOM and the formation of MDACs at the base of the ridges, related to the sulphate-methane
transition zone (SMTZ) (Boetius et al., 2000; Hinrichs and Boetius, 2002; González et al., 2012a). This is in good
accordance with the detection of methane (80 – 83 nM) at the Northern Pompeia Coral Ridge and the presence of
sulfide-oxidizing bacterial mats and shells of dead chemosynthetic bivalves at the western part of the ridge (Fig.
6, A). Likewise, the CWC Mounds Field surrounding the Southern Pompeia Coral Ridge (Fig. 3) is thoroughly
characterized by micro-seeps, due to ascending fluids from OPs through low-angle faults. This type of focused
seepage may promote formation of MDAC pavements in deeper layers of the sediments (Fig. 3), similar to coral
ridges along the Pen Duick Escarpment (Wehrmann et al., 2011). The generation of MDAC-hotspots at sites of
such seepage also explain the geometry of the downward tapering cones (Fig. 3).

4.2. Ecological meaning of hydrocarbon-rich seepage for CWCs

Our data suggests contemporaneous micro-seepage and CWC growth in the Pompeia Province (e.g. Fig. 4, B).
This relationship has also been observed elsewhere, e.g. in North Sea and off Mid Norway (Hovland, 1990;
Hovland & Thomsen, 1997), and the Angola margin (Le Guilloux et al., 2009). However, scleractinian fragments
recovered from the Al Gacel MV (embedded in carbonates D10-R3 and D11-R8, from the base and summit of the
volcano, respectively) and the Northern Pompeia Coral Ridge (D03-B1, necrotic part of a living Madrepora
oculata) displayed barely depleted δ13C values (ca. −8 to −1 ‰; Fig. 9: Table 2), close to the δ13C of marine
seawater (0 ± 3 ‰, e.g. Hoefs, 2015). This does not support a significant uptake of methane-derived carbon by the
CWCs and thus a direct trophic dependency as previously proposed (Hovland, 1990). Furthermore, the only DNA
in sample D03-B1 that could be attributed to a potential methanotrophic endosymbiont (ASV_189: Rodrigues et
al., 2013) occurred in minor amounts and most likely represents contamination from the environment or during
sampling. Taken together, there is no evidence that CWCs in the working area harbor microbial symbionts which
potentially could utilize the hydrocarbon-rich fluids. More likely, the CWCs feed on a mixture of phytoplankton,
zooplankton and dissolved organic matter as previously proposed for ones in other regions (Kiriakoulakis et al.,
2005; Duineveld et al., 2007; Becker et al., 2009; Liebetrau et al., 2010). This is in good accordance with the
presence of DNA from various common archaeal and bacterial taxa (e.g. Acidobacteria, Actinobacteria,
Candidatus Nitrosopumilus, Cenarchaeum sp.) and some potential members of the corals’ holobiont (e.g.
Enterobacteria, Verrucomicrobia, Nitrosococcus sp.) (Sorokin, 1995; Rädecker et al., 2015; Webster et al., 2016)
in sample D03-B1 (Fig. 11).

CWC development and hydrocarbon-rich seepage are consequently linked via the formation of MDAC deposits,
which provide the hard substrata needed for CWC larval settlement (e.g. Díaz-del-Rio et al., 2003; Van Rooij et
al., 2011; Magalhães et al., 2012; Le Bris et al., 2016; Rueda et al., 2016). If too severe, however, fluid flow and
associated metabolic processes can result in local conditions that are lethal to CWCs (see 4.3). Moreover, AOM
fueled by fluid flow can also cause an entombment of the CWCs by MDACs (Wienberg et al., 2009, Wienberg &
Titschack, 2015), as observed in D10-R3 and D11-R8 carbonates from the Al Gacel MV (Figs. 7 and 9: Tables 2
and 3). It is therefore not surprising that large CWC systems in the Pompeia Province are always linked to
structures that are affected by rather mild, non-eruptive seepage (i.e. the extinct MV, the coral ridges and the CWC
Mound Fields: Figs. 3 and 6). The observation that these systems are in large parts “coral graveyards” (Fig. 6, B–
D), similar to other areas in the Gulf of Cádiz (see Foubert et al., 2008; Wienberg et al., 2009), may be explained
by a post-glacial decrease in current strength (Foubert et al., 2008). In the light of our findings, however, they
could also have been negatively affected by periods of intensive seepage during higher tectonic activity. Future studies are important to test this hypothesis in greater detail.

4.3. Spatio-temporal co-existence of CWCs and chemosynthetic organisms — the buffer effect

As discussed above, MDAC deposits are ecologically beneficial for CWCs, as they served as optimal substrata even when seepage is still present (e.g. Hovland, 1990; Hovland & Thomsen, 1997; Le Guilloux et al., 2009; this study). Severe hydrocarbon-rich seepage, however, is ecologically stressful for the corals. Particularly, fluid- and AOM-derived hydrogen sulfide is considered problematic because of its role in coral necrosis (Myers & Richardson, 2009; García et al., 2016) and carbonate dissolution effects (Wehrmann et al., 2011).

Hydrogen sulfides can efficiently be buffered through the reaction with Fe-(oxyhydro)-oxides or Fe^{2+} dissolved in pore waters, ultimately forming pyrite (Wehrmann et al., 2011). Fe-(oxyhydro)-oxides nodules have previously been observed in the Iberian and Moroccan margins (González et al., 2009; 2012b), but not in the Pompeia Province. Instead, sulfide-oxidizing bacteria living in symbiosis with invertebrates (e.g. siboglinid worms: Petersen & Dubilier, 2009) (Fig. 5, D) and thriving in mats (Fig. 4, C; Fig. 6, A) were particularly prominent along this region. These microbes may form a biological buffer by withdrawing reduced sulfur species through their metabolic activity. Likewise, the consumption of methane and sulfate by AOM-microorganisms at active sites also contribute to CWCs colonization of the carbonates by reducing environmental acidification.

We propose that this biological buffer provides a further ecological linkage between hydrocarbon-rich seepage and cold-water corals along the Pompeia Province (“buffer effect model”: Fig. 12). This model explains the observed co-existence of non-chemosynthetic corals (e.g. on top of D10-R7 carbonate: Fig. 5) with AOM-microorganisms and chemosynthetic sulfide-oxidizing organisms at pockmark sites at the Al Gacel MV (Fig. 12, A). At the same time, it is in line with associations of sulfide-oxidizing bacterial mats, scleractinian corals, and other non-chemosynthetic octocorals at diapiric ridges and coral mounds in the Northern Pompeia Coral Ridge (Fig. 12, B, C). The impact and exact capacity of this biological buffer, however, remains elusive and must be evaluated in future studies.

5. Conclusions

The presence of cold-water corals related to hydrocarbon-seep structures like mud volcanoes and diapirs, is partly due to the irregular topography affecting bottom water-currents, which supply nutrients to the corals. Likewise, their tight-linkage to active hydrocarbon-rich seepage occurs by means of the production of methane-derived carbonates and how they provide the hard substrata cold-water corals need to develop. The discovery of methane-derived carbonates with embedded corals evidences the decline of coral colonization when the intensity of the fluid seepage increases or becomes more violent. Consequently, cold-water coral growth in these habitats depends directly on seepage intensity and how these fluids are drained onto the seafloor (i.e. eruptive, focused, diffused or dripping-like). Furthermore, cold-water corals rely on the microbial AOM-metabolism and sulfide oxidation to reduce seeped fluids in the environment, since they are harmful for the corals. This biological buffer is possibly crucial to keep conditions favorable for the growth of cold-water corals in the studied area, particularly in times of increased fluid seepage.

Author contribution
Blanca Rincón-Tomás, Dominik Schneider and Michael Hoppert carried out the microbial analysis. Jan-Peter Duda carried out the biomarker analysis. Luis Somoza and Teresa Medialdea processed seismic and bathymetric data. Pedro Madureira processed ROV data. Francisco Javier González and Joachim Reitner carried out the petrographic analysis. Joachim Reitner carried out the stable isotopic analysis. Blanca Rincón-Tomás prepared the manuscript with contributions from all co-authors.

Competing interests
The authors declare that they have no conflict of interest.

Acknowledgments
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References


Table 1. *In-situ* water variables measured during sampling with ROV sensors.

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<thead>
<tr>
<th></th>
<th>D10-R3</th>
<th>D10-R7</th>
<th>D11-R8</th>
<th>D03-B1</th>
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<tr>
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<td>Depth (m)</td>
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<td>39.05 – 39.43</td>
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<td>Dissolved oxygen (mg/l)</td>
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<td>4.71 – 5.09</td>
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Table 2. Stable carbon and oxygen isotopes ($\delta^{13}$C, $\delta^{18}$O) of samples from the Al Gacel MV and the Northern Pompeia Coral Ridge.

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<th>δ¹⁸O (‰)</th>
<th>δ¹³C (‰)</th>
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### Table 3. Stable carbon isotopic composition (δ¹³C) of selected lipid biomarkers (in Figure 10). (*) Please note that crocetane in D11-R8 coelutes with phytane. n.d. = not detected.

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<tr>
<th>Compound</th>
<th>D10-R7 (‰)</th>
<th>D11-R8 (‰)</th>
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<td>n-C₁₈</td>
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</tr>
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<td>n.d.</td>
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</tr>
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<td>Crocetane*</td>
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<td>PMI</td>
<td>−102.9</td>
<td>−74.3</td>
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Figure 1. Bathymetric map of the study area. A: location of the Gulf of Cádiz between Spain, Portugal and Morocco. The study area is marked with a red star; B: the Pompeia Province including its different morphological features. Red lines indicate ROV-paths, yellow stars mark sampling sites; C: detailed map of the Al Gacel MV including pathways of Dive 10 and 11 (black and blue lines, respectively). Further details of the area are provided in Figs. 2 and 3.
Figure 2. Bathymetric and seismic maps showing morphological features in northern Pompeia Province. A–B: bathymetric maps showing the Al Gacel MV, the Northern Pompeia Coral Ridge and the extinct MV. Yellow stars mark sampling sites. C: ultra-high seismic profile of the Pompeia Escarpment, westwards of the Northern Pompeia Coral Ridge.
Figure 3. Ultra-high resolution (A) and multichannel (B) seismic profiles showing geological features in southern Pompeia Province. Note mud diapirism has been described in this area (Vandorpe et al., 2017). OP = overpressure zone.
Figure 4: ROV still frames from the Al Gacel MV (Dives 10 and 11). A: eastern side of the volcano, displaying a field of sponges, corals and carbonates; B–C: active pockmark sites on the east side of the volcano, displaying authigenic carbonate surrounded by shells of chemosynthetic bivalves, fragments of scleractinian and octocorals, as well as sulfide-oxidizing bacterial mats; D: metric-sized carbonate blocks located in a slope at the summit of the volcano.
Figure 5: ROV still frames from the active pockmark site shown in Fig. 4. B–A: release of bubbles while sampling; C: detailed photograph of the octocorals on top of the carbonate; D: detailed still frame from siboglinid worms beneath the carbonate.
Figure 6. ROV still frames from the Northern Pompeia Coral Ridge and extinct MV (Dive 03), where there is currently a diffused seepage of fluids. A: abundant shells of chemosynthetic bivalves with sulfide-oxidizing bacterial mats at the western site of the Northern Pompeia Coral Ridge; B–D: field of dead scleractinian-corals colonized by living corals; D: still frame from the extinct MV.
Figure 7. Photographs of analyzed samples including sampling sites for stable carbon and oxygen isotope (δ13C, δ18O) analysis (crosses with numbers). Values of the stable isotopic analyses are found in Table 2. A–B: D10-R3 carbonate with embedded corals; C–D: D10-R7 carbonate with strong H2S odor; E–F: D11-R8 carbonate with embedded corals; G: D03-B1 scleractinian-coral fragment, Madrepora oculata.
Figure 8. Thin section photographs of MDACs. A–B: D10-R3 consisting of a micritic matrix with scattered foraminifers and oxidized frambooidal pyrites (reflected light); C–D: D10-R7 consisting of micritic and microsparitic carbonate with abundant unaltered frambooidal pyrites (C, transmitted light; D, reflected light). Please note open voids which represent potential pathways for fluid seepage (yellow circle in C).
Figure 9. Stable carbon and oxygen isotopes ($\delta^{13}$C, $\delta^{18}$O) of samples from the Al Gacel MV and the Northern Pompeia Coral Ridge (see Figure 3 for precise sampling points).

Figure 10. Total ion current (TIC) chromatograms of the analyzed samples. Isotopically depleted acyclic irregular isoprenoids such as Cr and PMI are typically found in settings influenced by the anaerobic oxidation of methane (AOM). Pr = pristane; Ph = phytane; Cr = crocetane; PMI = 2,6,10,15,19-pentamethylcosane; dots = n-alkanes; crosses = siloxanes (septum or column bleeding). Percentage values given on the vertical axes of chromatograms relate peak intensities to highest peak (Cr in D10-R7).
Figure 11. Bar charts representing relative abundances of prokaryotic taxa detected in each sample. A: bacterial taxa; B: archaeal taxa. In “others” aggregation is included taxa related to ubiquitous organism normally found in sea- and seepage-related environments, and unclassified organisms. Number of reads per taxa detailed in Table S1 (bacteria) and Table S2 (archaea).
Figure 12. The buffer effect model. A: Buffer effect at pockmark sites (e.g. sampling site of D10-R7) where carbonates are formed directly on the bubbling site acting as a cap; B: Buffer effect at diapiric ridges where MDAC slabs are formed on the base of the ridge; C: Buffer effect at coral mounds where MDAC slabs are formed in deeper layers of the sediment. Py = pyrite, SMTZ: sulfur-methane transition zone.