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METHODS
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Seep hunting in the Santos Basin, Southwest Atlantic: sampling strategy and employed methods of the multidisciplinary cruise BIOIL 1

Paulo Y. G. Sumida[1,*], Vivian H. Pellizari[1], Rafael A. Lourenço[1], Camila N. Signori[1], Amanda G. Bendia[1], Orlemir Carrerette[1], Fernanda M. Nakamura[1], Raissa B. Ramos[1], Gilberto Bergamo[1], Bruno H. M. Souza[1], Ana C. A. Butarelli[1], Júlia G. Passos[1], Rodolfo J. S. Dias[1], Mascimiliano Maly[1], Thomás N. S. Banha[1], Arthur Z. Güth[1], Luiza F. Soares[1], Paula D. N. Perugino[1], Felipe R. Santos[1], Frederico R. Santana[1], Michel M. de Mahiques[1]

[1] Instituto Oceanográfico, Universidade de São Paulo, Praça do Oceanográfico, 191 - CEP 05508-120, São Paulo-SP, Brazil

* Corresponding author: psumida@usp.br

ABSTRACT

The continental margin off the southeastern Brazilian coast is punctuated by a series of geological-geomorphological features, such as subsurface saline diapirs and pockmarks at the seafloor interface, which evidence the abundant presence of oil and gas in the region. In several of these sites, hydrocarbons can be naturally released into the water column, areas are cold seep areas. These are marked by the presence of oil- and gas-dependent ecosystems, where specific organisms are able to fix carbon from hydrocarbon chemosynthesis. In addition, light hydrocarbon fluid flow through the sediment may build up authigenic carbonates that can be further colonized by cold-water corals, generating large carbonate mounds over geological time, normally positioned at the border of these pockmark features. The present work reports on a multidisciplinary oceanographic cruise carried out in the Santos Basin, SW Atlantic, to seek, map, and collect geological, chemical, and biological data from different deep-sea habitats. The cruise occurred in November 2019 on the R/V Alpha Crucis of the Oceanographic Institute of the University of São Paulo (IOUSP). We intended to discover and detail different geomorphological features, characterize free-living and symbiotic microorganisms, determine the chemosynthetic rates in relation to heterotrophic microbial production, and characterize the fauna and study their ecological and evolutionary links within and across ocean basins. All discoveries made during the cruise and their respective results will be presented separately in several papers that comprise this special volume.

Descriptors: Pockmarks, Chemosynthesis, Macrobenthos, Carbonate mounds, and Microbial diversity.

INTRODUCTION

The deep seafloor is formed by a series of geomorphological features that ultimately define a mosaic of habitats where different bottom-associated communities thrive. One such feature, the pockmark, is abundant in the Santos Basin (SW Atlantic) (Leyden et al., 1976; Sumida et al., 2004; de Mahiques et al., 2017). Pockmarks are formed by the escape of gas from the subsurface that, in the Santos Basin, are tectonically conducted through salt diapirism (de Mahiques et al., 2017; Schattner et al., 2018). They are circular/elliptical-shaped depressions that can be over 1 km wide and 100 m deep (Schattner et al., 2016). Pockmarks can also act as particulate organic

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matter traps (Ramos et al., 2019) and alter near-bed circulation (Pau et al., 2014). Light hydrocarbon fluid flow through the sediment may also build up authigenic carbonates that can be further colonized by cold-water corals, generating large carbonate mounds over geological time (Hovland, 1990; Wheeler et al., 2007), normally positioned at the border of pockmarks (Sumida et al., 2004).

Depressions and mounds on the seafloor affect the benthic ecosystem in multiple ways in terms of water flow, type and amount of food availability, and sedimentation rates. The depositional environment formed at pockmarks tends to be colonized by invertebrate infauna mainly composed of annelids, mollusks, and crustaceans (Sumida et al., 2004; Sánchez et al., 2021). On the other hand, carbonate mounds are subject to stronger currents and are dominated by larger filter-feeding animals, such as cnidarians, sponges, and brittle stars (Henry and Roberts, 2007). In places where deep-sea corals abound, a higher proportion of macrofauna richness is associated with coral skeletons. These organisms seek shelter in the complex three-dimensional structure of dead coral fragments, frequently living among coral rubble in crevices and perforating the coral skeleton (Roberts et al., 2008). Coral rubble can occur both at the top and on the sides of carbonate mounds and within the pockmarks (Sumida et al., 2004).

In some active pockmarks, the seepage of hydrocarbons may completely alter the community composition and structure (Guillon et al., 2017; Olu et al., 2017). The flow of light hydrocarbons, especially methane, induces the microbial reduction of sulfate into sulfide through the anaerobic oxidation of methane. Both reduced compounds (i.e., methane and sulfide) fuel the chemosynthesis of organic compounds by microorganisms, entering the trophic cascade either through direct microbial cell consumption or through a series of complex symbiotic relations of microorganisms with their invertebrate hosts (Levin, 2005; Cordes et al., 2010). These environments (i.e., cold seeps) provide alternative evolutionary pathways for biota and are important locations for studying the connectivity and evolution of populations across basins (Levin, 2005).

Although seeps and associated communities are widespread on continental margins worldwide, little information is found for the eastern South American continental margin. In Brazil, seep fauna was only briefly described by Giongo et al. (2015) and Medina-Silva et al. (2017) off the southern margin. Other studies gathered evidence of seep organisms, but without a proper description of the environment (Tommasi, 1970; Domaneschi and Lopes, 1990; Sumida et al., 2004). Conversely, some geological and geophysical studies describe seeps without reporting a specialized fauna (Miller et al., 2015; Ketzer et al., 2018). In the Santos Basin, large pockmark fields are directly related to the intense salt tectonic activity in the subsurface, indicated by several exhumed salt diapirs (de Mahiques et al., 2017) and shallow and deep seismic records. These indications may suggest that natural seepage of hydrocarbon fluids through sediments occurs throughout this margin.

The present work reports on a multidisciplinary oceanographic cruise carried out in the Santos Basin (SW Atlantic) to seek, map, and collect geological, chemical, and biological data from seeps. The cruise occurred in November 2019 aboard the R/V Alpha Crucis of the Oceanographic Institute of the University of São Paulo (IOUSP). It is part of the ongoing Project 'Biology and Geochemistry of Oil and Gas Seepages, Southwest Atlantic (BIOIL)', coordinated by IOUSP and funded by Shell Brasil Petróleo LTDA through R&D levy regulation of Agência Nacional do Petróleo, Gás Natural e Biocombustível (ANP) of the Brazilian government. We intended to: discover and detail different geomorphological features; characterize free-living and symbiotic microorganisms; describe the microbiome of seep organisms in the SW Atlantic; assess the interaction between chemosynthetic communities and chemistry of the seep area; study the links between microbial communities and methane emissions (i.e., concerning global warming; Ruppel and Kessler, 2017); discover possible microbes capable of hydrocarbon degradation or altering the precipitation of methane hydrates; determine the chemosynthetic rates in relation to heterotrophic microbial production; characterize the fauna, and study its ecological and evolutionary links within and across ocean

basins. We present herein the sampling strategy and basic methodologies employed during the cruise BIOIL 1. All discoveries made during the cruise and their respective results will be presented separately in several papers that make up this special volume.

METHODS

The first oceanographic cruise of Project BIOIL occurred on November 11-24, 2019, with a total navigation distance of 1,550 nautical miles in the Santos Basin (SW Atlantic). We chose three areas of interest (A1, A2, A3) along the continental slope, between 400-800 m in depth (Figure 1). In each area, multibeam (MBES) and Chirp lines were performed, especially where features such as pockmarks and carbonate mounds were present. We also looked for acoustic signals potentially related to gas escape that guided sampling for geology, chemistry, microbiology, and macrobenthos for these sites, both on the seafloor and in the water column. Hundreds of miles of MBES + Chirp lines were collected, together with CTD-Rosette casts (Sea-Bird CTD/Carrousel 911 system with 12 10-L Niskin bottles) for hydrographic profile and water column microbiology, gravity corer samples for geology and microbiology, and box corer (Ocean Instruments – 0.25 m^2 of area) samples for geology, chemistry, microbiology, and benthic ecology (Table 1).

Box-corer samples were collected in triplicate at ten oceanographic sites, totaling 27 sediment samples. At some sites, triplicate sampling was not possible due to the nature of the seabed. After the recovery of the box corer, sediment samples were divided into two equal parts (~0.125 m^2 of area each). One half of the box corer sample was destined for macrofaunal study, while the other half was subsampled for geochemical, microbiological, and geological analyses. Sampling methods for each discipline are further detailed in the respective sections below and illustrated in Figure 2.

Geophysical data

Geophysical (acoustic) data were obtained in two survey areas. Multibeam data were obtained using a Reson Seabat 7160, with a central

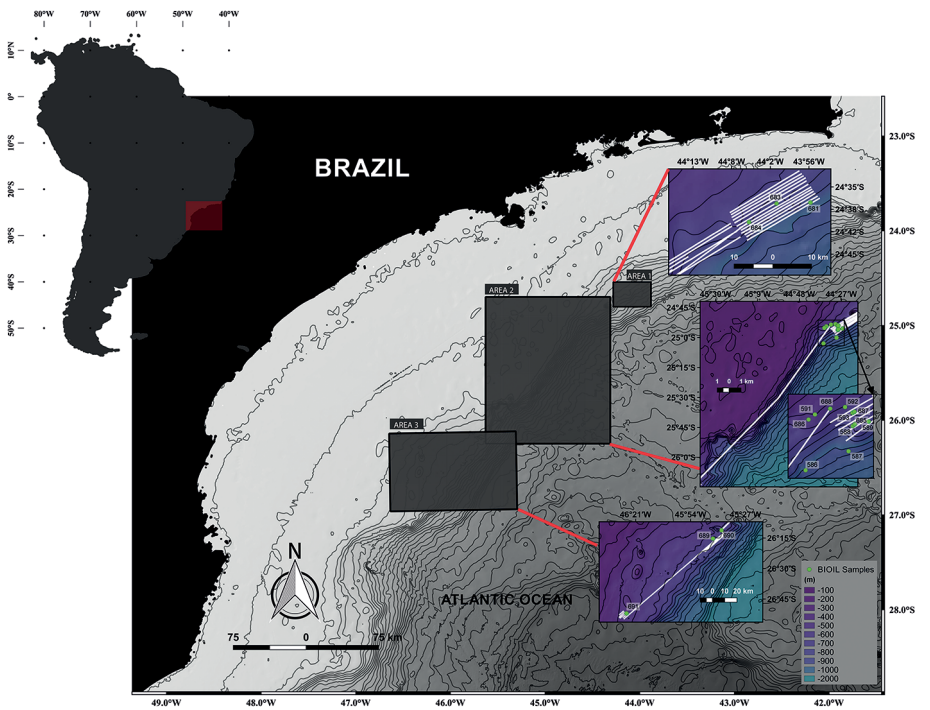

Figure 1. Acoustic survey tracks (white) and sampling sites (green markers) in the three sampling areas along the SE Brazilian continental margin investigated during Project BIOIL in November 2019 using the R/V Alpha Crucis.

Table 1. List of sites and collected samples by instruments of the First Survey of the Project BIOIL in November 2019 on board the R/V Alpha Crucis. CTD = Conductivity, Temperature and Depth sensor; ROV = Remotely Operated Vehicle; BC = Box Corer.

Area	Site	Sampling methods	Latitude	Longitude	Depth (m)	Sediment	Habitat
Area 1	681	CTD-Rosette; BC (3x)	-24.5221	-43.9308	~740	Carbonate sand	Sandy bottom
	682	CTD-Rosette; ROV Dive	-24.5214	-43.9285	~740	–	–
	683	CTD-Rosette; BC (3x)	-24.6246	-44.0147	850	Muddy carbonate sand	Base of carbonate mound
	684	CTD-Rosette; Gravity corer; BC (1x)	-24.6785	-44.0816	829	Muddy carbonate sand	Carbonate mound
Area 2	685	CTD-Rosette; BC (3x)	-24.4324	-44.4736	600	Carbonate	Carbonate mound
	686	CTD-Rosette; Gravity corer; BC (3x)	-24.9206	-44.5908	581	Sandy mud	Base of carbonate mound
	687	CTD-Rosette; BC (3x)	-24.8995	-44.4760	675	Mud	Carbonate mound
	688	CTD-Rosette; BC (3x)	-24.8921	-44.5351	564	Mud	Carbonate mound
Area 3	689	CTD-Rosette; BC (3x)	-26.2623	-45.7144	870	Mud	Pockmark
	690	CTD-Rosette; Gravity corer; BC (3x)	-26.1926	-45.6454	~730	Mud	Pockmark
	691	CTD-Rosette; BC (2x)	-26.8870	-46.4122	519	Mud	Diapir
	692	CTD-Rosette	-26.8834	-46.4177	–	–	–
	693	CTD-Rosette	-26.8482	-46.4235	–	–	–

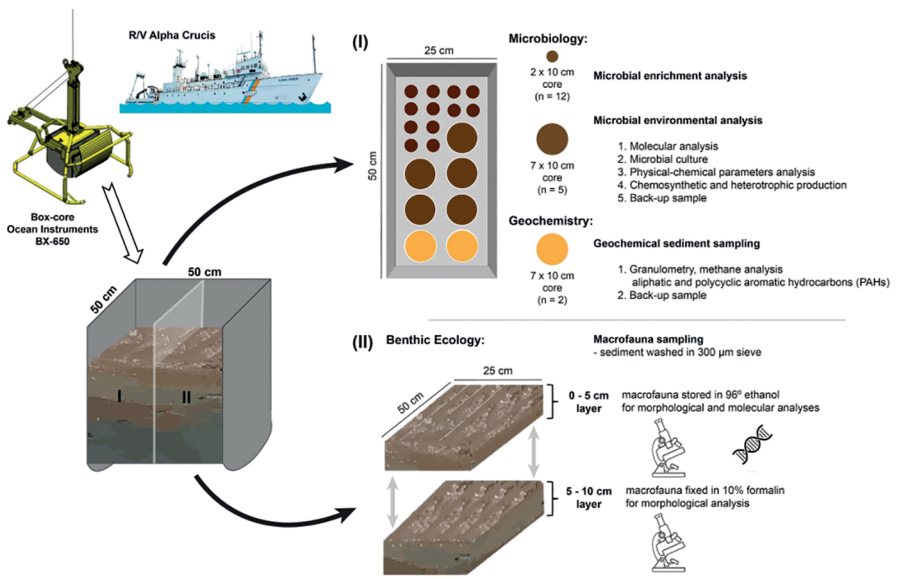

Figure 2. Box-core sample fate and analysis for each discipline during Project BIOIL cruise in November 2019 using the R/V Alpha Crucis.

frequency of 44 kHz along several lines parallel to the coast (Figure 1). Sub-bottom data were obtained with a 3260 Knudsen Chirp, with a central frequency of 3.5 kHz. Both instruments have their transducers installed in the hull of the R/V Alpha Crucis.

During the cruise, we revisited an area described in Maly et al. (2019) to improve acoustic mapping. This activity resulted in the discovery of a >40 km straight carbonate ridge (Figure 3) and a canyon-like depression, which is likely to be controlled by neotectonics (Figure 4).

A third sampling area corresponded to a giant pockmark field, previously described by Schattner et al. (2016), de Mahiques et al. (2017), and Ramos et al. (2019). In that area, evidence of residual and paleo-seepage was reported by dos Santos et al. (2018) and Portilho-Ramos et al. (2018), respectively. It also presents some of the few submerged exhumed salt diapirs in the South Atlantic Ocean (Schattner et al., 2018) (Figure 5).

Geological and chemical sediment sampling

The surface sediment from the box-corer samples (see 'Methods') was collected and stored in aluminum containers and kept frozen at -20°C for analyses of granulometry, aliphatic hydrocarbons, and polycyclic aromatic hydrocarbons (PAHs).

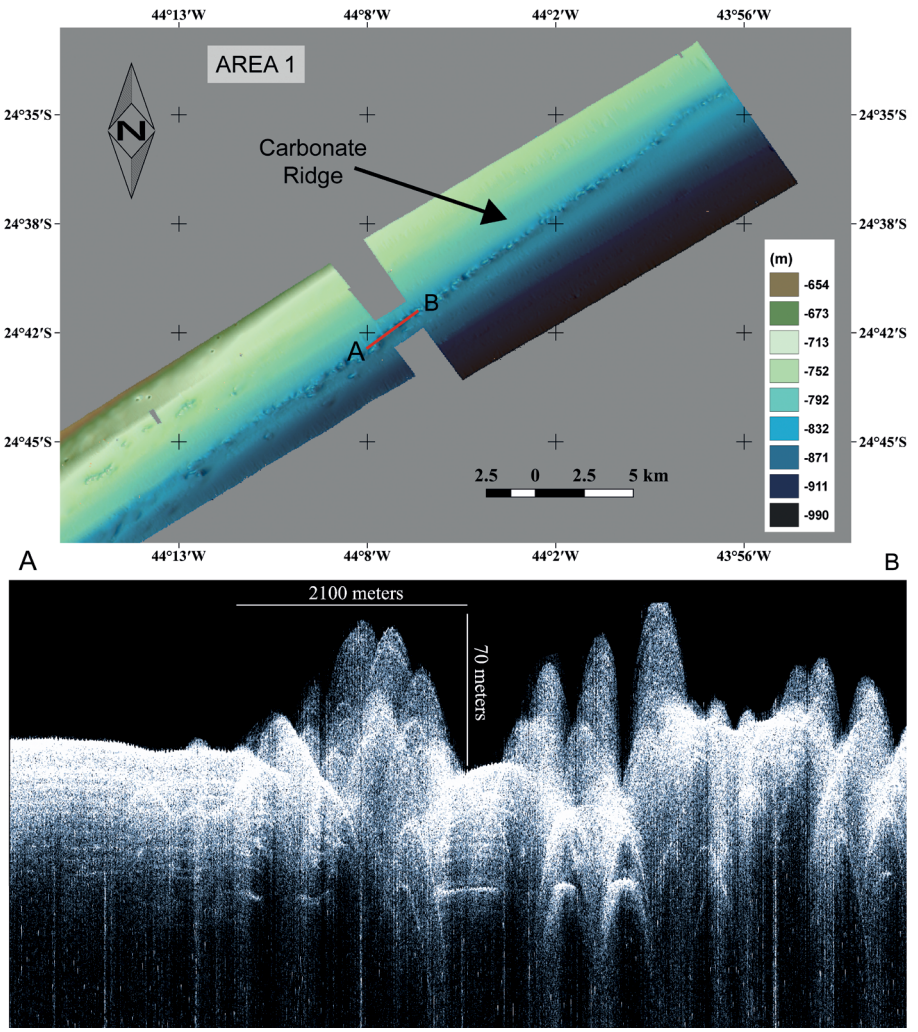

Figure 3. Multibeam (top panel) and chirp (bottom panel) data showing an aligned carbonate ridge recorded during the Project BIOIL cruise in Santos Basin (Area 2). The location of the chirp line is marked as A-B on the multibeam map.

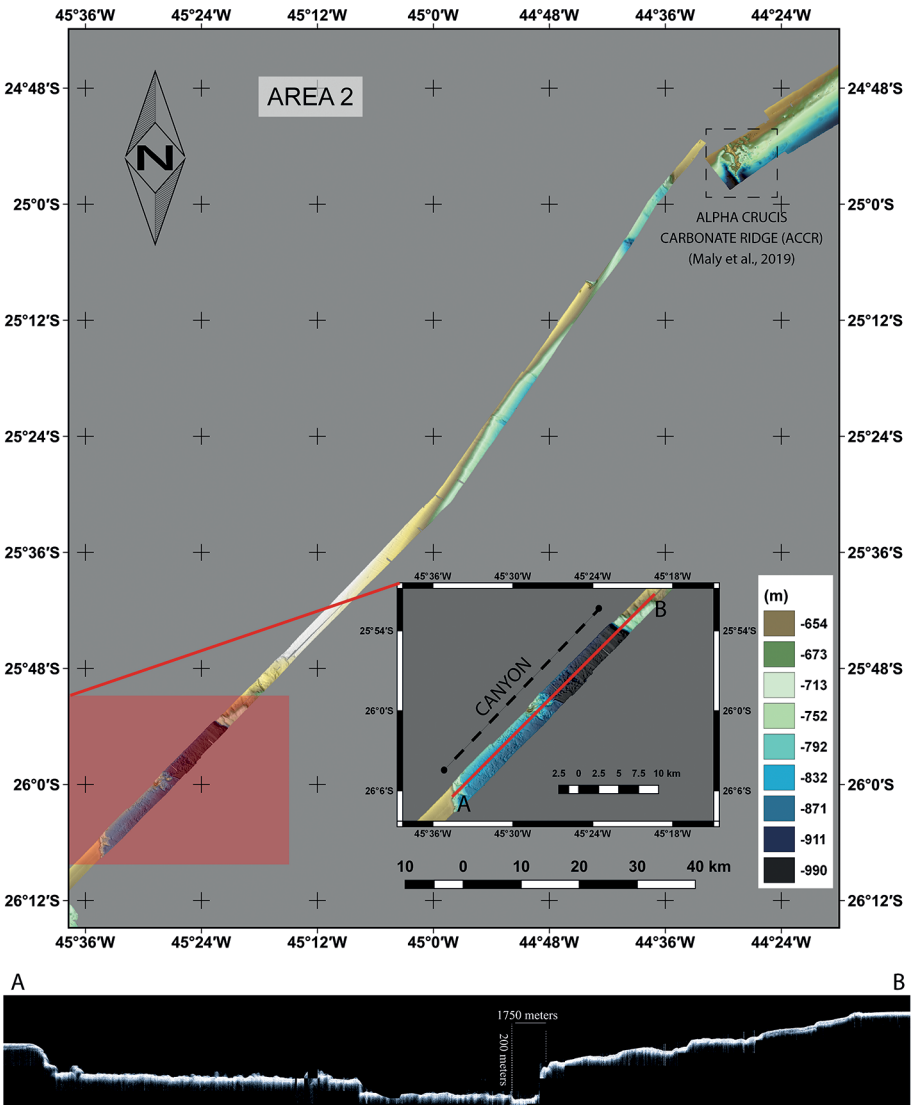

Figure 4. Multibeam (top) and chirp (bottom) records of tectonically-controlled canyon-like depression identified during the Project BIOIL cruise in Santos Basin (Area 2). The location of the chirp line is marked as A-B on the insert map.

Sampling for methane analysis was performed from a sediment PVC core (7 x 20 cm) subsampled from box corer samples at each site (Figure 2). The following sampling procedure was adopted from Marinho et al. (2012): a 5 cm^3 sediment sample was collected from a freshly exposed core section (every 10 cm, from the top up to 30 cm depth) using an open-ended plastic syringe. The sample was collected by inserting the syringe into the sediment, keeping the plunger next to the sediment surface to avoid contamination by atmospheric gases or trapped air bubbles. After sampling, 3 cm^3 of sediment was extruded into a 20 mL vial containing 6 mL of 1M NaOH 1M, and the excess sediment was discarded. The vial was immediately capped with a silicone/PTFE septum and shaken vigorously to induce methane equilibration with the headspace for later analysis in a gas chromatograph (5890A, Hewlett Packard) equipped with a packed, stainless steel Porapak-Q column (Agilent 3 m, 0.32 cm, 80-100 mesh) and a flame ionization detector.

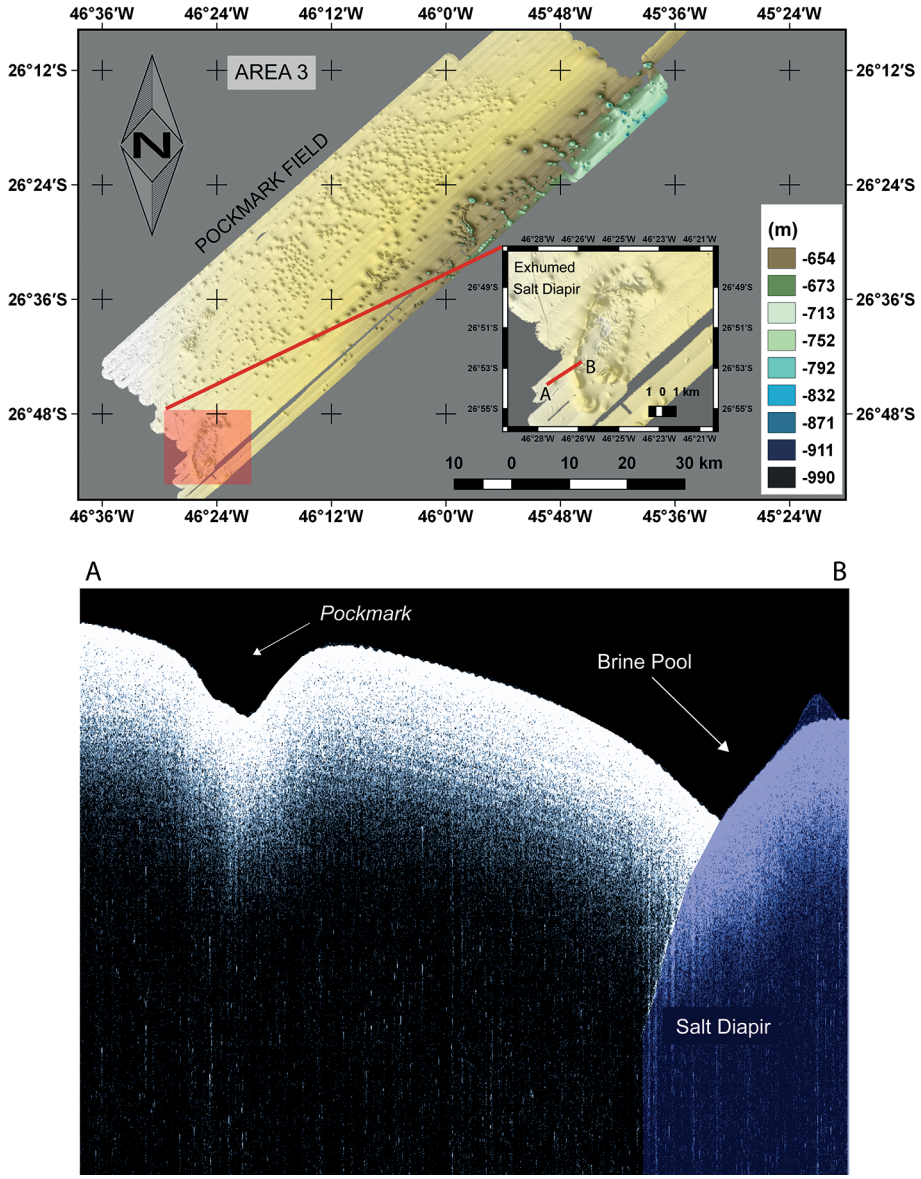

Figure 5. Top. Multibeam record of the southern part of a giant pockmark field, described by Schattner et al. (2016) and de Mahiques et al. (2017). In the southernmost part, an exhumed diapir, bordered by several pockmarks, is present. Bottom. 3.5 kHz chirp section showing the spatial relationship between the exhumed diapir and adjacent sediments. The location of a pockmark and a possible brine pool is shown.

Sediment and water sampling for microbiology

For the microbial environmental analyses, sediments were subsampled from the box corers using five 7 x 20 cm PVC cores, extruded and subdivided at layers of 0-5, 5-10, and 10-15 cm. Each PVC core was selected for the following analyses: (1) molecular; (2) culturing; (3) physical-chemical parameters; (4) chemosynthetic and heterotrophic production, and (5) to retain a back-up. In addition, two gravity-corer samples were collected at sites P684 and P686, comprising sediment cores 200 cm and 350 cm long. All sediment samples were stored in Whirl-pack bags at -80°C. For the study of pelagic microbial communities, seawater samples were collected with the CTD-Rosette at three depths in each station – surface, intermediate (halfway to the bottom), and bottom – comprising different pelagic zones and water masses. Approximately 20 L of each water sample were

filtered using a peristaltic pump through 0.22 µm-membrane Sterivex™ filters and frozen at -80˚C for molecular analysis. Molecular analyses of sediment and water samples will include 16S rRNA sequencing (metataxonomy) and shotgun metagenomics to reveal, respectively, the taxonomic and functional diversity of microbial communities.

For nutrient analyses, approximately 400 mL of filtered water samples were stored in amber flasks at room temperature or in bottles frozen at -20˚C. To assess the dissolved methane in water from all depths, 50 mL water samples were collected in 60 cc syringes, and the headspace equilibrium technique was applied as follows: 10 mL of argon flushing, syringe content mixing, and headspace gas harvesting. This gas was stored in airtight serum vials at room temperature for onshore gas chromatograph analysis.

Microbial culture samples

The sediments subsampled from the box corer were also destined for the culture of methanotrophic microorganisms. The three sediment layers (0-5 cm, 5-10 cm, and 10-15 cm) were homogenized prior to cultivation. For the culture of aerobic microbial consortia, 10 g of sediment was stored in Whirl-pack bags at 4˚C. For the culture of anaerobic microbial consortia, the sediment was stored in Hungate tubes, the headspace of the tubes was harvested by the vacuum formed using an attached 60 cc syringe and filled with argon, then stored at 4°C until inoculation in minimal NMS culture medium onshore.

Microbial enrichment samples

The DNA-based Stable Isotope Probing technique (DNA-SIP) was applied for methane enrichment analysis, using sediment samples from a single box core per site (681, 683, 685, 686, and 690). Twelve intact mini-cores (10 x 2 cm) with fifty grams of sediment were collected in 60 cc open-ended syringes using manual vacuum (generated by a 200 cc syringe), then sealed airtight with bottom caps and upper syringe 3-way taps. The headspace of the intact mini-cores was oxygen depleted by argon flushing, and cores were stored vertically at 4°C for further analyses onshore.

Chemosynthetic primary production and heterotrophic microbial production

To determine the chemosynthetic production (also referred to as dark carbon fixation) and the heterotrophic microbial production in the sediments and water column, *in situ*-simulated dark incubations were performed with ^{14}C-bicarbonate (e.g. Steemann-Nielsen, 1952, Reinthaler et al., 2010) and ^{3}H-leucine (Kirchman et al., 1985; Smith and Azam, 1992), respectively. After 6-12 h of incubation, depending on the sampling area and depth, the microbial activity was interrupted by adding formaldehyde 2%. Only the water samples for chemosynthesis were filtered through 0.22 µm membranes using a vacuum pump and a manifold system. All samples (sediment and water) for both processes (chemosynthesis and heterotrophic microbial production) were transferred into cryovials and stored at 4˚C for further analysis. To measure chemosynthesis, the membranes and sediment samples were exposed, in the laboratory, to concentrated HCl fumes to remove the remaining $^{14}CO_2$, and a scintillation cocktail was added to the vials. To determine heterotrophic production rates, sediment and water samples were treated with trichloroacetic acid, Mili-Q water, and ethanol through successive steps of centrifugation for protein extraction before adding the scintillation cocktail. Microbial production rates were measured in the scintillation counter (Packard Tri-Carb 2800TR), and the results obtained in disintegrations per minute were converted into production rates ($\mu C.mg^{-3}.h^{-1}$).

Benthic macrofauna

The 27 sediment samples collected with the box-corer and destined to macrofaunal studies (0.125 m^2) were elutriated and posteriorly sieved using a 500 µm and 300 µm mesh before preservation in ethanol or formaldehyde. Several macrofaunal specimens were sorted under a stereomicroscope and photographed alive immediately after collection (Figure 6). Part of the sample destined for morphological and taxonomic studies was fixed in formaldehyde 4% and stored at room temperature. The remaining samples were bulk preserved

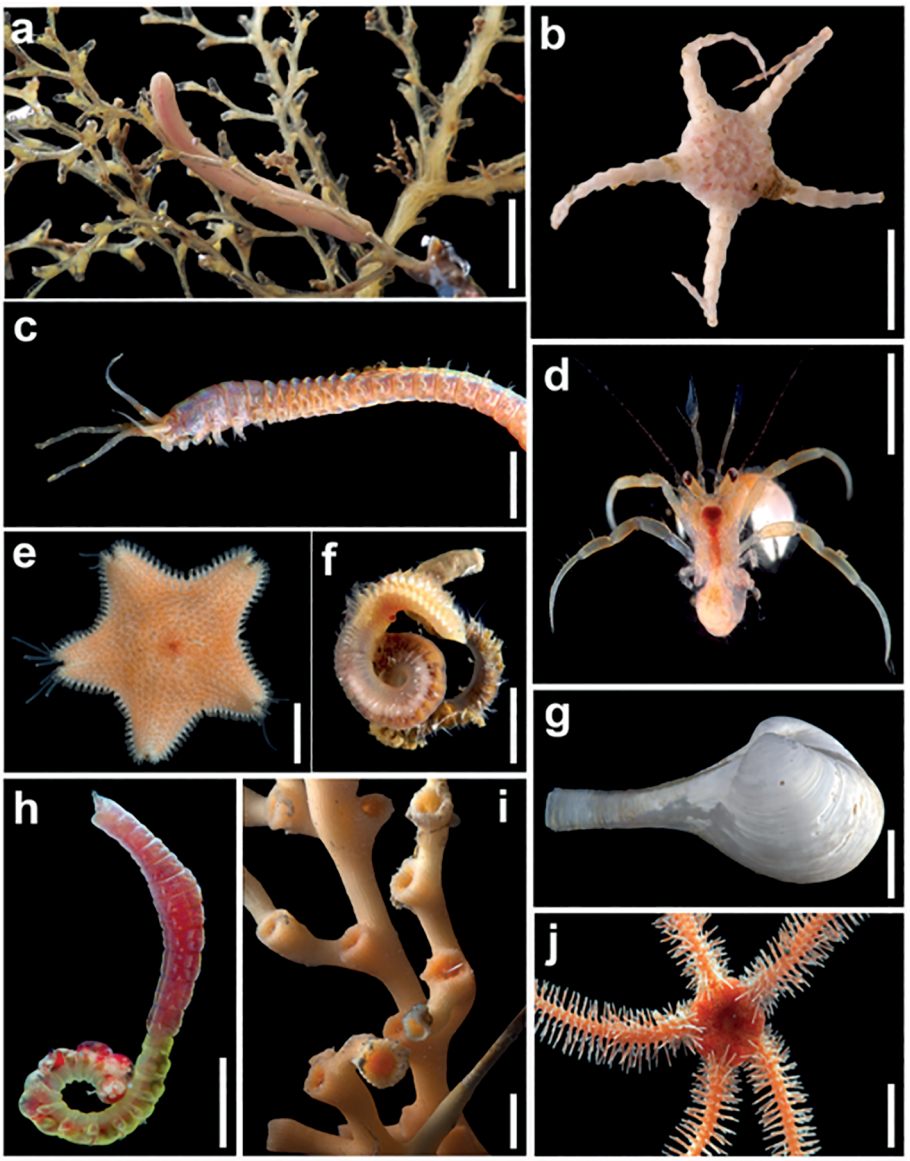

Figure 6. Benthic invertebrates collected with a box core during Project BIOIL Cruise I in Santos Basin, SW Atlantic. a) Solenogastres aplacophoran mollusk on a hydroid colony (scale bar: 0.5 mm); b) Oral view of the ophiuroid Ophiomisidium tommasii (scale bar: 0.5 mm); c) Onuphid polychaete (scale bar: 0.1 mm); d) Dorsal view of a pagurid crustacean (scale bar: 0.5 mm); e) Asterinid sea star, aboral view (scale bar: 0.5 mm); f) Paranoid polychaete (scale bar: 0.1 mm); g) Cuspidariid bivalve mollusk (scale bar: 0.1 mm); h) The polychaete Notomastus sp. (scale bar: 0.5 mm); i) The cold-water coral Solenosmilia variabilis (scale bar: 1.0 cm); j) Aboral view of the ophiuroid Ophiacantha sp. (scale bar: 0.5 mm).

in 96% ethanol and stored at 4ºC for further molecular analyses. The specimens selected for scanning electron microscopy (SEM) analysis were fixed in trialdehyde (glutaraldehyde + paraformaldehyde + sodium cacodylate) at 4ºC for 2 h and stored in sodium cacodylate at room temperature. To ensure the best results for DNA extraction and analyses of mitochondrial and nuclear DNA, the selected specimens were fixed in molecular-grade absolute ethanol and stored at -20 ºC.

CONCLUSION

Cold seeps are important ecosystems from ecological, environmental, and economic perspectives. Seep biota evolved under particular physical-chemical conditions, with organisms adapting to this

environment by acquiring new metabolic pathways and becoming new species to cope with this unique and challenging environment. In addition, the large volume of methane, a potent greenhouse gas, seeping from the seabed in these regions may generate a significant global impact. The presence of pockmarks and seeps also indicate high probabilities of oil and natural gas reserves in the area, of great interest to the oil and gas industry. This unique environment is likely to be a source of new discoveries and breakthroughs in biotechnology, as potential new microorganisms can be described and trigger new applications for the pharmaceutical and biotechnological industries, that derive innovation from the study of diverse and unique microbial metabolism. We have conducted a large survey on the continental slope of the SW Atlantic Ocean, and geophysical data revealed numerous potential gas seepages. The diversity of invertebrate species collected in our samples also suggests the presence of both active and inactive seeps. These seeps are mostly located at the base of big carbonate mounds, where soft sediment predominates. At the top, a thriving fauna associated with living and dead cold-water corals is present. Further, the combination of culture-dependent and independent approaches, with measurements of chemosynthetic and heterotrophic production rates, will allow us to reveal the microbial seeps indicators, as well as describe the active bacteria and archaea involved in chemosynthetic processes, especially methanotrophy. In this first cruise of Project BIOIL, we have mapped and gathered strong evidence of promising seep areas where methane seeps may be active. This initial data will provide direction to the forthcoming cruise, when we plan to use a suite of new equipment, including ROV imaging and sampling, an *in situ* methane analyzer, and a new USBL system.

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AUTHOR CONTRIBUTIONS

P.Y.G.S.: Conceptualization; Funding acquisition; Investigation; Resources; Supervision; Writing - original draft; Writing - review & editing.

V.H.P., R.A.L., C.N.S.: Conceptualization; Investigation; Resources; Writing - review & editing.

A.B.G., O.C., F.M.N., G.B., B.H.M.S., T.N.S.B., A.Z.G., P.D.N.P.: Investigation; Writing - review & editing.

R.B.R., A.C.A.B., J.G.P., R.J.S.D., M.M., L.F.S., F.R.S.: Investigation.

M.M.M.: Conceptualization; Funding acquisition; Investigation; Resources; Writing - review & editing.

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