

Multidisciplinary Scientific Cruises for Environmental Characterization in the Santos Basin – Methods and Sampling Design

Daniel L. Moreira¹, Adriana G. Dalto², Alberto G. Figueiredo JR³, Aline M. Valerio⁴, Amalia M. S. Detoni⁴, Ana C.T. Bonecker⁵, Camila N. Signori⁶, Cláudia Namiki⁶, Dalton K. Sasaki⁶, Daniel V. Pupo¹², Danilo A. Silva⁶, Deborah S. Kutner⁶, Diana C. Duque-Castaño⁶, Eduardo H. Marcon¹, Fabiana D.C. Gallotta¹, Fabiana S. Paula⁶, Fabiane Gallucci⁷, Gabriela C. F. Roque⁶, Giulia S. Campos⁶, Gustavo Fonseca⁷, Gustavo Mattos¹¹, Helena P. Lavrado², Ilson C. A. da Silveira⁶, Jessica O. Costa¹¹, João R. dos Santos Filho³, Juliane C. Carneiro³, Julio C.F. Moreira⁶, Laura Roza⁸, Leandro F.M. Araujo¹, Letícia Lazzari⁶, Letícia O. da Silva¹¹, Luan S. Michelazzo⁶, Luciano F. Fernandes⁹, Marcelo Dottori⁶, Marcus A. G. de Araújo Jr.¹, Mateus G. Chuqui⁶, Milena Ceccopieri⁶, Milton Borges-Silva⁶, Milton Kampel¹⁴, Natascha M. Bergo⁶, Paulo V. M. Silva⁴, Pedro M. Tura⁶, Rafael B. de Moura², Renato G. Romano⁶, Renato P. Martins¹, Renato S. Carreira⁸, Rodrigo G.A. Toledo¹⁰, Sérgio L.C. Bonecker⁵, Sibelle T. Disaró¹², Silvana V. Rodrigues¹³, Thais N. Corbisier⁶, Thaisa M. Vicente⁶, Vitor G. de Paiva⁴, Vivian H. Pellizari⁶, Wellington C. Belo¹, Frederico P. Brandini⁶, Sílvia H.M. Sousa⁶

¹ PETROBRAS Research Center. Centro de Pesquisas Leopoldo Américo Miguez de Mello (CENPES). Av. Horácio de Macedo, 950 - Cidade Universitária da Universidade Federal do Rio de Janeiro, CEP 21941-915, Rio de Janeiro, - RJ, Brazil

² Universidade Federal do Rio de Janeiro, Instituto de Biologia, Departamento de Biologia Marinha, Av. Carlos Chagas Filho, 373 Prédio do CCS - Bloco A, sala A1-089, CEP 21941-590, Rio de Janeiro, RJ, Brazil

³ Universidade Federal Fluminense (UFF), Programa de Pós-Graduação Dinâmica dos Oceanos e da Terra (DOT) Niterói, RJ, Brazil.

⁴ Instituto Nacional de Pesquisas Espaciais, Divisão de Observação da Terra e Geoinformática, Av. Dos Astronautas 1758, São José dos Campos, SP, Brazil CEP 12227-010

⁵ Universidade Federal do Rio de Janeiro, Instituto de Biologia, Departamento de Zoologia, Av. Carlos Chagas Filho, 373 Prédio do CCS - Bloco A, sala A0-084, CEP 21941-590, Rio de Janeiro, RJ, Brazil

⁶ Universidade de São Paulo, Instituto Oceanográfico, Praça do Oceanográfico, 191, CEP 05508-120, São Paulo, SP, Brazil

⁷ Universidade Federal de São Paulo, Instituto do Mar, - Campus Baixada Santista, Santos, SP, Brazil

⁸ PUC-Rio, Departamento de Química, Laboratório de Estudos Marinhos e Ambientais (LabMAM). Rua Marquês de São Vicente, 225. CEP 22451-900 Rio de Janeiro, RJ, Brazil

⁹ Universidade Federal do Paraná, Setor de Ciências Biológicas, Departamento de Botânica, Curitiba, PR, Brazil CEP 81531-990

¹⁰ SALT Engenharia e Meio Ambiente LTDA. Avenida Professor Lineu Prestes 2242, Sala 101. CEP 05508-000. Butantã, São Paulo SP, Brazil

¹¹ Federação das Indústrias do Estado do Rio de Janeiro/Serviço Nacional de Aprendizagem Industrial (FIRJAN/SENAI). R. Mariz e Barros, 678. CEP 20270-002 - Maracanã, Rio de Janeiro, RJ, Brazil

¹² Universidade Federal do Paraná, Museu de Ciências Naturais, Laboratório de Foraminíferos e Micropaleontologia Ambiental, Av. Cel. Francisco H. dos Santos, 100 – Jardim Américas, CEP 81530-000, Curitiba, PR, Brazil

¹³ Universidade Federal Fluminense, Centro de Estudos Gerais, Departamento de Química Analítica. Outeiro de São João Batista S/N, Centro, CEP 24020150 - Niterói, RJ - Brazil

* Corresponding author: danielmoreira@petrobras.com.br

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ABSTRACT

The Santos Basin (SB) is the main petroliferous basin in the Brazilian continental margin and one of the most studied marine areas in Brazil. However, historical data suggest that new efforts should be carried out to acquire quantitative biological data, especially in the deep sea, to establish the baseline of essential ocean variables in different ecosystems for future monitoring programs. The Brazilian energy company Petrobras planned and executed 24 oceanographic cruises over a period of 2 years to assess the benthic (SANSED cruise) and pelagic (SANAGU cruise) systems of the SB (356 days at sea in 2019 and 2021/2022). These efforts were part of the Santos Project, which comprised a comprehensive environmental study aimed at investigating benthic and pelagic variables to characterize ecology, biogeochemistry, thermohaline properties of water masses, and ocean circulation patterns, geomorphology, and sedimentology, as well as organic and inorganic chemistry. Here we present the detailed sampling designs and the field methods employed on board, during the SB scientific cruises. All sampling protocols were based on standardized approaches. For the benthos analyses, triplicate sediment samples were performed using a GOMEX-type box corer (0.25 m²) or a large modified Van Veen grab (0.75 m²) at 100 stations ranging from 25 to 2400 m depth. At each station, 25 geochemical and physico-chemical parameters were analyzed in addition to micro-, meio-, and macrofauna and living foraminifera samples. For the pelagic system, 60 stations were selected to investigate the plankton community, ranging in size from pico- to macroplankton, through vertical, horizontal, and oblique net hauls (20, 200, and 500 µm mesh size), as well as 25 biogeochemical parameters collected with an aid of a CTD-rosette sampler. Part of this scientific information also serves the Regional Environmental Characterization Project (PCR-BS) in support of Petrobras' Santos Basin drilling licensing process led by the Brazilian Environmental Agency – IBAMA. This project contributes to the sustainable development of the SB, in line with the guidelines of the United Nations Decade of Ocean Science for Sustainable Development.

Descriptors: Benthic and pelagic sampling, essential ocean variables, Santos Project, Regional Environmental Characterization, field methods

INTRODUCTION

The Santos Basin (SB) is the main petroliferous basin on the Brazilian continental margin. After the discovery of oil and gas reservoirs following the pre-salt layer, it has been the focus of major investments. Currently, the SB is responsible for 74% of Brazil's oil and gas production and holds the three largest oil and gas production fields, Tupi, Búzios, and Sapinhoá (ANP, 2022). The SB is one of the most studied marine areas in Brazil due to its historical and economic importance, having relevant port activities, fishing, tourism, national defense, and oil and gas exploration. All these activities impose a variety of threats to the environment and must be managed by governmental agencies, universities, and companies. The oil and gas exploration and production activities are considered potentially polluting by Brazilian legislation and requires environmental licensing to have their development duly authorized.

The Brazilian energy company Petrobras and the Oceanographic Institute of the University of São Paulo conducted the phase I of Santos Basin Regional Environmental Characterization Project (hereafter PCR-BS), which comprised the gathering

of historical data for the SB Petrobras drilling license (Petrobras, 2013). This study concluded there was a need for systematic quantitative biological data in marine areas, especially in the deep sea, despite the large amount of environmental data collected in the last few decades. To fill this gap, Petrobras and its research partners planned the phase II of this research project, with the aim of gathering new oceanographic data to supplement previous environmental studies and expand the knowledge on Ocean Physics, Marine Geology, Biogeochemistry and Marine Biology of the SB. This second phase of the PCR-BS focus on the biological composition of the benthic and pelagic communities and their ecological patterns in two different seasons, as well as establishing the baseline of hydrocarbons and metal compounds in the basin. Ocean physics has been addressed at both synoptic and climatological scales, and geological descriptions have been based on historical and new data. The new knowledge will support the ecosystem-based management of the region and the establishment of suitable environmental indicators for long-term monitoring. Part of these scientific data also serves the Regional Environmental Characterization Project in support

of Petrobras' Santos Basin drilling licensing process led by the Brazilian Environmental Agency – IBAMA.

Acquiring deep sea data is one of the top environmental challenges in the world given its immense dimension and generally remote nature, which imposes logistical and financial constraints (Ramirez-Llodra et al., 2010). The innumerable sampling approaches create a challenge in producing a standardized, comparable data needed to advance the acquisition of knowledge. The Census of Marine Life began to address issues on how to integrate national or regional datasets and creating sampling protocols for ocean studies (Snelgrove, 2016; Census of Marine Life, 2022). More recently, different initiatives, such as the Global Ocean Observing System (GOOS) and the Deep Ocean Observing Strategy (DOOS), have begun to develop a strategy for identifying and prioritizing essential ocean variables (EOVs; Lindstrom et al., 2012). These approaches are well established for physical and biogeochemical parameters, but it has yet to reach a consistent agreement for the biological parameters since research objectives are more diverse (Woodall et al., 2018). A formal framework was proposed by Woodall et al. (2018) to enable consistent data gathering. Petrobras has been acquiring standardized ocean data along the Brazilian margin since the early 2000s, when the company's environmental protocols initiative was established. Since the 2010s, these data have been assembled systematically in Coastal and Oceanic Environment Database (BDCO).

The Santos basin is located in the southern portion of the Brazilian continental margin, bordered in the north by the Cabo Frio High (22°S); in the south by the Florianópolis High (28.5°S); in the west, it covers the coast of the states of Rio de Janeiro, São Paulo, Paraná, and Santa Catarina; and in the east, it is limited by the 3,000 m isobath (Figure 1a). It is considered the largest Brazilian offshore sedimentary basin, occupying an area of approximately 350,000 km² (Moreira et al., 2007). The geomorphology and coastline shape of the Santos Basin play an important role in the dynamics of South Atlantic Central Water (SACW) upwelling (Rodrigues and Lorenzetti, 2001). Furthermore, the shape of the continental shelf break can induce

the Brazil Current (BC) eddy formation and favor coastal upwelling (Calado et al., 2010; Palóczy et al., 2014). The sedimentary pattern on the inner and middle continental shelves consists of Holocene sediments filling the irregular relief. On the outer shelf, the characteristics of the bottom are the result of an intense action of the Brazil Current, with the exposure of relict surfaces that extend at least to the 28°S parallel and the presence of bioclastic to bioliticlastic facies. The facies extend on the outer shelf and upper slope from Cabo Frio to near Santos (Figueiredo Júnior and Tessler, 2004; Mahiques et al., 2004).

The South Atlantic western boundary current system in the SB is composed by the Brazil Current (BC), the Intermediate Western Boundary Current (IWBC), and their associated mesoscale activities. The BC transports Tropical Water (TW) and South Atlantic Central Water (SACW) poleward. The IWBC transports both Antarctic Intermediate Water (AAIW) and Upper Circumpolar Water (UCPW) equatorward (Silveira et al., 2008, 2020). The hydrodynamics of the adjacent continental shelf are complex due to the diversity of physical forcing. The outermost region is influenced by the BC, while the innermost region is wind driven, mainly bidirectional and aligned along the isobaths. Near the coast, the hydrodynamics are also subjected to buoyancy forcing EOVs; (Dottori and Castro, 2009), forming a superficial hyaline front associated with the discharge of rivers. On the middle and inner shelves, the wind dynamics generate continental shelf waves that impact the sea surface height (Castro and Lee, 1995) and currents (Dottori and Castro, 2018).

The pelagic and benthic domains in the SB are subjected to many different oceanographic processes that influence the nutrient content of the euphotic zone and the seafloor, thus increasing the biomass of the whole food chain. In the northern portion, the coastal upwelling of the SACW around Cabo Frio supports phytoplankton primary production higher than 14 mgC m⁻³h⁻¹ and chlorophyll concentrations higher than 6.0 mg m⁻³ (Valentin, 2001; Gonzalez-Rodriguez et al., 2017). Meanders, eddies, and shelf-break upwelling in the SB are other processes that have the

potential to increase biomass productivity on a local scale (Gaeta et al., 1999; Campos et al., 2000; Kampel et al., 2000; Castro et al., 2006). At the southern portion of the basin, the La Plata River plume reaches 28°S parallel and a transition zone up to 24°S during the austral winter (Mahiques et al., 2004, 2008) publisher-place": "Belém", "title": "Nd and Pb isotope signatures on the Southeastern South American Upper Margin: implications for sediment transport and source rocks (2007, increasing the productivity in the area (Gonzalez-Silvera et al., 2006; Möller et al., 2008; Piola et al., 2008; Brandini et al., 2018).

Here we detail the geophysical, hydrographic, geological, oceanographic, and ecological surveys carried out onboard the R/V's Ocean Stalwart and Seward Johnson (Figures S01, S02) over 356 days in 2019 and 2021/2022. These cruises were conducted following the same protocols already employed by Petrobras with the aim of facilitating the comparison of oceanographic data between projects and improving oceanographic studies with Brazilian research institutions.

METHODS

SAMPLING DESIGN

The oceanographic cruises were divided into benthic and pelagic surveys and covered the whole basin in two different seasons to assess the temporal variation. The benthic surveys were split into two areas: the deep sea (up to 2,400 m), covering the continental slope and the São Paulo Plateau, and the continental shelf. The deep sea was sampled during the austral winter of 2019 and the austral summer of 2021 (Table 1). The continental shelf was sampled in the middle of austral spring of 2019 and in the autumn of 2021. The pelagic surveys covered the neritic and oceanic domains until 2,400 m depth, where semi-synoptic samples were collected along eight transects during the austral winter-early spring of 2019 and the summer of 2021/2022. The cruises are referred to as winter/summer deep sea benthic, spring/autumn shelf benthic, and winter/summer pelagic to facilitate communication.

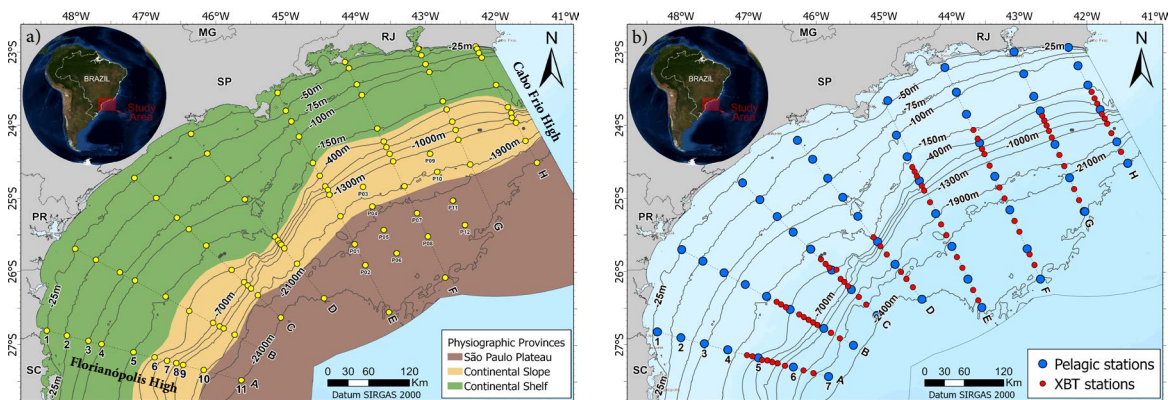


Figure 1. Map of sampling stations with an isobath distribution for the benthic survey (a) and the physiographic provinces based on Petrobras' geophysical data. Map of pelagic cruise sampling stations (b) spaced apart by approximately 20 and 30 nautical miles in distance over the neritic and oceanic zones, respectively. The red dots refer to XBT launches.

The sampling grid was set following the isobaths over eight transects to evaluate the benthic systems (Figure 1a). In addition, twelve stations were placed on the plateau area to fill the sampling gap with the lowest bathymetric gradient, which is where oil and gas exploration is concentrated. The sampling of the benthic system aims to map its environmental

heterogeneity and how it affects the faunal distribution. The sediments were sampled in triplicate at 100 stations along the 25, 50, 75, 100, and 150 m isobaths in the continental shelf, along the 400, 700, 1,000, 1,300, and 1,900 m isobaths in the continental slope, and approximately 2,200 and 2,400 m in the São Paulo Plateau (Figure 1a, Table S1).

Table 1. Campaigns, cruises, dates, seasons, and oceanographic provinces visited during the Santos Project.

Campaign	Cruises	Start	End	Season	Oceanographic Provinces
Winter Deep-sea Benthic	SANSED-01	06/11/2019	06/24/2019	Winter	Continental Slope/São Paulo Plateau
	SANSED-02	06/25/2019	07/08/2019	Winter	Continental Slope/São Paulo Plateau
	SANSED-03	07/09/2019	07/22/2019	Winter	Continental Slope/São Paulo Plateau
	SANSED-04	07/23/2019	08/03/2019	Winter	Continental Slope/São Paulo Plateau
Spring Shelf Benthic	SANSED-05	10/29/2019	11/11/2019	Spring	Continental Shelf
	SANSED-06	11/12/2019	11/25/2019	Spring	Continental Shelf
Summer Benthic Deep-sea	SANSED-07	02/16/2021	03/01/2021	Summer	Continental Slope/São Paulo Plateau
	SANSED-08	03/16/2021	04/02/2021	Summer	Continental Slope/São Paulo Plateau
Fall Shelf Benthic	SANSED-09	05/25/2021	06/25/2021	Fall	Continental Shelf
2019 Pelagic	SANAGU-01	08/05/2019	08/17/2019	Winter	Neritic/Oceanic (Transect A)
	SANAGU-02	08/20/2019	08/31/2019	Winter	Neritic/Oceanic (Transects B and C)
	SANAGU-03	09/01/2019	09/14/2019	Winter	Neritic/Oceanic (Transects C and D)
	SANAGU-04	09/16/2019	09/29/2019	Winter	Oceanic (Transects D and E)
	SANAGU-05	09/30/2019	10/13/2019	Spring	Neritic/Oceanic (Transect E and F)
	SANAGU-06	10/15/2019	10/28/2019	Spring	Neritic/Oceanic (Transects F, G and H)
2021 / 2022 Pelagic	SANAGU-08	12/12/2021	12/22/2021	Spring	Neritic/Oceanic (Transects A and B)
	SANAGU-09	12/22/2021	01/05/2022	Summer	Neritic/Oceanic (Transects B and C)
	SANAGU-10	01/06/2022	01/19/2022	Summer	Neritic (Transect D)
	SANAGU-11	01/22/2022	02/01/2022	Summer	Oceanic (Transects D and E)
	SANAGU-12	02/01/2022	02/15/2022	Summer	Neritic/Oceanic (Transects E and F)
	SANAGU-13	02/16/2022	03/01/2022	Summer	Oceanic (Transect F)
	SANAGU-14	03/01/2022	03/15/2022	Summer	Neritic/Oceanic (Transect G, H)
	SANAGU-15	03/15/2022	03/30/2022	Summer	Neritic/Oceanic (Transect G, H)

The pelagic sampling grid was set by stations spaced apart by approximately 20 and 30 nautical miles in distance over the neritic and oceanic zones, respectively. This approach aimed to obtain well-distributed water sampling over the whole basin, since the isobaths on the continental shelf and slope have different distributions in the southern and northern parts of SB (Figure 1b, Table S2). A total of 25 parameters were sampled at 60 stations with the CTD-Rosette system (conductivity, temperature, and depth) (Table S3). At each station, a vertical sampling scheme was performed in the upper ocean, based on the chlorophyll fluorescence profile, and in the water mass nucleus below 200 m depth to collect seawater (Figure 2a, b). In the euphotic zone, samples were obtained near the surface

(usually 5 m) at the beginning, maximum and at the end of the deep chlorophyll maximum layer (DCM). At shallow stations without DCM or with the presence of other water masses, such as SACW, the last sampling depth was set near the bottom (Figure 2a, Supplementary Table 3). Due to the large amount of water required for the analyses, two casts were eventually sampled at stations over 2,000 m deep. The first cast was used for the full profile, and the second one for the shallow profile.

Four to eight scientists, members of the academy, and a scientific chief joined the crew of the research vessels to perform the benthic (SANSED) and pelagic (SANAGU) cruises, usually composed of oceanographers, biologists, chemists, and geologists. They were responsible

for specific activities, such as sample processing for metagenomic analysis, water filtration, radiometric measurements, primary production

experiments, sedimentary descriptions and validation of all sampling, and laboratory procedures performed by ship crew members.

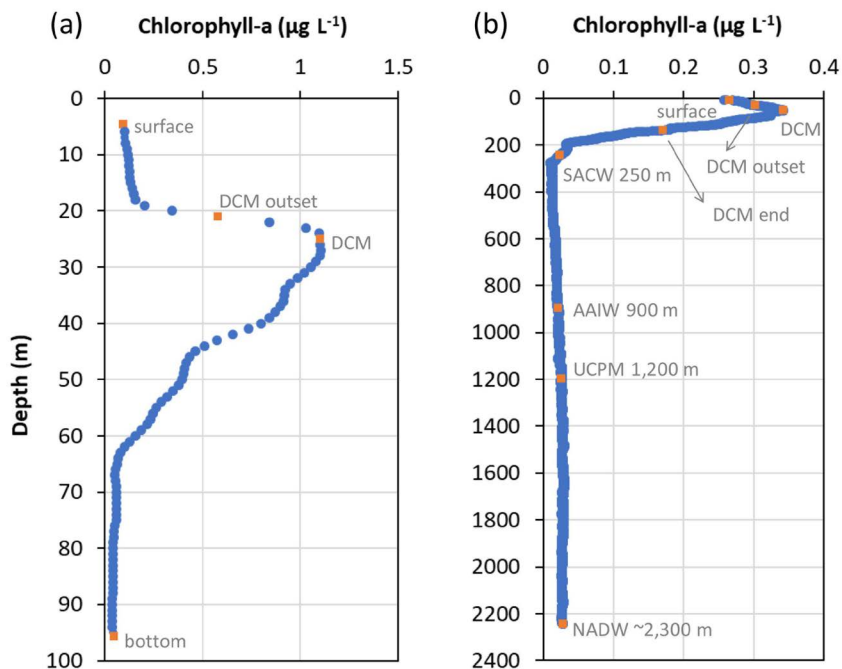


Figure 2. Vertical sampling design in neritic (a) and oceanic (b) domains based on CTD-Chlorophyll-a fluorescence depths (a, b) and water mass nuclei (b) during the pelagic cruises. DCM: deep chlorophyll-a maximum; SACW: South Atlantic Central Water; AAIW: Antarctic Intermediate Water; UCPW: Upper Circumpolar Water; NADW: North Atlantic Deep Water.

BENTHIC ORGANISMS

Sediment at the SB was collected using a GOMEX-type box corer 50 x 50 cm in area (0.25 m²) and 50 cm deep or using a large modified Van Veen grab with 231 L (80x92x40 cm, 0.75 m² surface area), according to bottom characteristics. The former was used in a silty and muddy matrix, generally on the slope, on the São Paulo Plateau, and in fewer stations over the continental shelf; and the latter was used in a sandy sediment or carbonate bioclastic gravel, generally in the continental shelf (Table S1). Using an Ultra Short Baseline (USBL) position system and a beacon (Kongsberg C-Node® SSBLTP) to limit a 150-m radius around the station, up to nine sampling attempts were made until three sediment samples were successfully retrieved to the deck.

The box corer and Van Veen grab collected the first 10 cm of the sediment layer, depending on the analyses (Figure 3). Only the box corer and Van

Veen grab samples that had a visually preserved surface sediment layer (no gross disturbance) and retained the overlying water were accepted for subsampling (Figures S03-S11). Once on deck, the overlying water was drained and sieved through a 45-µm mesh for future sampling quality control. Photographs of the sediment surface were taken and eventual megafaunal organisms were also collected. Temperature, pH, REDOX potential, and recovery depth were measured, and then 19 and 14 corers were retrieved from the continental slope and shelf, respectively (Figure 3), for prokaryote, foraminiferal, meiofauna, macrofauna, geological, hydrocarbon, organic matter, trace metal, and radioisotope analyses. A total of 43 L of sediment was obtained from triplicate sampling at each deep-sea station (over 400 m deep) and 28 L at each continental-shelf station.

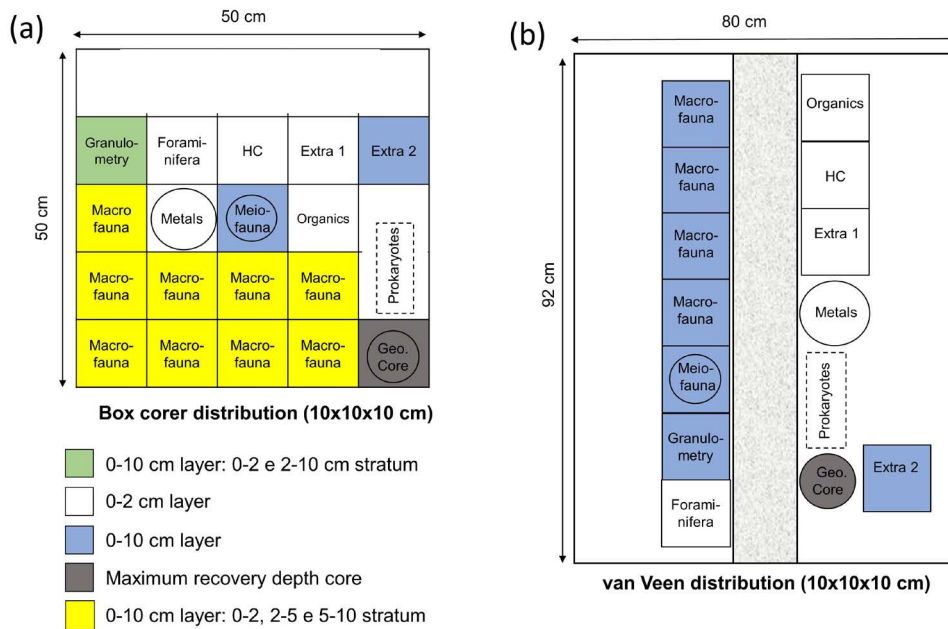


Figure 3. Subsample distribution and vertical stratification for box corer and Van Veen grabs in the deep sea (a) and continental shelf (b), respectively. HC is the hydrocarbon sample.

The first subsample (0-2 cm) carefully extracted from the box corer was used for prokaryotes to avoid contamination. Two samples with a minimum of 50 g of sediment were collected with a sterile spatula and stored in sterile Whirl-Pak for further processing in the laboratory. On board, one sample was immediately stored at $-80\text{ }^{\circ}\text{C}$ for molecular analyses. The other sample was preprocessed for bacterial and viral counts by flow cytometry. Briefly, two subsamples of 0.5 mL were added to 5 mL cryogenic tubes containing 3 mL of cell fixation solution (2% formaldehyde, 2.5% sodium chloride). The samples were incubated for 30 min at $4\text{ }^{\circ}\text{C}$ and stored at $-80\text{ }^{\circ}\text{C}$ until shore-based processing. Additional subsamples of 31 stations were separated into 15 mL Falcon tubes and conditioned at $4\text{ }^{\circ}\text{C}$ for microorganism cultivation in the 2021 campaign. Molecular analyses consisted of 16S rRNA gene and metagenomic sequencing. Despite the triplicate samples, the analyses were performed once at every station to obtain a comprehensive spatial analysis.

Deep-sea macrofauna (over 400 m deep) was analyzed in the top 10 cm of nine sediment cores (0.09 m^2) retrieved from the box corer. The cores were sliced into 0-2, 2-5, and 5-10 cm layers to improve macrofaunal fixation in 4% formaldehyde

seawater solution with neutral pH buffered with sodium tetraborate decahydrate. Continental shelf samples were also analyzed in the top 10 cm of four sediment cores (0.04 m^2) retrieved from a Van Veen grab without stratification and preserved with the same fixative solution. Macrofaunal samples were not sieved on board but preserved for shore-based analyses.

One subsample for the meiofauna was extracted from each box corer with a 5 cm diameter core tube pushed to a depth of 10 cm. The subsample was carefully transferred to a pot and fixed with the same macrofauna fixative.

The foraminiferal community was analyzed in the upper 2 cm from one corer of sediment ($10\times 10\times 5\text{ cm}$) which was treated and fixed with a 4% buffered formaldehyde and stained with Bengal rose (2 g of rose Bengal in 1000 mL of 4% buffered formaldehyde) to differentiate living from dead organisms (Walton, 1952). The color and pH were checked a day and a week after sampling and corrected if necessary. The samples for foraminiferal analyses collected in 2019 were sliced in a 1 cm layer for further vertical analyses, whereas the samples collected in 2021 were 2 cm integrated.

SEDIMENT AND WATER SAMPLING FOR CHEMICAL ANALYSES

Three sediment corers were subsampled from the box corer for the determination of hydrocarbons, trace metals, and organic matter, and two additional cores were separated for counterproof analyses. The top 2 cm of undisturbed surface sediment was placed in clean aluminum cans that were suitable for hydrocarbons and organic matter (USEPA, 2018b) and into double plastic bags for trace element analyses (USEPA, 2018a). These containers were decontaminated by the laboratories responsible for the analyses as follows: (1) the aluminum cans were washed with laboratory detergent (Extran®) and type 1 water, according to American Society for Testing and Materials (ASTM D1193), heated at 450 °C overnight, rinsed with pesticide grade dichloromethane, and kept closed until use in the field. The cans were considered decontaminated for 15 days after applying this protocol. (ii) The plastic bags were rinsed with running water, soaked in 20% v/v nitric acid solution for at least 2 hours, rinsed three times with type 1 water, dried at ambient temperature, and stored in a suitable plastic bag until use. One clean aluminum can was separated as trip blank every 14 days, and two others were separated as field blanks every 7 days. The field blank consisted of exposing an opened aluminum can filled with dry sodium sulfate to the same sampling time and environment of the regular sample (USEPA, 2014). All these samples were maintained at -20 °C until shore-based laboratory analyses (USEPA, 2018a, 2018b). The ship's wet laboratory stayed closed, and the crew was prohibited from smoking while processing samples on deck to avoid contamination.

Sediment samples for analyses of aliphatic hydrocarbons (n -C₁₀ to n -C₄₀), polycyclic aromatic hydrocarbons (PAH), total petroleum hydrocarbons (TPH), and petroleum biomarkers were lyophilized, Soxhlet extracted (USEPA 3540C) for 24 h, and fractionated (USEPA 3611B). The organic matter (OM) sample was separated for the determination of total organic carbon (TOC), total nitrogen (TN),

total and organic phosphorus (P-tot and P-org), total carbohydrates (CHO), total proteins (PRT), total lipids (LIP), biopolymeric carbon (BPC), carbon and nitrogen stable isotope ratios ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), chlorophyll-*a*, phaeopigments, and lipid biomarkers. Additionally, the radiocarbon of the bulk OM was also measured in selected samples. Trace metal samples were analyzed through their bioavailable and total fractions, and subsamples were selected for 226Ra and 228Ra analyses. All these analyses were related to sediments collected during benthic cruises.

Prior to the pelagic cruises, all glassware related to hydrocarbon analyses were decontaminated by washing with detergent (Extran®) and water, deionized water, acetone p.a., and finally rinsing with pesticide grade dichloromethane. During the pelagic cruises, surface water samples were collected by GO-FLO bottles for saturated and aromatic hydrocarbon analyses. These samples were extracted onboard without prior filtration by the separatory funnel liquid-liquid method 3510C (USEPA, 1996). Laboratory support stands, clamps, and rings were customized for use in field conditions. Briefly, each 4 L water sample was spiked with surrogate standard mixtures, containing n -C12 d_{26} , n -C20 d_{42} , n -C24 d_{50} , n -C30 d_{62} , and p -terphenyl- d_{14} ; the liquid-liquid extraction was performed 3 times with 30 mL of n -hexane. The combined extract was filtered through a funnel filled with anhydrous sodium sulfate and stored in an amber glass flask under refrigeration (< 6 °C) until analysis in the land-based laboratory (Figures S12a, S12b). Quality control requirements included field and method blanks prepared from organic-free water to monitor for potential sources of contamination and laboratory control samples to measure accuracy and method performance in each sample batch (USEPA, 2014).

From the SANAGU10 cruise onward, hydrocarbon samples were not extracted on board, but the ship returned to the port every seven days to meet the USEPA's holding time recommendation for semi-volatile analysis (USEPA, 2018b). These samples were then extracted in the onshore laboratory on the same day or the following day by the same method used onboard.

GEOLOGICAL AND GEOPHYSICAL SAMPLING

The sediment surface was visually described as soon as the sampler arrived onboard. Sediment characteristics were recorded, such as morphology, color (according to the Munsell Rock Color Chart), texture, sampler penetration, and sediment type (mud, sand and gravel). The presence or absence of carbonate contents, such as shells, carapaces and bioturbation, was described. Ferruginous sediment layers and shell debris deposits were also considered in the visual description.

After the visual descriptions, four different geological samplings were performed on board. Triplicate subsamples of a 10x10 cm core were sliced into 0-2, 2-10 cm, and 0-10 cm layers to provide grain size and carbonate content analyses. This partitioning was used to correlate the results with both biological and geochemical data. Additionally, one geological core was extracted from each station. These cores comprised a 75 mm diameter tube pushed

to maximum recovery depth of 50 cm. These cores had their top and bottom identified and stored upright in a refrigerated chamber. In the laboratory, these cores were analyzed through Multi-Sensor Core Logger. Once profiled, the cores were split into two halves, from which photographs were taken, and the interior was described. Cores were sliced into one cm-thick samples for geological characterization and sharing with other groups of interest.

The sub-bottom profiler (SBP) geophysical survey was designed to control the sampling station depth and acquire 3.5 kHz and 12 kHz chirp data (Knudsen Chirp 3260) along the ship tracks (Figure 4). The SBP was synchronized to DGPS VeriPos™ in RV Ocean Stalwart. Data were saved in SEG-Y file format for postprocessing of shallow seismic signals. Most legs acquired these data except SANSED 7, 8 and 9 carried out in 2021 by RV Seward Johnson, since this equipment was not available.

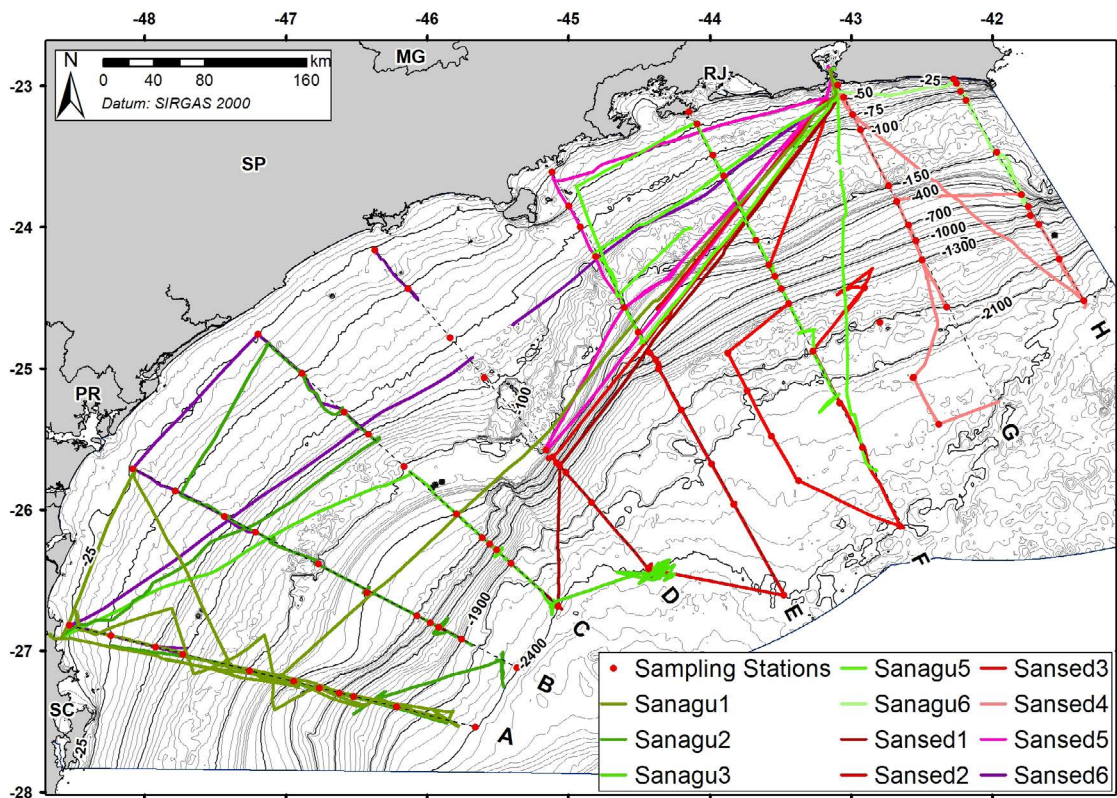


Figure 4. Map of sub-bottom profile data analyzed from 2019 benthic and pelagic cruises in the Santos Project (PCR-BS). This map serves for all underway measurements, such as ADCP and thermosalinograph.

HYDROGRAPHY DURING BENTHIC AND PELAGIC CRUISES

The goals of the hydrographic survey were to (1) measure current velocities to update our current knowledge on the regional circulation at the mesoscale; (2) map the main hydrographic features; (3) provide physical and biogeochemical data of the water column and near the bottom; and (4) define fixed vertical levels for water sampling based on the chlorophyll-*a* fluorescence profile in pelagic cruises. Currents, temperature, salinity, dissolved oxygen, and fluorescence measurements were carried out with unprecedented spatial resolution for the SB area, increasing at least 320 CTD and 156 Expendable BathyThermograph (XBT) profiles to the SB physical-biogeochemical climatology in different seasons and years. In total, the cruises spent 356 days at sea.

The hydrographical stations consisted of conductivity, temperature, and depth (CTD) profiles collected with a Sea-Bird Electronics 9plus equipped with pressure, conductivity, dissolved oxygen (DO), pH, turbidity, colored dissolved organic matter (CDOM), chlorophyll-*a* (Chl), and phycoerythrin fluorescence sensors (Figure S13). The sensor calibration followed the manufacturer's recommendations, and some sensors (e.g, DO and pH) underwent routinely field check-up. The biogeochemical data were calibrated by statistic regressions with *in situ* measurements of DO by Winkler titration, pH meter, and chlorophyll-*a* lab fluorometers. CTD profiles were obtained at all benthic and pelagic stations, and 78 expendable bathythermographs (XBTs) were launched in oceanic areas for a better scale resolution of physical parameters in the pelagic cruises, since the baroclinic radius is approximately 27-28 km in the SB (Figure 1b).

Two vessel-mounted Acoustic Doppler Current Profiler (ADCP) Teledyne RDI continuously acquired velocity profiles at 38 and 150 kHz and sampling at 20-m and 9-m vertical bins, respectively (Figure 4). Data were processed using the Common Ocean Data Access System (CODAS) software following the guidelines of Firing et al. (1995). The oceanographic features during the 2019 cruises are presented in Silveira et al. (2023 this issue) and Dottori et al. (2023 this issue).

PELAGIC BIOGEOCHEMISTRY

The pelagic ecosystem is complex and dynamic from a physical-chemical and biological point of view; therefore, several of its environmental and biotic components were assessed as the first step toward understanding the structure and functioning of the marine ecosystem from the base to the higher trophic levels in the Santos Basin. The relationships between the microbiota composition and its associated biogeochemical processes were analyzed based on measurements of *in situ* dissolved oxygen and pH, dissolved inorganic nutrients, colored dissolved organic matter (CDOM), concentrations of dissolved organic and total inorganic carbon, particulate material, and primary production (photosynthetic and chemosynthetic). Additionally, Secchi disk measurements were made to determine the euphotic zone depth.

For the analyses of dissolved oxygen (DO), 60 mL of water was collected directly from the Niskin bottle in Biological Oxygen Demand (BOD) glass bottles. The DO concentration in these aliquots was determined on board with a digital burette immediately after sampling and fixation with the Winkler technique reagents, according to the procedures described in Grasshoff et al. (2009). The pH of samples taken from Niskin bottles were analyzed in 50 mL aliquots using a benchtop pH meter (Hanna Instruments Inc. HI 98191) calibrated daily. This required waiting for the latest field reports with the pH and DO data generated in each leg to process together with the CTD data and generate continuous profiles of these variables.

Seawater retrieved directly from the Niskin bottles was filtered onto Whatman GF/F filters with a 60 ml polypropylene syringe coupled with a 25 mm filter holder, for later analyses of dissolved inorganic nutrients and dissolved organic carbon analyses. Prior to each station, the syringe was gently washed with Milli-Q ultrapure water, and in between Niskin bottles, the syringe was rinsed three times with sampling water. For nitrate, nitrite, phosphate, and silicate analyses, seawater was collected into 10 ml sterilized Falcon bottles, and ammonium was collected into 25 ml amber glass bottles, which were previously washed with 10% HCl solution. All samples were collected in

triplicate and kept frozen (-20 °C) until further analyses. In the shore-based laboratory, nitrate, nitrite, phosphate, and silicate concentrations were determined from an AA3-Seal autoanalyzer, following Grasshoff et al. (2009). Ammonium, on the other hand, was analyzed in a Hitachi U1100 spectrophotometer. For the dissolved organic carbon, approximately 50 ml of filtered seawater was collected in duplicate into prewashed (10% HCl) and precombusted (400 °C for 4 h) glass vials and kept frozen (-20 °C). The dissolved organic carbon (DOC) concentration was determined using the catalytic oxidation method, following the recommendations of Sugimura and Suzuki (1988), using an Elementar® Vario TOC CUBE.

The particulate material was investigated through gravimetry analysis. Prior to the cruises, Whatman GF/F filters were incinerated at 450 °C for four hours and were weighed to determine their initial weight (accuracy of 0.01 mg) (Grasshoff et al., 2009). On board, between 1 and 5 L of seawater were vacuum filtered. The volume varied according to the apparent particulate matter concentration. The filters were kept frozen (-20 °C) until further analysis. In the shore-based laboratory, the filters were dried at 60 °C for 24 hours and weighed again on a 0.01 mg precision electronic balance (Shimadzu AUW-220D-I) to determine the final weight. The concentration of the total particulate matter was calculated by the difference between the final weight and the initial weight divided by the filtered volume (Strickland and Parsons, 1972). A subsample of the filter was encapsulated in tin foil and analyzed with a Costech Elemental Combustion System analyzer coupled to the Thermo Scientific Delta V Advantage Isotope Ratio MS (EA-IRMS) to evaluate the carbon and nitrogen content and the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic indices. The carbon and nitrogen contents were corrected using the ratio of the subsampled area to the filtrated area (Turnewitsch et al., 2007) and divided by the filtered water volume.

In addition to the CTD chlorophyll fluorescence sensor, *in situ* chlorophyll-*a* concentrations were determined in 1 L water samples filtrated onto Whatman GF/F filters (average porosity of 0.7 μm). After filtration, the filters were stored at -80 °C and

analyzed with a 10-AU Turner Design benchtop fluorometer according to the protocol established by Welschmeyer (1994). For pigment extraction, 10 mL of a mixed solution of acetone with dimethyl sulfoxide (DMSO) was used, with 60% acetone (90% pure acetone + 10% Milli-Q ultrapure water) and 40% pure DMSO. The filters were extracted in a freezer for 24 hours. Additionally, photosynthetic pigments were analyzed with HPLC-CHEMTAX techniques to better characterize the phytoplankton community throughout the stations and depth layers. Five liters of water were collected at 4 or 3 fluorescence sampling depths in the euphotic zone during the SANAGU 2019 and 2021/22 cruises, respectively. These were filtered onto 25 mm diameter GF/F membrane filters (Millipore, MA) and stored in liquid nitrogen. All laboratory procedures were performed under green light to avoid photodegradation.

Primary production experiments were performed at selected stations according to weather conditions and the available photoperiod (for photosynthesis). Water samples (1 L) were collected using the CTD-Rosette system at the surface and deep chlorophyll maximum for photosynthesis, in addition to depths of 250 m, 900 m, 1200 m, and 2300 m for chemosynthesis. On board, 5 μCi of ^{14}C -bicarbonate was inoculated into 70 mL aliquots of water samples and incubated from 5 to 8 h (e.g., Nielsen, 1952; Casamayor et al., 2008; Reinthaler et al., 2010; Signori et al., 2017). For photosynthesis, the aliquots were conditioned in eight different levels of incident sunlight (100%, 55%, 37%, 16%, 8%, 4%, 1%, 0%), and surface water was continuously pumped through the incubation system to control the *in situ* simulated temperature (Figure S14). For chemosynthesis, two replicates and one killed control (with 2% formaldehyde to inhibit microbial growth) were placed in the dark, using room temperature, air conditioning temperature, and even the refrigerator to simulate the *in situ* water temperature. After incubations, the samples were filtered onto a 0.22 μm pore-size and 25 mm diameter membrane filters (Millipore, MA) using a vacuum pump, after which they were stored in cryogenic tubes at -20 °C until further laboratory analyses. In the shore-based laboratory, the membranes were exposed

to concentrated HCl fumes for 30 s and transferred to a scintillation vial with 5 mL of liquid scintillation cocktail (Ultima Gold, PerkinElmer). After 24 h in the fridge, the radiation of the samples was finally counted in a liquid scintillation counter (PerkinElmer Tricarb 2810 TR), and the results given in disintegrations per minute were converted into rates of primary production ($\text{mgC m}^{-3}\text{h}^{-1}$) (Teixeira, 1973).

Additional data was collected during the second 2021/2022 pelagic cruise with a Laser In-Situ Scattering and Transmissometry equipment (LISST-Deep®). This equipment uses the laser diffraction technique to obtain the particle-size distribution (PSD) in an aquatic environment. The suspended particle spectrum profiles were performed at all stations along transects E, F, and G, in addition to test Stations G1 and A7. To our knowledge, this is the first time these data have been acquired as a continuum profile in Brazilian waters.

REMOTE SENSING

In situ bio-optical and radiometric measurements were acquired during the pelagic cruises with the aim of characterizing the spatiotemporal distribution of bio-optical properties and their relationships with biogeochemical properties, considering two contrasting seasons. The performance of ocean color remote sensors (MODIS, VIIRS, and OLCI) for the estimation of bio-optical and biogeochemical properties was analyzed in relation to *in situ* measurements obtained during the sampling campaigns. Remotely sensed meteo-oceanographic variables, such as sea surface temperature, ocean surface winds, chlorophyll-*a* concentration, sea surface height, and geostrophic surface circulation – acquired from a suite of different orbital sensors (MODIS/Aqua, VIIRS/SNPP, VIIRS/NOAA20, ABI/GOES16, ASCAT-A,B,C/MetOp, OLCI/Sentinel-3A/-3B, and radar altimeters Jason-2, Jason-3, Saral/AltiKa, SRAL) – were analyzed in conjunction with synoptic weather charts for contextualizing the meteo-oceanographic conditions in relation to environmental variability at synoptic and seasonal scales. Interannual variability and temporal trends were analyzed based on remote sensing and reanalysis time series validated regionally, including

sea surface temperature, sea surface height, ocean surface winds, chlorophyll-*a* concentration, mixed layer depth, heat flux, photosynthetically available radiation, precipitation, and climatic indices (Southern Oscillation Index and Multivariate ENSO Index).

During the pelagic cruises, seawater samples (5 L) were collected at the surface and at the depth of the DCM at 60 stations for the measurement of light absorption coefficients by CDOM and particulate matter (phytoplankton and nonalgal detritus or particles). Duplicate subsamples for CDOM analysis were filtered by gravity directly from Niskin/Go-Flow bottles using a Whatman Polycap Aqueous Solution filter device with a 0.2 μm pore size, stored in precombusted glass bottles wrapped with aluminum foil and kept under refrigeration (4 °C) until further laboratory analysis (Manino et al., 2019). The CDOM absorption coefficient was calculated in a shore-based laboratory via spectrophotometer absorbance measurements of the filtered CDOM samples at room temperature in a 0.1 m optical pathway quartz cell. The absorption spectral shape of CDOM was estimated using a linear least-squares regression (Bricaud et al., 1981). Particulate matter samples were filtered on board, in duplicate, through 25 mm Whatman GF/F (0.7 μm nominal pore size) until reaching a volume of 2 L or for up to 40 min. The filters were stored in liquid nitrogen. Particulate matter absorption coefficient spectra were calculated in shore-based laboratory using the transmittance-reflectance method (Tassan and Ferrari, 2002). The absorption coefficients of CDOM and particulate matter were analyzed using a dual-beam Shimadzu UV-2450 spectrophotometer equipped with an integration sphere.

Above-water radiometric measurements were obtained using an ASD FieldSpec Handheld Pro spectroradiometer at 60 stations during the SANAGU campaigns following the NASA protocol (Mueller et al., 2003). The remote sensing reflectance above the sea surface, which is the ratio between the water leaving radiance and the above surface downwelling solar irradiance (E_d), was compared with MODIS, VIIRS, and OLCI sensor bands. An approximately 100% reflecting Spectralon reference panel was used to measure E_d .

PLANKTONIC ORGANISMS

The taxonomic composition, carbon biomass, and metabolic diversity of different size classes of autotrophic and heterotrophic plankton organisms were assessed with high-throughput techniques, 16S rRNA sequencing, metagenomics, flow cytometry, or with both optical and electronic microscopy. In addition to prokaryotes and virioplankton, nanoplankton, microplankton, zooplankton and ichthyoplankton communities were investigated in the Santos Project.

Seawater samples were collected in a vertical profile at all oceanographic stations to characterize the SB microbiome. Water samples (15 L) from six discrete depths (surface, DCM, 250, 900, 1200, 2300 m) were filtered by a peristaltic pump through a 0.22 μm SterivexTM filter (Millipore, MA), for environmental DNA analyses. The Sterivex filter was immediately treated with RNA later (Qiagen) and stored. Quintupled samples (4 mL) were collected at all stations and depths for flow cytometry. Samples were stored in cryovials, immediately preserved with Sigma–Aldrich glutaraldehyde solution (0.2% final concentration), and flash-frozen in liquid nitrogen for a few minutes for fixation. In 2021/22 cruises, 68 additional subsamples of 15 stations were cryopreserved for microorganism cultivation, and individual 1.5 mL of raw water samples were added to 375 μL of 50% glycerol solution and mixed. All samples for microbiological research were stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

The microplankton and nanoplankton were collected at the four upper sampling depths defined by the chlorophyll-*a* fluorescence profiling at all 60 oceanographic stations. Two liters of water were poured into two 1-L dark flasks and preserved with 5 mL of Lugol's alkaline solution buffered with sodium acetate. In the shore-based laboratory, each sample (total = 240) was gently filtrated onto a 20 μm net, concentrating the cells by reverse filtration until a final volume of 130 to 200 mL. An aliquot of 100 mL of the original sample was used to count nanoplankton cells. At least 400 cells were counted under an inverted microscope (200x and 400x), keeping the estimate error approximately 10% (Verick, 1979). Additionally, 100 mL of seawater was collected with Niskin bottles near the surface and at the level of the DCM to distinguish autotrophic nanophytoplankton cells from nanoheterotrophs. Samples were poured

into 100 mL dark flasks, preserved with 5 mL of 4% formaldehyde seawater solution buffered with sodium tetraborate decahydrate, and stored in the dark and at ambient temperature. At the shore-based laboratory, samples were filtered onto a Nucleopore filter with 0.2 μm porosity, stained with the fluorochrome Proflavine, and counted with an immunofluorescence microscope at 1,000x. Additionally, these samples were also used to count coccolithophores since the cells lose the coccoliths when preserved in Lugol solution. In addition to cell counting, the carbon content of the nano- and microplankton cells was estimated based on adapted geometric figures using conversion factors available in the literature (Verity et al., 1992; Montagnes et al., 1994).

Net microplankton were sampled in daytime with two Bongo nets (mouth diameter of 0.6 m and mesh size of 20 μm) towed vertically along the 200 m water column and 5.0 m above the bottom at shallow stations (Figure S15). Bongo net samples were preserved either by adding 4 mL of Lugol's alkaline solution into a 250 mL dark flask or with buffered 4% formaldehyde seawater solution (total = 120 samples). Torn nets were replaced by identical one or by another bongo net but with a 30 cm mouth diameter aperture to minimize loss in heavy seas. The net sampling of microplankton was conducted only during the daytime. To investigate the plankton composition, aliquots of both Lugol and formalin preserved samples (due to selective destruction of cells) were examined under a light microscope and prepared for electron microscopy, especially diatoms and coccolithophores (Hasle and Fryxell, 1970). Tomas (1997) and the taxonomic references therein were consulted for species identification, in addition to up-to-date papers published more recently.

The mesozooplankton were collected using a Multinet[®] Maxi type (Hidrobios) with 0.5 m^2 (Figure S16) towed obliquely at 2.0 knots, in nine target depth layers (0-bottom, 2,400 (or bottom)-1,500 m, 1,500-1,100 m, 1,100-550 m, 550-150 m, 150-100 m, 100-50 m, 50-25 m, 25-0 m) (Figure 5). At each station, two tows were performed: the first one fitted with the 500 μm mesh size net and the other with the 200 μm mesh size to collect ichthyoplankton and zooplankton, respectively. This activity was always carried out at night, usually

between 6:00 pm and 6:00 am, with some trawls ending later due to operational problems. In deep ocean trawls, the multinet was launched into the sea before 6 pm, since the equipment takes 2 hours to reach the bottom and the trawls take approximately 5 to 6 hours to reach the surface.

One flowmeter installed at the Multinet frame measured the water volume filtered in each net. The samples of the first net, which integrated the water column, were fixed in alcohol p.a. 99.8% and kept in the refrigerator for further genetic analyses. Stratified samples were fixed in a buffered 4% formaldehyde-seawater solution. To avoid organism contamination, each layer had its own set of nets. If a net was required for reuse at different depths, a decontamination protocol was performed consisting of washing the net with powdered soap and diluted bleach for 30 minutes and rinsing with plenty of water. The zooplankton samples were deposited in the Zooplankton Collection of the Integrated Laboratory of Zooplankton and Ichthyoplankton (*Laboratório*

Integrado de Zooplâncton e Ictioplâncton) of the Biology Institute, Federal University of Rio de Janeiro, and the ichthyoplankton samples were deposited in the Biological Collection “Prof. Edmundo F. Nonato” of the Oceanographic Institute of the University of São Paulo.

Epineuston and hyponeuston were collected using a Hydro-Bios model 300 neuston net, which is composed of a catamaran swimmer body of PVC, a double net frame for surface (150 mm depth) and subsurface nets (450 mm depth), two superposed nets of 500 µm mesh size (with a mouth opening of 15 cm high x 30 cm wide), and a net length of 400 cm (Figure S17). The lower net was equipped with a General Oceanics flowmeter to estimate the volume of water filtered. Samples were immediately fixed in a buffered 4% formaldehyde-seawater solution. The zoo- and ichthyoneuston identified were deposited in the zoo and ichthyoplankton Collection of Integrated Laboratory of Zooplankton and Ichthyoplankton of the Biology Institute, Federal University of Rio de Janeiro.

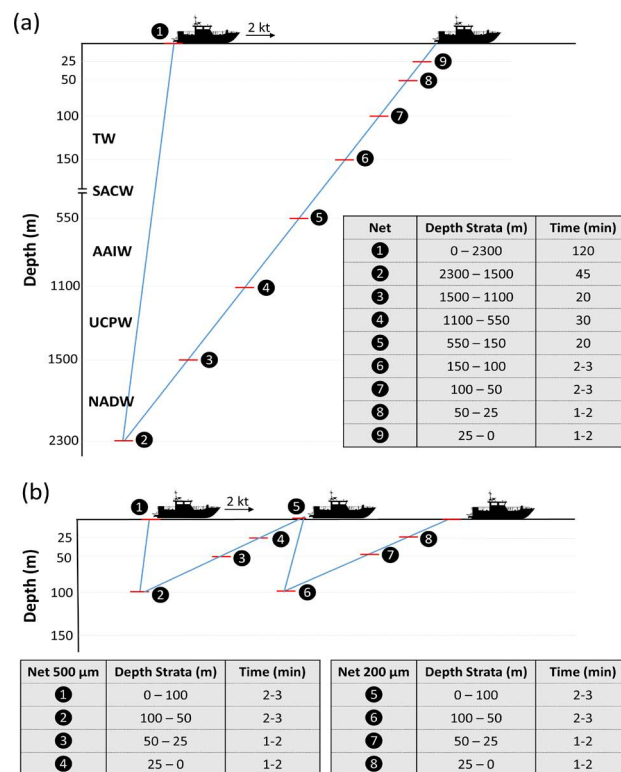


Figure 5. Schematic Multinet tow showing the target layer depths in deep and shallow waters and their approximate trawl times. TW: Tropical Water; SACW: South Atlantic Central Water; AAIW: Antarctic Intermediate Water; UCPW: Upper Circumpolar Water; NADW: North Atlantic Deep Water.

SUMMARY AND FINAL REMARKS

An extensive amount of multidisciplinary data was acquired during the Santos Basin Regional Environmental Characterization cruises. The oceanographic cruises were divided into benthic (SANSSED) and pelagic (SANAGU) surveys and covered the whole basin, from the coastal and surface waters to a depth of 2,400 m, in two different seasons. During the pelagic cruises, water and planktonic samples were collected using several equipment, such as CTD-rosette

with biogeochemical sensors for turbidity, pH, DO, Chl-a fluorescence; LISST-Deep equipment; bongo multinet; and neuston nets, from the surface until 2,400-m depth. During the benthic cruises, sediment samples were collected to investigate biogeochemical, geochemical, and geological parameters and to assess bacterial, foraminifera, meiofaunal, and macrofaunal communities. During all the cruises, physical and geophysical parameters were acquired by CTD, XBT, ADCP, thermosalinograph, SBP, and meteorological station (Figure 6).

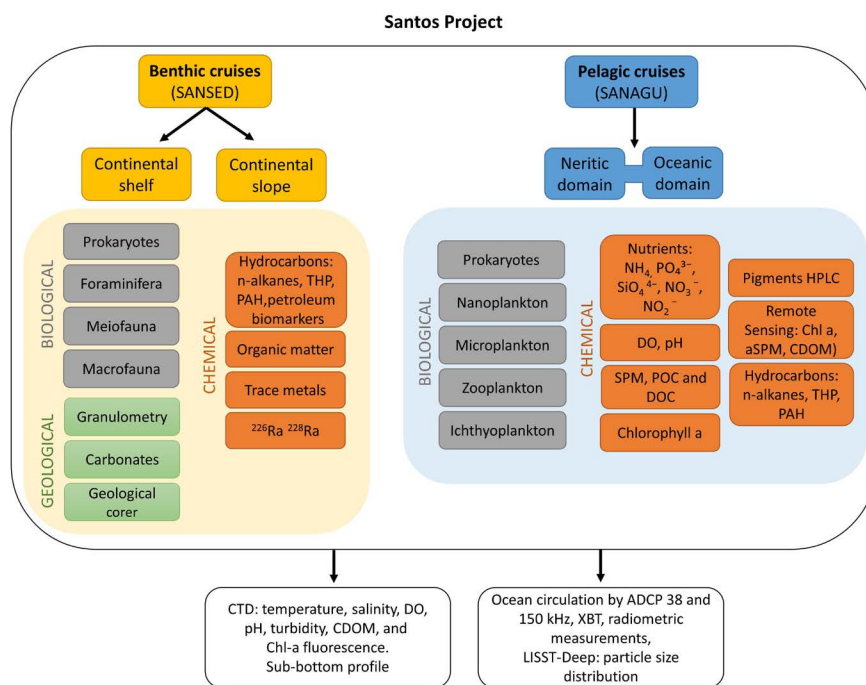


Figure 6. Synthesis of environmental samples collected during the benthic and pelagic cruises in the Santos Project.

These data supplement and expand previous environmental studies and provide the scientific baseline information needed to enhance the understanding of oceanographic processes in the region and its interactions with marine organisms and ecosystems. We detected different sediments and ecosystems on the continental shelf and slope up to 2,400 m depth with distinct grain size, carbonate contents, and at different water depths (Ferreira et al., 2023), as well as all water masses in the SB pelagic system. During the 356 days at sea, we had the opportunity to sample and measure the main oceanographic features in the region,

such as coastal upwelling and other phytoplankton blooms, southern coastal water intrusion (Dottori et al., 2023 - this issue), the Brazil Current and its mesoscale eddies (Silveira et al., 2023 - this issue), the role of chemosynthesis to the pelagic realm, the photosynthesis and CDOM relationship (Kutner et al, 2023 – this issue), the spatial trends in the distribution of natural radioisotopes in the bottom sediments (Ferreira et al., 2023 - this issue), and other curious features and organisms that need further investigation. To give a brief result that scales the research effort, a total of 35,674 individuals belonging to 203 taxa of the

macrofauna *stricto-sensu* were found at continental slope just in Winter Deep-sea Benthic Campaign (SANSED1-4), with average densities ranging from 241 to 12,959 ind m⁻² (de Moura, et al, 2023 – this issue) and a total of 669 benthic foraminiferal species were identified at the same stations. Thus, the multidisciplinary cruises for environmental characterization in the Santos Basin achieved the goal of increasing scientific data in deep sea and oceanic region of the SB, in alignment with the best standardized sampling approaches (Woodall et al., 2018), emphasizing the need for ocean observation (Lindstrom et al., 2012) and all challenges stressed by the United Nations Decade of Ocean Science for Sustainable Development (UNESCO, 2021).

The next phase consists of transforming these data into information, which has the potential to enhance the environmental management of IBAMA, Petrobras, and the oil and gas industry and to support stakeholders' data-driven decision-making. A machine learning pipeline was also developed to overcome data integration challenges in ecosystem studies (Fonseca and Vieira, 2023 – this issue). Despite the SARS-CoV-2 pandemic, the Santos Project accomplished an unprecedented step toward gathering scientific environmental data that will enable data-driven decision management.

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AUTHOR CONTRIBUTIONS

- D.L.M.: Conceptualization; Data curation; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.
- R.G.A.T.: Data curation; Investigation; Methodology; Validation; Visualization; Writing – original draft; Writing – review & editing.
- L.F.M.A.: Data curation; Investigation; Methodology; Resources; Visualization; Writing – original draft; Writing – review & editing.
- J.C.C.: Data curation; Formal Analysis; Investigation; Methodology; Visualization; Writing – original draft; Writing – review & editing.
- J.R.S.F.: Data curation; Formal Analysis; Investigation; Visualization; Writing – original draft; Writing – review & editing.
- M.D., M.K.: Conceptualization; Formal Analysis; Funding acquisition; Investigation; Methodology; Resources; Software; Supervision; Validation; Writing – original draft; Writing – review & editing.
- C.N.: Conceptualization; Data curation; Formal Analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing – original draft; Writing – review & editing.
- A. G. F. Jr., A. C. T. B., C. N. S., H. P. L., L. F. F., R. S. C., S. L. C. B., S. T. D., S. V. R., V. H. P.: Conceptualization; Data curation; Formal Analysis; Funding acquisition; Investigation; Methodology; Resources; Supervision; Validation; Writing – original draft; Writing – review & editing.
- F. D. C. G., M. A. G. A. J.: Conceptualization; Formal Analysis; Investigation; Methodology; Resources; Supervision; Validation; Writing – original draft; Writing – review & editing.
- M. G. C.: Conceptualization; Data curation; Formal Analysis; Investigation; Methodology; Project administration; Resources; Software; Validation; Writing – original draft; Writing – review & editing.
- N. M. B.: Data curation; Formal Analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing.
- R. B. M.: Data curation; Formal Analysis; Investigation; Writing – original draft; Writing – review & editing.
- I. C. A. S.: Conceptualization; Formal Analysis; Funding acquisition; Investigation; Methodology; Resources; Software; Supervision; Validation; Writing – review & editing.
- F. P. B., S. H. M. S.: Conceptualization; Data curation; Formal Analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing – review & editing.
- E. H. M.: Conceptualization; Data curation; Methodology; Project administration; Resources; Supervision; Validation; Writing – review & editing.
- T. N. C.: Conceptualization; Data curation; Formal Analysis; Funding acquisition; Investigation; Methodology; Resources; Supervision; Validation; Writing – review & editing.

A.M.V.: Data curation; Formal Analysis; Investigation; Software; Validation; Writing – review & editing.

R.P.M., W.C.B.: Conceptualization; Data curation; Methodology; Project administration; Resources; Validation; Writing – review & editing.

F.S.P.: Data curation; Formal Analysis; Investigation; Validation; Writing – review & editing.

F.G., G.F.: Conceptualization; Data curation; Formal Analysis; Funding acquisition; Investigation; Methodology; Resources; Software; Supervision; Writing – review & editing.

D.K.S., D.A.S., M.B.S., V.G.P.: Data curation; Formal Analysis; Investigation; Methodology; Software; Writing – review & editing.

A.M.S.D.: Data curation; Formal Analysis; Investigation; Software; Writing – review & editing.

P.V.M.S.: Formal Analysis; Investigation; Software; Writing – review & editing.

T.M.V.: Conceptualization; Data curation; Formal Analysis; Investigation; Methodology; Project administration; Resources; Writing – review & editing.

D.V.P., J.C.F.M., P.M.T.: Data curation; Formal Analysis; Investigation; Methodology; Writing – review & editing.

A.G.D., D.S.K., D.C.D.C., G.C.F.R., G.S.C., L.L., M.C.: Data curation; Formal Analysis; Investigation; Writing – review & editing.

R.G.R.: Formal Analysis; Investigation; Writing – review & editing.

G.M., J.O.C., L.R., L.O.S., L.S.M.: Data curation; Investigation; Writing – review & editing.

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