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Diversity, trophic dynamics, resilience, and value of hardground invertebrates at methane seeps
and on mineral-rich slopes in the deep Eastern Pacific

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Oceanography with a Specialization in Interdisciplinary Environmental Research

by

Olivia Soares Pereira

Committee in charge:

Professor Lisa A. Levin, Chair
Professor Douglas H. Bartlett
Professor Paterno R. Castillo
Professor Mark R. Jacobsen
Professor Paul R. Jensen
Professor Gregory W. Rouse

2024

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University of California San Diego

2024

DEDICATION

To my Família Sapo.

EPIGRAPH

In the deep sea, some encounters are so rare
that they last for a lifetime.

If we are leaving part of ourselves behind,
then we are not doing the best science we can be doing.

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ABSTRACT OF THE DISSERTATION

Diversity, trophic dynamics, resilience, and value of hardground invertebrates at methane seeps and on mineral-rich slopes in the deep Eastern Pacific

by

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Hard substrates in the deep sea create habitat heterogeneity for diverse invertebrate communities through the provision of substrate and cues for attachment, food, and refuge. However, the same environmental conditions that allow for the formation of these substrates are influencing the biological communities. By analyzing community and trophic dynamics of invertebrates associated with carbonate rocks at methane seeps on the Costa Rica Pacific margin

and on mineral-rich hardgrounds off Southern California, I observed that low dissolved oxygen concentration and depth play a role in structuring these communities, as well as seepage activity at seeps and substrate type on mineral hardgrounds. Low oxygen decreases community abundance and community and trophic diversity at both ecosystems and creates complex trophic interactions for fauna on mineral-rich substrates at intermediate depths within the oxygen minimum zone. At seeps, low oxygen seems to override the effects of seepage activity, and the dependence of macrofauna on chemosynthetic production increases with depth. Manipulative experiments with carbonate rocks at seeps demonstrated how the environment influences recovery and resilience of macrofauna. Low oxygen limits colonization and resilience, but seepage activity favors bacterial-grazing seep fauna and food supply. Some macrofaunal species are associated with specific microbial operational taxonomic units (OTUs) for provision of food and habitat, and the responses of these OTUs to changes in seepage activity are correlated to macrofaunal assemblages. Thus, microbial activity seems to play a role in the resilience of macrofaunal at seeps. These findings highlight how the environment and animal-microbe interactions influence macrofaunal communities and their responses to physical and environmental disturbances. They can provide insights for future management and conservation efforts as seeps and mineral-rich hardgrounds provide many ecosystems services but are threatened by human activities and climate change. A first economic valuation analysis of methane seeps reveals that Costa Ricans are willing to pay for protection of seeps and, most importantly, seem to have an affection and care about deep-sea ecosystems (especially endemic species) that become stronger when there is more knowledge of these ecosystems. This finding highlights the importance of biodiversity research, engaging with the public and communicating our findings to facilitate management of deep-sea ecosystems.

Chapter 1 INTRODUCTION

Although most of the deep ocean is covered by sediments (Snelgrove 1999), many benthic habitats in the deep sea are considered heterogeneous, with both soft and hard substrates (Lacharité and Metaxas 2017). These include methane seeps, canyons, and seamounts, among others, where we find authigenic carbonates, mineral precipitates, and basalts.

At methane seeps, carbonate precipitation is a by-product of anaerobic oxidation of methane (AOM) carried out by microbial consortia of sulfate reducing bacteria and methane oxidizing archaea (Aloisi et al. 2000, Boetius et al. 2000, Orphan et al. 2002). The reaction of combined AOM and sulphate reduction increases alkalinity, promoting the precipitation of authigenic carbonates (Marlow et al. 2014b). Seeps and associated carbonate rocks can be found on active and passive continental margins globally, from shallow shelf to deep waters (400-7,326 m) (Fujikura et al. 1999), making these rocks an important global carbon reservoir. Seeps also play an important role in global biogeochemical cycling and elemental transformation of carbon by sequestering methane and CO₂ in active seep settings (Boetius and Wenzhoefer 2013).

Among chemical precipitates, ferromanganese (FeMn) crusts and phosphorites are found on the deep seafloor (González et al. 2016). FeMn crusts occur throughout ocean basins at depths from 400 to 7,000 m on seamounts, ridges, and plateaus (Hein and Koschinsky 2014), and are typically formed in the mixing zone between oxygen-minimum waters and the oxygen-rich deep waters (Wang et al. 2009). In areas where there is a slow sedimentation rate, Fe and Mn can precipitate onto basalt and other types of rocks, forming a thin layer (patina). Phosphorites occur on continental margins and seamounts, typically at shallower depths (< 2,500 m) and in upwelling zones (Hein et al. 2016). They are formed just below the sediment-water interface with interstitial fluid rich in phosphate in suboxic to anoxic environments (Arning et al. 2009) with low sediment

accumulation rates (Schöllhorn et al. 2020). Phosphorite deposits can also form through early diagenetic processes where carbonates are replaced by carbonate fluorapatite, a phosphate mineral (Hein et al. 2016). Both phosphorites and FeMn crusts are targeted for mining in various regions of the world ocean (Hein et al. 2000, 2016, Levin et al. 2016).

These hard substrates in the deep ocean create habitat heterogeneity for diverse invertebrate communities and provide biofilm cues for settlement, substrate for attachment, reproductive sites, refuge, and, in some cases, food supply (Case et al. 2015, Levin et al. 2015, Marlow et al. 2014a). However, the same environmental conditions that allow for the formation of carbonate rocks, FeMn crusts, and phosphorites also influence biological communities and can drive benthic patterns of community structure. For example, seepage activity has been shown to dictate community composition at methane seeps (see Levin et al. 2015, Pereira et al. 2021), and effects of dissolved oxygen concentration have been reported for megafaunal communities on mineral precipitates (see Corrêa et al. 2022, Vlach 2023).

In addition to community structure, trophic structure is an important factor that affects ecosystem functioning, as energy is transferred throughout food webs (Choy et al. 2017). Trophic linkages are often identified through stable isotope (usually carbon and nitrogen) analyses (Thurber 2014) by taking advantage of natural variation in stable isotope ratios (Layman et al. 2007). Generally, there is an isotopic change with each successive trophic level, as animals preferentially respire ^{12}C over ^{13}C and excrete ^{14}N over ^{15}N (McCutchan et al. 2003, Peterson and Fry 1987). The carbon isotope shift between a species' diet and its tissue is small (0 to 1‰) but initial fractionation varies substantially among primary producers, and, therefore, $\delta^{13}\text{C}$ is an excellent indicator of carbon sources at the base of the food web (Layman et al. 2007; DeNiro and Epstein 1978). The diet-tissue shift for the nitrogen isotopes, however, is greater among successive

trophic levels, and $\delta^{15}\text{N}$ often increases by 2‰ to 4‰ with each trophic level, making the nitrogen isotope a useful tool to estimate trophic position (McCutchan et al. 2003, Post 2002). Initial $\delta^{15}\text{N}$ value is influenced by local N_2 fixation, which can be carried out by benthic deep-sea bacteria in sediments, archaea syntrophic consortia, and by chemosynthetic symbionts of invertebrates (Dekas et al. 2009, Petersen et al. 2016). An individual's stable isotope composition derives then from its food sources, and isotope composition of multiple individuals can be used to assess the trophic role of that species, i.e., its trophic niche. The position of the species in $\delta^{13}\text{C}$ - $\delta^{15}\text{N}$ bi-plots with species (or individuals and populations) is used to infer aspects of the food web structure. Community-wide metrics were developed to help illustrate how individual species' niches and dispersion of those niches drive variation in important aspects of trophic structure (Layman et al. 2007).

Methane seeps and mineral-rich hardgrounds provide many ecosystem services (i.e., contributions to human well-being from ecosystems), such as carbon sequestration, nutrient cycling, specialized communities including endemic species, nursery and feeding habitats, as well as cultural and spiritual significance and have inspired literature, film, art, dance, and education (Thurber et al. 2014, Levin et al. 2016, Turner et al. 2019, Armstrong et al. 2022). However, with increasing interest in deep-sea resources, these ecosystems are threatened by bottom trawling, oil and gas extraction, and deep-seabed mining (Ramirez-Llodra et al. 2011, Levin et al. 2016). Adding to these, as the ocean warms, the consequences of ocean deoxygenation have already been reported in the deep sea (Schmidtke et al. 2017). Animals living associated with these hard substrates are most likely sensitive to such environmental changes and physical disturbances.

The main objective of this dissertation is to characterize and understand the distributions and lifestyles of macrofaunal invertebrates living on hard substrates (authigenic carbonate rocks,

FeMn crusts, and phosphorites) on continental margins at bathyal depths across environmental gradients. This dissertation generates information that can inform the response of deep-sea ecosystems on continental margins to changes in environmental conditions associated with climate change and deep-sea resource extraction and provide insights for future management and conservation efforts. Such efforts will most likely have increased public support, and consequently have a higher potential for success, if there is more awareness of the research and science that is being done; this in turn can lead to a higher engagement and input to science policy (LaRiviere et al. 2014, O'Connor et al. 2021, Escobar-Briones and Álvarez-Sánchez, 2023). Thus, this dissertation also incorporates socio-economic approaches to understand people's concern about and valuation of the deep sea and motivation toward deep-sea conservation.

The main questions to be answered are:

- (1) How do environmental conditions (i.e., seepage activity, depth, temperature, and dissolved oxygen concentration) influence the macrofaunal community on hard substrates?
- (2) How quickly does the macrofaunal community on hard substrates respond to changes in environmental conditions (e.g., seepage)?
- (3) How does substrate type influence the macrofaunal community on hard substrates?
- (4) Is macrofaunal response to changes in environmental conditions (seepage) correlated with changes in the microbial community and are macrofaunal and microbial responses the same in terms of diversity?
- (5) What aspects of the deep sea do people care about and are they willing to pay for deep-sea conservation?

To answer these questions, research was conducted at bathyal depths in the eastern Pacific Ocean, at methane seeps off Costa Rica (Fig. 1.1A-C,E), and on mineral-rich hardgrounds in the Southern California Borderland (Fig. 1.1D,F).

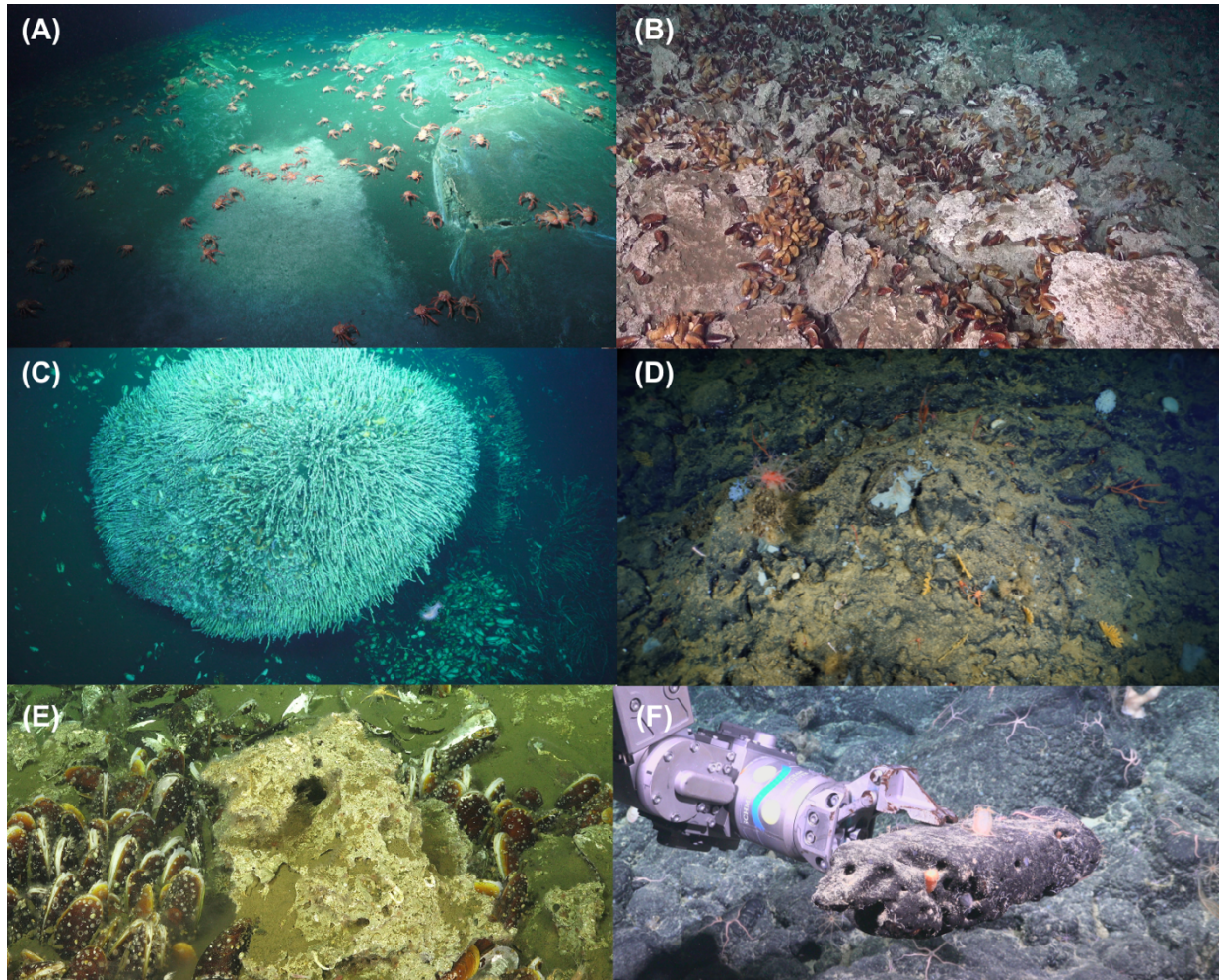


Figure 1.1: Hardground ecosystems in the deep sea and their rocks: (A) Quepos Landslide, (B) Mound 12, and (C) Jaco Scar, three methane seeps off the Costa Rican Pacific margin, and (D) Southern California Borderland hardgrounds. (E) Carbonate rock at Mound 12. (F) FeMn encrusted rock at Patton Escarpment, Southern California Borderland.

The following dissertation data chapters were produced:

Chapter 2 involves an examination of the macrofaunal community and associated trophic structure on *in situ* and experimental (17-months transplant experiment and 3-month colonization

experiment) carbonate rocks at three methane seep sites in Costa Rica with different environmental conditions. New data are presented for (i) Quepos Landslide: at 380-400 m, within the oxygen minimum zone (OMZ); and (ii) Jaco Scar: at 1,850 m, well below the OMZ, a hydrothermal seep with a thermal anomaly. Both were compared to Mound 12 at 990-1,000 m with intermediate oxygen (described in Pereira et al. 2021, 2022). This chapter helped answer Questions 1 and 2.

Chapter 3 investigates whether macrofaunal assemblage patterns and their dynamics with changing seepage activity at Mound 12 correspond to specific microbial communities living on the same rocks and their dynamics. Studies were carried out on both *in situ* and transplanted rocks described in Pereira et al. (2021) and Mullin (2020). This chapter addressed Question 4.

Chapter 4 examines the effects of two mineral-rich substrate types (phosphorite, FeMn crusts), depth, dissolved oxygen concentration, and proximity to shore on macro- and megafaunal invertebrate trophic structure on hard substrates off Southern California. This chapter addressed Questions 1 and 3, and it is published in Limnology and Oceanography (Pereira et al. 2024a).

Chapter 5 employs discrete choice experiments, an economics approach, for valuation of seep ecosystem services in order to provide insights, recommendations, and guidelines for seep management and conservation efforts. An economic valuation study of Costa Rican methane seeps was conducted in a survey format, which included questions to investigate people's connection to the deep sea and the importance of deep-sea ecosystems to people. This chapter addressed Question 5 and is published in Ecological Economics (Pereira et al. 2024b).

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Chapter 2 HETEROGENEITY AND RESILIENCE OF MACROFAUNAL COMMUNITIES ON CARBONATE ROCKS AT METHANE SEEPS ACROSS DEPTH AND OXYGEN GRADIENTS ON THE COSTA RICA MARGIN

Olivia S. Pereira, Greg. W. Rouse, Erik Cordes, Jorge Cortés, Lisa A. Levin

ABSTRACT

Much of the variability in methane seep macrofaunal communities has been attributed to seepage activity, however, more attention is needed to other environmental conditions that might be playing a role in defining methane seep communities. With the goal of understanding how depth and oxygen concentration affect the influence of seepage activity on macrofaunal diversity and trophic structure and their recovery and resilience, we conducted mensurative experiments and manipulative experiments that exploit natural seepage and hydrographic conditions at three seeps on the Costa Rican Pacific margin: (1) Quepos Landslide, at 400 m, within the oxygen minimum zone (OMZ), (2) Mound 12, at 1,000 m, just below the OMZ, and (3) Jaco Scar, at 2,000 m, well below the OMZ. We observed that seepage activity is the main factor influencing macrofauna at the deepest seep, where chemosynthetic production is the main food source, and active fauna shows faster responses to colonization and transplant experiments. At intermediate depth, where the seep is under the influence of both photo- and chemosynthetic production, transition fauna seems to have higher recovery and resilience rates. In contrast, within the OMZ, low oxygen conditions override the seepage activity, yielding low densities and low diversity, and the macrofauna might not be established enough to colonize new substrates or survive changes in seepage activity. Our study highlights the heterogeneity of macrofaunal communities created by environmental conditions (i.e., seepage activity, depth, and oxygen concentration). With the expansion of OMZs due to ocean deoxygenation and possible expansion of seepage due to ocean

warming, it is important we understand how environmental conditions affect the recovery and resilience of these communities.

INTRODUCTION

Since the initial discovery of methane seeps, they have been found on continental margins all over the world in many geological and environmental settings (Sibuet and Olu 1998). Seeps occur at depths from 60 to 7,326 m (Fujikura et al. 1999, Vedenin et al. 2020) and under various dissolved oxygen concentrations, including within oxygen minimum zones (OMZs) (Levin et al. 2015, Seabrook et al. 2024). In the central East Pacific, more than 100 seep sites have been found associated with mounds, faults, subduction scars, and landslides in the offshore margin off southern Nicaragua and Costa Rica (Sahling et al. 2008). Seep habitats are frequently associated with authigenic carbonate rocks, a by-product of microbial anaerobic methane oxidation, that host diverse macrofaunal communities (Levin et al. 2015, Pereira et al. 2021). The increased heterogeneity provided by seeps – created by methane and sulfide fluxes and provision of hard substrate – interacting with hydrographic features such as depth and oxygen gradients, may shape these unique assemblages.

Zonation of deep-sea benthic communities due to environmental gradients has been long documented (Levin et al. 2001, Carney 2005). High species richness has been reported for megafaunal assemblages at intermediate depths between 1,000-3,000 m (Rex et al. 1981, Rex and Etter 2010, De Leo et al. 2017). Depth also affects the delivery of photosynthetic organic material from surface waters to the deep ocean; The export flux to deeper waters declines with depth as the organic matter is consumed by midwater organisms (Rex et al. 2005).

A well-developed OMZ at bathyal depths of 500-800 m in the Northeast Pacific also creates an important environmental gradient (Levin et al. 2016). Diversity, density, and biomass of megafaunal and macrofaunal communities have been shown to be reduced under low oxygen conditions within OMZs (Levin 2003). OMZs occur in highly productive continental margins, where water stability allows for accumulation of organic matter leading to reduced oxygen concentrations (below 0.5 mL/L) (Levin 2003, Guilini et al. 2012). However, observations of community patterns across environmental gradients come mainly from soft sediment studies. Presence of hard substrates increases ecosystem complexity, generally supporting higher megafaunal diversity and altered community composition but not abundance necessarily (Lacharité and Metaxas 2017). Benthic megafaunal abundances on seamounts have been shown to be low within the OMZ, and the highest in the lower boundary of OMZs, where sediment organic matter (i.e., food supply) was the highest (Wishner et al. 1990, 1995).

Seep macrofaunal communities have been studied in the Eastern Pacific from Alaska to Costa Rica (Levin et al. 2015, Pereira et al. 2021, Gerard et al. in prep). Macrofaunal communities show distinct characteristics associated with seepage activity, while OMZs tend to homogenize assemblages by favoring species that are more adapted to low oxygen conditions (Levin et al. 2000, Guilini et al. 2012). However, a comparison of macrofaunal community composition in seep vs non-seep sediments between seeps under different oxygen conditions showed that seeps exposed to higher oxygen concentrations showed higher diversity and species accumulation curves (Levin et al. 2010). Notably, sensitivity of hard substrate seep fauna, associated with carbonate rocks, to environmental gradients has received little attention. Ocean deoxygenation caused by increased global temperatures will expand OMZs, influencing benthic communities including methane seeps (Stramma et al. 2008, Breitburg et al. 2018). Understanding how such communities

are affected by low oxygen conditions gives us better knowledge for predicting changes in distribution patterns and resilience of the benthos in future climate scenarios (Venturelli et al. 2018).

Here, we examined how species diversity, density, composition and trophic diversities of macroinvertebrates on carbonate rocks vary across three methane seep sites with different environmental context (depth and oxygen level). We ask how these differences affect the dynamics and resilience of such communities. By conducting mensurative experiments that exploit natural seepage and hydrologic gradients, and two kinds of manipulative experiments (colonization and transplants across seepage gradients) we aimed to understand how the influence of seepage activity on community dynamics is affected by depth, temperature, and oxygen concentrations (1) in naturally occurring (i.e., *in situ*) macrofaunal communities on carbonate rocks, (2) in newly colonized assemblages on carbonate substrates, and (3) for the recovery and resilience capacity of macrofaunal communities on carbonate rocks. Specific questions addressed are: (i) Are macrofaunal responses to seepage activity affected by hydrologic conditions? (ii) Is the resilience of seep and transition fauna to a decline in seepage affected by hydrologic conditions? (iii) What features of community structure are recovered in 3 months? In 17 months? And do these responses change with different hydrographic conditions? (iv) What taxa and what attributes of these taxa confer good colonization ability and resilience? Is colonization ability and resilience affected by hydrologic conditions?

METHODS

Study area

The convergent margin of Costa Rica in the Pacific Ocean, where the Cocos Plate subducts beneath the Caribbean Plate (Kimura et al. 1997), hosts hundreds of seep sites at depths of 400-2,400 m (Sahling et al. 2008), and new sites are continually being discovered. For example, a new seep named The Thumb was discovered in January 2019 during a research cruise aboard the *R/V Falkor* (FK190106) (personal observation). These seeps have been found associated with mounds, faults, subduction scars, and landslides, and almost all the rock samples from previous studies were methane-related authigenic carbonates (Sahling et al. 2008). This study examines macrofaunal invertebrates on carbonate rocks from three bathyal seep sites that span from within to below the Costa Rica oxygen minimum zone (OMZ; $[O_2] < 22 \mu M$) (Fig. 2.1, Table 2.1):

Quepos Landslide is a low-oxygen ($4.25 \mu M O_2 kg^{-1}$) site at 389-400 m within the OMZ, with a head wall 160 m high (Harders 2011), and fluids may originate from buried hydrates within the gas hydrate stability zone (Steeb et al. 2015). Bacterial mats are present throughout, covering the sediment, with a few carbonate and mudstone rocks outcrops (Karaca et al. 2014), as well as abundant tuna crabs *Pleurocondes* sp. and fishes (personal observation).

Mound 12 is located at 990-1000 m water depth (Mau et al. 2006), just below the oxygen minimum zone ($27.1 \mu M O_2 kg^{-1}$) (Levin et al. 2015). Mudflows suggest that it is a frequently active site (Niemann et al. 2013) with an alternation of seepage with low- activity phases within 12 months (Niemann et al. 2013, Mau et al. 2007). The bottom is characterized by carbonates hosting the Costa Rican yeti crabs *Kiwa puravida* (Thurber et al. 2011) and bathymodiolin mussel beds (Sahling et al. 2008), as well as smaller animals such as neolepetopsid limpets and provannid snails (Pereira et al. 2021).

Jaco Scar is a large landslide caused by seamount subduction with warm fluid ($\sim 6^\circ C$) at the active seeping area, and, thus, is considered a hydrothermal seep with assemblages of species

associated with hydrothermal vent and seep ecosystems (Levin et al. 2012). It is located well below the OMZ ($79.07 \mu\text{M O}_2 \text{ kg}^{-1}$) at 1,850 m, with very high, deeply sourced methane fluxes (Tryon et al. 2010, Mau et al. 2014). Siboglinid polychaetes *Lamellibrachia barhamia* and *Escarpia spicata* form large spherical bushes (1-2 m in diameter), supporting bathymodiolin mussels and lepetodrilid limpets in high densities, as well as zoarcid fishes nestling within the worm tubes (Levin et al. 2012, Frable et al. 2023). Dense beds of the clam *Archivesica gigas* also support gastropods and galatheid crabs, and frenulate siboglinids (*Siboglinum* sp.) form another distinct microhabitat (Levin et al. 2012).

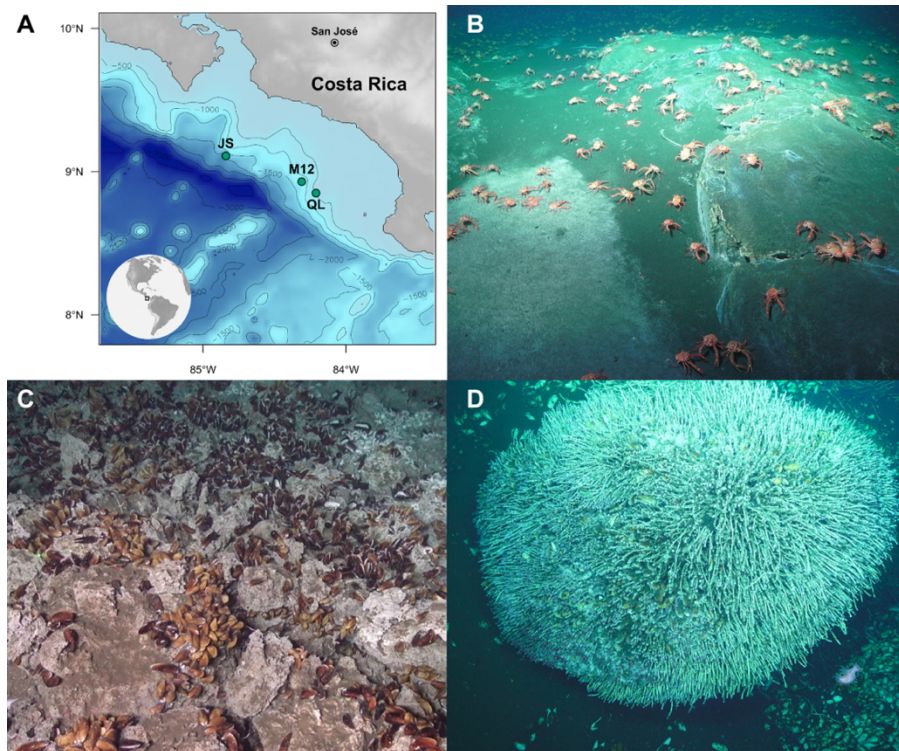


Figure 2.1: (A) Location and photos of the three methane seep sites off the Pacific margin of Costa Rica: (B) Quepos Landslide (QL), (C) Mound 12 (M12), and (D) Jaco Scar (JS).

Table 2.1: Environmental settings of methane seep sites off the Pacific margin of Costa Rica.

Seep site	Latitude	Longitude	Depth range (m)	Average bottom temperature (°C)	Average bottom salinity	Average bottom oxygen (µM)
Quepos Landslide	8°51.051'N	84°12.984'W	308-399	11.31	34.79	4.25
Mound 12	8°55.827'N	84°18.757'W	991-1003	4.91	34.56	27.1
Jaco Scar	8°05.389'N	84°49.620'W	1708-1887	2.48 ¹	34.71	79.07

¹ A thermal anomaly was measured of up to 8°C in certain seeping areas.

Shipboard sampling

Carbonate rocks were sampled for faunal studies at three seep sites (Quepos Landslide, Mound 12, and Jaco Scar) off the Pacific margin of Costa Rica (Table 2.1) during two research cruises aboard the *R/V Atlantis* (May to June 2017 – AT37-13 – and October 2018 – AT42-03) using the HOV Alvin, and a third research cruise aboard *R/V Falkor* (January 2019) using the ROV SuBastian. Unmanipulated samples (*in situ*) were taken at sites with different seepage activity when possible (Table 2.1). High activity level (i.e., active sites) was defined visually by the presence of microbial mats, methane bubbles or seep megafauna (bathymodiolin mussels, vesicomyid clams, and/or siboglinid tube worms), as previously done by Levin et al. (2015). Background sites were defined by not having seep megafauna and carbonates but occasional deep-sea megafauna (e.g., paralomid crabs). Transition sites were defined by having carbonates and a mix of species from the surrounding, background deep sea and seep area (see Ashford et al. 2021). The rocks were placed into individual containers of Delrin bioboxes on the Alvin basket to avoid cross contamination of fauna during recovery, and they were used as control samples for two experiments:

Colonization experiment: To compare the dynamics and colonization rate of seep and non-seep communities at the different sites, defaunated rocks were deployed at sites with active seepage for 3 months (deployed in October 2018, recovered in January 2019) and 17 months (deployed in

May 2017, recovered in October 2018) at Quepos Landslide and Jaco Scar. A 17-month and 7-year colonization experiment were also performed at Mound 12.

Transplant experiment: To compare biological patterns and dynamics of methane seep fauna, the rates and trajectory of community response, and the effect of hydrographic conditions on such dynamics under different seepage activity, carbonate rocks hosting animals were moved by the HOV to sites with different seepage activity for 17 months (start in May 2017, recovered in October 2018) at Quepos Landslide and Jaco Scar. A 17-month transplant experiment was also performed at Mound 12. Rocks were moved from active sites to lesser seepage activity and from transition or background sites to active sites.

Although it was not possible to perform identical experiments at all sites, we tried to keep the experimental design as similar as possible. The 17-month and 7-year colonization experiments at Mound 12 and the 17-month transplant experiment at Mound 12 were previously reported in Pereira et al. (2021). However, the invertebrate communities were described and discussed at the family and order levels there. Here, we describe and discuss the results at the species level and include only rocks for which we have density values.

In situ and experimental carbonate rocks were photographed intact upon recovery. The associated fauna was handpicked and identified to the lowest taxonomic level possible. Tissue samples for stable isotope analyses were collected from at least three specimens of each species on each rock to examine reliance on seep production and frozen at -20°C. The rocks were wrapped in aluminum foil to determine the approximate rock surface area later in the laboratory, and then left overnight in a bucket with filtered seawater at room temperature for additional infauna to crawl out. On the next day, the water in the buckets with the remaining fauna was sieved on a 0.3 μm mesh and preserved in 96% ethanol in Nalgene bottles to be sorted in the laboratory.

To characterize particulate organic matter (POM) stable isotope composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) for constraining nutritional sources, surface (0-5 m) and bottom waters (< 15 m above the seafloor) were sampled at each seep using 2-4 L of water collected in a Niskin Rosette on a CTD and filtered through a glass microfiber filter. The filter with retained material was frozen at -20°C for later processing in the laboratory.

Laboratory processing

In the laboratory at Scripps Institution of Oceanography, the aluminum foil used to wrap the rocks was weighed using a top-loading balance to an accuracy of two decimal places in milligrams. The total weight of the foil was divided by the average weight of a 1 cm^2 piece of foil to determine the approximate rock surface area in cm^2 . Samples in Nalgene bottles were re-sieved using a $0.3\text{-}\mu\text{m}$ mesh and sorted in freshwater at 12x magnification under a dissecting microscope. The specimens were identified to species level and counted. The numbers of animals that were removed at sea upon recovery for isotope analyses were added to the laboratory total counts. The faunal tissue and POM samples collected for stable isotope analyses ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) were processed as in Pereira et al. (2021, 2022) and sent to Washington State University for stable isotope measurements. Precision for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses were $\pm 0.02\text{‰}$ and $\pm 0.05\text{‰}$, respectively (standard deviation of 10 replicate organic standards).

Data synthesis and statistical analyses

Densities of invertebrate communities were standardized to number of individuals per 200 cm^2 , the average size of a rock, as previously done in Levin et al. (2015) and Pereira et al. (2021). PERMANOVA analysis was performed to check for variability in densities among seeps, seepage

activity, and sampling year (i.e., cruise) for *in situ* communities. To compare densities between *in situ* communities and experimental communities (including colonization and transplant), Kruskal-Wallis tests followed by Dunn's tests using the Benjamini-Hochberg adjustment (Benjamini and Hochberg 1995) were used for datasets with three factors or more, and Wilcoxon tests were used for datasets with two factors.

Three-way ANOSIM and SIMPER analyses were performed on Primer v.7 using Bray-Curtis dissimilarity matrices of species density values, after fourth-root transformation, to assess variability in community composition among seeps, seepage activity, and sampling year. One-way ANOSIM and SIMPER analyses were performed on Primer v.7 to assess differences in community composition between *in situ* rocks and experimental rocks, including transplant and colonization experiments. Cluster and non-multidimensional scaling (nMDS) analyses were also performed on Primer v.7 with the same dataset to provide a visualization of the results.

Shannon-Wiener diversity (H'), Pielou's evenness (J), richness (S), and rarefaction curves were calculated using the R package 'vegan' for the seeps and different seepage activity, including for *in situ* and experimental communities, by pooling total counts of all samples at each seep or seepage activity.

Mean isotope values of species were calculated before calculating the averages by seep, seepage activity, and experiment to avoid sampling bias. PERMANOVA tests were performed to check for variability in isotopic composition among seeps, seepage activity, and sampling year for *in situ* communities. To compare isotopic values between *in situ* communities and experimental communities (including colonization and transplant), Kruskal-Wallis tests followed by Dunn's tests using the Benjamini-Hochberg adjustment (Benjamini and Hochberg 1995) or Wilcoxon tests were used as described above. Community-level metrics (Layman et al. 2007) were calculated

using the ‘SIBER’ package in R (Jackson et al. 2011), following Pereira et al. (2021, 2022, 2024). Trophic diversity was assessed using standard elliptical area corrected for sample size (SEAc) in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ biplots. Pairwise trophic overlap based on Bayesian posterior estimates of ellipses was calculated using 2 chains of 20,000 iterations with a burn-in of 1000 and thinning of 10, as previously done in Pereira et al. (2022, 2024).

RESULTS

Naturally occurring macrofaunal communities

Densities of macrofaunal invertebrates on carbonate rocks varied among seep locations (Fig. 2.2A-C); Jaco Scar showed the highest densities, and Quepos Landslide, the lowest (PERMANOVA, $R^2 = 0.2087$, $p = 0.0001$). Across all seeps, densities were the highest at transition sites ($R^2 = 0.1040$, $p = 0.05$). However, within seep, active sites showed the highest densities at Mound 12 in 2017 (Kruskal-Wallis, $\chi^2_4 = 12.3087$, $p = 0.02$), and there was no significant difference in densities with seepage activity at Quepos Landslide (Kruskal-Wallis, $\chi^2_3 = 1.5128$, $p = 0.70$). These might have led to the significant, but weak interaction between cruise (i.e., sampling year) and seep site that was found ($R^2 = 0.0896$, $p = 0.008$), since there was no significant interannual difference in densities ($R^2 = 0.0141$, $p = 0.37$) and no significant interaction between seep and habitat ($R^2 = 0.0286$, $p = 0.40$).

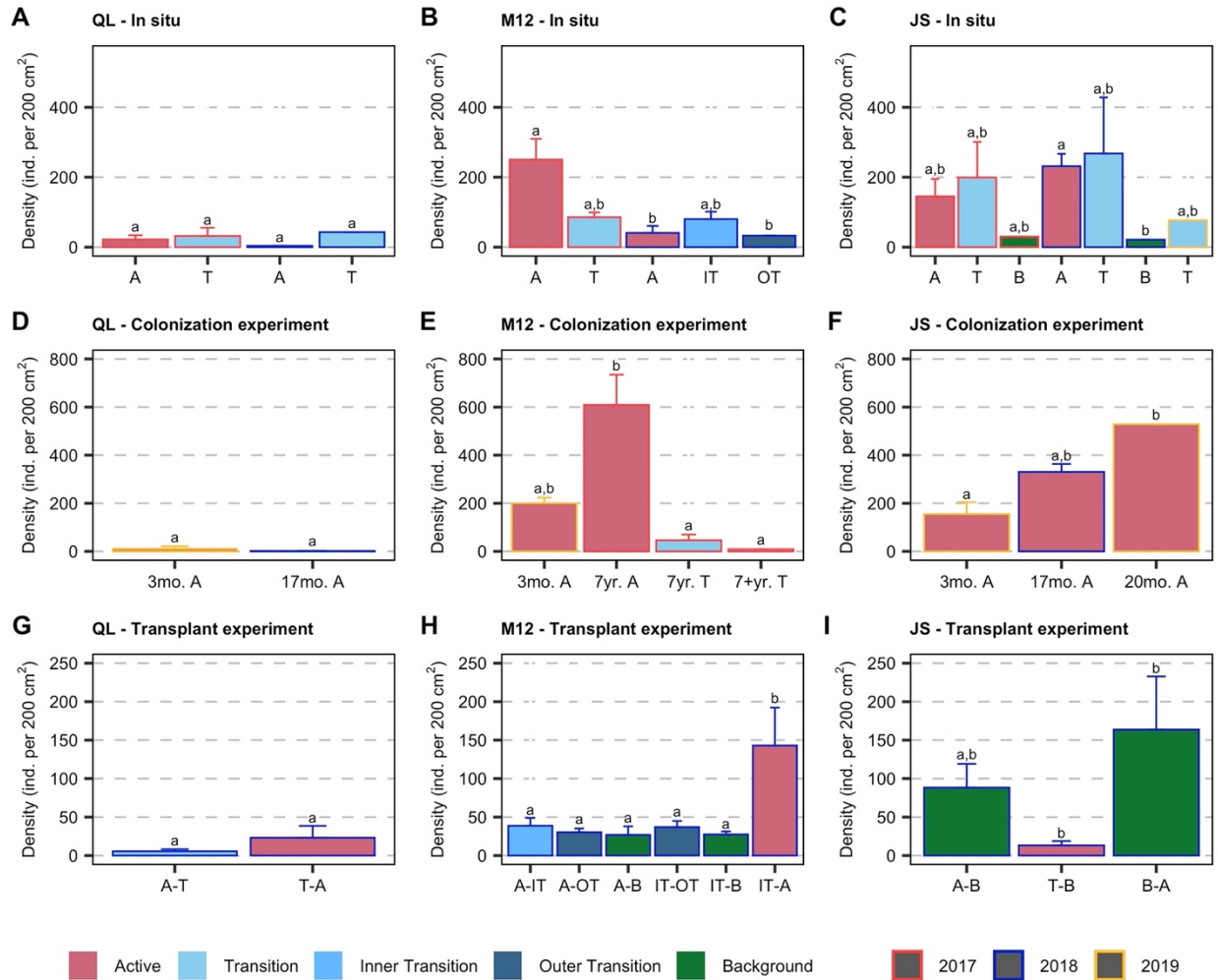


Figure 2.2: Density (number of individuals per 200 cm²) of macrofaunal invertebrates on (A-C) in situ carbonate rocks, (D-F) colonization rocks, and (G-I) transplanted rocks at Quepos Landslide (QL), Mound 12 (M12), and Jaco Scar (JS) across a seepage gradient. Fill colors indicate seepage activity (A: Active, T: Transition, IT: Inner transition, OT: Outer transition, B: Background) and border colors indicate sampling year.

Community composition was also different among seep locations (three-way ANOSIM, Global $R = 0.671$, $p = 0.001$; Table 2.2), seepage activity (Global $R = 0.355$, $p = 0.006$; Table 2.3), and year (Global $R = 0.355$, $p = 0.006$) (Fig. 2.3A-C, Fig. 2.4A, Supp. Fig. 2.1-2.4). Among seeps, Jaco Scar macrofaunal communities on carbonates showed the highest dissimilarity to those at the other seeps (JS vs QL: $R = 0.775$, $p = 0.001$; JS vs M12: $R = 0.709$, $p = 0.001$), and Mound 12 and

Quepos Landslide showed the lowest dissimilarity, although still high ($R = 0.662$, $p = 0.001$) (Fig. 2.4A, Supp. Fig. 2.1). Jaco Scar was dominated by the tanaids *Parakanthophoreus* sp. 1 and *Typhlotanoides* sp. 1 (27%), annelids *Chrysopetallum* sp. and *Laminatubus paulbrooksi* (23%), and the snail *Provanna goniata* (13%). The tanaids and *L. paulbrooksi* contributed the most (14%) to the dissimilarity between Jaco Scar and Quepos Landslide (SIMPER, average dissimilarity = 96.97). These five most abundant species at Jaco Scar were also the species that contributed the most (17%) to the dissimilarity between Jaco Scar and Mound 12, together with the snail *Provanna laevis*, which was more abundant at Mound 12 (SIMPER, average dissimilarity = 87.09). In addition to the abundance of *P. laevis* (19%), Mound 12 was dominated by *Chrysopetallum* sp. (15%) and the limpets *Lepetodrilus ovalis* (8%), Neolepetopsidae (6%), *Pyropelta musaica* (3%), and *Pyropelta wakefieldi* (3%). *P. laevis* and *L. ovalis* contributed to 10% of the dissimilarity between Mound 12 and Quepos Landslide (SIMPER, average dissimilarity = 93.13). Finally, Quepos Landslide was dominated by a species in the hydrozoan order Anthoathecata (27%).

Across all seeps, communities at active sites were different than at inner transition ($R = 0.323$, $p = 0.035$), outer transition ($R = 0.75$, $p = 0.036$), and background sites ($R = 1.000$, $p = 0.033$), and more similar to communities at transition sites, although not significantly so ($R = 0.151$, $p = 0.053$). In general, active sites were dominated by *P. goniata* (16%) and *P. laevis* (12%). Transition sites (including transition, inner transition, and outer transition) were dominated by *Chrysopetallum* sp. (24%) and *Parakanthophoreus* sp. 1 (12%). Surprisingly, background sites were dominated by *L. paulbrooksi* (18%). However, the dominance of species and major taxa within seepage activity varied among seeps (Table 2.3, Fig. 2.3A-C).

Finally, there was a shift in community composition across all seeps from 2017 to 2018 ($R = 0.351$, $p = 0.003$) and no significant difference between 2017 vs 2019 ($R = 0.333$, $p = 0.40$) and

2018 vs 2019 ($R = 0$, $p = 0.67$), possibly due to the low sample size in 2019 (only one *in situ* rock collected at Jaco Scar at a transition site). The 2017 to 2018 interannual difference was higher at active sites (SIMPER, average dissimilarity = 84.15), where seep species (e.g., *P. laevis*, *L. ovalis*, *Lepetodrilus stet.*, *P. musaica*, *P. wakefieldi*, and *Kiwa puravida*) were more abundant in 2017, than at transition sites (SIMPER, average dissimilarity = 75.71).

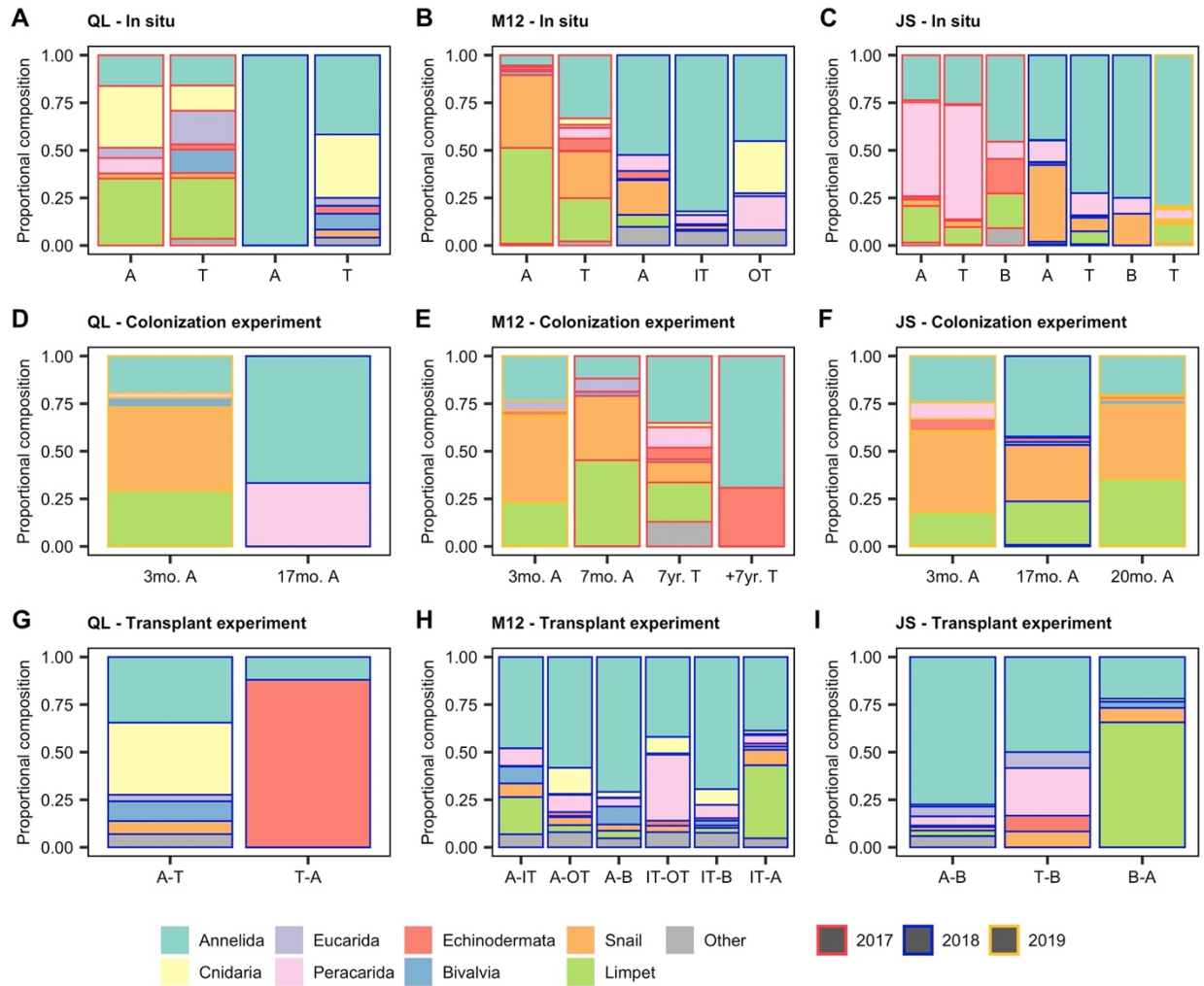


Figure 2.3: Proportional community composition of macrofaunal invertebrates on (A-C) *in situ* carbonate rocks, (D-F) colonization rocks, and (G-I) transplanted rocks at Quepos Landslide (QL), Mound 12 (M12), and Jaco Scar (JS) across a seepage gradient. A: Active, T: Transition, IT: Inner transition, OT: Outer transition, B: Background.

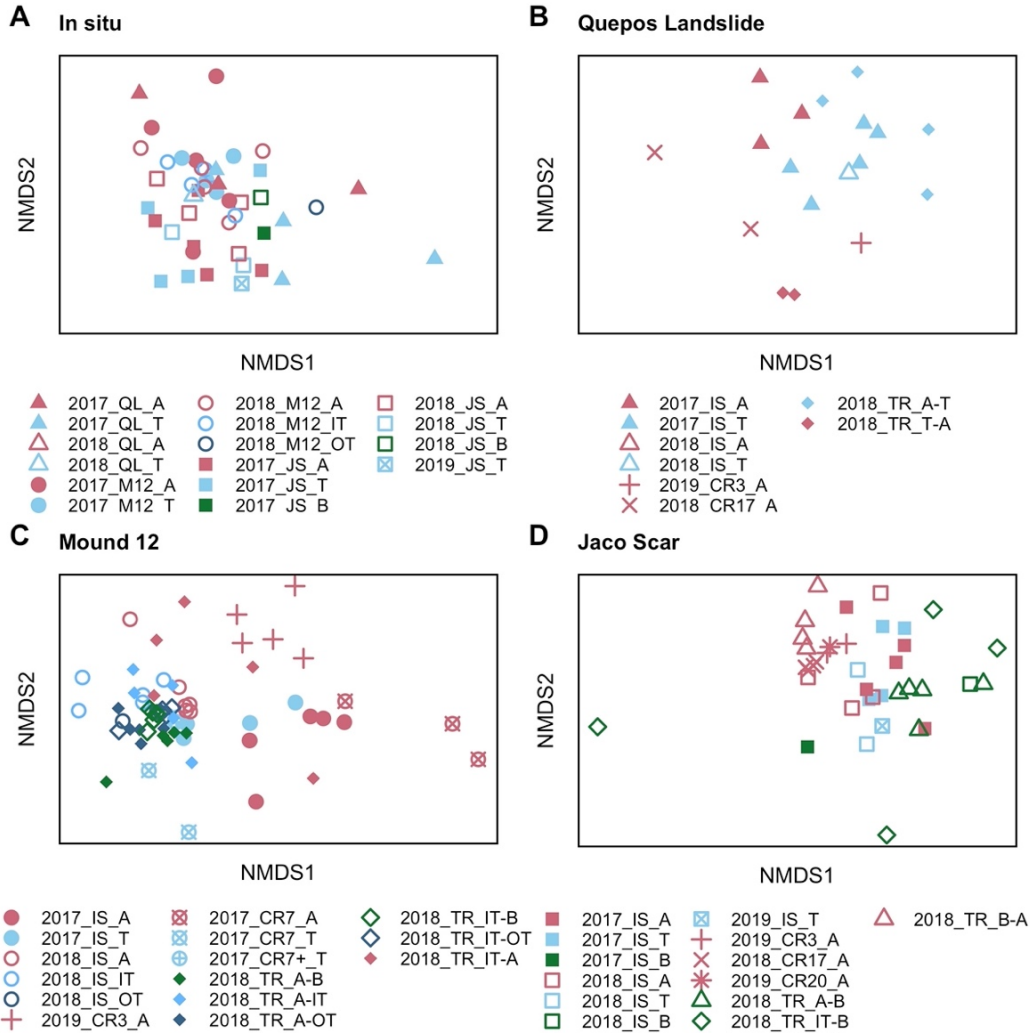


Figure 2.4: Non-multidimensional scaling analyses of macrofaunal invertebrates on (A) in situ carbonate rocks at the three seep locations, and on in situ and experimental carbonate rocks at (B) Quepos Landslide, (C) Mound 12, and (D) Jaco Scar.

Among seeps, Mound 12 showed the highest Shannon Wiener diversity ($H' = 2.83$), richness ($S = 52$), and Pielou's evenness ($J = 0.73$), followed by Jaco Scar ($H' = 2.44$, $S = 47$, $J = 0.71$), and Quepos Landslide ($H' = 1.75$, $S = 14$, $J = \text{n.a.}$ due to a sample having 1 specimen) (Table 2.4). However, Jaco Scar showed a steeper slope of the species rarefaction curve (Fig. 2.5A). Among seepage activity across all seeps, transition sites showed the highest Shannon-Wiener diversity ($H' = 2.36\text{-}2.63$) and background sites had the lowest ($H' = 2.08$) (Fig. 2.5B). Richness

decreased with increasing distance from the seep (S at active sites = 47.5, transition sites = 26-69, background = 11), and evenness was the highest at background sites (J = 0.90) followed by transition sites (J = 0.56-0.81). These general patterns of diversity metrics with seepage activity were also observed within each seep location, except for Jaco Scar, where richness was the highest at active sites (Table 2.4, Fig. 2.5C).

Table 2.2: Top 10 most abundant species of macrofaunal invertebrates on carbonate rocks at Quepos Landslide, Mound 12, and Jaco Scar. Superscripts indicate species that individually contributed to more than 10% of the community at sites with different seepage activity (A: active, T = transition, B = background).

Quepos Landslide	%	Mound 12	%	Jaco Scar	%
<i>Anthoathecata</i> ^{A+T}	27	<i>Provanna laevis</i> ^{A+T}	19	<i>Chrysopetallum</i> sp. ^{A+T}	17
<i>Eudaronia</i> sp. ^A	8	<i>Chrysopetallum</i> sp. ^{IT+OT+T}	14	<i>Parakanthophoreus</i> sp. 1 ^T	14
<i>Placopecten</i> sp. ^T	8	<i>Lepetodrilus ovalis</i> ^A	8	<i>Provanna goniata</i> ^A	13
<i>Pyropelta musaica</i>	8	Neolepetopsidae ^A	6	<i>Typhlotanoides</i> sp. 1 ^A	7
<i>Branchinotogluma</i> sp.	6	<i>Pyropelta musaica</i>	3	<i>Laminatubus paulbrooksi</i> ^B	6
Serpulidae sp. 1	6	<i>Pyropelta wakefieldi</i>	3	<i>Bispira</i> sp.	5
<i>Pleuroncodes monodon</i>	6	<i>Lepetodrilus stet.</i>	3	<i>Archinome levinae</i>	3
<i>Lepetodrilus stet.</i>	6	Fucaria sp. 1	3	<i>Neolepetopsis</i> sp. 2	2
<i>Dorvillea</i> sp.	4	<i>Anguillosyllis</i> sp.	2	<i>Neolepetopsis</i> sp. 3	2
Galatheididae sp.	4	<i>Laminatubus</i> sp.	2	<i>Gammaropsis</i> sp. 2	2

Table 2.3: Top 10 most abundant species of macrofaunal invertebrates on carbonate rocks at active, transition (including transition, inner transition, and outer transition), and background sites. Superscripts indicate species that individually contributed to more than 10% of the community at the different seeps (JS: Jaco Scar, M12: Mound 12, QL: Quepos Landslide).

Active	%	Transition	%	Background	%
<i>Provanna goniata</i> ^{JS}	16	<i>Chrysopetallum</i> sp. ^{JS+M12}	24	<i>Laminatubus paulbrooksi</i> ^{JS}	18
<i>Provanna laevis</i> ^{M12}	12	<i>Parakanthophoreus</i> sp. 1 ^{JS}	12	<i>Chrysopetallum</i> sp.	9
<i>Chrysopetallum</i> sp. ^{JS}	7	<i>Bispira</i> sp.	4	Hesionidae	6
<i>Typhlotanoides</i> sp. 1 ^{JS}	6	<i>Anthoathecata</i> ^{M12+QL}	3	Ophiuroidea	6
<i>Parakanthophoreus</i> sp. 1	6	<i>Laminatubus paulbrooksi</i>	3	<i>Ophiura</i> cf. <i>scutellata</i>	6
<i>Lepetodrilus ovalis</i> ^{M12}	6	<i>Provanna laevis</i> ^{M12}	3	<i>Lepetodrilus stet.</i>	6
Neolepetopsidae ^{M12}	5	<i>Typhlotanoides</i> sp. 1	3	<i>Fucaria</i> sp. 2	6
<i>Laminatubus paulbrooksi</i>	4	<i>Archinome levinae</i>	2	Amphinomidae sp. 1	3
<i>Pyropelta musaica</i> ^{QL}	2	<i>Gammaropsis</i> sp. 2	2	<i>Cirriiformia</i> sp.	3
<i>Pyropelta wakefieldi</i>	2	<i>Neolepetopsis</i> sp. 3	2	Phyllodocidae	3

Table 2.4: Community and trophic diversity metrics of macrofaunal invertebrates on in situ and experimental carbonate rocks at Quepos Landslide, Mound 12, and Jaco Scar across a seepage gradient. Community metrics are Shannon-Wiener diversity (H'), richness (S), and Pielou's evenness(J). Trophic metrics are mean $\pm$ 1 standard error $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (‰), total niche area (TA), and standard ellipse area corrected for sample size (SEAc).

	H'	S	J	Mean $\delta^{13}\text{C}$ (‰)	Mean $\delta^{15}\text{N}$ (‰)	TA	SEAc
Quepos Landslide In situ rocks							
2017 Active	2.13	12	0.77	-22.4 ± 2.0	5.2 ± 2.3	89.5	73.4
2017 Transition	2.76	29	0.82	-20.1 ± 0.8	7.9 ± 1.0	142.1	59.2
2018 Active	2.13	16	0.77	n.a.			
2018 Transition	2.09	11	0.87	-22.9	10.2	35.3	26.2
Mound 12 In situ rocks							
2017 Active	2.51	52	0.63	-38.2 ± 2.4	4.6 ± 0.7	342.7	111.1
2017 Transition	3.19	67	0.76	-33.9 ± 3.4	6.5 ± 1.5	633.6	181.2
2018 Active	3.44	46	0.9	-38.4 ± 1.0	5.1 ± 0.7	250.5	86.3
2018 Inner transition	2.36	69	0.56	-33.7 ± 3.3	8.1 ± 0.7	158	59.1
2018 Outer transition	2.63	26	0.81	-25.7 ± 0.1	9.1 ± 0.6	13.1	12.4
Jaco Scar In situ rocks							
2017 Active	2.88	77	0.66	-39.8 ± 2.1	3.8 ± 0.5	520.5	158.5
2017 Transition	2.4	63	0.58	-32.9 ± 1.3	4.5 ± 1.3	367.9	134.7
2017 Background	2.69	16	0.97	-24.5	3.8	15.9	29.4
2018 Active	2.59	93	0.57	-39.3 ± 2.1	4.9 ± 0.4	561.5	101.7
2018 Transition	2.66	47	0.69	-38.0 ± 4.1	8.0 ± 2.0	97.3	73.6
2018 Background	1.47	6	0.82	-29.9	10	13.5	48.9
2019 Transition	2.39	29	0.71	-44	7.1	1.9	7.1
Quepos Landslide Transplant experiment – 17 months (2017-2018)							
Active to transition	2.04	12	0.82	-21.2 ± 0.3	10.2 ± 0.4	1.6	2
Transition to active	0.42	3	0.38	-20.6	11.7	n.a.	
Mound 12 Transplant experiment – 17 months (2017-2018)							
Active to background	3.61	76	0.83	-45.3 ± 9.2	6.2 ± 1.2	470.8	273.7
Active to inner transition	3.49	76	0.83	-32.5 ± 5.4	8.5 ± 2.0	406.9	169.9
Active to outer transition	3.36	65	0.81	-37.7 ± 4.3	8.3 ± 1.0	104	62.2
Inner transition to active	2.88	71	0.67	-39.5 ± 2.8	5.4 ± 1.1	164.5	50.3
Inner transition to background	3.04	52	0.77	-23.0 ± 0.9	10.5 ± 1.7	60.7	48.2
Inner transition to outer transition	3.05	45	0.8	-29.4 ± 5.1	9.3 ± 0.8	117.4	73.9
Jaco Scar Transplant experiment – 17 months (2017-2018)							
Active to background	2.24	41	0.6	-38.4 ± 3.8	7.6 ± 1.1	223.1	152.8
Transition to background	2.37	11	0.99	-37.3 ± 6.6	5.5 ± 2.0	81.1	155.5
Background to active	2.68	55	0.67	-40.8 ± 1.0	2.6 ± 0.6	260.3	76.7
Colonization experiment – 3 months (2018-2019)							
Quepos Landslide active	2.29	15	0.85	-22.1	11.1	n.a.	
Mound 12 active	2.63	69	0.59	-35.8 ± 0.9	7.5 ± 0.4	203.6	57.2
Jaco Scar active	2.98	54	0.75	-38.5 ± 1.8	6.8 ± 0.6	226.1	66.8
Colonization experiment – 17 months (2017-2018)							
Quepos Landslide active	1.1	3	1	-33.5	5.5	n.a.	
Jaco Scar active	2.82	87	0.63	-39.2 ± 0.8	6.0 ± 0.6	197.3	53.9
Colonization experiment – 7.4 years (2010-2017)							
Mound 12 active	2.25	43	0.6	-34.6 ± 2.5	1.3 ± 0.4	365	96.2
Mound 12 transition	3.41	45	0.8	-29.8 ± 1.8	7.4 ± 0.7	173.8	83.4
Other colonization experiments							
Mound 12 transition – 7+ years	1.92	8	0.93				
Jaco Scar active – 20 months	2.3	48	0.63				

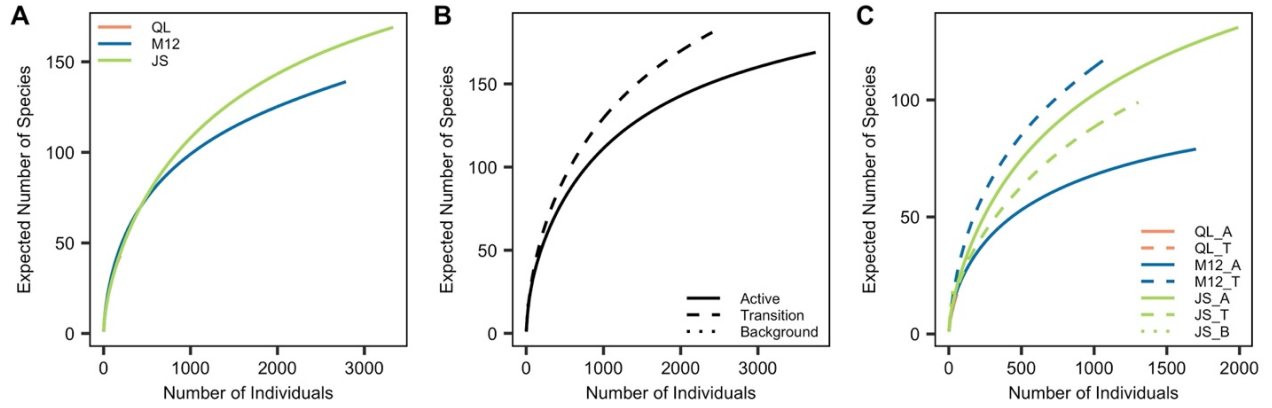


Figure 2.5: Rarefaction curves of macrofaunal invertebrates on in situ carbonate rocks by (A) seep location, (B) seepage activity, and (C) seepage activity within seep location. Seep locations: Quepos Landslide (QL), Mound 12 (M12), and Jaco Scar (JS). Seepage activities: Active (A), transition (T), and background (B).

Stable carbon isotope values ($\delta^{13}\text{C}$) of macrofaunal invertebrates (Table 2.4) were the lowest at Jaco Scar and highest at Quepos Landslide (PERMANOVA, $R^2 = 32.4361$, $p = 0.0001$) and increased with increasing distance from each seep (i.e., along a gradient of decreasing seepage activity) ($R^2 = 6.3869$, $p = 0.0005$). There was also a significant interannual difference in $\delta^{13}\text{C}$ values ($R^2 = 4.9926$, $p = 0.0067$), possibly due to low sample size (two rocks were collected at Quepos in 2018 and one rock was collected at Jaco Scar in 2019). There was no significant interaction between seep location and sites with different seepage activity ($R^2 = 0.1638$, $p = 0.87$) and between cruise (year) and seep location ($R^2 = 0.2431$, $p = 0.82$) in $\delta^{13}\text{C}$ values. For the stable nitrogen isotopes ($\delta^{15}\text{N}$), there was no difference in values of macrofaunal invertebrates across seep location (PERMANOVA, $R^2 = 0.0829$, $p = 0.07$), seepage activity ($R^2 = 0.1324$, $p = 0.11$), and cruise ($R^2 = 0.0875$, $p = 0.06$) (Table 2.4). There was also no significant interaction between seep location and seepage activity ($R^2 = 0.0372$, $p = 0.38$) and between cruise and seep location ($R^2 = 0.0161$, $p = 0.75$) in $\delta^{15}\text{N}$ values. However, there was a pattern of decreasing $\delta^{15}\text{N}$ values with depth, with the shallowest seep, Quepos Landslide, showing the highest values, and the

deepest seep, Jaco Scar, the lowest. There was also a pattern of increasing $\delta^{15}\text{N}$ values with increasing distance from the seep, with active sites across all seeps showing lower values and background sites, higher values (Table 2.4). Trophic diversity based on SEAc and trophic niche based on TA increase with increasing seepage activity, except at Mound 12 in 2017 when these were higher at transition sites (Table 2.4, Fig. 2.6A-C). Trophic overlap at active and transition was the highest between Jaco Scar and Mound 12 (61% and 35%, respectively), and the lowest between Jaco Scar and Quepos Landslide (23% and 20%, respectively) (Fig. 2.7A). Interestingly, trophic overlap across seep location and seepage activity was the highest between Jaco Scar transition sites and Mound 12 active sites (48%) and between Jaco Scar background sites and Quepos Landslide active sites (46%) (Fig. 2.7D).

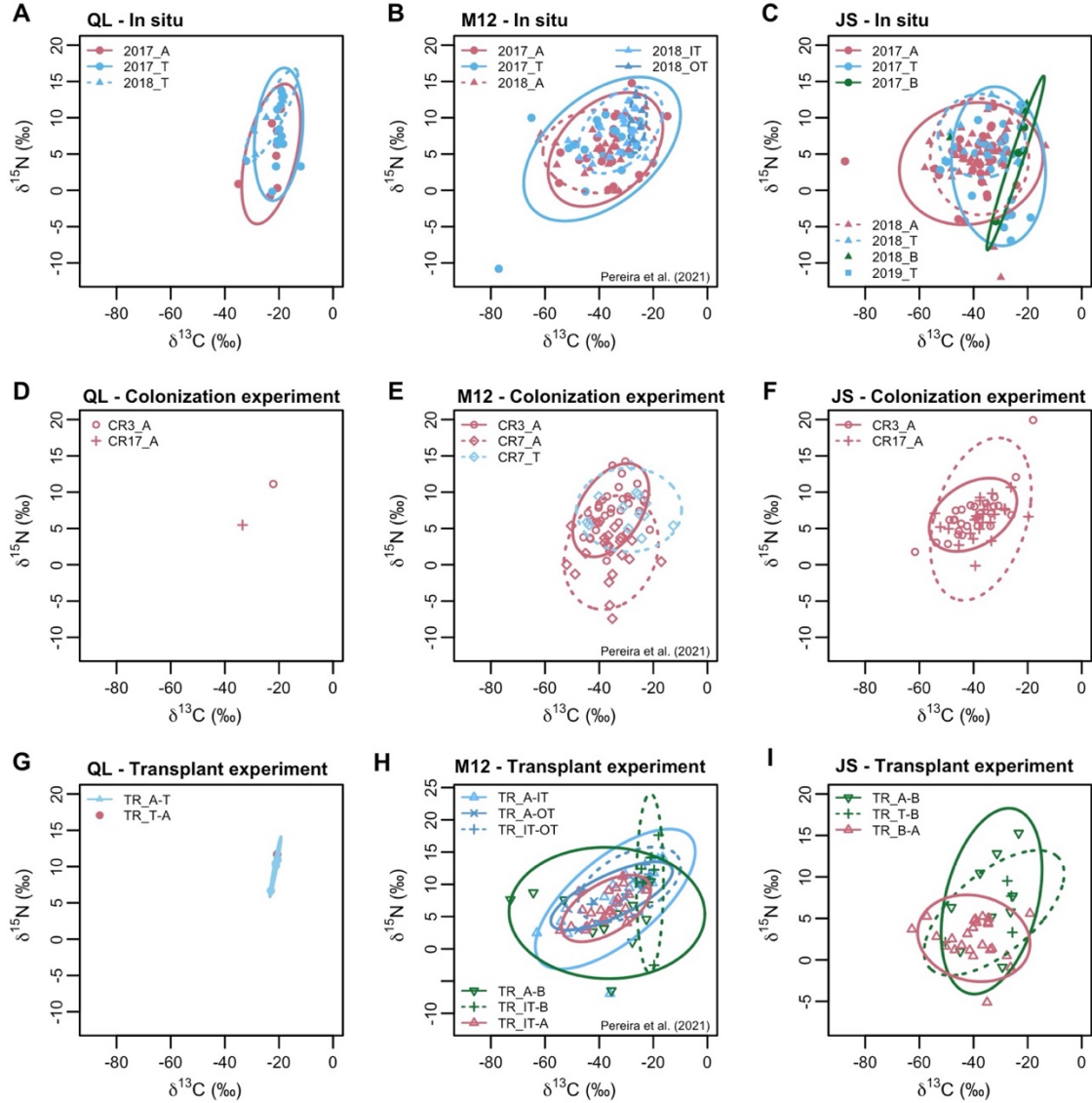


Figure 2.6: Trophic niche diversity of macrofaunal invertebrates on (A-C) in situ carbonate rocks, (D-F) colonization rocks, and (G-I) transplanted rocks at Quepos Landslide (QL), Mound 12 (M12), and Jaco Scar (JS) across a seepage gradient. Each point represents the average for one species. Ellipses represent standard ellipse areas corrected by sample size (SEAc). A: Active, T: Transition, IT: Inner transition, OT: Outer transition, B: Background.

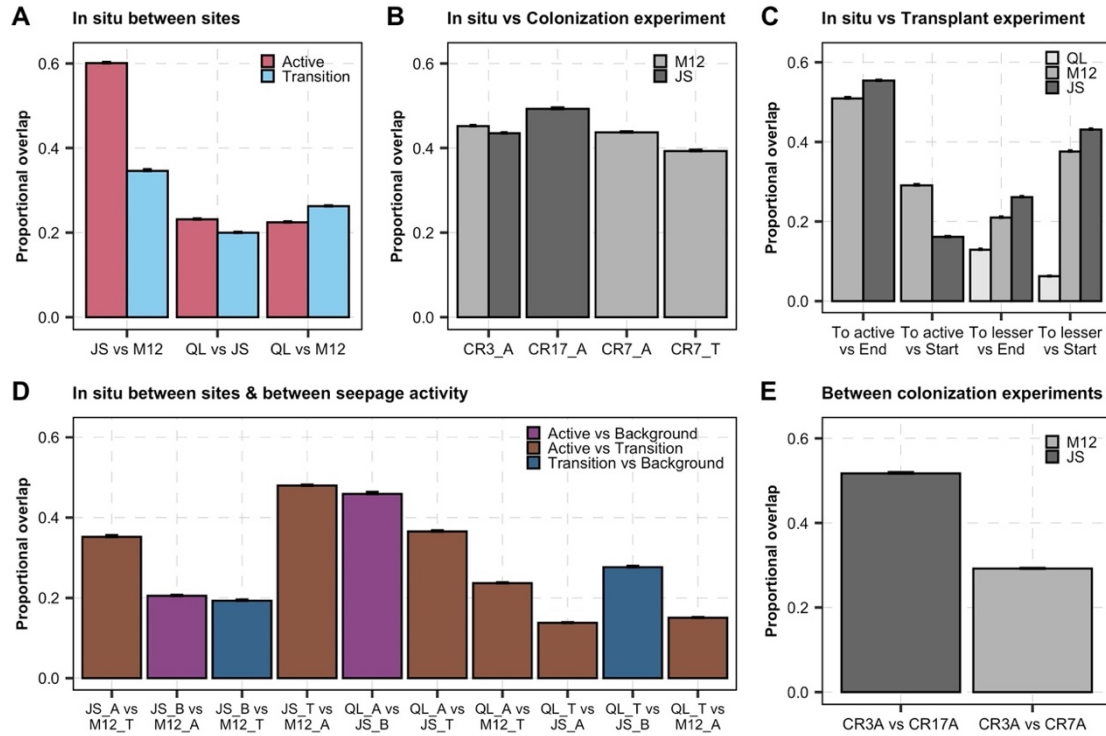


Figure 2.7: Proportional Bayesian overlap of standard ellipse areas (SEAc) of macrofaunal invertebrates on (A) *in situ* carbonate rocks between seeps at active and transition sites, (B) *in situ* carbonate rocks vs colonization experiments, (C) *in situ* carbonate rocks vs transplanted rocks, (D) *in situ* carbonate rocks between seeps and seepage activity, and (E) colonization rocks with different exposure duration. Seeps are Quepos Landslide (QL), Mound 12 (M12), and Jaco Scar (JS). Seepage active sites are A: Active, T: Transition, IT: Inner transition, OT: Outer transition, and B: Background. Colonization experiments were conducted for 3 months (CR3), 17 months (CR17), and 7 years (CR7).

Macrofaunal colonization patterns

Colonizing invertebrate communities at active sites did not achieve *in situ* densities in 3 months at Quepos Landslide (11 ± 10 individuals/200 cm²; $W = 4$, $p = 0.40$) and Mound 12 (200 ± 23 individuals/200 cm²; $W = 1$, $p = 0.14$), but did so at Jaco Scar (155 ± 50 individuals/200 cm²; $W = 7$, $p = 0.27$) (Fig. 2.2D-F). After 17 months, colonizer densities were still low at Quepos Landslide (2 ± 1 individuals/200 cm²; $W = 2$, $p = 1.00$), but increased with time at Jaco Scar with values 2-4x greater than on *in situ* rocks (17 months: 330 ± 2 individuals/200 cm², $W = 3$, $p = 0.20$; 20 months: 529 individuals/200 cm², $W = 0$, $p = 0.40$). This overshoot in colonizer density with

time was also observed in the longer-term experiment (7 years) at active sites at Mound 12, where colonizer densities were 2.5x higher than on *in situ* rocks, although not significant (609 ± 126 individuals/200 cm²; $W = 1$, $p = 0.07$). However, at transition sites at Mound 12, colonizer densities did not achieve *in situ* values in 7 years (46 ± 24 individuals/200 cm²; $W = 12$, $p = 0.34$) and were even lower within 7+ years (9 individuals/200 cm²; $W = 4$, $p = 0.40$).

There was no significant difference in community composition between colonization rocks and *in situ* rocks at Quepos Landslide within 3 months ($R = 0.5$, $p = 0.40$) and 17 months ($R = 0$, $p = 1.00$), although SIMPER analyses showed high dissimilarities (100%), which is also apparent in the MDS and cluster analyses (Fig. 2.3D, 2.4B, Supp. Fig. 2.2). However, these statistics could be a result of the low number of colonizers (two 3-months colonization rocks had 0 individuals, and 17-months colonization rocks had 0-1 individuals) that were also species that were not commonly found on *in situ* rocks (the annelids *Dorvillea* sp. and Spionidae sp., and the amphipod *Harpinae* sp.) (Supp. Table 2.1). At both Jaco Scar and Mound 12 (Fig. 2.3E-F, Fig. 2.4C-D), colonizer communities became more similar to *in situ* communities with time, but they never fully recovered, not even after 7 years at Mound 12 (SIMPER, average dissimilarity = 75.8% and 79.94% within 3-months at JS and M12, respectively, 72.35% within 17 months at JS, 72.96% within 20 months at JS, and 54.22% within 7 years at M12). Early colonizers (3-20 months) at Jaco Scar were *P. goniata*, *Neolepetopsis* limpets, *Archinome levinae*, and *Fucaria* snails (Supp. Table 2.3). Snails and limpets were also the most successful colonizers at Mound 12 at active sites both in the short- and long-term, including *P. laevis*, *L. ovalis*, *P. corymba*, and *P. musaica* (Supp. Table 2.2). These were also the top species that contributed the most to the dissimilarity between colonization rocks and *in situ* rocks at Mound 12 and Jaco Scar, being more abundant on

colonization rocks, in addition to *L. paulbrooksi* at Jaco Scar, which was more dominant on *in situ* rocks.

Shannon-Wiener diversity (H'), richness (S), and Pielou's evenness (J) on colonization rocks at all seep locations were higher than on *in situ* rocks, but Shannon-Wiener diversity and richness decreased with time of colonization exposure, and evenness increased with time, except at Jaco Scar, where evenness was highest within 3 months (Table 2.4).

There was no significant difference between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of 17-month colonizers and *in situ* communities at Quepos Landslide ($\delta^{13}\text{C}$: $W = 1$, $p = 0.44$; $\delta^{15}\text{N}$: $W = 4$, $p = 1.00$). At Jaco Scar and Mound 12, there was no significant difference in $\delta^{13}\text{C}$ between colonizers and *in situ* communities (pairwise Wilcoxon tests, $p > 0.05$). $\delta^{15}\text{N}$ values of colonizers were 1.1‰ higher than *in situ* animals within 17 months at Jaco Scar ($W = 435$, $p = 0.02$) and 2.4‰ higher at Mound 12 within 3 months ($W = 219$, $p = 0.005$), but 3.3‰ lower within 7 years ($W = 349$, $p = 0.001$). Trophic diversity based on TA and SEAc was lower on colonization rocks than on *in situ* rocks at both Jaco Scar and Mound 12, but it decreased with time of experiment at Jaco Scar (3 to 17 months) and increased at Mound 12 (3 months to 7 years) (Table 2.4, Fig. 2.6). Trophic overlap at active sites between colonizers and *in situ* animals within 3 months was higher at Mound 12 (45%) than at Jaco Scar (43%), but it increased within 17 months at Jaco Scar (49%) and decreased within 7 years at Mound 12 (44%) (Fig. 2.7B).

Transplanted macrofaunal patterns

Although differences in densities between transplanted communities and the *in situ* communities at the start and end sites were not statistically significant (Kruskal-Wallis and Wilcoxon tests, $p > 0.05$), there were some interesting patterns and trends (Fig. 2.2G-I). Densities of

macrofaunal invertebrates at Jaco Scar and Mound 12 increased within 17 months when rocks were moved to active sites but decreased when moved to less seepage activity. In contrast, at Quepos Landslide, densities always decreased after transplant. However, at all seeps, transplanted communities did not achieve densities equal to *in situ* rocks at the end site, except at Mound 12, where densities on rocks that were transplanted from transition to active sites were 3.5x higher than the *in situ* active community.

Composition of communities on rocks transplanted to active sites looked more similar to *in situ* seep communities at Jaco Scar ($R = 0.688$, $p = 0.03$) than at Quepos Landslide ($R = 0.333$, $p = 0.50$) and Mound 12 ($R = 0.301$, $p = 0.011$) (Fig. 2.3G-I), gaining seep species such as *Neolepetopsis* sp. 2, *Lepetodrilus* stet., and *P. goniata* (Supp. Table 2.6). At Quepos Landslide, transition to active communities were different ($R = 0.404$, $p = 0.04$) and with lower diversity metrics than the original, transition community. They had abundant holothurians not found anywhere else that accounted for 88% of the total individuals and 17% of the dissimilarity between transplanted and *in situ* communities (SIMPER, average dissimilarity = 90.46%) (Supp. Table 2.4). At Mound 12, inner transition to active communities were similar to the original, transition community (although not significant, $R = 0.175$, $p = 0.13$) and relatively different than the end, active community ($R = 0.301$, $p = 0.001$). These rocks also showed the highest richness values, hosting both seep and transition species (Supp. Table 2.5). Diversity metrics were higher for communities on the transplanted rocks than communities at the start site (i.e., transition or background) but lower than communities at the end site (i.e., active) at all seeps (Table 2.4).

Communities on rocks transplanted from active to transition sites at Mound 12 showed the most similarity between transplanted rock and initial active communities (M12: $R = 0.78$ to 0.85 , $p < 0.005$) and low similarity to the end, transition communities, although not significant ($R =$

0.156 to 0.235, $p > 0.05$). At Quepos Landslide, there was no significant difference in community composition between active to transition rocks and the start ($R = 0.26$, $p = 0.08$) and end *in situ* communities ($R = 0.091$, $p = 25$). Diversity metrics increased at Mound 12 following transplant from active to transition sites but decreased at Quepos Landslide (except for evenness). However, at both locations, transplanted rocks gained transition species such as hydroids, *Branchinotogluma* sp., and *Placopecten* sp. at Quepos Landslide and peracarid crustaceans, *Chrysopetallum* sp., and chitons at Mound 12, and retained some seep species including *Fucaria* sp. 1, *Kanoia myronfeinbergi*, and *L. paulbrooksi* at Quepos Landslide, and *Neolepetopsis* limpets, *Archinome levinae*, and *Bathymodiolus earlougheri* at Mound 12 (Supp. Tables 2.4-2.5).

Communities on rocks transplanted to background sites at Jaco Scar and Mound 12 were relatively similar to the original communities. This was especially true when rocks were moved from active sites (JS: $R = 0.552$, $p = 0.008$; M12: $R = 0.74$, $p = 0.008$), and less so when moved from transition sites (JS: $R = 0.494$, $p = 0.008$; M12: $R = 0.519$, $p = 0.008$). However, all rocks transplanted to background sites at Jaco Scar were also somewhat similar to the end, background communities, although not significant (A-B: $R = 0.72$, $p = 0.17$; IT-B: $R = 0.458$, $p = 1.00$). Transition/background species (e.g., *Chrysopetallum* sp., dorvilleid and cirratulid annelids, and peracarid crustaceans) colonized these rocks at Jaco Scar. Some seep species persisted after 17 months on rocks transplanted to background sites including *L. paulbrooksi*, *Amphisamytha fauchaldi*, and *P. goniata* at Jaco Scar (Supp. Table 2.6), and *A. fauchaldi*, *Paralepetopsis* sp., *Lamellibrachia barhami*, and *Bathymodiolus* mussels at Mound 12 (Supp. Table 2.5). Diversity metrics were higher on the transplanted rocks than end, background communities. Interestingly, they were lower than from the initial, active or transition communities at Jaco Scar. At Mound 12, diversity metrics were higher than those of the initial communities when moved from active sites

but lower when moved from transition sites. In other words, Mound 12 diversity metrics persisted through the transplant to background sites when coming from an active site.

For most transplants there was no significant difference between mean $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values of communities on transplanted rocks and those at the start and end *in situ* communities (Kruskal-Wallis and Wilcox tests, $p > 0.05$; Table 2.4). Exceptions were communities transplanted from background to active sites at Jaco Scar, which showed 16.3‰ lower $\delta^{13}\text{C}$ values than background communities ($Z = 2.9738$, $p = 0.009$) but were similar to active communities ($Z = 0.7656$, $p = 0.44$) and 2.3‰ lower $\delta^{15}\text{N}$ values than active communities ($Z = 3.5782$, $p = 0.001$). Also, communities transplanted from active to transition sites at Mound 12 showed 1.9-2.2‰ higher $\delta^{15}\text{N}$ values than at active sites (Dunn's tests, $p < 0.05$), and communities transplanted from transition to background sites at Mound 12 showed 10.9‰ higher $\delta^{13}\text{C}$ values ($W = 4$, $p = 0.0002$) and 4‰ higher $\delta^{15}\text{N}$ values ($W = 30$, $p = 0.02$) than at transition sites. At both Jaco Scar and Mound 12, trophic diversity based on TA and SEAc was the highest on rocks transplanted from active to background sites and the lowest on rocks transplanted to active sites (Table 2.4, Fig.2.7). However, trophic overlap was the highest between rocks transplanted to active sites and the *in situ* active communities. For these rocks, Jaco Scar showed highest trophic overlap between transplanted rock and end site (i.e., active) but Mound 12 showed highest trophic overlap between transplanted rock and start site (i.e., transition). For rocks transplanted to lesser seepage activity, Jaco Scar showed the highest trophic overlap between transplanted rock vs start and end communities, and Quepos Landslide, the lowest overlap (Fig. 2.7C).

DISCUSSION

Much of the variability in methane seep macrofaunal communities documented previously has been attributed to seepage activity (Dando et al. 1994, Levin 2005, Levin et al. 2017). Indeed, here we observed that seepage activity exhibits a strong influence in densities, community composition, diversity metrics, and trophic diversity across different methane seeps on the Costa Rican margin. However, we see evidence that other environmental variables (e.g., depth and oxygen concentration) play a role in shaping faunal and trophic diversities of these communities, as well as their recovery and resilience abilities.

An increase in density at active sites is expected due to the increased availability of organic matter by chemosynthetic production (Levin 2005) with a change in community composition, where carbonates at active sites are generally dominated by grazing gastropods and symbiont-bearing annelids and bivalves, and background (i.e., non-seep) sites are generally dominated by echinoderms (Levin et al. 2015, Rybakova et al. 2022). Here, however, the highest densities occurred at transition sites at Mound 12 and Jaco Scar (Fig. 2.2), where seep species are coexisting with background species. Densities at these seeps were on average 3-6x higher than densities of macrofauna associated with carbonate rocks at shallower seeps at Hydrate Ridge, OR (Levin et al. 2017), 1.3-2.5x higher than on carbonates at the Amon Mud Volcano and Pockmark areas of the Nile Deep-sea Fan at 1,000-1,700 m (Ritt et al. 2011), and 20-26x higher than at the Del Mar Seep off California at 1,020 m (Grupe et al. 2015). However, compared to a previous study at Mound 12 in 2009, densities were 1.4x lower at active sites and similar at transition sites (Levin et al. 2015). Community composition also changed with seepage activity, but there was variability in densities and community composition across seep locations (Fig. 2.2-2.3). Densities were the highest at Jaco Scar, which was dominated by tanaids and annelids, at our deepest site at 2,000 m. Tanaids have been reported as a common taxon in reducing habitats, with several species restricted

to seeps (Blazewicz-Paszkowycz and Bamber 2011, Zeppilli et al. 2011), greater diversity with depth (Blazewicz-Paszkowycz et al. 2012), and reaching high densities at seeps at 3,280 m in Alaska (Levin and Mendoza 2007). Jaco Scar also showed great abundance of the snail *Provanna goniata*, while Mound 12 was dominated by *Provanna laevis* and other grazing gastropods. This depth-specific preference within the *Provanna* genus has also been reported for communities associated with tubeworms and mussel beds at Jaco Scar and Mound 12 (Betters et al. 2023).

The patterns are very different at Quepos Landslide, which is our shallowest site at 400 m, and within the OMZ. Quepos Landslide is dominated by hydroids, but showed the lowest macrofaunal densities, which did not vary with seepage activity (Fig 2.2-2.3). Densities at Quepos Landslide were 4-8x and 2-3x lower than at seeps in the California and Oregon OMZs, respectively, which have similar oxygen concentration to Quepos Landslide (Levin et al. 2010). They were also 2.5-5x lower than at seeps under low oxygen conditions at Hydrate Ridge, OR, although the oxygen concentration at Quepos Landslide was 2x lower than at Hydrate Ridge (Levin et al. 2017). Other seeps at similar, relatively shallow depth range (400-600 m) in the Bering Sea are inhabited by “hydroid bushes”, with numerous hydroids taking advantage of the hard substrate and potential food provided by seeps. Notably, the diversity of hydroids in the North Pacific decreases with depth below 1,000 m (Prudkovsky et al. 2023). Oxygen concentration has been known to decrease macrofaunal densities at seeps and other deep-sea hardground ecosystems (Wishner et al. 1990, 1995, Levin 2003). It seems that, at Quepos Landslide, oxygen concentration is overriding the effect of seepage activity to lower densities. Moreover, the transitional gradient with seepage activity has been reported to be less pronounced at shallow seeps (Seabrook et al. 2024). However, predation might be another factor influencing the macrofaunal community at Quepos Landslide, which generally decreases with depth (Sahling et al. 2003). Quepos Landslide

is inhabited by a dense community of tuna crabs *Pleuroncodes monodon* that might be feeding on the macrofauna (personal observation) (Fig. 2.1B).

Diversity metrics across all seeps also varied with seepage activity (Table 2.4). Transition sites showed the highest Shannon-Wiener diversity, but active sites showed higher species richness, and background sites, higher evenness, reflecting the dominance of selected species at active sites compared to background sites (Vedenin et al. 2020). Sediment macrofauna from these same locations also showed higher evenness at background sites and lower Shannon-Wiener diversity and richness at active sites (Ashford et al. 2021). This highlights the heterogeneity provided by carbonate rocks at active sites. Among seeps, Mound 12 showed the highest Shannon-Wiener diversity, but species rarefaction rate was higher at Jaco Scar, especially after around 500 specimens, when Jaco Scar curve crosses Mound 12 (Fig. 2.5). This may suggest that Jaco Scar was under-sampled and might reflect an even higher habitat heterogeneity at this seep location (Vedenin et al. 2020).

Depth also indirectly influences trophic structure at seeps; shallow-water seeps receive more organic matter from photosynthetic origin due to stronger benthic-pelagic coupling (Levin 2005), while deep-water seeps receive less of that production and chemosynthetic organic matter is more significant (Dando et al. 1991, 1994, Levin and Mendoza 2007). Indeed, Quepos Landslide showed the highest $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, and Jaco Scar, the lowest, demonstrating the higher dependence on chemosynthetic production with increasing depth (Table 2.4). Still, isotope values of macrofauna at actively seeping sites at Quepos Landslide were lower than values typical of non-chemosynthetic ecosystems (e.g., Levin et al. 2012, Colaço et al. 2013, Pereira et al. 2024), and animals might be feeding on a mixture of photosynthetic and chemosynthetic production. This use

of chemosynthesis-based carbon and photosynthesis-based carbon into the diet of seep animals has been reported for other shallow seep sites (~300 m) (Levin et al. 2002, Åström et al. 2019).

Regarding seepage activity, the higher isotope values of animals at background sites suggest they depend more on photosynthetic production than animals at active sites, as has been reported in other studies at seeps on the Pacific margin (Levin et al. 2002, 2015, 2017) and for sediment fauna (Portail et al. 2016, Åström et al. 2017, Ashford et al. 2021). However, trophic diversity was higher at active sites at Mound 12 and Jaco Scar than at transition and background sites (Table 2.4, Fig. 2.6), suggesting that, although animals are more dependent on chemosynthetic production, they are feeding on a greater variety of food sources than at background sites. At Quepos Landslide, the combined effects of seepage activity and oxygen on the community, as described above, might explain the higher trophic diversity at transition sites than at active sites. Finally, the larger photosynthetic input at Quepos Landslide and higher dependence on chemosynthetic production at Mound 12 and Jaco Scar might also explain the high trophic overlap between Jaco Scar and Mound 12 at both active and transition sites and the high trophic overlap between Quepos Landslide active sites and Jaco Scar background sites (Fig. 2.7).

Macrofaunal colonization patterns under different hydrologic conditions

Colonizers showed faster recovery in density values at Jaco Scar than at the other two seep locations, achieving natural densities within 3 months and an overshoot of 2-4x greater densities in 17 months (Fig. 2.2). This overshoot was also observed at Mound 12 to a lesser extent, where colonization densities were 1.5x higher than *in situ* densities in 10.5-months (Grupe 2014) and 2.5x higher in 7 years (this study, Pereira et al. 2021). This overshoot in densities was mainly due to high densities of bacterial grazing gastropods, which were dominant colonizers at both seep

locations. These gastropods, including *Provanna* snails and neolepetopsid limpets, consistently dominate seep habitats (Levin et al. 2015), and their presence impacts the settlement of other animals (Grupe 2014) in the short- and long-term. Indeed, tanaids that were abundant on *in situ* rocks at Jaco Scar were not good colonizers, perhaps due to competition for space with these gastropods or direct-development limitations to recruitment. The response of bacterial grazers suggests a rapid growth of the microbial community that provides their food within as little as 3 months. A quick response of the microbial community to changing seepage has been observed within 17 months at Hydrate Ridge, OR (Case et al. 2015).

Additionally, it seems that the dominance of gastropods also affected the trophic structure in the long-term (Table 2.4, Fig. 2.6). The lower trophic diversity on colonization rocks than on *in situ* rocks at both Jaco Scar and Mound 12 and the higher trophic overlap between colonizers and *in situ* communities in 3 months suggest that there was less variability in feeding modes on colonization than on *in situ* rocks, and that the grazer, gastropod-dominated community was feeding on similar food sources as *in situ* animals. Moreover, trophic diversity decreased with time at Jaco Scar, consistent with the overshoot in gastropod densities within 17 months. However, in 7 years, trophic diversity increased at Mound 12, and there was a lower trophic overlap, suggesting that dietary resources change as succession proceeds (Grupe 2014).

While hydrographic conditions (i.e., depth and oxygen concentration) at Jaco Scar and Mound 12 seemed not to play a major role in colonization patterns, and a bacterial-grazing feeding mode conferred good colonization ability at these seep locations, the hydrographic conditions at Quepos Landslide seem to limit the colonization capacity of macrofaunal animals. After 17 months exposure, rocks were colonized by single animals (or none) of species that were not commonly found in any other rocks (Fig. 2.3). These were the annelids *Dorvillea* sp. and Spionidae sp., and

the amphipod *Harpinae* sp. Disturbances, including changes in environmental conditions, can increase mortality rate and reduce reproduction rates for resident species (Mouillot et al. 2013). Low oxygen conditions within the OMZ can also limit larval recruitment and connectivity across margins (Seabrook et al. 2024), and predation of juveniles can affect recruitment success (Sahling et al. 2003). Some bacterial-grazing, seep species were actually able to colonize the rocks in 3 months (e.g., *P. goniata*, *Neolepetopsis* sp. 2, and *P. corymba*) but were likely predated upon and disappeared from the 17-month colonization rocks. Alternatively, the absence of colonizers could be explained by a lack of biofilm that is necessary for macrofaunal recruitment (Hadfield 2011), where the microbial community did not develop within the time frame of the experiments. However, hypoxia or near anoxia is common at E. Pacific seeps, where microbial activity is taking up oxygen, and the impact of oxygen availability on microbes at seeps within OMZs may be hard to observe (Seabrook et al. 2024). If microbial growth was the only factor influencing macrofaunal colonization, we would not expect to see such a difference in colonization patterns between Quepos Landslide and the other seep locations. Quepos Landslide has been reported as one of the most active areas in terms of seepage activity based on anaerobic oxidation of methane activity and methane emissions (Karaca et al. 2014). In addition, Quepos Landslide is mostly covered in bacterial mats with less exposed carbonate rocks than at the other seep locations (Karaca et al. 2014, personal observation). High environmental stress may limit colonization and reduce species (Alfaro-Lucas et al. 2020), and it is possible then that multiple stressors (e.g., seepage activity, oxygen, and availability of hard substrate) are influencing the *in situ* community, yielding the low densities and low diversity discussed above, to a point where the macrofaunal community is not well-established enough to be able to colonize new substrates with new recruits or motile adults.

Macrofauna resilience against changes in seepage activity under different hydrographic conditions

The results from the transplant experiment from transition or background sites to active sites suggest that the resilience of seep and transition fauna to a decline in seepage activity is affected by hydrographic conditions, and seep and transition fauna respond differently depending on the availability and dependence of chemosynthetic production (which increases with increasing depth). At Jaco Scar, active, seep communities respond faster to changes in seepage activity and are more resilient than at Mound 12. Community composition, isotope values, and trophic overlap of the communities transplanted to active sites were more similar to active, *in situ* communities at Jaco Scar than at Mound 12 (Fig. 2.3, 2.8, Table 2.4). The higher dependence on chemosynthetic production by Jaco Scar macrofauna described above could also lead to a more dynamic response of this seep community. A 13-month transplant experiment at Hydrate Ridge, OR, also revealed that seep fauna responds more quickly and can persist longer under less seepage than transition fauna, possibly due to their motility and opportunistic feeding on microbes (Levin et al. 2017). In addition, the boundary between active, transition, and background sites is sharper with increasing depth, as chemosynthetic production becomes more relevant (Rybakova et al. 2022). In contrast, communities on rocks transplanted from transition to active sites at Mound 12 retained greater composition similarity and higher trophic overlap to *in situ*, transition communities (Fig. 2.3, 2.8). Mound 12 might be under a greater influence of photosynthetic production than at Jaco Scar (but not as much as at Quepos Landslide), and the transition community at Mound 12, consisting of both seep and transition species using both photo- and chemosynthetic production, might respond faster and be more resilient to changes in seepage activity than the active, seep community.

Strikingly, active, seep fauna showed higher resilience than transition fauna when transplanted to background sites at both Mound 12 and Jaco Scar in terms of community diversity and trophic structure (Table 2.4, Fig. 2.6). Carbonates recovered from outside a seep area retained significant microbial biomass capable of methanotrophic activity, suggesting the viability of chemosynthetic production even under low seepage activity (Marlow et al. 2014). It is possible that the rocks transplanted from active to background sites retained their chemosynthetic microbial community, which supported seep species through the 17 months of the transplant. Among these seep species, the survival of the bacterial grazer *Chrysopetallum* sp. and the symbiont-bearing annelid *L. paulbrooksi* support this hypothesis. In contrast, the seep provannid snails (*P. laevis* and *P. goniata*) and pyropeltid limpets (*P. corymba*, *P. musaica*, and *P. wakefieldi*) seemed to not survive the transplant to lesser seepage. The same pattern was observed on a 13-month transplant at Hydrate Ridge, OR, and it was hypothesized that these species feed on microbial taxa that did not survive the transplant (Levin et al. 2017). In addition, *P. corymba* is commonly found on provannid snails (personal observation), and it could be a matter of substrate availability rather than food availability for these limpets, when provannid snails do not survive the transplant.

We were particularly interested in the transplant experiment at Quepos Landslide as it mimics a possible future scenario of expansion of methane seepage (via gas hydrate destabilization) due to ocean warming and expansion of OMZs due to ocean deoxygenation. However, the patterns were not as clear as at Jaco Scar and Mound 12. Densities always decreased after transplant (Fig. 2.2), possibly due to the limited *in situ* community available for survival or recruitment in the first place, as with the colonization experiments. When rocks were moved to active sites, the community was different than on any other rock, dominated by holothurians and with lower diversity metrics (Fig. 2.3, Table 2.4). We are still unsure the reason for this, but it is

clear that these holothurians took advantage of the provision of substrate and chemosynthetic production when no other animal would. However, the dynamics and resilience patterns on transplants to lesser seepage activity at Quepos Landslide were more similar to patterns observed at Jaco Scar and Mound 12 on the same transplant types; Quepos Landslide rocks transplanted to transition sites retained some seep species (e.g., hydroids Anthoathecata and *L. paulbrooksi*) but gained transition species (e.g., *Branchinotogluma* sp. and *Placopecten* sp.). We expected, however, that trophic overlap between the community transplanted to transition sites and the *in situ*, transition community would be higher at Quepos Landslide, since animals depend less on chemosynthetic production and the transition between seepage activity is less clear (see discussion above). This was not observed; transplants to transition sites at Quepos Landslide led to the lowest trophic overlap among all transplant experiments (Fig. 2.7). The slightly higher isotope values of animals on rocks transplanted to transition sites indicate an even higher dependency on photosynthetic production (Table 2.4). It is possible that the resilience of the microbial community is also affected by the low oxygen conditions, and chemosynthetic production under low seepage activity is not as viable at Quepos Landslide under low oxygen, as it is at Jaco Scar and Mound 12 (described above).

CONCLUSION

We observed high heterogeneity on *in situ* and experimental communities at three methane seeps under different hydrographic conditions on the Costa Rican margin. Quepos Landslide, the shallowest seep, within the OMZ, showed unique macrofaunal communities with low diversity metrics, and low recovery and resilience rates, where low oxygen concentration seems to override the effect of seepage activity, and photosynthetic production is a more important food source. At

Jaco Scar, the deepest seep, seepage activity plays a larger role in defining the macrofaunal communities. Seep animals seem to be more resilient at Jaco Scar and respond faster to colonization and transplant experiments, possibly due to greater dependence on chemosynthetic production, which appears to increase with increasing depth. At Mound 12, at an intermediate depth and just below the OMZ, the macrofaunal assemblages at transition sites, which are more adapted to use photo- and chemosynthetic sources, show greater recovery and resilience rates instead. We also observed changes on *in situ* community composition between Jaco Scar and Mound 12 due to depth preferences (e.g., higher abundance of tanaids with increasing depth and higher abundance of *P. goniata* at Jaco Scar instead of *P. laevis* as in Mound 12). Finally, feeding mode seems to confer good colonization ability at Jaco Scar and Mound 12 in the short- and long-term, but low oxygen plays a more important role at Quepos Landslide, limiting colonization ability of macrofauna.

Our work highlights the role of environmental conditions in defining macrofaunal communities at methane seeps, beyond seepage activity, but also how hydrographic conditions might affect the role of seepage activity and the colonization and resilience ability of seep and transition macrofauna. Understanding how these environmental conditions are shaping these communities might give us a glimpse of what these communities might look like in the future due to expansion of OMZs and methane seepage.

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SUPPLEMENTAL FILES

Supplemental Table 2.1: Top 10 most abundant species of macrofaunal invertebrates on carbonate rocks deployed at active sites at Quepos Landslide for 3 months and 17 months.

3 months	%	17 months	%
<i>Provanna goniata</i>	29	<i>Dorvillea</i> sp.	33
<i>Neolepetopsis</i> sp. 2	17	Spionidae sp.	33
<i>Pyropelta corymba</i>	10	<i>Harpinae</i> sp.	33
<i>Paraonidae</i> sp. 1	7		
<i>Provanna laevis</i>	7		
Serpulidae sp. 1	5		
<i>Gyptis</i> sp.	5		
<i>Laminatubus</i> sp.	2		
<i>Parougia</i> sp.	2		
Amphipoda sp. 2	2		

Supplemental Table 2.2: Top 10 most abundant species of macrofaunal invertebrates on carbonate rocks deployed at Mound 12 at active sites for 3 months and 7 years and at transition sites for 7 years.

3 months Active	%	7 years Active	%	7 years Transition	%
<i>Provanna laevis</i>	39	<i>Provanna laevis</i>	27	<i>Neolepetopsis</i> sp. 3	12
<i>Pyropelta corymba</i>	9	<i>Lepetodrilus ovalis</i>	26	<i>Laminatubus</i> sp.	8
<i>Gyptis roberscipssi</i>	7	<i>Amphisamytha fauchaldi</i>	8	<i>Aplacophora</i> sp. 1	6
<i>Ophryotrocha</i> sp. 2	5	<i>Lepetodrilus</i> stet.	7	<i>Anguillosyllis</i> sp.	5
<i>Kiwa puravida</i>	4	<i>Kiwa puravida</i>	7	<i>Setenula</i> c.f. <i>ussuriensis</i>	5
<i>Pyropelta musaica</i>	4	<i>Hyalogyrina</i> sp.	6	<i>Kanoia myronfeinbergi</i>	5
<i>Lacydonia</i> sp.	4	<i>Pyropelta musaica</i>	4	Serpulidae	4
<i>Ophiuroglypha</i> c.f. <i>meridionalis</i>	4	<i>Pyropelta corymba</i>	3	Ophiuroidea	4
<i>Kanoia myronfeinbergi</i>	2	<i>Pyropelta wakefieldi</i>	2	<i>Hyalogyrina</i> sp.	4
<i>Provanna goniata</i>	2	<i>Neolepetopsis</i> sp. 4	1	<i>Brada</i> sp. 2	4

Supplemental Table 2.3: Top 10 most abundant species of macrofaunal invertebrates on carbonate rocks deployed at active sites at Jaco Scar for 3 months and 17 months.

3 months	%	17 months	%
<i>Provanna goniata</i>	21	<i>Provanna goniata</i>	27
<i>Fucaria</i> sp. 2	10	<i>Neolepetopsis</i> sp. 3	16
<i>Neolepetopsis</i> sp. 2	8	<i>Archinome levinae</i>	9
<i>Fucaria</i> sp. 3	8	Hesionidae	7
<i>Neolepetopsis</i> sp. 3	6	<i>Neolepetopsis</i> sp. 2	4
<i>Archinome levinae</i>	6	<i>Parougia</i> sp.	4
<i>Gyptis robertscrippi</i>	6	<i>Gyptis robertscrippi</i>	4
<i>Parakanthophoreus</i> sp. 1	5	<i>Lacydonia</i> sp.	4
<i>Ophiura</i> c.f. <i>scutellata</i>	5	<i>Aphelochaeta</i> sp.	2
<i>Lacydonia</i> sp.	2	<i>Brada</i> sp. 2	2

Supplemental Table 2.4: Top 10 most abundant species of macrofaunal invertebrates on carbonate rocks transplanted across seepage activity at Quepos Landslide for 17 months. A: Active, T: Transition.

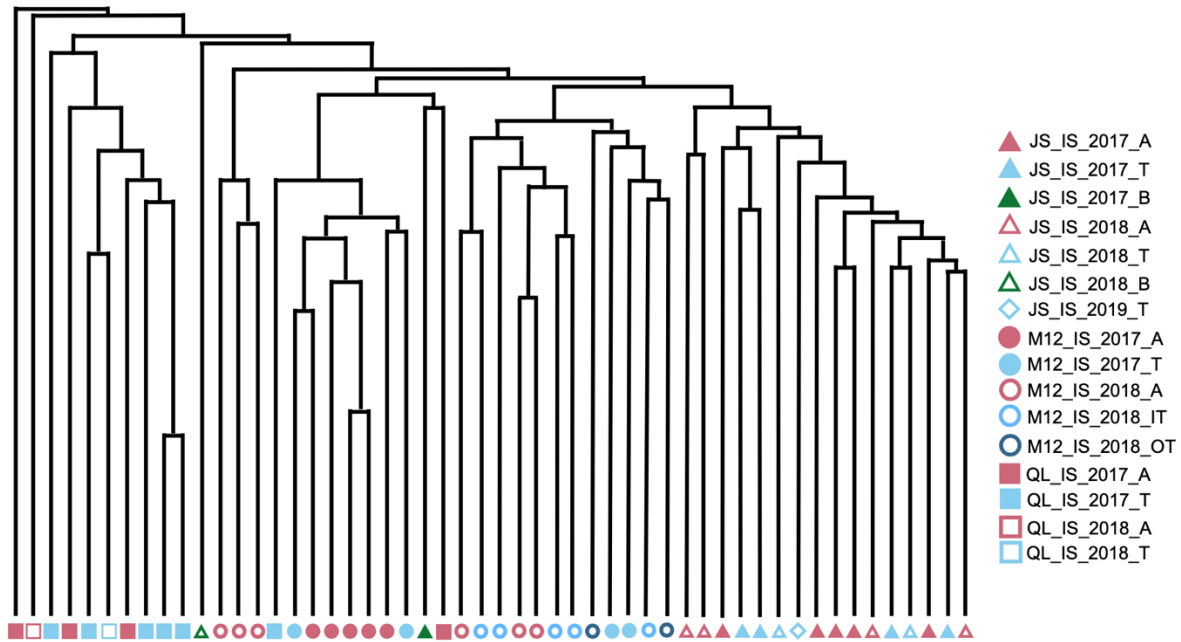
A-T	%	T-A	%
Anthoathecata stet.	38	Holothuroidea sp. 1	88
<i>Branchinotogluma</i> sp.	14	<i>Pseudopotamilla</i> sp.	10
Annelida	10	Ampharetidae sp. 1	2
<i>Placopecten</i> sp.	10		
<i>Pseudopotamilla</i> sp.	3		
<i>Laminatubus paulbrooksi</i>	3		
Spionidae sp.	3		
<i>Pleuroncodes monodon</i>	3		
<i>Solenogastres</i> sp. 2	3		
<i>Kanoia myronfeinbergi</i>	3		

Supplemental Table 2.5: Top 10 most abundant species of macrofaunal invertebrates on carbonate rocks transplanted across seepage activity at Mound 12 for 17 months. A: Active, IT: Inner transition, OT: Outer transition, B: Background.

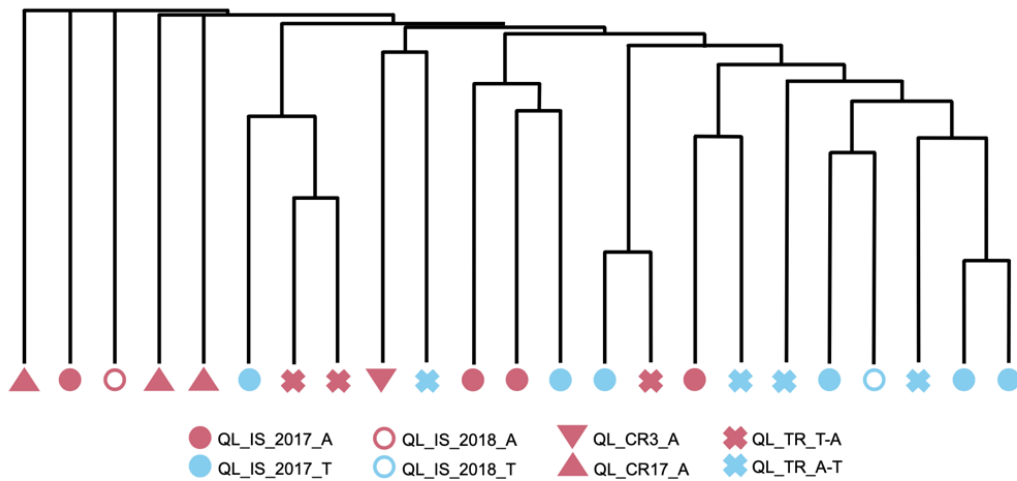
IT-A	%	IT-OT	%	IT-B	%
<i>Pyropelta musaica</i>	18	<i>Elaphognathia</i> c.f. <i>rangifer</i>	22	<i>Chrysopetallum</i> sp.	33
<i>Chrysopetallum</i> sp.	16	<i>Chrysopetallum</i> sp.	19	Anthoathecata stet.	7
<i>Neolepetopsis</i> sp. 3	13	Anthoathecata stet.	5	Amphinomidae sp. 1	6
<i>Pyropelta wakefieldi</i>	13	<i>Monoculodes</i> sp.1	4	<i>Galapagomystides patricki</i>	6
<i>Neolepetopsis</i> sp. 1	4	<i>Nannoniscoides</i> sp.	4	<i>Parakanthophoreus</i> sp. 1	4
<i>Provanna laevis</i>	4	Amphinomidae sp. 1	3	<i>Apheloachaeta</i> sp.	3
<i>Lepetodrilus ovalis</i>	3	Paraonidae sp. 1	3	Paraonidae sp. 1	2
<i>Lacydonia</i> sp.	3	Actiniaria sp. 2	3	Phyllodocidae sp. 3	2
<i>Parakanthophoreus</i> sp. 1	2	<i>Apheloachaeta</i> sp.	2	Gammaridae sp.	2
<i>Anguillosyllis</i> sp.	2	Polynoidae	2	Acari sp. 2	2
A-IT	%	A-OT	%	A-B	%
<i>Chrysopetallum</i> sp.	17	Amphipoda sp. 4	21	<i>Galapagomystides patricki</i>	13
<i>Neolepetopsis</i> sp. 1	8	<i>Chrysopetallum</i> sp.	11	<i>Amphisamytha fauchaldi</i>	12
<i>Neolepetopsis</i> sp.3	7	<i>Archinome levinae</i>	6	Amphinomidae sp. 1	5
<i>Bathymodiolus earlougheri</i>	5	Actiniaria sp. 2	6	Amphipoda sp. 3	5
<i>Kanoia myronfeinbergi</i>	5	Polynoidae	4	Nereididae sp. 1	4
<i>Amphisamytha fauchaldi</i>	4	Anthoathecata sp.	4	<i>Paralepetopsis</i> sp.	4
Acari	4	Amphinomidae sp. 1	2	<i>Sirsoe</i> sp.	3
Chrysopetallidae	3	<i>Nereis piscesae</i>	2	<i>Apheloachaeta</i> sp.	3
Polyplacophora sp. 1	3	<i>Neolepetopsis</i> sp. 1	2	<i>Lamellibrachia barhami</i>	3
<i>Archinome levinae</i>	2	<i>Elaphognathia</i> c.f. <i>rangifer</i>	2	Sessilia sp.	3

Supplemental Table 2.6: Top 10 most abundant species of macrofaunal invertebrates on carbonate rocks transplanted across seepage activity at Jaco Scar for 17 months. A: Active, T: Transition, B: Background.

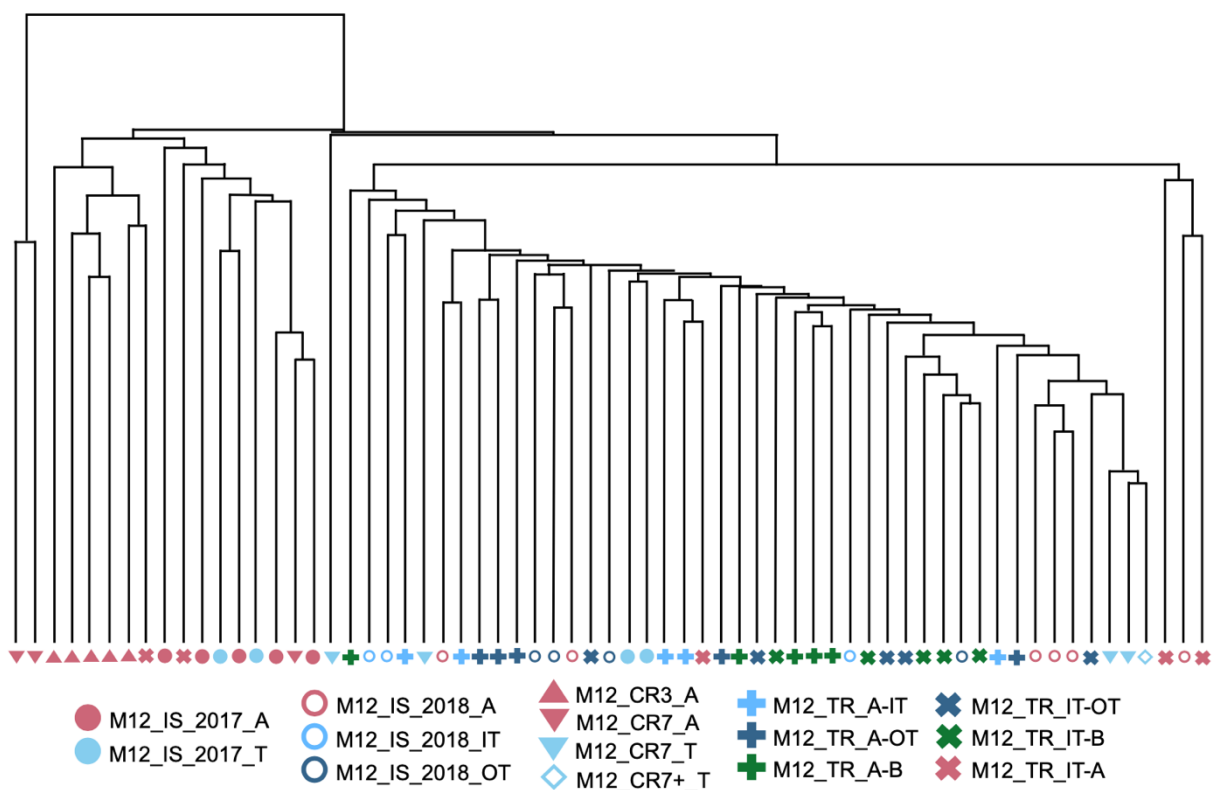
B-A	%	A-B	%	T-B	%
<i>Neolepetopsis</i> sp. 2	37	<i>Chrysopetallum</i> sp.	38	<i>Laminatubus paulbrooksi</i>	17
<i>Lepetodrilus</i> stet.	13	<i>Laminatubus paulbrooksi</i>	26	<i>Amphisamytha fauchaldi</i>	8
<i>Provanna goniata</i>	6	Juvenile shrimp	5	Cirriformia sp.	8
<i>Paralepetopsis</i> sp.	6	Porifera sp.	4	Dorvilleidae sp.	8
<i>Amphisamytha fauchaldi</i>	4	<i>Synmerosyllis</i> sp.	3	Phyllodocidae sp. 1	8
<i>Galapagomystides patricki</i>	3	<i>Parakanthophoreus</i> sp. 1	2	Juvenile shrimp	8
<i>Neolepetopsis</i> sp. 3	3	Syllidae sp. 4	1	Isopoda sp.	8
Hesionidae sp.	3	<i>Archinome levinae</i>	1	<i>Nannoniscoides</i> sp.	8
<i>Apheloachaeta</i> sp.	2	Phyllodocidae	1	Tanaidacea	8
<i>Lepetodrilus ovalis</i>	2	<i>Nannoniscoides</i> ps.	1	<i>Ophiambix</i> sp.	8



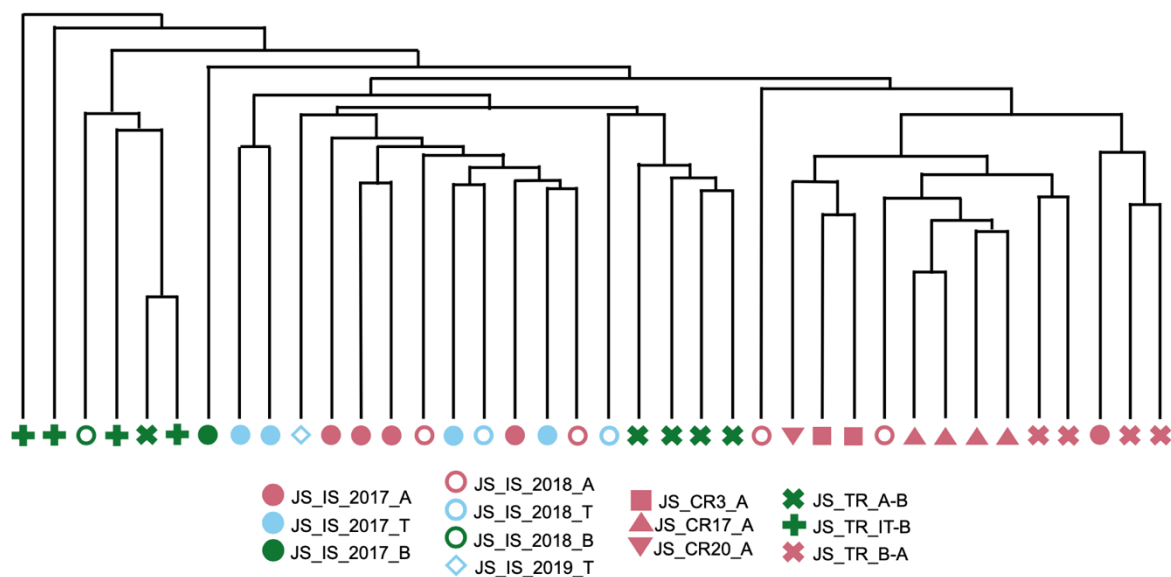
Supplemental Figure 2.1: Cluster of *in situ* communities across seep location and seepage activity. Cluster analysis was performed using a Bray-Curtis dissimilarity matrix of densities after fourth-root transformation. Seep locations: Quepos Landslide (QL), Mound 12 (M12), and Jaco Scar (JS). Seepage activity: Active (A), Transition (T), Inner transition (IT), Outer transition (OT), Background (B).



Supplemental Figure 2.2: Cluster of macrofaunal communities on in situ and experimental carbonate rocks at Quepos Landslide (QL). Cluster analysis was performed using a Bray-Curtis dissimilarity matrix of densities after fourth-root transformation. Seepage activity: Active (A), and Transition (T). Experiments: 3-month colonization experiment (CR3), 17-month colonization experiment (CR17), and 17-month transplant experiment (TR).



Supplemental Figure 2.3: Cluster of macrofaunal communities on in situ and experimental carbonate rocks at Mound 12 (M12). Cluster analysis was performed using a Bray-Curtis dissimilarity matrix of densities after fourth-root transformation. Seepage activity: Active (A), Transition (T), Inner transition (IT), Outer transition (OT), and Background (B). Experiments: 17-month colonization experiment (CR17), 7-year colonization experiment (CR7), +7-month colonization experiment (CR7+), and 17-month transplant experiment (TR).



Supplemental Figure 2.4: Cluster of macrofaunal communities on in situ and experimental carbonate rocks at Jaco Scar (JS). Cluster analysis was performed using a Bray-Curtis dissimilarity matrix of densities after fourth-root transformation. Seepage activity: Active (A), Transition (T), and Background (B). Experiments: 3-month colonization experiment (CR3), 17-month colonization experiment (CR17), 20-month colonization experiment (CR20), and 17-month transplant experiment (TR).

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Chapter 2, in full, is currently being prepared for submission for publication of the material.

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Chapter 3 MICROBIAL MEDIATION OF MACROFAUNAL RESPONSE TO METHANE SEEPAGE

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ABSTRACT

Animal-microbe interactions at methane seeps may take many forms and can involve settlement cues, trophic interactions, detoxification, biogeochemical modification, and substrate and shelter provision. There has been extensive work describing the microbial and macrofaunal communities associated with carbonate rocks at seeps and community patterns under natural and manipulated gradients in seepage activity. However, the question that remains unanswered is whether there are interactions tying the macrofaunal responses to the microbial community or the macrofaunal patterns are generated by seepage activity purely. We analyzed macrofaunal and microbial communities on carbonate rocks at methane seeps off the Costa Rican Pacific margin to understand whether there are strong relationships between the communities and correlations between specific macrofaunal species and microbial operational taxonomic units (OTUs). We observed that macrofaunal Shannon-Wiener diversity and species richness depend on microbial richness and that macrofaunal community composition patterns with seepage activity are not aligned with microbial composition patterns, suggesting that macrofaunal species might depend on specific OTUs. Indeed, by performing co-inertia and db-RDA analyses, we found significant associations between macrofaunal species and specific OTUs that reflect patterns of these OTUs with environmental drivers. In addition, animal responses to changes in seepage activity follow the responses of the OTUs they depend on for food and habitat provision. This work sheds a light

into the complexity of animal-microbe associations at methane seeps and highlights the importance of these interactions to the structure and functioning, and health of the ecosystem.

INTRODUCTION

Interactions between animals and microbes are fundamentally important aspects of animal biology and system ecology (McFall-Ngai et al. 2013). In the deep sea, microbes are critical for the establishment of animal communities in extreme environments (Osman and Weinnig 2022), either by association with a distinct microbial community (Thurber et al. 2013, Levin et al. 2016) or through a symbiotic relationship with a microbial partner (Dubilier et al. 2008, Petersen and Dubilier 2009). These interactions are not isolated but nested within communities of other organisms that coexist in larger, more complex assemblages (McFall-Ngai et al. 2013). Deep-sea chemosynthetic ecosystems are examples of such nested communities where chemoautotrophic production of organic matter by microbes contributes to larger communities that include non-symbiotic and symbiotic animal and microbial species. An ecological perspective of animal-microbe interactions can help us understand their greater role in the ecosystem and predict and manipulate community structure and functioning to promote ecosystem health (McFall-Ngai et al. 2013).

Among chemosynthetic ecosystems, at methane seeps animal-microbe interactions may take many forms and can involve settlement cues, trophic interactions, detoxification, biogeochemical modification, and substrate and shelter provision (Danovaro et al. 2024). Microbial biofilms may provide cues for settlement of some macrofaunal invertebrates (Hadfield 2011). Grazing, deposit feeding and suspension-feeding invertebrates consume the free-living microbial community (Van Dover and Fry 1994), and many other species are now known to have

symbiotic bacteria (Dubilier et al. 2008). This chemosynthetic production is carried up to higher consumers through predator-prey interactions. Besides providing nutrition to animals, microbial activity leads to the formation of carbonate rocks, which provide hard substrate for attachment, shelter, and reproduction (Levin et al. 2016, Bowden et al. 2013). On the other hand, the sulfidic environment and the microbes that produce sulfide and methane can also function as settlement inhibitors of other animals that do not tolerate such conditions at active seepage. But sulfide oxidizers remove sulfide and help detoxify the environment, being an important facilitator for some invertebrates.

There has been extensive work describing the microbial and macrofaunal communities associated with carbonate rocks at seeps and how seepage activity influences these communities (e.g., Marlow et al. 2014a, Case et al. 2015, Grupe et al. 2015, Levin et al. 2015, 2017, Mullin 2020, Pereira et al. 2021, among others). The most common groups of Archaea on and in carbonate rocks at methane seeps are anaerobic methane-oxidizing archaea (ANME), and the most common bacteria are Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, and Chloroflexi (Case et al. 2015, Mullin 2020). Microbial assemblages on naturally occurring carbonates change across seepage activity gradients and have different roles in the environment (summarized in Table S1). However, operational taxonomic unit (OTU) richness and evenness does not change with seepage, meaning that microbial assemblages at transition sites are as diverse as at active sites (Case et al. 2015). This change in composition and diversity with seepage activity is also observed for the macrofaunal community. Carbonates at active sites are generally dominated by grazing gastropods and symbiont-bearing annelids and bivalves, background (i.e., non-seep) sites are generally dominated by echinoderms, and both seep and background species are found coexisting at transition sites (Levin et al. 2015, Pereira et al. 2021, Rybakova et al. 2022).

In addition to patterns in microbial and macrofaunal communities with natural seepage gradients, previous work provides background information on how these communities respond to manipulated changes in seepage activity. A 13-month transplant experiment of carbonate rocks from active sites to low-activity sites at Hydrate Ridge, OR, showed more similar microbial assemblage to *in situ* rocks at active sites (i.e., where they were transplanted from) than to *in situ* rocks at transition sites (i.e., where they were transplanted to) (Case et al. 2015). After transplanting away from active sites, OTU richness was 30% lower than *in situ* rocks at active sites, but the lost OTUs characteristic of active sites were replaced by OTUs characteristic of low-activity sites. High-abundance OTUs at the start of the experiment (e.g., ANME-1 and Gammaproteobacterial *Thiotrichaceae*) were more resilient than low-abundance OTUs (e.g., Epsilonproteobacterial *Helicobacteriaceae* mostly disappeared after 13 months). For transplants in the opposite direction, simulation of seep activation by transplanting rocks from transition sites to active sites showed that microbial assemblages are quick to respond to the new active conditions. *Helicobacteriaceae* showed the quickest response and recovered after 13 months to high abundance in transplanted carbonates, while *Thiotrichaceae* showed a slower response. The bidirectional transplant experiments at Hydrate Ridge provide evidence that, in general, microbial assemblages can adapt to changes in seepage activity, although OTUs show different responses (Case et al. 2015).

A 17-month transplant experiment showed that macrofaunal invertebrates are also able to adapt to new seepage activity conditions, but some species were not as resilient as others (Pereira et al. 2021, Chapter 2). Parallel to the responses observed for microbial communities in Case et al. (2015), macrofaunal densities on rocks transplanted away from active sites were lower than on *in situ* rocks at active sites, and the transplanted rocks gained taxa characteristic of low-activity sites.

Moreover, in opposite direction transplants, active taxa were acquired on rocks moved to active sites at a higher rate than the acquisition of low-seepage taxa. Bacterial grazers such as the snail *Provanna laevis* and the limpets *Paralepetopsis* sp. and *Neolepetopsis* sp. rapidly colonized these rocks in high abundances, suggesting a rapid establishment of the microbial community that provides their food or settlement cues.

Building upon these works, the question that remains unanswered is: are there interactions between microbial and macrofaunal communities or are macrofaunal patterns generated by seepage activity purely? By analyzing whether the macrofaunal community composition and dynamic response to changes in seepage activity correspond to shifts in microbial communities living on the same *in situ* and transplanted carbonate rocks at methane seeps on the Costa Rican margin, we aim to address the following questions: (1) Is macrofaunal diversity and/or richness correlated with microbial diversity and/or richness? (2) Do microbial and macrofaunal communities show similar response patterns to changes in seepage activity and with seep location with respect to Shannon-Wiener diversity, richness, and community composition? (3) Is the abundance (or lack) of specific macrofaunal species linked to the dominance of certain OTUs? (4) Do these interactions between macrofaunal and microbial communities change at different successional stages and under manipulated changes in seepage activity? Understanding how animals interact with and respond to microbial communities will improve overall knowledge of seep dynamics and improve the ability to manage these relationships in the face of climate change and direct human disturbance. Expanding oxygen minima under ocean deoxygenation (which intercept the Eastern Pacific margin being studied here) may differentially affect seep microbes depending on their oxygen preferences, with consequences for animal assemblages. In addition, ocean warming may lead to gas hydrate dissociation, enhancing seepage and altering seep

communities, and bottom trawling may cause physical disturbance on the seafloor (Levin et al. 2016), affecting both microbial and macrofaunal communities. This study provides information on whether or not macrofaunal communities are more resilient depending on the microbial community, and identifies interactions that might maintain ecosystem structure and functioning.

METHODS

Study area and approach

The macrofaunal dataset used in this chapter is a subset of the data from Chapter 2. Briefly, *in situ* carbonate rocks were collected using the submersible Alvin in 2017, 2018, and 2019 at three methane seep sites off the Pacific coast of Costa Rica: (1) Quepos Landslide, at 400 m, within the oxygen minimum zone (OMZ); (2) Mound 12, at 1,000 m, just below the OMZ, and (3), Jaco Scar, at 2,000 m, well below the OMZ with a thermal anomaly. These *in situ* rocks were used as control for 3-month, 17-month, and 7 year- colonization experiments, and 17-month transplant experiments (see Chapter 2). The data subset used in this chapter includes *in situ* rocks from all three seeps, and 7-year colonization experiments and 17-month transplant experiments at Mound 12. The associated macrofaunal invertebrates were separated from the rocks upon recovery, sorted, identified to the lowest taxonomic level possible, and counted using a microscope in the laboratory (see Chapter 2 for full methods description).

The microbial community dataset is a subset from Mullin (2020), and it was provided in the form of OTU relative abundance after DNA extraction and sequencing by S. Mullin using methods briefly described as follows. After faunal removal, the carbonate rocks were subsampled for microbial analysis using an ethanol-sterilized chisel and frozen in liquid nitrogen before storage at -80°C on board ship. In the laboratory, microbial DNA was extracted from rock subsamples

using the Qiagen PowerSoil DNA Extraction Kit according to the kit protocol. The subsamples were washed and sonicated to remove external sediment-associated microorganisms (Mason et al. 2015). Samples were powdered with a sterile mortar and pestle, and 0.25-0.5 g of powder was added to the lysis tubes. The tubes were flash frozen in liquid nitrogen and thawed in a 25°C water bath. C1 detergent was then added to the samples before samples were vortexed and incubated in a 65°C water bath for 30 minutes. The heated samples were then placed in the beater and processed for DNA extraction following the rest of the manufacturer’s instructions. The V4-V5 region of the 16S rRNA gene was amplified using archaeal/bacterial primers with Illumina adapters using Q5 Hot Start PCR mixture (see Mullin 2020 for mixture and cycling details). Sequenced samples were processed in QIIME version 1.8.0 following Mason et al. (2015). 16s RNA gene sequences were clustered *de novo* into operational taxonomic units (OTUs) with 99% similarity using UCLUST, and the most abundant sequence was chosen as representative for each *de novo* OTU (Edgar 2018). Taxonomic identification for each representative sequence was assigned with a modified SILVA-132 database, requiring minimum similarity of 90% to assign a taxonomy (see Mullin 2020 for full methods description).

We were able to gather macrofaunal and microbial data simultaneously from 55 rocks, which is summarized in Table 3.1.

Table 3.1: Number of rocks by seep location and seepage activity (A: Active, T: Transition, IT: Inner transition, OT: Outer transition, B: Background).

	Number of rocks by seepage activity
In situ rocks	
Quepos Landslide	5 A, 3 T
Mound 12	6 A, 8 T
Jaco Scar	2 A
Experimental rocks at Mound 12	
7-year colonization experiment	2 A, 2 T
+7-year colonization experiment	2 A
Transplant experiment	4 A-IT, 5 A-OT, 5 A-B, 5 IT-OT, 5 IT-B, 4 IT-A

Statistical analyses

Shannon-Wiener diversity ($H' \log e$) and richness (S) were calculated for the macrofaunal and microbial communities on each rock. Richness is expressed as number of species for macrofauna and number of OTUs for the microbial community. Generalized linear models were performed to test the statistical relationship between macrofaunal Shannon-Wiener diversity and species richness versus microbial Shannon-Wiener diversity and OTU richness. Variability in Shannon-Wiener diversity of macrofaunal and microbial communities between seepage activity level and across seep locations was checked with Kruskal-Wallis tests followed by post-hoc Dunn's tests using the Benjamini-Hochberg adjustment (Benjamini-Hochberg 1995) to examine whether macrofaunal and microbial Shannon-Wiener diversity and species/OTU richness follow the same patterns with seepage activity and seep location.

To examine whether macrofaunal and microbial communities show similar response patterns to changes in seepage activity and seep location with respect to community composition, we performed a tanglegram. The tanglegram consists of two clusters based on Euclidean dissimilarity matrixes, one for the macrofaunal community composition and the other for the microbial community composition, and a measure of entanglement that indicates how good the alignment between clusters is. Entanglement varies between 0 (no entanglement) to 1 (full entanglement). The closest to 0, the better the alignment between clusters, i.e., the communities show more similar patterns.

Total counts of macrofaunal species and microbial OTUs were calculated to identify the most common species and OTUs across all samples and create a subsample with species and OTUs that accounted for at least 1% of the total communities. This subsample consisted of 18 macrofaunal species that accounted for 70% of the total macrofaunal community, and 17 OTUs

that accounted for 80% of the total microbial community (described in the Results section). Heatmaps were created with this subsample to identify changes in the abundance of these most dominant species and OTUs across seepage activity and seep locations.

Co-inertia analyses were conducted using the subsample to examine multivariate relationships between macrofaunal species and microbial OTUs and examine whether the abundance (or lack) of specific species is associated with the dominance of certain OTUs. We performed three co-inertia analyses considering three data sets: (1) all samples, (2) *in situ* samples only, and (3) experimental samples only. By analyzing these data sets separately, we aimed to evaluate whether relationships change at different successional stages (*in situ* vs colonization rocks) and under a manipulated change in seepage activity (*in situ* vs transplanted rocks).

Finally, to examine the contribution of specific OTUs to similarity patterns of the macrofaunal communities based on community composition, a distance-based redundancy analysis (db-RDA) was performed using the subsample considering the three data sets as above. We checked rank correlations between dissimilarity indices and gradient separation to decide which distance measure to use in the db-RDA analyses (Faith et al. 1987). For the data set with all samples, we used the Euclidean distance measure. For the data set with *in situ* samples, we used the Gower's distance measure. And for the data set with experimental samples, we used the Euclidean distance measure.

All statistical analyses were performed in RStudio Version 2023.06.0+421. Diversity metrics were calculated using the functions 'diversity()' and 'specnumber()' in the 'vegan' package. Generalized linear models and diversity metrics were plotted using the function 'ggplot()' in the 'ggplot2' package. The tanglegram was performed using the function 'tanglegram()' in the 'dendextend' package. Co-inertia analyses were conducted using the functions 'dudi.pca()' and

‘`coinertia()`’ in the ‘`ade4`’ package, and the resulting X and Y canonical weights were plotted using the function ‘`s.arrow()`’. Rank correlation between dissimilarity indices was calculated using the function ‘`rankindex()`’ in the ‘`vegan`’ package, and db-RDA analyses were performed using the function ‘`dbrda()`’ and transformed into tidy data so it could be plotted using the function ‘`ggplot()`’ in the ‘`ggplot2`’ package.

RESULTS

There was no linear relationship between microbial Shannon-Wiener diversity and macrofaunal Shannon-Wiener diversity ($t = -0.013$, $p = 0.99$) and between microbial Shannon-Wiener diversity and macrofaunal richness across all seeps ($t = 0.151$, $p = 0.88$) (Fig. 3.1A,C). However, macrofaunal Shannon-Wiener diversity and richness increased with increasing microbial richness ($t = 2.207$, $p = 0.03$; $t = 2.336$, $p = 0.02$, respectively) (Fig. 3.1B,D). Among seep locations, macrofaunal Shannon-Wiener diversity and species richness were the highest at Mound 12 and the lowest at Quepos Landslide, at both active and transition sites ($\chi^2_{10} = 25.68$, $p = 0.004$; $\chi^2_{10} = 27.35$, $p = 0.002$) (Fig. 3.2A,C). Microbial Shannon-Wiener diversity was generally the highest at Quepos Landslide and the lowest at Jaco Scar at both active and transition sites, although not significant ($\chi^2_{10} = 7.57$, $p = 0.67$) (Fig. 3.2B). There was also no significant difference in microbial richness among seeps ($\chi^2_{10} = 16.78$, $p = 0.08$), but there was a pattern of highest richness at Quepos Landslide and lowest richness at Jaco Scar at active sites, and highest richness at Mound 12 followed by Quepos Landslide at transition sites (Fig. 3.2D). Tanglegram analysis did not show similar clustering patterns based on macrofaunal composition and microbial composition with seepage activity and seep location, yielding a relatively high entanglement of 0.60 (Fig. 3.3).

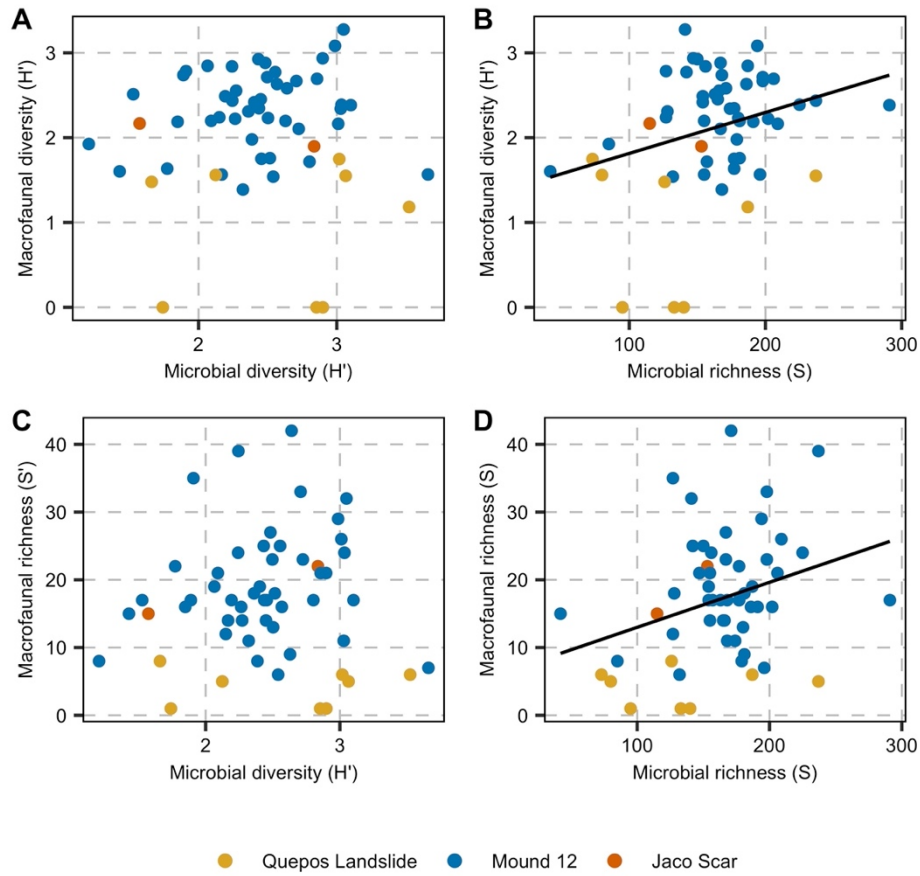


Figure 3.1: Relationship between (A) macrofaunal Shannon-Wiener diversity and microbial Shannon-Wiener diversity, (B) macrofaunal Shannon-Wiener diversity and microbial richness, (C) macrofaunal richness and microbial Shannon-Wiener diversity, and (D) macrofaunal richness and microbial richness. Generalized linear models were used to fit the data, and regression lines are plotted when significant ($p < 0.05$).

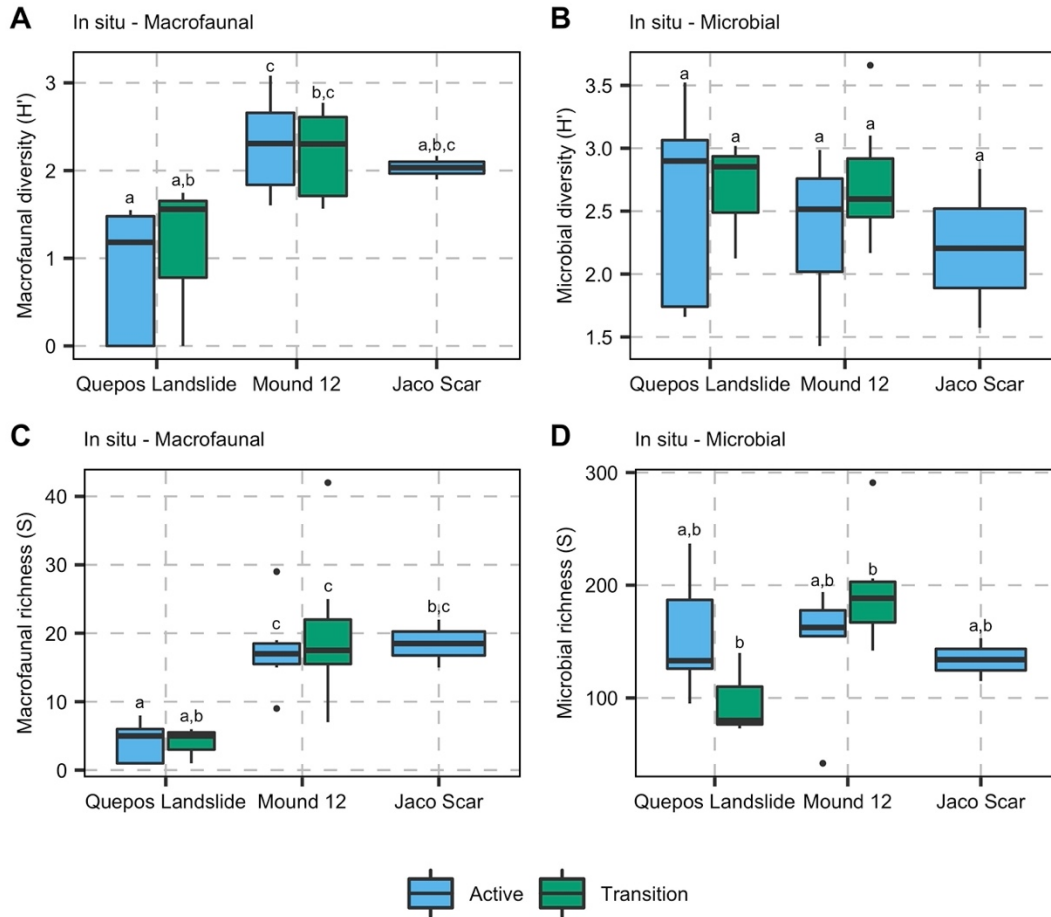


Figure 3.2: (A-B) Shannon-Wiener diversity and (C-D) richness of macrofaunal and microbial communities on in situ rocks across methane seeps.

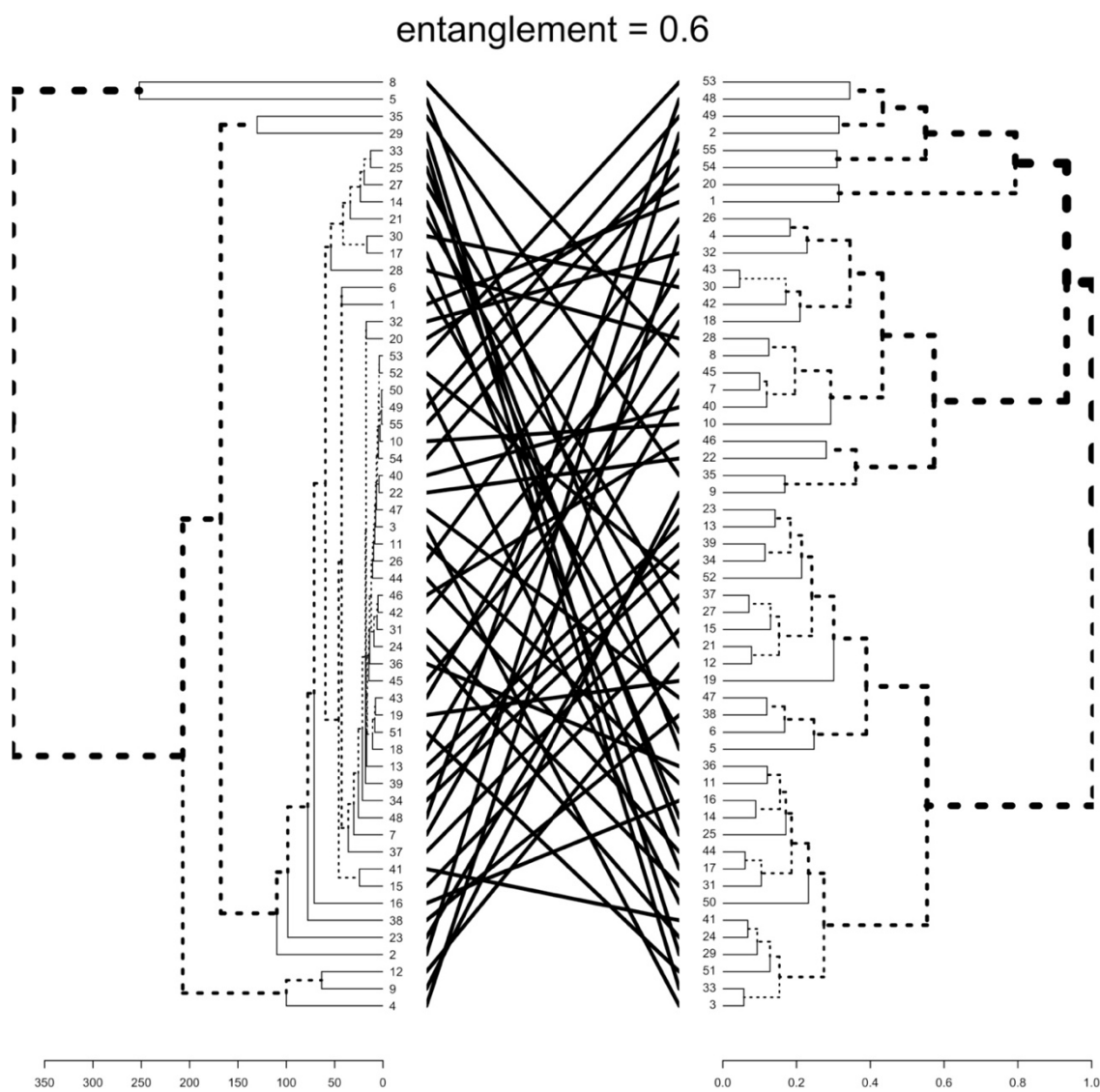


Figure 3.3: Tanglegram of macrofaunal (left) and microbial (right) clustering based on community composition. Entanglement varies between 0 (no entanglement and a good alignment) to 1 (full entanglement). Dashed lines represent differences between the two clusters – all branches were different between clusters. Samples information can be found in Table S2.

Eighteen species composed 70% of the macrofaunal community across all samples (Table 3.2), and their abundance varied across seepage activity and seep location (Fig. 3.4A). Hydroids Anthoathecata were more common at active sites at Quepos Landslide, transition sites at Mound 12, and on most of the transplanted rocks at Mound 12. The tanaids *Parakanthophoreus* sp. 1 and *Typhlotanoides* sp. 1 were more abundant at Jaco Scar. The bacterial grazers neolepetopsid

limpets, provannid snails, and pyropeltid limpets were more abundant on *in situ* rocks at Mound 12 at active sites and on rocks transplanted from transition to active sites at Mound 12.

Sixteen OTUs composed 80% of the microbial community across all samples (Table 3.2), and their relative abundance varied across seepage activity and seep location (Fig. 3.4B). Thermodesulfovibrionia and Gammaproteobacteria showed higher relative abundance on *in situ* rocks at transition sites at Quepos Landslide. Anaerolineaceae showed higher relative abundance on *in situ* rocks at active sites at Jaco Scar. Interestingly, SEEP SRBs showed higher relative abundance on rocks transplanted from active to background sites at Mound 12. ANME-2 showed higher relative abundance on *in situ* rocks at active sites at Mound 12, as well as on rocks transplanted from active sites to lesser seepage activity and rocks transplanted from transition to active sites at Mound 12. ANME-1 showed slightly high relative abundance at transition sites at Mound 12, both on *in situ* and transplanted rocks.

Table 3.2: Species and OTUs that accounted for at least 1% of the total communities. These contributed to 70% of the total macrofaunal community and 80% of the total microbial community.

Macrofaunal community		Microbial community	
Species	%	OTU	%
<i>Provanna laevis</i>	18	ANME-1b	28
<i>Lepetodrilus ovalis</i>	9	ANME-2b	12
<i>Pyropelta musaica</i>	6	ANME-1a	7
<i>Chrysopetallum</i> sp.	6	ANME-2c	6
<i>Pyropelta wakefieldi</i>	4	ANME-2a 2b	6
<i>Amphisamytha fauchaldi</i>	4	Thiohalophilus	5
<i>Neolepetopsis</i> sp. 3	3	Gammaproteobacteria	3
<i>Hyalogyrina</i> sp.	3	ANME-1	2
<i>Lepetodrilus</i> stet.	2	Lokiarchaea	2
<i>Kiwa puravida</i>	2	Unassigned	2
<i>Parakanthophoreus</i> sp. 1	2	Thermodesulfovibrionia	2
<i>Galapagomystides patricki</i>	2	Anaerolineaceae	1
<i>Pyropelta corymba</i>	2	SEEP SRB-1	1
<i>Fucaria</i> sp. 1	2	JS1	1
Anthoathecata	1	SEEP SRB-2	1
<i>Laminatubus</i> sp.	1	Archaea	1
<i>Neolepetopsis</i> sp. 1	1		
<i>Typhlotanoides</i> sp. 1	1		

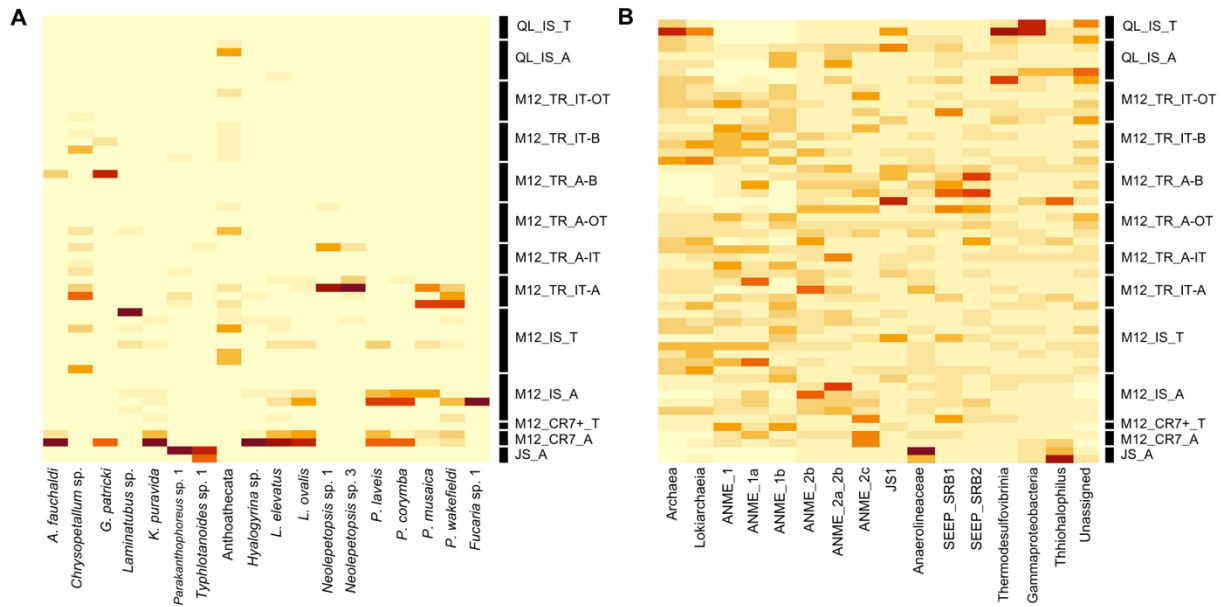


Figure 3.4: Heatmaps based on total counts of (A) macrofaunal species and (B) microbial OTUs on in situ rocks (IS) at Quepos Landslide (QL), Mound 12 (M12), and Jaco Scar (JS), 7-years colonization experiment (CR7) at Mound 12, and 17-months transplant experiments (TR) at Mound 12. Seepage activity: Active (A), Transition (T), Inner transition (IT), Outer transition (OT), Background (B).

Co-inertia analysis with all samples, including *in situ* and experimental communities, yielded a correlation of 13% (Fig. 3.5A). The tanaids *Parakanthophoreus* sp. 1 and *Typhlotanoides* sp. 1 were positively associated with Anaerolineaceae and negatively associated with some ANME-1b. Seep species (e.g., *Fucaria* sp. 1, *P. corymba*, *P. musaica*, *L. ovalis*, and *Neolepetopsis* limpets) were positively associated with ANME-1a, ANME-2b, ANME-2c, and SRB1.

Co-inertia analysis with only *in situ* communities yielded a higher correlation of 34% (Fig. 3.5B). The tanaids *Parakanthophoreus* sp. 1 and *Typhlotanoides* sp. 1 were positively associated with Anaerolineaceae and *Thiohalophilus*, and negatively associated with ANME-1, ANME-2a_2b, and SRB1. Seep species (*P. corymba*, *P. musica*, *P. wakefieldi*, *Fucaria* sp. 1, *Neolepetopsis* sp. 3, *Lepetodrilus* stet., and *Laminatubus* sp.) were positively associated with

ANME-1, ANME-2, ANME-2c, and SRB2. The hydroid Anthoathecata was positively associated with ANME-1b, Lokiarchaea, and JS1.

Co-inertia analysis with only communities on experimental rocks yielded a correlation of 16%, and some of the patterns observed for the *in situ* communities changed (Fig. 3.5C). Limpets *Lepetodrilus* stet. and *P. corymba* and the snail *P. laevis* were positively associated with ANME-1, as well as *A. fauchaldi* and *Typhlotanoides* sp. Other seep species, *P. wakefieldi*, *P. musaica*, *Neolepetopsis* limpets, and *Laminatubus* sp. and the tanaid *Parakanthophoreus* sp. 1 were positively associated with ANME-1a and ANME-2c, and negatively associated with ANME-1b. The hydroid Anthoathecata stet. was positively associated with *Thiohalophilus*, *Therrmodesulfovibrionia*, *Gammaproteobacteria*, and *Lokiarchaea*.

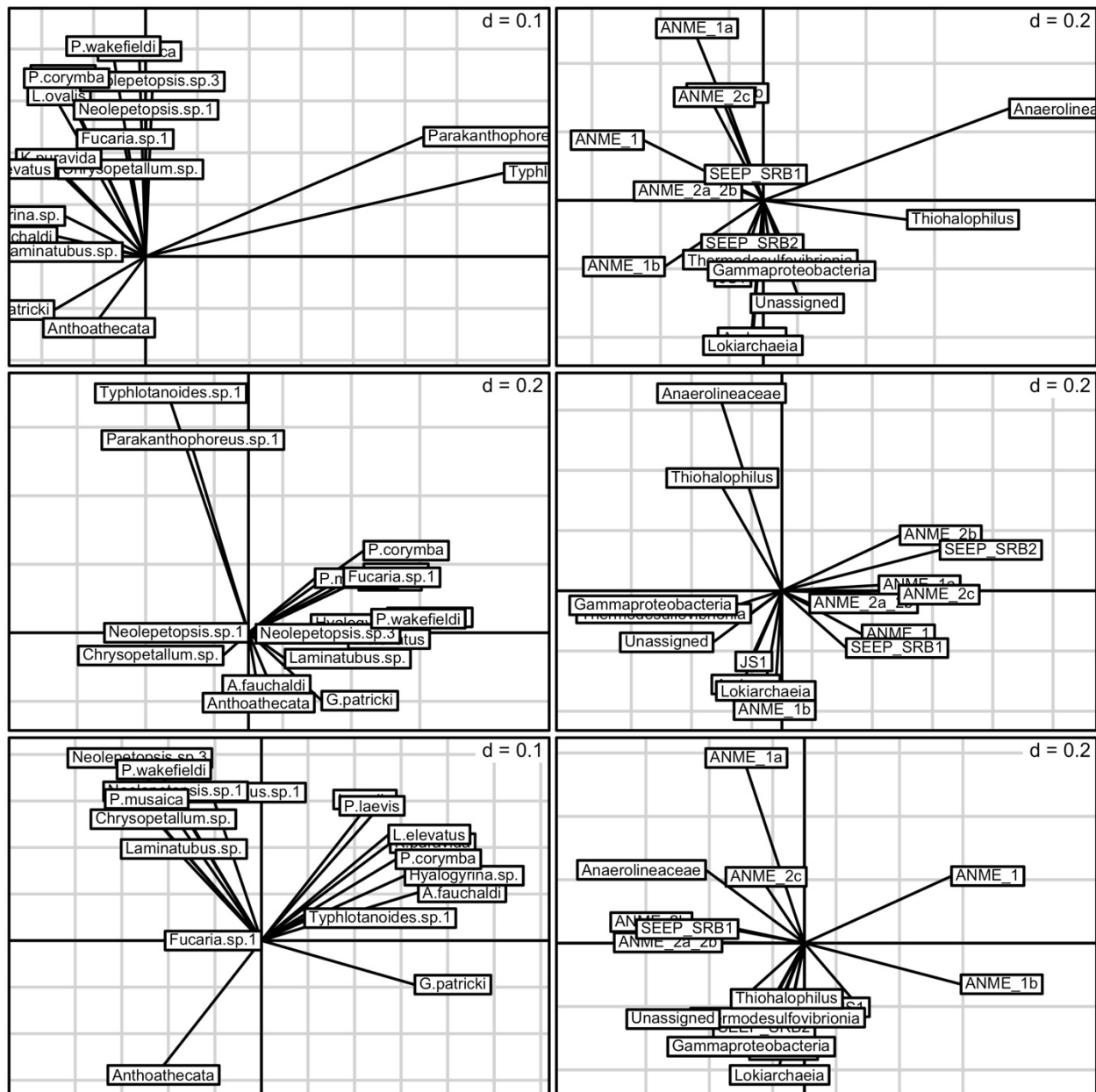


Figure 3.5: Co-inertia analyses of macrofaunal species and microbial OTUs on (A) both in situ and experimental rocks, and (B) in situ rocks only, and (C) experimental rocks only. Arrows indicate the magnitude and direction of variation for each species/OTU.

Results of the db-RDA analysis considering all samples revealed that ANME-1 significantly explains some of the variations observed in macrofaunal community composition among treatments ($F = 4.13$, $p = 0.04$) (Fig. 3.6A). However, ANME-1 was only positively correlated to the 7-year colonization macrofaunal communities at Mound 12.

Results of the db-RDA analysis considering *in situ* rocks only revealed that SEEP SRB-2, ANME-2b, and ANME-2c significantly explain some of the variation observed for *in situ* macrofaunal community composition ($F = 6.66$, $p = 0.02$; $F = 5.13$, $p = 0.03$; $F = 5.09$, $p = 0.03$, respectively) (Fig. 3.6B). These OTUs were positively correlated to macrofaunal communities on *in situ* rocks at Mound 12, mainly at active sites. SEEP SRB-2 was negatively correlated to macrofaunal communities on *in situ* rocks at transition sites at Mound 12, and other ANME-2b and ANME-2c were negatively correlated to macrofaunal communities on *in situ* rocks at Quepos Landslide active and transition sites and at Jaco Scar active sites. Additionally, ANME-1a, ANME-1, and SEEP SRB-1 were also significant at the 0.1 significance level ($F = 3.84$, $p = 0.05$; $F = 2.73$, $p = 0.09$; $F = 3.11$, $p = 0.06$) and positively correlated to macrofaunal communities on *in situ* rocks at Mound 12 at both active and transition sites but negatively correlated to macrofaunal communities on *in situ* rocks at Quepos Landslide and Jaco Scar. Two other interesting positive correlations, although not significant, were between Quepos Landslide and Jaco Scar *in situ* macrofaunal communities and the OTUs Anaerolineaceae ($F = 2.47$, $p = 0.14$), *Thiohalophilus* ($F = 0.70$, $p = 0.45$), *Thermodesulfobibrionia* ($F = 0.94$, $p = 0.43$), and Gammaproteobacteria ($F = 0.58$, $p = 0.61$).

Finally, results of the db-RDA analysis considering experimental rocks revealed some interesting correlations between OTUs and macrofaunal species on colonization and transplanted rocks, although none were significant (Fig. 3.6C-D). ANME-1 was positively correlated to 7-year colonization macrofaunal communities at active sites at Mound 12 ($F = 2.35$, $p = 0.12$). ANME-1a, ANME-2c, and Anaerolineaceae were positively correlated to macrofaunal communities transplanted from transition to active sites ($F = 0.92$, $p = 0.34$; $F = 0.25$, $p = 0.73$; $F = 0.24$, $p = 0.83$, respectively). These were in the opposite direction than macrofaunal communities on rocks

transplanted to lesser seepage activity, which were positively correlated to *Thiohalophilus* ($F = 0.89$, $p = 0.41$), *Thermodesulfovibronia* ($F = 0.10$, $p = 0.85$), *Lokiarchaea* ($F = 0.28$, $p = 0.74$), Gammaproteobacteria ($F = 0.23$, $p = 0.86$), and SEEP SRB-2 ($F = 0.32$, $p = 0.68$).

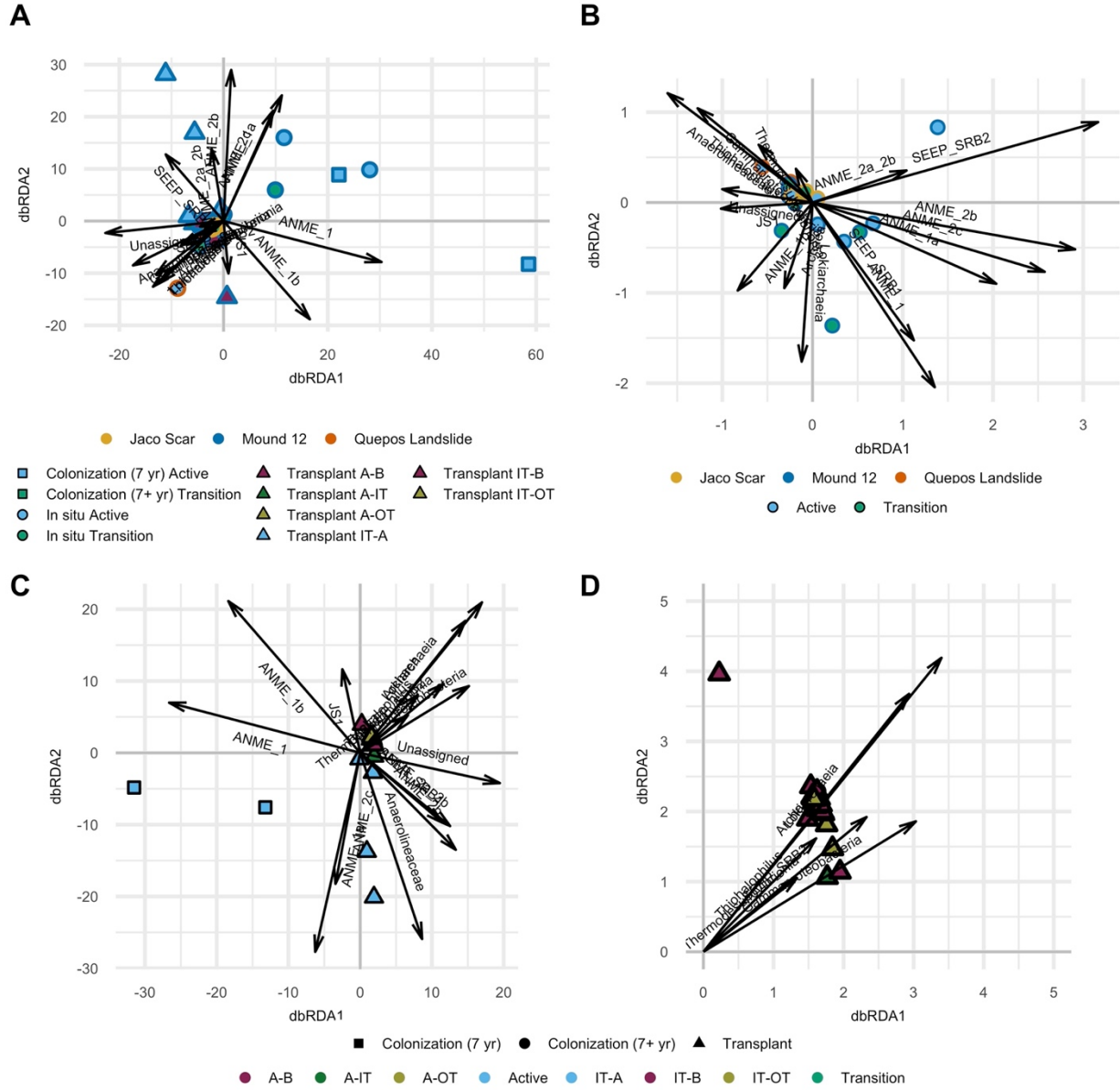


Figure 3.6: Distance-based redundancy analysis (db-RDA) of microbial OTUs and communities on (A) both in situ and experimental rocks, and (B) in situ rocks only, and (C-D) experimental rocks only (panel D is a zoom into the top right part of panel C). Arrows indicate the magnitude and direction of variation for each species/OTU. A: Active, T: Transition, IT: Inner transition, OT: Outer transition, B: Background.

DISCUSSION

Microbial activity at methane seeps provides substrate for attachment, nutrition, and settlement cues for invertebrate macrofaunal communities (Danovaro et al. 2024). In seep sediments, the methanotroph microbial community can support unique animal communities (Thurber et al. 2013), and we expected to see strong correlations between macrofaunal and free-living microbial communities associated with carbonate rocks. We observed a positive linear relationship between macrofaunal Shannon-Wiener diversity and microbial richness, but there was no relationship between macrofaunal Shannon-Wiener diversity and microbial diversity (Fig. 3.1). This suggests that macrofaunal species might depend on specific OTUs rather than on microbial diversity. In addition, microbial Shannon-Wiener diversity and richness were the highest at Quepos Landslide and did not lead to the highest macrofaunal Shannon-Wiener diversity and richness, but the lowest instead (Fig. 3.2), and perhaps the microbes that have higher relative abundance and are more adapted to the low oxygen conditions at Quepos Landslide are not the OTUs that the macrofauna might be associated with. The microbial OTUs *Thermodesulfovibrionia* and *Gammaproteobacteria* showed higher relative abundance at Quepos Landslide. *Thermodesulfovibrionia* are anaerobic sulfate reducers that have been reported in high abundance in low-oxygen groundwater communities (Yan et al. 2020, Rogers et al. 2022), and some *Gammaproteobacterial* methanotrophs have been shown to have adaptations to oxygen limitation (Skenner et al. 2015). However, there were no strong associations between macrofaunal species at Quepos Landslide and these OTUs based on co-inertia analyses. The high entanglement between clusters based on community composition of the macrofauna and microbial communities also support this idea that macrofaunal patterns might be more related to specific OTUs and not to the microbial community as a whole (Fig. 3.3). A study comparing macrofaunal communities to

archaeal and bacterial communities at hydrothermal vents also showed that high archaeal and bacterial diversity does not necessarily lead to high macrofaunal diversity, but functional partnerships between macrofaunal species and OTUs form core communities, which are different under different fluid temperatures (Murdock et al. 2021). Here, we also observed some relevant associations between macrofaunal species and specific OTUs at the Costa Rican seeps, and that these interactions seem to dictate macrofaunal responses to natural and manipulated changes in seepage activity. We discuss these associations below.

At Mound 12, the dominance of ANME-1, ANME-2, and SEEP SRB-2 at active sites might be favoring macrofaunal seep species (Fig. 3.4). Cell aggregates of archaea belonging to ANME-2 and sulfate-reducing bacteria (SRB) are involved in AOM (Orphan et al. 2001), a process that leads to the formation of the carbonate rocks at active seepage (Marlow et al. 2014b), where these macrofaunal seep species are abundant. In addition, the AOM consortia provides food for many seep species including the limpets *P. corymba*, *P. musaica*, *P. wakefieldi*, *Lepetodrilus* sp., and *Neolepetopsis* sp. and the snail *P. laevis*, which graze on microbes (Levin et al. 2015, Govenar et al. 2015). Thus, the observed positive association between seep macrofauna and these OTUs was expected (Fig. 3.5A-B). However, in sediments, it is common to see a top-down control of macrofauna on the microbial community due to grazing activity (Baldrighi et al. 2013, Stief 2013, Broman et al. 2024). Thus, we also expected to see some negative associations between bacterial-grazer species and OTUs that these animals might be grazing upon. Gut contents of some vent limpets showed that they specialize on Epsilonproteobacteria, while others have a more diversified diet (Govenar et al. 2015). Previous studies hypothesized that some of the seep grazers might also be specialists, especially *P. laevis* and *P. wakefieldi* (Levin et al. 2015, Pereira et al. 2021, Chapter 2). Here, we observed a negative association between *P. laevis*, *P. corymba*, *P. wakefieldi*,

Lepetodrilus stet., and *Fucaria* sp. 1 with Gammaproteobacteria on *in situ* rocks (Fig. 3.5B). However, as mentioned above, Gammaproteobacteria showed higher relative abundance at Quepos Landslide than at Mound 12, where we do not find these species, and, thus, this negative correlation might not be an effect of grazing pressure, but rather an effect of low oxygen on the macrofaunal communities (Chapter 2).

At Jaco Scar, tanaids were more abundant at active sites (Fig. 3.4A), and positively associated with Anaerolineaceae and *Thiohalophilus* on *in situ* rocks (Fig. 3.5B), which possibly led to the community-wide correlations between these OTUs and the communities at Jaco Scar (Fig. 3.6A-B). A recent study showed that the order of Anaerolineales bacteria can be physically associated with sediment-hosted AOM consortia, especially when ANME-2a is the most abundant ANME lineage (and not ANME-1a and ANME-2c) and hypothesized that the microorganisms physically associated with the AOM consortia are different depending on the ANME lineage (Yu et al. 2022). *Thiohalophilus* are obligate sulfur oxidizing bacteria that can replace other Gammaproteobacteria with more versatile metabolisms (Sorokin et al. 2008). Anaerolineaceae and *Thiohalophilus* showed higher relative abundance on *in situ* rocks at Jaco Scar (Fig. 3.4B), and we hypothesize that there is a possible preference by tanaids for AOM consortia with Anaerolineaceae and *Thiohalophilus*, perhaps because the association between AOM consortia and Anaerolineales might alter the methanotrophic capacity of the consortia given the potential methanogenic metabolism of Anaerolineales (Yu et al. 2022). This association with specific OTUs and lineages by tanaids might also explain the differences in tanaid abundance observed at Mound 12. At this seep location, tanaids are more abundant at transition sites rather than at active sites as at Jaco Scar (Fig. 3.4A). Communities on *in situ* rocks at active sites Mound 12 were more positively correlated

to ANME-1a, ANME-2b, ANME-2c, and SEEP SRB-1 than at Jaco Scar (Fig. 3.6B), which are OTUs that tanaids were negatively associated with on *in situ* rocks (Fig. 3.5B).

On transplanted rocks, however, it seems that the macrofaunal communities are following the responses of the microbial community after transplant. Macrofaunal communities on rocks transplanted to active sites were positively correlated to ANME-1a, ANME-2c, and Anaerolineaceae (Fig. 3.6C-D). This is striking because previous transplant experiments showed a slow response by Anaerolineaceae to seep activation, but they have high tolerance to low-activity conditions (Case et al. 2015). Still, the persistence of Anaerolineaceae might have led to the resilience of the tanaid *Parakanthophoreus* sp. 1 on these rocks (Fig. 3.4A) given their strong association (Fig. 3.5C-D).

The seep species that colonized these rocks, including *P. musaica*, *P. wakefieldi*, *Neolepetopsis* limpets, and *Chrysopetallum* sp., are bacterial grazers (Wiklund et al. 2009, Levin et al. 2015, Govenar et al. 2015), and were positively associated with ANME-1a and ANME-2c on rocks transplanted to active sites (Fig. 3.5C). ANME-1 and ANME-2c are commonly found in higher abundance at lesser seepage activity than ANME-2a/b (Marlow et al. 2014b, Case et al. 2015), and ANME-1 is known to be relatively insensitive to seepage activity and may perform methanotrophy even under low activity (Marlow et al. 2014b, Case et al. 2015). Thus, it seems that ANME-1 and ANME-2c were able to survive through the transplant to active sites, and the persistence of these OTUs might have attracted the macrofaunal seep species that feed on the microbial community. With longer exposure, ANME-1, which grows more slowly than ANME-2 (Orphan et al. 2009, Girguis et al. 2003) and seems to colonize carbonates seeded by the underlying sediments (Case et al. 2015), was able to establish on the 7-year colonization rocks (Fig. 3.4B,

3.6C); on these we find more *P. laevis*, *P. corymba*, and *P. musaica*, which were strongly associated with ANME-1 (Fig. 3.4A, 3.5B).

Communities on rocks transplanted to transition and background sites were positively correlated to *Thiohalophilus*, *Thermodesulfovibrinia*, Gammaproteobacteria, Lokiarchaea, other Archaea, and SEEP SRB-2 (Fig. 3.6C-D). In a previous transplant experiment at Hydrate Ridge, OR, some Gammaproteobacterial OTUs were able to remain at a relative high abundance after transplant to low seepage after 13 months (Case et al. 2015). In addition, some Gammaproteobacteria might have more diverse metabolisms (Sorokin et al. 2008) that could enable them to grow and establish on the experimental rocks moved to lesser seepage activity. The correlation and presence of SRB2 and gammaproteobacterial on these rocks suggests the persistence of methanotrophy after transplant, supporting macrofaunal seep species through the 17 months of the transplant (Chapter 2). However, a general lack of association between macrofaunal species and SRBs might indicate that seep species are more strongly associated with archaeal lineages, or that the animals are feeding on the bacteria. Isotopic values of neolepetopsid limpets on rocks transplanted to lesser seepage activity indicate reliance on methane-derived carbon, with $\delta^{13}\text{C}$ ranging from -63.0‰ to -46.6‰ (Chapter 2), which are typical of archaeal/bacterial AOM consortia (Orphan et al. 2001). Other bacterial grazer taxa with low $\delta^{13}\text{C}$ values (-55.1‰ to -42.4‰) on these rocks were chitons, although they were not included in the analyses here given their low abundance (Chapter 2). Feeding on archaeal-bacterial aggregates at seeps has been reported before for dorvilleids (Thurber et al. 2012). This further supports the hypothesis of persistent chemosynthetic production after transplant providing nutrition to selected macrofaunal seep species that were able to survive the transplant. Lokiarchaea also showed a positive correlation to communities on rocks transplanted to lesser seepage activity. Little is known about

the ecological distribution, metabolism, and role in biogeochemical cycling of Lokiarchaea, but given their close affiliation to eukaryotes, it is thought that eukaryotes evolved from an archaeal ancestor and Lokiarchaeota might be the “missing link” in the formation of the eukaryotic lineage (Manoharan et al. 2019).

Finally, at Quepos Landslide, where the hydrographic conditions are influencing the macrofaunal community (Chapter 2), hydroids were positively associated with ANME-1b, Lokiarchaea, and JS1 on *in situ* rocks. JS1 is a heterotrophic anaerobic bacteria found mainly in anoxic sediments, and especially in organic-rich sediments, and an important group of bacteria in shallow seeps (Li et al. 2023, Miyake et al. 2023). It has high relative abundance at Quepos Landslide (Fig. 3.4B). On experimental rocks at Mound 12, hydroids were positively associated with OTUs correlated to communities on rocks transplanted to lesser seepage activity such as ANME-1b that is more common at low seepage (as described above) and negatively associated with ANME-1 on 7-year colonization rocks, where they could have been competing for space and food with bacterial grazers. Hydroids are known to live in high densities at shallow seeps taking advantage of the substrate and food provision (Prudkovsky et al. 2023, Chapter 2). However, they may have methanotrophic symbiotic bacteria (Goffredi and others, unpublished data), and further studies are needed to reveal whether hydroid patterns are purely related to the environment or to food provision by either free-living or symbiotic microbial activity.

CONCLUSION

By analyzing associations between macrofaunal species and microbial OTUs at methane seeps under natural and manipulated changes in seepage activity, we observed that animal-microbial correlations are playing a major role in community structure and functioning. Natural

environmental drivers of microbial community patterns are reflected in animal-microbial associations. For example, the dominance of Anaerolineales bacteria at Jaco Scar and their persistence through transplant to active sites at Mound 12 could have led to high abundance of tanaids, which seem to thrive where Anaerolineales is associated with the AOM consortia. In addition, it seems that animal responses to changes in seepage activity mirror the microbial responses. When methanotrophic microbes are able to persist through a quiescence in seepage activity, seep animals that depend on chemosynthetic production as a source and provision of habitat also survive and thrive under low seepage conditions. In the case of seepage activation, ANME-1a and ANME-2c, typically more abundant at lower seepage activity, are able to persist in the short term (17 months), providing food for bacterial grazer that quickly colonize the rocks, and, in the long term (7 years), slower-growing microbes also provide food for a more established macrofaunal community. However, it is important to note that positive associations do not necessarily indicate causation. For example, if low oxygen is a driving factor of community structure, the distribution of aerobic animals and aerobic microbes could show positive associations but that does not mean that the microbial patterns are responsible for macrofaunal distribution. We also did not consider other microbial roles in habitat creation besides carbonate formation (e.g., bacterial mats, detoxifying, among others), which can also have impacts on macrofaunal distribution and could be incorporated into future studies. In addition, using amplicon sequence variant (ASV) approach instead of OTUs could provide a better resolution of microbial diversity.

We conclude that while environmental drivers (i.e., seepage activity and low oxygen) seem to be dictating the microbial community patterns, the macrofaunal community seems to have associations with specific OTUs, which they depend on for food and habitat provision. Thus, the

macrofauna appear to respond to the resilience and resistance of these OTUs under natural and manipulated changes in seepage activity. This work indicates the complexity of animal-microbe associations at methane seeps, suggesting the importance of these interactions to the structure, functioning, and health of the ecosystem. The critical function of methane filtration, a key ecosystem service at seeps, may be mediated by microbe-macrofauna linkages. With increasing interest in deep-sea resources, seeps are threatened by oil and gas extraction, bottom trawling, and ocean warming and deoxygenation. It is important we consider animal-microbial interactions when planning for deep-sea ecosystem management and conservation.

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SUPPLEMENTAL FILES

Supplemental Table 3.1: Summary of archaeal and bacterial OTU affiliations, their physiology and location at the seep relative to seepage activity, from Orphan et al. (2001), Yamamoto and Takai (2011), Bruckner et al. (2013), Case et al. (2015), Taubert et al. (2019), Mullin (2020), and Beckmann et al. (2021).

OTU	Physiology	Seepage activity location
Anaerobic methane-oxidizing archaea ANME-1	Methane oxidizers ($CH_4 \rightarrow HCO_3^-$); Involved in the anaerobic oxidation of methane in association with SRBs or other bacteria via nitrate and nitrite reduction.	Abundant at active and low-activity sites in association with SRBs and without bacterial partners in mono-species cell clusters; Perform methanotrophy even at low-activity sites; ANME-1b are more abundant at lesser seepage activity than ANME-1a.
Anaerobic methane-oxidizing archaea ANME-2	Methane oxidizers ($CH_4 \rightarrow HCO_3^-$).	Found in association with SRBs and without bacterial partners as individual filaments; More abundant at active sites than ANME-1; ANME-2a/b are more abundant at active sites than ANME-2c, which is more abundant at lesser seepage activity.
Nitrosopumilaceae (e.g., <i>Nitrosopumilus</i>)	Ammonium oxidizer.	Higher abundance at low-activity sites.
Deltaproteobacterial sulfate-reducing bacteria (SRB, e.g., SEEP-SRB1)	Sulfate reducers ($SO_4^{2-} \rightarrow HS^-$); Involved in the anaerobic oxidation of methane. Not all SRBs are coupled with ANMEs, some are free-living.	Surprisingly, some SRBs involved in AOM are relatively insensitive to seepage activity.
Epsilonproteobacteria (e.g., <i>Helicobacteraceae</i>)	Sulfur oxidizers ($4S_2O_3^{2-} \rightarrow 8SO_4^{2-}$)	Increased abundance at active seepage sites, where there are high sulfide concentrations; Adapted to dynamic seepage activity.
Gammaproteobacteria (e.g., <i>Thiotrichaceae</i>)	Sulfur oxidizers; Some groups perform oxidation of sulfur or methane.	Increased abundance at active seepage sites, where there are high reduced sulfur compounds; More restricted habitat than for Epsilonproteobacteria.
Gammaproteobacteria <i>Methylococcaceae</i> (type I methanotrophs)	Aerobic methane-oxidizing with particulate methane monooxygenase (pMMO) ($CH_4 \rightarrow CH_3OH$)	Higher abundance at active sites.
Verrucomicrobia (type III methanotroph)	Aerobic methane oxidizers.	Higher abundance at active sites.
Gammaproteobacteria (e.g., <i>Nitrospina</i> , <i>Nitrosomonas</i>)	Ammonium and nitrite oxidizers.	Higher abundance at low-activity sites.
Leptospirales	Heterotrophs.	Higher abundance at low-activity sites.

* Coupled with SRB to realize the overall reaction $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$.

Supplemental Table 3.2: Sample identification from tanglegram.

Number in tanglegram	Cruise	Experiment	Seep location	Seepage activity or Transplant
1	2017	In situ	Jaco Scar	Active
2	2017	In situ	Jaco Scar	Active
3	2017	Colonization (7+ years)	Mound 12	Transition
4	2017	Colonization (7 years)	Mound 12	Active
5	2017	Colonization (7 years)	Mound 12	Active
6	2017	In situ	Mound 12	Active
7	2017	In situ	Mound 12	Active
8	2017	In situ	Mound 12	Active
9	2017	In situ	Mound 12	Active
10	2018	In situ	Mound 12	Active
11	2018	In situ	Mound 12	Active
12	2017	In situ	Mound 12	Transition
13	2017	In situ	Mound 12	Transition
14	2017	In situ	Mound 12	Transition
15	2017	In situ	Mound 12	Transition
16	2017	In situ	Mound 12	Transition
17	2018	In situ	Mound 12	Inner transition
18	2018	In situ	Mound 12	Outer transition
19	2018	In situ	Mound 12	Outer transition
20	2018	Transplant (17 months)	Mound 12	A-B
21	2018	Transplant (17 months)	Mound 12	A-IT
22	2018	Transplant (17 months)	Mound 12	A-OT
23	2018	Transplant (17 months)	Mound 12	IT-A
24	2018	Transplant (17 months)	Mound 12	IT-B
25	2018	Transplant (17 months)	Mound 12	IT-OT
26	2018	Transplant (17 months)	Mound 12	A-B
27	2018	Transplant (17 months)	Mound 12	A-IT
28	2018	Transplant (17 months)	Mound 12	A-OT
29	2018	Transplant (17 months)	Mound 12	IT-A
30	2018	Transplant (17 months)	Mound 12	IT-B
31	2018	Transplant (17 months)	Mound 12	IT-OT
32	2018	Transplant (17 months)	Mound 12	A-B
33	2018	Transplant (17 months)	Mound 12	A-IT
34	2018	Transplant (17 months)	Mound 12	A-OT
35	2018	Transplant (17 months)	Mound 12	IT-A
36	2018	Transplant (17 months)	Mound 12	IT-B
37	2018	Transplant (17 months)	Mound 12	IT-OT
38	2018	Transplant (17 months)	Mound 12	A-B
39	2018	Transplant (17 months)	Mound 12	A-IT
40	2018	Transplant (17 months)	Mound 12	A-OT
41	2018	Transplant (17 months)	Mound 12	IT-A
42	2018	Transplant (17 months)	Mound 12	IT-B
43	2018	Transplant (17 months)	Mound 12	IT-OT
44	2018	Transplant (17 months)	Mound 12	A-B
45	2018	Transplant (17 months)	Mound 12	A-OT
46	2018	Transplant (17 months)	Mound 12	IT-B
47	2018	Transplant (17 months)	Mound 12	IT-OT
48	2017	In situ	Quepos Landslide	Active
49	2017	In situ	Quepos Landslide	Active
50	2017	In situ	Quepos Landslide	Active
51	2017	In situ	Quepos Landslide	Active
52	2017	In situ	Quepos Landslide	Active
53	2017	In situ	Quepos Landslide	Transition
54	2017	In situ	Quepos Landslide	Transition
55	2017	In situ	Quepos Landslide	Transition

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Chapter 4 INVERTEBRATE TROPHIC STRUCTURE ON MARINE FERROMANGANESE AND PHOSPHORITE HARDGROUNDS

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Levin

Invertebrate trophic structure on marine ferromanganese and phosphorite hardgrounds

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Abstract

The Southern California Borderland hosts a variety of geologic and oceanographic features that allow for diverse habitats to occur in a restricted region with a strong oxygen minimum zone (OMZ) and hard substrates. These include ferromanganese (FeMn) crusts and phosphorites targeted for deep-seabed mining in other regions. Baseline studies regarding hardground macro- (> 0.3 mm) and megafaunal (> 2 cm) invertebrates are lacking, although they contribute to understanding nutrient cycling and resilience of deep-sea communities to ocean deoxygenation, fishing, or mineral extraction. With the goal of understanding how substrate type, depth, and dissolved oxygen concentration influence invertebrate trophic structure, we surveyed $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of invertebrates on hard substrates on the Southern California Borderland margin along a depth gradient (120–2400 m) through the OMZ at inshore (< 100 km from shore) and offshore (100–250 km from shore) sites, using generalized additive models and community-level metrics. Macrofaunal isotopic values correlate with substrate type, exhibiting higher trophic diversity on FeMn crusts and specialized communities on phosphorites. Megafaunal isotopic values correlate with proximity to shore; animals offshore seem to depend more on phytoplanktonic production than animals inshore. In general, $\delta^{15}\text{N}$ increased with decreasing dissolved oxygen and increasing depth, possibly due to remineralization processes within the OMZ and with depth. We discuss how feeding modes and community composition might influence the observed patterns. This study elucidates the importance of the environmental context in shaping invertebrate trophic structure on continental margins and provides baseline knowledge that may be useful in regions where these minerals are targeted for extraction.

The Southern California Borderland is an active continental margin uniquely characterized by a network of deep basins interrupted by a series of banks, ridges, knolls, seamounts, and escarpments, which create diverse hard substrates (Emery 1960; Vedder et al. 1974). The area experiences tectonic movement, affecting

onshore and canyon erosion, influencing sediment transport and deposition from terrestrial sources into the ocean (Covault et al. 2011). In addition, the strong seasonal upwelling and high productivity lead to a well-developed oxygen minimum zone (OMZ) (Ulloa and Pantoja 2009), where mid-water oxygen minima ($[\text{O}_2] \leq 22 \mu\text{mol L}^{-1}$) intercept the continental margins (Levin 2003). The geology is characterized by numerous rock types, including phosphorites and ferromanganese (FeMn) crusts, minerals of deep-sea mining interest (Hein et al. 2016; Petersen et al. 2016). Phosphorites are common along continental margins. While present in the Southern California Borderland, phosphorite rocks are not actively forming (Loebner et al. 1987). Farther offshore and on the seamounts just beyond the California continental margin, the reduced sedimentation due to strong currents allows for the formation of FeMn crusts (Conrad et al. 2017). FeMn crusts, composed primarily of iron and manganese oxides, slowly precipitate from seawater at depths from 400 to 7000 m. They can concentrate cobalt, nickel, tellurium, titanium, platinum, and rare earth elements (Hein et al. 2000).

Both phosphorites and FeMn crusts are targeted for mining in various regions of the world ocean (Hein et al. 2000, 2016; Levin et al. 2016). Most studies of fauna on these substrates

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initially focused on megafauna (> 2 cm), but growing interest in deep-sea minerals has led to a flurry of work on smaller macrofaunal taxa (> 0.3 mm). These studies have demonstrated that marine minerals host diverse benthic communities and that the environmental setting of the marine minerals influences this community (see Schlacher et al. 2014; Morgan et al. 2015; Delavenne et al. 2019; Corrêa et al. 2022; Vlach 2022; Guraieb 2024). In addition, depth, organic matter input, dissolved oxygen concentration, upwelling events, temperature, and substrate type can create zonation of deep-sea benthic communities (Levin and Sibuet 2012).

As the ocean warms, the consequences of ocean deoxygenation driven by changes in gas solubility, ocean ventilation, and biological consumption (Levin 2018) are manifested as expanding OMZs (Stramma et al. 2008). The global ocean has lost more than 2% of its dissolved oxygen content (Schmidt et al. 2017), and the Southern California Borderland region has experienced even greater dissolved oxygen losses (Bograd et al. 2008). Animals exhibit different biological and physiological tolerances and limitations to dissolved oxygen concentration, and trophic linkages might be sensitive to such environmental changes (Gallo 2018).

The Southern California Borderland is an ideal site to assess the influence of low dissolved oxygen and substrate heterogeneity on the structure and functioning of hard-substrate invertebrate communities. Trophic interactions underlie ecosystem functioning, and changes in the trophic structure of macrofauna and megafauna represent a large and often overlooked aspect of environmental dynamics. Studies on the trophic structure of fauna associated with other marine minerals of potential economic interest, such as sulfides at hydrothermal vents and polymetallic nodules, have shown the importance of these substrates for the food-web integrity of associated fauna, for energy export to the surrounding deep sea (Salcedo et al. 2021; Stratmann et al. 2021) and for trophic flexibility of species (Portail et al. 2016, 2018). Moreover, benthic invertebrates are an important food source for demersal fisheries off California, playing a role in benthopelagic coupling in the region (Gallo 2018). Thus, baseline studies that inform on ecosystem functioning are valuable, particularly in the face of potential alterations from climate change or mineral resource extraction.

Stable isotope ratios have long been applied to evaluate the trophic structure of deep-sea smaller invertebrate fauna (e.g., Levin et al. 2000a; Iken et al. 2001; Demopoulos et al. 2010; Fanelli et al. 2011; Thurber 2014), enabled by natural variation in stable isotope ratios of different taxa at the base of the food chain and the fact that faunal signatures reflect their food sources (Layman et al. 2007). Carbon isotope fractionation varies substantially among primary producers (DeNiro and Epstein 1978): $\delta^{13}\text{C}$ values of estuarine and marine littoral sources (averaging -8‰ and -23‰ , respectively) are higher than terrestrial and riverine sources (averaging -30‰ and -26‰ , respectively), and, thus, high riverine inputs of

terrestrial organic matter may lower $\delta^{13}\text{C}$ values of organic matter on continental margins closer to shore (Simenstad and Wissmar 1985). Local N_2 fixation causes lower $\delta^{15}\text{N}$ values, and often $\delta^{15}\text{N}$ increases by 2–4‰ with each trophic level (Post 2002; McCutchan et al. 2003). In addition, as particulate organic matter sinks to the deep sea and through OMZs, microbial processes in the water column, including remineralization, denitrification, and anaerobic ammonium oxidation, can increase $\delta^{15}\text{N}$ (Mollier-Vogel et al. 2012). In the Southern California Borderland, upwelling supplies surface waters with organic matter enriched in ^{15}N (Mollier-Vogel et al. 2012). Close to shore or near sewer outfalls, wastewater is also enriched in ^{15}N (McClelland et al. 1997).

This study investigates trophic structure based on stable isotope analyses ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of macro- and megafaunal invertebrates associated with different types of hard substrates collected in the Southern Borderland California. To understand whether trophic structure changes with substrate type and if this response is modified by substrate type or instead due to depth, dissolved oxygen concentration, and proximity to shore, rocks were collected along a depth gradient passing through the OMZ at 6 inshore (< 100 km from shore) and 8 offshore (100–250 km from shore) sites. Here, trophic structure is examined using stable isotope means (± 1 standard error) and ranges to identify the source and variability of primary production at the base of the food chain and a number of trophic levels and community-wide metrics (Layman et al. 2007) to investigate trophic diversity (see *Methods* section). Specific questions addressed are: (1) Are there differences in invertebrate trophic structure with substrate type (phosphorites, FeMn crusts, basalt, and other rocks)? (2) How do (a) water depth, (b) dissolved oxygen concentration, and (c) proximity to shore (inshore vs. offshore) influence invertebrate trophic structure? (3) Do macro- and megafaunal invertebrates rely on the same food sources? Do they exhibit similar trends with substrate type, depth, dissolved oxygen, and proximity to the coast? And (4) Are trophic patterns identified in 1–3 consistent across taxa and feeding modes?

Methods

Sampling and sample processing

Fourteen sites off the Southern California Borderland (Fig. 1) spanning depths of ~ 120 –2400 m (Table 1) were visited by ROVs Hercules and Argus in November 2020 onboard the EV *Nautilus* (NA124) and by ROV SuBastian in July 2021 onboard the RV *Falkor* (FK210726). CTD casts were made over each site during the research cruises to collect temperature, salinity, and dissolved oxygen concentration data. Surface (0–5 m) and near-bottom (< 15 m above the bottom) water samples were collected with a CTD Rosette for stable isotope analyses of particulate organic matter (POM). About 2–4 L of seawater from each Niskin bottle sample were filtered through a GE Healthcare Whatman Grade 0.7 μm ,

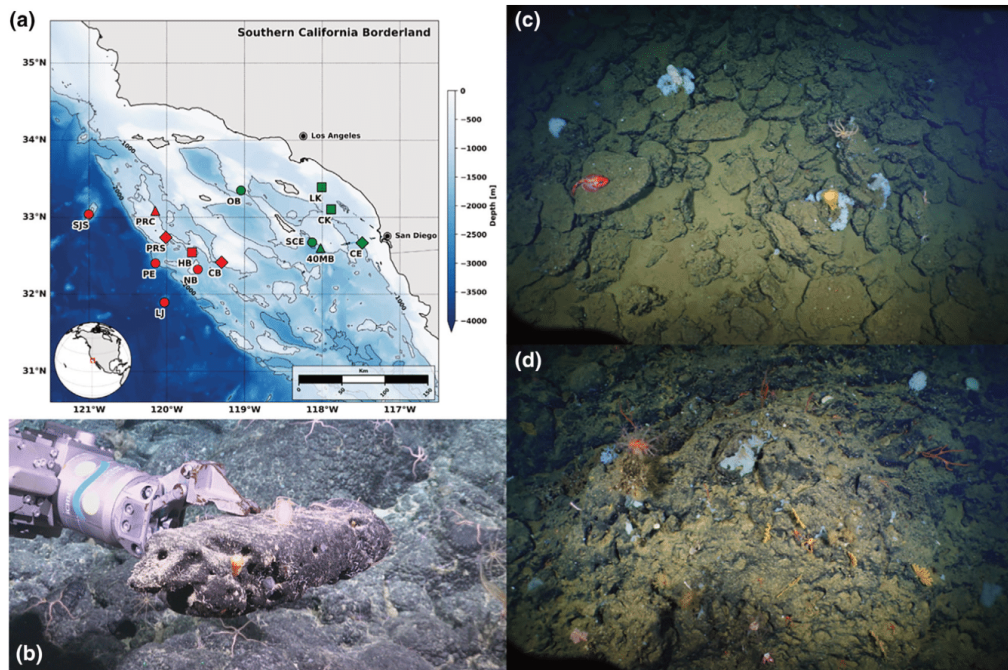


Fig. 1. (a) Location of the sampling sites off the Southern California Borderland. Offshore sites (100–250 km from shore, red): SJS: San Juan Seamount; PRC: Patton Ridge Central; PRS: Patton Ridge South; PE: Patton Escarpment, HB: Hancock Bank, NB: Northeast Bank; CB: Cortes Bank, LJ: Little Joe. Inshore sites (< 100 km from shore, green): OB: Osborn Bank; LK: Lasuen Knoll, CK: Crespi Knoll, SCE: San Clemente Escarpment; 40MB: 40-Mile Bank, CE: Coronado Escarpment. Symbols represent the main type of rock found at each site: FeMn (circle), phosphorites (diamond), basalt (square), and other rock (triangle). (b) FeMn encrusted rock with associated macrofauna being collected at Patton Escarpment. (c) Phosphorite deposits at Patton Ridge South. (d) FeMn crusts at San Juan Seamount.

47 mm diameter glass microfiber filter, and the filter with retained material was frozen at -20°C for subsequent stable isotopic analysis.

Samples were collected by the ROVs at different locations, encompassing a range of depths and dissolved oxygen concentrations (Table 1). The ROVs were equipped with SeaBird FastCAT 49 (NA124) and SBE49 (FK210726) CTDs, and depth, temperature, and dissolved oxygen concentration were registered for each sample collected. The distinction between macrofaunal and megafaunal invertebrates was based on size (macro: > 0.3 mm but < 2 cm, mega: > 2 cm). Rocks were collected for macro- and megafaunal sampling across the 14 sites and placed into separate biobox compartments to avoid cross-contamination of fauna. Once on deck, the rocks were photographed, the associated fauna on the rocks were handpicked, and the water in the recovery container was sieved through a $300\ \mu\text{m}$ sieve. Additional megafauna samples were collected using the ROV manipulator arm or the multi-chamber suction sampler. Both megafaunal and macrofaunal

individuals were identified to the lowest possible taxonomic level. On board ship tissue subsamples for stable isotope analyses ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) from three specimens of each macrofaunal morpho-species were washed in milli-Q water, placed in pre-weighed tin capsules or glass vials (pre-combusted at 500°C for 4 h) and frozen at -20°C (Levin and Mendoza 2007; Pereira et al. 2021). Megafaunal tissues were frozen in plastic bags at -20°C for further laboratory processing. Rock types were determined based on visual properties and x-ray diffraction, as described in Vlach (2022).

In the laboratory at Scripps Institution of Oceanography, tissue in glass vials and plastic bags was subsampled in pre-weighed tin capsules. All samples in tin capsules were then oven-dried at 60°C overnight, weighed, and double-boated for acidification with phosphoric acid. After acidification, samples were left in a desiccator overnight. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ measurements were made on 0.2–2.5 mg dry-weight samples combusted using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer at the Stable Isotope

Table 1. Site, proximity to shore, dive number, sampling depth range, and type and number of samples collected for macrofaunal and megafaunal isotope analyses in the Southern California Borderland in 2020 and 2021. Macrofaunal samples column indicates the type and number of rocks collected, and megafaunal samples column indicates the type of rock and number of megafaunal individuals sampled. Inshore sites were < 100 km far from shore, and offshore sites were between 100 and 250 km from shore. FeMn includes pure FeMn cobbles/crust and carbonate or basalt with FeMn patina/crust, and other rocks include granitic, mudstone, sandstone, sedimentary, siltstone, and barite. Locations are shown in Fig. 1.

Site	Proximity to shore	Dive number	Sampling depth range (m)	Macrofaunal samples	Megafaunal samples
40-Mile Bank	Inshore	H1845, S0446, S0447	657–1039	FeMn (1), basalt (5), and other rocks (4)	Other rocks (29)
Coronado Escarpment	Inshore	S0452	443–467	Phosphorites (2)	Phosphorite (5)
Cortes Bank	Offshore	H1843	436–529	Phosphorites (6)	Phosphorite (4)
Crespi Knoll	Inshore	S0448	443–525	Basalt (5), other rocks (1)	Basalt (28)
Hancock Bank	Offshore	S0440	317–595	Phosphorite (1), basalt (6)	Basalt (14)
Lasuen Knoll	Inshore	S0449	120–452	Other rocks (5)	Basalt (10)
Little Joe Seamount	Offshore	S0445	2368–2377	FeMn (4)	FeMn (11)
Northeast Bank	Offshore	H1842	552–1132	FeMn (2), basalt (1)	FeMn (15)
Osborn Bank	Inshore	H1847	396	Basalt (1)	FeMn (5)
Patton Escarpment	Offshore	S0444	1453–1701	FeMn (7)	FeMn (14)
Patton Ridge Central	Offshore	H1840	686–821	Phosphorite (1), basalt (1), other rocks (3)	Other rocks (6)
Patton Ridge South	Offshore	H1844	562–726	Phosphorite (7)	Phosphorite (7)
San Clemente Escarpment	Inshore	H1846	1189–1718	FeMn (4)	FeMn (5)
San Juan Seamount	Offshore	H1841, S0441, S0442, S0443	689–1443	FeMn (13), basalt (7)	FeMn (29)

Facility at the University of California, Davis (SIF-UCD). The glass microfiber filters were scraped into tin capsules and sent to SIF-UCD for POM stable isotope analyses. The long-term standard deviation for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses conducted by SIF-UCD are 0.2‰ and 0.3‰, respectively.

Data analyses

We divided our POM and invertebrate samples into three categories according to depth and dissolved oxygen concentration based on CTD profiles (see Results below): above OMZ (< 400 m and $[\text{O}_2] > 22 \mu\text{mol L}^{-1}$), within OMZ (400–1100 m and $[\text{O}_2] \leq 22 \mu\text{mol L}^{-1}$), and below OMZ (> 1100 m and $[\text{O}_2] > 22 \mu\text{mol L}^{-1}$). We refer to these as OMZ categories for simplicity, but they represent an interaction between depth and dissolved oxygen. We calculated the average isotopic composition of macro- and megafaunal invertebrates for each taxon within a sample before calculating the averages by site, rock type, proximity to shore, and OMZ category to avoid sampling bias.

Statistical tests were performed to check for variability in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of POM between site, proximity to shore, and OMZ category. The data were not normally distributed, and the variance was not homogeneous even after transformation, and, thus, we used Wilcoxon tests when comparing between inshore vs. offshore, and Kruskal–Wallis tests followed by post hoc Dunn's tests using the Benjamini–Hochberg adjustment

(Benjamini and Hochberg 1995) when comparing among sites and OMZ categories. $\text{POM}_{\text{aboveOMZ}}$, $\text{POM}_{\text{withinOMZ}}$ and $\text{POM}_{\text{belowOMZ}}$ all are near-bottom samples (< 15 m above the bottom), different from $\text{POM}_{\text{surface}}$ (0 m).

Generalized additive models (GAMs) were used to analyze the relationship between macro- and megafaunal isotopic values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and environmental variables (site, depth, dissolved oxygen, rock type, and proximity to shore) using the R package “mgcv.” The same model was applied initially, including a tensor product interaction term (ti) between depth and dissolved oxygen:

$$\delta^{13}\text{C} = s(\text{Depth}) + s(\text{Oxygen}) + \text{ti}(\text{Depth}, \text{Oxygen}) + (\text{Proximity to shore}) + (\text{Rock type}) + (\text{Site}),$$

$$\delta^{15}\text{N} = s(\text{Depth}) + s(\text{Oxygen}) + \text{ti}(\text{Depth}, \text{Oxygen}) + (\text{Proximity to shore}) + (\text{Rock type}) + (\text{Site}).$$

A backward model selection procedure was used to identify the best model, following Gallo et al. (2020) and Richards et al. (2023). Models were assessed using the Akaike information criterion (AIC) (Akaike 1974), and through percent variance explained (R^2 adj) and generalized cross-validation (GCV) when AIC values were similar. Variables found to be non-significant ($p > 0.05$) were removed when model performance (lower AIC) was improved following removal but retained when model performance did not improve. The normality of

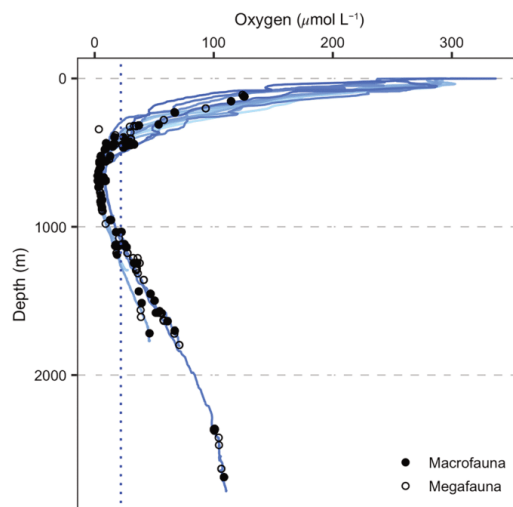


Fig. 2. Dissolved oxygen ($\mu\text{mol O}_2 \text{L}^{-1}$) vertical profile (blue lines) from CTD casts in the Southern California Borderland. Macrofauna (closed circles) and megafauna (open circles) collections depths are shown relative to dissolved oxygen concentration. Sampling occurred in October–November 2020 with ROV Hercules and July 2021 with ROV SuBastian. Dashed line represents the oxygen minimum zone threshold of $[\text{O}_2] = 22 \mu\text{mol L}^{-1}$.

the models was assessed visually using *qqplots*. Plots of the final models were visualized with the R package “visreg.” Generalized linear models were also plotted for each taxon to

check for taxon-specific responses to depth and dissolved oxygen gradients.

PERMANOVA tests were conducted to analyze whether there was a difference in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between macro- and megafauna, among taxa, and among feeding modes. We found a significant effect (PERMANOVA results reported below)—hence, the generalized additive models described above were conducted separately for macro- and megafauna. Wilcoxon tests and Kruskal–Wallis tests followed by post hoc Dunn’s tests using the Benjamini–Hochberg adjustment (Benjamini and Hochberg 1995) were conducted to examine how trophic structure of each taxon and feeding mode changes with rock type, proximity to shore, and OMZ categories. These results were used to discuss whether generalized additive model results were due to differences in faunal composition.

Community-level metrics (Layman et al. 2007) were calculated using Stable Isotope Bayesian Ellipses in R (SIBER) package (Jackson et al. 2011), following Pereira et al. (2021, 2022), to examine the trophic structure of the megafaunal and macrofaunal communities on different rock types, proximity to shore, and OMZ categories. Source and variability of primary production at the base of the food chain was identified through $\delta^{13}\text{C}$ values and ranges. Source of nitrogen and number of trophic levels were identified based on $\delta^{15}\text{N}$ values and ranges. Relative isotopic niche areas in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ biplots were determined through standard elliptical areas corrected for sample size. Trophic diversity was calculated as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ total hull areas in this same bivariate space, and species packing was determined through measurement of the mean

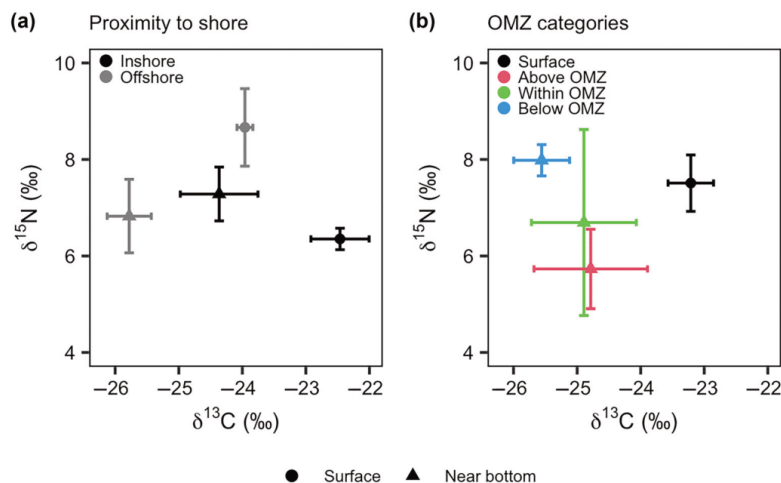


Fig. 3. Dual isotope plots ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ average ± 1 standard error) of surface (circles) and near-bottom (triangles) particulate organic carbon off the Southern California Borderland. Data are presented with respect to (a) proximity to shore and (b) near-bottom dissolved oxygen concentrations. Off-shore sites were $> 100 \text{ km}$ away from the coastline. Oxygen minimum zone categories were defined with an OMZ threshold of $[\text{O}_2] < 22 \mu\text{mol L}^{-1}$ and water depth. Data are available in Supporting Information Table S1.

distance to the centroid and mean nearest neighbor distance. Pairwise ellipse overlaps based on Bayesian posterior estimates were calculated using 2 chains of 20,000 iterations with a burn-in of 1000 and thinning of 10 (Pereira et al. 2022) to examine whether animals are utilizing the same food sources on different rock types, proximity to shore, and OMZ categories.

Results

Environmental context and particulate organic carbon

Using a dissolved oxygen concentration threshold of $[O_2] = 22 \mu\text{mol L}^{-1}$, the OMZ in the Southern California Borderland is at approximately 400–1100 m, with a variability of ± 100 m across the 14 sites profiled with CTD casts (Fig. 2).

The isotopic signature of particulate organic matter (POM) was examined across sites, proximity to shore, and OMZ categories (Fig. 3). There was no significant difference across sites (Kruskal–Wallis, $p > 0.05$ for all tests), and, thus, we did not

differentiate POM samples by site in our analyses. In relation to proximity to shore, there was no significant difference in $\delta^{13}\text{C}$ of POM surface inshore ($-22.5\text{‰} \pm 0.4\text{‰}$) and > 100 km offshore ($-23.9\text{‰} \pm 0.1\text{‰}$) ($W = 1$, $p = 0.06$), but $\delta^{15}\text{N}$ was higher offshore ($8.7\text{‰} \pm 0.8\text{‰}$) than inshore ($6.3\text{‰} \pm 0.2\text{‰}$) ($W = 16$, $p = 0.03$). Near-bottom PON $\delta^{13}\text{C}$ was slightly higher inshore ($-24.4\text{‰} \pm 0.6\text{‰}$) than offshore ($-25.8\text{‰} \pm 0.3\text{‰}$) ($W = 5$, $p = 0.048$), but there was no significant difference in $\delta^{15}\text{N}$ (inshore: $7.3\text{‰} \pm 0.5\text{‰}$, offshore: $6.8\text{‰} \pm 0.8\text{‰}$) ($W = 16$, $p = 0.88$). In a comparison of OMZ categories, $\delta^{13}\text{C}$ POM_{surface} ($-23.2\text{‰} \pm 0.4\text{‰}$) was significantly higher than POM_{belowOMZ} near-bottom ($-25.6\text{‰} \pm 0.9\text{‰}$) ($z = -3.0125$, $p = 0.02$).

Invertebrate trophic patterns with environmental variables

A total of 572 individuals (400 macrofauna, 172 megafauna) were sampled from 166 invertebrate species (96 macrofauna, 44 megafauna, 26 macro- and megafauna species). Macrofaunal $\delta^{13}\text{C}$ ranged from -21.8‰ (brittle star) to -10.4‰ (hydroid

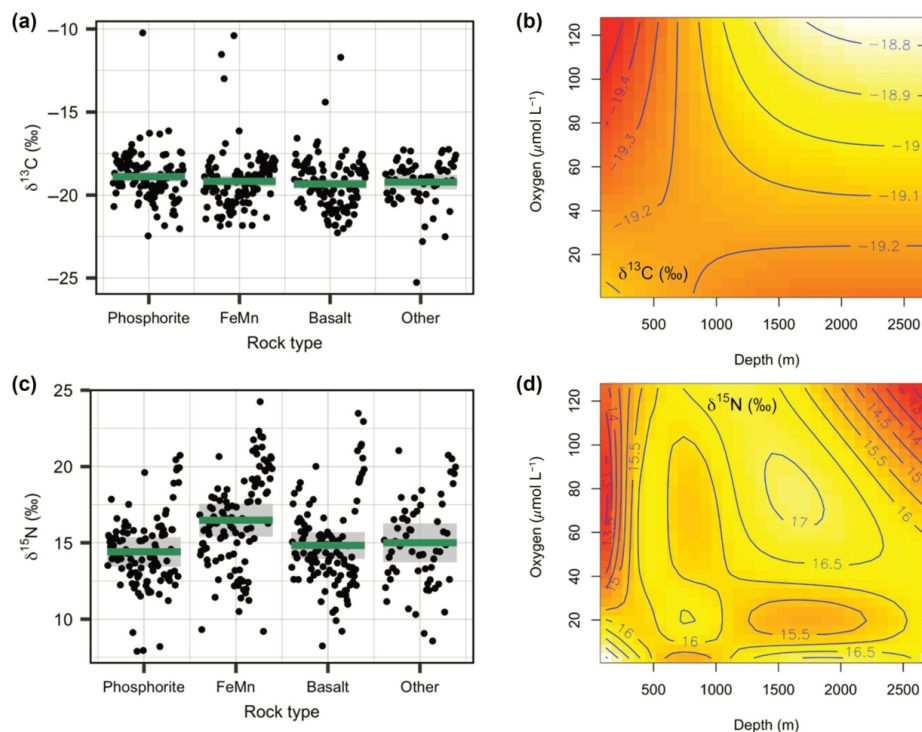


Fig. 4. Generalized additive model (GAM) results for (a, b) $\delta^{13}\text{C}$ and (c, d) $\delta^{15}\text{N}$ of macrofaunal invertebrates associated with hard substrates off the Southern California Borderland relative to rock type and the interaction between depth and dissolved oxygen. Trend lines represent best fit lines for GAMs, and gray bands represent 95% confidence intervals. Order of subplots reflects the relative importance of the variables in the model (i.e., a and c subplots show the most important variables in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ models, respectively).

Stylaster sp.) with an average of $-19.1\text{‰} \pm 0.1\text{‰}$, and $\delta^{15}\text{N}$ ranged from 7.0‰ (brittle star) to 23.1‰ (sponge) with an average of $15.1\text{‰} \pm 0.3\text{‰}$. Megafaunal $\delta^{13}\text{C}$ ranged from -21.8‰ (galatheid crab) to -10.2‰ (brittle star *Ophiacantha diplasia*) with an average of $-18.7\text{‰} \pm 0.2\text{‰}$, and $\delta^{15}\text{N}$ ranged from 9.5‰ (holothurian) to 23.0‰ (tunicate) with an average of $15.3\text{‰} \pm 0.3\text{‰}$. In general, the sampled macrofaunal communities showed a higher variety of feeding modes with more detritivores, deposit feeders, and grazers (36% of total number of individuals) than sampled megafaunal communities, which were dominated by filter feeders and suspension feeders (61% of total number of individuals). Annelids and echinoderms accounted for 53% of the macrofaunal individuals, whereas cnidarians and poriferans dominated the megafaunal individuals (62%) (see Supporting Information Figs. S1, S2).

Results of the generalized additive model of $\delta^{13}\text{C}$ values for macrofauna yielded a best-fit model, which included rock type and the interaction between depth and dissolved oxygen (GCV = 2.572, $R^2 = 0.008$, deviance explained = 1.95%,

AIC = 1465.8), although the latter was not significant. Macrofaunal $\delta^{13}\text{C}$ was the lowest on basalt ($t = -2.032$, $p = 0.04$) (Fig. 4a,b). The best-fit model of $\delta^{15}\text{N}$ values for macrofauna also included rock type and the interaction between depth and dissolved oxygen (GCV = 8.2490, $R^2 = 0.117$, deviance explained = 13.8%, AIC = 1916.6). Macrofaunal $\delta^{15}\text{N}$ was the highest on FeMn rocks ($t = 3.968$, $p < 0.0001$) and increased with increasing depth and decreasing dissolved oxygen concentration ($F = 2.103$, $p = 0.03$) (Fig. 4c,d).

The best-fit model of $\delta^{13}\text{C}$ for megafauna included proximity to shore and the interaction between depth and dissolved oxygen (GCV = 3.792, $R^2 = 0.075$, deviance explained = 11.8%, AIC = 652.2), although the latter one was not significant. Megafaunal $\delta^{13}\text{C}$ was lower offshore than inshore ($t = -2.199$, $p = 0.03$) (Fig. 5a,b). The best-fit model of $\delta^{15}\text{N}$ for megafauna included dissolved oxygen concentration and depth (not as an interaction term) (GCV = 7.878, $R^2 = 0.024$, deviance explained = 3.71%, AIC = 766.6). Megafaunal $\delta^{15}\text{N}$ increased with decreasing dissolved oxygen

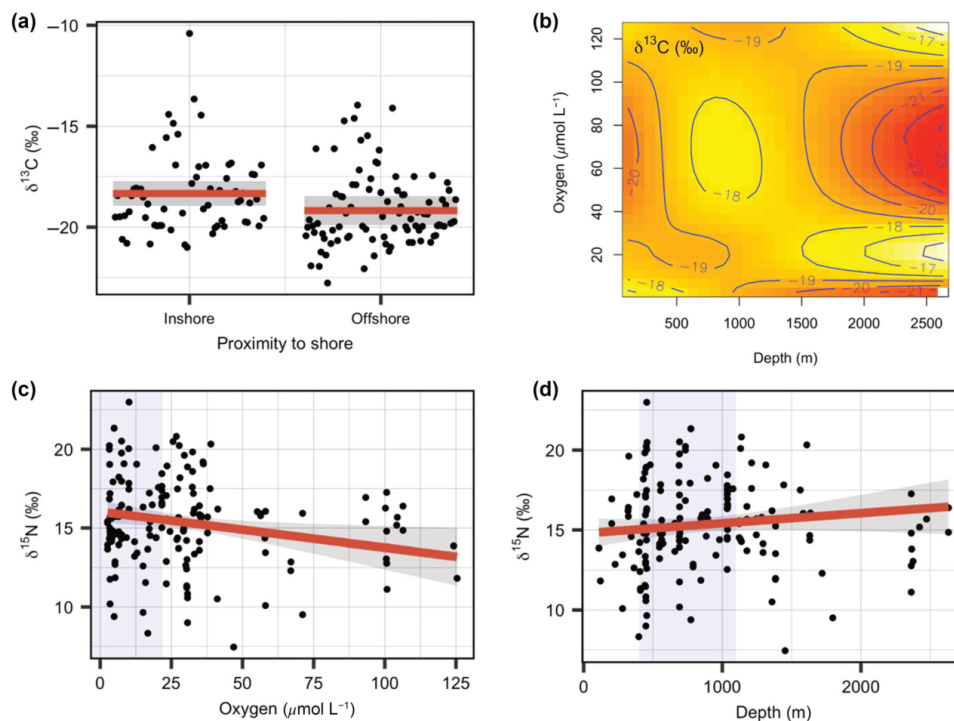


Fig. 5. Generalized additive model (GAMs) results for (a, b) $\delta^{13}\text{C}$ and (c, d) $\delta^{15}\text{N}$ of megafaunal invertebrates associated with hard substrates off the Southern California Borderland relative to proximity to shore, depth, dissolved oxygen concentration, and the interaction between depth and dissolved oxygen. Trend lines represent best fit lines for GAMs, and gray bands represent 95% confidence intervals. Order of subplots reflects the relative importance of the variables in the model (i.e., a and c subplots show the most important variables in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ models, respectively).

($F = 5.887$, $p = 0.02$) and depth was not significant ($F = 1.780$, $p = 0.18$) (Fig. 5c,d).

In general, macrofaunal $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were, respectively, 0.4‰ and 0.2‰ lower than megafaunal values ($\delta^{13}\text{C}$: $R^2 = 0.030$, $p = 0.0002$; $\delta^{15}\text{N}$: $R^2 = 0.021$, $p < 0.0001$); however, these values are close to the standard deviation of the analytical precision. There was no interaction between taxa and feeding modes for both $\delta^{13}\text{C}$ ($R^2 = 0.030$, $p = 0.05$) and $\delta^{15}\text{N}$ ($R^2 = 0.006$, $p = 0.52$). Cnidarians and annelids exhibited the lowest $\delta^{13}\text{C}$ for macro- and megafauna, respectively, and bryozoans and echinoderms the highest

$\delta^{13}\text{C}$ for macro- and megafauna, respectively ($R^2 = 0.041$, $p = 0.001$). Among feeding modes, suspension feeders and detritivores exhibited the lowest $\delta^{13}\text{C}$ for macro- and megafauna, respectively, and deposit feeders and grazers the highest $\delta^{13}\text{C}$ for macro- and megafauna, respectively ($R^2 = 0.044$, $p = 0.004$). Bryozoans and echinoderms exhibited the lowest $\delta^{15}\text{N}$ for macro- and megafauna, respectively, and, among feeding modes, deposit feeders exhibited the lowest $\delta^{15}\text{N}$ for both macro- and megafauna (among taxa: $R^2 = 0.327$, $p < 0.0001$; among feeding modes: $R^2 = 0.085$, $p < 0.0001$) (Fig. 6; Supporting Information

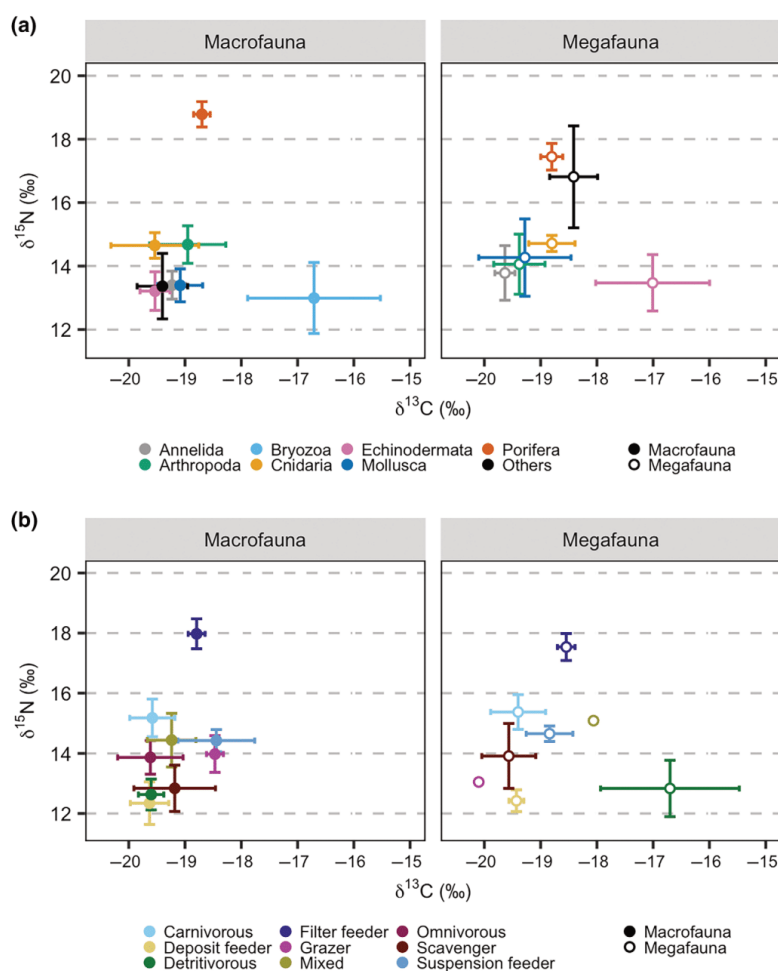


Fig. 6. Dual isotope plots ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ average ± 1 standard error) of macro- and megafaunal invertebrates on mineral-rich rocks off the Southern California Borderland by (a) taxa and (b) feeding modes. Data are available in Supporting Information Table S3.

Table S3). Poriferans and filter feeders showed the highest $\delta^{15}\text{N}$ for both macro- and megafauna. Values were surprisingly high for all different sponge classes with average $\delta^{15}\text{N}$ ranging from 14.7–19.1‰ (Supporting Information Fig. S3; Supporting Information Table S4), but there was no significant difference between the classes Demospongiae, Hexactinellida, and unknown sponges (pairwise Dunn's tests, $p > 0.05$).

Within each taxon, only the following isotopic values were significantly different across rock type, proximity to shore, and OMZ categories: macrofaunal annelid $\delta^{15}\text{N}$ was higher offshore ($14.3\text{‰} \pm 0.3\text{‰}$) than inshore ($13.1\text{‰} \pm 0.5\text{‰}$) ($W = 180$, $p = 0.04$); macrofaunal arthropod $\delta^{15}\text{N}$ was higher offshore ($15.9\text{‰} \pm 1.0\text{‰}$) than inshore ($13.4\text{‰} \pm 0.4\text{‰}$) ($W = 58$, $p = 0.02$); macrofaunal echinoderm $\delta^{15}\text{N}$ was higher on FeMn ($14.0\text{‰} \pm 0.8\text{‰}$) and phosphorite ($13.1\text{‰} \pm 0.9\text{‰}$) than on other rocks ($11.2\text{‰} \pm 0.2\text{‰}$) ($z = 3.0817$, $p = 0.01$ and $z = -2.8801$, $p = 0.01$, respectively); and macrofaunal poriferan $\delta^{15}\text{N}$ was higher on phosphorite ($18.7\text{‰} \pm 0.8\text{‰}$) than on basalt ($17.0\text{‰} \pm 0.5\text{‰}$) ($z = 2.9130$, $p = 0.02$) (Supporting Information Figs. S4–S6).

Invertebrate trophic diversity

The macrofaunal and megafaunal communities on phosphorites and FeMn, respectively, exhibited the lowest trophic diversity (SEAc) among all rock types. Total niche space (TA) was the highest on FeMn crusts for macrofauna and on phosphorites for megafauna (Fig. 7a,d; Supporting Information Tables S5, S6). The lowest $\delta^{13}\text{C}$ range was observed on phosphorites for macrofauna and on FeMn for megafauna, but $\delta^{15}\text{N}$ range was similar among rock types (Supporting Information Tables S5, S6). Offshore macrofaunal communities had higher trophic diversity (TA, SEAc) and lower trophic redundancy (CD, MNND) than inshore communities (Fig. 7b; Supporting Information Table S5), but the opposite trend was observed for megafaunal communities (Fig. 7e; Supporting Information Table S6). Regarding OMZ categories, macro- and megafauna showed the highest $\delta^{13}\text{C}$ range and highest trophic diversity (TA, SEAc) below the OMZ and above the OMZ, respectively, but both macro- and megafauna showed the most variable food sources ($\delta^{15}\text{N}$) within the OMZ (Fig. 7c,f; Supporting information Tables S5, S6). In general, SEAc were statistically different across rock types, proximity to shore, and OMZ categories (Kruskal–Wallis tests, $p < 0.05$).

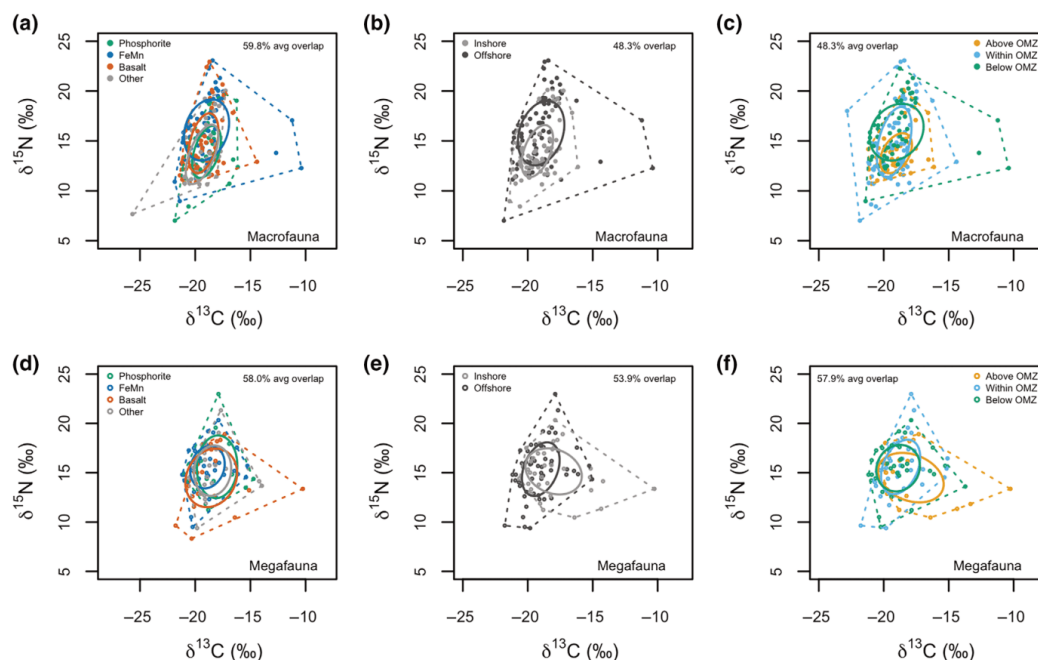


Fig. 7. Trophic niche diversity of mega- and macrofaunal invertebrates on mineral-rich rocks off the Southern California Borderland by (a, d) rock type, (b, e) proximity to shore, and (c, f) oxygen minimum zone (OMZ) categories. Each point represents the average for one species. Circles represent standard ellipse areas corrected by size (SEAc). Dashed lines represent the total niche area (TA). Data available in Supporting Information Tables S4, S5.

Table 2. Bayesian overlap (%) of standard ellipse areas (SEAc) of invertebrate macro- (below the diagonal) and megafaunal (above the diagonal) invertebrates on mineral-rich rocks off the Southern California Borderland by rock type, proximity to shore, and oxygen minimum zone (OMZ) categories. Values on the diagonal in bold represent overlap between macrofauna and megafauna.

SEAc Bayesian overlap (%)				
Rock type				
	Phosphorite	Ferromanganese	Basalt	Other rocks
Phosphorite	43.8	51.7	60.4	61.8
Ferromanganese	44.9	54.5	48.0	66.8
Basalt	68.6	56.0	50.7	59.3
Other rocks	70.9	47.7	70.7	54.0
Proximity to shore				
	Inshore	Offshore		
Inshore	40.5	53.9		
Offshore	48.3	66.5		
Oxygen minimum zone categories				
	Above OMZ	Within OMZ	Below OMZ	
Above OMZ	33.3	48.1	54.9	
Within OMZ	50.0	68.6	70.8	
Below OMZ	37.6	57.3	66.3	

(Supporting Information Fig. S7), and isotopic overlap was low, ranging from 44.9–70.9% (Table 2). Isotopic overlap between macro- and megafauna by rock type, proximity to shore, and OMZ categories was on average 50.8%, 53.5%, and 56.3%, respectively (Table 2).

Discussion

We analyzed $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of macro- and megafaunal invertebrates associated with hard substrates off the Southern California Borderland across environmental gradients (depth, dissolved oxygen concentration, and proximity to shore). Macro- and megafauna exhibited different trophic structures that reflect community composition and feeding modes. The communities sampled for stable isotope analyses reflect the composition of macrofaunal invertebrate communities in the Southern California Borderland based on data from Guraieb (2024), which are dominated by annelids and echinoderms (sample = 53%, entire community = 61%). The sampled megafaunal community was somewhat distinct to the composition of megafaunal invertebrate communities in the region based on data from Vlach (2022), which is dominated by echinoderms (48%), but the sample was dominated by cnidarians and poriferans (sample = 62%, entire community = 37%). The dominant feeding modes were macrofaunal detritivores, deposit feeders, and grazers, with low $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, and megafaunal filter feeders and

suspension feeders, with the highest $\delta^{15}\text{N}$ values. Suspension and filter feeders (sponges and gorgonians) also dominate megafauna on seamounts in the Azores with the highest trophic position values in sponges (3–5), close to that of top predators (Colaço et al. 2013). Sponges might feed on more recycled and degraded POM, yielding higher $\delta^{15}\text{N}$ values (Iken et al. 2001; Fanelli et al. 2011). Deposit feeders, however, might feed on POM that has been further recycled in the sediment, yielding even higher $\delta^{15}\text{N}$ values than filter feeders (Fanelli et al. 2013). This was not what we observed; deposit feeders had $\delta^{15}\text{N}$ values ~5–6‰ lower than filter feeders. A previous study reported the same pattern on soft bottoms in the deep Arctic (1080 m), where deposit-feeding annelids showed $\delta^{15}\text{N}$ values 6–8‰ lower than sponges (Gontikaki et al. 2011). Sponges might then be feeding on resuspended material that is more highly degraded from processes occurring on the seafloor (Parzanini et al. 2018). Carnivory and symbiotic bacteria could also explain their higher $\delta^{15}\text{N}$ (Bergmann et al. 2009). Shallow-water sponges have been reported to assimilate symbiont-derived N from symbiotic cyanobacteria, driving an enrichment in $\delta^{15}\text{N}$ of sponge cells (Freeman and Thacker 2011). Interestingly, a study in the Arctic Mid-Ocean Ridge reported that deep-sea sponges with low microbial abundances showed higher $\delta^{15}\text{N}$ values (14.6–19.4‰, comparable to our values) than sponges with high microbial abundances ($\delta^{15}\text{N}$ = ~10‰) and suggested that low microbial abundance sponges recycle nitrogen internally within the sponge tissue in the sponge microbiome through an as yet unknown pathway (Hanz et al. 2022).

Invertebrate trophic patterns with substrate type

Trophic structure based on community metrics was also dependent on substrate type for both macro- and megafaunal invertebrates. However, macrofaunal isotopic composition was more correlated with substrate type than megafauna, possibly due to closer association of substrate and feeding modes; phosphorites and FeMn crusts might support different macrofaunal niches as suggested by the low (44–51%) niche overlap between them. Phosphorites are the rocks with highest rugosity (personal observation), possibly allowing for higher POM accumulation, and we find a more specialized macrofaunal community, with more grazers scraping organic matter off the substrate. Animals might also be grazing on the bacterial community on the substrates, which seems to be more homogeneous on phosphorites than on FeMn crusts (J. Gutleben, pers. oral comm.). Macrofaunal trophic diversity based on total niche area and standard ellipse area was also the lowest on phosphorites, suggesting that the macrofaunal species on phosphorites might be feeding on the same food sources or at least on fewer food sources than on the other substrates, supporting the idea of a niche specialization on phosphorites.

FeMn crusts, in contrast, showed highest total niche area for both macro- and megafaunal invertebrates. Interestingly,

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the macrofaunal community sampled on FeMn crusts is distinct from the communities on the other rock types, with FeMn hosting more filter feeders (mainly poriferans) and carnivores (mainly cnidarians) at higher trophic levels, more similar to the megafaunal community. Even though these results are from individuals sampled for stable isotope analysis, they reflect the trends in the entire community. Macrofaunal communities on FeMn in the Southern California Borderland showed the greatest number of unique taxa compared to phosphorites, basalt, and sedimentary rocks and are more diverse than phosphorites (Guraieb 2024). Notably, FeMn crusts greater than 1 mm thick and phosphorites were never collected from the same location in our study and therefore the unique and complex environmental setting at each location where crusts or phosphorites were collected may be influencing fauna and isotopic composition just as much or more than the substrate composition itself. Most of the FeMn crusts were sampled from the two seamounts (Little Joe and San Juan seamounts), where environmental conditions (i.e., depth, light levels, productivity of the overlying water, hydrodynamic regime, deposition dynamics) can determine invertebrate communities and even favor specific taxa, such as corals and other filter feeders that take advantage of the faster currents (see review by Clark et al. 2010). Moreover, thicker FeMn crusts are typically found deeper in the Southern California Borderland (Supporting Information Table S7). Changes in basal or baseline shifts in isotope values due to environmental conditions (e.g., eutrophication and input of terrestrial/riverine POM) can be reflected in isotope niche and isotope overlap among rock type, rather than reflecting changes in consumer behavior (Fry and Davis 2015). Indeed, we observed that depth, dissolved oxygen, and proximity to shore are important factors influencing isotope values (see discussion below).

Invertebrate trophic patterns with depth and dissolved oxygen concentration

The increased $\delta^{15}\text{N}$ with increasing depth was an expected result due to remineralization of organic matter as it falls through the water column, as well as the higher $\delta^{15}\text{N}$ with decreasing dissolved oxygen concentration due to denitrification and anaerobic ammonium oxidation (Mollier-Vogel et al. 2012). These trends have been reported for crustaceans and fish on the continental slope off Spain (Fanelli et al. 2013), corals and sponges on seamounts in the Azores (Colaço et al. 2013), deep-pelagic micronekton in the Gulf of Mexico (Richards et al. 2023), and for demersal fish on the southern California slope (Gallo 2018). There are strong combined effects of dissolved oxygen and depth in shaping isotope values, as indicated by the significant interaction term between depth and dissolved oxygen in the generalized additive models. This interaction explained more of the isotopic variance than the individual variables alone (except for megafaunal $\delta^{15}\text{N}$), possibly due to the presence of the OMZ at

intermediate depths. This combined effect might also explain the trophic structure of macrofauna within the OMZ, where there were the most variable N sources but lowest species spacing. Low dissolved oxygen conditions tend to homogenize communities by favoring more adapted species (Levin et al. 2000b). Below the OMZ, the higher macrofaunal position is likely due to reliance on isotopically enriched food sources at depths (Richards et al. 2023), where POM $\delta^{15}\text{N}$ was higher. In addition, the higher macrofaunal trophic diversity below the OMZ might be an edge effect of the OMZ, where more biologically available organic matter accumulates at the lower boundary of the OMZ due to microbial remineralization within the OMZ (Mullins et al. 1985). For megafauna, however, trophic diversity was higher above the OMZ, and, perhaps, their trophic structure is more sensitive to low dissolved oxygen conditions. Effects of dissolved oxygen have been reported for megafaunal community composition in the Southern California Borderland, with more homogenized communities found under low dissolved oxygen conditions (Vlach 2022), and on FeMn deposits in the Southwest Atlantic (Corrêa et al. 2022).

Invertebrate trophic patterns with proximity to shore

Macrofaunal trophic diversity based on community metrics was higher offshore than inshore, possibly due to more diverse taxa, and, thus, more variability in feeding modes, that were sampled offshore. Megafauna, in contrast, were dominated by filter-feeding poriferans and carnivorous cnidarians and showed lower trophic complexity offshore than inshore. At offshore sites, animals might depend more exclusively on phytoplankton production in the form of marine snow (Polunin et al. 2001) vs. inshore, where there are other sources of photosynthetic primary production with higher $\delta^{13}\text{C}$ (e.g., algae, kelp, seagrass, marsh grasses, etc.). The higher megafaunal $\delta^{13}\text{C}$ values inshore might indicate that they are indeed feeding on sources derived from this other production with higher $\delta^{13}\text{C}$ than phytoplankton. We expected to see the opposite (lower invertebrate $\delta^{13}\text{C}$ at inshore sites) due to riverine inputs of terrestrial organic matter that could affect initial $\delta^{13}\text{C}$ values (Simenstad and Wissmar 1985). In addition, nearshore phytoplanktonic communities in the region are dominated by diatoms, with lower $\delta^{13}\text{C}$ and higher $\delta^{15}\text{N}$ values, than offshore communities that are dominated by cyanobacteria (James et al. 2022), with higher but more variable $\delta^{13}\text{C}$ and lower $\delta^{15}\text{N}$ (Vuorio et al. 2006; Wada et al. 2012). Even though POM $\delta^{13}\text{C}$ was higher inshore ($-24.4\text{‰} \pm 0.6\text{‰}$) than offshore ($-25.8\text{‰} \pm 0.3\text{‰}$) in our study, it was still 6‰ lower than megafaunal $\delta^{13}\text{C}$ ($-18.0\text{‰} \pm 0.4\text{‰}$), supporting our hypothesis that animals inshore might be feeding directly on algae, kelp, seagrasses, etc., with higher $\delta^{13}\text{C}$ values (-18‰ to -14‰ ; see von Biela et al. 2016; Velázquez-Ochoa et al. 2022), and to a lesser extent on POM since $\delta^{13}\text{C}$ shifts 0–1‰ with trophic level (DeNiro and Epstein 1978). Moreover, even though we observed higher initial values of POM $\delta^{15}\text{N}$

inshore, possibly due to upwelling and wastewater input (McClelland et al. 1997; Mollier-Vogel et al. 2012), there was no difference in invertebrate $\delta^{15}\text{N}$ with proximity to shore. A previous study comparing deep-sea fish and crustaceans off Spain also observed higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values on the continental slope (inshore), where terrestrial material and algae remains sink to the seafloor, than on the insular slope (offshore), characterized by oceanic conditions where animals depend mainly on marine snow (Fanelli et al. 2013). Trophic diversity based on total niche area and SEAc was higher on the “offshore,” insular slope, and the food chain was shorter (Fanelli et al. 2013), as we also observed in the Southern California Borderland. Shorter food chains may compensate for the lower productivity that happens offshore (see James et al. 2022).

Implications for future ecosystem disturbance

One of the main challenges for deep-sea management is the lack of data, making it hard to define ecological thresholds for precautionary approaches to preserve the marine environment and, most importantly, to detect changes that might result from disturbances such as climate change or marine mining operations. Previous studies have reported the importance of hard substrates for invertebrate communities on marine minerals and other hardgrounds (Corrêa et al. 2022), but mainly for megafauna, and with a focus on community composition and little attention to trophic structure across substrates. Alteration of trophic linkages has been described as a potential impact of deep-seabed mining (Levin et al. 2016). For example, the removal of nodules could have major consequences for invertebrate food-web integrity such as reduced food-web complexity and biodiversity loss (Stratmann et al. 2021). In addition, mining activities will likely increase the sediment loads and sedimentation rates that might affect feeding modes of hardground fauna and the quality of the organic material that sinks to the seafloor (Monserrat et al. 2019). Here, we not only provide novel baseline trophic data for invertebrate communities associated with FeMn crusts and phosphorites, but we also show the importance of substrate in shaping these trophic linkages and feeding modes. Although there is no current mining interest in the Southern California Borderland, our study elucidates the usefulness of baseline trophic data for management and conservation efforts in areas where these minerals are being actively targeted for marine mineral extraction. For example, exploration contracts were approved by the International Seabed Authority for FeMn crusts in the Prime Crust Zone of the western Pacific and on the Rio Grande Rise in the southwestern Atlantic (Petersen et al. 2016). Zones off Namibia, New Zealand, and Mexico were targeted for phosphorite mining (Hein et al. 2016), although exploitation contracts were not approved in the latter two countries. In addition, we expect to see cumulative effects from deoxygenation, which is predicted to progress with continued climate change

(Schmidtke et al. 2017), as dissolved oxygen is another important variable shaping macro- and megafaunal trophic structure.

Our study demonstrates that environmental context (substrate type, depth, dissolved oxygen, and proximity to shore) shapes macro- and megafaunal invertebrate trophic structure on continental margins. FeMn crust and phosphorite substrates in the Southern California Borderland support enhanced trophic biodiversity and specific trophic niches compared with other rock types (basalt, sedimentary), which can inform decision-making regarding environmental thresholds for trophic disturbance during deep-seabed mining activities. We also observed significant differences between macro- and megafaunal trophic structures, showcasing that we cannot generalize trophic patterns across size classes, and that changes in body size might dictate trophic relationships. Although macrofauna has received less attention in management discussions and plans in areas where these substrates are being targeted for mineral exploration, our data suggest that macrofauna could exhibit more specific associations to substrate type than megafauna. Much remains to be uncovered about macrofaunal diets on marine mineral hardgrounds and their possible reliance on microbes specific to marine mineral substrates. Finally, environmental conditions (depth, dissolved oxygen, proximity to shore) create complex and heterogeneous trophic relationships that can interact with substrate reliance.

Data availability statement

Data are available on request from authors.

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Conflict of Interest

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Table S1: Mean $\pm$ 1 standard error carbon and nitrogen stable isotope values (‰) of particulate organic carbon off the Southern California Borderland in 2020 and 2021 with proximity to shore and across oxygen minimum zone (OMZ) categories. Oxygen minimum zone categories were defined based on oxygen concentration (considering an OMZ threshold of $[O_2] < 22 \mu\text{mol/L}$) and depth. Offshore sites were >100 km away from the coastline.

POC samples	n	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
Proximity to shore			
Inshore (surface)	4	-22.9 ± 0.1	6.3 ± 0.2
Offshore (surface)	4	-23.9 ± 0.1	8.7 ± 0.8
Inshore (near bottom)	5	-24.4 ± 0.6	7.3 ± 0.5
Offshore (near bottom)	7	-25.8 ± 0.3	6.8 ± 0.8
OMZ categories			
Surface (0 m)	8	-23.2 ± 0.3	7.5 ± 0.6
Above OMZ (near bottom)	2	-24.8 ± 0.9	5.7 ± 0.8
Within OMZ (near bottom)	4	-24.9 ± 0.8	6.7 ± 1.9
Below OMZ (near bottom)	6	-25.5 ± 0.4	8.0 ± 0.3
Near bottom average	12	-25.2 ± 0.4	7.0 ± 0.5

Table S2: Mean $\pm$ 1 standard error carbon and nitrogen stable isotope values (‰) number of morphospecies (n) of macrofauna on mineral-rich rocks collected off the Southern California Borderland in 2020 and 2021 by site, rock type, proximity to shore, and oxygen minimum zone (OMZ) categories. Oxygen minimum zone categories were defined based on oxygen concentration (considering an OMZ threshold of $[O_2] < 22 \mu\text{mol/L}$) and depth. Offshore sites were >100 km away from the coastline. Species isotope means were calculated prior to calculating the means by site, rock type, proximity to shore, and OMZ.

	Macrofauna			Megafauna		
	n	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	n	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
Site						
40-Mile Bank	37	-19.5 ± 0.2	14.6 ± 0.5	18	-18.3 ± 0.4	15.7 ± 0.4
Coronado Escarpment	23	-18.9 ± 0.3	14.0 ± 0.2	3	-17.6 ± 1.7	12.0 ± 0.7
Cortes Bank	16	-18.7 ± 0.3	14.6 ± 0.8	3	-18.5 ± 0.5	19.6 ± 2.0
Crespi Knoll	16	-19.2 ± 0.3	14.0 ± 0.7	12	-17.1 ± 0.8	15.5 ± 0.8
Hancock Bank	18	-19.7 ± 0.3	14.0 ± 0.7	11	-19.7 ± 0.3	14.3 ± 1.0
Lasuen Knoll	9	-19.7 ± 0.4	12.7 ± 0.6	4	-17.5 ± 1.5	13.5 ± 0.7
Little Joe Seamount	10	-19.0 ± 0.9	14.4 ± 1.1	11	-19.8 ± 0.7	14.6 ± 0.5
Northeast Bank	11	-19.0 ± 0.3	14.8 ± 1.1	8	-18.3 ± 0.7	15.8 ± 0.5
Osborn Bank	2	-17.8 ± 0.1	15.1 ± 3.3	5	-19.4 ± 0.4	13.6 ± 0.5
Patton Escarpment	21	-19.2 ± 0.2	16.6 ± 0.7	11	-19.4 ± 0.2	13.6 ± 0.9
Patton Ridge Central	18	-19.1 ± 0.2	13.9 ± 0.6	6	-18.9 ± 0.4	14.6 ± 1.9
Patton Ridge South	18	-18.9 ± 0.4	13.1 ± 0.9	7	-17.6 ± 0.8	15.1 ± 0.5
San Clemente Escarpment	6	-19.1 ± 0.6	15.1 ± 1.8	5	-18.8 ± 0.7	16.8 ± 1.0
San Juan Seamount	47	-19.2 ± 0.3	16.0 ± 0.5	18	-18.2 ± 0.4	16.0 ± 0.4
Rock type						
Phosphorite	43	-19.0 ± 0.2	13.9 ± 0.4	12	-18.0 ± 0.5	15.6 ± 0.9
FeMn	63	-19.0 ± 0.2	16.1 ± 0.4	40	-18.9 ± 0.2	15.5 ± 0.3
Basalt	55	-19.2 ± 0.2	14.8 ± 0.4	25	-18.5 ± 0.5	14.5 ± 0.6
Other rocks	45	-19.4 ± 0.2	13.6 ± 0.5	27	-18.4 ± 0.3	15.2 ± 0.5
Proximity to shore						
Inshore	77	-19.3 ± 0.1	14.0 ± 0.3	41	-18.0 ± 0.4	15.1 ± 0.4
Offshore	99	-19.0 ± 0.2	15.8 ± 0.3	61	-19.0 ± 0.2	15.3 ± 0.4
OMZ categories						
Above OMZ	47	-19.0 ± 0.2	13.8 ± 0.3	24	-17.9 ± 0.5	14.5 ± 0.5
Within OMZ	78	-19.2 ± 0.2	14.9 ± 0.2	47	-18.7 ± 0.2	15.6 ± 0.4
Below OMZ	68	-19.0 ± 0.3	15.9 ± 0.4	36	-18.9 ± 0.3	15.5 ± 0.4
O₂ ranges ($\mu\text{mol/L}$)						
< 10	62	-19.1 ± 0.2	14.8 ± 0.5	29	-18.4 ± 0.3	15.5 ± 0.5
10 to 22	27	-19.3 ± 0.3	14.5 ± 0.7	25	-19.0 ± 0.3	15.7 ± 0.6
22 to 40	66	-19.0 ± 0.2	14.9 ± 0.3	29	-18.0 ± 0.4	15.7 ± 0.5
40 to 60	22	-19.1 ± 0.3	16.4 ± 0.7	9	-19.3 ± 0.3	13.7 ± 1.0
60 to 80	6	-18.8 ± 0.3	16.2 ± 1.3	5	-19.6 ± 0.3	12.6 ± 1.3
> 100	25	-19.3 ± 0.5	13.4 ± 0.4	13	-19.7 ± 0.7	14.4 ± 0.5

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Table S3: Mean $\pm$ 1 standard error carbon and nitrogen stable isotope values (‰) of macro- and megafaunal invertebrates on mineral-rich rocks collected off the Southern California Borderland in 2020 and 2021 by taxa and feeding mode. Species isotope means were calculated prior to calculating the means.

	Macrofauna		Megafauna	
	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
Taxa				
Annelida	-19.2 $\pm$ 0.2	13.4 $\pm$ 0.4	-19.6 $\pm$ 0.2	13.8 $\pm$ 0.8
Arthropoda	-18.9 $\pm$ 0.7	14.7 $\pm$ 0.6	-19.4 $\pm$ 0.4	14.0 $\pm$ 0.9
Bryozoa	-16.7 $\pm$ 1.2	13.0 $\pm$ 1.1	NA	NA
Cnidaria	-19.5 $\pm$ 0.8	14.6 $\pm$ 0.4	-18.8 $\pm$ 0.4	14.7 $\pm$ 0.2
Echinodermata	-19.5 $\pm$ 0.3	13.2 $\pm$ 0.6	-17.0 $\pm$ 1.0	13.5 $\pm$ 0.9
Mollusca	-19.1 $\pm$ 0.4	13.4 $\pm$ 0.5	-19.3 $\pm$ 0.8	14.3 $\pm$ 1.2
Porifera	-18.7 $\pm$ 0.1	18.8 $\pm$ 0.4	-18.8 $\pm$ 0.2	17.4 $\pm$ 0.4
Others	-19.4 $\pm$ 0.4	13.4 $\pm$ 1.0	-18.4 $\pm$ 0.4	16.8 $\pm$ 1.6
Feeding mode				
Carnivorous	-19.6 $\pm$ 0.4	15.2 $\pm$ 0.6	-19.4 $\pm$ 0.5	15.4 $\pm$ 0.6
Deposit feeder	-19.6 $\pm$ 0.3	12.3 $\pm$ 0.7	-19.4 $\pm$ 0.1	12.4 $\pm$ 0.4
Detritivorous	-19.6 $\pm$ 0.2	12.6 $\pm$ 0.5	-16.7 $\pm$ 1.2	12.8 $\pm$ 0.9
Filter feeder	-18.8 $\pm$ 0.1	18.0 $\pm$ 0.5	-18.5 $\pm$ 0.1	17.5 $\pm$ 0.4
Grazer	-18.5 $\pm$ 0.1	14.0 $\pm$ 0.6	-20.1	13.0
Mixed	-19.2 $\pm$ 0.4	14.4 $\pm$ 0.9	-18.1	15.1
Omnivorous	-19.6 $\pm$ 0.6	13.9 $\pm$ 0.6	NA	NA
Scavenger	-19.2 $\pm$ 0.7	12.8 $\pm$ 0.8	-19.6 $\pm$ 0.5	13.9 $\pm$ 1.1
Suspension feeder	-18.4 $\pm$ 0.7	14.4 $\pm$ 0.4	-18.8 $\pm$ 0.4	14.6 $\pm$ 0.2

Table S4: Mean $\pm$ 1 standard error carbon and nitrogen stable isotope values (‰) of macro- and megafaunal sponges on mineral-rich rocks collected off the Southern California Borderland in 2020 and 2022. Species isotope means were calculated prior to calculating the means.

	Macrofauna		Megafauna	
	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
Demospongiae	-20.2	14.7	-18.9 $\pm$ 0.6	17.2 $\pm$ 1.0
Hexactinellida	-19.7 $\pm$ 1.3	16.4 $\pm$ 1.1	-18.9 $\pm$ 0.1	17.1 $\pm$ 0.5
Unknown	-18.6 $\pm$ 0.1	19.1 $\pm$ 0.4	-18.5 $\pm$ 0.4	18.2 $\pm$ 0.8

Table S5: Community metrics of macrofaunal invertebrates associated with hard substrates off the Southern California Borderland across rock types, proximity to shore, and OMZ categories. TA: Total area; CD: Mean distance to centroid; MNND: Mean nearest neighbor distance; SDNND: Standard deviation of nearest neighbor distance; SEA: Standard ellipse area; SEAc: Standard ellipse area corrected for sample size.

Macrofauna	$\delta^{13}\text{C}$ range	$\delta^{15}\text{N}$ range	TA	SEA	SEAc	CD	MNND	SDNND
Rock types								
Phosphorite	5.7	12.8	42.6	9.5	9.7	2.4	0.6	0.5
FeMn	11.5	14.1	97.4	19.2	19.5	3.1	0.6	0.6
Basalt	7.0	12.2	46.3	11.8	12.0	2.7	0.5	0.4
Other rocks	8.4	12.4	41.3	11.1	11.4	2.8	0.6	0.9
Proximity to shore								
Inshore	5.3	11.7	41.3	8.7	8.9	2.4	0.4	0.3
Offshore	11.5	16.1	107.4	17.5	17.7	3.1	0.6	0.8
OMZ categories								
Above OMZ	5.0	9.4	30.7	6.9	7.1	1.9	0.5	0.4
Within OMZ	8.4	16.1	79.7	13.4	13.6	3.2	0.5	0.6
Below OMZ	11.0	13.3	91.7	18.2	18.5	2.9	0.6	0.7

Table S6: Community metrics of megafaunal invertebrates associated with hard substrates off the Southern California Borderland across rock types, proximity to shore, and OMZ categories. TA: Total area; CD: Mean distance to centroid; MNND: Mean nearest neighbor distance; SDNND: Standard deviation of nearest neighbor distance; SEA: Standard ellipse area; SEAc: Standard ellipse area corrected for sample size.

Megafauna	$\delta^{13}\text{C}$ range	$\delta^{15}\text{N}$ range	TA	SEA	SEAc	CD	MNND	SDNND
Rock types								
Phosphorite	6.4	11.7	38.4	18.3	20.1	3.1	1.7	0.8
FeMn	5.9	10.8	36.0	10.4	10.7	2.3	0.5	0.3
Basalt	14.2	12.3	62.5	21.5	22.6	4.1	1.3	0.9
Other rocks	6.7	11.9	41.3	13.5	14.1	2.6	0.8	0.6
Proximity to shore								
Inshore	10.4	9.9	56.5	15.6	16.1	2.8	0.8	0.7
Offshore	6.7	13.6	49.7	11.7	11.9	2.6	0.5	0.4
OMZ categories								
Above OMZ	9.7	8.4	49.4	19.2	20.2	3.1	0.9	0.8
Within OMZ	6.7	13.6	46.0	12.2	12.6	2.6	0.7	0.4
Below OMZ	7.4	10.8	44.2	12.4	12.7	2.4	0.6	0.5

Table S7: Average $\pm$ 1 standard error, minimum, and maximum sampling depths for each rock type.

Rock type	Average $\pm$ 1 standard error depth (m)	Minimum depth (m)	Maximum depth (m)
Phosphorite	526 $\pm$ 9	406	790
Ferromanganese	1332 $\pm$ 29	110	2688
Basalt	599 $\pm$ 17	315	1124
Other rocks	600 $\pm$ 32	120	1037

Figure S1: Taxon composition (% based on number of individuals) by (A-B) rock type, (C-D) proximity to shore, and (E-F) oxygen minimum zone categories for macro- and megafaunal invertebrates. Note that these represent the taxa sampled for stable isotope analyses and not the total community on the rocks. See Guraieb (2024) and Vlach (2022) for total community composition analyses.

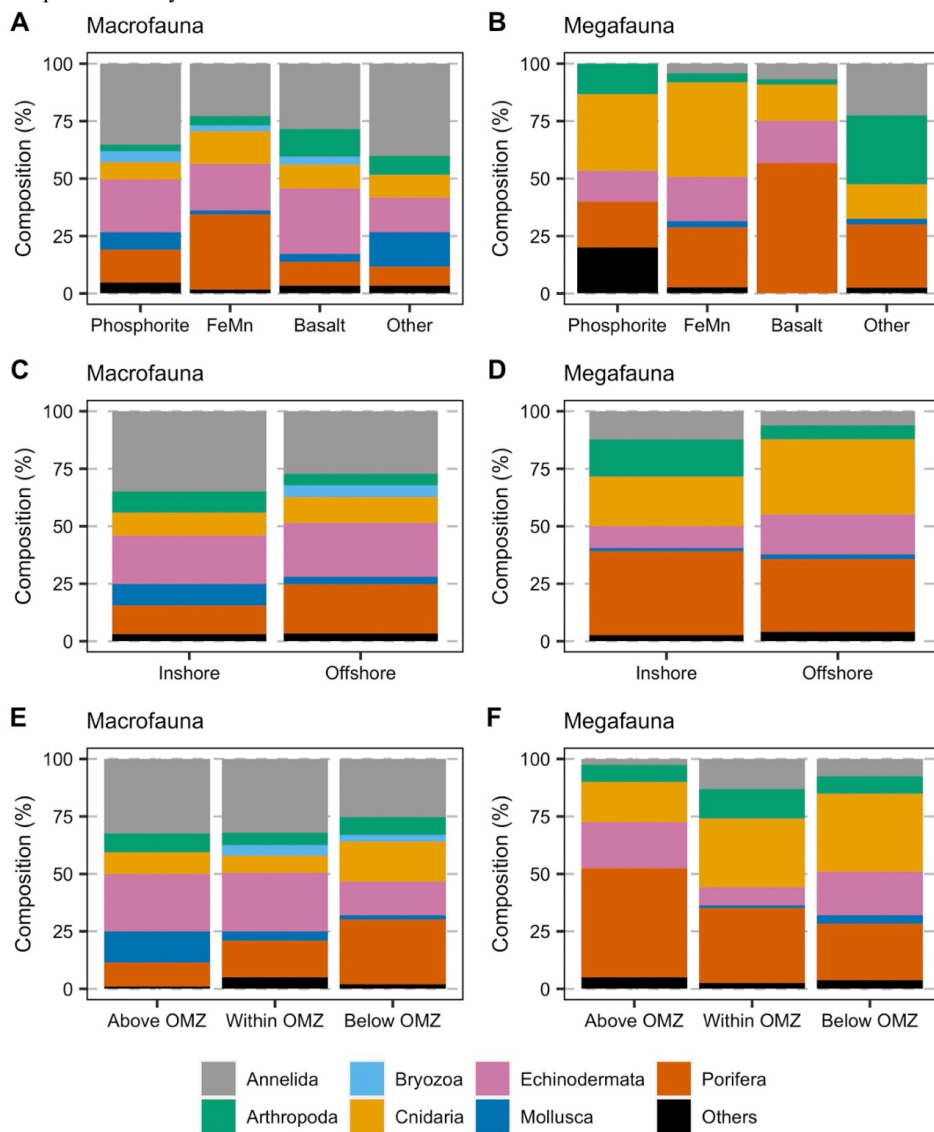


Figure S2: Feeding mode composition (% based on number of individuals) by (A-B) rock type, (C-D) proximity to shore, and (E-F) oxygen minimum zone categories for macro- and megafaunal invertebrates. Note that these represent the taxa sampled for stable isotope analyses and not the total community on the rocks.

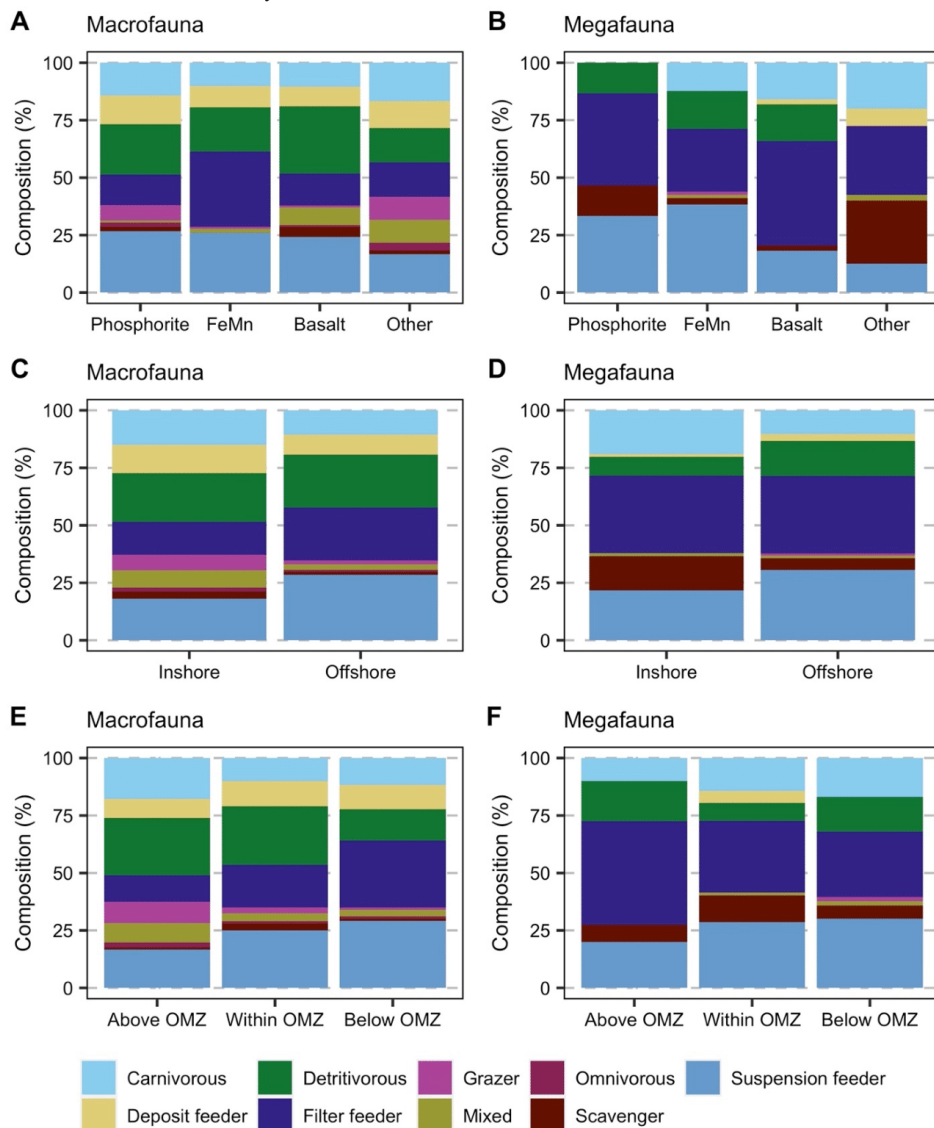


Figure S3: Dual isotope plots ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ average ± 1 standard error) of macrofaunal (filled circles) and megafaunal (open circles) sponges by class associated with hard substrates off the Southern California Borderland with depth.

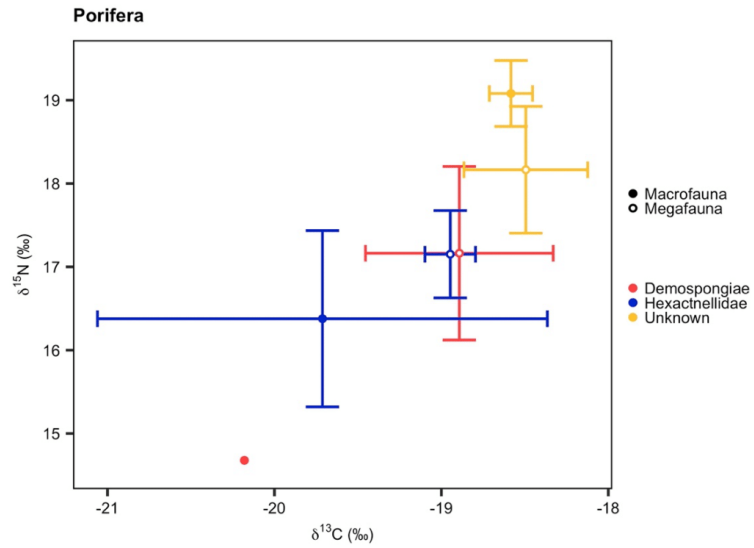


Figure S4: Dual isotope plots ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ average ± 1 standard error) of macrofaunal (filled circles) and megafaunal (open circles) invertebrates on (A) different rock types, (B) with proximity to shore, and (C) with oxygen minimum zone categories off the Southern California Borderland.

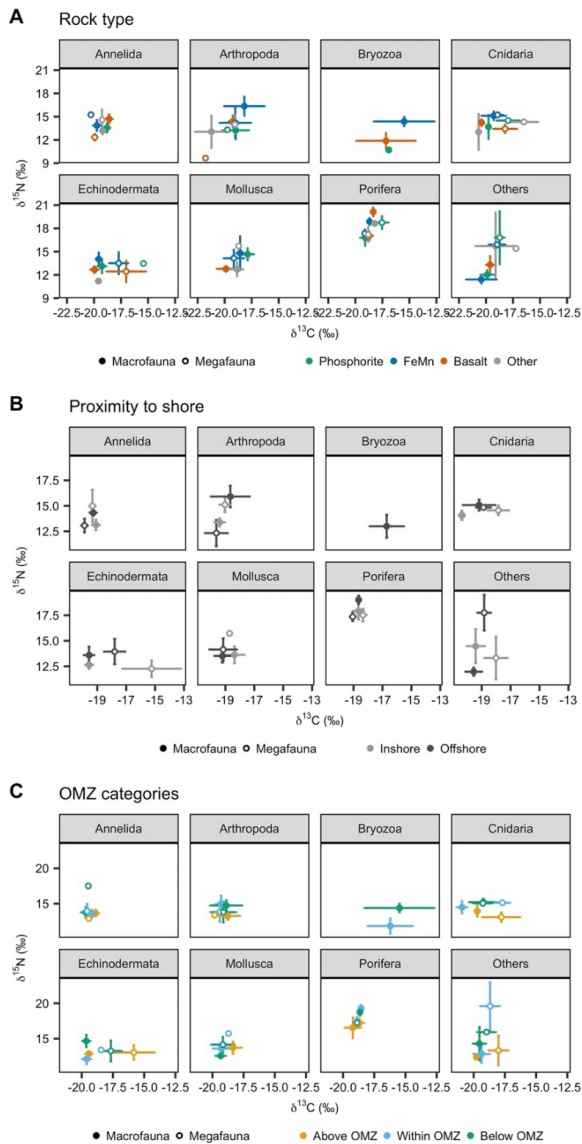


Figure S5: Trends in (A) $\delta^{13}\text{C}$ and (B) $\delta^{15}\text{N}$ of macrofaunal (black) and megafaunal (gray) invertebrates (gray) associated with hard substrates off the Southern California Borderland with depth. Data were fitted in linear regression models.

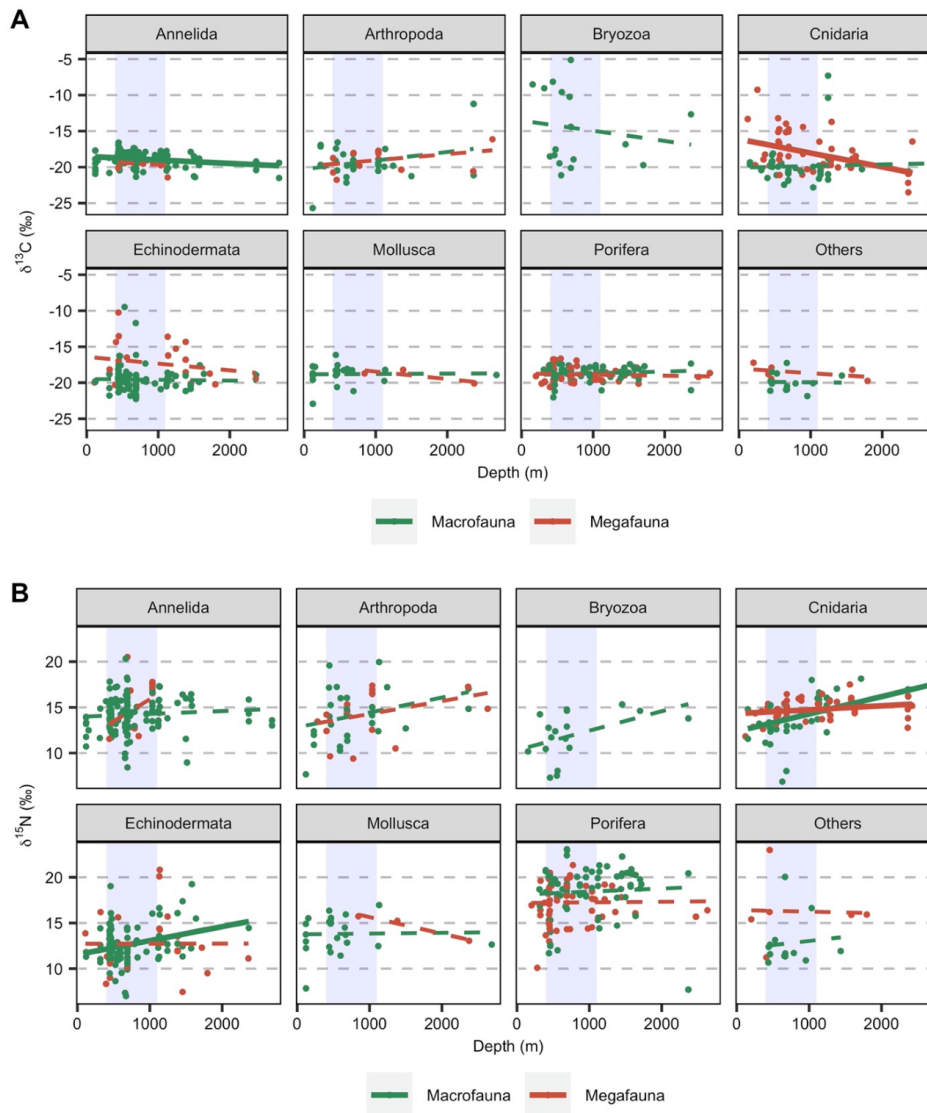


Figure S6: Trends in (A) $\delta^{13}\text{C}$ and (B) $\delta^{15}\text{N}$ of macrofaunal (black) and megafaunal (gray) invertebrates associated with hard substrates off the Southern California Borderland with oxygen concentration. Data were fitted in linear regression models.

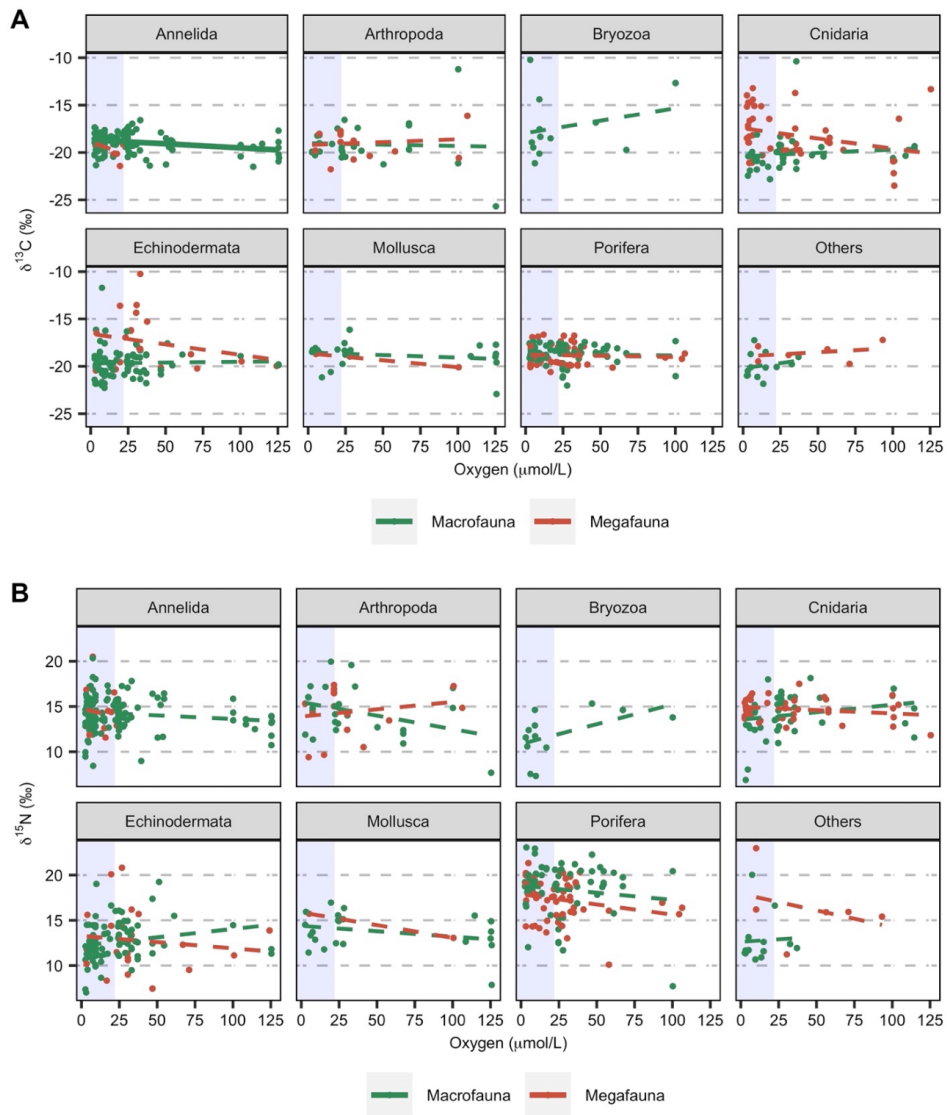
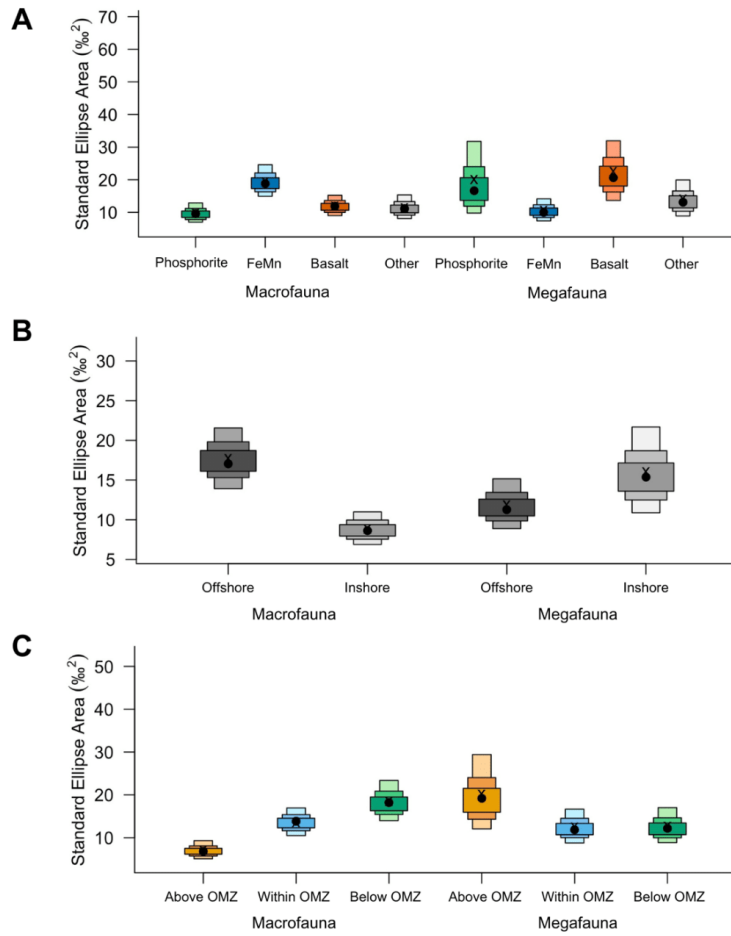


Figure S7: Bayesian standard ellipse areas of invertebrate macro- and megafaunal communities on (A) different rock types, (B) with proximity to shore, and (C) with oxygen minimum zone categories off the Southern California Borderland. X's represent the maximum likelihood estimated standard ellipse area corrected for sample size. All comparisons were statistically different (Dunn's test, $p < 0.05$).



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Chapter 4, in full, is a reprint of the material as it appears in Invertebrate trophic structure on marine ferromanganese and phosphorite hardgrounds. Pereira, Olívia S.; Vlach, Devin; Bradley, Angelica; Gonzalez, Jennifer; Mizell, Kira; Levin, Lisa A. *Limnology and Oceanography*, 2024. The dissertation author was the primary investigator and author of this paper.

Chapter 5 UNDERSTANDING AND VALUING HUMAN CONNECTIONS TO DEEP-SEA METHANE SEEPS OFF COSTA RICA

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Understanding and valuing human connections to deep-sea methane seeps off Costa Rica

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ABSTRACT

Methane seeps are highly productive ecosystems that provide carbon sequestration services, host diverse communities including endemic species, and serve as habitats for commercial fisheries. Little is known about the economic value the public places on them. Discrete Choice Experiments (DCEs) are administered to a sample of Costa Rican taxpayers to evaluate their willingness to pay (WTP) in monetary terms using tradeoffs made in a survey context involving three of the main attributes of methane seep ecosystems to provide insights for future conservation and management efforts. Extensive effort is devoted to understanding how Costa Ricans view different aspects of the deep sea. We find that they associate it with strange animals, natural resources, the unknown, and being far from reach. Perhaps surprisingly, they underestimate how much they know about the deep sea. We find that WTP for methane seep protection is the highest for programs that protect seeps with endemic species, followed by seeps with high climate change mitigation potential and commercial fishing habitat. Higher-income groups and women are more likely to prefer options that increase the current level of protection. We discuss how science communication and community engagement contribute to care expressed toward the deep sea.

1. Introduction

Although there is an increasing interest in deep-sea resources there is still a critical knowledge gap in understanding how people value such resources. Values include direct and indirect use values, non-use values, and option values (Folkersen et al., 2018), as well as the value of the associated ecosystem services, i.e., the contributions to human well-being from ecosystems. Deep-sea environments are critical to nutrient cycling, carbon absorption and sequestration, and contain genetic and biological assets that are unique to these ecosystems (Thurber et al., 2014). They also hold cultural and spiritual significance and have inspired literature, film, art, dance, and education (Levin et al., 2016; Turner et al., 2019; Armstrong et al., 2022). Among the many deep-sea ecosystems that have been discovered since Western exploration began are chemosynthetic ecosystems, including methane seeps, that rely on chemical energy rather than photosynthesis (Ramirez-Llodra et al.,

2010). Methane seeps are common on continental margins globally, where fluid enriched in reducing gas (mainly methane and hydrogen sulfide) ascends from the seafloor due to tectonic activity (Ritt et al., 2010). Microorganisms consume the methane and reduced compounds in the seeping fluids and synthesize organic matter fueling a diverse, chemosynthetic community (see Orphan et al., 2001; Ritt et al., 2010; Marlow et al., 2014; Ashford et al., 2021). Methane seeps are known to provide important climate services such as carbon cycling and carbon sequestration (indirect use value), host diverse specialized animal communities including endemic species (with potential option value and non-use values), and serve as nursery and feeding habitats for important fisheries species (see Marlow et al., 2014; Levin et al., 2015, 2016; Le et al., 2017, 2022; Seabrook et al., 2019). Seeps may also be important potential systems for genetic and biopharmaceutical studies and were considered a promising alternative energy source (Desbruyères et al., 2007; Liu et al., 2019).

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In spite of many possible reasons why the public values protecting deep-sea ecosystems, relatively little work exists evaluating economic willingness to pay (WTP) for preservation of these systems. We fill this gap by conducting a discrete choice experiment (DCE)¹ to investigate (i) perceptions about the deep sea and methane seeps, (ii) what aspects of the deep sea people most value, and (iii) how much people are willing to pay for methane seep protection in Costa Rica. Our goal is to provide insights and recommendations for future management and conservation efforts; the DCE allows us to separate the importance of several different attributes of deep-sea ecosystems. The relative novelty of deep-sea ecosystem services, defined here as contributions to human well-being including supporting services, provisioning services, regulating services, and cultural services (Millennium Ecosystem Assessment, 2005, see Le et al., 2017), complicates their economic valuation (Le et al., 2017). An important reason is that many of these services represent non-use values; They involve benefits derived from the existence of the ecosystem (for example the desire to preserve a resource for its own sake) but not from direct use.

While few deep-sea ecosystems have been studied in an economic valuation context, deep-water coral reefs are an important exception: Glenn et al. (2010) estimate WTP of the public for the conservation of cold-water corals and find strong public support for banning trawling. They observe that lower scientific knowledge on the part of respondents reduces WTP. Jobstovogt et al. (2014) also consider cold-water corals and find that preferences for cold-water coral conservation are linked to the non-use value of preserving particular species like deep-sea fish, starfish, lobsters, anemones, and sponges. Injuries to deep-sea corals are a component of the economic damage assessment conducted for the BP oil spill (Bishop et al., 2017). More recently, Xuan et al. (2021) investigate WTP in Norway, Scotland, and Canada for policy to protect ecosystems in the North Atlantic. We observe that most deep-sea economic valuation studies have been conducted in Europe and that very few estimates (either marine or terrestrial) are available from Latin America. Our study is among the first to gather data and develop WTP estimates for marine ecosystems in Latin America. We are also the first to consider WTP related to methane seeps or any chemosynthetic ecosystem.

Finally, a range of questions in the conservation literature revolve around the idea of care (Armstrong et al., 2022) and whether knowledge, for example of the deep sea, strengthens care.² Jamieson et al. (2020), Armstrong et al. (2022), and Jamieson et al. (2022) debate whether perceptions and care toward the 'deep' are primarily associated with fear. Given this background, we also incorporate questions in our survey about perceptions, examining initial reactions to the deep sea (before we work to strengthen understanding and conduct the DCE itself). Using these initial reactions we also offer additional interpretation and evidence in the discussion of care and human motivation toward conservation generally.

2. Study area and methods

2.1. Costa Rican methane seeps

Costa Rica is one of the 20 most biodiverse countries in the world due to its geographic location; it experiences tropical weather, is surrounded by the Pacific and Atlantic Ocean, and includes the oceanic Cocos Island

(Alvarado et al., 2012). With 92% of its total territory being marine area, Costa Rica possesses a great variety of marine ecosystems, including gulfs, bays, rocky shores, beaches, islands, and deep-sea ecosystems (e. g., seamounts, methane seeps, and mesophotic corals) hosting high marine richness (Alvarado et al., 2012; Levin et al., 2015; Cortés, 2019; Maestro et al., 2022). The country also stands out for its commitment to conservation and has recently expanded its marine protected area to ~30% of the country's marine territory (MINAE, 2022). Although public participation in management efforts has been increasing in recent years (Maestro et al., 2022), the lack of adequate stakeholder participation, limited coordination among governmental agencies, and a shortage of economic resources are the main threats to marine management in the country (Alvarado et al., 2012).

In deep waters, methane seeps are common features along the Pacific margin of Costa Rica (Fig. 1). This is a convergent margin characterized by subduction erosion, where >100 fluid seeps have been discovered, corresponding to a seep site every 4 km along the continental slope. These seeps show diverse structures, associated with landslide scars, seamounts, fractures, mounds, and faults, at depths from 400 to 2200 m and occur <100 km away from the Costa Rican shoreline (Sahling et al., 2008). Since the discovery of Costa Rica's methane seeps, research has been conducted describing the chemosynthetic communities associated with these methane-rich environments (see Sahling et al., 2008; Levin et al., 2012, 2015; Goffredi et al., 2014, 2020; Ashford et al., 2021; Pereira et al., 2021, 2022; Azofeifa-Solano et al., 2022). Costa Rican seeps support 3 to 30 times higher hardground faunal densities than seeps in the northeast Pacific off California and in the Mediterranean Sea (Levin et al., 2015). However, the intense oxygen minimum zone (OMZ) off Costa Rica between ~200–500 m deep, where oxygen concentration is lower than 22 $\mu\text{mol/L}$, yields much lower diversity in comparison to seep sites below the OMZ (Levin et al., 2015). The megafauna (larger animals) at these seeps varies among sites, but is mainly dominated by bathymodiolin mussels, the Costa Rican yeti crab *Kiwa puravida*, siboglinid polychaetes *Lamellibrachia barhamia* and *Escarpa spicata*, as well as lepetodrilid and neolepetopsid limpets and provannid snails (Levin et al., 2015; Pereira et al., 2021). Recently, new species have been described, including the new deep-sea eelpout fish *Pyrolyscus jaco* nestling within the tubeworms (Frale et al., 2023), nine new ribbon worm species (Sagorny et al., 2022), five new species of scale worms (Lindgren et al., 2019; Hatch et al., 2020), and a new deep-sea coral species (Breed et al., 2019). Many more remain to be described (G.W. Rouse, personal communication).

2.2. Survey

The survey was conducted in Spanish and the complete version can be found in Supplementary File 1, along with a translation into English. It consisted of background questions to evaluate perceptions and prior knowledge about the deep sea, a two-minute long informative video about methane seeps (Supplementary File 2, in Spanish), six choice sets (see section below), and demographic questions. If participants chose a "no conservation" option for any of the six cards they were also asked the reason why they picked that option. Prior to the final design of the survey, a focus group provided feedback on the questions, selection of attributes, and accompanying options.

Participants for the main survey were recruited by Qualtrics (www.qualtrics.com), a major online panel provider, where the sampling frame was specified as current Costa Rican taxpayers who were 18 years and older. As is standard practice, participants received a small compensation payment from Qualtrics. A sample of 502 respondents was obtained.

2.3. Discrete choice experiment

The survey respondents were given a discrete choice experiment. This offered three options per choice set: A status quo option where seeps were not explicitly protected which involved zero new costs and

¹ DCEs can be used to investigate direct economic use and non-use values connected to ecosystem existence (Glenn et al., 2010). DCEs aim to estimate the structure of an individual's preferences by establishing the relative importance of different attributes as incorporated within a set of alternatives presented in a survey questionnaire format (Adamowicz et al., 1994; Carson and Czajkowski, 2014; Johnston et al., 2017).

² In the economic literature on non-market valuation, this notion of care would be categorized as a stewardship motivation for the class of economic benefits related to the resource's existence (Mitchell and Carson, 1989).

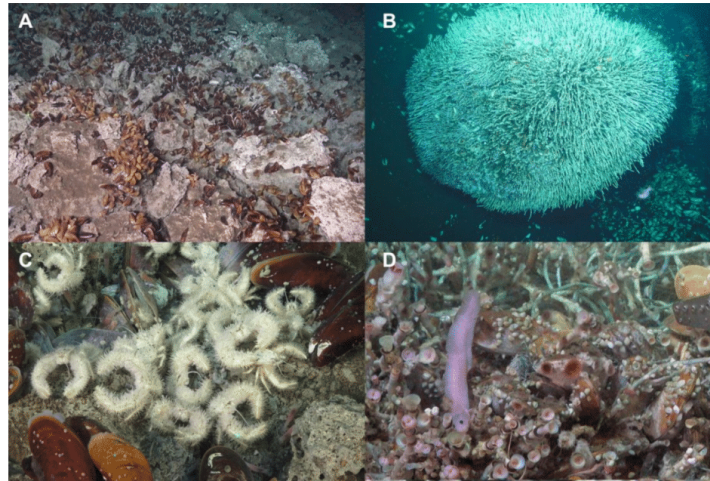


Fig. 1. Methane seeps off the Costa Rican Pacific margin and some of their endemic species. (A) Mussel bed at Mound 12 at 1000 m deep, (B) Tubeworms at Jaco Scar at 1800 m deep, (C) *Kiwa puravida*, yeti crab from Mound 12, (D) *Pyrolycus jaco*, eelpout from Jaco Scar, within *Lamellibrachia barhamia* and *Escarpia spicata* tubeworms (from [Frale et al., 2023](#)).

two options that would increase the level of protection in different ways and incur a specified increase in cost. The questionnaire reminded participants to account for budget constraints and to consider their other expenses to avoid hypothetical bias from people ignoring their budget constraints ([Jobstvig et al., 2014](#)).

The increased protection options described to participants on the choice cards (see [Fig. 2](#)) were built around existing science describing ecosystem services offered by methane seeps and, more specifically, by methane seeps on the Costa Rican margin. The attributes and accompanying options are described in [Table 1](#).

The zero-cost option was described as a scenario where the seep offers basic ecosystem services (i.e., there are no unique species, no special benefits to commercial fisheries, and little potential for climate regulation), and no conservation policy would be implemented. The cost attribute for the other options was chosen from six levels in the local currency, the Costa Rican colón (₡ 685 = US\$ 1 at the time of the survey). Magnitudes were chosen considering local annual income, tax rates, and bill denominations. The question frames the amount in the form of an annual payment. The cost levels were: ₡500, ₡1000, ₡2000, ₡5000, ₡7000, and ₡10,000 (\$0.73 to \$14.64 at the time the survey was conducted).

There was a total of 36 choice sets, each with three options 1, 2, and 3 (see Supplementary File 3 for all cards in Spanish). Option 1 was the status quo option, and it was kept the same for all choice sets to ensure that the respondent could always afford one of the options. An example choice set is provided in [Fig. 2](#) (card 17 in Supplementary File 3). The order of the 36 choice sets was randomized so there was no methodical structure, and they were divided into blocks of 6 choice sets. Each block of choice sets was randomly assigned to an equal number of respondents. A more in-depth explanation of the DCE methodology can be found in Supplementary File 4.

2.4. Surveying prior background knowledge and affiliation with the marine sector

To understand respondent prior knowledge about the deep sea we provided a list of statements (all of which are considered scientifically correct) and asked if the respondent agreed or disagreed. This allowed us to categorize knowledge of the deep sea. For each statement where a

respondent selected “Completely agree” we assigned 1 point, 0.75 point for “Agree”, 0.5 point for “Neutral”, 0.25 point for “Disagree” and 0 points for “Completely disagree”. There were 10 statements for a total of 10 points allowing us to create a calculated index of knowledge categories (CalcKnow). We did the same with a series of statements asking for level of agreement with conservation actions (CalcConserv), allowing us to see how this connected with respondent’s knowledge and WTP.³

We also included a dummy variable based on the respondents’ affiliation with the marine sector (Sector), including but not limited to tourism, fishing industry, marine shipping, other marine resources (oil, gas), education (student or professor), non-governmental organizations (NGOs), and governmental institutions.

2.5. Data synthesis and statistical analyses

Our main analysis used conditional and mixed (random parameters) logit models ([Train, 2009](#)) of the DCE results and were estimated using STATA 17. Our random parameter’s models included both mean and standard deviation parameters on the three ecosystem attributes (*Endemic species*, *Fisheries*, and *Climate change*) experimentally assigned by the DCE. The cost parameter following [Train and Weeks \(2005\)](#) is specified as fixed in order to ensure that the WTP ratio is well-behaved. We provide estimates for the sample itself and the sample weighted to make the median age, percent male and percent college educated match the 2021 Costa Rican Census ([INEC, 2021](#)). Results for our preferred model without weights and after weighing are shown in [Table 2](#) which uses an alternative specific constant (ASC) attached to the status quo option. The ASC captures respondents’ preference for a zero cost, business as usual outcome relative to any conservation option. We explore interactions between the ASC and each of the demographic variables in [Table 2](#). In the mixed logit model, we specify the parameters on attributes and ASC as random. WTP estimates are similar across the fixed and random parameters models. We note a likelihood ratio test prefers the random parameters model and so refer to those results in what follows (fixed-coefficient model results can be found in Supplementary File 5).

³ CalcConserv ranges from 0 to 5 based on a series of 5 statements.

(1) Presence of seep unique species: Whether there are unique species at the seep that would be protected. These could be species that are unique to seeps around the world, and/or species that are unique to Costa Rican seeps.

(2) Importance of the seep for commercial fish: Whether there are commercial fish at the seep that would be protected and if so, whether they are using the seep as feeding grounds or as reproduction sites. Fish, tuna crabs, and giant crabs have been seen using seeps as reproduction and feeding sites.

(3) Potential for climate regulation: How much potential the seep has to help regulate the climate by keeping methane from being released to the atmosphere.

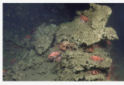
CONDITIONS	OPTION 1 (no conservation measures applied)	OPTION 2	OPTION 3
Presence of seep unique species	No seep unique species 	Unique species from seeps worldwide 	Unique species from seeps worldwide + seeps in Costa Rica 
Importance of the seep for commercial fisheries	No commercial fisheries 	Feeding grounds for commercial fisheries 	Reproduction sites for commercial fisheries 
Potential for climate regulation	Little potential 	Little potential 	Some potential 
Additional annual cost per person for protection, management, and monitoring	€0	€5 000 	€500 

Fig. 2. Choice card example in English. All choice cards can be found in Supplementary File 3 in Spanish.

WTP values are expressed in US\$/per unit increase in the attribute throughout, and we also calculated WTP as a fraction of average annual income for reference.

Linear regression models were estimated to evaluate whether there is a correlation between knowledge and level of agreement with conservation actions. Answers to the open-ended question of what people think of when they hear the words “deep sea” were coded into a set of categorical variables.

Finally, we compare our results to other economic valuation studies of deep-sea ecosystems. We used exchange rates and average annual income⁴ at the time the studies were conducted to provide the most meaningful comparisons.

3. Results

3.1. Socio-demographics characteristics of survey respondents

The sociodemographic characteristics of our survey respondents were compared to their population characteristics as reported by the Instituto Nacional de Estadística y Censos Costa Rica (INEC, 2021). Our sample was reasonably representative along some dimensions, but had the divergences common to many internet panels. It is younger (sample = 59% under median age, INEC = 26%), had a higher proportion of males (sample = 57%, INEC = 46%), and better educated (sample = 39% university degree, INEC = 10%). For the choice models estimated

below, we provide estimates using the unweighted sample and a weighted sample where the inverse probability weights result in our sample matching INEC on the eight cells defined by these three variables. If younger, female, and college graduate are positively related to willingness to pay, applying the weights should result in lower estimate and that is the observed result below.

Most income brackets were well represented in the sample, except for people earning less than US\$ 5600 annually who, not unexpectedly, were underrepresented (sample = 29%, INEC = 60–70%).⁵ The same pattern was observed in employment status, where 77% of the sample was employed (INEC = 49%). Considering respondents with dependents, the most common number of dependents (2–4) in the sample was similar to the Costa Rican population (sample = 37.8%, INEC = 34.3% based on number of people in a household), but there were fewer respondents with no dependents in the sample than in the population (sample = 27.3%, INEC = 39.3%). 79% of respondents said they are not associated with the marine sector, and of the 21% who are, they work mainly in tourism (56% of respondents associated with the marine sector), followed by the fishing industry (15% of respondents associated with the marine sector) and academia/research (12.5% of respondents associated with the marine sector). We interacted these sociodemographic variables with the ASC in our conditional logit models and explored subsamples of the data restricted to particular income and education groups. See Supplementary File 5 for more complete characterization of the socio-demographic data.

⁴ Average annual income data from Organization for Economic Co-operation and Development via Statista.

⁵ Weighting directly on income is not typically done due its strong relationship with the other three variables, which temporally precede.

Table 1
Attributes and accompanying levels used in the discrete choice experiment.

Attribute	Attribute description	Levels
Presence of seep unique species	This attribute assesses whether there are unique species (i.e., endemic, however we used the term “unique” to keep the survey in non-scientific language) at the seep that would be protected. These could be species that are unique to seep ecosystems and occur at seeps around the world (worldwide seep unique species) and/or species that are unique to Costa Rican seeps (Costa Rican seep unique species). For example, the giant clams <i>Calypptogena</i> occur globally at seeps, but the yeti crab <i>Kiwa puravida</i> is only found in Costa Rica.	1. No unique species (status quo) 2. Worldwide seep unique species 3. Worldwide and Costa Rican seep unique species
Importance of seep for commercial fish	This attribute assesses whether there are commercial fish at the seep and if so, whether they are using the seep as feeding grounds or as reproduction areas. Fish, tuna crabs, and giant crabs have been seen using seeps as reproduction and feeding sites.	1. No commercial fish (status quo) 2. Commercial fish use the seep as feeding grounds 3. Commercial fish use the seep as reproduction sites
Climate regulation potential	This attribute assesses how much potential the seep has to help regulate the climate by keeping methane from being released to the atmosphere.	1. Little potential (status quo) 2. Some potential 3. Great potential

Table 2
Variables and levels used in conditional and mixed logit models.

Attribute	Levels
Endemic species	Presence of endemic species (0 = No endemic species, 1 = Worldwide seep endemic species, 2 = Worldwide and Costa Rican seep endemic species).
Fisheries	Relevance for fisheries (0 = No commercial fisheries, 1 = Feeding grounds to commercial fisheries, 2 = Reproductive sites for commercial fisheries).
Climate change	Climate mitigation potential (0 = Little potential, 1 = Some potential, 2 = Great potential).
Cost	Cost attribute (€500, €1000, €2000, €5000, €7000, and €10,000).
STR	Respondent's identification number and question number (specific to each response of each individual).
Card	Choice set number
Gender	0 = Prefer not to say, 1 = Woman, 2 = Men, 3 = Transgender, 4 = Non-binary.
Income*	0 = Prefer not to say, 1 = Up to US\$5600, 2 = More than US\$ 5600 and less than US\$ 8400, 3 = More than US\$ 8400 and less than US\$ 13,900, 4 = More than US\$13,900 and less than US\$ 28,000, 5 = More than US\$ 28,000.
Education	0 = Prefer not to say, 1 = Pre-school, 2 = Sixth grade, 3 = High school, 4 = College, 5 = Masters, 6 = PhD, 7 = Technical degree.
Work	0 = Prefer not to say, 1 = Part-time worker, 2 = Full time worker, 3 = Student, 4 = Independent, 5 = Looking for a job, 6 = Retired, 7 = Other.
Marine sector	Whether the respondent works in the marine sector (1 = yes).
Deep sea knowledge	Respondent's own perception of their knowledge about the deep sea (1 = Little knowledge to 5 = Excellent knowledge).
Conservation index	Category of level of agreement with conservation actions based on a point system (1 = Little knowledge to 5 = Excellent knowledge).

* € 685 = US\$ 1 at the time of the survey; US\$ rounded to hundreds.

3.2. Background knowledge about the deep sea

In an open-ended question asking people about the first thing they think of when they hear the words ‘deep sea’, people associated the deep sea with biodiversity, marine resources, science, environmental characteristics, specific ecosystems, and emotions (Fig. 3). Some interesting patterns emerged when analyzing these answers by sociodemographic characteristics; biodiversity terms were mentioned more often by lower income people, emotion terms were mentioned more with lower education level, while environmental aspects were mentioned more by those with higher education level (Supplementary File 5). More than 50% of the respondents put “habitat for unique animals” as the first or second most important connection they have to the deep sea, and >75% of the respondents ranked “provision of mineral resources” and “biomedical potential”, respectively, as the least important connections they have to the deep sea. “Provision of food” and “climate regulation” were ranked second and third (Fig. 4).

Respondents were asked whether they agreed or not with statements that are considered scientifically correct (Fig. 5). Overall, respondents agreed or totally agreed with most of the scientific statements regarding general aspects of the deep sea. But they tended to be more neutral toward statements regarding the specifics of methane seeps and the role of the deep sea in climate mitigation, suggesting most respondents are less informed prior to the survey on these issues. We also compared respondents' self-assessment of knowledge about the deep sea to calculations based on their responses to these scientific statements: 10% vs 4% (self-assessment vs calculated) had no knowledge about the deep sea, 49% vs 50% had little knowledge, 35% vs 5% had some knowledge, 4% vs 4% had good knowledge, and 2% vs 37% had excellent knowledge.

3.3. Willingness to pay for methane seep protection

Table 3 reports coefficient estimates from the mixed logit models we fit for the sample both unweighted and weighted, as well as the corresponding estimates of willingness to pay (WTP) for conservation for a one-unit improvements in three attributes: Endemic species, fisheries, and climate change mitigation. For the weighted sample, appropriate when estimates for Costa Rican taxpayers are desired, increasing the importance of the endemic species attribute in the specified manner (Table 2) one level raises WTP to protect methane seeps by US\$ 22.86/year. Increasing the importance of seeps for commercial fisheries raise WTP by US\$ 9.25/year. And finally increasing the importance of climate regulation provided by seeps increases WTP for their protection by US\$ 15.10/year. All three WTP values were significantly different from zero at the 1% level.

With respect to the parameters on the random components, there is considerable preference heterogeneity with respect to endemic species and climate change but not fisheries. The random component on the ASC, itself, is also insignificant suggesting that variation here is adequately captured by the ASC interaction terms. Interactions between the ASC and social demographic characteristics were generally not strongly statistically significant. This is not an uncommon phenomenon in a DCE; without interaction with the ASC they fall out of the model as the demographic factor is held constant across choices. Perhaps more important here though is that there were non-trivial correlations between them. For instance, education and income have the expected positive correlation. The point estimates for income and gender suggest that women and higher income groups were less likely to choose the status quo, instead favoring one of the other options, although the point estimates are individual. The major reason for inclusion of these variables is to absorb some of the sources of unobserved heterogeneity. It is



Fig. 3. Responses when asked about the first thing respondents think of when they hear the words deep sea. Responses to this open-ended question were categorized for analysis and this visual representation. Numbers and shading represent the number of times each word or phrase was mentioned.

also worth noting that, as expected, WTP decreases between unweighted sample and the sampled weighted to match Costa Rica's INEC with respect to median age, gender, and college graduate. With the down weighting of the younger, male, and college graduates, we find that for individual attributes WTP declines by a fairly small (6%) to moderate (22%) degree. Estimates of the magnitudes of the random components generally increase by a small to moderate amount and all become insignificant due to noisier estimates of standard deviation parameters.

3.4. Attitudes toward conservation

Respondent's attitudes toward conservation actions were positive overall (Fig. 6), and they tended to become more favorable with greater knowledge (Fig. 7). Respondents mostly agreed that deep-sea ecosystems should be protected (63% agreed that the government should do

more to protect them), but were more negative when it came to conservation actions justified through the provision of fish habitat and climate change mitigation (only 21% agreed that the deep sea should be protected for providing such services). Interestingly, respondents seemed to value the deep sea mostly for its existence and bequest values: 52% would like to have the option to use deep-sea ecosystems in the future, 77% agreed that they provide benefits for their children and future generations, and 85% valued the uniqueness and fragility of deep-sea ecosystems even though they don't intend to use them.

In spite of these generally positive attitudes, we also found that nearly half of respondents (226) were not willing to pay (i.e., chose the zero-cost status quo option) in at least one choice set. The main reason given for this, when asked in a follow-up question, was that they believe the Costa Rican government should pay out of its existing budget (39%), a common response of those in surveys who are happy to have the good

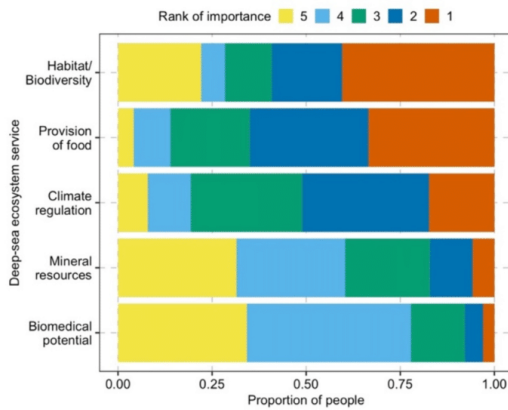


Fig. 4. Ranked importance of deep-sea ecosystem services by respondents.

provided at no direct cost to them. Other common reasons provided were that resource users should pay (18%), an inability to afford payments (14%), or they would rather pay into a conservation trust fund rather than directly pay a tax (13%) (see Supplementary File 4 for details).

4. Discussion

4.1. Willingness to pay for methane seep protection

We first compare the magnitudes of our WTP results with related studies conducted in other countries and settings (Table 4). Our estimated WTP for methane seep conservation in Costa Rica is lower than WTP for deep-sea ecosystems in Norway (LaRivière et al., 2014; Aanesen et al., 2015; Xuan et al., 2021), Canada (Xuan et al., 2021), and Italy (O'Connor et al., 2020). It exceeds that for a marine ecosystem (cold-water corals) in Ireland (Glenn et al., 2010) and for certain terrestrial ecosystems in Chile (Cerdeña et al., 2013). However, Costa Rica also has lower average income than countries in nearly all prior studies. When we consider WTP as a fraction of average annual income we find that WTP in our setting is comparable to related studies in Table 4. This supports the idea that environmental concerns as measured by WTP may be fairly high in some middle-income countries (Vincent et al., 2014). Our results here are consistent with Costa Rica's emergence as the leader among developing Latin American countries in environmental conservation (Steinberg, 2001) and it is well-known for its attention to tropical forest (Biénabe and Hearne, 2006). Costa Rica was one of the first countries to voice concerns about deep-seabed mining when it did so in 2022.⁶

In several related studies of deep-sea ecosystems, attributes associated with existence value had the highest WTP, including protection of species living there (Jobsvogt et al. 2014), protection of habitat (LaRivière et al., 2014; Aanesen et al., 2015), and existence of endemic species (Cerdeña et al., 2013, this study). In our study, respondents were

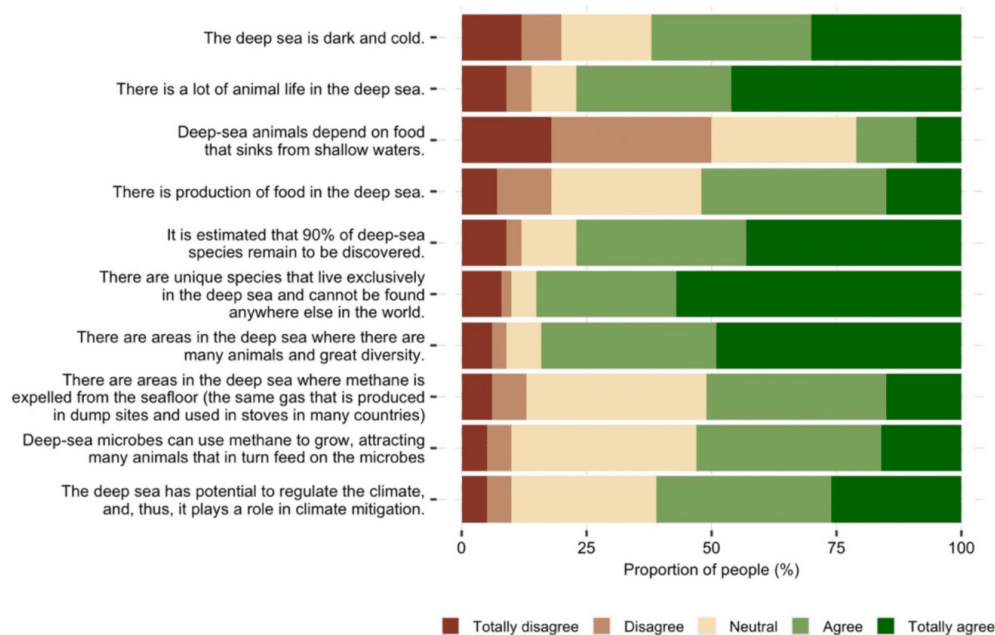


Fig. 5. Respondents' level of agreement (%) on deep-sea science questions. Data are available in Supplementary File 5.

⁶ <https://savethehighseas.org/voices-calling-for-a-moratorium-governments-and-parliamentarians/>

Table 3

Attribute coefficients and WTP estimates for methane seep conservation for the mixed logit models without weighting the data and after weighting the data to make the sample statistically equivalent to the population considering gender, age, and education.

	Without weights		With weights	
Random parameters	Mean of coefficient	WTP (US\$)	Mean of coefficient	WTP (US\$)
ASC	-0.664 (1.070)	–	-0.459 (0.953)	–
Endemic species	0.470 (0.089)***	24.30	0.609 (0.147)***	22.86
Fisheries	0.228 (0.059)***	11.80	0.246 (0.077)***	9.25
Climate change	0.309 (0.069)***	16.00	0.402 (0.125)***	15.10
	SD of coefficient		SD of coefficient	
ASC	0.926 (2.524)		1.390 (2.104)	
Endemic species	0.841 (0.271)***		1.066 (0.512)	
Fisheries	0.090 (0.669)		0.065 (0.236)	
Climate change	0.797 (0.296)***		0.867 (0.381)	
Non-random parameters	Mean of coefficient		Mean of coefficient	
Cost	-0.019 (0.007)***		-0.027 (0.010)**	
ASC x Income group	-0.217 (0.193)		-0.385 (0.299)	
ASC x Education group	0.545 (0.327)*		0.476 (0.342)	
ASC x Female	-0.291 (0.194)		-0.036 (0.215)	
ASC x Full-time worker	-0.003 (0.145)		-0.126 (0.243)	
ASC x Marine sector	0.018 (0.167)		-0.594 (0.404)	
ASC x Knowledge of deep sea	-0.074 (0.097)		0.144 (0.166)	
ASC x Conservation index	-0.013 (0.062)		-0.117 (0.118)	

Standard errors in parentheses. Significance levels are indicated as ***, **, and * for 1%, 5%, and 10%, respectively. A negative ASC interaction coefficient indicates preference for one of the improvement options over the status quo option. Choice sets: 6 per $N = 502$ respondents.

willing to pay the most for protection of endemic species and the least for protection of commercial fish habitat. The fish habitat attribute contains both an existence motivation (as with the endemic species attribute) and a value from the direct consumption of commercial fish (Aanesen et al., 2015). Expectations might have been that in a low-income country there would be a greater emphasis on direct fisheries value; it is interesting to see here that the global climate mitigation value, along with protection of endemic seep species, dominates over those associated with commercial fishing.

4.2. Limitations of surveys and DCEs

Some common limitations associated with DCEs and valuation surveys more generally include the need for the survey to present the material in such a way as to enable almost all respondents including those

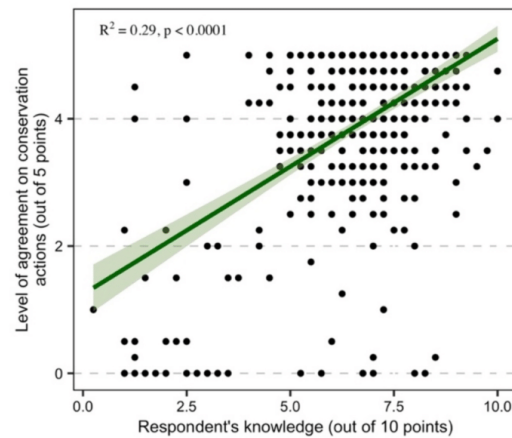


Fig. 7. Linear regression between respondent's knowledge and level of agreement on conservation actions.

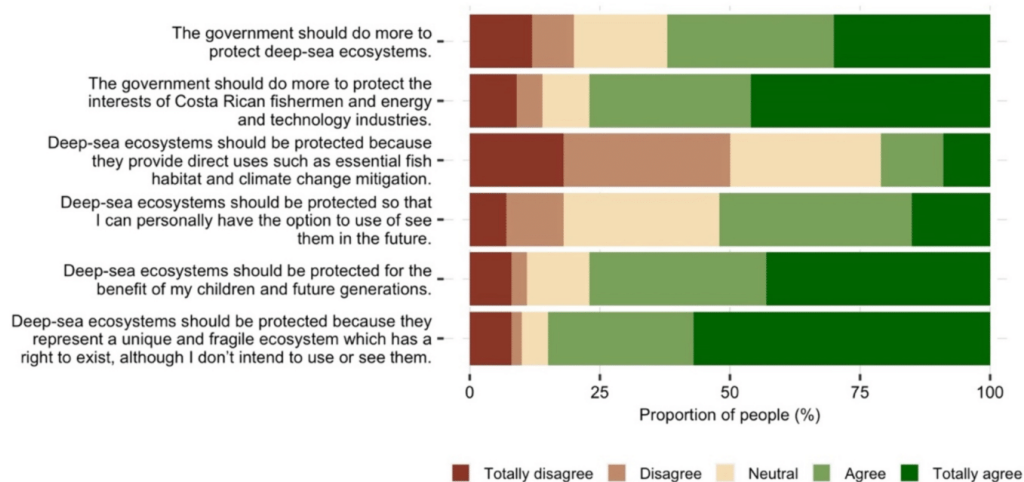


Fig. 6. Respondents' level of agreement (%) on conservation actions. Data are available on Supplementary File 4.

Table 4

Comparison of willingness to pay (WTP) for protection or restoration of deep-sea ecosystems around the world and other ecosystems in Latin America: WTP in US\$ per year, and as a percentage of the country's average annual income. Currency converted to US\$ with exchange rates at the time the study was conducted. Average annual income at the time the study was conducted.

Country	Goal of the study	Attribute	WTP (US \$/year)	WTP (% of average annual income)	Reference
Costa Rica	Conservation of methane seeps	Protect seeps with endemic species	22.86	0.19	This study
		Protect seeps with potential for climate regulation	15.10	0.12	
		Protect seeps that are habitat and/or nursery for economically important fisheries	9.25	0.07	
		High potential for medicinal products from deep-sea organisms	62.19	0.14	
United Kingdom	Protection of deep-sea biodiversity				Jobstvøgt et al. (2014)
		High level of species protection	47.28	0.11	
		Intermediate level of species protection	43.29	0.10	
		Ban trawling in all coral areas	0	0	
Ireland	Protection of cold-water corals				Glenn et al. (2010)
		Ban trawling in all known coral areas	12.27	0.02	
		Ban all fishing in all coral areas	1.33	<0.001	
		Protect areas that are habitat/nursery for many fish species	267.16	0.45	
Norway	Extension of current MPAs with the goal of including cold-water corals				LaRivière et al. (2014)
		Protect areas attractive for the fish industry	41.23	0.07	
		Increase total protected area to 10,000 km ²	41.10	0.07	
		Protect areas attractive for oil and gas industry	22.02	0.04	
		Protect areas that are important habitat for fish	187.58	0.40	
Norway	Protection of cold-water coral				Aanesen et al. (2015)
		Protect areas attractive for the fish industry	44.07	0.09	
		Protect areas attractive for the oil industry	18.08	0.04	
		Extend protected area to 5,000 km ²	59.89	0.13	
Italy	Restoration of a deep-water canyon	Extend protected area to 10,000 km ²	74.58	0.16	O'Connor et al. (2020)
			39.62	0.11	
Norway	Protection of high-seas ecosystems in the North Atlantic	Reduction in marine litter density	35.04–52.51	0.05–0.07	Xuan et al. (2021)
		Improvement of health of fish stock	30.33–72.29	0.04–0.05	
		Increase in protected area	3.30–28.32	0.004–0.04	
		Number of marine economy jobs created	14.28–18.41	0.02–0.03	
Scotland	Protection of high-seas ecosystems in the North Atlantic	Reduction in marine litter density	83.19–128.27	0.20–0.30	Xuan et al. (2021)
		Improvement of health of fish stock	50.86–86.97	0.10–0.20	
		Increase in protected area	25.13–50.62	0.07–0.14	
		Number of marine economy jobs created	26.67–44.72	0.07–0.12	
Canada	Protection of high-seas ecosystems in the North Atlantic	Reduction in marine litter density	38.59–57.23	0.05–0.08	Xuan et al. (2021)
		Improvement of health of fish stock	38.94–50.62	0.06–0.07	
		Increase in protected area	22.54–25.02	0.03–0.04	
		Number of marine economy jobs created	20.18–31.03	0.03–0.05	
Chile	Valuation of environmental benefits provided by a terrestrial reserve	Availability of drinkable water	6.60–8.90	0.04–0.06	Cerdeira et al. (2013)
		Existence of endemic amphibians in the reserve	6.70–8.20	0.04–0.05	
		Observe animals in a visit to the reserve	4.40–4.70	0.02–0.03	
		Existence of endemic orchids in the reserve	1.70–3.00	0.01–0.02	
Costa Rica	Public preferences for biodiversity conservation and scenic beauty	Increased protection of biodiversity in relatively remote areas	3.98	0.08	Biénabe and Hearne (2006)
		Improved protection of scenic beauty in relatively accessible forest areas	3.00	0.06	

not originally that familiar with the ecosystem and the changes of interest to it to understand the choice task they are being asked about. Further, they need to do so in a manner that makes the survey consequential in the sense that they are providing information about the tradeoffs they are willing to make and that policymakers may take the public's preferences into account in making decisions concerning the ecosystem. Since directly assessing understanding via surveys can often be challenging (Aanesen et al., 2015), we undertook several initial focus groups and in-depth interviews where we gathered feedback in-person with respect to specific issues. This allowed us to closely tailor the final survey, with detailed instructions especially for the DCE component.

Although respondents showed more knowledge about the deep sea than we originally expected, they were substantially less familiar with methane seeps, and in particular with the role of the deep sea in climate mitigation. However, previous studies show that even though respondents may not have pre-existing preferences on a subject they are unfamiliar with, they are still able to build preferences within the survey if the information they need for making an informed decision is provided (Jobstvogt et al., 2014). A related issue is whether respondents perceive the information provided in the survey to be accurate as this can influence (typically in a negative direction) both preferences and WTP (LaRiviere et al., 2014). Our finding that WTP for improvements in the protection of methane seeps despite the relative unfamiliarity with these ecosystems compared to the other two experimentally assigned attributes (i.e., fisheries and climate) is suggestive evidence that the survey presentation with respect to endemic seep species was largely successful.

Another potential challenge related to the information provided in our survey is that we cannot compare results to alternative approaches (for example, to a survey where no information is provided at all). However, based on our analyses using the calculated deep-sea knowledge index, it seems that this does affect WTP (see discussion below). In addition, a prior study observed that when respondents were informed of how well they scored on background knowledge questions, their WTP for an extension of current marine protected areas in Norway was 1.5× higher (LaRiviere et al., 2014). Thus, we recommend that future studies further consider how background knowledge questions may impact responses within a DCE.

Protest votes from respondents who reject some aspect of the survey's scenario in DCEs can be identified, to some extent, by adding background and follow-up questions to understand the reasoning behind their choice. Often, protest voters believe that others should bear the costs of a policy and so vote 'no' even when they like the policy itself (Mariel et al., 2021). In our study, the set of potential protesters were initially identified by looking at the set of respondents who choose the zero-cost status quo option in all six choice sets and gave a reason for doing so other than being unable to afford payment (5 respondents out of 502). We also looked at two patterns of choices that may be reflective of quickly going through the DCE without paying much attention to the attribute levels – picking all of their options from the second column (2 respondents out of 502) or picking all of their options from the third column (4 respondents out of 502). Here random assignment of attribute levels to options makes it likely that only a very small fraction of respondents finds this pattern of choices optimal. We also looked at what fraction of respondents disagreed with all of the conservation actions statements (19 out of 502 respondents), but none of these respondents chose the status quo option in all six choice sets nor disagreed with all of the deep-sea science statements, which both seems plausible and suggestive that these were not necessarily protest voters.

4.3. Management application to methane seeps, the deep sea, and in Costa Rica

Global protection of methane seeps is limited, especially in deep waters, and at present is mostly based on area-based management tools (ABMTs, also known as spatial management strategies). These include

twenty-seven marine protected areas (MPAs) that have been designated in European shallow waters (< 200 m) for methane seeps, known as Special Areas of Conservation (SACs) (Noble-James et al., 2019). Methane-derived authigenic carbonates associated with methane seepage are termed 'submarine structures made by leaking gases' and together with their biological communities are considered 'habitats of community importance' under the EC Habitats Directive (European Commission, 2013). These SACs are located within the EEZs of the UK, Ireland, Denmark, France, Romania, Greece, and Spain (Noble-James et al., 2019). Outside of Europe, the uThukela Banks MPA off the east coast of South Africa was designated to protect coastal, shelf, and slope ecosystems (e.g., sand, gravel, reefs, and submarine canyons) spanning an area of 4094 km², and recently gas (likely methane) leakage at depths of <100 m was mapped for the first time in the region (Green et al., 2022). Within deep waters, Canada recently established the Tang-e-gwan-ħačx-wiqak-Tsigis MPA, a 133,000 km² area off the west coast of Vancouver Island featuring methane seeps, hydrothermal vents, and >40 seamounts at depths of up to 2250 m (Times Colonist, 2023). The region of the Finike Seamount (880–1000 m) in the Mediterranean Sea has been identified as a fragile and unique ecosystem with a distinct methane seep community where trawling should be banned or controlled (Öztürk, 2009). ABMTs have also been proposed for deep-sea ecosystems in the vicinity of oil and gas industrial activity, including methane seeps, where protected areas would be established around ecologically or biologically significant areas (EBSAs) of representative communities with a buffer zone at least 2 km away from the protected area (Cordes et al., 2016). The Pacific Fishery Management Council also recognized methane seeps and associated biological communities as essential fish habitat due to trophic and reproductive support and incorporated seeps into fisheries management (NOAA, 2020).

Other types of ABMTs consider fundamental ecological characteristics of the ecosystem, such as those we describe in the policy attributes for our DCE. Intergovernmental organizations may develop criteria to identify vulnerable, sensitive, and ecologically or biologically significant ecosystems in need of protection (Gollner et al., 2021). These alternative types of ABMT include (i) Vulnerable Marine Ecosystems (VMEs), developed in the context of deep-sea fishing by Regional Fisheries Management Organizations within the Food and Agriculture Organization (FAO), (ii) Ecologically and Biologically Significant Areas (EBSAs), developed in the context of the Convention on Biological Diversity (CBD), and (iii) Particularly Sensitive Sea Areas (PSSAs), developed in the context of international shipping activities by the International Maritime Organization. These three tools use similar criteria to those we study, making more precise the concepts of uniqueness/rarity, vulnerability, biological diversity, structural complexity, importance for threatened, endangered or declining species, and others (Blanchard and Gollner, 2022). Regarding deep-sea ecosystems, hydrothermal vents are considered VMEs by FAO, four have been described as EBSAs, and seven additional vents should also be considered for inclusion (see Gollner et al., 2021). Cold-water corals ecosystems have also been recognized as VMEs (FAO, 2009). Although methane seeps have not yet been officially recognized by such tools, they have been considered for several possible VMEs (FAO, 2009), and studies show their potential to fit these criteria (FAO, 2018).

Notably, among these three tools, PSSAs are the only one to have social, cultural, and economic criteria (Gollner et al., 2021), mentioning the use of living marine resources of social or economic importance and cultural and historical importance (IMO, 2005). Gollner et al. (2021) deemed these not relevant for hydrothermal vents and suggested an ecosystem service criterion instead to identify hydrothermal ecosystems in need of protection, considering provisioning (e.g., genetic resources), regulating and supporting (e.g., carbon sequestration), and cultural services (inspiration for arts, science, and technology), as well as importance for scientific research. However, these are all mainly direct or indirect use values since contribution to art through inspiration is potentially monetizable and is not necessarily connected to existence or

bequest motivations for non-use value (Chan et al., 2011). Perhaps, more importantly, it is the public who will ultimately be asked to pay for any protection effort in a world of competing demands and limited resources. Thus, management tools still need to incorporate non-use values for marine management programs to be successful (O'Connor et al., 2021). Including non-use values in management tools increases the overall value of ecosystems and their services (Xuan et al., 2021) and better measures welfare losses arising from damages (see Bastian-Olvera and Moore, 2021).

In general, in Costa Rica, MPA management tends to be adaptive with high public participation (Maestro et al., 2022), and the existence value of biodiversity given by Costa Ricans in our survey is reflected in the country's position toward marine conservation. Costa Rica recently expanded the Cocos Island National Park and the Bicentennial Marine Management Area from 2.7% to ~30% of the country's marine territory (MINAE, 2022), achieving for Costa Rican waters the global goal of protecting 30% of the world's ocean by 2030. The expanded protected area is centered around Cocos Island and includes deep seamounts located south of the island.

However, offshore MPAs are difficult to keep and patrol due to logistics and cost, especially when they are large and do not have easily identifiable boundaries (e.g., in the case of Cocos MPA, the boundaries are represented by territorial sea). In addition, enforcement can be quite expensive, and other tools such as social norms and inclusion of all stakeholders can help with compliance and, consequently, the success of the MPA (Arias et al., 2014). The incorporation of local communities into protected area management is emerging in Costa Rica (Maestro et al., 2022). Marine Areas for Responsible Fishing (AMPR) were recognized in 2009 as a new model for participatory management of small-scale fisheries in the country, and, although its effectiveness remains to be determined, collaborations with NGOs and the government have strengthened the participation of small-scale fishers (Lozano and Heinen, 2016). The Santa Rosa National Park (northwest coast of Costa Rica) has a strong local component based on education and awareness-raising, following a bottom-up approach with the inclusion of the population at all stages (Maestro et al., 2022).

The inclusion of stakeholder consultation in conservation strategic planning processes through ecotourism provides dual benefits for conservation and local communities (Hunt and Vargas, 2018). In addition, tourism activities can be sources of income for conservation efforts in the form of entrance fees to protected areas and national park stamps, for example (Maestro et al., 2022). Tourism is one of the main sources of foreign income in Costa Rica (Biénabe and Hearne, 2006), and it is estimated that up to 80% of tourists visit the country for ecotourism activities. A previous study by Biénabe and Hearne (2006) reported that foreign tourists were willing to pay a one-time fee of \$6.77 in the form of an airport tax, as well as voluntary contributions at hotels, for improved biodiversity conservation of Costa Rican tropical forests. Similarly, governmental institutions and NGOs could propose an additional fee to the national income tax paid by Costa Rican citizens paired with voluntary contribution scheme to fund offshore, deep-sea management efforts⁷. However, it is important to consider people's preferences when developing such projects to ensure public awareness and support (O'Connor et al., 2021). Here, we observed that people in Costa Rica tend to care the most about the biodiversity value of deep-sea ecosystems. This is important because while the value of a seep as a commercial fisheries habitat might be estimable by a production function approach, as might be the value of the seep in terms of mitigating carbon, an analysis that fails to consider existence value defined in terms of endemic species, omits the biggest of these three factors expressed in monetary terms. Our more qualitative work helps illustrate that respondents perceived the good they would get if the protection policy

was implemented: a fascination with or the wonderment of the strange creatures of the dark, deep sea and the thought that society should also be willing to give up something monetarily to help ensure they continued to thrive offshore in Costa Rica. Specific wordings by respondents and information about the knowledge held by different segments of the population can be helpful in developing programs concerning methane seeps, and effectively communicating with those segments of the public.

Finally, WTP values can be used as inputs in cost-benefit analyses associated with policies affecting marine biodiversity and resources. The cost-benefit framework informs trade-offs between the costs of an activity and the benefits to the public given different levels of protection (Lew, 2015). For example, a study estimated that the costs and time required to restore the habitat and fauna associated with deep-seabed mining of polymetallic nodules would result in a significant reduction in profitability (Sumaila et al., 2023).⁸ The benefits of such restoration, however, could also be large and would be informed by the WTP values estimated here. Related, WTP values of the type we provide also play a key role in natural resource damage assessment, where restoration action needs to provide services of the same type, quality, and value of those lost (NOAA, 1996).

4.4. People do care about the deep sea, and the importance of science communication

Although WTP studies help us understand whether (and how much) people are willing to pay for deep-sea conservation, it is unclear how they contribute to people's perception of the deep sea (Jamieson et al., 2022) and to what degree WTP is largely motivated by the notion of care advanced by some researchers looking at deep-sea protection issues (Armstrong et al., 2022). To try to elucidate these aspects, we incorporated other sets of questions in our survey. First, we followed a common survey practice for qualitative exploratory analysis by asking respondents for the first thing they thought about when they hear the words "deep sea" before we had provided respondents with any specific information about the deep sea. Jamieson et al. (2020) argues that the deep sea is often associated with feelings of thalassophobia, "deepest darkest fears", monsters, the unknown, and that there is an anthropocentric disconnect. Indeed, these were concepts mentioned by respondents as their first thoughts of the deep sea, including "dangerous waters" [*agua peligrosa*] and "fear" [*miedo*]. However, these were in the minority ($n = 10$), and the answers regarding mysteries and the unknown ($n = 31$) were rather positive, e.g., "a very respected place in the world due to its mysteries" [*un lugar del mundo muy respectable debido a su enorme misterio*], "a world to be discovered" [*un mundo por descubrir*], "an adventure with many mysteries" [*una aventura con muchos misterios*], and "habitat for many species that remain to be discovered" [*habitat de muchas especies por conocer*]. The anthropocentric disconnect was also present but rather as a place that is distant ("away from the coast" [*más adentro que de la costa*], "into the ocean" [*el mar adentro*]), deep ("very deep" [*muy hondo, gran profundidad*], "below the waves" [*debajo de las olas*]), and dark ("where you cannot see" [*no se ve nada*], [*oscuro*]), which are not wrong in their essence. Some even recognized the need for specialized equipment to reach the deep ocean ("where it is hard to get to" [*donde es difícil llegar*], "where humans can't reach under their own means" [*donde el ser humano no es capaz de llegar bajo sus propios medios*]). A lack of intimacy, relatability, and common ground that people tend to feel toward deep-sea animals (Jamieson et al., 2020) also proved not to be the case with our respondents. Animals mentioned included sharks, crabs, tuna, and whales, which are more familiar than

⁷ In practice, done through executive/legislative actions that sets agency budgets and mandate actions.

⁸ The restoration costs here were based on shallow-water ecosystem restoration and on the costs to develop and deploy artificial nodules; there is no evidence that such restoration would be successful. <https://planet-tracker.org/the-sky-high-cost-of-deep-sea-mining/>

some other deep-sea species examples portrayed in the media. Lastly, it seems that the idea that the deep sea is to be feared might be fading, and people are seeing it for its “beauties” [*belleza, increíble, hermoso*], “valuable assets” [*son nuestras riquezas marinas*], “diversity and natural richness” [*diversidad y riquezas naturales*], as an “exciting adventure” [*aventura emocionante*], “peaceful” [*paz*], and in a respondent’s own words, “each sense that it [the deep sea] captures is a door of well-being, but it is imperative that the sea becomes that infinite balm for our mind” [*cada sentido que lo capta es una puerta de bienestar pero se impone que el mar se convierte en ese bálsamo infinito para nuestra mente*].

From these responses, it is clear that perceptions about the deep sea are not necessarily negative. This impression is later supported by the results from our DCE tradeoff analysis reported above which shows that most respondents are willing to pay non-trivial amounts for helping to ensure deep-sea conservation. But is this the same thing as “care”? The definition of “care” in the Oxford dictionary is “the provision of what is necessary for the health, welfare, maintenance, and protection of someone, or something”; “feel concern or interest; attach importance to something”, and “look after and provide for the needs of”. In the economics/environmental philosophy literature, “care” has been used to define “environmental stewardship”:

The actions taken by individuals, groups, or networks of actors, with various motivations and levels of capacity, to protect, care for or responsibly use the environment in pursuit of environmental and/or social outcomes in diverse social-ecological contexts (Bennett et al., 2018).

Stewardship actions can operate directly (e.g., establishment of protected areas) or indirectly (e.g., environmental education) and happen at different scales, from individual species to entire ecosystems (Bennett et al., 2018). Motivation for stewardship actions has been increasingly connected to relationships between human and non-human life, or “relational values”. These refer to a sense of connection, kinship, and identity that people develop to other living things and the desire to look after something (West et al., 2018). Respondents in our survey ranked provision of habitat and biodiversity as their most important connection to the deep sea, and the majority (> 80%) agreed with statements that deep-sea ecosystems should be protected so they can personally have the option to use or see them in the future, for the benefit of their children and future generations, and because they represent a unique and fragile ecosystem that has a right to exist. What is valuing the protection of an ecosystem and attaching importance and a connection to it if not care and stewardship?

The question that remains open is whether “knowledge of the deep sea encourages care, or at least reduces fear” (Armstrong et al., 2022). Fear of the deep sea and lack of care may be attributed to lack of knowledge. At the same time, fear can be a barrier for effective deep-sea science communication (Jamieson et al., 2022). Our survey results indicate that people tend to be more prone to conservation actions and have a higher WTP when they have more knowledge, but interestingly, also that their self-perception of their own knowledge might be underestimated. Jamieson et al. (2020) introduces an interesting discussion of how deep-sea scientists tend to diminish how much we know about the deep sea. It is understandable then that people would also think that they do not know much about the deep sea, especially when science is traditionally communicated through peer-reviewed publications written in technical language and not always with free access (Mea et al., 2016).

Science communication, outreach efforts, community engagement and incorporation of traditional knowledge can help scientists amplify the impact of the research, reach key audiences, improve access to science, and increase literacy (Shanley and López, 2009; Escobar-Briones and Álvarez-Sánchez, 2023). Engagement of the public in deep-sea research can also contribute to informed citizen input to policy, have a positive effect on generating support for deep-sea conservation, and can influence economic decisions (LaRivière et al., 2014; O’Connor et al., 2021; Escobar-Briones and Álvarez-Sánchez, 2023). In past years,

there has been a general increased interest in communication efforts from the academic community (Rose et al., 2020). Within the deep-sea science community, scientists are also changing how they communicate, highlighting the importance, values, and fascinating aspects of the deep sea, and encouraging people to create a connection to the deep sea (Jamieson et al., 2020; Jamieson et al., 2022).

Recent advancements in technology allow for deep-sea scientists to engage with the public in real-time exploration of the deep sea through livestream of remotely operated vehicle video (e.g., telepresence by NOAA Ocean Exploration Trust and Schmidt Ocean Institute) (Gallaudet et al., 2020), and for the public to interact with live deep-sea animals in aquariums (e.g., Monterey Bay Aquarium “Into the Deep: Exploring Our Undiscovered Ocean” exhibition that opened in 2022). Virtual reality, ocean digital twins, and virtual representations of the environments have also been evolving as tools that can be used in deep-sea science communication (see Burke and Crocker, 2020; Hruby et al., 2020). Finally, deep-sea scientists and institutions are also engaging more with social media and featuring art exhibitions showcasing the deep sea including a photographic exhibition in metro stations in Bilbao, Spain, in 2023, and the exhibition “Painting the Deep” by the artist Lily Simonson at the Harvard Museum of Natural History in 2019. These virtual and in-person forms of communication are effective to improve feelings of thalassophobia, perspectivism, and phenomenology described by Jamieson et al. (2020), and to create relatability and help develop personal connections to the deep sea.

5. Conclusion

Our study is the first to estimate Costa Rican’s WTP for protection of a marine ecosystem. It is also the first WTP study, globally, for a methane seep or for any chemosynthetic ecosystem. A key demonstration here is the possibility of designing effective valuation surveys for novel marine ecosystems that have thus far been neglected. For future studies in countries where very little prior research exists, we recommend that the survey and DCE include other ecosystems that may be more well-studied, and more relatable to respondents, as a means for additional comparison.

The work reported here shows Costa Ricans support protection of methane seeps off their coast. Strikingly, our respondents had the highest willingness to pay for programs that protect endemic seep species. Changes that enhanced climate change mitigation and commercial fishing habitat were also associated with substantial economic value, although notably smaller than that for protecting endemic seep species. Respondent willingness to pay increases with prior knowledge about the deep sea, suggesting a key role for science education in driving public willingness to monetarily support government actions to protect key ecosystem services in Costa Rica. Current management tools used for deep-sea ecosystems such as EBSAs and VMEs still need to incorporate non-use values as a criterion. Our approach can be seen as one way to rigorously demonstrate the value of a cultural resource in the deep-sea environment. Moreover, continued science communication and outreach efforts from the deep-sea science community can contribute to a more engaged and informed population that will in turn provide further input to policy. Our results indicate that both policymakers and scientists should put new weight on the role of deep-sea literacy and existence values, which together underpin economic willingness to pay and the potential success of deep-sea management programs.

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CRedit authorship contribution statement

Olivia S. Pereira: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mark Jacobsen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Data curation. **Richard Carson:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Data curation. **Jorge Cortés:** Writing – review & editing, Validation, Resources. **Lisa A. Levin:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Chapter 5, in full, is a reprint of the material as it appears in Understanding and valuing human connections to deep-sea methane seeps off Costa Rica. Pereira, Olívia S.; Jacobsen, Mark; Carson, Richard; Cortés, Jorge; Levin, Lisa A., Ecological Economics, 2024. The dissertation author was the primary investigator and author of this paper.

Chapter 6 CONCLUSION

Hard substrates in the deep ocean create habitat heterogeneity for diverse invertebrate communities and provide biofilm cues for settlement, substrate for attachment, reproductive sites, refuge, and, in some cases, food supply (Case et al. 2015, Levin et al. 2015, Marlow et al. 2014a). However, the same environmental conditions that allow for the formation of these hard substrates also influence biological communities and can drive benthic patterns of community structure. This dissertation focused on authigenic carbonates at methane seeps, ferromanganese crusts, and phosphorites. It investigated how environmental conditions such as seepage and dissolved oxygen concentration influence macrofaunal community and trophic dynamics in two ecosystems on bathyal continental margins: methane seeps off the Costa Rica Pacific margin and mineral-rich hardgrounds off the Southern California Borderland.

Methane seep macrofaunal communities rely mainly on chemosynthetic production by microbial activity, while those on mineral-rich hardground rely on photosynthetic production that sinks from shallow waters into the deep sea. However, the provision of hard substrate in the form of carbonate rocks at methane seeps and phosphorites and ferromanganese crusts on the Southern California margin support community and trophic diversity of macrofauna. At seeps, the substrate (i.e., carbonate rocks) is a means for food supply access and habitat, and the higher macrofaunal richness at active sites highlights the heterogeneity provided by carbonate rocks at active seepage (Chapter 2), compared to sediment fauna, which is less diverse and has lower richness at active sites (Ashford et al. 2021). On phosphorites and ferromanganese crusts substrate type correlates with invertebrate trophic structure, with ferromanganese crusts supporting distinct macrofaunal niches and phosphorites supporting a specialized community (Chapter 4). Studies have reported distinct macro- and megafaunal communities associated with ferromanganese compared to other

hard substrates (Corrêa et al. 2022, Vlach 2022, Guraieb 2023), and that substrate type may drive the structure of benthic communities, as seen in the case of cobalt-rich crusts (Schlacher et al. 2014). However, it remains unclear whether these differences are a direct effect of substrate (for example, due to rugosity or chemical composition), and we still know relatively little about the intersections of organisms with substrate. A recent study showed that abiotic seawater electrolysis produces oxygen from polymetallic nodules (Sweetman et al. 2024). If processes like this ‘geo-battery’, as it was called, are true for other substrates like ferromanganese crusts, they could also influence the macrofaunal relationship with substrate.

In addition to substrate type, environmental conditions influence both ecosystems studied here – methane seeps and mineral-rich hardgrounds. There are combined effects of low oxygen and depth; at shallow methane seeps, low oxygen seems to override the effect of seepage activity, creating unique macrofaunal communities (Chapter 2). Microbial community composition and distribution at seeps also significantly co-varies with depth and associated variables, including oxygen and habitat type (Seabrook et al. 2024). On the Southern California margin, the oxygen minimum zone at intermediate depths creates complex macrofaunal trophic relationships (Chapter 4 (Pereira et al. 2024a)). Effects of low oxygen have been reported for megafaunal community composition off the coast of Southern California and on ferromanganese deposits in the Southwest Atlantic (Corrêa et al. 2022, Vlach, 2022). Additionally, at methane seeps, I observed a stronger influence of seepage activity on macrofaunal communities with increasing depth, with macrofauna at deeper seeps relying more on chemosynthetic production (Chapter 2). Seepage activity also influences sediment fauna at Costa Rican seeps, creating a ‘chemotone’ with distinct community composition but similar density and diversity to lower seepage neighboring habitat (Ashford et al. 2021).

These findings highlight the heterogeneity of macrofaunal communities associated with hard substrates in the deep sea and their dependence on environmental variables. With manipulative experiments at methane seeps, I could examine whether the observed environmental variables also influence the dynamics and resilience of these communities. Low oxygen conditions seem to influence the *in situ* community to the point where the macrofaunal community is not sufficiently established to be able to colonize new substrates with new recruits or motile adults or even survive through changes in seepage activity. At deeper seeps, where macrofauna depend more on chemosynthetic production, seep fauna show high resilience, and the dominant feeding mode is associated with good colonization ability (Chapter 2). These results align with what is known about chemosynthetic communities; manipulative experiments at hydrothermal vents and with whale bones and wood pieces mimicking organic falls show that hydrothermal activity and chemosynthetic production lead to elevated functional diversity, improved colonization ability, and enhanced deep-sea biodiversity (Levin et al. 2017, Saeedi et al. 2019, Alfaro-Lucas et al. 2020, Young et al. 2022, Pereira et al. 2022). This dissertation provides a new perspective on the resilience of these communities in the face of environmental changes due to ocean deoxygenation and possible seepage expansion.

Since microbial activity provides a food source to macrofauna through chemosynthetic production, and the reliance on chemosynthetic production and feeding modes seem to play a large role in macrofaunal resilience and colonization at methane seeps (Chapter 2), I hypothesized that macrofaunal dynamics would also correlate with microbial communities. By examining relationships between macrofaunal and microbial communities and associations between macrofaunal species and microbial operational taxonomic units (OTUs), it seems that seepage activity and oxygen concentration may dictate the microbial community patterns, and in turn

specific microbial OTUs may dictate the patterns of those macrofaunal species which they depend on for food and habitat (Chapter 3). Thus, the responses of macrofauna to manipulative experiments observed in Chapter 2 appear to reflect the resistance and resilience of these specific OTUs (Chapter 3). A next step is to examine animal-microbial interactions on the mineral-rich hardgrounds off Southern California and compare whether a more photosynthetic-reliant macrofaunal community might (or might not) have similar correlations to the microbial community as observed for chemosynthetic ecosystems. I hypothesize that the latter exhibit a stronger dependence of macrofauna on microorganisms for food supply and not just substrate provision.

It would also be interesting to examine whether the dynamics observed in Chapters 2 and 3 remain the same when the different substrates (i.e., carbonate rocks, ferromanganese crusts, and phosphorites) are in proximity within and away from methane seeps, since the systems studied here are geographically separated. A 7-year colonization experiment with carbonate rocks, wood, and bone deployed in close proximity to a seep in the Costa Rica Pacific margin showed that seepage activity exerts control on the communities at active sites, but at transition sites, the communities on wood and bone were more similar to organic-fall communities (Pereira et al. 2022). These studies exemplify the complexity of macrofaunal dynamics among different systems.

The findings from Chapters 2-4 characterize and inform the distributions of macrofaunal invertebrates living on hard substrates at methane seeps and mineral-rich hardgrounds across environmental gradients, including low oxygen and depth, as well as substrate type. They highlight that environmental conditions influence these communities including their responses to physical disturbances and to changes in environmental conditions. For example, low oxygen conditions within the oxygen minimum zone limit the recovery and resilience of seep animals, and changes

in seepage activity affect the microbial community that provides food and habitat for the macrofaunal community. Methane seeps and mineral-rich hardgrounds provide many ecosystems services but are threatened by bottom trawling, oil and gas extraction, deep-seabed mining, ocean deoxygenation, and ocean warming (Ramirez-Llodra et al. 2011, Thurber et al. 2014, Levin et al. 2016, Schmidtke et al. 2017, Turner et al. 2019, Armstrong et al. 2022, Le et al. 2022). The findings of Chapter 2-4 provide insights for future seafloor management and conservation efforts.

Stewardship efforts will likely have a higher potential for success with increased public support. By incorporating an interdisciplinary approach and conducting an economic valuation study, Chapter 5 (Pereira et al. 2024b) demonstrates that deep-sea management efforts should incorporate people's preferences in order to be well-received by the public. People are willing to pay for protection of methane seeps and, most importantly, seem to have an affection and concern about deep-sea ecosystems that becomes stronger when there is more knowledge of these ecosystems. For example, endemic species were most valued by Costa Ricans, even above fisheries and climate services. This highlights the importance of biodiversity research.

There has been an increase in science communication efforts by the deep-sea science community, encouraging people to create a connection to the deep sea (Jamieson et al. 2020, Jamieson et al. 2022). However, from my personal experience as a science communicator, I see that while the deep sea is reaching more people, the perception that the deep sea is stable rather than dynamic still lingers. Studies like Chapter 2-4 of this dissertation and other recent studies (Alfaro-Lucas et al. 2020, Stratmann et al. 2021, Corrêa et al. 2022, Rybakova et al. 2022, Seabrook et al. 2024, among others) show the opposite. We need to constantly communicate the significance of these new findings to the public.

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