



# Chemosymbiotic species from the Gulf of Cadiz (NE Atlantic): distribution, life styles and nutritional patterns

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**Abstract.** Previous work in the mud volcanoes from the Gulf of Cadiz (South Iberian Margin) revealed a high number of chemosymbiotic species, namely bivalves and siboglinid polychaetes. In this study we give an overview of the distribution and life styles of these species in the Gulf of Cadiz, determine the role of autotrophic symbionts in the nutrition of selected species using stable isotope analyses ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$ ) and investigate the intra-specific variation of isotope signatures within and between study sites. During our studies, we identified twenty siboglinidae and nine bivalve chemosymbiotic species living in fifteen mud volcanoes. Solemyid bivalves and tubeworms of the genus *Siboglinum* are widespread in the study area, whereas other species were found in a single mud volcano (e.g. “*Bathymodiolus mauritanicus*”) or restricted to deeper mud volcanoes (e.g. *Polybrachia* sp., *Lamelisabella denticulata*). Species distribution suggests that different species may adjust their position within the sediment according to their particular needs, and to the intensity and variability of the chemical substrata supply. Tissue stable isotope signatures for selected species are in accordance with values found in other studies, with thiotrophy as the dominant nutritional pathway, and with methanotrophy and mixotrophy emerging as secondary strategies. The heterogeneity in terms of nutrient sources (expressed in the high variance of nitrogen and sulphur values) and the ability to exploit different resources by the different species may explain the high diversity of chemosymbiotic species found in the Gulf of Cadiz. This study increases the knowledge on distributional patterns and resource partitioning of chemosymbiotic species and highlights how trophic fuelling varies on spatial scales with direct implications to seep assemblages and potentially to the biodiversity of continental margin.

## 1 Introduction

The finding that hydrothermal vent mussels have a significantly depleted  $\delta^{13}\text{C}$  signature, very distinct from the values of other typical deep-sea invertebrates, led Rau and Hedges (1979) to postulate that these mussels rely on some non-photosynthetic food source. Later this finding was repeated for several cold seep invertebrates, and confirmed by the observation that they host symbiotic chemoautotrophic bacteria (Conway et al., 1994; Cavanaugh et al., 1992). In this symbiotic relationship, the invertebrate host facilitates the access to inorganic carbon, oxygen and reduced sulphur-compounds, and in return it uptakes the bacterial metabolic byproducts or digests symbiont tissue for its nutrition (Fisher, 1990; MacAvoy et al., 2002). The carbon source for thiotrophic and methanotrophic bacteria is different, with the former using pore water or near-bottom water dissolved inorganic carbon (DIC) and the latter using  $\text{CH}_4$  (Fisher, 1990; Conway et al., 1994).

The different carbon fixation pathways involve distinct isotopic fractionation (e.g. carbon, sulphur and nitrogen), which makes stable isotope approaches particularly useful for elucidating the nutritional status of organisms in vent and seep environments (Conway et al., 1994; Van Dover and Fry, 1994). Carbon isotopic values have been used to differentiate animals with thiotrophic symbionts from those with methanotrophic symbionts (Brooks et al., 1987; Kennicutt et al., 1992), and to identify the source methane pool as either thermogenic or biogenic (Sassen et al., 1999). Chemoautotrophs tend to have lower  $\delta^{15}\text{N}$  values than heterotrophs or marine phytoplankton, and the  $\delta^{34}\text{S}$  values of animals hosting thiotrophic bacteria are depleted relative to animals with methanotrophic symbionts and to heterotrophs

(Brooks et al., 1987). In fact, values of  $\delta^{34}\text{S}$  below 5 ‰ can be used to infer a thiotrophic mode of nutrition (Vetter and Fry, 1998) reflecting the isotopic signature of the sulphide source (Fisher, 1995). Stable isotope signatures can therefore provide information on food resource use and partitioning, both inter- and intraspecifically (Levesque et al., 2003).

Previous work in the Gulf of Cadiz mud volcanoes (South Iberian Margin) found a high number of chemosymbiotic species, namely bivalves and siboglinid polychaetes (Rodrigues et al., 2008; Hilário and Cunha, 2008; Hilário et al., 2010; Oliver et al., 2011), and studies on the faunal assemblages in the area show high biodiversity and high variability in structure, composition and density, which are likely to result from a combination of biogeographic, historical and environmental factors (Cunha et al., 2013). Especially relevant for the distribution of chemosymbiotic species is the variability in the fluid sources and transport mechanisms determining the intensity of the fluxes and the geochemistry of the porewater and sediments.

The Gulf of Cadiz is an extensive cold seepage area including over 40 mud volcanoes at depths between 200 and 4000 m (Pinheiro et al., 2003; Van Rensbergen et al., 2005). The molecular and isotopic composition of the hydrocarbon gases present in the different mud volcanoes reveal a predominance of thermogenic sources with varying degrees of thermal maturity (Mazurenko et al., 2002, 2003; Stadnitskaia et al., 2006; Hensen et al., 2007; Nuzzo et al. 2008) and, in some cases, partial recycling of thermogenic methane and other hydrocarbons mediated by anaerobic oxidation of methane (AOM)-related methanogenic archaea in the shallow sediments (Nuzzo et al., 2009). Differences in both fluid geochemistry and composition of the microbial assemblages responsible for the AOM activity have a major influence on the quantity and chemical composition of the hydrocarbons reaching near-surface sediments or the water column and offer a multitude of physicochemical niches that can be exploited by species with different anatomical features, physiology and symbiotic associations.

Symbiotic bivalves in the Gulf of Cadiz include members of five of the six families known to host chemoautotrophic bacteria: Solemyidae, Lucinidae, Thyasiridae, Vesicomysiidae, and Mytilidae (Génio et al., 2008; Rodrigues et al., 2008; Oliver et al., 2011). These bivalves have different anatomical organization, particularly in relation to the structure of the gill that in all of them are modified to house the symbionts (reviewed by Taylor and Glover, 2010). They also have very different life styles ranging from deep sediment burrowers to epibenthic byssus-tethered species. Bathymodiolin mussels (family Mytilidae) further differ from the other chemosymbiotic bivalves in the way that they have retained their filter feeding ability and are capable of co-hosting sulphur- and methane-oxidising symbionts; the former are also able to utilise hydrogen as energy source (Fisher et al., 1993; Duperron et al., 2008; Petersen et al., 2011).

Siboglinid polychaetes are represented in the Gulf of Cadiz by a high diversity of frenulates (Hilário et al., 2010). Frenulata, the most speciose clade of siboglinids, include a wide range of body sizes and tube morphologies. They live in a variety of reducing environments, including shelf and slope sediments, cold seeps, hydrothermal vents and organic falls, with their tube spanning oxic–anoxic boundaries (Hilário et al., 2011). The anterior end of the tube remains at the sediment surface or extends into the oxygenated bottom water, where oxygen is absorbed by a gill-like structure. Hydrogen sulphide, or methane in the case of *Siboglinum poseidoni* that harbours methane-oxidising endosymbionts (Schmaljohann and Flügel, 1987), is transported across the posterior tube and body wall into the trophosome, a specialized tissue where the endosymbiotic bacteria are housed (reviewed by Hilário et al., 2011). In addition to the nutrition provided by their symbionts, species of the genus *Siboglinum* can take up and metabolise dissolved organic compounds at a rate sufficient to sustain respiration but not enough for growth and reproduction (Southward and Southward, 1981).

Due to its characteristics (biogeographical location, large number of seeps, seep regime variability), the Gulf of Cadiz provides a unique setting for the study of chemosynthesis-based assemblages. The high diversity of chemosymbiotic species found in the Gulf of Cadiz led us to hypothesize that (1) at small spatial scales different morphological and life style adaptations allow the co-occurrence of species and trophic niche partitioning, and (2) trophic diversity under differential geochemical conditions results in high biodiversity at local and regional scales. Herein, we determine the trophic range of chemosymbiotic species using stable isotope analyses ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$ ) and analyse the results in relation to their distributions at different spatial scales. Furthermore, we assess intra and interspecific differences in the trophic range of chemosymbiotic species occurring at the same and at different sites and discuss them in the light of sediment geochemistry.

## 2 Material and methods

Chemosymbiotic species were obtained from macrofaunal samples collected during 14 cruises carried out between 2000 and 2009 from numerous locations in the Gulf of Cadiz using a variety of quantitative and non-quantitative sampling devices and processed on board as described by Rodrigues et al. (2011a). Chemosymbiotic bivalves and siboglinid tubeworms were sorted and identified; *Siboglinum* and *Polybrachia* specimens were ascribed to species according to Hilário et al. (2010) or given new codenames consistent throughout the samples when they did not yet match a known species. Specimens from the largest and more abundant species (five bivalve and seven tubeworm species) were dissected on board and the tissues (e.g. gill and foot for



**Table 1.** Sampling details of specimens collected for stable isotope analysis. Different quantitative and non-quantitative samplers were used. Station code is composed by the cruise name and station number as used in PANGAEA database ([www.pangaea.de](http://www.pangaea.de))

MV Station code	Depth (m)	Latitude (N)	Longitude (W)	Specimens code
Mercator				
TTR15_AT569	358	35°17.92'	06°38.72'	25.1Mr, 27.1Mr
Gemini				
M2007_15	444	35°09.60'	06°49.47'	12.1Ge
M2007_19	430	35°06.44'	06°59.92'	12.2Ge, 25.2Ge, 25.3Ge
Kidd				
TT-R14_AT528	489	35°25.30'	06°43.97'	25.4K
Lazarillo de Tornos				
M2006_38A	494	35°19.09'	06°46.40'	12.3LT
Meknès				
TTR15_AT586	701	34°59.15'	07°04.38'	16.1Mk, 25.5Mk, 25.6Mk
Yuma				
TTR14_AT524	960	35°24.97'	07°05.46'	24.1Y
TTR16_AT604	1030	35°25.82'	07°06.33'	24.2Y, 25.7Y
TTR16_AT605	975	35°25.05'	07°05.45'	11.1Y, 24.3Y, 24.4Y
Ginsburg				
TTR16_AT607	983	35°22.68'	07°04.98'	24.5Gi
Jesus Baraza				
TTR12_AT391	1105	35°35.44'	07°12.26'	24.6JB
Darwin				
TTR16_AT608	1115	35°23.53'	07°11.47'	26.1D, 26.2D
TTR17_AT664	1128	35°23.52'	07°11.48'	25.8D, 26.3D, 26.4D
JC10_032	1109	35°23.52'	07°11.51'	25.9D, 26.5D, 26.6D
Captain Arutyunov				
TTR14_AT544	1330	35°39.71'	07°20.01'	10.1CA
TTR14_AT546	1345	35°39.69'	07°20.05'	10.2CA
MSM01/03_190#1	1322	35°39.66'	07°19.97'	14.1CA
MSM01/03_217#1	1321	35°39.64'	07°20.05'	28.1CA
MSM01/03_225	1320	35°39.71'	07°20.02'	28.2CA
Sagres				
TTR17_AT667	1562	36°02.20'	08°05.54'	12.4S, 24.7S, 25.10S, 28.3S
Carlos Ribeiro				
TTR16_AT615	2200	35°47.24'	08°25.27'	28.4CR, 28.5CR
JC10_057	2175	35°47.25'	08°25.32'	28.6CR
MSM01/03_157	2200	35°47.27'	08°25.36'	28.7CR
MSM01/03_169	2199	35°47.26'	08°25.36'	24.8CR, 28.8CR
Bonjardim				
TTR15_AT597	3061	35°27.56'	09°00.03'	4.1B
Porto				
MSM01/03_145	3860	35°33.70'	09°30.44'	3.1P, 24.9P

The solemyid *Solemya elairrachensis* was the most widespread chemosymbiotic bivalve (Fig. 1 and Supplement Fig. S1) occurring at eight MVs in the shallower El Ar-raiche field (Mercator, Gemini, Kidd) and in the Carbonate Province (Meknès, Yuma, Ginsburg, Darwin and Sagres). Another solemyid *Acharax gadirae* was found in seven MVs at greater water depths in the Carbonate Province (Yuma, Ginsburg, Jesus Baraza) and Deep Field (Capt. Arutyunov, Sagres, Carlos Ribeiro and Porto), with both solemyid species co-occurring at three mud volcanoes. The thyasirids

were represented by two chemosymbiotic species occurring in MVs of the Deep Field – *Thyasira vulcolutre* (Capt. Arutyunov, Sagres and Carlos Ribeiro) and *Spinaxinus sentosus* (Capt. Arutyunov) – and two mixotrophic species occurring mainly at shallower water depths: *Axinulus croulinensis* (Mercator, Meknès and Capt. Arutyunov) and *Thyasira granulosa* (Mercator and Meknès). The three vesicomid species were always found at water depths greater than 1000 m: two small *Isorropodon* species, *I. megadesmus* (Capt. Arutyunov and Darwin MVs) and *I. perplexum* (Capt. Arutyunov), and

the larger *Christenoconcha regab* (Bonjardim MV). Lucinids and mytilids were each represented by a single species in only one MV (*Lucinoma asapheus* at Mercator MV and “*Bathymodiolus*” *mauritanicus* at Darwin MV, respectively).

The unitentaculate Frenulata genus *Siboglinum* was found in 14 MVs. From the nine *Siboglinum* species, *Siboglinum* Ib has the widest distribution in the Gulf of Cadiz, being present in seven MVs from approximately 350 to 3060 m water depth (Fig. 1 and Supplement Fig. S1). *Siboglinum* Ia is present in three MVs and three other species are found in two different MVs. The four remaining *Siboglinum* species are only found in one MV. It is worth noting the presence of the methanotrophic species *Siboglinum* cf. *poseidoni* (Rodrigues et al., 2011b) in the Capt. Arutyunov MV.

With the exception of one species of the genus *Polybrachia*, multitentaculate species are only present in MVs below 1300 m water depth. Only three of the eleven species are found in more than one MV: *Polybrachia* sp.1 is found in Mercator and Bonjardim, *Polybrachia* Va in Carlos Ribeiro and Semenovich, and one species of an undetermined genus (Undetermined Ila) in Carlos Ribeiro and Porto MVs (Fig. 1). The species *Lamelisabella denticulata* and *Spirobrachia tripeira* form conspicuous clumps of dark long tubes that cover the crater of Porto MV. The tubes of *Bobmarleya gadensis* are less conspicuous and more regularly spaced but were also observed forming a continuous field in the crater of Carlos Ribeiro MV.

### 3.2 Stable isotope signatures – interspecific variation

Stable isotope values were obtained for five bivalve species and seven siboglinid species (Table 2; Figs. 2 and 3). Although analyses were carried out in the gill and foot tissues for some bivalve specimens, no coherent differences in the isotopic values of these two tissues were observed and therefore only the average results per specimen are presented. Chemosymbiotic bivalves presented  $\delta^{13}\text{C}$  average values between  $-54.8$  and  $-27.2$ ‰; the most depleted  $\delta^{13}\text{C}$  values were measured in “*Bathymodiolus*” *mauritanicus* from Darwin MV and the least depleted for *Acharax gadirae* from Sagres MV. There was a clear segregation between the mytilids and the other families (Solemyidae, Lucinidae and Thyasiridae) that never showed  $\delta^{13}\text{C}$  values below  $-36.8$ ‰ (Fig. 2, Table 2). Despite some overlap of individual values, the different species showed distinct average  $\delta^{13}\text{C}$  signatures (Figs. 2 and 3). The overall  $\delta^{15}\text{N}$  range for the chemosymbiotic bivalves (10.5‰) was lower than the  $\delta^{13}\text{C}$  range (27.5‰); mean  $\delta^{15}\text{N}$  values varied between  $-1.2$  and  $7.0$ ‰ with *Solemya elarraichensis*, *Lucinoma asapheus* and *Thyasira vulcolutre* showing less depleted values than “*B.*” *mauritanicus* and *A. gadirae* (Fig. 2, Table 2). The  $\delta^{34}\text{S}$  values were the most variable with an overall range of 45.3‰. “*Bathymodiolus*” *mauritanicus* presented lighter  $\delta^{34}\text{S}$  signatures and a relatively narrow range (values between 11.5 and 17.7‰). Thyasirids and solemyids showed

more depleted signatures and much wider  $\delta^{34}\text{S}$  ranges, especially *A. gadirae* (between  $-25.7$  and  $11.9$ ‰) (Fig. 3, Table 2).

The  $\delta^{13}\text{C}$  values for Siboglinidae worms varied between  $-49.8$ ‰ (*Siboglinum* Id from Capt. Arutyunov MV) and  $-33.1$ ‰ (*Siboglinum* Ib, Gemini MV). Values of  $\delta^{13}\text{C}$  lower than  $-40$ ‰ were found for *Siboglinum* cf. *poseidoni* and *Siboglinum* Id, both in the Capt. Arutyunov MV, and for *Lamelisabella denticulata* from Porto MV (Figs. 2 and 3, Table 2). Except for *Siboglinum* Ib specimens from the shallow Gemini and Lazarillo de Tormes MVs that had values ranging from 8.2 to 12.2‰, the majority of the species showed  $\delta^{15}\text{N}$  values below 6‰. Similarly, these two *Siboglinum* Ib samples also presented relatively high values of  $\delta^{34}\text{S}$  (6.5 and 5.7‰). The lowest value of  $\delta^{34}\text{S}$  ( $-16.8$ ‰) was obtained for *Siboglinum* If from Meknès MV (Fig. 3, Table 2).

Overall, the set of different bivalve and tubeworm species in each MV show segregated trophic ranges; there is almost no overlap in  $\delta^{13}\text{C}$  values of co-occurring species and the cases of overlap in  $\delta^{34}\text{S}$  ranges usually involve one tubeworm and one bivalve species (Fig. 3). Also, trophic diversity is lower at the shallower MVs than at the deeper ones where there is a higher number of chemosymbiotic species spanning a wider range of trophic signatures (Fig. 3).

### 3.3 Intra-specific variation within and between study sites

The mean  $\delta^{13}\text{C}$  values for *Acharax gadirae* specimens collected from six different mud volcanoes ranged from  $-27.2$  to  $-34.7$ ‰ (Table 2), with a clear separation of values from specimens collected above ( $-27.2$  to  $-29.3$ ‰ in Ginsburg, Yuma, Jesus Baraza and Sagres MVs) and below 2000 m water depths ( $-31.6$  to  $-34.7$ ‰ in Carlos Ribeiro and Porto MVs). Within the same site, variability was much smaller (1.3‰ in four specimens from Yuma MV). *Solemya elarraichensis* presented small intraspecific variation between sites (2.0‰) and negligible variation within the same site (0.2 to 0.6‰). The  $\delta^{13}\text{C}$  signatures for the thyasirid *Thyasira vulcolutre* varied by 2.7‰, between specimens from Sagres and Carlos Ribeiro MVs, but most of the variation was observed among specimens from Carlos Ribeiro (range of 2.4‰). “*Bathymodiolus*” *mauritanicus* showed the greatest variability within the same site (7.1‰ in Darwin MV).

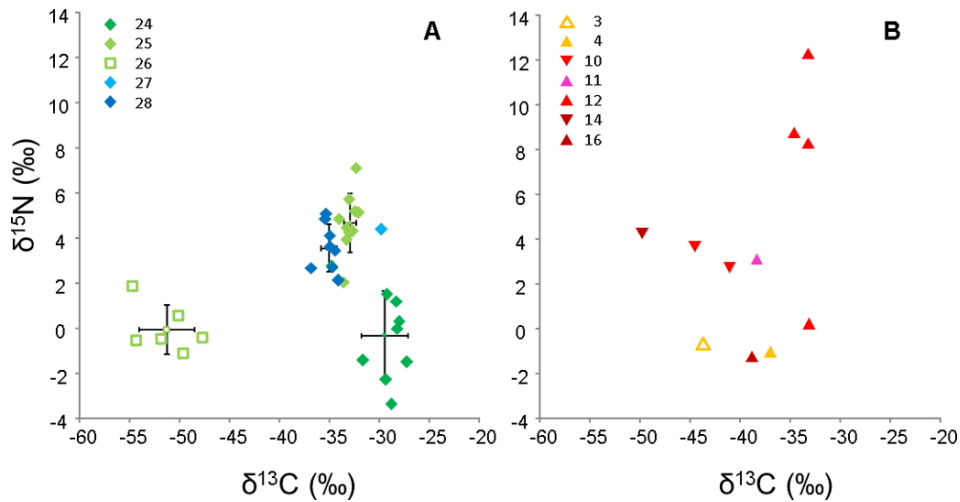
The intraspecific range of mean  $\delta^{15}\text{N}$  values in different sites varied from 3.0‰ (*T. vulcolutre*) to 6.1‰ (*A. gadirae*). Within the same site *S. elarraichensis* showed ranges of 2, 0.5 and 0.4‰ in the Gemini, Meknès and Darwin MVs, respectively. The four specimens of *A. gadirae* collected in the Yuma MV showed the widest range of  $\delta^{15}\text{N}$  values within the same species and site for all studied species (3.8‰). The  $\delta^{15}\text{N}$  ranges were 0.5‰ in Capt. Arutyunov MV and 2.4‰ in Carlos Ribeiro MV for *T. vulcolutre* and 3.0‰ in Darwin MV for “*B.*” *mauritanicus*.

**Table 2.** Stable isotope values of  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$  (mean, standard error – SE and number of samples –  $n$ ) for the different specimens studied here. Specimen codes according to Table 1. \* indicates that several specimens were pooled together

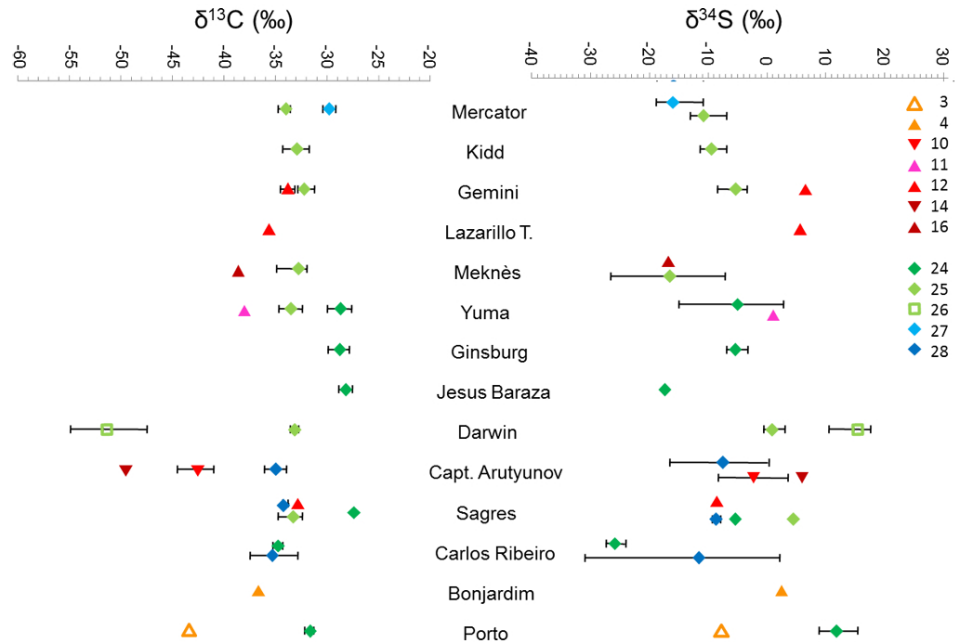
Specimens code	Species name	$\delta^{13}\text{C}$			$\delta^{15}\text{N}$			$\delta^{34}\text{S}$		
		Mean	SE	$n$	Mean	SE	$n$	Mean	SE	$n$
	<b>Siboglinidae – Frenulata</b>									
3.1P	<i>Lamelisabella denticulata</i> *	–43.7		1	–0.6		1	–7.7		1
4.1B	<i>Polybrachia</i> sp.1*	–36.9		1	–1.1		1	2.5		1
10.1CA	<i>Siboglinum</i> cf. <i>poseidoni</i> *	–41.0		1	2.7		1	3.6		1
10.2CA	<i>Siboglinum</i> cf. <i>poseidoni</i> *	–44.5		1	3.6		1	–8.2		1
11.1Y	<i>Siboglinum</i> Ia*	–38.2		1	3.0		1	1.1		1
12.1Ge	<i>Siboglinum</i> Ib*	–34.6		1	8.7		1	6.5		1
12.2Ge	<i>Siboglinum</i> Ib*	–33.1		1	12.2		1			
12.3LT	<i>Siboglinum</i> Ib*	–35.9		1	8.2		1	5.7		1
12.4S	<i>Siboglinum</i> Ib*	–33.0		1	0.2		1	–8.4		1
16.1Mk	<i>Siboglinum</i> If*	–38.7		1	–1.3		1	–16.8		1
14.1CA	<i>Siboglinum</i> Id*	–49.8		1	4.2		1	6.0		1
	<b>Bivalvia</b>									
24.1Y	<i>Acharax gadirae</i> *	–28.0	0.3	3	0.3	0.3	3	–9.2	1.2	3
24.2Y	<i>Acharax gadirae</i>	–29.3	0.6	3	–2.3	0.3	3	–12.3	1.4	3
24.3Y	<i>Acharax gadirae</i>	–28.3	0.3	3	1.2	0.2	3	–0.4	0.9	3
24.4Y	<i>Acharax gadirae</i>	–29.2	0.1	3	1.5	0.4	3	1.9	0.7	3
24.5Gi	<i>Acharax gadirae</i>	–28.8	0.6	3	–3.4	0.3	3	–5.4	1.0	3
24.6JB	<i>Acharax gadirae</i>	–28.2	0.4	3	–0.1	0.1	3	–17.3	0.2	3
24.7S	<i>Acharax gadirae</i>	–27.2		1	–1.5		1	–5.3		1
24.8CR	<i>Acharax gadirae</i>	–34.7	0.3	3	2.7	0.2	3	–25.7	1.7	3
24.9P	<i>Acharax gadirae</i>	–31.6	0.3	3	–1.4	0.2	3	11.9	1.9	3
25.1Mr	<i>Solemya elarraichensis</i> *	–34.0	0.4	3	4.8	0.3	3	–10.7	1.9	3
25.2Ge	<i>Solemya elarraichensis</i>	–32.0	0.5	3	5.1	0.4	3	–4.2	0.5	3
25.3Ge	<i>Solemya elarraichensis</i>	–32.3	0.3	3	7.1	0.5	3	–6.4	1.4	3
25.4K	<i>Solemya elarraichensis</i> *	–32.9	0.8	3	4.2	0.3	3	–9.4	1.3	3
25.5Mk	<i>Solemya elarraichensis</i>	–33.0	0.9	3	5.7	0.2	3	–21.8	4.0	3
25.6Mk	<i>Solemya elarraichensis</i>	–32.4	0.4	3	5.2	0.2	3	–11.2	2.0	3
25.7Y	<i>Solemya elarraichensis</i>	–33.5	1.1	3	2.0	0.4	3			1
25.8D	<i>Solemya elarraichensis</i>	–32.7		1	4.3		1	–0.8		
25.9D	<i>Solemya elarraichensis</i>	–33.2	0.2	3	3.9	0.2	3	2.2	1.0	3
25.10S	<i>Solemya elarraichensis</i>	–33.2	0.7	3	4.4	0.2	3	4.3	0.3	3
26.1D	<i>“Bathymodiolus” mauritanicus</i>	–54.3		1	–0.7		1	17.2		1
26.2D	<i>“Bathymodiolus” mauritanicus</i>	–51.4	0.4	3	–0.4	0.1		16.6	0.0	3
26.3D	<i>“Bathymodiolus” mauritanicus</i>	–54.8		1	1.8		1	11.5		1
26.4D	<i>“Bathymodiolus” mauritanicus</i>	–50.0	0.7	3	0.5	0.1	3	11.6	0.6	3
26.5D	<i>“Bathymodiolus” mauritanicus</i>	–47.7		1	–0.5		1	17.7		1
26.6D	<i>“Bathymodiolus” mauritanicus</i>	–49.5	2.1	3	–1.2	0.3	3	17.7		1
27.1Mr	<i>Lucinoma asapheus</i> *	–29.8	0.4	3	4.4	0.3	3	–16.0	2.5	3
28.1CA	<i>Thyasira vulcolutre</i> *	–34.9	0.6	3	4.1	0.9	3	–14.0	2.4	3
28.2CA	<i>Thyasira vulcolutre</i> *	–34.9	0.0	3	3.6	1.3	3	–0.6	0.6	3
28.3S	<i>Thyasira vulcolutre</i>	–34.1	0.2	3	2.1	0.7	3	–8.4	0.5	3
28.4CR	<i>Thyasira vulcolutre</i>	–36.8	0.4	3	2.6	1.4	3	1.0	0.5	3
28.5CR	<i>Thyasira vulcolutre</i>	–34.6	1.8	3	2.7	2.2	3	2.2	0.0	3
28.6CR	<i>Thyasira vulcolutre</i>	–35.4	1.0	3	4.8	0.9	3			1
28.7CR	<i>Thyasira vulcolutre</i>	–34.4	0.3	3	3.4	0.7	3	–27.6	1.7	3
28.8CR	<i>Thyasira vulcolutre</i>	–35.3	0.4	3	5.1	0.8	3	–21.9		1

Isotopic signatures of  $\delta^{34}\text{S}$  measured in *A. gadirae* showed a wide range (37.6 ‰) with high variability within the same site (14.2 ‰ in Yuma MV) and between different sites (more than 20 ‰). In *S. elarraichensis* the  $\delta^{34}\text{S}$  overall range was

lower: 26.1 ‰ as well as within the same volcano (2.2, 10.6 and 3.0 ‰ in Gemini, Meknès and Darwin MVs, respectively). *Thyasira vulcolutre* presented  $\delta^{34}\text{S}$  ranges of 13.4 ‰ in Capt. Arutyunov MV and 29.8 ‰ in Carlos Ribeiro MV



**Fig. 2.** Mean values (from Table 2) of  $\delta^{13}\text{C}$  vs.  $\delta^{15}\text{N}$  for *Bivalvia* (A) and *Frenulata* (B) from the Gulf of Cadiz. Global mean values of species and respective standard error are also represented in (A). Species codes as defined in Fig. 1.



**Fig. 3.** Total ranges and average values (symbol) of  $\delta^{13}\text{C}$  and  $\delta^{34}\text{S}$  of the different chemosymbiotic species co-occurring in each mud volcano. Species codes as defined in Fig. 1.

(comparable to the overall range for the species). The six specimens of “*B.* *mauritanicus*”, all from Darwin MV, varied by 5.2 ‰.

Intra-specific variation of frenulate isotopic signatures could only be examined for the species *Siboglinum* Ib. The  $\delta^{13}\text{C}$  values varied from  $-35.9$  ‰ in the Lazarillo de Tormes MV to  $-33$  ‰ in the Sagres MV;  $\delta^{15}\text{N}$  values varied from  $0.2$  ‰ (Sagres MV) to  $12.2$  ‰ (Gemini MV) and the  $\delta^{34}\text{S}$  varied from  $-8.4$  (Sagres MV) to  $6.5$  ‰ (Gemini MV).

Within Yuma MV, ranges of  $1.5$  and  $3.5$  ‰ were registered for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , respectively.

Overall, intraspecific  $\delta^{13}\text{C}$  ranges were narrower within the same MV than between different MVs while  $\delta^{34}\text{S}$  ranges were sometimes higher within the same MV than between different MVs (Fig. 3). For each given species  $\delta^{34}\text{S}$  ranges in different MVs were much more variable and usually wider than their  $\delta^{13}\text{C}$  ranges.

## 4 Discussion

### 4.1 Distribution and life styles

Studies that combine form and function are essential to understand the ways in which chemosymbiotic species have evolved to exploit their environment and to interpret their current distribution. Because the association between a metazoan and its symbionts can be seen as an adaptation to bridge oxic–anoxic interfaces (Cavanaugh, 1994), the chemistry profile of the sediments is expected to play a role in determining which species are able to inhabit a particular site (Dando et al., 2008).

Solemyid, lucinid and thyasirid bivalves are sediment dwellers whose burrows span oxic–anoxic interfaces in the seafloor. Whereas solemyids form “U”- or “Y”-shaped burrows (Stewart and Cavanaugh, 2006), lucinids and thyasirids maintain an anterior inhalant connection to the surface (Taylor and Glover, 2000; Dufour, 2005) and produce long ramified burrows. In some thyasirids the length and number of burrows show a negative relationship with the concentration of hydrogen sulphide in the sediment (Dufour and Felbeck, 2003) and it has been proposed that lucinids and thyasirids are able to “mine” insoluble sulphides (Dando et al., 1994, 2004). Details of sulphur acquisition strategies are available for some lucinid and thyasirid species but both groups exhibit such a diversity of morphologies and live in such a wide variety of habitats that it is likely that they utilize a number of different behaviours and chemical pathways to acquire reduced sulphur from the environment (Taylor and Glover, 2000; Dufour, 2005). Regarding Vesicomidae, most species live shallowly burrowed in sediment usually with the posterior half of the shell protruding (Krylova and Sahling, 2010); sulphide uptake is through the foot that protrudes into the sediment, while oxygen uptake is through the gill (Goffredi and Barry, 2002; Taylor and Glover, 2010). Finally, bathymodiolin mussels (family Mytilidae) are epibenthic and live attached by byssus threads to hard substrates, sometimes forming tight aggregates. Their reliance on chemosynthetic symbionts located in their gills implies that reduced compounds are needed in the surrounding fluid, as they do not have access to the anoxic sediment (Duperron, 2010). Bathymodiolin mussels occur in a broad range of environments, which may be attributed to their nutritional flexibility: some species are capable of hosting multiple symbioses, including the co-occurrence of sulphur- and methane-oxidizers, and can obtain additional nutrition from filter feeding (Fisher et al., 1993; Duperron et al., 2008).

Frenulata are generally described as tubeworms that extend over oxic–anoxic boundaries in the sediment, with the anterior end on the top layer of the sediment and the posterior tube and body buried in the sediment. However, this simplified description hides the variety of morphologies of different species that can show wide ranges of sizes (length and width), depth penetration in the sediment and tube thick-

ness, all important features that may influence their ability to exploit different sources of nutrients.

In the Gulf of Cadiz, the methane–sulphate zone, where most microbial activity occurs, is usually more than 30 cm below the seafloor and often at 80 cm or more below the seafloor (Niemann et al., 2006; Hensen et al., 2007). An exception is Capt. Arutyunov MV where high methane concentrations were measured near the surface (Hensen et al., 2007). Burrowing bivalves and frenulates with long bodies were expected in mud volcanoes with wider redox boundaries, living in sediments where high sulphide concentrations are deep below the sediment interface. This holds true for frenulate species; for example the long frenulates *Lamellisabella denticulata*, *Spirobrachia tripeira* and *Bobmarleya gadensis* have only been found in Porto, Bonjardim and Carlos Ribeiro MVs, where high sulphide concentrations are deep (> 50 cm) below the seafloor (Hensen et al., 2007; Nuzzo et al., 2008) and the presence of gas hydrate reservoirs is likely to ensure fluxes capable of sustaining larger body sizes (and consequently higher biomass). In Capt. Arutyunov MV geochemical gradients are steep but highly variable; the sediments are frequently disturbed by gas hydrate dissociation and sustain very high densities of smaller frenulates (*Siboglinum* spp.). At shallower MVs only the slim *Siboglinum* species and also *Polybrachia* sp.1 (at Mercator MV only) are found, but always in more modest densities (Cunha et al., 2013). The presence of small frenulates in MVs with a variety of geochemical settings suggests that smaller and shorter species may have more flexible requirements of reduced compounds, allowing them to exploit even lower concentrations that diffuse to the upper sediments.

The only epibenthic chemosymbiotic bivalve known in the Gulf of Cadiz, “*Bathymodiolus*” *mauritanicus*, was found in dense aggregations inhabiting the narrow fissures between the large carbonate slabs that pave the crater of Darwin MV; aggregations of small (young) individuals are rare, suggesting a strong intraspecific competition for the limited amount of favourable habitat where higher concentrations of reduced compounds reach the seafloor. In the Gulf of Cadiz, unlike many other cold seeps, chemosymbiotic bivalves are predominantly burrowing species that are found in a variety of MVs with very different geochemical settings. Solemyids are widespread but were always found at relatively low densities. Thyasirids are represented by several species; the juveniles and the smaller mixotrophic species may occur in relatively high densities and are often found closer to the sediment surface, while the larger species *Thyasira vulcolutre* is only found at the deeper (more active) MVs. The small vesicomid *Isorropodon megadesmus* is one of the dominant species found in the near surface of gas-saturated sediments in Capt. Arutyunov MV (Cunha et al., 2013). Also for the vesicomids, the larger species *Cristineconcha regab* was only found in one of the deeper MVs.

These distributions suggest that different species and/or ontogenic phases may adjust their position within the



sediment according to their particular needs and to the intensity and variability in the supply of the chemical substrata. Little is known about the distributional patterns at the MV scale but up to five chemosymbiotic species were collected in a single boxcore sample (Cunha et al., 2013). The co-existence of such high numbers of bivalve and tubeworm species differing in size and shape at small spatial scales are a conspicuous manifestation of niche differentiation.

#### 4.2 Methanotrophy vs. thiotrophy

Overall, the isotopic signatures of the chemosymbiotic species indicate the prevalence of a thiotrophic mode of nutrition for most species with the exception of *S. cf. poseidoni* and “*B.*” *mauritanicus*. The presence of microbial symbionts was confirmed in the majority of the studied species with molecular characterization and fluorescent in situ hybridization (Rodrigues et al., 2010, 2011b, 2012, 2013; Rodrigues and Duperron, 2011). The isotopic signatures of the two bivalve tissues analysed (foot and gill) did not show coherent differences in any of the studied species, which as suggested in previous studies (Cary et al., 1989) is an indication of a high degree of carbon flow from the bacterial symbiont to the host.

Carbon isotopic values have been used to identify the source of methane as either biogenic ( $\delta^{13}\text{C} \leq -64\text{‰}$  when purely biogenic) or thermogenic ( $-30 \geq \delta^{13}\text{C} \geq -50\text{‰}$  when purely thermogenic) methane (Schoell, 1980). The  $\delta^{13}\text{C}$  signature of the species harbouring methanotrophic symbionts, *S. cf. poseidoni* and *Siboglinum* Id from Capt. Arutyunov MV, and “*B.*” *mauritanicus* from Darwin MV (Rodrigues et al., 2011b, 2013), are consistent with the inferred thermogenic origin of methane in the Capt. Arutyunov MV in particular and the Gulf of Cadiz in general (Nuzzo et al., 2008).

Average  $\delta^{13}\text{C}$  values for *S.cf. poseidoni* and *Siboglinum* Id were less depleted than values previously reported for *S. poseidoni* collected in Norwegian fjords which are known to harbour methane-oxidising bacteria (Schmaljohann and Flügel, 1987; Supplement Table S2). Nevertheless, the values reported here are compatible with methanotrophic nutrition, and molecular studies have already shown the occurrence of methane-oxidising bacteria in *S.cf. poseidoni* from the Capt. Arutyunov MV (Rodrigues et al., 2011b). The average of  $\delta^{34}\text{S}$  value below 6‰ found in *S.cf. poseidoni* was unexpected as it may indicate thiotrophy. A possible explanation for this value is a putative contamination of the sample by specimens belonging to other species. Because of their small size and morphological similarity, it is possible that co-occurring juveniles of *Polybrachia* sp. 2 may have been pooled together in the analysis, which complicates the interpretation of the results and may explain this apparent contradiction.

The highly depleted  $\delta^{13}\text{C}$  isotope signature of “*B.*” *mauritanicus*, (species confirmed as part of the “childressi” group;

Génio et al., 2008) is in accordance with values previously reported for *B. childressi* (a known methanotrophic mussel; see Table S2). Molecular studies for “*B.*” *mauritanicus* revealed the occurrence of a dual symbiosis with the presence of two phylotypes of methane-oxidising bacteria and a less abundant phylotype of a sulphur-oxidising bacterium (Rodrigues et al., 2013). Although thiotrophy is usually inferred from  $\delta^{34}\text{S}$  values below 5‰ (Vetter and Fry 1998), the low abundance of sulphur-oxidising bacteria can explain the  $\delta^{34}\text{S}$  values above 5‰ found in our study. Isotopic values for mytilids are variable and dependent upon their nutrition, symbionts, ontogeny and local environmental conditions.

The isotope values of other bivalve and siboglinid species are consistent with the use of seawater  $\text{CO}_2$  fixed via sulphide oxidation and are in line with values reported for other species elsewhere. The  $\delta^{13}\text{C}$  values measured in *L. asapheus* are in the range of values reported for lucinids from other locations that are known to harbour thiotrophic symbionts (Table S2). The stable carbon isotope composition of *T. vulcolutre* is also similar to that registered for other *Thyasira* species (*T. methanophila* and *T. sarsi*; Table S2) and indicates that the autotrophic bacteria make a substantial contribution to the nutrition of the host. Chemoautotrophy is usually considered to be more important in the nutrition of thyasirids than of lucinids (Van Dover and Fry, 1989), which also seems to be the case in the Gulf of Cadiz, since *T. vulcolutre* have more depleted values of  $\delta^{13}\text{C}$  than *L. asapheus*. However, more depleted values may reflect local isotopic signatures of methane due to assimilation of pore water bicarbonate derived from microbial sulphate reduction coupled to methane oxidation (Scott et al., 2004; Becker et al., 2011). Methane isotopic signature (Table S1) is considerably less depleted at shallower MVs (higher thermal maturity of methane; Nuzzo et al., 2009) where *L. asphaeus* occurs than in the deeper MVs where *T. vulcolutre* is found.

#### 4.3 Resource partitioning

The high diversity of chemosymbiotic species found in the Gulf of Cadiz may be explained by a combination of historical and contemporary factors (Cunha et al., 2013). The number of species coexisting in a single MV may be as high as nine (e.g. Capt. Arutyunov MV) and usually with no apparent zonation pattern, which suggests that there is resource partitioning. This may occur by means of differentiated life styles, differentiated metabolic pathways and strategies or by a combination of these. If chemical resource partitioning occurs, consistent differences in isotope values between species may be expected (Becker et al., 2011). Co-occurrence of phylogenetically close species is particularly interesting and in the Gulf of Cadiz it may be observed both for thyasirids and solemyids.

Overall, *Solemya elarraichensis* presented more depleted  $\delta^{13}\text{C}$  and more enriched  $\delta^{15}\text{N}$  signatures than *Acharax gadi-rae*, which are probably consequences of metabolic and

morphological differences between the two species. Symbionts of co-generics of both species are known to use different forms of the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) for CO<sub>2</sub> fixation. Whereas symbionts of *Solemya* species present RubisCO form I (Scott et al., 2004), symbionts of *Acharax* present the form II of the enzyme (Duperron et al., 2012), which has lower discrimination factors against <sup>13</sup>CO<sub>2</sub>. Previous studies have shown that the difference in discrimination against <sup>13</sup>C isotopes by the two forms of RubisCO can explain the disproportion in the <sup>13</sup>C values between two groups of chemosymbiotic invertebrates: the “−30‰ δ<sup>13</sup>C group” with a form I RubisCO and the “−11‰ δ<sup>13</sup>C group” expressing a form II (Robinson and Cavanaugh, 1995).

When compared with other taxa that also present form II RubisCO (e.g. vent vestimentiferans), the values found for *A. gadirae* are more depleted but are in accordance with the values found for the bivalves *Calymene magnifica* and *Calymene pacifica* (Southward et al., 1994; Fisher, 1995), which suggests that bivalves expressing RubisCO form II may have factors other than this enzyme structure and kinetics contributing to the carbon fractionation.

It is worth noting that within our samples of *A. gadirae*, specimens from deeper MVs presented more depleted δ<sup>13</sup>C values and in the same range of values found for *S. elarraichensis*. Oliver et al. (2011) ascribed *Acharax* specimens from Porto and Carlos Ribeiro mud volcanoes to the new species *A. gadirae* but because of the small size of the available specimens the same authors raised the possibility of the existence of a different species in these mud volcanoes, which could also explain the difference in the isotopic signatures.

When compared with previous data, the δ<sup>13</sup>C values of *S. elarraichensis* and of *A. gadirae* from the deeper mud volcanoes were similar to those found for other species of the same genera (Table S2). However, several differences were found in the δ<sup>15</sup>N values between solemyid species, suggesting that they are using different chemical species of nitrogen, tapping different pools of nitrogen, or discriminating differently after acquisition of their nitrogen source. Furthermore, the δ<sup>15</sup>N range may likely reflect a dilution of the very high <sup>15</sup>N ammonium values occurring at the sediment water interface (Lee and Childress, 1996; Carlier et al., 2010).

Nitrogen isotope values measured in various species of chemosymbiotic bivalves and frenulate tubeworms showed a wide range of values (Table S1), including one species with δ<sup>15</sup>N values above 6‰ (value indicated by Levin and Michener (2002) as upper limit for the presence of chemoautotrophic symbionts). Similar relatively high values were found in *Siboglinum* Ib collected from three of the shallowest mud volcanoes and are probably related to the fact that these animals can exploit different sources of nitrogen. *Siboglinum* species are known to be able of take up and metabolize dissolved organic compounds in addition to the nutrition provided by their symbionts (Southward and Southward, 1981).

Furthermore, because of the shallower water depth they may have access to organic nitrogen derived from surface waters. On the contrary, low δ<sup>15</sup>N values indicate that organic nitrogen is of local origin, presumably resulting from the activity of autotrophic bacteria (Levin, 2005; MacAvoy et al., 2008), and are possibly due to assimilation of isotopically light nitrate or ammonium by the symbionts or, in the case of the very negative values, possibly fixation of N<sub>2</sub> (Fisher, 1995). As proposed by other authors (Carlier et al., 2010), we hypothesize that the inter-specific variations of δ<sup>15</sup>N found in this study may result from species-specific types of symbionts characterized by different fractionation factors during the assimilation of dissolved inorganic nitrogen, and that in the particular case of frenulate tubeworms, different species may have different degrees of access and abilities to exploit dissolved organic compounds.

In general, there was considerably less intra-specific δ<sup>13</sup>C variation between specimens collected from the same volcano than from different MVs, which is plausibly explained by a higher variation in the composition of hydrocarbon pools at larger spatial scales (from one MV to another). These differences may be lower at shallow MVs as high thermal maturity of the gases is consistent with more limited differences in carbon isotopic composition (Nuzzo et al., 2009). However, there were relevant intra-specific differences in the isotopic signatures of chemosymbiotic species both within and between MVs. The observed intra-specific variation in the δ<sup>13</sup>C may result from the assimilation of DIC of diverse origins depending on the MV, and in variable proportions, but also from the variable δ<sup>13</sup>C fractionation occurring during DIC fixation by endosymbionts, with the isotopic fractionation depending on the growth rate, and therefore on the size of each individual, as reported by other studies (Carlier et al., 2010).

Similar arguments can be used to explain the differences in the δ<sup>34</sup>S signatures. There is little fractionation between sulphide and sulphate or organic sulphur as a result of sulphide oxidation by chemoautolithotrophic bacteria (reviewed in Canfield, 2001). Therefore, δ<sup>34</sup>S values of animals with chemoautolithotrophic sulphur-oxidising symbionts reflect their reduced sulphur source (MacAvoy et al., 2005) and probably the relative abundance of reduced compounds available. δ<sup>34</sup>S values were highly variable among individuals of the same species, either in different MVs or within the same MV, reflecting high variability in the sulphide pool on a very small spatial scale. According to Becker et al. (2011), this pattern of high variability is consistent with the cycling of sulphate and sulphide between chemosymbiotic invertebrates and sediment microbial consortia. It is known that in the Gulf of Cadiz, the variations in environmental setting and AOM activity are reflected by very diverse microbial community compositions and that the three microbial consortia known to perform this reaction (ANME-1, -2, and -3) are active with different distribution patterns (Vanreusel et al., 2009). It is therefore probable that a diversity of sulphide sources

is available locally for exploitation by the chemosymbiotic metazoans.

## 5 Conclusions

The number of chemosymbiotic species found in the Gulf of Cadiz is high when compared with other seep locations such as the mud volcanoes in the Eastern Mediterranean Sea and the Gulf of Guinea (Hilário et al., 2010; Oliver et al., 2011). Here we report for the first time on the distribution and life styles of these species and investigate resource partitioning at multiple spatial scales. Our results highlight the relevance of seep chemoautolithotrophic production in this area and provide a starting point for future trophic ecology studies. Long term observations on the biology of chemosymbiotic species coupled with geochemical measurements are still needed to better understand the interplay between environmental variability and symbiont dynamics.

In the Gulf of Cadiz thiotrophy is the dominant nutritional pathway, with methanotrophy and mixotrophy emerging as secondary strategies. We found a wide span of  $\delta^{13}\text{C}$  isotopic signatures that show well-segregated ranges between species and are relatively consistent within species as a result of species-specific metabolic pathways. Contrastingly, the  $\delta^{34}\text{S}$  values are highly variable, both within and between species, and are likely the result of the great variability of the sulphide pool at local and regional scales. The diversity and the distribution of chemosymbiotic species in the Gulf of Cadiz reflect the environmental heterogeneity in terms of nutrient sources at local and regional scales. The mild seepage regimes at the shallower MVs favour the occurrence of mixotroph species that are capable of shifting opportunistically their feeding mode, while the deeper MVs with more intense although highly variable seep regimes sustain a higher trophic diversity (and biomass). The coexistence of an assortment of life styles is a conspicuous manifestation of niche differentiation and allows resource partitioning at multiple spatial scales. Overall, the variety of nutrient sources, metabolic pathways, feeding strategies and life styles translates into a unique taxonomical diversity with a direct impact on the seep assemblage and a relevant contribution to the regional biodiversity.

**Supplementary material related to this article is available online at: <http://www.biogeosciences.net/10/2569/2013/bg-10-2569-2013-supplement.pdf>.**

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