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no Golfo de Cadiz**

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica da Doutora Maria Marina Ribeiro Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Frenulata, vulcões de lama, Golfo de Cadis, isótopos estáveis

resumo

Os Siboglinideos são anelídeos tubícolas, componentes importantes de comunidades quimiossintéticas profundas como fontes hidrotermais, fontes frias, carcaças de baleias e sedimentos reduzidos. Em adultos, estes poliqueta não possuem um sistema digestivo funcional, sendo dependentes de endossimbiontes microbianos para as suas necessidades energéticas. Atualmente são reconhecidas quatro linhagens principais dentro da família Siboglinidae: Frenulata, Vestimentifera, Sclerolinum e Osedax, dos quais os Frenulata são os menos estudados. A descoberta de comunidades quimiossintéticas dominadas por Frenulata em vários vulcões de lama no Golfo de Cádiz e a possibilidade de explorar estes ecossistemas através de diversos programas de pesquisa tem sido uma boa oportunidade para expandir o conhecimentos neste fascinate grupo de animais.

Como outros animais quimiossintéticos, os Frenulata tem valores de $\delta^{13}\text{C}$ empobrecidos resultantes das vias metabólicas das suas bactérias associadas. Neste estudo as assinaturas de isótopos estáveis foram utilizadas para determinar (1) a fonte de energia (metano ou enxofre) das bactérias endossimbiontes das diferentes espécies de Frenulata, (2) a ocorrência de partição de recursos entre espécies que habitam o mesmo local e (3) diferenças nas fontes de energia da mesma espécie proveniente de diferentes locais. Adicionalmente os efeitos de diferentes métodos de conservação (congelamento, álcool e formaldeído) nas assinaturas isotópicas foram comparados.

O valor de $\delta^{13}\text{C}$ de ambas as espécies colhidas no vulcão de lama Porto sugere que as mesmas podem hospedar bactérias metanotróficas, e que são capazes de explorar microhabitats diferentes num mesmo vulcão de lama. As assinaturas isotópicas de carbono foram mais (*Spirobranchia tripeira* e *Lamelisabella denticulata*) ou menos (*Siboglinum* cf. *poseidoni*) baixas do que o esperado sugerindo a ocorrência de uma simbiose dupla, sugerindo que este tipo de simbiose pode ser mais frequente do que se pensava em Frenulata. Hipoteticamente a simbiose dupla pode permitir aos Frenulata sobreviver em ambientes químicos temporal e espacialmente variáveis como é o caso dos vulcões de lama.

Os efeitos dos métodos de preservação nos valores de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$ estão de acordo com estudos anteriores, sugerindo que amostras de Frenulata preservadas quimicamente podem ser utilizadas para estudos de ecologia trófica. Porém, os resultados obtidos para amostras que incluem o tubo de quitina característico destes animais devem ser interpretados com cautela.

keywords

Frenulate, mud volcanoes, Gulf of Cadiz, Stable isotopes

abstract

Siboglinids are tube-dwelling annelids that are important members of deep-sea chemosynthetic communities, which include hydrothermal vents, cold seeps, whale-falls and reduced sediments. As adults, they lack a functional digestive system and rely on microbial endosymbionts for their energetic needs. Currently most researchers recognize four main lineages within Siboglinidae, Frenulata, Vestimentifera, *Sclerolinum* and *Osedax*, of which Frenulata is the most understudied. The discovery of chemosynthetic-based communities dominated by frenulates in several mud volcanoes in the Gulf of Cadiz, and the possibility to explore these habitats through several research programmes has been an excellent opportunity to extend our knowledge on these fascinating tubeworms. As other chemosymbiotic animals, frenulates have depleted values of $\delta^{13}\text{C}$ that result from the metabolic pathways of their associated bacteria. In this study, stable isotope signatures were used to determine (1) the energy source (methane or sulphur) of the endosymbionts of different species of frenulates, (2) the occurrence of resource partitioning between species inhabiting the same site and (3) differences in the energy sources of the same species inhabiting different sites. Additionally, the effect of different sample preservation methodologies (frozen, ethanol, and formaldehyde) in the isotopic signatures was also compared.

The $\delta^{13}\text{C}$ values of both species from Porto mud volcano indicate that these species may host methanotrophic symbionts and that they are able to exploit different microhabitats within the same seep site. The carbon isotopic signatures were more (*Spirobrachia tripeira* and *Lamellisabella denticulata*) or less (*Siboglinum* cf. *poseidoni*) depleted than expected suggesting that dual symbiosis may be more prevalent than previously thought in frenulates. It is hypothesized that dual symbioses allow frenulates to survive in the spatially and temporally variable chemical environment of mud volcanoes.

The effects of the preservation method on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values is in agreement with previous studies and suggest that frenulate samples preserved chemically can be used in trophic ecology studies. However results obtained with samples including the chitinous tube of the animals should be interpreted with caution.

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I. Introduction

The history of deep-sea biology is littered with generalisations abandoned after the discovery of exceptions to their “rules”. One of the most striking revelations in the recent history of deep-sea biology was the discovery of exceptions to the exponential decrease in biomass along a depth gradient in the form of luxuriant animal communities at deep-sea hydrothermal vents. First discovered along the Galapagos Rift in 1977 (Corliss and Ballard 1977), these communities are remarkable for their use of geothermal energy as an alternative to solar radiation in the preservation of inorganic carbon. Supported by high levels of *in situ* microbial-mediated chemosynthesis, hydrothermal vents, in contrast to “normal” deep-sea, have very high biomass and relatively low diversity with highly adapted, mainly novel, megafaunal and macrofaunal species (Tunnicliffe 1991). This discovery led to one of the most active programmes in deep-sea exploration and biology.

Soon after chemoautotrophic communities were discovered at hydrothermal vents, dense communities of animals were encountered at brine seeps along the base of the Florida Escarpment in the Gulf of Mexico (Paull *et al.* 1984) and then at sites of crustal destruction in subduction zones along convergent margins (Sibuet and Olu 1998). Many of the invertebrates in these “cold seep” communities are taxonomically allied to those of hydrothermal vents and the nutritional basis for their high biomass also have a chemoautotrophic basis. More recently, the study of chemosynthetic fauna has extended to the communities that develop in other reducing habitats such as whale falls (Smith *et al.* 1989), sunken wood and areas of oxygen minima when they intersect with the margin or seamounts (Levin 2003).

Although the domain of cold seeps has received considerably less attention compared to the investigations of deep-sea hydrothermal vents, cold seeps are now known to be among the most geologically diverse and widely distributed of the deep-sea reducing environments explored to date, and new sites are still being discovered every year. Also, it has been suggested that the diversity is greater at cold seeps and that the interactions between geological and

biological systems are more complex at low than high temperatures (Aharon 1994; Tunnicliffe *et al.* 1996).

I.1. Cold seeps geological settings and fluid flow

Since their initial discovery, active seeps have been reported from shallow to hadal (> 6000 m) depths (Sibuet and Olu-Le Roy 2002; Levin 2005, and references therein), along other active and passive margins, and from all parts of the global ocean, even Antarctic regions (Domack *et al.* 2005) (Figure 1). Cold seeps are related to geological processes such as tectonically-induced high-fluid pressures, petroleum or natural gas escape, artesian flow or catastrophic erosion and submarine slides.

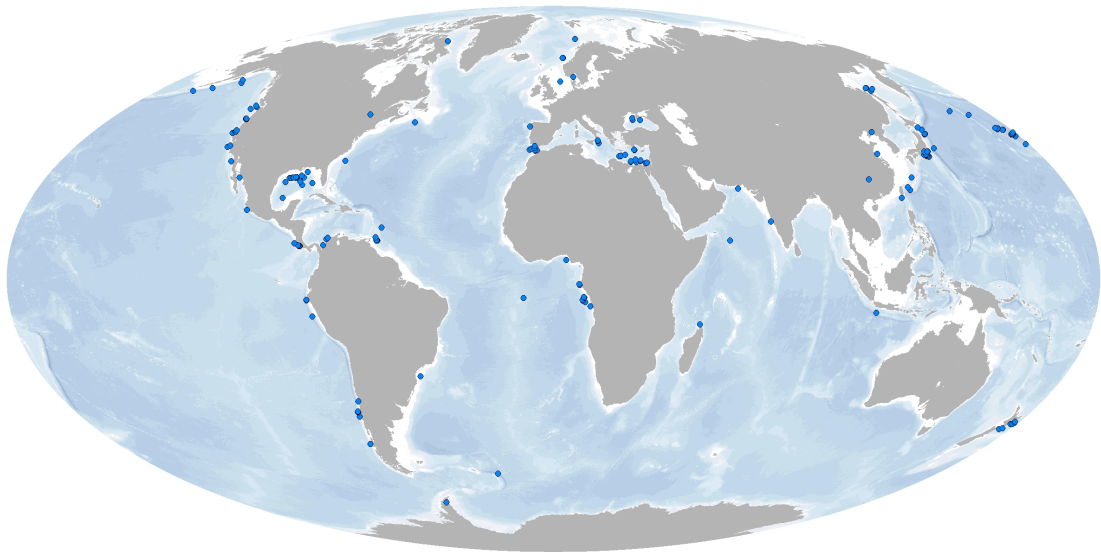


Figure 1. Map showing all known deep-water cold seep sites that have been studied with respect to their fauna. This map was produced by Maria Baker of the ChEss programme in September 2010.

On passive margins seeping is usually associated with oil and gas reservoirs. In some passive margins, salt tectonics creates the conduits for the seeping fluids. Ancient salt deposits below the sediments where hydrocarbons and methane have accumulated push upward (because they are less dense), forming deep cracks in the sediments through which gases and petroleum escape (Kennicutt *et al.* 1985). Fluids may also arise from sulphide-rich brine seeps, as described from the Florida Escarpment, located at the juncture of the escarpment base and the abyssal sediment plain (Paull *et al.* 1984). Seepage of methane and hydrocarbons of thermogenic may also occur through shallow pockmarks along the eroding crest of a steep anticline belonging to the transform fault (Simoneit *et al.* 1990). These cold seeps are usually located a few kilometers from a hydrothermal vent field and are colonised by chemosynthetic animals similar to those found at other cold seeps but also by species usually found at hydrothermal vents.

In subduction areas, seeps occur both on well-developed accretionary prisms and along erosive margins to a depth of at least 6000 m (Tunnicliffe *et al.* 2003). On accretionary prisms faults develop in the compacting sediment, providing conduits for trapped fluid, which may be of either continental or oceanic origin. Compressional forces yield not only active thrust faults near the deformation front, but can also yield diapiric structures like mud volcanoes that are created by an influx of water from deep over-pressured zones (Olu *et al.* 1997). At some sites the subduction trench is pulling apart, and the associated seismic activity forces out fluids. In erosive margins and landslides seepage occurs on the head scars of large-scale debris slides probably linked to earthquake occurrences (Olu *et al.* 1996), and along canyon walls where sediment piles have been removed by tectonic motions and faults on seamounts entering subduction zones (Sibuet and Olu 1998).

Understanding of the different sources and forms of seep systems continues to grow as new seep settings are encountered. Interactions between hydrothermal venting, methane seepage and carbonate precipitation have led to several new constructs in both shallow (Michaelis *et al.* 2002; Canet *et al.* 2003) and deep water (Kelly *et al.* 2001). New settings may be discovered where spreading

ridges (e.g., Chile Triple Junction) or seamounts (e.g., Aleutian Archipelago) encounter subduction zones, or when seepage occurs within oxygen minima (Schmaljohann *et al.* 2001; Salas and Woodside 2002). Mass wasting from earthquakes, tsunamis or turbidity currents may generate or expose reduced sediments and yield seep communities as well (e.g., Mayer *et al.* 1988).

Unlike hydrothermal vent systems, where aerobic sulphide oxidation was identified as the most important potential energy source for chemotrophy, both aerobic and anaerobic methane oxidation are probably the most important chemosynthetic processes at cold seeps. However, in association with organic remains, two anaerobic processes are important to chemosynthesis based on sulphide oxidation. At active margins seeps, sulphide is produced in near-surface sediments as a result of bacterial reduction of sulphate, which utilizes methane or other hydrocarbons as energy sources (Carney 1994). Reduction of sulphate in seawater also occurs as a terminal metabolic process in the anaerobic degradation of organic remains, providing sulphide for development of microbial mats and invertebrate symbioses. The carbon sources for chemosynthesis in cold seeps are organic: methane, petroleum, other hydrocarbon gases, or solid gas hydrate that derive from accumulated sedimentary organic carbon (Tunnicliffe *et al.* 2003).

Large variation of fluid flow rates within a single cold seep area and between different sites have been observed, with direct and indirect measurements of fluid flow on some active seep sites have shown values ranging from 86 to 1765 ml^2d^{-1} (Sibuet and Olu 1998). Spatial and temporal variations in the fluid supply are thought to be responsible for the patchy and ephemeral occurrence of chemosynthetic fauna in seep areas (Olu *et al.* 1996, 1997). However, it remains difficult to relate biological production to flow rates together with the chemical composition of the fluids because the biological and geochemical analysis and measurement of fluid flow are seldom undertaken together.

I.2. Cold seep macrofaunal communities

The physical and chemical conditions of reducing environments select for a small pool of inhabitants compared to the huge diversity in the deep-sea. The adaptations required to partake of the enhanced productivity of these environments have greatly limited the dominant inhabitants to a relatively few groups. While the oasis analogy is used to describe these islands of plenty (e.g., Carney 1994), few taxa have found it beneficial. Most of the general kinds of animals that populate reducing environments live nowhere else in the deep-sea except in other reducing environments (Hessler and Kaharl 1995). Most significantly, not only the same species, but entire groups of animals are found only at reducing environments showing that vent and seep fauna are endemic at high taxonomic levels, including class and order.

At cold seeps the dominant macrofaunal species are large symbiont-containing species that rely on sulphide or methane oxidation, or both, carried out by their chemoautotrophic bacterial symbionts (Sibuet and Olu 1998). Symbiont containing species include Vesicomidae, Mytilidae, Solemyidae, Thyasiridae and Lucinidae bivalves, Siboglinidae tubeworms and Cladorhizidae and Hymedesmiidae sponges. The occurrence and distribution of these taxa in different seepage areas are thought to be related to the depth of methane/sulphide gradients in the sediment (Rodrigues 2009). It has been suggested that in most cold seeps dissolved sulphide does not escape from near-surface sediments, restricting the utilization of sulphide to animals that extend part of their body into the sediment (e.g. clams and some siboglinid tubeworms) and to sediment microorganisms. The type of symbiont-containing invertebrate that dominates a site may depend on which compound(s) the symbionts can exploit.

For benthic organisms in reducing environments, substratum and supply of reducing substances are intimately linked. Substrata are colonized because they serve as a medium for molecular diffusion or fluid flow or are located in the path of fluid discharge providing access to reducing substances or the products of chemosynthesis. Thus, the availability of appropriate substrata may influence

faunal community composition. Cold seeps are predominantly soft-bottom environments, but hard substrata exist in the form of carbonate concretions, rocky outcrops, clam shells, siboglinid tubes and even methane hydrates. While there are a considerable variety of hard surfaces at seeps, understanding of their importance to seep-community composition and diversity is still very incomplete (Tunnicliffe *et al.* 2003).

Apart from "obligate" species that are restricted to sites in direct proximity to fluids rich in sulphide, methane, or other reducing inorganic compounds (e.g. ammonia; Fisher 1990), cold seeps macrofaunal communities are also made up of "regional" species which occur in the seepage area and also in neighbouring non-seep habitats. This regional fauna may forage on chemosynthetic biota (e.g. *Neptunea amianta*, lithodid crabs), but typically are not dependent on chemosynthetic production (Barry *et al.* 1996). Colonization of seeps by species not containing symbionts, endemic or otherwise, contributes to the development of a complex ecosystem. Seeps represent localized perturbations of the vast and well-established soft-bottom benthic environment of the deep-sea. The participation of non-symbiont-containing deep-sea species in seep food webs is an important feature at several sites where extremely high densities of meiofauna, suspension feeders, deposit feeders and carnivorous occur (Carney 1994).

It is unlikely that there is a complete accounting of macrofaunal diversity within any single seep ecosystem but there is evidence that diversity is high relative to hydrothermal vent habitats (Levin 2005). In relation to the "normal" deep-sea, cold seeps, due to food availability for local fauna, present higher biomass and abundance (Agard *et al.* 1993). In a review of the ecology of cold seeps Levin (2005) showed that total macrofaunal densities at seeps may be impoverished (North Sea: Dando *et al.* 1991), enhanced (Santa Barbara: 1980; Oregon; Gulf of Mexico: Levin *et al.* 2003), or identical to those in nearby non-seep sediments.

I.2.1. Siboglinid tubeworms

Siboglinid tubeworms are ecologically important members of deep-sea chemosynthetic communities, including hydrothermal vents and cold seeps. Some are community dominants and others are primary colonists of new vent sites (Shank *et al.* 1998); they include some of the longest living (Fisher *et al.* 1997) and fastest growing marine invertebrates (Lutz *et al.* 1994).

The fact that siboglinids tend to be found in deep-sea sediments resulted in the first member of this group, *Siboglinum weberi* Caullery, 1914 (Caullery 1914), not being described until early in the 20th century. There are now more than 100 nominal species described (Rouse 2001), most from abyssal regions, though exceptionally they are found in depth of less than 100 m (Southward and Culter 1986; Miura *et al.* 1997).

The varied and complex taxonomic history of Siboglinidae (previously known as Pogonophora) represents one of the more fascinating tales in animal systematics: they have been placed in both Deutorostomia (Ivanov and Petrunkevitch 1955; Southward and Southward 1963) and Protostomia (van der Land and Nörrevang 1975; Southward 1988) and have been assigned to all taxonomic ranks from family to phylum. The first member of the group was found, Caullery (1914) named the genus *Siboglinum* and the family Siboglinidae, without assigning the animal to a particular phylum. Annelid affinities are now supported by both morphological and genetic studies. Rouse and Fauchald 1997 conducted a series of cladistic analysis of the morphology of polychaetes and stated that: "The Pogonophora should now be reclassified as members of the clade Sabellida". They argued that "since the name Pogonophora was misleading at this level, the name of the group should revert to that of the first family name originally formulated for members of the group, that of Siboglinidae Caullery, 1914". This name change was also proposed by (McHugh 1997), it has since then been used by several authors (Schulze 2003; Halanych 2005; Hilario *et al.* 2011). Currently most researchers recognize four main lineages within Siboglinidae: Frenulata, Vestimentifera, *Sclerolinum* and *Osedax*.

As adults all siboglinids lack a digestive track and live in an obligatory association with endosymbiotic bacteria housed in a specialized tissue called trophosome (Cavanaugh *et al.* 1981; Felbeck 1981; Southward *et al.* 1981; Southward *et al.* 1986). Given the conspicuous absence of a digestive system, many functional studies of siboglinids have concentrated on the question of nutrition. Early hypotheses centred on the possibility of dissolved organic matter (DOM) uptake across the body wall (Southward and Southward 1980). The twin papers of Cavanaugh *et al.* (1981) and Felbeck (1981) revolutionized this viewpoint by showing that larger siboglinids utilized symbiosis with chemoautotrophic bacteria.

Although biological generalizations are often problematic, each siboglinid clade is, in general, found in a certain type of habitat. Frenulates are typically found in muddy (often deep) environments; vestimentiferans typically occur in hydrothermal vent and hydrocarbon seep areas; *Sclerolinum* is known to live on decaying organic matter (e.g., wood and rope) but also occurs free-living in mud; whereas *Osedax* is found exclusively on vertebrate bones (Figure 2).

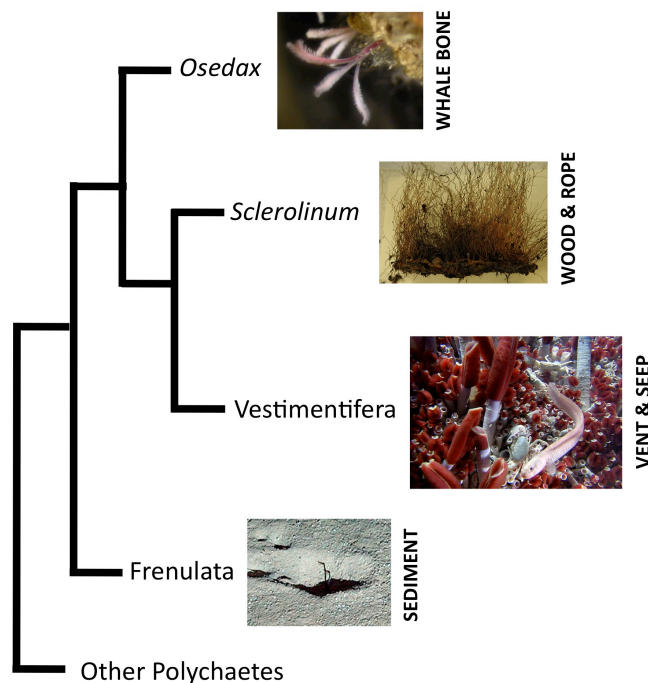


Figure 2. Phylogenetic relationships amongst Siboglinidae (adapted from Hilário *et al.* 2011).

The critical environmental feature in common between all these habitats is the presence of a reduction-oxidation (REDOX) boundary. Living at the REDOX boundary, vent, seep and anoxic mud siboglinids fuel their bacterial symbionts with oxygen, sulphide and carbon dioxide via some unique adaptations to their circulatory system (Southward *et al.* 2005). Bacterial symbionts then fix CO₂ into organic molecules using sulphide as the energy source (Arp *et al.* 1987; Fisher 1990). At the whale-bone habitat of *Osedax*, less is known about the chemical milieu but a REDOX boundary and high levels of sulphide are also present reviewed by (Hilario *et al.* 2011). Siboglinids living in different environments have evolved adaptations to exploit differences in food and sulphide (or in some cases methane) availability. Whereas vestimentiferans living on hydrothermal vent chimneys absorb sulphide through a branchial plume that extends up to 2 m into the water column (Andersen *et al.* 2002), vestimentiferans living in cold seeps obtain sulphide from the sediment, across the wall of the buried tube (Julian *et al.* 1999). Frenulates, notwithstanding some exceptions, are found mainly in organic-rich, reduced sediments. Because frenulates can transport dissolved organic matter across their tube and body wall (Southward and Southward 1981), sulphide is presumably transported across the thin tube that is buried in the sediment, but data supporting this are scarce. In the case of the frenulate *Siboglinum poseidoni*, methanogenesis is reported (Schmaljohann *et al.* 1990). Sulphide levels or uptake location have not yet been investigated for *Sclerolinum* species, and for *Osedax*.

One of the biggest issues in the study of siboglinid ecology and evolution is the lack of sampling of frenulates (Halanych 2005; Hilário *et al.* 2011). Sampling constraints, a shortage of taxonomic expertise, and the fact that for a long time specimens were taken as bycatch and often poorly preserved have all contributed to the current situation of Frenulata being the least-studied group of siboglinids. In spite of being the first described (Caullery 1914), the most diverse and widely distributed siboglinid lineage. The discovery of chemosynthetic communities in mud volcanoes in the Gulf of Cadiz, often dominated by frenulates, and explorations run by several research programmes have provided excellent opportunities to sample these fascinating worms. At

least 21 species of several genera of frenulates are found in this area (A. Hilário personal communication), some new to science (Hilário and Cunha 2008; Hilario *et al.* 2010). This remarkable diversity of frenulates is unprecedented and is hypothesized to result from environmental heterogeneity associated with the bathymetric and geochemical settings of these mud volcanoes.

Because frenulates absorb reduced compounds through the posterior parts of their tubes, the REDOX profile of the sediments it expected to play a role in determining which species are able to inhabit a particular site (Dando *et al.* 2008). Shorter species are expected to live in mud volcanoes with higher concentrations of reduced compounds in upper layers of the sediment and vice-versa. Larger frenulates whit long bodies are expected to bridge wider REDOX boundaries, living in sediments where reduced compounds are expelled in seep fluids or diffused to the upper sediment layers. Nonetheless, shorter and smaller species may demand less of these nutrient gases, allowing them to exploit lower concentrations that diffuse to the upper sediment. These hypotheses might help to explain the presence of the long-bodied (> 30 cm) some species in Porto and others mud volcanoes where high sulphide concentrations are deep below the sediment interface (Hensen *et al.* 2007; Nuzzo *et al.* 2008). However, these mud volcanoes are the deepest presently known in the Gulf of Cadiz suggesting that bathymetry may also play a role in the distribution of these species.

I.3. Trophic ecology

The presence of hydrogen sulphide in hydrothermal fluids and an abundance of sulphide oxidizing bacteria were the first clues leading to the hypothesis that faunal communities at deep-sea reducing habitats are sustained by microorganisms that chemosynthesize organic matter from carbon dioxide and mineral nutrients. The microbes catalyse the oxidation of hydrogen sulphide and other reducing substances and use the chemical energy released to produce adenosine triphosphate (ATP) required for carbon dioxide reduction.

Chemosynthesis is a process that has been microbiologically and biochemically

well studied. However, its quantitative role in the carbon cycle of the photosynthetically dominated Earth's surface has never been considered to be significant until the discovery of deep-sea reducing habitats (Jannasch 1995). At cold seeps, several compartments for primary production can be recognised including endo- and ectosymbiont production, near bottom and subsurface-derived suspended microbial production, microbial production on inanimate surfaces, but also sinking photosynthetically derived production (Tunnicliffe 1991; MacAvoy *et al.* 2005). In spite of being fuelled by chemosynthesis, reducing environments are closely linked to the photosynthetic ecosystems in the upper layer of the ocean. All animals and many microorganisms require dissolved oxygen for their metabolism, which is a by-product of photosynthesis. At seeps, in addition to the requirement of dissolved oxygen for respiration, the methane that powers seep chemosynthesis is derived from photosynthetically-produced organic matter.

Although both aerobic and anaerobic methane oxidation are important chemosynthetic processes at cold seeps, where methane is initially the most abundant reducing substance in migrating fluids, the key process in these systems is the microbially mediated anaerobic oxidation of methane (AOM) coupled to sulphate reduction (Boetius *et al.* 2000). One product of this process is dissolved sulphide that is used by free-living thiotrophic bacteria and invertebrate (e.g. siboglinids, mytilids, lucinids and vesicomysids) symbioses. These symbioses allow the bacteria to have a better access to reduced compounds such as methane or sulphide and to oxidants enabling them to fix CO₂, while the eukaryotic hosts acquire carbon from symbionts either through translocation of nutrients or direct digestion of bacteria (Krueger and Cavanaugh 1997).

Despite their chemosynthetic base, deep-sea reducing habitats present a trophic structure comparable in many ways to food webs of shallow-water ecosystems. In addition to primary producers, there are a variety of consumer types among macro- and megafauna (i.e., grazers, suspension-feeders, deposit-feeders, predators, parasites, commensals) (Van Dover 2000). Nutrition of seep fauna varies, with sulphur-oxidizing and methano-oxidizing symbiotic

bacteria fuelling most of the megafaunal forms but macrofauna and most meiofauna are mainly heterotrophic. Macrofaunal food sources are largely photosynthesis based at shallower seeps but reflect carbon preservation by chemosynthesis and considerable incorporation of methane derived carbon at deeper sites (Levin *et al.* 2000). Mechanisms of export benthic production from cold seeps are often related to activities of marauding predators, including fish and crabs that are not endemic residents of the seep environment.

Traditional approaches to food web studies usually include gut content analysis, together with field and laboratory observations. However, these approaches are difficult to apply when studying the deep-sea because of restricted accessibility (low temporal and spatial resolution) as well as technical problems: live organisms for experimental studies are difficult to obtain and gut content analyses are hampered by specimens being damaged during sampling and pressure effects (Hobson and Welch 1992; Iken *et al.* 2001). Biochemical markers such as stable isotope can provide temporally integrated signatures of trophic relationships and offer distinct advantages over conventional dietary techniques once (1) trophic-level information is based on assimilates, not just ingest foods and (2) trophic positions represent long-term averages, and depending on the metabolic rate of the tissue that is being measured (Tieszen *et al.* 1983; Iken *et al.* 2001).

I.3.1. Stable isotope Technique

I.3.1.1 Notation

Natural abundances of stable (non decaying) isotopes of tissues in organisms have proven to be useful biomarkers in studies of trophic ecology because large functional groups of primary producers have distinctive ratios (R) of $^{13}\text{C}:^{12}\text{C}$.

Isotopes differ in the number of neutrons in the atom. The atomic weight of each natural element is the weighted average of its isotopes. The isotopes might be divided in two fundamental types, the stable and the unstable (radioactive).

Isotopic composition is the relative distribution of isotopes of a given element, usually expressed in the form of the ratio of a less common isotope to the most common (e.g. $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$, $^{34}\text{S}/^{32}\text{S}$). Ratios of stable isotopes are typically expressed as delta values in “per mil” notation (‰):

$$\delta X = \left[\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right] \times 10^3 (\text{‰})$$

Where δX is the isotope ratio in δ units relative to a standard, and R_{sample} and R_{standard} are the absolute isotope ratios of the sample and standard, respectively.

In ecology studies, the stable isotopes most frequently used are carbon ($^{13}\text{C}/^{12}\text{C}$), nitrogen ($^{15}\text{N}/^{14}\text{N}$) and sulphur ($^{34}\text{S}/^{32}\text{S}$). All isotope values reported in literature are referenced to primary standards. The standard for carbon is a marine limestone fossil (the Pee Dee Belemnite) (Craig 1953), for nitrogen, the atmospheric air (Mariotti 1983), and for sulphur the standard is a troilite from the Canyon Diablo iron meteorite (CTD) (Krouse 1980).

Because they are relative numbers, isotope ratios, expressed as ‰, can be positive or negative. Positive values of δX indicate enrichment of the sample in the heavy isotope compared to the standard, and negative values imply depletion of the heavy isotope in the sample relative to the standard.

In primary producers, both physical and enzymatic processes discriminate against the heavier carbon isotope during preservation of CO_2 . During most trophic interactions in the marine environment, carbon isotope ratios increase only by about 1‰ (Peterson and Fry 1987), lending credence to the notion that, for carbon isotopes at least, “you are what you eat”.

While carbon isotopes display the direct relationship between diet and consumers, nitrogen isotope ratios increase by ~3 to 4‰ with each successive trophic level (DeNiro and Epstein 1981; Minagawa and Wada 1984). In addition Nitrogen isotopes can also be used to differentiate nutrient sources. Using a dual isotope approach, the carbon and nitrogen isotopic compositions of the consumers can be used together with estimated trophic shifts to outline what

must have been the isotopic characteristics of consumer invertebrate diets (Van Dover 2000).

Sulphur stable isotopes, albeit much less used, are most useful in discriminating between thiotrophic and non-thiotrophic modes of nutrition in marine invertebrates (Brooks *et al.* 1987; Vetter and Fry 1998).

I.3.1.2. Stable isotopes in food webs

The autochthonous, non photosynthetic nature of the food web base in deep-sea reducing ecosystems was established for the first time in the eastern Pacific vents due to the very distinct $\delta^{13}\text{C}$ values of tube worms, clams and mussel tissues (Rau and Hedges 1979). These values were either far more positive ($\sim -12\text{‰}$ in tubeworms) or far more negative ($\sim -35\text{‰}$ in bivalves) than photosynthetically derived carbon reaching the seafloor ($\sim -18\text{‰}$). Carbon isotope analyses have since then been used to demonstrate autochthonous, chemoautotrophic-based food webs in a variety of deep-sea and shallow water ecosystems, including hydrothermal vents (Van Dover and Fry 1989, 1994; Vetter and Fry 1998; Van Dover 2002; Colaço *et al.* 2002; Yamanaka *et al.* 2003; Levesque *et al.* 2006; De Busserolles *et al.* 2009), whale falls (Deming *et al.* 1997) and seep sites from California (Levin and Michener 2001, Levin *et al.* 2003), Gulf of Mexico (MacAvoy *et al.* 2002, 2005; Levin and Mendoza 2007), Haakon Mosby Mud volcano (Gebruk *et al.* 2003) and Eastern Mediterranean (Werne *et al.* 2002; Olu-Le Roy *et al.* 2004; Carlier *et al.* 2010).

Phytoplankton-derived organic matter typically has $\delta^{13}\text{C}$ signatures between -15‰ and -25‰ (Fry and Sherr 1984). Organisms with a diet based on carbon produced by marine chemoautotrophs will be expected to have $\delta^{13}\text{C}$ values considerably more negative than organisms whose diet is based on photosynthetically fixed carbon, or less negative if in presence of Rubisco form II. In fact, carbon preservation fuelled by energy derived from sulphide oxidation that involves Rubisco form I often produces $\delta^{13}\text{C}$ values between -27‰ and -37‰ , but alternative pathways that involve Rubisco form II can yield much heavier $\delta^{13}\text{C}$ values (-9 to -16‰) (Brooks *et al.* 1987; Fisher 1990; Robinson

and Cavanaugh 1995). In addition, bacteria that use methane, both as energy and carbon sources, are more ^{13}C -depleted (usually $<40\%$) than sulphur-oxidising bacteria that fix dissolved inorganic carbon from the water column ($-35 < \delta^{13}\text{C} < -27\%$) (Paull *et al.* 1985). This is because methane is much more ^{13}C -depleted (usually $<-40\%$) (Whiticar 1999; Milkov 2005) than water column dissolved inorganic carbon ($\sim 0\%$) and also because methane is assimilated by bacteria with a small carbon-isotopic fractionation (Alperin *et al.* 1988). Carbon values can also help to identify the source of methane pool as either thermogenic ($\delta^{13}\text{C} = -40$ to -45%) or biogenic ($\delta^{13}\text{C} \leq -45\%$) (Sassen *et al.* 1999).

Specimens in reduced settings that bear chemoautotrophic symbionts often exhibit light, sometimes negative $\delta^{15}\text{N}$ signatures if local inorganic nitrogen is assimilated or fixed (Conway *et al.* 1994). Chemoautotrophs tend to have lower $\delta^{15}\text{N}$ values (-5 to -12%) than heterotrophs (2.8 to 13%) or marine phytoplankton (Levin and Michener 2001; MacAvoy *et al.* 2002).

To support growth based on sulphide oxidation and carbon dioxide preservation, inorganic nitrogen (e.g. nitrate and ammonia) must also be assimilated, the mechanisms by which these symbioses assimilate ammonia and nitrate is not well characterized, but many of the transformations are probably localized in the bacterial symbionts. Many species of bacteria assimilate nitrate and ammonia into amino acids. In some species, nitrate serves both as an alternative electron acceptor and as a source of nitrogen. In both cases, nitrate is first reduced by nitrate reductase to nitrite which can subsequently be reduced to ammonia (Lee *et al.* 1999).

Sulphur stable isotopes discriminate between organic matter produced in the water column (by phytoplankton) and organic matter synthesised in reduced sediments by chemosynthetic microorganisms (Brooks *et al.* 1987; Vetter and Fry 1998). Indeed, sulphates in the water column show homogeneous $\delta^{34}\text{S}$ values ($\sim +21\%$), and their preservation by phytoplankton occurs with a small negative fractionation, resulting in oceanic particulate organic matter with similar signatures ($+17 < \delta^{34}\text{S} < +21\%$; Peterson and Fry 1987). Benthic fauna that only depend on phytoplanktonic production for their food show $\delta^{34}\text{S}$ values in the

same range (Fry 1988; Peterson 1999) because sulphur isotopes do not significantly fractionate between trophic levels (McCutchan *et al.* 2003). Dissimilatory sulphate reduction by bacteria within the sediment results in a strong fractionation and much more ^{34}S - depleted (-25 to ~5%) sulphides. Consequently, organisms that assimilate these reduced compounds (e.g. sulphur-oxidising bacteria and organisms that depend on them) exhibit low $\delta^{34}\text{S}$ values as well (<5%). Therefore, in cases where the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values alone fail to unambiguously identify energetic pathways and carbon sources, additional $\delta^{34}\text{S}$ analyses may allow to differentiate between input of photosynthetic and chemosynthetic (or methanotrophic vs. thiotrophic) material for seep-related organisms (Brooks *et al.* 1987; Kennicutt *et al.* 1992; MacAvoy *et al.* 2005).

Three important aspects must be considered when comparing the isotopic compositions of different individuals or different species. First, if two organisms use the same dietary carbon, nitrogen and sulphur sources they will have similar isotopic compositions. Second, organisms having the same isotopic compositions do not necessarily have the same diet, since they can have different diets with the same averaged isotopic compositions. Third, a difference in the isotopic composition of two organisms reflects a difference in their diets. Stable isotopic compositions can therefore provide information on food resource use and partitioning, both interspecifically and intraspecifically (Levesque *et al.* 2003).

I.4. Objectives

The discovery of a remarkable diversity of frenulates in several seepage areas in the Gulf of Cadiz (GoC), allied to the possibility to explore these habitats through several research programmes, present an excellent opportunity to extend our knowledge on the ecology of this understudied group of siboglinids.

Frenulates, as other chemosymbiotic animals, have depleted values of $\delta^{13}\text{C}$ that result from the metabolic pathways of their symbionts. In this study, stable isotope signatures are used to:

1. establish the sources of C, N and S for the nutrition of different species of frenulates;
2. determine the occurrence of resource partitioning between frenulate species inhabiting the same site;
3. determine differences in C, N and S isotopic signatures of frenulate species inhabiting different sites;

In addition, the effect of different sample preservation methodologies (frozen, ethanol, and formaldehyde) in the isotopic signatures will also be compared.

II. Material and methods

II.1. Study area - Gulf of Cadiz

The Gulf of Cadiz, located between 34°N and 37°15'N and 6°W to 9°45'W is enclosed by the South Iberian Atlantic Margin and the North African Atlantic Margin of Morocco, west of the Gibraltar Strait (Figure 3). For more than a decade, the international marine scientific community has deployed considerable efforts in studying the occurrence of cold seepage, mainly in the form of mud volcanism, and gas hydrates in the Gulf of Cadiz (Baraza and Ercilla 1996; Pinheiro *et al.* 2003; Somoza *et al.* 2003). Since the discovery of the gulf's first mud volcano in 1999, research cruises have steadily unveiled one mud volcano after another (Gardner 2001; Pinheiro *et al.* 2003; Somoza *et al.* 2003; Van Rensbergen *et al.* 2005).

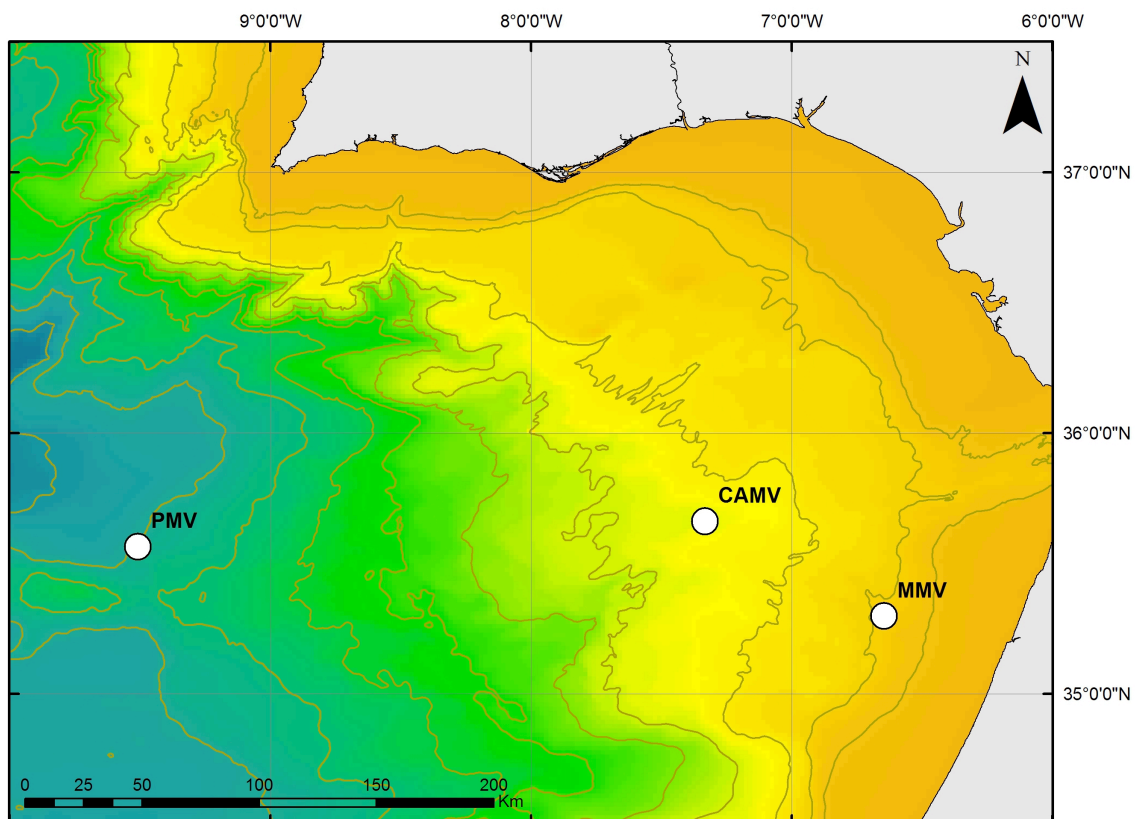


Figure 3. Geographic location of the study area (Por – Porto MV, CA – Captain Arutyunov MV, Mer – Mercator MV).

The geological settings of the Gulf of Cadiz are extremely complex and still under debate (Sartori *et al.* 1994; Maldonado *et al.* 1999; Gutscher *et al.* 2002). However, it is now broadly accepted that this region is deeply faulted and seismically active (Buforn *et al.* 1995; Ribeiro *et al.* 1996) because of two major geodynamic driver mechanisms, the subduction associated with the westward emplacement of the Gibraltar Arc and formation of the Gulf of Cadiz accretionary wedge, probably not active at present, and oblique lithosphere collision between Iberia and Nubia, active at present and causing active thrusting (Zitellini *et al.* 2009).

Because of its location in a compressional tectonic province, active mud volcanism in the Gulf of Cadiz is a widespread phenomenon, extending from the Iberian-Moroccan shelf to the deeper regions in the centre of the gulf (Foucher *et al.* 2009). This mud volcanism is characterized by a wide diversity of processes and environmental settings, such as different types of fluid migration pathways, tectonic activity and/or salt diapirism, migration velocity, fluid composition and alteration processes, depth, sea bottom temperature (from 4°C to 13°C if under the influence of the Mediterranean outflow water), and the presence of gas hydrate (Niemann *et al.* 2006). Structurally, mud volcanoes are equally diverse, ranging in shape from amorphous mud pies to conical structures, and in size from a few meters to kilometres in diameter, attaining heights of up to 100m (Dimitrov 2002). Ground truthing evidence of the active mud volcanoes include the presence of hydrocarbons of thermogenic origin (Stadnitskaia *et al.* 2006) and fluids derived from clay mineral dehydration, indicating active processes of fluid migration from depths up to 5 km below the seafloor (Hensen *et al.* 2007).

Biogeographically, the Gulf of Cadiz is located in the North East Atlantic warm temperate zone at the boundary of the Mediterranean Sea zone. Together with the Azores, Madeira and Canary Islands, known by their high biodiversity and level of endemism, West Iberia and the Gulf of Cadiz belong to the Lusitanian province that connects at north to the Biscay region and at south to the Senegal West African region. This privileged location, in a biogeographic crossroad where the oceanographic circulation establishes pathways for organism

dispersal and favours the links to the Mediterranean, African and European regions, together with a high environmental heterogeneity is thought to favour speciation and to sustain the high biodiversity of the region.

Like in other seepage areas, factors affecting the biodiversity and species distributional patterns in the mud volcanoes of the Gulf of Cadiz are likely to include pore water hydrogen sulphide concentrations and fluxes, oxygen availability and sediment structural characteristics (Sahling *et al.* 2002; Levin *et al.* 2003). The environmental heterogeneity related to bathymetry and differences in activity and fluid composition between mud volcanoes (Pinheiro *et al.* 2003; Van Rensbergen *et al.* 2005; Hensen *et al.* 2007; Niemann *et al.* 2006) is likely to lead to high diversity of several taxa, including Frenulata (Rodrigues 2009, Hilário *et al.* 2010).

In the shallower mud volcanoes the typical seep fauna is represented by a lower number of inconspicuous species such as the small-sized *Siboglinum spp.* and buried bivalves (Solemyidae and Lucinidae). In the deeper mud volcanoes, however, species richness and density of the assemblages are generally much lower but the degree of endemism is clearly higher and mud volcanoes are colonized by a high diversity of typical seep fauna. Here, large-sized Frenulata sometimes forming conspicuous clumps and fields of Thyasiridae or Vesicomidae bivalves may reach high densities in patchy aggregations (Rodrigues 2009).

Samples were collected from three mud volcanoes the Gulf of Cadiz: the Mercator mud volcano in the El Arraiche mud volcano field, and the Porto and Captain Arutyunov mud volcanoes in the deep-water mud volcano field.

Mercator mud volcano (MMV)

The Mercator mud volcano, at 350 m depth is one of the shallowest mud volcanoes in the Gulf of Cadiz. MMV is set at the southern flank of the Vernadsky Ridge within a 2 km wide, N–S oriented, collapse zone; it is asymmetric with a moat along its southern and western side, it has a maximum diameter of 2.45 km at the base and 1.1 km at its top and its highest point (141 m) is at the southern side. Mercator has a semi-concentric pattern related to the

fronts of mudflow lobes, rather than a radial outward mudflow pattern, a 2 m deep rimmed-crater occurs at the northern side but is absent at the southern side. The top of the mud volcano consists of a crater and a 38 m high central dome with patches of disturbed sediments from which gas venting is occasionally observed. A smaller buried structure, interpreted as a buried mud volcano occurs south of MMV within the same collapse zone (Van Rensbergen *et al.* 2005).

Chemically, the sediment pore fluids from the MMV are extremely enriched in chloride reaching up to 5.3 M (thus, exceeding normal seawater values by a factor of 9), and the Na/Cl ratios are close to 1 suggesting halite dissolution by the ascending fluid (Haeckel *et al.* 2007).

The most conspicuous organisms seen during video surveys of the MMV crater were solitary corals (*Caryophyllia* sp.), accompanied by Cidaridae echinoids and Onuphidae polychaetes (*Hyalinoecia*) (Oliver *et al.* 2011).

Captain Arutyunov mud volcano (CAMV)

The Captain Arutyunov mud volcano was discovered during the TTR12 (Training Through Research) cruise on board the research vessel *Prof. Logachev*. It is a cone shaped structure with a relief of up to 200 m and a maximum diameter of 4.9 km at approximately 1320 m depth.

The fluids originate from clay mineral dehydration at a sediment depth of about 5000 m (Hensen *et al.* 2007). However these fluids are enriched in both Na and Cl and the approximately constant Na/Cl ratio of 1 is a signal of halite dissolution (Hensen *et al.* 2007).

Seafloor observations revealed distinct habitats on top of CAMV that were closely associated with methane seepage and high methane pore water concentrations close to the sediment surface. These habitats comprise seafloor areas densely populated by frenulates and sediments where frenulates occurred only sporadically, but numerous unsorted clasts of mainly mudstones were present. On the flanks of the CAMV, seabed morphology changed and no further indications of fluid or gas seepage were found (Sommer *et al.* 2009). The total seabed methane emission from CAMV is small compared to the

overall methane release from other known mud volcanoes (Foucher *et al.* 2009), which appears to be related to slow fluid flow. This slow fluid flow provides enough time for anaerobic methanotrophic bacteria to consume a large proportion of the ascending methane, and for the settlement of Frenulata species, which directly and indirectly affects methane turnover. However, there are localized sites with enhanced and probably periodic, methane release where concentrations of this compound can reach 20 μM up to 1 m above the seafloor (Sommer *et al.* 2009).

Porto mud volcano (PMV)

The Porto mud volcano, at approximately 3900 m depth, was discovered during the TTR15 (Training Through Research) cruise on board the research vessel *Prof. Logachev*. It is located at the toe of the active sedimentary wedge in association with a major deep strike-slip fault that marks the Africa-Eurasia Plate Boundary Zone (Zitellini *et al.* 2009). Pore water and sediment chemical analyses suggest that the methane in PMV is produced in deep in sediment and has a biogenic rather than a thermogenic origin (Nuzzo *et al.* 2009), which is contrasting with all other mud volcanoes studied in the Gulf of Cadiz.

The flat top of the PMV is covered by a continuous field of clumps of 20 to 50 individuals of frenulates that are accompanied mainly by stalked hexactinellid sponges and crinoids. Many old tubes and sponge stalks are colonised by epifaunal organisms (hydrozoans, actinarians, cirripeds and other). Mobile fauna (galatheid lobsters, ophiuroids and holothurians) and scattered shells of Solemyidae bivalves were also observed among the tubes (Hilário and Cunha 2008).

II.2. Study species

In this study four species of Frenulata were analyzed (Table 1), two species from the genus *Siboglinum*, one unidentified species (Figure 4A) and *Siboglinum* cf. *poseidoni* (Figure 4B), *Spirobrachia tripeira* (Figure 4C) and *Lamellisabella denticulata* (Figure 4D).

Table 1. Collection and preservation data for the specimens used in this study. PMV (Porto mud volcano), CAMV (Captain Arutyunov mud volcano), MMV (Mercator mud volcano), E (96% Ethanol), F (4% seawater formaldehyde), Fr (-20°C freeze), GR (Tv Grab), MUC (multiple core), GKG (Box corer).

Species	Mud volcano	Cruise	Station	Latitude (N)	Longitude (W)	Depth (m)	Preservation method
<i>Spirobrachia tripeira</i>	PMV	TTR17	AT683 GR	35°33.750'	09°30.365'	3890	E, F
<i>Spirobrachia tripeira</i>	PMV	TTR16	AT622 GR	35°33.773'	09°30.416'	3902	E
<i>Lamellisabella denticulata</i>	PMV	TTR17	AT683 GR	35°33.750'	09°30.365'	3890	E, F
<i>Lamellisabella denticulata</i>	PMV	MSM01-03	162-MUC05	35°33.738'	09°30.492'	3863	Fr
<i>Siboglinum sp.</i>	CAMV	MSM01-03	180-GKG7	35°39.740'	07°19.960'	1323	E
<i>Siboglinum sp.</i>	MMV	MSM01-03	242-GKG13	35°17.870'	06°38.810'	350	E
<i>Siboglinum cf. poseidoni</i>	CAMV	MSM01-03	217-GKG10	35°39.642'	07°20.049'	1321	E
Miscellaneous	CAMV	TTR14	AT544 GR	35°39.692'	07°20.046'	1345	Fr
Miscellaneous	CAMV	MSM01-03	190-MUC09	35°39.668'	07°19.970'	1320	Fr

Genus *Siboglinum* (Caullery, 1914)

Siboglinum is the most widespread genus of Frenulata, with 69 described species occurring in all oceans in depths ranging from 22 to 8000 m. The genus is characterized by a single anterior tentacle, with or without pinnules and without epidermal glands; the anterior part of the trunk carries two rows of papillae, containing pyriform glands, almost always without adhesive plaques; there are two or three girdles of chaetae; the postannular part of the trunk carries ventral papillae, singly or in small groups, opposed by large dorsal glandular shields; the tube is usually ringed, and sometimes also segmented (Ivanov 1963). Two species of *Siboglinum* were analyzed in this study: *Siboglinum cf. poseidoni* and one unidentified species.

The unidentified species of *Siboglinum* (Figure 4A) used here was collected from the Captain Arutyunov and Mercator mud volcanoes. This species, although not yet described has previously been used in molecular studies (Hilário *et al.* 2010).

Siboglinum poseidoni is the only frenulate known to be hermaphroditic and to harbour methanotrophic endosymbionts (Flügel and Langhof 1983; Schmaljohann and Flügel 1987). *Siboglinum poseidoni* was described from methane seeps in the Skagerrak (Flügel and Langhof 1983) and its presence

has been reported from the Captain Arutyunov mud volcano in the Gulf of Cadiz (Figure 4B; Hilário *et al.* 2010) and the Carmen mud volcano in the Alboran Sea (Hilário pers. comm.).

Genus *Spirobrachia* (Ivanov, 1952)

The genus *Spirobrachia* is characterized by the presence of multiple (39-223) tentacles united into a plate that is spirally wound; the bases of the tentacles are arranged in a screw spiral that may be attached to a lophophore; the protosoma is combined with the mesosoma and a median furrow runs along the dorsal side of the mesosoma; in the front part of the trunk there are cuticular plaques shaped like bows or hoops that lie on pulvilli; the spermatophores are narrow, with a pair of wings at the base of the filament; the tube is hard with a soft and flimsy anterior end, having traces of segments with delicate collars (Ivanov 1952; Smirnov 2000).

Spirobrachia tripeira (Hilário and Cunha 2008), described from specimens collected in the Porto mud volcano is the first species of this genus to be reported from the Atlantic Ocean and from mid latitudes. Previously *Spirobrachia* species had only been known from high latitudes, both South and North (Hilário and Cunha 2008). *Spirobrachia tripeira* is found in the Porto mud volcano in clumps of 20 to 50 individuals accompanied by an undetermined number of other frenulate species (*Lamellisabella denticulata* and *Polybrachia* sp.)

Genus *Lamellisabella* (Ushakov, 1933)

Species of genus *Lamellisabella* are characterized by the presence of multiple tentacles arranged side-by-side forming a hollow cylinder and horseshoe-shaped metameric plaques in the forepart of the trunk; the protosoma is joined to the mesosoma and the cephalic lobe to the protosoma; the bridles are well developed with pronounced keels tapering strongly towards the dorsal ends; the spermatophores are leaf-shaped and broad; the tube may be smooth or segmented with funnel-like mouth (Ivanov 1963).

Lamellisabella denticulata is the only species of this genus described from the

Atlantic Ocean (Southward 1978). It was first found in the Bay of Biscay at 4000 m depth, but Southward (1978) proposed that its range extends along the continental rise as far south as the Gulf of Guinea. In the Gulf of Cadiz it was found on the Porto mud volcano (Hilário and Cunha 2008; Hilário *et al.* 2010).

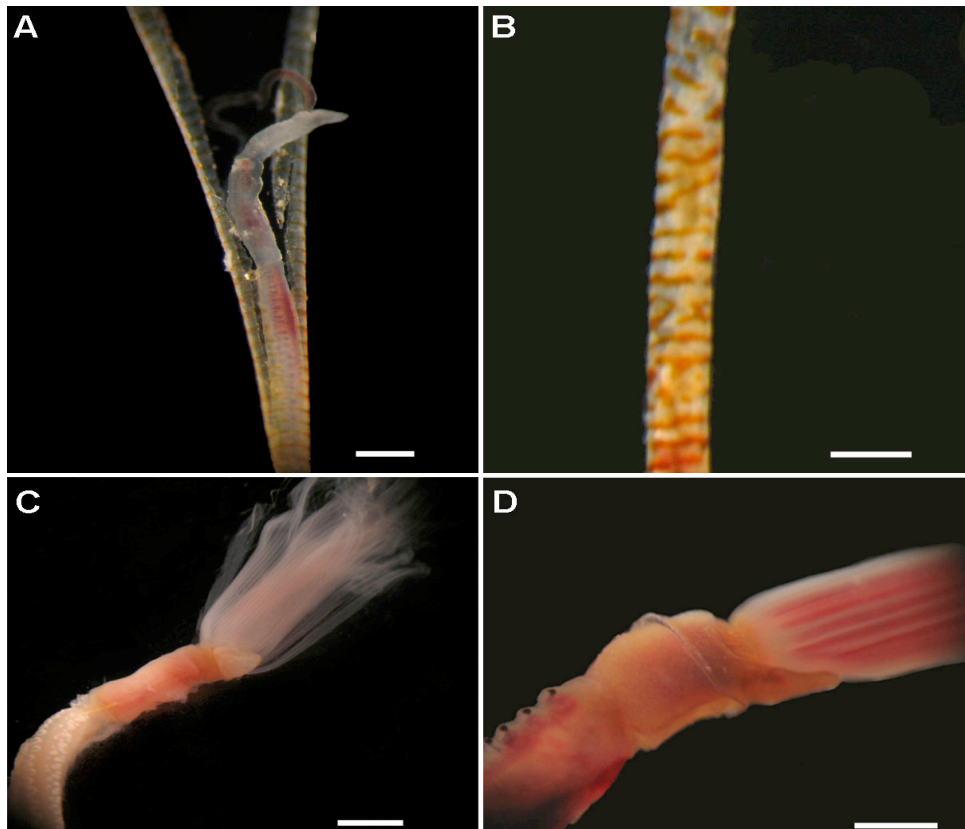


Figure 4. A- *Siboglinum sp1* (scale 0,5mm), B- *Siboglinum poseidoni* (scale 0,5mm), C- *Spirobrachia tripeira* (scale1mm), D- *Lamellisabella denticulata* (scale1mm). Hilário, A.

II.3 Sample collection and processing

Specimens of the four species were obtained from TV-guided grab (GR), multiple corer (MUC) and box corer (GKG) samples collected on Porto, Captain Arutyunov and Mercator mud volcanoes. Specimens of *Spirobrachia tripeira* and *Lamellisabella denticulata* were collected on the Porto mud volcano; specimens of *Siboglinum cf. poseidoni* were collected on the Captain Arutyunov mud volcano and *Siboglinum sp.* were collected on the Mercator and Captain Arutyunov mud volcanoes. The specimens were carefully picked from the

sediment, and preserved in 96% ethanol or 4% seawater formaldehyde. A summary of the collection and preservation data is presented in Table 1. In all cruises the sample processing was initiated onboard with different procedures according to the specific samplers.

Specimens of the four species were identified under a stereomicroscope and the specimens of *Spirobrachia tripeira* and *Lamellisabella denticulata* were dissected out of their tubes. Because of the minute size of *Siboglinum* species, the animals were not dissected. Single specimens (*Spirobrachia tripeira* and *Lamellisabella denticulata*) or groups of approximately 40 specimens (*Siboglinum* sp. and *Siboglinum* cf. *poseidoni*) were used for stable isotope analyses.

All samples were lyophilized and homogenized in a fine powder using a mortar-and-pestle grinding tool. Tissue samples were separated in subsamples devoted to $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ analyses.

Due to the small amount of material, sub-samples for $\delta^{13}\text{C}$ analyses were not decarbonated by immersion in liquid HCL (10%), instead $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were measured directly. For $\delta^{34}\text{S}$ analyses sub-samples of the same species were pooled together and were re-suspended in distilled water, shaken for 5 min and centrifuged, and the supernatant discarded. This procedure was repeated twice, and the samples were finally dried at 60°C and re-ground. All samples were analyzed at ISO-Analytical Laboratory (Cheshire, UK) using the elemental analysis-isotope ratio MS method.

Frozen samples from CAMV containing *Siboglinum* sp. and *Siboglinum* cf. *poseidoni* and one frozen specimen of *Lamellisabella denticulata* from PMV that were previously processed following the above protocol were also included in this study.

II.4. Statistical analysis

Differences in $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ signatures between preservation method, taxa or mud volcanoes were tested using the non-parametric Mann-Whitney test. In all cases the null hypothesis was tested at a 5% significance level.

The statistical analyses were performed using the free open source PAST software (<http://folk.uio.no/ohammer/past>) and all graphs were plotted using Microsoft Excel software.

III. Results

Table 2 summarizes the results obtained from the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ stable isotope analyses performed in this study. The $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values found for all studied species indicate that chemoautotrophy is a significant source of nutrition for these animals.

Due to the small number of samples available it was only possible to statistically test the following differences:

- between preservation methods for *Spirobrachia tripeira* preserved in ethanol and formaldehyde;
- between *Spirobrachia tripeira* and *Lamellisabella denticulata* from PMV, and *Siboglinum* sp. and *Siboglinum* cf. *poseidoni* from CAMV;
- between *Siboglinum* sp. from CAMV and MMV.

The $\delta^{13}\text{C}$ signature of the *Spirobrachia tripeira* ranged from -37.3 to -46.8‰ in the ethanol-preserved specimens and from -45.4 to -48.4‰ in the formaldehyde-preserved specimens. For the same species, the $\delta^{15}\text{N}$ values ranged from 2.1 to 5.5‰ in the ethanol-fixed specimens and from 2.8 to 3.8‰ in the formaldehyde-fixed specimens (Figure 5). A single $\delta^{34}\text{S}$ value of -8.6‰ was measured for specimens of *S. tripeira* after ethanol preservation.

The results of the Mann-Whitney U test performed to investigate the differences in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between the two preservation methods (ethanol and formaldehyde) in *Spirobrachia tripeira* show that the differences found are not statistically significant ($p > 0.05$; Table 3).

Table 2. Summary of the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values used in this study. E (96% Ethanol), F (4% seawater formaldehyde), Fr (-20°C freeze), N (number of samples), se (standard error), *only one measurement.

Species	MV	Preservation	N	$\delta^{13}\text{C}$		$\delta^{15}\text{N}$		$\delta^{34}\text{S}$	
				mean	se	mean	se	mean	se
<i>S. tripeira</i>	PMV	E	6	-44.0	1.6	3.4	0.5		
<i>S. tripeira</i>	PMV	E	1					-8.62*	
<i>S. tripeira</i>	PMV	F	5	-46.6	0.5	3.3	0.2		
<i>L. denticulata</i>	PMV	E	4	-48.6	0.5	0.2	1.9		
<i>L. denticulata</i>	PMV	F	1	-51.4*		6.2*			
<i>L. denticulata</i>	PMV	Fr	1	-43.1*		-0.6		-7.7*	
<i>Siboglinum</i> sp.	CAMV	E	5	-35.2	0.1	5.2	0.1		
<i>Siboglinum</i> sp.	MMV	E	6	-41.0	0.5	-3.7	0.1		
<i>Siboglinum</i> sp.	MMV	E	1					-27.14*	
<i>S. cf. poseidoni</i>	CAMV	E	6	-39.2	0.8	4.8	0.2		
<i>S. cf. poseidoni</i>	CAMV	E	1					-13.0*	
Miscellaneous	CAMV	Fr	3	-45.1	2.6	3.5	0.4	0.4	4.4

The specimens of *Lamelisabella denticulata* preserved in ethanol presented $\delta^{13}\text{C}$ values between -47.3 and -49.6‰; a single value of -51.4‰ was obtained for the formaldehyde-preserved specimen. The $\delta^{15}\text{N}$ signatures of this species ranged from -3.4 to 2.6‰ in ethanol-preserved specimens and a single value of 6.2‰ was obtained for the formaldehyde-preserved specimen (Figure 5).

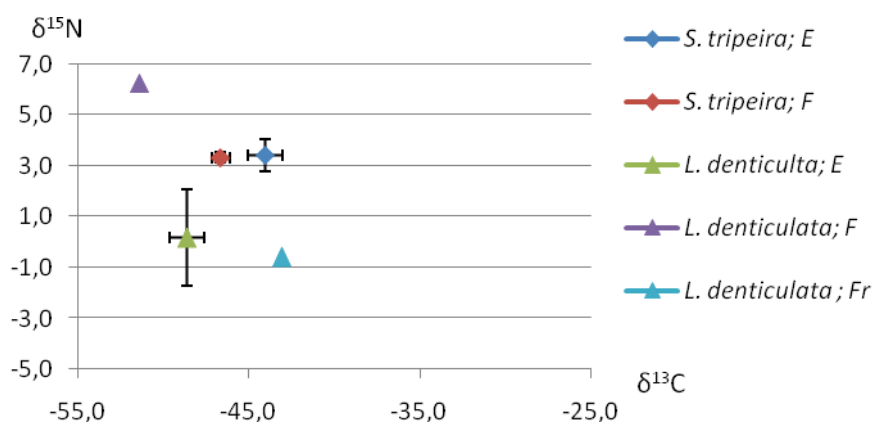


Figure 5. Isotopic signatures ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of *Spirobrachia tripeira* and *Lamelisabella denticulata* sampled on PMV. E (96% Ethanol), F (4% seawater formaldehyde), Fr (-20°C freeze).

The results of the Mann-Whitney U test performed to investigate the differences between the $\delta^{13}\text{C}$ values of *S. tripeira* and *L. denticulata* show that these are statistically significant ($p < 0.05$), with the former being more depleted. In contrast, $\delta^{15}\text{N}$ values show no significant differences between these two species (Table 3), which can be due to the high variability found within *Lamellisabella denticulata*.

The $\delta^{13}\text{C}$ signatures of the specimens collected in the Captain Arutyunov mud volcano ranged from -34.9 to -35.7‰ for *Siboglinum* sp. and -35.4 and -40.4‰ for *Siboglinum* cf. *poseidoni*. The more depleted values found in *Siboglinum* cf. *poseidoni* were found to be significantly different from those of the other species of *Siboglinum* (Mann-Whitney U test, $p < 0.05$, Table 3). The $\delta^{15}\text{N}$ signatures of both species did not significantly differ with *Siboglinum* sp. presenting $\delta^{15}\text{N}$ values between 4.9 and 5.7‰ and *S. poseidoni* between 4.5 and 5.2‰ (Figure 6). A single $\delta^{34}\text{S}$ value of -13.0‰ was measured for specimens of *S. poseidoni*.

For *Siboglinum* sp. specimens collected in the Mercator mud volcano, $\delta^{13}\text{C}$ values ranged from -39.1 to -42.0‰ and $\delta^{15}\text{N}$ values ranged between -3.4 and -4.3‰. *Siboglinum* sp. collected in this mud volcano presented a $\delta^{34}\text{S}$ value of -27.1‰.

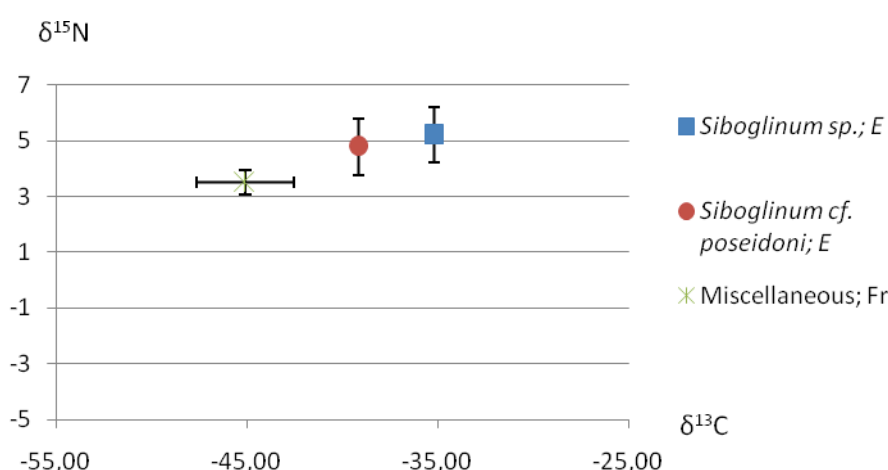


Figure 6. Isotopic signatures ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of *Siboglinum* sp. and *Siboglinum* cf. *poseidoni* and mixed sample containing both species sampled on CAMV. E (96% Ethanol), Fr (-20°C freeze).

The results of the Mann-Whitney U test performed to investigate the differences in the isotope signatures of *Siboglinum* sp. collected from different mud volcanoes (MMV and CAMV) show that for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values these differences are statistically significant ($p > 0.05$, Table 3). For both isotope signatures the specimens collected from the Mercator mud volcano show more depleted values than those collected from the Captain Arutyunov mud volcano (Figure 7).

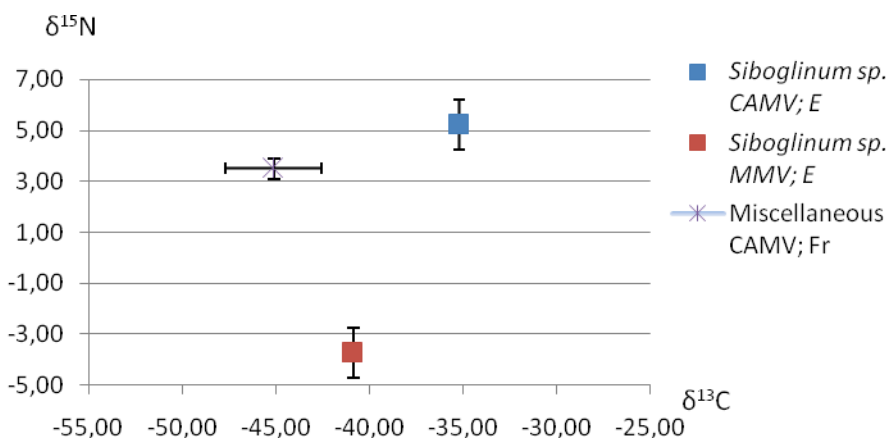


Figure 7. Isotopic signatures ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of *Siboglinum* sp. sampled on MMV and CAMV and a mixed sample containing *Siboglinum* sp. and *Siboglinum* cf. *poseidoni* sampled on CAMV. E (96% Ethanol), Fr (-20°C freeze).

Table 3. Summary of the results of the Mann-Whitney U tests performed to investigate the differences in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values obtained (p - probability, ns - non significant). E (96% Ethanol), F (4% seawater formaldehyde).

Test	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
<i>Spirobrachia tripeira</i> (E) vs <i>Spirobrachia tripeira</i> (F); PMV	ns	ns
<i>Siboglinum</i> cf. <i>poseidoni</i> vs <i>Siboglinum</i> sp.; CAMV	$p \leq 0,05$	ns
<i>Spirobrachia tripeira</i> vs <i>Lamellisabella denticulata</i> ; PMV	$p \leq 0,05$	ns
<i>Siboglinum</i> sp. (CAMV) vs <i>Siboglinum</i> sp. (MMV)	$p \leq 0,05$	$p \leq 0,05$

When possible the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values obtained in this study were compared with previous results obtained from frozen samples. For a frozen specimen of *Lamelisabella denticulata* the $\delta^{13}\text{C}$ signature (-43.1‰) was less depleted than those obtained for specimens preserved in both ethanol and formaldehyde; the $\delta^{15}\text{N}$ value (-0.6‰) was within the range of those obtained from samples preserved in ethanol, but more depleted than the value obtained from the specimen preserved in formaldehyde (Figure 5).

For specimens of the genus *Siboglinum* from the Captain Arutyunov mud volcano, the $\delta^{13}\text{C}$ signature of frozen samples (-41.0 to -49.8‰) were more depleted than those found for the specimens of *Siboglinum* sp. and *Siboglinum* cf. *poseidoni* preserved in ethanol; the $\delta^{15}\text{N}$ values of the same frozen samples (2.7 to 4.2‰) were lower than those found in specimens of both species preserved in ethanol (Figure 6).

Regarding the $\delta^{34}\text{S}$ signatures, the frozen samples from Captain Arutyunov mud volcano presented less depleted values than those found for a specimen of *Siboglinum* cf. *poseidoni* from the same mud volcano and a specimen of *Siboglinum* sp. collected from the Mercator mud volcano, both preserved in ethanol (Figure 8).

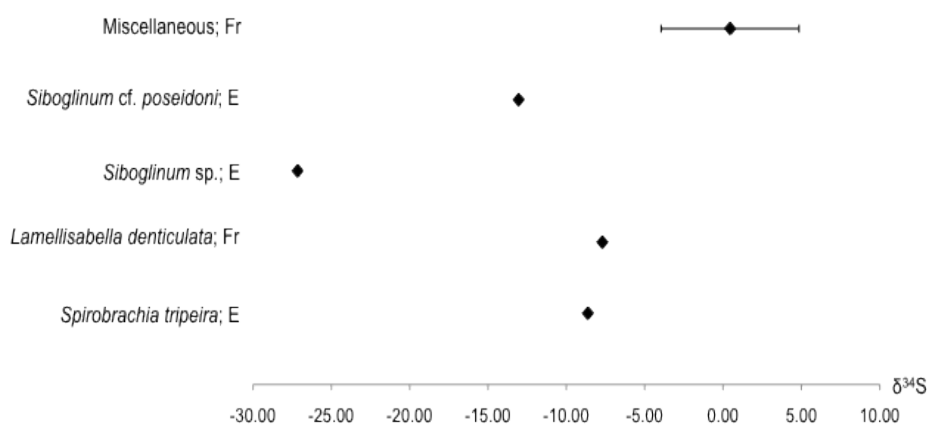


Figure 8. $\delta^{34}\text{S}$ isotopic signatures of the different species. E (96% Ethanol), Fr (-20°C freeze); Error bars represent the standard error.

IV. Discussion

Effects of the preservation on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values

Given the potential usefulness of stable isotope measures from preserved samples, and also the advantages of chemically preserving small, fragile benthic species to prevent their damage and rupture, the potential effects of sample preservation on carbon and nitrogen isotopic values of Frenulata species from different mud volcanoes in the Gulf of Cadiz were evaluated. Values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of frozen samples and of samples preserved in the most often used preservation chemicals for marine specimens, 4% seawater formaldehyde and ethanol, were compared. Our results on the effects of formaldehyde and ethanol preservation in Frenulata confirm the results of previous authors (e.g. Fanelli *et al.* 2010; Sarakinos *et al.* 2001), showing that there are no significant differences in the isotopic values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between samples preserved in ethanol and formaldehyde (Table 3; Figure 5). Previous studies comparing different preservation times in both formaldehyde and ethanol found no significant effect of the preservation for up to 6 months and suggested that samples preserved for less than this period of time can be used to identify trophic levels (Fanelli *et al.* 2010).

Because of the small number of samples it was not possible to statistically test the differences found in the isotopic signatures between samples preserved in ethanol and formaldehyde with those that were frozen. However contradicting results were obtained and are worthwhile discussing. While the values of $\delta^{13}\text{C}$ of ethanol- and formaldehyde preserved specimens of *Lamellisabella denticulata* were more depleted than that of a frozen specimens (Figure 5), in the case of the two species of *Siboglinum*, the $\delta^{13}\text{C}$ value of frozen samples of a mixture of the two species were more depleted than those of the individual species preserved in ethanol (Figure 6). Previous authors have shown that formaldehyde preservation causes depletion of $\delta^{13}\text{C}$ values and have suggested a number of mechanisms to explain it including the exchange of light isotopes for heavier ones (Gearing 1991; Hobson *et al.* 1997; Edwards *et al.* 2002), the addition of lightweight carbon of medium preservation (Gearing 1991;

Edwards *et al.* 2002), and the extraction of lipids (Syväranta *et al.* 2008) and protein hydrolysis during preservation (Arrington and Winemiller, 2002; Sarakinos *et al.* 2002). More studies are needed to identify which of these, if any, caused the depletion of $\delta^{13}\text{C}$ values found in the ethanol- and formaldehyde preserved specimens of *Lamellisabella denticulata* but the most likely and supported theory is that of the direct incorporation of lighter carbon from the formaldehyde, since formaldehyde can bind to certain biochemical constituents of the tissue and carry its own signature (Hobson *et al.* 1997).

The depletion of the $\delta^{13}\text{C}$ values found in the frozen samples containing a mixture of the two species of *Siboglinum* in comparison with those of the individual species preserved in ethanol is in agreement with several previous studies (e.g. DeNiro and Epstein 1976, 1978; McConnauey and McRoy 1979; Gloutney and Hobson 1998; Doucett *et al.* 1999) that suggest that ethanol can act as a solvent of isotopically lighter compounds present in the samples. In the case of differences found in this study they are likely related with the use of whole animals, including the chitinous tube. If the chitin in the tube is isotopically lighter than the ethanol it is possible that the latter acts as a solvent resulting in an overall enriched sample. However, more studies are needed to confirm our results and to compare the isotopic signatures of *Siboglinum* species with and without their chitinous tubes.

Regarding the $\delta^{15}\text{N}$ values, the main difference was found between the frozen and the formaldehyde preserved samples of *Lamellisabella denticulata*, with the formaldehyde preserved sample showing a higher value (Figure 5). In previous studies (e.g. Fanelli *et al.* 2010) no significant effects of formaldehyde or ethanol preservation were found on $\delta^{15}\text{N}$; since only one frozen specimen was used in this study it is not possible to assess the significance of these results.

Food sources of different species of Frenulata

It has been suggested that the high diversity of frenulates found in mud volcanoes in the Gulf of Cadiz is related with habitat heterogeneity at regional and local scales (Hilário *et al.* 2010). Where two species co-exist *Lamellisabella denticulata* and *Spirobrachia tripeira* (Porto MV) and *Siboglinum* cf. *poseidoni*

and *Siboglinum* sp. there was no apparent zonation patterns, which led to speculation that the two species co-exist by partitioning one or more resources. If resource partitioning was occurring, we would expect to see consistent differences in isotope values between species.

In order to test the hypotheses that different species of frenulates inhabiting the same mud volcano can exploit different sources of energy the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of different species in the Porto and Captain Arutyunov mud volcanoes were compared and in both cases significant differences in the $\delta^{13}\text{C}$ were found (Table 3).

Generally, values of $\delta^{13}\text{C}$ below -40‰ are characteristic of species housing methanotrophic endosymbionts (Brooks *et al.* 1987) and therefore the $\delta^{13}\text{C}$ signatures found in this study suggest that *Lamellisabella denticulata* relies on metanotrophs, which could explain the differences found between this species and *Spirobrachia tripeira*. However, the molecular characterization of the endosymbionts of both species revealed only the presence of thiotrophs (Rodrigues *et al.* 2011). Regarding the two species of *Siboglinum* collected in CAMV, the relatively enriched (> 40‰) values of $\delta^{13}\text{C}$ found in *Siboglinum* cf. *poseidoni* are unexpected, since it has been shown that *Siboglinum poseidoni* hosts methanotrophic endosymbionts (Flügel and Langhof 1983; Schmaljohann and Flügel 1987; Rodrigues *et al.* 2011).

The presence of both methane- and sulphur-oxidising symbionts, known as dual symbiosis, has been reported from several species of invertebrates from hydrothermal vents and cold seeps (e.g. Fisher *et al.* 1995; Duperron *et al.* 2011), including *Siboglinum* sp. from CAMV (Rodrigues *et al.* 2011). Dual symbioses has been mostly studied in chemosymbiotic mussels and has been interpreted as an adaptation to survive in environments that are highly variable in both time and space (Distel *et al.* 1995) with the contribution of the different symbionts being influenced by the availability of methane and reduced sulphur (Duperron *et al.* 2011). Although there are no studies on frenulates it is likely that dual symbioses function in a similar way and may be more usual than currently known. Dual symbioses may explain the contradicting results between the $\delta^{13}\text{C}$ signatures found in this study and previous molecular characterization

of endosymbionts of *Lamellisabella denticulata*, *Siboglinum* cf. *poseidoni* and *Siboglinum* sp. (Rodrigues *et al.* 2011). Another possible explanation for these contradicting results is that the sediment of the mud volcanoes may be rich in C-depleted organic matter, which can be incorporated across the body wall as shown in the early studies of Southward and Southward (1980).

Statistically significant differences were found when $\delta^{13}\text{C}$ signatures of *Siboglinum* sp. collected in different mud volcanoes, MMV and CAMV, were compared: specimens from MMV presented values near the border of values characteristic of methanotrophic species and specimens from CAMV presented values typical of thiotrophic species, suggesting that the same species can use one or the other type of symbionts depending on the environmental conditions. However this result has to be interpreted cautiously since the $\delta^{34}\text{S}$ value shown by *Siboglinum* sp. from MMV is noticeable more negative than for any of the other samples (Figure 8), suggesting an "obvious" symbiosis with thiotrophs. As demonstrated for mussels, it is possible that frenulate species can dynamically adapt the abundances of methanotrophs and thiotrophs in the trophosome as a response to different and fluctuating environmental conditions but to test this hypotheses more detailed studies are needed and in the future endosymbiont characterization, stable isotope analyses of the host, and measurements of methane and sulphur concentrations in the environment should be done in an integrative way.

The $\delta^{13}\text{C}$ values found for *Spirobrachia tripeira* ($-43.57 \pm 1.91\text{‰}$) are on the limit of the characteristic for methanotrophs and one can argue that like *L. denticulata*, *S. tripeira* can host both methane- and sulphur-oxidising symbionts. If that is the case, the differences found in the carbon isotopic signatures of the two species from PMV can be explained by different origins of the methane being used by the endosymbionts. Values between -40‰ and -45‰ are usually indicative of methane of thermogenic origin while more negative values are associated with a biogenic origin (Sassen *et al.* 1999). Detailed analyses of the seepage fluids in the Porto mud volcano showed a predominantly deep microbial origin of methane but not exclude a partial thermal origin (Nuzzo *et al.* 2009) and therefore it is possible that the two species exploit microhabitats with

different sources of methane.

Unfortunately there were not enough samples to statistically test the differences found in the sulphur isotopic signatures between the different species of Frenulata, however the value found for *Siboglinum* sp. from MMV (Figure 8) is apparently more negative than for any of the other samples. Future studies should further investigate differences in the $\delta^{34}\text{S}$ values and, if possible, relate them with environmental conditions.

V. Conclusion

For many years, mainly because of sampling constraints, frenulates have been overlooked and thought to be of limited ecological importance. The present study adds new and useful information on preservation methods and trophic ecology of this understudied group of Siboglinidae tubeworms.

The results herein presented show that, similarly to other marine vertebrates and invertebrates (e.g. Fanelli *et al.* 2010; Sarakinos *et al.* 2001), Frenulata preserved in ethanol and formaldehyde can be used in trophic ecology studies, which is of extreme importance since it enables the utilization of samples preserved in ethanol and formaldehyde primarily collected for molecular and morphological studies. Moreover, because most species are either very small or have a very hard tube, frenulates are extremely hard to dissect on board research vessels and chemical preservation of the whole animal is the most common and convenient practice. However, the isotopic signatures of samples including non-dissected animals should be interpreted with caution since the chitin that compose the tube can influence the $\delta^{13}\text{C}$ results. It is therefore recommended that in future studies animals used in isotopic analyses must be dissected out of their tubes, which can be done under a stereomicroscope once the preserved samples are in the laboratory.

In this study frozen and chemically preserved samples were used to determine the energy source (methane or sulphur) of the endosymbionts of different species of frenulates and the potential occurrence of resource partitioning between species inhabiting the same mud volcano. The carbon isotopic signatures were more (*Spirobrachia tripeira* and *Lamellisabella denticulata*) or less (*Siboglinum* cf. *poseidoni*) depleted than expected for thio- and methanotrophic species, respectively, suggesting that dual symbiosis may occur in these species and may be more prevalent than previously known in frenulate tubeworms. However, it is also possible that the sediment of the mud volcanoes may be rich in C-depleted organic matter, which can be incorporated across the animal's body wall.

The isotopic signatures obtained for the analysed specimens suggest that different species can exploit different microhabitats in the same mud volcano, which is in agreement with the previous hypotheses that the high diversity of frenulates found in the Gulf of Cadiz is related to the spatially and temporally variable chemical environment of mud volcanoes (Hilário *et al.* 2010). Nevertheless these are only preliminary results and in future trophic ecology studies of frenulates stable isotope analyses of the host, endosymbiont characterization, and measurements of methane and sulphur concentrations in the environment should be done in an integrated way.

VI- References

- Agard, J. B. R., Gobin, J., Warwick, R. M., 1993. Analysis of marine macrobenthic community structure in relation to pollution, natural oil seepage and seasonal disturbance in a tropical environment (Trinidad, West Indies). *Marine Ecology Progress Series*. 92, 233-243.
- Aharon, P., 1994. Geology and biology of modern and ancient submarine hydrocarbon seeps and vents. An introduction. *Geology Marine Letters*. 14, 69-73.
- Alperin, M. J., Reeburgh, W. S., Whiticar, M. J., 1988. Carbon and hydrogen isotope fractionation resulting from anaerobic methane oxidation. *Global Biogeochemical Cycles*. 3, 279-288.
- Andersen, A. C., Jolivet, S., Claudinot, S., Lallier, F. H., 2002. Biometry of the branchial plume in the hydrothermal vent tubeworm *Riftia pachytila* (Vestimentifera; Annelida). *Canadian Journal of Zoology-Revue Canadienne De Zoologie*. 80, 320-332.
- Arp, A. J., Childress, J. J., Vetter, R. D., 1987. The sulfide-binding protein in the blood of the vestimentiferan tube-worm, *Riftia*, is the extracellular hemoglobin. *Journal of Experimental Biology*. 128, 139-158.
- Baraza, J., Ercilla, G., 1996. Gas-charged sediments and large pockmark-like features on the Gulf of Cadiz slope (SW Spain). *Marine and Petroleum Geology*. 13, 253-261.
- Barry, J. P., Greene, G. G., Orange, D. L., Baxter, C. H., Robison, B. H., Kochevar, R. E., Nybakken, J. W., Reed, D. L., McHugh, C. M., 1996. Biologic and Geologic characteristics of cold seeps in Monterey Bay, California. *Deep-Sea Research I*. 43(11-12), 1739-1762.
- Boetius, A., Ravensschlag, K., Schubert, C. J., Rickert, D., Widdel, F., Gieseke, A., Amann, R., Jorgensen, B. B., Witte, U., Pfannkuche, O., 2000. A marine microbial consortium apparently mediating anaerobic oxidation. 407, 623-626.
- Brooks, J. M., Kennicutt, M. C., Fisher, C. R., Macko, S. A., Cole, K., Childress, J. J., Bidigare, R. R., Vetter, R. D., 1987. Deep-sea hydrocarbon seep communities: evidence for energy and nutritional carbon sources. *Science*. 238, 1138-1142.
- Bufo, E., Degaldeano, C. S., Udias, A., 1995. Seismotectonics of the Ibero-Maghrebian Region. *Tectonophysics*. 248, 247-261.
- Canet, C., Prol-Ledesma, R.M., Melgarejo, J.C. & Reyes, A., 2003. Methane-related carbonates formed at submarine hydrothermal springs: a new setting for microbially-derived carbonates? *Marine Geology*. 199, 245–261.
- Carlier, A., Ritt, B., Rodrigues, C. F., Sarrazin, J., Olu, K., Grall, J., Clavier, J., 2010. Heterogeneous energetic pathways and carbon sources on deep eastern Mediterranean cold seep communities. *Marine Biology*. 157, 2545-2565.
- Carney, R. S., 1994. Consideration of the oasis analogy for chemosynthetic communities at Gulf of Mexico hydrocarbon vents. *Geology Marine Letters*. 14, 149-

159.

Caullery, M., 1914. Sur les Siboglinidae, type nouveau d'invertébrés recueillis par l'expédition du Siboga. *Comptes rendus de l'Académie des sciences, Série, III.* 158, 2014-2017.

Cavanaugh, C. M., Gardiner, S. L., Jones, M. L., Jannasch, H. W., Waterbury, J. B., 1981. Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila*: Possible chemoautotrophic symbionts. *Science.* 213, 340—342.

Colaço, A., Dehairs, F., Desbruyères, D., 2002. Nutritional relations of deep-sea hydrothermal fields at the Mid-Atlantic Ridge: a stable isotope approach. *Deep-Sea Research I.* 49, 395 - 412.

Conway, N. M., Kennicutt, M. C., Van Dover, C. L., 1994. Stable isotopes in the study of marine chemosynthetic-based ecosystems. In Lajtha K & Michener RH (eds) *Stable Isotopes in Ecology and Environmental Science.* Oxford: Blackwell Scientific. 158-186.

Corliss, J. B., Ballard, R. D., 1977. Oasis of life in the cold abyss. *National Geographic.* 152,441-452.

Craig, H., 1953. The geochemistry of the stable isotopes. *Geochimica et Cosmochimica Acta.* 3, 53-92.

Dando, P. R., Austen, M. C., Burke Jr., R. A., Kendall, M. A., Kennicutt II, M. C., Judd, A. G., Moore, D. C., O'Hara, S. C. M., Schmaljohann, R., Southward, A. J., 1991. Ecology of a north sea pockmark with an active methane seep. *Marine Ecology Progress Series.* 70, 49-63.

Dando, P. R., Southward, A. J., Southward, E. C., Lamont, P., Harvey, R., 2008. Interactions between sediment chemistry and frenulate pogonophores (Annelida) in the north-east Atlantic. *Deep-Sea Research I.* 55, 966-996.

de Busserolles, F., Sarrazin, J., Gauthier, O., Gélinas, Y., Fabri, M. C., Sarradin, P. M., Desbruyères, D., 2009. Are spatial variations in the diets of hydrothermal fauna linked to local environmental conditions? *Deep-Sea Research II.* 56, 1649-1664.

Deming, J. W., Reysenbach, A. L., Macko, S., Smith, C. R., 1997. Evidence for the microbial basis of a chemoautotrophic invertebrate community at a whale fall on the Deep seafloor: bone-colonizing bacteria and invertebrate endosymbionts. *Microscopy Research and Technique.* 37, 162 - 170.

DeNiro, M. J., Epstein, S., 1976. You are what you eat (plus a few ‰) the carbon isotope cycle in food chains. *Geochimica et Cosmochimica Acta.* 6, 834.

DeNiro, M. J. and Epstein, S., 1978. Influence of diet on distribution of carbon isotopes in animals. *Geochimica et Cosmochimica Acta.* 42(5), 495-506.

DeNiro, M. J., Epstein, S., 1981. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica et Cosmochimica Acta.* 45.

Dimitrov, L. I., 2002. Mud volcanoes - the most important pathway for degassing deeply buried sediments. *Earth-Science Reviews.* 59, 49-76.

Distel, D. L., Lee, H. K. W., Cavanaugh, C. M., 1995. Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel. *Proceedings of the National Academy of Sciences of the United States of America*. 92, 9598–9602.

Domack E., Ishman S., Leventer A., Sylva S., Willmot V., Huber B., 2005. A Chemotrophic Ecosystem Found Beneath Antarctic Ice Shelf. *EOS* 86, 269-276.

Doucett, R.R., Hooper, W. and Power, G., 1999. Identification of anadromous and nonanadromous adult brook trout and their progeny in the Tabusintac River, New Brunswick, by means of multiple-stable-isotope analysis. *Trans. America Fisher Society*. 128, 278–288.

Duperron, S., Guezi, H., Gaudron, M. S., Ristova, P. P., Wenzhofer, F., Boetius, A., 2011. Relative abundances of methane – and sulphur – oxidizing in the gills of a cold seep mussel and link to their potential energy sources. *Geobiology*. 9, 491-491.

Fanelli, E., Cartes, J. E., Papiol, V., Rumolo, P., Sprovieri, M., 2010. Effects of preservation on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of deep sea macrofauna. *Journal of Experimental Marine Biology and Ecology*. 395, 93-97.

Felbeck, H., 1981. Chemoautotrophic Potential of the Hydrothermal Vent Tube Worm, *Riftia-Pachyptila Jones* (Vestimentifera). *Science*. 213, 336-338.

Fisher, C. R., 1990. Chemoautotrophic and methanotrophic symbioses in marine invertebrates. *Aquatic sciences*. 2, 399-436.

Fisher, C. R., 1995. Toward an appreciation of hydrothermal-vent animals: their environment, physiological ecology, and tissue stable isotope values. In: Humphris SE, et al (eds) *Seafloor hydrothermal systems: physical, chemical, biological, and geological interactions*. American Geophysical Union. 297–316.

Fisher, C. R., Urcuyo, I. A., Simpkins, M. A., Nix, E., 1997. Life in the slow lane: Growth and longevity of cold-seep vestimentiferans. *Marine Ecology*, 18, 83-94.

Flügel, H. J., Langhof, I., 1983. A new hermaphroditic pogonophore from the Skagerrak. *Sarsia*. 68, 131–138.

Foucher, J., Westbrook, K. G., Boetius, A., Ceramicola, S., Dupre, S., Mascle, J., Mienert, J., Pfannkuche, O., Pierre, C., Praeg, D., 2009. Structure and Drivers of Cold Seep Ecosystems. *Oceanography*. 22 (1), 92-109.

Fry, B., Sherr, E. B., 1984. ^{13}C Measurements as indicators of carbon flow in marine and freshwater ecosystems. *Contribution in Marine Science*. 27, 13-46.

Fry, B., Sherr, E. B., 1988. $\delta^{13}\text{C}$ measurements as indicators of carbon flow in marine and freshwater ecosystems. In: *Ecological studies analysis and synthesis*, PW Rundel, JR Ehleringer & KA Nagy (eds). 28, 196-229.

Gardner, J. M., 2001. Mud volcanoes revealed and sampled on the Western Moroccan continental margin. *Geophysica Research Letters*. 28, 339-342.

Gearing, J. N., 1991. The study of diet and trophic relationships through natural abundance ^{13}C . *Carbon Isotope Techniques*. In: *Carbon isotope techniques*, Coleman

DC & Fry B (eds). Academic Press. 201-218.

Gebruk, A. V., Krylova, E. M., Lein, A. Y., Vinogradov, G. M., Anderson, E., Pimenov, N. V., Cherkashev, G. A., Crane, K., 2003. Methane seep community of the Hakon Mosby mud volcano (the Norwegian Sea): composition and trophic aspects. *Sarsia*. 88, 394-403.

Gloutney, M. L. and Hobson, K. A., 1998. Field preservation techniques for the analysis of stable-carbon and nitrogen isotope ratios in eggs. *J. Field Ornithology*. 69, 223–227.

Gutscher, M. A., J. Malod, J. P. Rehault, I., Contrucci, F. K. I., Mendes V., Spakman, W., 2002. Evidence for active subduction beneath Gibraltar. *Geology*. 30, 1071-1074.

Haeckel, M.; Liebetrau, V.; Linke, P.; Reitz, A.; Schneider v. Deimling, J.; Schönfeld, J.; Vanneste, H. 2007. Sources and Rates of Fluid Flow at Mud Volcanoes - Examples from the Gulf of Cadiz. *American Geophysical Union*. 22, 04.

Halanych, K. M., 2005. Molecular phylogeny of siboglinid annelids (a.k.a. pogonophorans): a review. *Hydrobiologia*. 535/536, 297-307.

Hensen, C., Nuzzo, M., Hornibrook, E., Pinheiro, L. M., Bock, B., Magalhaes, V. H., Bruckmann, W., 2007. Sources of mud volcano fluids in the Gulf of Cadiz - indicators for hydrothermal imprint. *Geochimica et Cosmochimica Acta*.

Hessler, R. R., Kaharl, V. A., 1995. The deep-sea hydrothermal community: an overview. In: Humphris, S. E., Zierenberg, R., Mullineaux, L., Thomson, R. (eds). *Seafloor Hydrothermal Systems: Physical, {C}hemical, {B}iological, and {G}eological {I}nteractions*. American Geophysical Union. 91, 72-84.

Hilário, A., Capa, M., Dahlgren, T. G., Halanych, K. M., Little, C. T. S., Thornhill, D. J., Verna, C., Glover, A. G., 2011. New Perspectives on the Ecology and Evolution of Siboglinid Tubeworms. *Plos One*. 6, e16309.

Hilario, A., Cunha, M. R., 2008. On some frenulate species (Annelida: Polychaeta: Siboglinidae) from mud volcanoes in the Gulf of Cadiz (NE Atlantic). *Scientia Marina*. 72, 361-371.

Hilário, A., Johnson, S. B., Cunha, M. R., Vrijenhoek, R. C., 2010. High diversity of frenulates (Polychaeta: Siboglinidae) in the Gulf of Cadiz mud volcanoes: a DNA taxonomy analysis. *Deep-Sea Research I*. 57, 143-150.

Hobson, K. A., Welch, H. E., 1992. Determination of trophic relationships within a high marine food web using ¹³C and ¹⁵N analysis. *Marine Ecology Progress Series*. 84, 9-18.

Iken, K., Brey, T., Wand, U., Voigt, J., Junghans, P., 2001. Food web structure of the benthic community at the Porcupine Abyssal Plain (NE Atlantic): a stable isotope analysis. *Progress in Oceanography*. 50, 383-405.

Ivanov, A.V., 1952. New Pogonophora from far Eastern Seas. *Systematic Zool*. 3, 69-79.

Ivanov, A.V., 1963. *Pogonophora*. Academic Press. London.

Ivanov, A. V., and Petrunkevitch A., 1955. On the assignment of the Class

Pogonophora to a separate Phylum of Deuterostomia Brachiata A. Ivanov, phyl. nov. Syst. Zool. 4, 177-178.

Jannasch, H. W., Humphris, S. E., Zierenberg, R., Mullineaux, L., Thomson, R. 1995. Microbial interactions with hydrothermal fluids. In: Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions. American Geophysical Union. 91, p 273-296.

Julian, D., Gaill, F., Wood, E., Arp, A. J., Fisher, C. R., 1999. Roots as a site of hydrogen sulfide uptake in the hydrocarbon seep vestimentiferan *Lamellibrachia* sp. The Journal of Experimental Biology. 202, 2245—2257.

Kelly, D.S., Karson, J.A., Blackman, D.K., Früh-Green, G.L., Butterfield, D.A., Lilley, M.D., Olson, E.J., Schrenk, M.O., Roe, K.K., Lebon, G.T., Rivizzigno, P. & The AT3-60 Shipboard Party. 2001. An off-axis hydrothermal-vent field near the Mid-Atlantic Ridge at 30°N. Nature. 412, 145–149.

Kennicutt, M. C., Brooks, J. M., Bidigare, R. R., MacDonald, T. J., 1985. Vent-type taxa in a hydrocarbon seep region on the Louisiana slope. Nature. 317, 352-353.

Kennicutt, M. C., Bueke, R. A., MacDonald, J. R., Brooks, J. M., Denoux, G. J., Macko, S. A., Stable isotope partitioning in seep and vent organisms: chemical and ecological significance. In: S. A. Macko, M. H. Engel, (Eds.), Isotope Fractionations in Organic Matter: Biosynthetic and Diagenetic Processes., Vol. 101, 1992, pp. 293-310.

Krouse, R., 1980. Sulphur isotopes in our environment. In: Handbook of Environmental Isotope Geochemistry. 1, 435–472.

Krueger, D. M., Cavanaugh, C. M., 1997. Phylogenetic diversity of bacterial symbionts of *Solemya* hosts based on comparative sequence analysis of 16S rRNA genes. Applied Environmental Microbiology. 63(1), 91-98.

Lee, R. W., Robinson, J. J., Cavanaugh, C. M., 1999. Pathways of inorganic nitrogen assimilation in chemoautotrophic bacteria-marine invertebrates symbioses: expression of host and symbiont glutamine synthetase. The Journal of Experimental Biology. 202, 283-300.

Levesque, C., Juniper, S. K., Marcus, J., 2003. Food resource partitioning and competition among alvinellid polychaetes of Juan de Fuca Ridge hydrothermal vents. Marine Ecology Progress Series. 246, 173-182.

Levesque, C., Kim Juniper, S., Limen, H., 2006. Spatial organization of food webs along habitat gradients at deep-sea hydrothermal vents on Axial Volcano, Northeast Pacific. Deep Sea Research I. 53, 726-739.

Levin, L., 2005. Ecology of cold seep sediments interactions of fauna with flow chemistry and microbes. Oceanogr. Mar. Biol. Ann. Rev. 43, 1-46.

Levin, L. A., Barry, J. P., Felbeck, H., Smith, C. R., Young, C. M., 2007. Advances in Vent, Seep, Whale- and Wood-Fall Biology. Marine Ecology. 28, 1-2.

Levin, L. A., Etter, R. J., Rex, M. A., Gooday, A. J., Smith, C. R., Pineda, J., Stuart, C. T., Hessler, R. R., Pawson, D., 2001. Environmental influences on regional deep-sea species diversity. Annual Review of Ecology and Systematics. 32, 51-93.

- Levin, L. A., Gage, J. D., Martin, C., Lamont, P. A., 2000. Macrobenthic community structure within and beneath the oxygen minimum zone, NW Arabian Sea. *Deep-Sea Research I*. 2, 189-226.
- Levin, L. A., Ziebis, W., Mendoza, G. F., Growney, V. A., Tyyon, M. D., Brown, K. M., Mahn, C., Gieskes, J. M., Rathburn, 2003. Spatial heterogeneity of macrofauna at northern California methane seeps: influence of sulfide concentration and fluid flow. *Marine Ecology Progress Series*. 265, 123-139.
- Lutz, R. A., Shank, T. M., Fornari, D. J., Haymon, R. M., Lilley, M. D., Damm, K. L. V., 1994. Rapid growth at deep-sea vents. *Nature*. 371, 663-664.
- MacAvoy, S. E., Fisher, C. R., Carney, R. S., Macko, S. A., 2005. Nutritional associations among fauna at hydrocarbon seep communities in the Gulf of Mexico. *Marine Ecology Progress Series*. 292, 51-60.
- MacAvoy, S. E., Macko, S. A., Joye, S. B., 2002. Fatty acid carbon isotope signatures in chemosynthetic mussels and tube worms from gulf of Mexico hydrocarbon seep communities. *Chemical Geology*. 185, 1-8.
- Maldonado, A., Somoza, I., Pallarés I., 1999. The Betic orogen and the Iberian-African boundary in the Gulf of Cadiz: geological evolution (central north Atlantic). *Mar. Geol.* 155, 9-43.
- Mariotti, A., 1983. Atmospheric nitrogen is a reliable standard for natural ^{15}N abundance measurements. *Nature (Lond.)*. 303, 685-687.
- Mayer, L. A., Shor, A. N., Hughes C. J., Piper, D. J. W., 1988. Dense biological communities at 3850 m on the Laurentian Fan and their relationship to the deposits of the 1929 Grand Banks earthquake, *Deep-sea Research*. 35, 1235-1246.
- McConnaughey, T., McRoy, C. P., 1979. Food-web structure and the fractionation of carbon isotopes in the Bering Sea. *Marine Biology*. 53, 257-262.
- McCutchan, J. H., Lewis, W. M., Kendall, C., McGrath, C. C., 2003. Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulphur. *Oikos*. 102, 378-390.
- McHugh, D., 1997. Molecular evidence that echiurans and pogonophorans are derived annelids. *Proc. Natl. Acad. Sci. U.S.A.* 94, 8006-8009.
- Michaelis, W., Seifert, R., Nauhaus, K., Treude, T., Thiel, V., Blumenberg, M., Knittel, K., Gieseke, A., Peterknecht, K., Pape, T., Boetius, A., Amann, R., Jorgensen, B.B., Widdel, F., Peckmann, J., Pimenov, N.V. & Gulin, M.B., 2002. Microbial reefs in the Black Sea fueled by anaerobic oxidation of methane. *Science* 299, 1013-1015.
- Milkov, A. V., 2005. Molecular and stable isotope compositions of natural gas hydrates: a revised global dataset and basic interpretations in the context of geological settings. *Organic Geochemistry*. 36, 681-702.
- Minagawa, M., Wada, E., 1984. Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}\text{N}$ and animal age. *Geochimical et Cosmochimical Acta*. 48, 1135-1140.

- Miura, T., Tsukahara, J., Hashimoto, J., 1997. *Lamellibrachia satsuma*, a new species of vestimentiferan worms (Annelida: Pogonophora) from a shallow hydrothermal vent in Kagoshima Bay, Japan. *Proceedings of the Biological Society of Washington*. 110, 447-456.
- Niemann, H., Duarte, J., Hensen, C., Omoregie, E., Magalhães, V. H., Elvert, M., L.M., P., Kopf, A., Boetius, A., 2006. Microbial methane turnover at mud volcanoes of the Gulf of Cadiz. *Geochimica et Cosmochimica Acta*. 70, 5336-5355.
- Nuzzo, M., Hornibrook, E. R. C., Gill, F., Hensen, C., Pancost, R. D., Haeckel, M., Reitz, A., Scholz, F., Magalhaes, V. H., Bruckmann, W., Pinheiro, L. M., 2009. Origin of light volatile hydrocarbon gases in mud volcano fluids, Gulf of Cadiz - evidence for multiple sources and transport mechanisms in active sedimentary wedges. *Chemical Geology*. 266, 350-363.
- Nuzzo, M., Hornibrook, E. R. C., Hensen, C., Parkes, R. J., Cragg, B. A., Rinna, J., von Deimling, J. S., Sommer, S., Magalhaes, V. H., Reitz, A., Bruckmann, W., Pinheiro, L. M., 2008. Shallow Microbial Recycling of Deep-Sourced Carbon in Gulf of Cadiz Mud Volcanoes. *Geomicrobiology Journal*. 25, 283-295.
- Oliver, G., Rodrigues, C., Cunha, M. R., 2011. Chemosymbiotic bivalves from the mud volcanoes of the Gulf of Cadiz, NE Atlantic, with descriptions of new species of Solemyidae, Lucinidae and Vesicomidae. *ZooKeys*. 113, 1–38.
- Olu-Le Roy, K., Sibuet, M., Fiala-Médioni, A., Gofas, S., Salas, C., Mariotti, A., Foucher, J. P., Woodside, J., 2004. Cold seep communities in the deep eastern Mediterranean Sea: composition, symbiosis and spatial distribution on mud volcanoes. *Deep-Sea Research I*. 51, 1915 - 1936.
- Olu, K., Duperret, A., Sibuet, M., Foucher, J. P., Fiala-Médioni, A., 1996. Structure and distribution of cold seep communities along the Peruvian active margin: relationship to geological and fluid patterns. *Marine Ecology Progress Series*. 132, 109-125.
- Olu, K., Lance, S., Sibuet, M., Henry, P., Fiala-Médioni, A., Dinét, A., 1997. Cold seeps communities as indicators of fluid expulsion patterns through mud volcanoes seaward of the Barbados accretionary prism. *Deep-sea Research I*. 44, 811-841.
- Paull, C. K., Hecker, B., Commeu, R., Freeman-Lynde, R. P., Neumann, C., Corso, W. P., Golubic, S., Hook, J. E., Sikes, E., Curry, J., 1984. Biological communities at the Florida Escarpment resemble hydrothermal vent taxa. *Science*. 226, 965-967.
- Paull, C. K., Jull, A. J. T., Toolin, L. J., Linick, T., 1985. Stable isotope evidence for chemosynthesis in an abyssal seep community. *Nature*. 317, 709-711.
- Peterson, B. J., Fry, B., 1987. Stable isotopes in ecosystem studies. *Annual Review Ecology System*. 18, 293-320.
- Peterson, B. J., 1999. Stable isotopes as tracers of organic matter input and transfer in benthic food webs: A review. *Acta Oecologica*. 20, 479-487.
- Peterson, B. J., Fry, B., 1987. Stable isotopes in ecosystem studies. *Annual Review Ecology System*. 18, 293-320.
- Pinheiro, L. M., Ivanov, M. K., Sautkin, A., Akmanov, G., Magalhães, V. H.,

- Volkonskya, A., Monteiro, J. H., Somoza, L., Gardner, J., Hamouni, N., Cunha, M. R., 2003. Mud volcanism in the Gulf of Cadiz: results from the TTR-10 cruise. *Marine Geology*. 195, 131-151.
- Rau, G. H., Hedges, J. I., 1979. Carbon-13 depletion in a hydrothermal vent mussel: suggestion of a chemosynthetic food source. *Science*, 203 (4381): 648-649.
- Ribeiro, A., Cabral, J., Baptista, R., Matias, L., 1996. Stress pattern in Portugal mainland and the adjacent Atlantic region, West Iberia. *Tectonics*. 15 (2), 641-659.
- Robinson, J. J., Cavanaugh, C. M., 1995. Expression of form I and form II Rubisco in chemoautotrophic symbioses: Implications for the interpretation of stable carbon isotope values. *Limnology Oceanography*. 46, 1496-1502.
- Rodrigues, C. F., 2009. Macrofaunal communities from mud volcanoes in the Gulf of Cadiz. PhD thesis, University of Aveiro.
- Rodrigues, C. F., Hilário, A., Cunha, M. R., Weightman, A. J., Webster, G. 2011. Microbial diversity in Frenulata (Siboglinidae, Polychaeta) species from the mud volcanoes of the Gulf of Cadiz (NE Atlantic). *Antoine van Leeuwenhoek*. 100, 1, 83-98.
- Rouse, G. W., Fauchald, K., 1997. Cladistics and polychaetes. *Zoologica Scripta*. 26 (2), 139-204.
- Rouse, G. W., 2001. A cladistic analysis of Siboglinidae Caullery, 1914 (Polychaeta, Annelida): formerly the phyla Pogonophora and Vestimentifera. *Zoological Journal of the Linnean Society*. 132, 55-80.
- Sahling, H., Rickert, D., Lee, R. W., Linke, P., Suess, E., 2002. Macrofaunal community structure and sulfide flux at gas hydrate deposits from the Cascadia convergent margin, NE. *Marine Ecology Progress Series*. 231, 121-138.
- Salas, C., Woodside, J., 2002. *Lucionoma kazani* n. sp. (Mollusca: Bivalvia): evidence of a living benthic community associated with a cold seep in the Eastern Mediterranean Sea. *Deep-Sea Research I*. 49, 991 - 1005.
- Sartori, R., Torelli, I., Zitellini, N., Peis D., Iodolo, E., 1994. Eastern segment of the Azores-Gibraltar line (central-eastern Atlantic): an oceanic plate boundary with diffuse compressional deformation. *Geology*. 22, 555-558.
- Sassen, R., Joye, S., Sweet, S. T., DeFreitas, D. A., Milkov, A. V., MacDonald, I. R., 1999. Thermogenic gas hydrates and hydrocarbon gases in complex chemosynthetic communities, Gulf of Mexico continental slope. *Organic Geochemistry*. 30, 485-497.
- Schmaljohann, R., Drews, M., Walter, S., Linke, P. & von Rad, U., 2001. Oxygen-minimum zone sediments in the northeastern Arabian Sea off Pakistan: a habitat for the bacterium *Thioploca*. *Marine Ecology Progress Series*. 211, 27-42.
- Schmaljohann, R., Faber, E., Whiticar, M. J., Dando, P. R., 1990. Coexistence of methane-based and sulfur-based endosymbioses between bacteria and invertebrates at a site in the Skagerrak. *Marine Ecology-Progress Series*. 61, 119-124.
- Schmaljohann, R., Flugel, H.J., 1987. Methane-oxidizing bacteria in Pogonophora. *Sarsia*. 72, 91-98.

- Schulze, A., 2003. Phylogeny of Vestimentifera (Siboglinidae, Annelida) inferred from morphology. *Zoologica Scripta*. 32, 321-342.
- Shank, T. M., Fornari, D. J., Damm, K. L. V., Lilley, M. D., Haymon, R. M., Lutz, R. A., 1998. Temporal and spatial patterns of biological community development at nascent deep-sea hydrothermal vents (9°N, East Pacific Rise). *Deep-Sea Research II*. 45, 465-515.
- Sibuet, M., Olu, K., 1998. Biogeographic, biodiversity and fluid dependence of deep-sea cold-seep communities at active and passive margins. *Deep-Sea Research Part II*. 45, 517-567.
- Sibuet, M., Olu-LeRoy, K., 2002. Cold seep communities on continental margins: structure and quantitative distribution relative to geological and fluid venting patterns. In *Ocean Margin Systems*, G. Wefer et al. eds). Berlin: Springer-Verlag, 235–251.
- Simoneit, B. R. T., Lonsdale, P. F., Edmond, J. M., Shanks, I. W. C., 1990. Deep-water hydrocarbon seeps in Guaymas Basin, Gulf of California. *Applied Geochemistry*. 5, 41-49.
- Smirnov, R. V., 2000. A new species of Spirobrachia (Pogonophora) from the orkney Trench (Antarctica). *Polar Biol*. 23, 567-570.
- Smith, C. R., Kukert, H., Wheatcroft, R. A., Jumars, P. A., Deming, J. W., 1989. Vent fauna on whale carcasses. *Nature*. 341, 27-28
- Sommer, S., Linke, P., Pfannkuche, O., Schleicher, T., J., S. v. D., Reitz, A., Haeckel, M., Flögel, S., C., H., 2009. Seabed methane emissions and the habitat of frenulate tubeworms on the Captain Arutyunov mud volcano (Gulf of Cadiz). *Marine Ecology Progress Series*. 382, 69-86.
- Somoza, L., Díaz-del-Río, V., León, R., Ivanov, M., Fernández-Puga, M. C., Gardner, J. M., Hernández-Molina, F. J., Pinheiro, L. M., Rodero, J., Lobato, A., Maestro, A., Vázquez, J. T., Medialdea, T., Fernández-Salas, L. M., 2003. Seabed morphology and hydrocarbon seepage in the Gulf of Cádiz mud volcano area: acoustic imagery, multibeam and ultra-high resolution seismic data. *Marine Geology*. 195, 153-176.
- Southward, A. J., Southward, E. C., 1981. Dissolved organic matter and the nutrition of the Pogonophora: a reassessment based on recent studies of their morphology and biology. *Kieler Meeresforschung*. 5, 445-453.
- Southward, A. J., Southward, E. C., Dando, P. R., Barrett, R. L., Ling, R., 1986. Chemoautotrophic function of bacterial symbionts in small pogonophora. *J Mar Biol Ass*. 66, 415–437.
- Southward, A. J., Southward, E. C., Dando, P. R., Rau, G. H., Felbeck, H., Flugel, H., 1981. Bacterial Symbionts and Low C-13-C-12 Ratios in Tissues of Pogonophora Indicate Unusual Nutrition and Metabolism. *Nature*. 293, 616-620.
- Southward, A. J., Southward, E. C., 1980. The significance of dissolved organic-compounds in the nutrition of siboglinum-ekmani and other small species of Pogonophora. *Journal of the Marine Biological Association of the U. K.* 60, 1005-1034.

- Southward, A. J., 1988. Distribution of Pogonophora in canyons of the Bay of Biscay: Factors controlling abundance and depth range. *J. Mar. Biol. Ass. U.K.* 68, 627 - 638.
- Southward, E., 1986. Gill symbionts in Thyasirids and other bivalve molluscs. *J. Mar. Biol. Ass. U.K.*, 889-914.
- Southward, E. C., 1963. On a new species of Siboglinun (Pogonophora), found on both sides of the North Atlantic. *J. Mar. Biol. Ass. U.K.* 43, 513-517.
- Southward, E. C., 1978. Description of a new species of Oligobrachia (Pogonophora) from the North Atlantic, with a survey of the Oligobrachiidae. *J. Mar. Biol. Ass. U.K.* 58, 357 - 365.
- Southward, E. C., Schulze, A., Gardiner, S. L., 2005. Pogonophora (Annelida): form and function. *Hydrobiologia*. 535/556, 227-251.
- Stadnitskaia, A., Ivanov, M. K., Blinova, V., Kreulen, R., van Weering, T. C. E., 2006. Molecular and carbon isotopic variability of hydrocarbon gases from mud volcanoes in the Gulf of Cadiz, NE Atlantic. *Marine and Petroleum Geology*. 23, 281-296.
- Tieszen, L. L., Boutton, T. W., Tesdahl, K. G., Slade, N. A., 1983. Fractionation and turnover of stable carbon isotopes in animal tissues – implication for $\delta^{13}C$ analysis of diet. *Oecologia*. 57(1-2), 32-37.
- Tunnicliffe, V., 1991. The biology of hydrothermal vents: Ecology and evolution. *Oceanography and Marine Biology Annual Review*. 29, 319-407.
- Tunnicliffe, V., Fowler, C. M. R., McArthur, A. G., 1996. Plate tectonic history and hot vent biogeography. In: MacLeod CJ, Tyler PA, Walker CL (eds) *Tectonic, magmatic, hydrothermal and biological segmentation of Mid-Ocean ridges*. Geological Society. 225-238.
- Tunnicliffe, V., Juniper, S. K., Sibuet, M., 2003. Reducing environments of the deep-sea floor. In: Tyler PA (ed) *Ecosystems of the World: The Deep-Sea*. Elsevier.
- Van der Land, J., Nørrevang, A., 1975. The systematic position of Lamellibrachia (Annelida, Vestimentifera). *Z. Zool. Syst. Evolforsch.* 1, 86-101.
- Van Dover, C. L., 2000. *The Ecology of Deep-Sea Hydrothermal Vents*. New Jersey: Princeton University Press.
- Van Dover, C. L., 2002. Trophic relationships among invertebrates at the Kairei hydrothermal vent field (Central Indian Ridge). *Marine Biology*. 141, 761-772.
- Van Dover, C. L., Fry, B., 1989. Stable isotopic compositions of hydrothermal vent organisms. *Marine Biology*. 102, 257-263.
- Van Dover, C. L., Fry, B., 1994. Microorganisms as food resources at deep-sea hydrothermal vents. *Limnology Oceanography*. 39, 51-57.
- Van Rensbergen, P., Depreiter, D., Pannemans, B., Henriët, J. P. 2005. Seafloor expression of sediment extrusion and intrusion at the El Arraiche mud volcano field, Gulf of Cadiz *J. Geophys. Res.* 110.

Van Rensbergen, P., Depreiter, D., Pannemans, B., Moerkerke, G., Van Rooij, D., Marsset, B., Akhmanov, G., Blinova, V., Ivanov, M., Rachidi, M., Magalhaes, V., L., P., Cunha, M., Henriët, J. P., 2005. The El Arraiche mud volcano field at the Moroccan Atlantic slope, Gulf of Cadiz. *Marine Geology*. 219, 1-17.

Vetter, R. D., Fry, B., 1998. Sulfur contents and sulfur-isotope compositions of thiotrophic symbioses in bivalve molluscs and vestimentiferan worms. *Marine Biology*. 132, 453-460.

Werne, J., Baas, M., Sinninghe Damsté, J., 2002. Molecular isotopic tracing of carbon flow and trophic relationships in a methane-supported benthic microbial community. *Limnology Oceanography*. 47, 1694-1701.

Whiticar, M. J., 1999. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chemical Geology*. 161, 291-314.

Yamanaka, T., Mizota, C., Satake, H., Kouzuma, F., Gamo, T., 2003. Stable Isotope Evidence for a Putative Endosymbiont-Based Lithotrophic *Bathymodiolus* sp. Mussel Community Atop a Serpentine Seamount. *Geomicrobiology Journal*. 20, 185-197.

Zitellini, N., Gràcia, E., Matias, L., Terrinha, P., Abreu, M., DeAlteriis, G., Henriët, J., Dañobeitia, J., Masson, D., Mulder, T., Ramella, R., Somoza, L., Diez, S., 2009. The quest for the Africa–Eurasia plate boundary west of the Strait of Gibraltar. *Earth and Planetary Science Letters*. 208, 13-50.