



Deep-sea bacterial communities in sediments and guts of deposit-feeding holothurians in Portuguese canyons (NE Atlantic)

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ABSTRACT

Deposit-feeding holothurians often dominate the megafauna in bathyal deep-sea settings, in terms of both abundance and biomass. *Molpadia musculus* is particularly abundant at about 3400 m depth in the Nazaré Canyon on the NE Atlantic Continental Margin. However, these high abundances are unusual for burrowing species at this depth. The objective of this research was to understand the reasons of the massive occurrence of these molpadiid holothurians in the Nazaré Canyon. To address this question we investigated possible trophic interactions with bacteria at sites where the organic content of the sediment was different (Setúbal and Cascais Canyons, NE Atlantic Continental Margin). The molecular fingerprinting technique of Denaturing Gradient Gel Electrophoresis (DGGE) with band sequencing, combined with non-metric multi-dimensional scaling and statistical analyses, was used to compare the bacterial community diversity in canyon sediments and holothurian gut contents. Our results suggest that *M. musculus* does not need to develop a specialised gut bacterial community to aid digestion where the sediment is rich in organic matter (Nazaré Canyon); in contrast, such a community may be developed where the sediment is poorer in organic matter (Cascais Canyon).

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1. Introduction

Submarine canyons, deep incisions of the continental shelf and continental slope, are abundant along the European NE Atlantic Ocean and Mediterranean margins (Weaver et al., 2004; Canals et al., 2004). Over the last two decades, submarine canyons have received increasing attention as hotspots of benthic production (Vetter, 1994) and key habitats of commercially exploited species

(Pagès et al., 2007). Compared to the surrounding continental slope areas, submarine canyons are characterized by a higher content of organic C and relatively high biogeochemical activity (Gage et al., 1995; Vetter and Dayton, 1998; Schmidt et al., 2001; Epping et al., 2002). The elevated benthic metabolic activity in canyons compared to adjacent regions is caused by the high delivery rate of organic matter (OM) (including fresh material) from the shelf directly to the deep sea (van Weering et al., 2002). These episodic inputs play an important role in deep-sea processes and also have an important effect on faunal distributions.

The composition and diversity of benthic communities reflect the stability of the systems they inhabit. Episodic events (slumps and turbidity flows) and tidal currents

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(reaching velocities of up to 2 m/s – Vetter and Dayton, 1998), cause resuspension and down-slope transport of sediments, creating significant disturbance to benthic ecosystems (Thorne-Miller and Catena, 1991). Faunal abundance and biomass in canyon systems are higher (e.g. Gage et al., 1995; Vetter and Dayton, 1998; Duineveld et al., 2001), and faunal diversity lower (e.g. Gage et al., 1995; Vetter and Dayton, 1998; Curdia et al., 2004), than in adjacent slope habitats. These differences have been attributed to the higher organic content of canyon systems (Gage et al., 1995; Duineveld et al., 2001).

The Portuguese margin has some of Europe's largest submarine canyon systems (Nazaré Canyon, Setúbal Canyon and Cascais Canyon – Fig. 1). As part of the EU Integrated Project HERMES (Hotspot Ecosystem Research on the Margins of European Seas), a number of research cruises have been undertaken to the Portuguese margin to investigate the composition, biomass and activity of benthic communities inhabiting these canyons. A large number of burrowing sea cucumbers (holothurians) is present in the central part of the Nazaré Canyon (3200–3400 m depth) (Weaver, 2005). On the other hand, the abundance of other benthic fauna commonly found at bathyal depths at continental margins is low. Burrowing sea cucumbers were also found in Setúbal and Cascais Canyons, albeit in low abundance. Two species of deposit-feeding holothurians were sampled in these canyons. One, *Molpadia musculus* Risso, 1826, is a sub-surface burrowing deposit-feeder with a mean abundance of 125 ind/m². The other, *Ypsilothuria bitentaculata* Ludwig, 1893, is a sedentary surface-feeder with a mean abundance of 136 ind/m². Their high densities are unusual for deep-sea burrowing species at this depth (Weaver, 2005).

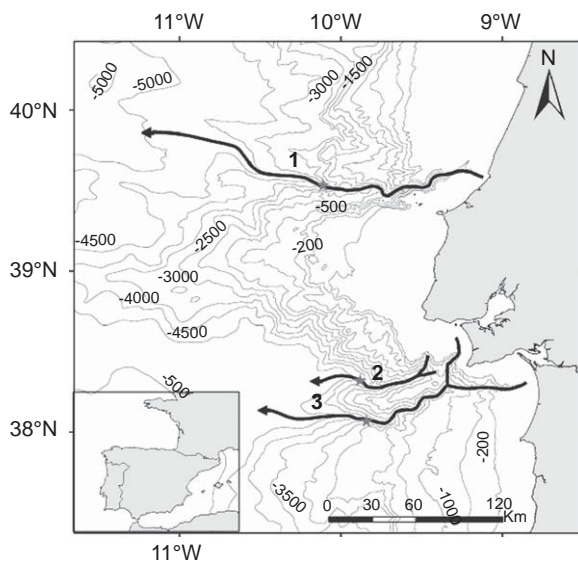


Fig. 1. Overview chart of the bathymetry of the Portuguese margin showing the location of the canyons: 1—Nazaré Canyon, 2—Cascais Canyon, 3—Setúbal Canyon. The ★ represents the stations (3500 m depth) sampled and the small square in the inset of Spain–Portugal the area zoomed in. Bathymetry from GEBCO, 2003.

Deposit-feeding holothurians dominate the deep-sea megabenthos in many areas, in terms of both biomass and abundance (Sibuet et al., 1982; Billett, 1991; Roberts et al., 2000). At the Porcupine Abyssal Plain (PAP; NE Atlantic Ocean), for example, holothurians account for ~76 and 93% of the megabenthos abundance and biomass, respectively (Billett et al., 2001). According to Sibuet (1985), food supply is an important factor controlling the abundance and the composition of the holothurian assemblages. The various species have each developed a different way of exploiting food sources. Individual holothurian species show both behavioural and morphological adaptations including habitat selection (Billett, 1991), differences in tentacle morphology (Roberts et al., 2000; Roberts and Moore, 1997) and gut diversification (Moore et al., 1995). The material ingested from the surface sediment by these deposit-feeding holothurians includes microorganisms, meiofauna, decaying organic debris and inorganic components, as well as dissolved organic matter (DOM) (Roberts et al., 2001). The nutritional value of the sediments varies seasonally (Billett et al., 1983) and with distance from the source of organic material, which is generally produced photosynthetically in surface waters and sinks to the ocean floor as phytodetritus. The overall nutritional value of the phytodetritus is reflected by the biomass of both bacteria and invertebrate megafauna colonizing it (Roberts et al., 2001). High bacterial abundances have been recorded in the guts of deep-sea holothurians (Deming and Colwell, 1982; Sibuet et al., 1982; Roberts et al., 2001) and there is often a proliferation of microbes in their hindguts, suggesting that sediment and/or enteric bacteria play an important role in their nutrition. The deep-sea holothurians may use the bacteria directly as food source or they can use the bacteria indirectly to provide them with the essential nutrients not available otherwise (Deming and Colwell, 1982; Deming et al., 1981; Eardly et al., 2001).

This aim of this study is to understand how the high abundance of the holothurian *M. musculus* can be maintained in the bathyal Portuguese canyons (Nazaré, Setúbal and Cascais). We hypothesized that trophic interactions with enteric bacteria make the high organic content of the canyon sediment available to the holothurians. To address these issues, sediment samples and holothurian gut contents were collected in the three canyons and their composition analysed for bacterial diversity. We used denaturing gradient gel electrophoresis (DGGE) as a fingerprinting technique to characterize the bacterial community composition and sequencing, followed by multi-dimensional scaling and other statistical analyses, to evaluate bacterial diversity and potential differences between sample types.

2. Materials and methods

2.1. Study site

The Nazaré Canyon (Fig. 1) is a very narrow and deep canyon without a connection to a major river outflow. By

contrast, Setúbal Canyon (Fig. 1) is connected to two rivers (Tagus, Sado) and is situated just offshore of the most densely populated and industrialized area of Portugal (De Stigter et al., 2007). The Nazaré Canyon is characterized by the presence of moderately strong tidal currents (max. currents reach peaks of 35 cm/s) in the upper and middle canyon where they cause resuspension, transport and redistribution of particulate matter (Quaresma et al., 2007). Resuspension is also evident from the nepheloid layers found in the head of the canyon. Transport of sediments towards the lower canyon takes place predominantly by sediment gravity flows occurring on annual or longer timescale (De Stigter et al., 2007). Activity in the Nazaré Canyon is particularly vigorous during periods of winter storms (De Stigter et al., 2007). The Cascais Canyon (Fig. 1) is not connected to any river flow but it is thought to have acted as the major conduit of sediment from the continental shelf to the abyssal plain at the time of the Lisbon earthquake in 1755.

2.2. Species studied

Molpadia musculus (Fig. 2) is a deep-sea holothurian (Phylum Echinodermata) with a sausage-shaped body that narrows into a posterior caudal appendage. This species is red to purple in colour. It possesses reduced or absent tube feet along the body wall and an oral disk bearing 10 to 15 small digitate tentacles arranged in a circle around the mouth. Ossicles always include fusiform rods present in the tail and usually in the body wall. Dark orange phosphate deposits are abundant and aggregated, especially in the mid-body section. These organisms feed head down in the sediment and maintain contact with the sediment surface through the tail. In this way, this species maintains the flow of oxygenated water through its respiratory trees connected to the cloaca in the tail (Pawson et al., 2001).

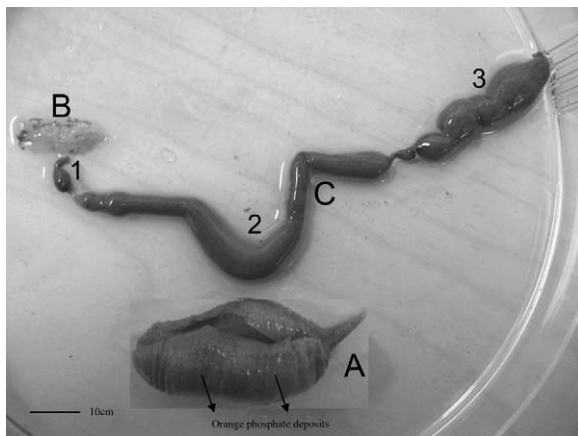


Fig. 2. Photograph of the deep-sea holothurian *Molpadia musculus* (A) and its dissected gonad (B) and gut (C), including the oesophagus (1), the mid gut (2) and the hind gut (3).

2.3. Sampling

Molpadia musculus was collected during RSS *Charles Darwin* cruise 179 between 12 April and 17 May 2006. Three Agassiz trawls were taken at depths of 3200 to –3300 m in the Cascais, Setúbal and Nazaré Canyons (Fig. 1). The water temperature at the sampling depth was around 3 °C. A total of 9 specimens was examined from the Nazaré Canyon, but only 3 specimens from each of the other two canyons. Only intact animals, packed in cold mud and thus thermally insulated during retrieval, were selected for the study. Upon retrieval, *Molpadia musculus* was put immediately in a temperature-controlled laboratory at 4 °C. Each specimen was dissected in a sterilized Petri dish. The gut was opened and sediment samples were taken from three different parts of the gut (the oesophagus, the mid gut and the hind gut) with a sterilized spatula (Fig. 2). Each sediment sample was transferred in a vial and stored at –80 °C.

At the same stations, sediment samples were taken using a megacorer fitted with 100 mm diameter core tubes. From each of three cores per canyon, a sample from 0–1 cm sediment depth was collected and stored at –80 °C.

2.4. DNA extraction and PCR

Extraction of DNA, amplification of 16S rRNA gene fragments, DGGE analysis and sequencing of excised DGGE bands were carried out as described by Schäfer et al. (2002). Bacterial DNA was extracted from 1 g sediment using the UltraClean soil DNA isolation kit (a commercial kit for DNA recovery from soil samples, MOBio laboratories, CA, USA). The primers used for PCR amplification of 16S rRNA gene fragments were 341F-GC (containing a 40-bp GC-rich sequence at the 5'-end) and 907RM, which is an equimolar mixture of the primers 907RC (5'-CCGTCAATTCCTTTGAGTTT-3') and primer 907RA (5'-CCGTCAATTCATTTGAGTTT-3') (Schäfer and Muyzer, 2001).

All PCR reactions were conducted in 50 µl aliquots containing both primers at 20 pM, 50 mM KCL, 10 mM Tris-HCL (pH 9.0), 1.5 mM MgCL₂, 100 mM of each deoxynucleoside triphosphate, 1 U of Taq polymerase biotherm+ (Genecraft, Germany), 1 µl of template DNA (Moeseneder et al., 1999). Each 50 µl contained 10–50 ng template DNA. The PCR reactions were carried out with a denaturation step of 3 min at 94 °C, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 1 min extension at 72 °C, followed by a final extension step of 10 min at 72 °C. PCR products were inspected on 1% (w/v) agarose gels and quantified by comparison of band intensities with a DNA ladder (Precision Molecular Mass Standard; Bio-Rad, Hercules, CA, USA) using the software Multianalyst (Bio-Rad) after scanning gels with a Fluor-S imaging system (Bio-Rad). DGGE analyses were carried out with a DCode system (Bio-Rad) using a 6% acrylamide/bisacrylamide gel with a denaturing gradients of 20–80% denaturants (see Schäfer and Muyzer, 2001; Muyzer et al., 1993 for further details).

2.5. Multivariate analysis

DGGE bands were analyzed with PRIMER v.6 (Plymouth Routines in Multivariate Ecological Research; Clarke and Warwick, 2001). Bands were scored as present/absent prior to the statistical analysis. A similarity matrix based on Bray Curtis similarity was applied to produce a non-metric multidimensional scale two dimensional plot (MDS) (Clarke and Warwick, 2001). The results give a stress value that indicates how the points are placed in two dimensions in such a way as to satisfy the similarity ranking. Stress values indicate the adequacy of the nMDS representation (Clarke and Warwick, 2001). To assess the similarity composition of the bacterial assemblages we performed randomization/permutation tests (ANOSIM) on the nMDS results. One-way ANOSIM tests were used to assess the significance of differences in the composition of the bacterial assemblages of groups of samples defined a priori (e.g. samples from different canyons). A statistic R was also determined as a measure of the degree of dissimilarity of bacterial composition between samples. R -values range from -1 and 1 and approach zero if there are no differences in the community composition. The analysis of similarity is a permutation-based statistical test. It is an analogue of the univariate ANOVA, which tests the differences between groups of multivariate samples from different locations, experimental treatments, etc. In this study, it was used to test the differences between canyons.

2.6. Comparative sequence analysis

DGGE bands were excised for re-amplification and sequencing according to Schäfer et al. (2002). The sequencing was carried out by the Cambridge sequencing team (UK) (www.geneservice.co.uk/services/sequencing). The same set of primers used for the PCR reaction was used for sequencing. Sequence data were automatically collated and analyzed using the ABI sequencing analysis software and were subsequently verified manually. Similarities between these sequences and the published sequences were determined using BLAST (Basic Local Alignment Search Tool) of the National Centre for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/BLAST>). Searches were undertaken at the nucleotide level (BLASTn) to identify similar sequences, as well as identifying any potential chimera. Significantly similar sequences and the associated BLAST “scores” were recorded. Further phylogenetic analyses were restricted to nucleotide positions that were unambiguously alignable in all sequences (600 nucleotides on average). Multiple bootstrapped data sets (1000 samplings) of the aligned data were exported as a PHYLIP 3.5 interleaved file type to run the SEQBOOT program. Least-squares distance matrix analyses, based on the evolutionary distances, were estimated from similarity values using the maximum likelihood analysis with multiple data sets option. Phylogenetic analyses using neighbour joining NEIGHBOR and parsimony DNA-PARS methods were performed. Random order input of

sequences, single jumbling and the global rearrangement option were used to avoid potential bias introduced by the order of sequence addition. The resulting tree files were analyzed using the CONSENSE program to provide confidence estimates for phylogenetic tree topologies and to make a majority rule consensus tree. All phylogenetic programs used were from the Phylogenetic Interference Package (PHYLIP) version 3.6 (Felsenstein, 2001).

3. Results

Analysis of the bacterial community composition, based on DGGE patterns of the PCR-amplified 16S rDNA gene fragments, revealed highly diverse bacterial communities within the canyon sediments and within the gut compartments of *M. musculus* in all canyons. This diversity was indicated by the presence of many distinguishable bands (Fig. 3A–D).

3.1. Bacterial community composition in sediments

Analyses of the DGGE-fingerprints by nMDS clearly identified distinctly different bacterial communities in the sediments of the Nazaré Canyon and the other two canyons (statistical $R = 1.000$, when comparing Nazaré Canyon vs Cascais Canyon and statistical $R = 1.000$ when comparing Nazaré Canyon vs Setúbal Canyon) (Fig. 4A). However, ANOSIM revealed no significant difference between bacterial assemblages in sediments from the Setúbal Canyon and Cascais Canyon ($R = -0.250$). The similarity among the sediment samples obtained from each canyon was high (average similarity Nazaré Canyon = 82%; Setúbal Canyon = 83%; Cascais Canyon = 77%).

3.2. Bacterial community composition in guts

Analyses by nMDS of the DGGE-fingerprints obtained from gut sections of *M. musculus* collected in the three canyons revealed that those from the Nazaré Canyon were statistically different from the other two canyons ($R = 0.520$, $p = 0.01$) (Fig. 4B). The similarity among the gut samples from each canyon was low (average similarity Nazaré Canyon = 34%; Setúbal Canyon = 54%; Cascais Canyon = 65%).

For each canyon site, no significant differences in bacterial community composition in the 3 gut sections of *M. musculus* were detected (Nazaré Canyon, $R = 0.086$, $p = 0.2$, Setúbal Canyon $R = -0.222$, $p = 0.8$, and Cascais Canyon $R = -0.279$, $p = 0.9$) (Fig. 4B).

3.3. Bacterial community composition in sediments and guts

For each of the canyons, we evaluated whether the sediment bacterial community composition was different from that in the gut of *M. musculus*. The nMDS plots showed segregation between sediment and gut samples in the Cascais and Setúbal Canyons whereas in the Nazaré Canyon the sediment and gut samples are intermingled (Figs. 4C, D). In both Nazaré and Setúbal Canyons, the ANOSIM did not reveal a significant difference between

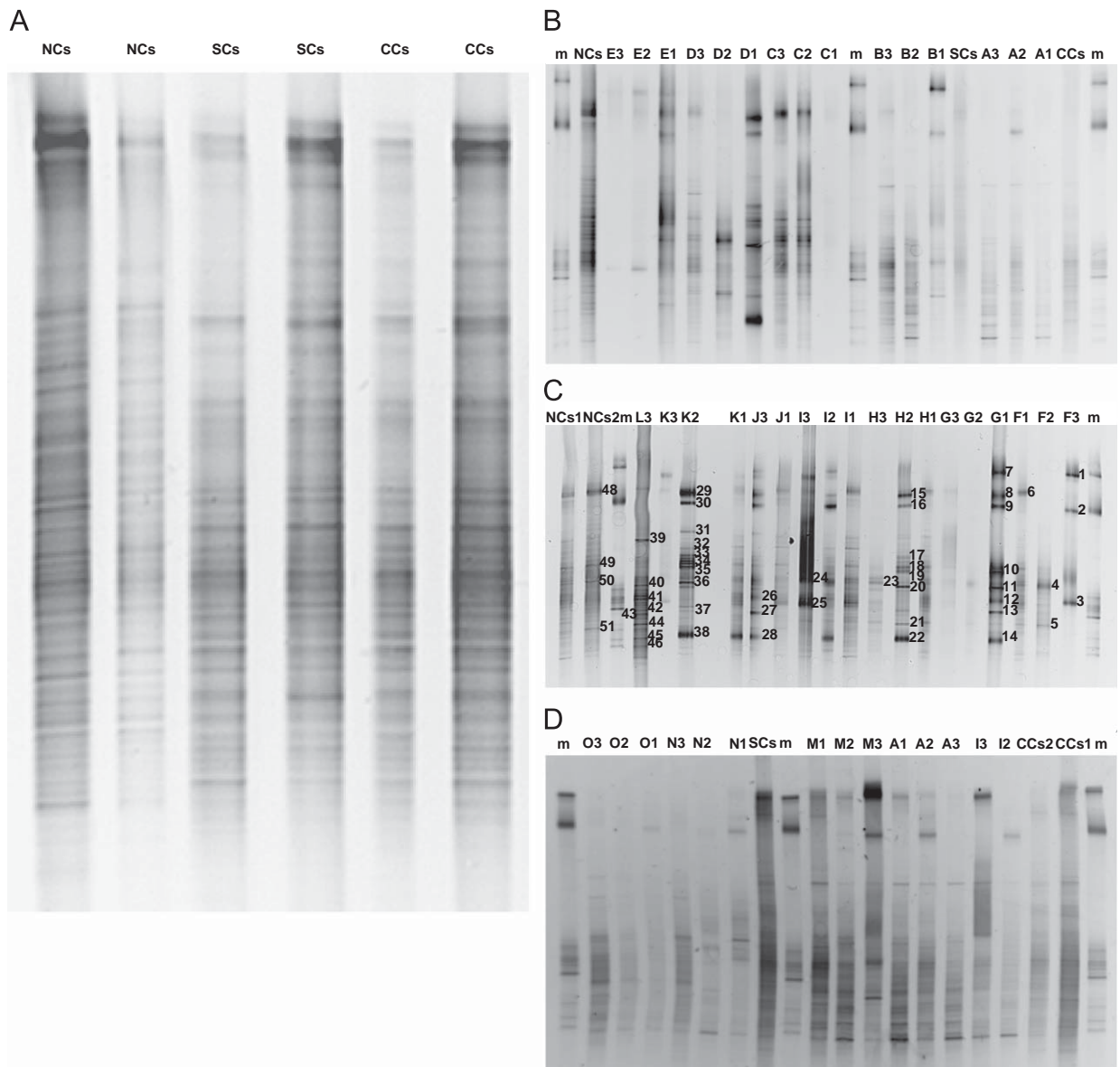


Fig. 3. (A) DGGE patterns of PCR-amplified 16S rRNA gene fragments from bacterial DNA in superficial sediments from the Nazaré Canyon sediment (NCs), Setúbal Canyon sediment (SSs) and Cascais Canyon sediment (CCs). (B) DGGE patterns of PCR-amplified 16S rRNA gene fragments from bacterial DNA in the oesophagus (1), mid gut (2), and hind gut (3) sections of five specimens of *M. musculus*, one from Cascais Canyon (A), one from Setúbal Canyon (B), and three from Nazaré Canyon (C–E). Results from superficial canyon sediment appear in lanes marked CCs, SCs, and NCs (see (A)) for comparison. Standard markers (a mixture of 10 sequenced bacteria) appear in lanes marked “m”. (C) DGGE patterns of PCR-amplified 16S rRNA gene fragments from bacterial DNA in two samples of Nazaré Canyon sediment (NCs1 and NCs2) and in the oesophagus (1), midgut (2), and hindgut (3) sections of seven specimens of *M. musculus* (F–L) from the same canyon. Standard markers as in (B). Numbers next to bands key to sequences in Fig. 5. (D) DGGE patterns of PCR amplified 16S rRNA gene fragments from bacterial DNA in samples of Cascais Canyon sediment (CCs) and Setúbal Canyon sediment (SCs) and in the oesophagus (1), midgut (2), and hindgut (3) sections of five specimens of *M. musculus* from these canyons, three from Cascais Canyon (I, M, and A; see (B)) and two from Setúbal Canyon (N, O). Standard markers as in (B).

sediment and gut bacterial community composition (Nazaré Canyon sediments vs Nazaré Canyon gut content, $R = -0.368$, $p = 0.1$; Setúbal Canyon sediment vs Setúbal Canyon gut content, $R = 0.689$, $p = 0.143$). However, for the Cascais Canyon, ANOSIM revealed significant differences between the bacterial community composition in the sediments and in the gut of *M. musculus* (Cascais

Canyon sediment vs Cascais Canyon gut content, $R = 0.556$, $p = 0.04$).

In total, 46 DGGE bands were excised and sequenced (Fig. 3C) and 20 distinct bacterial phylogenetic affiliations were defined (Fig. 5). The lengths of the 46 obtained sequences (GeneBank codes accession numbers from FJ822664 to FJ822683) were from 403 to 478 bp. All the

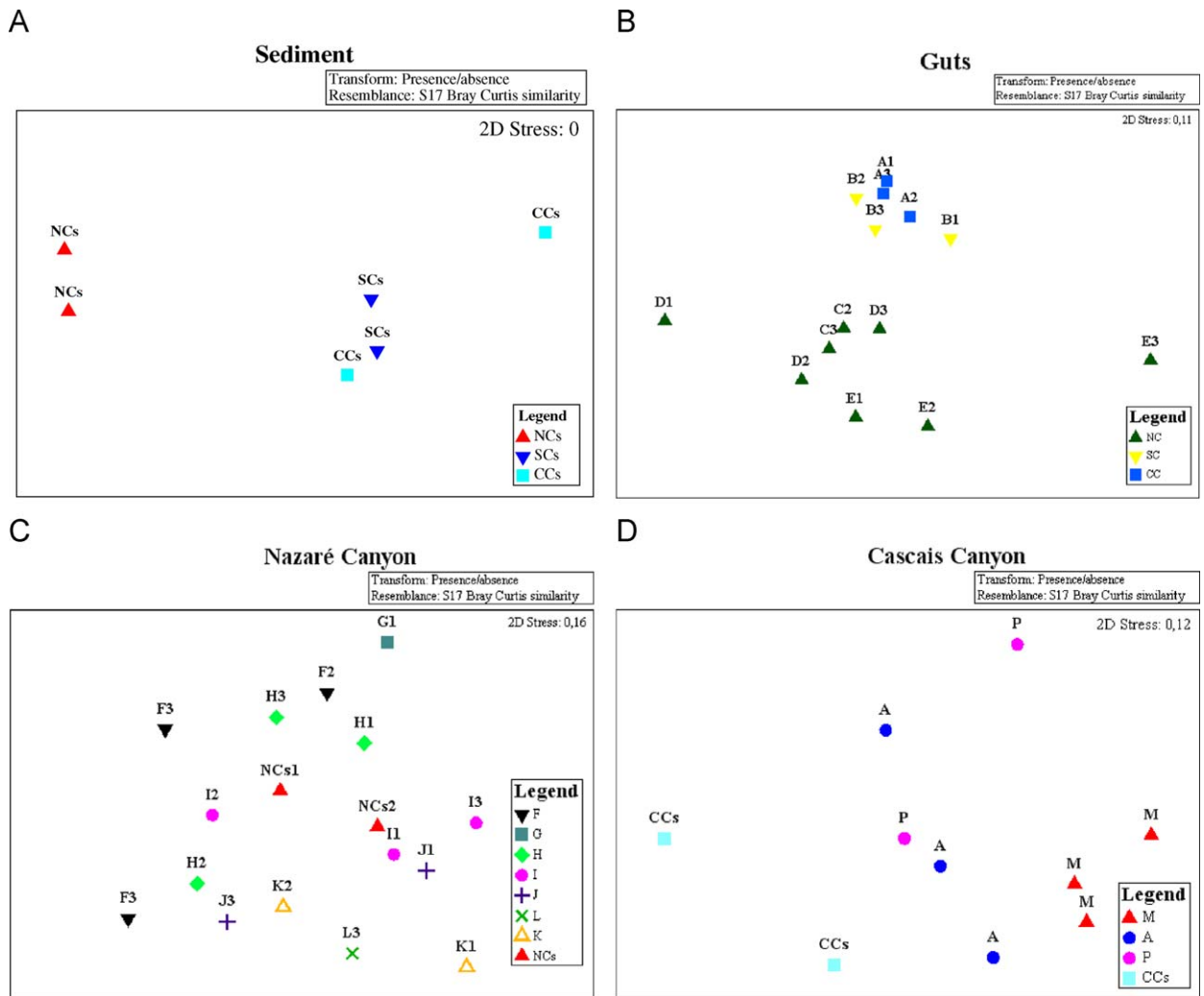


Fig. 4. (A) Non-metric multi-dimensional scaling plot of the presence/absence of bands from bacterial communities in two samples of Nazaré Canyon sediment (NCs), Setúbal Canyon sediment (SCs), and Cascais Canyon sediment (CCs). (B) Non-metric multi-dimensional scaling plot of the presence/absence of bands from bacterial communities in gut sections of *M. musculus* from Nazaré Canyon (NC), Setúbal Canyon (SC), and Cascais Canyon (CC); other designations as in Fig. 3B. (C) Non-metric multi-dimensional scaling plot of the presence/absence of bands from bacterial communities in two samples of Nazaré Canyon sediment (NCs1 and NCs2), and in the oesophagus (1), mid gut (2), and hind gut (3) sections of six specimens of *M. musculus* (F–K) from the same canyon. (D) Non-metric multi-dimensional scaling plot of the presence/absence the bands from bacterial communities in Cascais Canyon sediments (CCs) and in the oesophagus (1), mid gut (2), and the hind gut (3) sections of three specimens of *M. musculus* (L, M, A) from the same canyon.

bacteria identified from the various DGGE bands were closely related to either Bacteroidetes, formerly termed Cytophaga-Flavobacterium-Bacteroides (CFB), Proteobacteria, Fibrobacter/Acidobacter, Spirochaetes groups. Most of the DGGE bands were affiliated with the CFB group. Closely related bacteria were obtained from each of the three sections along the gut of *M. musculus*. The other bands excised from the DGGE gels illustrated in Figs. 3B and D were also affiliated with the groups mentioned above with the exception of the presence of a Gram positive group (data not shown). It was not possible to sequence any bacteria from the sediment samples because the separation of the bands in the DGGE gel was not sufficient for interpretation.

4. Discussion

The nutrition of deep-sea holothurians has received considerable attention since the early 1980s (Deming and Colwell, 1982; Sibuet et al., 1982; Billett et al., 1988; Roberts et al., 1991, 2000; Witbaard et al., 2001; Ginger et al., 2001; Hudson et al., 2005). Evidence that some deep-sea holothurians feed selectively, whether on organic-rich particles from the seabed or on enriched superficial sediment layers, has been obtained by comparing the organic content of ingested sediments in the oesophagus and that of sediments in the vicinity of the organism (Sibuet et al., 1982; Billett et al., 1988). Bacteria might play an important role in this process via their

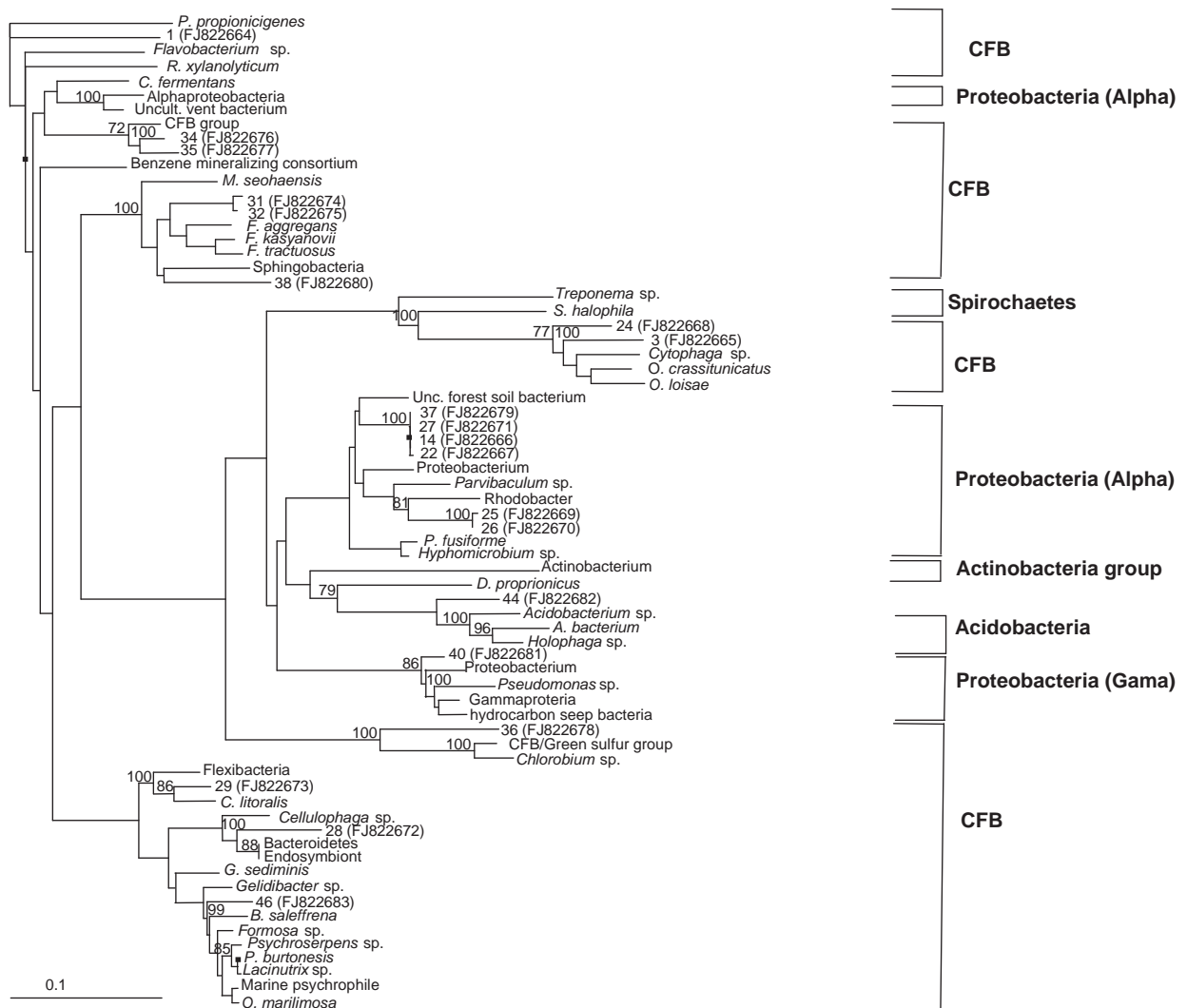


Fig. 5. Phylogenetic tree based on bacterial 16S rRNA gene fragments from excised DGGE bands marked in Fig. 3C from gut sections of holothurians from Nazaré Canyon. The tree was drawn from ClustalW generated multiple sequences alignment of nucleotide sequences. The numbers at the nodes are percentages indicating levels of bootstrap support, based on a neighbour-joining analysis of 1000 resample data sets. Only values > 75% are shown. (e.g. 1, 36, etc.), refer to the numbers in the denaturing gradient gel (Fig. 3C). Scale bar represents 0.1 substitutions per nucleotide position.

hydrolytic ectoenzymes, which make high molecular weight dissolved organic matter (DOM) available to bacteria (Roberts et al., 1991). The existence of abundant enteric bacteria in the guts of deep-sea holothurians has been showed repeatedly (Deming and Colwell, 1982; Danovaro et al., 1998; Roberts et al., 2001), but a link between bacteria and the host nutrition remains poorly demonstrated. Yet, several studies point to a decline in bacterial numbers in the foregut of deep-sea holothurians, suggesting the digestion of bacteria (Deming and Colwell, 1982). For example, Moore et al. (1995) observed that the abundance of bacteria in *Oneirophanta mutabilis* decreased between the oesophagus and the anterior intestine but increased again in the posterior intestine and rectum/cloaca. According to Roberts et al. (2001), the differences

in bacterial activity and abundances in different parts of the guts of abyssal holothurians point to different bacterial environments along their guts and the potential bacterial breakdown of a broad range of substrates (Plante and Jumars, 1992).

The ultimate goal of the present study was to better understand the role of bacteria in the nutrition of *M. musculus* in the Portuguese canyons. Analyses of the bacterial community structure in sediments ingested by holothurians and in the surrounding sediments should provide clues about the trophic interactions between the bacteria and their holothurian hosts, which potentially can enhance the bioavailability of sediment organic matter for the holothurian. To our knowledge this is the first attempt to compare the structure of bacterial

communities associated with a bathyal organism and the surrounding sediments.

We found that the bacterial community is highly diverse both within the canyon sediments and within the gut compartments of *M. musculus* (Fig. 3A–D). Previous studies have demonstrated the existence of abundant enteric bacteria in the sediments (Danovaro et al., 1998) and in guts of deep-sea holothurians (Deming and Colwell, 1982; Roberts et al., 2001), but a link between bacteria and host nutrition remains poorly demonstrated. Furthermore, the composition of bacterial communities in the sediments and guts of *M. musculus* from the Nazaré Canyon were distinctly different from those found in the other two canyons (Fig. 4A and B). The existence of a distinct bacterial community in the Nazaré Canyon might be due to a combination of high organic carbon content of the sediment and specific physical parameters (e.g., hydrodynamic regimes, granulometry) in this canyon system. Kiriakoulakis et al. (2008) concludes that, compared to the other two canyons, sediments in the Nazaré Canyon are enriched in organic carbon (mean TOC concentrations $2.08 \pm 0.06\%$ of dry sediment in Nazaré Canyon, 1.3% in Setúbal Canyon, $1.3 \pm 0.04\%$ in Cascais Canyon; mean TON $0.21 \pm 0.01\%$ of dry sediment in Nazaré Canyon, 0.2% in Setúbal Canyon, $0.19 \pm 0.07\%$ in Cascais Canyon). However, food availability is not the only factor that can explain the distribution of organisms. Heterogeneity in substrate characteristics, e.g., sediment texture and grain size, might also explain the overall variability of the distribution of benthic organisms (Jumars and Eckman, 1983; Flach et al., 2002; Gooday, 2002).

According to De Stigter et al. (2007), the sediments from both Cascais and Nazaré Canyon are dominated by the mud fraction ($<63 \mu\text{m}$ grain size), which is finer grained in the Cascais Canyon than in the Nazaré Canyon. The difference in grain-size distribution between the two canyons reflects differences in their sediment supply. In Cascais Canyon, sedimentation is more continuous than in the Nazaré Canyon. The sediment of the Nazaré Canyon is predominantly terrigenously-derived material originating from the adjacent shelf and actively transported through the canyon to the bathyal plain. The finer sediment resuspended by bottom currents settles on the adjacent terraces, whereas some sand or even coarser material generally travels through the canyon. For example, at 3200 to 3300 m depth (the location where the molpadiids are abundant), the sediment deposition rate and the OM content of the mud are high (12 mm/y), while OM degradation is only moderate (Weaver, 2005). As for the Setúbal Canyon, sediments in the upper canyon at around 1800 m depth are finer grained than those of the Nazaré Canyon but coarser than those of the Cascais Canyon. Sediment from the lower part of the Setúbal Canyon at 4400 m depth has a comparable particle size distribution to the deep Cascais sediment. This indicates a less dynamic sediment transport regime in the Setúbal Canyon, than in the other canyons. The sediment rates from both of these canyons are 1 mm/y (De Stigter et al., 2007).

In the Nazaré Canyon, the bacterial community composition in the sediments cannot be distinguished

from that in the guts of *M. musculus* (Fig. 4C). Thus, from our study, there is no evidence for a specialised enteric bacterial community in *M. musculus* in this canyon. Also, no differences in the bacterial community composition among the different regions of the gut of *M. musculus* were detected in Nazaré Canyon. This absence of evidence may indicate that the digestive strategies of *M. musculus* do not involve the hydrolytic assistance of a gut flora but only the consumption of sedimentary bacteria (also suggested by Roberts et al. (2001) ingested incidentally when feeding on organic-rich sediments as in the Nazaré Canyon. Members of the Cytophaga-Flavobacteria-Bacteroides (CFB) group dominated the composition of the bacterial community while less important groups belonged to the Proteobacteria, Fibrobacter/Acidobacter and Spirochaetes groups. All these groups are commonly found in the marine environment (Yang et al., 2007) again indicating the absence of a specialised enteric bacterial community.

MDS and ANOSIM reveal that the sediment bacterial community in the Cascais Canyon is significantly different to that in the guts of *M. musculus* (Fig. 4D). As the sediment here is poorer in organic matter than in the Nazaré Canyon (mean TOC concentrations $2.08 \pm 0.06\%$ of dry sediment in Nazaré Canyon, $1.3 \pm 0.04\%$ in Cascais Canyon; mean TON $0.21 \pm 0.01\%$ of dry sediment in Nazaré Canyon, $0.19 \pm 0.07\%$ in Cascais Canyon), the gut bacterial community may reflect the elevated organic matter content in the gut as compared to the more nutrient-poor ambient sediment. It is possible that this specific gut bacterial community supports *M. musculus* in making refractory organic matter available as suggested by Jumars et al. (1990). Our data for the Setúbal Canyon are inconclusive. A larger data set is needed to determine the nature of the bacterial communities in this canyon.

This study used comparative analyses of bacterial community structure to suggest that the organic content of the sedimentary environment may determine whether or not the holothurian species *M. musculus* develops a specific gut flora to aid digestion. We found that the higher the organic content of the sediment, the less likely it is that a specific gut community is developed. More specific molecular fingerprinting techniques (ARISA, T-RFLP and CARD-FISH) are required in order to further investigate the trophic interactions between bacteria and *M. musculus*. The bioavailability of potential food sources, such as proteins, carbohydrates and lipids, also needs to be studied in order to understand the nutritional requirements of these organisms in the Portuguese canyons.

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