

The trophic biology of the holothurian *Molpadia musculus*: implications for organic matter cycling and ecosystem functioning in a deep submarine canyon

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Abstract. Megafaunal organisms play a key role in ecosystem functioning in the deep-sea through bioturbation, bioirrigation and organic matter cycling. At 3500 m water depth in the Nazaré Canyon, NE Atlantic, very high abundances of the infaunal holothurian *Molpadia musculus* were observed. To quantify the role of *M. musculus* in sediment cycling, sediment samples and holothurians were collected using an ROV and in situ experiments were conducted with incubation chambers. The biochemical composition of the sediment (in terms of proteins, carbohydrates and lipids), the holothurians' gut contents and holothurians' faecal material were analysed. In the sediments, proteins were the dominant organic compound, followed by carbohydrates and lipids. In the holothurian's gut contents, protein concentrations were higher than the other compounds, decreasing significantly as the material passed through the digestive tract. Approximately $33 \pm 1\%$ of the proteins were digested by the time sediment reached the mid gut, with a total digestion rate equal to $67 \pm 1\%$. Carbohydrates and lipids were ingested in smaller amounts and digested with lower efficiencies ($23 \pm 11\%$ and $50 \pm 11\%$, respectively). As a result, the biopolymeric C digestion rate was on average $62 \pm 3\%$. We estimated that the population of *M. musculus* could remove approximately 0.49 ± 0.13 g biopolymeric C and 0.13 ± 0.03 g N m⁻² d⁻¹ from the sediments. These results suggest that *M. musculus* plays a key role in the benthic tropho-dynamics and biogeochemical processes in the Nazaré Canyon.

1 Introduction

The deep sea is the largest ecosystem on Earth, covering about 60% of the globe's surface. Deep-sea ecosystems provide the largest reservoirs of biomass and non-renewable resources (Gage and Tyler, 1991), which are linked to their biodiversity (Danovaro et al., 2008b). They act as the ultimate sink for organic material derived from the upper ocean's primary production and are essential in the biogeochemical cycling of organic matter (OM) on a global scale (Dell'Anno and Danovaro, 2005; Danovaro et al., 2008a).

Deep-sea submarine canyons host hotspots of OM and biomass (Gage et al., 1995; Vetter and Dayton, 1998; Duineveld et al., 2001; Bianchelli et al., 2008; Ingels et al., 2009; Tyler et al., 2009; de Leo et al., 2010; Pusceddu et al., 2010; Vetter et al., 2010). Within canyons, biodiversity is high as a result of their topographic complexity and spatio-temporal variability (Bianchelli et al., 2008; Ingels et al., 2009; Tyler et al., 2009; Vetter et al., 2010). The topographic complexity of canyons makes them difficult ecosystems to investigate. As a result, knowledge of their ecological functioning (e.g., community respiration, productivity, nutrient cycling, OM supply and remineralisation rates) are largely unknown.

The Nazaré Canyon is a major conduit of organic matter and sediment on the Portuguese margin, and one of the most spectacular topographic features of the NE Atlantic margin (de Stigter et al., 2007; Arzola et al., 2008; Masson et al., 2010). Large concentrations of organic C and biopolymeric C (BPC) have been observed within the middle section of the Nazaré Canyon, suggesting this canyon may act as a sink for OM burial (Schmidt et al., 2001; de Stigter et al.,



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2007; Masson et al., 2010; Pusceddu et al., 2010). Sediments and OM are transported from the continental shelf down the canyon and into the deep sea (de Stigter et al., 2007; Oliveira et al., 2007; de Leo et al., 2010; Vetter et al., 2010). Enrichment of OM in canyon sediments leads to enhanced benthic abundance and biomass (Rowe et al., 1982; Soetaert et al., 1991; Vetter and Dayton, 1998, 1999; Palanques et al., 2005; Bianchelli et al., 2008), and increased metabolic activities (Duineveld et al., 2001; Accornero et al., 2003).

In the central part of the Nazaré Canyon (3500 m depth) the deep-sea burrowing sea cucumber, *Molpadia musculus* (Riso, 1826), occurs in consistently high abundances, which is unusual for a deep-sea burrowing species at this depth (Amaro et al., 2009). The reasons for such high abundances remain unknown. The particulate organic carbon (POC, e.g. the food supply) that arrives on the seabed is an important factor controlling the abundance and composition of macrofauna and megafauna (Sibuet, 1985; Ruhl, 2007; Smith et al., 2008; Ruhl et al., 2008; de Leo et al., 2010). Ruhl and Smith (2004) found significant correlations between food supply and megafaunal abundance, in particular holothurians, over a 14-year time series at an abyssal site in the NE Pacific Ocean. They found that the abundance of some species increase in abundance during periods of high food availability, whereas the abundance of others increase with periods of low food supply. Similar correlations have also been reported in the Porcupine Abyssal Plain (PAP) in the northeast Atlantic (Billett et al., 2001). Food sources for benthic organisms may include organic detritus (including seagrass/algae debris and dead or decaying animals), microorganisms (prokaryotes, diatoms, protozoans) and faecal pellets of other animals (Massin, 1982; Moriarity, 1982; Jumars, 1993; Roberts et al., 2001). The relative proportions of these potential food items and their biochemical composition determine the nutritional value of the ingested material, which, in turn, regulates the potential utilization rates by benthic fauna (Fabiano et al., 1999) and, in particular, by deposit-feeding holothurians (Purinton et al., 2008). A proportion of the material ingested by benthic heterotrophs is still fresh (i.e., recently deposited detritus from the surface ocean), but some is already degraded either by planktonic organisms during the particles descent to the sea bottom (Turley and Mackie, 1995; Wakeham et al., 1997) or by other benthic metazoans and/or prokaryotes (Smith et al., 1993; Levin et al., 1997; Miller et al., 2000). The largest fraction of OM in deep-sea sediments is composed of refractory compounds and becomes available to higher benthic organisms only after ageing and microbial breakdown (Danovaro et al., 1993; Pusceddu et al., 2009). Although there have been several investigations of the nutrition of deposit-feeding holothurians (Deming and Colwell, 1982; Sibuet et al., 1982; Billett et al., 1988; Roberts et al., 1991; Roberts et al., 2000; Ginger et al., 2001; Witbaard et al., 2001; Hudson et al., 2005), the debate concerning the biological and chemical composition of the material ingested and assimilated by these organisms is still

open. Khrifounoff and Sibuet et al. (1980) suggested assimilation efficiencies of 15% for total organic carbon (TOC) and 22% for total nitrogen (TN) in some abyssal species. Likewise, Ginger et al. (2001) estimated assimilation efficiencies from 15 to 46% for TOC and from 11 to 53% for TON in four abyssal holothurians with different feeding strategies. Moreover, it is still largely unknown the contribution these large organisms make to the biogeochemical processes and organic matter cycling in deep-sea ecosystems.

The aim of our study is to understand the role of *M. musculus* in the ecosystem functioning (organic matter cycling) of the Nazaré Canyon at 3500 m. To do so, we investigate the biochemical composition of potential food sources (in terms of proteins, carbohydrates and lipids) and estimate enzymatic digestion and potential utilization by *M. musculus*. In particular the following hypotheses were tested: a) is there a selective feeding on specific components of the sediment organic matter? b) Does the holothurian feeding play an important role in the biogeochemical cycling of organic matter in deep-sea sediments? In addition, owing to the high abundances of this deep-sea holothurian, we quantify the impact of holothurians on C and N cycling at mid-canyon depths.

2 Material and methods

2.1 Study area

The Nazaré Canyon is a very narrow and elongated system. It extends for more than 210 km, from the canyon head less than 1 km from the Portuguese coastline to the abyssal plain at 5000 m depth (Fig. 1). Unlike many submarine canyons, the Nazaré Canyon is not connected to a major river system. However, it is a major sediment pathway on the Portuguese margin (de Stigter et al., 2007). The sediment is predominantly of terrigenous silt and clay origin (Alt-Epping et al., 2007). The sediment is actively transported from the upper continental shelf to the canyon and out onto the abyssal plain by sediment gravity flows that occur on annual or longer timescales and are particularly vigorous during periods of winter storms (de Stigter et al., 2007; Masson et al., 2010). The currents flowing down the canyon lead to cycles of resuspension and deposition, alternating with intervals during which the sediment is deposited on the sea bed (de Stigter et al., 2007). The finer sediment resuspended by bottom currents, settles on the terraces adjacent to the thalweg, whereas sand and even coarser material generally travels through the canyon. In the upper and middle canyon there are moderately strong tidal currents (max. currents speeds up to 35 cm s^{-1}) (de Stigter et al., 2007), which can cause sediment resuspension and consequently, transport and redistribution of particulate matter (Quaresma et al., 2007).

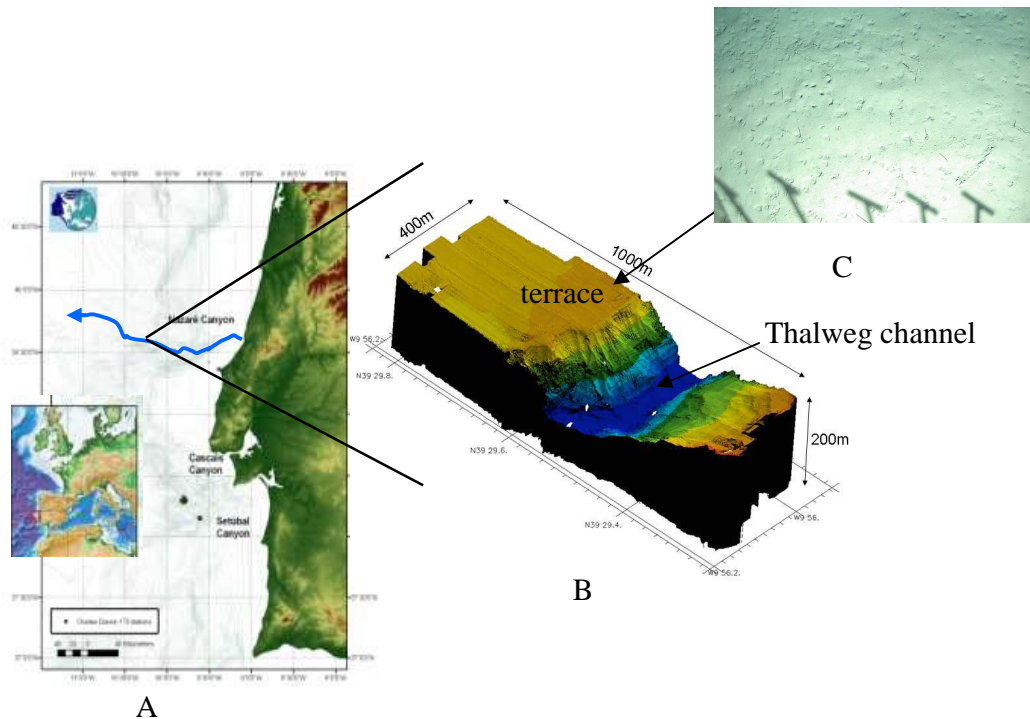


Fig. 1. Nazaré Canyon, 3500 m site. **(A)** Chart of the bathymetry of the Portuguese margin (GEBCO, 2003), showing the location the Nazaré Canyon; **(B)** ROV high resolution swath bathymetry of the central thalweg channel of the Nazaré canyon at 3500 m. The steep channel wall is composed of rock outcrop. The channel floor has boulders with a thin drape of mud. **(C)** Terrace to the north of the thalweg is characterised by burrowing animals as seen by the burrows and feeding pits in the photograph (Modified after, Masson et al., 2010).

2.2 Species studied

Molpadia musculus (Phylum Echinodermata) is a sub-surface deposit feeder holothurian and a mound builder. It spends its life orientated vertically or obliquely in the substrate, head downward, ingests the sediment at depth and excretes at the sea floor, thereby causing a significant upward transport of particles (i.e., a “conveyor belt feeder”; Rhoads, 1974). It has a stout body and a posterior region which narrows to form a “tail”. There are no tube feet (Fig. 2). Species of the genus *Molpadia* inhabits muddy environments from shallow waters to abyssal depths (Rhoads and Young, 1971). These organisms feed head down in the sediment and use their tails to keep contact with the sediment surface (bioirrigation) (Pawson et al., 2001).

2.3 Sampling

Densities of *M. musculus* were determined using data collected during RRS *Charles Darwin* cruise 179 from 12 April to 17 May 2006. Five deployments were taken using an UKORS Megacorer (twelve multiple cylindrical cores of 0.008 m² internal area each, for a total of approximately 0.1 m² sampled per deployment) at ca. 3500 m (Table 1) in the Nazaré Canyon. Molpadiids were counted and their den-

sity per m² determined from replicated multicorers. Furthermore, the specimens were weighted and total biomass estimated as wet weight per m².

Sediment samples, holothurians and their faecal material were collected using the *Isis* Remotely Operated Vehicle (ROV) at 3500 m in the Nazaré Canyon during the RRS *James Cook* cruise 10, from 3 June to 7 July 2007 (Table 1). Undisturbed sediment samples were collected with the push cores ($n = 3$) fitted with 57 mm inner diameter core tubes. Upon recovery, all cores were sliced at the following intervals: 0–1, 1–3, 3–5, 5–10, 10–15 cm and deep frozen at -80°C until analysis. The water temperature at the sampling depth was approximately 3°C .

As *M. musculus* individuals were not visible in ROV video footage of the seafloor, these specimens were collected with a ROV scoop that dug into the sediment. The mud collected at the same time as the holothurians was removed by shaking the scoop until the sediment fell away. A total of 10 holothurians were collected at the same location as the sediment samples (Table 1) and returned to the ship inside the bio-box attached to a retractable tool tray. Only intact animals were selected for the study. Once the ROV was back on board, *M. musculus* specimens were placed immediately at in situ temperature (3°C), in a temperature-controlled laboratory. Each specimen was dissected in a sterilized Petri dish.

Table 1. List of the stations data outlining the samples taken during cruises carried out on board the RV *Charles Darwin* and RV *James Cook*.

Station	Gear	Dive	Date	Latitude	Longitude	Depth (m)
CD56848#1	Megacorer	–	08-05-2006	39°30'00	09°56'01	3517
CD56848#2	Megacorer	–	08-05-2006	39°30'00	09°55'60	3523
CD56848#3	Megacorer	–	09-05-2006	39°29'24	09°56'03	3512
CD56851#1	Megacorer	–	09-05-2006	39°29'24	09°55'58	3517
CD56851#2	Megacorer	–	09-05-2006	39°29'24	09°56'01	3517
CD56851#3	Megacorer	–	10-05-2006	39°29'24	09°56'01	3522
CD56856#1	Megacorer	–	11-05-2006	39°29'24	09°55'60	3519
CD56856#2	Megacorer	–	11-05-2006	39°30'00	09°55'60	3522
JC10-090	ISIS experiment	46	09-06-2007	39°29'82	09°55'85	3534
JC10-091	ISIS experiment	48	12-06-2007	39°29'82	09°55'89	3534
JC10-099	ISIS suction sampler	50	13-06-2007	39°29.85	09°56'91	3535
JC10-099	ISIS suction sampler	50	14-06-2007	39°29.52	09°56'14	3648
JC10-099	ISIS suction sampler	50	14-06-2007	39°29.53	09°56'14	3649
JC10-099	ISIS suction sampler	50	14-06-2007	39°29.53	09°56'14	3651
JC10-106	ISIS Push core	51	15-06-2007	39°29.76	09°55'94	3529
JC10-106	ISIS Push core	51	15-06-2007	39°29.76	09°55'94	3529
JC10-106	ISIS Push core	51	15-06-2007	39°29.76	09°55'93	3529
JC10-106	ISIS Push core	51	15-06-2007	39°29.77	09°55'92	3529
JC10-106	ISIS Push core	51	15-06-2007	39°29.76	09°55'93	3529

**Fig. 2.** A picture of a specimen of *Molpadia musculus* immediately after collection, when analysed for biometric variables. (A) – mouth, (B) – tail.

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) making certain to exclude all gut tissues (see details in Roberts et al., 1996). Each sediment sample from the gut was transferred to a vial and stored at -80°C until analysis.

Faecal material was collected during an in situ experiment placed on the seabed. The in situ experiment consisted of a circular chamber with 6 individual funnels that allowed each specimen to defecate to an individual sterilized vial. Five specimens were collected with the same method described above. At 3500 m, each *M. musculus* was placed into an individual funnel. A sixth funnel was used as a control. Once each specimen was on the funnel, no evisceration or defecation was evident during the time of the set up of the experiment. The experiment lasted three days, the time necessary

for these organisms to defecate (Amaro, personal observation). Once on board, the faecal material was collected in a sterilized vial attached to the end of each funnel and stored at -80°C .

2.4 Analysis of potential food sources

The protein, carbohydrate and lipid contents of 1) the sediments, 2) the material extracted from the gut and 3) the faecal material, were determined spectrophotometrically, and their concentrations were calculated from calibration curves of serum albumin, D-glucose and tripalmitine equivalents, respectively, and normalised to sediment dry weight (Pusceddu et al., 2009). For each biochemical assay, blanks were obtained using pre-combusted sediments (450°C for 4 h). The detailed protocols are described in Danovaro (2010). All of the analyses were performed on three replicates, with approximately 0.2–1 g of wet sediment per sample.

Biopolymeric carbon was defined as the sum of the carbon equivalents of total proteins, carbohydrates and lipids (using conversion factors of 0.49, 0.40 and 0.75, respectively) and has been often reported as the fraction of total organic C potentially available to benthic consumers (Fabiano et al., 1995; Danovaro et al., 2001; Dell'Anno et al., 2002, 2003).

As *M. musculus* feeds head down in the sediment, it was assumed that all of the biochemical compounds ingested by these holothurians occurred in the top 15 cm depth of the sediment.

For all of the biochemical compounds, the digestion rate was estimated as the percentage of the difference in concentration between the oesophagus and hind gut. Based on the concentrations of the different biochemical compounds in the gut, faeces and sediment layers deeper than 1 cm, we determined the concentration factor as the ratio between concentrations in the oesophagus and in the sediment.

2.5 Analyses of exo-enzymatic activities

Extracellular enzyme activities (aminopeptidase, β -glucosidase and alkaline phosphatase) were determined by cleavage of analogue fluorogenic substrates (L-Leucine-4-methylcoumarinyl-7-amide, Leu-MCA; 4-MUF- β -D-glucopyranoside, MUF-Glu and 4-MUF-P-phosphate, MUF-P respectively; all substrates from SIGMA) as described in Danovaro et al. (1998). After evaluation of saturation concentrations, measurements were carried out in triplicate by adding 100 μ l of MUF-Glu and Leu-MCA (final concentration 200 μ M) and 50 μ l of MUF-P (final concentration 50 μ M) in a final volume of 5 ml of seawater. Substrate incubations were performed in the dark at in situ temperature for 1 h. Samples were analyzed fluorometrically (at 380 nm excitation, 440 nm emission for Leu-MCA and 365 nm excitation, 455 nm emission for MUF-Glu and MUF-P) as reported in Danovaro et al. (2005). The detailed protocols of all enzymatic analyses are described in Danovaro (2010). Data were normalized to dry weight (60°C, 24 h) and reported as nanomoles of fluorescein released per gram of sediment per hour.

2.6 Data analysis

Differences in the quantity of the different potential food sources between different sediment layers were investigated by means of one-way analyses of variance (ANOVA). When significant differences were observed, then post-hoc Tukey's tests were performed. For all ANOVA and Tukey's tests, an alpha level of 0.05 was used as the threshold for statistical significance. Before the analyses, the homogeneity of variances was checked using the Cochran's C test. The Cochran test was not significant for any of the variables considered, so that the data were not transformed before analysis.

Since the measurements from the different trait guts and the faeces were most likely dependent on one another, thus hampering the application of parametric ANOVA tests, differences in the quantity of the different potential food sources (protein, carbohydrate, and lipid) in the different traits of the holothurians' gut and the faeces and the differences in the rates of extracellular enzymatic activities among the different traits of the holothurians' gut and the sediment were separately investigated by means of non-parametric Kruskal-Wallis analyses of variance. The significant results were tested using a Mann-Whitney test, and the P-values corrected for multiple tests using a Bonferroni correction. Kruskal-

Wallis and Mann-Whitney tests were carried out using the Software STATISTICA 6.0.

The differences in 1) the biochemical composition (protein, carbohydrate and lipid) of the different sediment layers, 2) the biochemical composition and enzymatic activities within the holothurians gut material and 3) the top cm of the sediment, were then investigated by a distance-based permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001; McArdle and Anderson, 2001). PERMANOVA was carried out using the PERMANOVA package included in the Primer 6+ software. The analysis was based on Euclidean distances of normalized data using 4999 random permutations of the appropriate units (Anderson and Ter Braak, 2003). The pseudo-multivariate variance components for each term in the model were calculated using direct multivariate analogues to the univariate ANOVA estimators (e.g., Searle et al., 1992).

3 Results

3.1 Abundance and biomass of molpadiids holothurians

Based on the number of organisms present in the total area sampled by the cores, we estimated a total abundance of 220 ± 75.4 (standard error) individuals per m^2 and a total biomass of 473.1 ± 16.9 (standard error) g wet weight per m^2 .

3.2 Analyses of potential food sources in sediments and in holothurian gut contents

Vertical profiles of protein, carbohydrate, lipid and biopolymeric C concentrations in the top 15 cm of sediment are illustrated in Fig. 3. Proteins were the dominant biochemical compound (70%, on average of all sediment layers), followed by lipids (18%) and carbohydrates (12%). Total protein concentrations ranged from 10.76 ± 0.63 mg g^{-1} in the top cm of the sediment to 5.11 ± 0.80 mg g^{-1} in the 10–15 cm layer.

Sedimentary protein content decreased significantly from the 0–1 cm layer to the deeper sediment layers (Table 2). No significant differences were observed between all other layers. Carbohydrate concentrations in the sediment ranged from 1.29 ± 0.44 mg g^{-1} in the top 0–1 cm layer to 1.51 ± 0.31 mg g^{-1} in the 10–15 cm layer. There were no significant changes with increasing depth in the sediment (Table 2). Lipid concentrations ranged from 0.92 ± 0.09 mg g^{-1} in the top cm of the sediment to 1.21 ± 0.16 mg g^{-1} in the deepest sediment layer. No significant changes with increasing depth in the sediment were detected (Table 2). Biopolymeric C sediment content displayed the same trend as proteins with a significant difference between the top 1 cm and the rest of the sediment core

Table 2. Results of the ANOVA (a) and PERMANOVA (b) tests carried out to ascertain differences in the quantity (ANOVA) and biochemical composition (PERMANOVA) of sediment OM with changing depth in the sediment of the Nazaré Canyon at 3500 m depth. DF = degrees of freedom, SS = sum of squares; MS = mean square; F = F statistic; P = probability level; *** $P < 0.001$; ** = $P < 0.01$; SNK = Student-Newman-Keuls post-hoc comparisons tests; ns = not significant; na = not applicable. Reported are also the results of the Cochran's C test: ns = not significant.

	Source	SS	DF	MS	F	P	SNK
a) ANOVA							
Protein Cochran's C = 0.5549 (ns)	Depth	71.94	4	17.99	30.92	***	1>2–5
	Residual	5.82	10	0.58			
	Total	77.76	14				
Carbohydrate Cochran's C = 0.4102 (ns)	Depth	0.25	4	0.06	0.21	ns	na
	Residual	2.96	10	0.30			
	Total	3.21	14				
Lipid Cochran's C = 0.3340 (ns)	Depth	0.20	4	0.05	1.23	ns	na
	Residual	0.41	10	0.04			
	Total	0.61	14				
Biopolymeric C Cochran's C = 0.4990 (ns)	Depth	16.20	4	4.05	7.45	**	1>2–5
	Residual	5.44	10	0.54			
	Total	21.63	14				
b) PERMANOVA							
Depth in the sediment	4	1025.10	256.27	16.61	**		
Residual	10	154.28	15.428				
Total	14	1179.30					

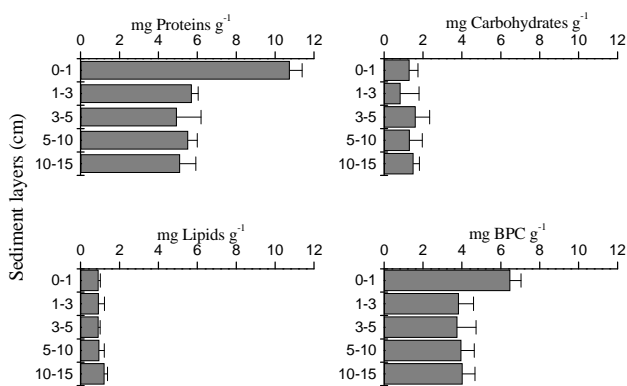


Fig. 3. Vertical distributions of the main biochemical classes of organic compounds (proteins, carbohydrates, lipids) and biopolymeric C (the sum of carbon within these compounds) in the sediment. Standard deviations are indicated. Data are expressed in milligrams per g of sediment dry weight ($n = 3$).

(Table 2). As for the different classes of organic compounds, no significant differences were observed between the layers from 1 to 15 cm depth in the sediment core.

3.3 Organic matter in holothurians guts and faeces

Changes in protein, carbohydrate and lipid contents through the gut and in the faeces of *M. musculus* are illustrated in Fig. 4. Protein concentrations ranged from $17.05 \pm 3.26 \text{ mg g}^{-1}$ in the oesophagus to $5.57 \pm 0.83 \text{ mg g}^{-1}$ in the faeces. In all holothurians gut tracts and in the faeces, protein content was 5 to 17 times greater than the other biochemical compounds. Protein concentrations decreased progressively from the oesophagus to the mid- and end-gut and the faeces (Table 3, Mann-Whitney test). Carbohydrate content ranged from $1.85 \pm 0.64 \text{ mg g}^{-1}$ in the oesophagus to $2.24 \pm 0.34 \text{ mg g}^{-1}$ in the mid gut, and $1.38 \pm 0.29 \text{ mg g}^{-1}$ in the faeces, but there were no significant differences between the different gut sections and the faeces (Table 3). Lipid concentrations varied from $2.14 \pm 0.68 \text{ mg g}^{-1}$ in the oesophagus to $3.97 \pm 0.75 \text{ mg g}^{-1}$ in the mid gut and to $1.02 \pm 0.11 \text{ mg g}^{-1}$ in the faeces, and displayed values in the mid-gut significantly higher than in the other holothurians gut tracts and in the faeces (Table 3, Mann-Whitney test). Biopolymeric C concentrations ranged from $10.70 \pm 2.24 \text{ mg g}^{-1}$ in the oesophagus to $4.04 \pm 0.61 \text{ mg g}^{-1}$ in the faeces.

Table 3. Results of the Kruskal-Wallis ANOVA (a) and PERMANOVA (b) tests carried out to ascertain differences in the quantity (ANOVA) and biochemical composition (PERMANOVA) of sediment OM among the various sections of the holothurians' gut and the faeces. DF = degrees of freedom, H = Kruskal-Wallis H , P = P-level, SS = sum of squares; MS = mean square; F = F statistic; P = probability level; *** = $P < 0.001$ Oesophagus: first part of the gut; mid gut: second part of the gut; others: nd gut and faeces (see material and methods for details). P-level for non parametric ANOVA were Bonferroni corrected.

Variable	DF	H	P	Mann-Whitney test	
a) Kruskal-Wallis ANOVA					
Protein	3	9.462	0.024	Oesophagus>mid-gut>End-gut=Faeces	
Carbohydrate	3	5.051	ns	Not applicable	
Lipid	3	9.359	0.025	Mid gut>Oesophagus=End-gut>Faeces	
Source	DF	SS	MS	Pseudo-F	P
b) PERMANOVA					
Gut section/faeces	3	267.15	89.052	17.161	***
Residual	8	41.513	51.891		
Total	11	308.67			

Based on the concentrations of the different biochemical compounds in the gut, faeces and sediment layers deeper than 1 cm, we determined the concentration factor as the ratio between concentrations in the oesophagus and in the sediment. The concentration factor of proteins in the oesophagus was approximately 3.1. For carbohydrate and lipid the concentration factors were approximately 1.3 and 2.0, respectively. The mean concentration of biopolymeric C in the oesophagus was approximately 2.7 greater than in the sediment.

Digestion rates of the different biochemical compounds were then calculated as the difference in concentration between the material in the oesophagus and that in the faeces. These calculations revealed that proteins had the highest digestion rate ($67 \pm 1\%$), followed by carbohydrates ($50 \pm 11\%$) and lipids ($23 \pm 11\%$). Overall, biopolymeric C digestion rate was $62 \pm 3\%$ (Fig. 5).

3.4 Enzymatic activities

Changes in extracellular enzymatic activities within the holothurians gut are presented in Fig. 6. The Kruskal-Wallis tests revealed that the rates of the three investigated activities displayed significant differences between the different gut traits and the sediment (Table 4). In particular, rates of aminopeptidase activity in the mid gut were significantly higher than in the other traits and the sediment (Mann-Whitney test, $P < 0.05$ for all comparisons, after Bonferroni correction), whereas β -glucosidase activity rates were lower in the end gut than in the other gut traits and in the sediment (Mann-Whitney test, $P < 0.05$ for all comparisons, after Bonferroni correction). Alkaline phosphatase activity rates decreased progressively from the oesophagus-mid gut to the end gut and the sediment (Mann-Whitney test, $P < 0.05$ for all comparisons, after Bonferroni correction).

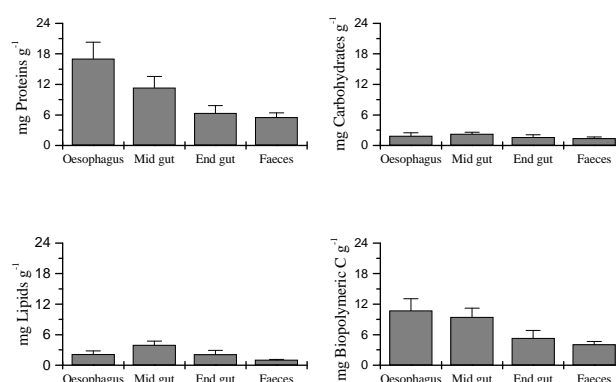


Fig. 4. Distributions of the main biochemical classes of organic compounds (proteins, carbohydrates, lipids) and biopolymeric C through the gut of *M. musculus* (oesophagus, mid gut, hind gut and faeces). Standard deviations are indicated. Data are expressed in milligrams per g of sediment dry weight ($n = 3$).

3.5 Potential contamination of holothurian gut contents

Previous studies pointed out that the operations of gut content extraction can determine the contamination of subsequent biochemical analyses (FitzGeorge-Balfour et al., 2010), as a result of unregulated lipolysis of phospholipids within the digestive tissue resulting from the death of organisms on recovery (Ginger et al., 2001). This would limit the use of biomarkers in gut contents, which are also present in the organisms tissues, to determine feeding selectivity in deep-sea organisms (Hudson et al., 2003; Wigham et al., 2003; Howell et al., 2004). However, it is worthy of notation that in the present study, we report that the biochemistry of the gut contents of *M. musculus* closely reflects that of the sediments,

Table 4. Results of the Kruskal-Wallis ANOVA (a) and PERMANOVA (b) tests carried out to ascertain differences in extracellular enzymatic activities among the various sections of the holothurians' gut (ANOVA and SNK tests) and between the gut and the sediment. DF = degrees of freedom, SS = sum of squares; MS = mean square; F = F statistic; P = probability level; ** = $P < 0.01$; ns = not significant; na = not applicable.

Variable	DF	H	P	Mann-Whitney test	
a) Kruskal-Wallis ANOVA					
Aminopeptidase	3	9.154	0.027	Mid gut>Oesophagus=End gut=sediments	
β -glucosidase	3	8.128	0.043	End gut<Oesophagus=Mid gut=Faeces	
Alkaline phosphatase	3	9.492	0.023	Oesophagus=Mid gut>End gut>Faeces	
b) PERMANOVA					
Source	DF	SS	MS	Pseudo-F	P
Gut/sediment	3	2.22E+10	7.39E+09	64.123	**
Residual	8	9.21E+09	1.15E+09		
Total	11	3.14E+10			

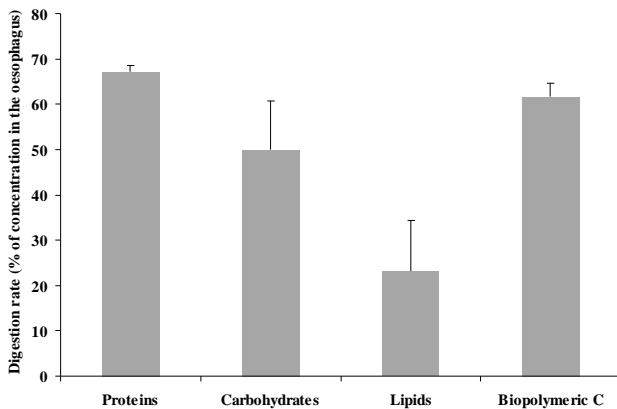


Fig. 5. Digestion rate in % of the main biochemical classes of organic compounds (proteins, carbohydrates, lipids) and biopolymeric C in *M. musculus*. Standard deviations are indicated. Data are mg per g of sediment dry weight ($n = 3$).

suggesting a negligible effects of contamination induced by gut content extraction.

4 Discussion

Deep-sea holothurians are common in most deep-sea soft bottoms and dominate the megafaunal abundance and biomass over large parts of the deep-sea floor (Sibuet et al., 1982; Billett, 1991; Roberts et al., 2000). In recent years, large scale changes have been noticed in the density of abyssal megafauna species both in the NE Atlantic (Billett et al., 2001) and NE Pacific (Lauermann et al., 1996; Ruhl and Smith, 2004). Significant effects have been observed in the process of OM on the seabed when species

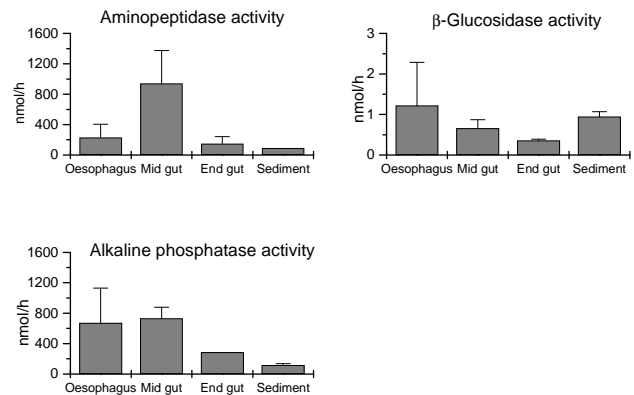


Fig. 6. Distributions of aminopeptidase, β -glucosidase and alkaline phosphatase activity through the gut of *M. musculus* and in sediment samples. Standard deviations are indicated. Data are expressed in nanomole of substrated released per g of sediment dry weight per hour ($n = 3$).

changed in density (Bett et al., 2001; de Leo, et al., 2010). Although the reason for such a rise in the holothurians abundance is still uncertain, it is possible to hypothesise that this phenomenon is associated with changes in phytoplankton community structure and changes in quantity and quality of the POC flux to the abyssal seafloor (Wigham et al., 2003; Ruhl et al., 2004; Smith et al., 2006, 2008; Billett et al., 2010). Such changes will in turn affect the structure and function of deep-sea ecosystems (Smith et al., 2008). In the present study, very high abundances of the infaunal holothurian *M. musculus* were found at 3500 m in the Nazaré Canyon. For comparison, *Molpadia blakei* was found with abundances of ca. 0.0044 ind. per m^2 at depths of ca. 3900 m at the PAP site (Billett, 1991). In contrast, the

abundances here were 220 ± 75.4 ind. per m^2 with a biomass of 0.473 ± 0.017 kg per m^2 , which is unprecedented in deep-sea infaunal holothurians. In the Kaikoura Canyon, molpadiids biomass was similar to the biomass estimated here, averaging 0.65 kg per m^2 (de Leo et al., 2010). To investigate the potential food sources that might sustain such a large population, the biochemical composition of the sediment, the holothurians' gut contents and holothurians' faecal material were analysed. We used the labile portion of OM in the sediment as the possible food source for these organisms. This labile portion of OM mainly consists of simple and/or combined compounds (i.e. biopolymers) like proteins, carbohydrates and lipids, which are rapidly mineralised (Danovaro et al., 2001; Pusceddu et al., 2009). Organic matter in the sediments where *M. musculus* exists in high densities is composed mainly by proteins, followed by carbohydrates and lipids (Fig. 3). High concentrations of proteins (and biopolymeric C) were found in the top 0–1 cm layer and despite a significant decrease to 1–3 cm layer, large quantities were observed also down to 15-cm depth in the sediment core. For comparison, at the PAP site, sedimentary protein concentrations (up to approximately 1.5 mg g^{-1} in the top 0.5 cm; Danovaro et al., 2001) were about seven-folds lower than those reported in the present study (10.7 ± 0.6 mg g^{-1} in the top 1st centimetre). In other studies, the availability of proteins in deep-sea sediments was even lower than the one mentioned above (Sibuet, 1984; Pfannkuche and Thiel, 1987; Danovaro et al., 1993; Boetius et al., 1996; Tselepides et al., 2000). Carbohydrates and lipids were in the same range as for the PAP deep-sea sediments (Danovaro et al., 2001). Although we did not measure C and N sediment contents, having thus no possibility to ascertain the “freshness” of the organic material in the sediment using C/N ratios (Meyers, 1994), we report here that values of the protein to carbohydrate ratio in the Nazaré Canyon at 3500 m depth were 9.07 ± 0.97 in the top centimeter. Values of the protein to carbohydrate ratio in deep-sea sediments range from <0.1 in the oligotrophic Eastern Mediterranean Sea (500–2400 m depth, Danovaro et al., 1993) to 0.7–0.9 at the PAP (at 4850 m depth; Danovaro et al., 2001). Values of this ratio in marine sediments display also generally higher values immediately after the deposition of phyto-detritus on the seafloor and decrease when highest flux rates of carbohydrate-enriched material are observed (Tselepides et al., 2000; Fabiano et al., 2001).

This indicates that the subsurface sediment layers of the Nazaré Canyon are characterised by a relatively large availability of compounds with high nutritional quality (i.e. N rich). These results were consistent with previous studies that reported that sediments here are enriched in OM and chlorophyll-a and present a significant accumulation of labile and high quality OM, compared with other sites along the Portuguese margin and canyons in the Mediterranean regions (Danovaro et al., 1999; Tselepides et al., 2000; García and Thomsen, 2008; García et al., 2008; Amaro et al., 2009;

Pusceddu et al., 2010). The Nazaré Canyon displayed sediment accumulation rates higher than those found in other regions located along the west Iberian continental slope (between 5 and 17 $mm\ y^{-1}$ on various different terraces) (de Stigter et al., 2007; Arzola et al., 2008; Masson et al., 2010; Pusceddu et al., 2010). The rates found at ca. 3,500 m are higher than expected for these depths (14 $mm\ y^{-1}$) and are closer to those found in the canyon head (de Stigter et al., 2007). This suggests this part of the canyon to be a mid-canyon depocenter of sediment and OM (Arzola et al., 2008; Masson et al., 2010). The cause for this is still unknown, however it is thought to be related to a number of regional oceanographic and local climatic processes like wind, internal waves, tidal currents (Schmidt et al., 2001; Vitorino et al., 2002a, b; Canals et al., 2006; Masson et al., 2010), floods and storms (de Stigter et al., 2007), which have been observed in other deep-sea canyons along the European continental margins (Pusceddu et al., 2010). The export of organic C from the upper to the mid part of the canyon is a recurrent mechanism fuelling the benthos of this area with a significant amount of OM of high nutritional quality. For example, an accumulation of labile organic compounds on the seabed is often associated with unprecedented abundance, biomass and biodiversity of metazoan meiofauna (Danovaro et al., 2002, 2003; Gambi et al., 2003).

The gut of a holothurian can be considered as a bioreactor (Penry and Jumars, 1986, 1987; Jumars, 2000), in which the animals quickly extract assimilable food from the ingested sediments. A wide spectrum of hydrolytic gut enzymes has evolved to fit the diet of each species, reflecting the types of food items that are available in the sediment (Féral, 1989).

Sibuet et al. (1982), investigating the trophodynamics of the holothurians *Deima validum validum* and *Pseudostichopus villosus* from the Demera Abyssal Plain, estimated assimilation efficiencies of ca. 18% for proteins, ca. 2.7% for carbohydrates and ca. 1.1% for lipids. A total of ca. 22.3% and 20.5% of the ingested material was assimilated by *D. validum validum* and *P. villosus*, respectively. In our study we found high concentrations of proteins in the oesophagus of *M. musculus* (5–17 times higher than the other biochemical compounds) while lipids and carbohydrates were present in low concentrations (Fig. 4). Protein concentrations decreased significantly between the oesophagus and the rest of the holothurian gut, concomitantly with the highest aminopeptidase activities found in the mid gut (Fig. 6). This enzyme is responsible for the hydrolysis of proteins, breaking them down into oligomers which are more easily assimilated by the animal (Roberts et al., 2001). We believe that the mid gut is the portion of the holothurians where the highest fraction (approximately $33 \pm 1\%$) of the proteins is digested, with a final digestion rate equal to $67 \pm 1\%$. Carbohydrates and lipids were not removed in the mid gut, but were preferentially digested in the end gut, with final digestion rates equal to $50 \pm 11\%$ and $23 \pm 11\%$, respectively (Figs. 4, 5, 6). This result reflects the preferential utilization of organic

N and lipids in the dietary requirement of these holothurians. Moreover, the significantly higher alkaline phosphatase activity in the oesophagus and mid gut when compared to the hind gut (Figs. 4, 6) supports the hypothesis of a potential and quick exploitation of organic P in the mid gut. Phosphatase activity contributes to degradation and cycling of organic phosphorus. The activity of this enzyme has been found to be consistently high in abyssal holothurians (Roberts et al., 2001). Therefore holothurians could also play an important role in phosphorus cycling in deep-sea sediments.

Previous studies have hypothesized a key role of the prokaryotic biomass in the diet of deep-sea holothurians (Deming and Colwell, 1982; Sibuet et al., 1982; Roberts et al., 2001; Amaro et al., 2009). However, overall prokaryotic biomass contributed for less than 0.1% of the total protein absorbed (Amaro, personal observation). Holothurians would appear not to rely on microbes for their direct nutrition. However, prokaryote activity on organic substrates might indirectly provide essential nutrients (Deming and Colwell, 1982; Eardly et al., 2001).

Deposit-feeders may have two ways to ingest and assimilate food using foraging and digestive strategies, which involve: 1) particle selection, in which the organism preferentially selects food-rich particles during the pickup and ingestion (Billett et al., 1988; Levin et al., 1997; Ferner and Jumars, 1999), and 2) assimilatory selection in which the organism selectively digests and/or assimilates food in its guts (Penry and Jumars, 1990; Mayer et al., 1997; Jumars, 2000). Based on the composition and concentration of biochemical compounds in the sediment and in the oesophagus, we determined the average food selection. In our study, we estimated the average concentration of biopolymeric C in the oesophagus of *M. musculus* as being approximately 3-times greater than in the sediment. However, this concentration factor might depend on season (Billett et al., 1988). Radio-carbon data (a proxy for labile OM) suggest that *M. musculus* has a low particle selection index and high digestive selection index (McClintic et al., 2008; Puriton et al., 2008). This observation is in agreement with, Miller et al. (2000), who found labile material in the guts of molpadiids using the tracer ^{234}Th . These authors concluded that molpadiids either feed on very rich subsurface sediments or are able to catch labile surface sediments down into their burrows. Assuming that *M. musculus* is a head-down feeder like *Molpadia oolitica* (Rhoads and Young, 1971) we hypothesize that in the Nazaré Canyon, *M. musculus* has a high efficiency in exploiting sedimentary proteins and complements its feeding requirements with an optimal foraging strategy.

At the time of sampling, given the average abundance to be $220 \pm 75.4 \text{ ind. m}^{-2}$, the population of *M. musculus* at 3500 m depth in the Nazaré Canyon could remove ca. $0.49 \pm 0.13 \text{ g C}$ and $0.13 \pm 0.03 \text{ g N m}^{-2} \text{ d}^{-1}$ from the sediment. However, we cannot disregard the importance of other deep-sea benthic species that have been found in large numbers in this system. For instance, the holothurian *Yp-*

silothuria bitentaculata (Ludwig, 1893) has abundances of 136 ind. m^{-2} in the same location as *M. musculus* (Amaro, personal observation). *Ypsilothuria bitentaculata* is a surface-deposit feeder that could be responsible for exploiting food-rich particles from surficial sediments and may have higher particle selectivity than *M. musculus*. The presence of large numbers of these two species in the same deep-sea system can be explained either by the establishment of an ecological coexistence permitted by their different feeding strategies (horizontal vs. vertical feeding of *Y. bitentaculata* and *M. musculus*, respectively), either by an excess of food availability. Whatever the factor or combination of factors allowing coexistence, these two deposit-feeding holothurians have therefore the potential to impact the remaining benthic community through the depletion of available sources to other organisms while influencing the trophic strategy of one other. Deep-sea megafauna are the primary consumers of fresh food and phytodetritus and may possess a competitive advantage over taxa when foraging for it (Billett et al., 2010). Macrofauna preferentially rework the food that arrives on the seabed, ahead of the meiofauna and bacteria (Witte et al., 2003). These animals can be very active when contributing to sediment reworking and potentially influencing other infaunal assemblages through bioturbation and remineralization (Ruhl and Smith, 2004). Smallwood et al. (1999), Ginger et al. (2001) and Witbaard et al. (2001), suggested that megafauna can significantly affect the food resources available to the rest of the benthos. McClain and Barry (2010) also suggested that megafauna are important habitat modifiers in the deep sea affecting macrofauna biodiversity. They showed a high fauna turnover and changes in the community structure at scales $<100 \text{ m}$, and often $<10 \text{ m}$ related to geographic features on the Monterey Canyon. Macrofauna biodiversity values are lower in the sediments characterised by the presence of *M. musculus* and *Y. bitentaculata* than in other Nazaré Canyon areas (Cunha, personal observation). We conclude that the impact of *M. musculus* affects not only the degradation of OM, but its redistribution and availability for other fauna at specific locations of the seafloor in the largest submarine canyon of the European margins. Our data also show that the sediment from the faeces still contain a considerable amount of biopolymeric C ($4.04 \pm 0.61 \text{ mg g}^{-1}$). Although this value may be overestimated, as the faecal material may contain artifacts due to disturbance or handling of the experiment, it is still important to note that faeces may be important in the redistribution of OM. This consequently, will affect the infaunal community. In summary we suggest that whilst feeding, these deep-sea holothurians, play a potentially important role in sediment mixing and modify the structure and geochemistry, mineralisation of sediments. Thus, they appear to be key ecosystem engineers within the Nazaré Canyon.

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