
Relationship between the occurrence of filamentous bacteria on *Bathymodiolus azoricus* shell and the physiological and toxicological status of the vent mussel

I. Martins^{a,*}, A. Colaço^a, R. Serrão Santos^a, F. Lesongeur^b, A. Godfroy^b, P.-M. Sarradin^c and R.P. Cosson^d

^a IMAR, DOP — Department of Oceanography and Fisheries, University of the Azores, 9901-862 Horta, Portugal

^b Ifremer DEEP/Laboratoire de Microbiologie des Environnements Extrêmes, UMR6197, BP 70, 29280 Plouzané, France

^c Ifremer, DEEP/Laboratoire Environnement Profond, BP 70, 29280 Plouzané, France

^d Université de Nantes, Laboratoire de Biologie Marine, ISOMer, EA 2160, BP 92208, 44322 Nantes, France

*: Corresponding author : I. Martins, Tel.: +351 292200457; fax: +351 292200411, email address : imartins@uac.pt

Abstract:

The edifice walls of the Eiffel Tower hydrothermal vent site (Mid-Atlantic Ridge, Lucky Strike vent field) are populated with dense communities of dual symbioses harboring vent mussel *Bathymodiolus azoricus*, some of which are covered by white filamentous mats belonging to sulfur-oxidizing bacteria. Mussels were collected in both the presence and absence of the filamentous bacteria. A sample of the filamentous bacteria was collected and water measurements of temperature, CH₄ and H₂S were recorded at the collection area. The whole soft tissues were analyzed for total lipid, carbohydrate and total protein. Metallothioneins and metals (Cu, Fe and Zn) levels were determined in the major organs. The results showed no significant physiological and toxicological evidence that emphasizes the influence of associated sulfur-oxidizing filamentous bacteria on *B. azoricus* mussel shells. However, *B. azoricus* mussel seems to be well adapted to the assorted physico-chemical characteristics from the surrounding environment since it is able to manage the constant fluctuation of physico-chemical compounds.

Keywords: Biomarkers; Eiffel Tower; Filamentous bacteria; Metallothioneins; Metals; Vent mussel

1. Introduction

Lucky Strike is one of the largest known active vent fields (37° 18' N, 32° 16' W), located in the Mid-Atlantic Ridge between 1730 and 1736 m depth. The hydrothermal fluid, emitted at temperature ranging between 170 and 324 °C, presents characteristics (temperature, chlorinity, gas and metal concentration) that vary from site to site within the field (Charlou et al., 2000 J.L. Charlou, J.P. Donval, E. Douville, P. Jean-Baptiste, J. Radford-Knoery, Y. Fouquet, A. Dapoigny and M. Stievenard, Compared geochemical signatures and the evolution of Menez Gwen (37°50'N) and Lucky Strike (37°17'N) hydrothermal fluids, south of the Azores triple junction on the Mid-Atlantic Ridge, *Chem. Geol.* 171 (2000), pp. 49–75. Article | PDF (602 K) | View Record in Scopus | Cited By in Scopus (71)Charlou et al., 2000). The hydrothermal vent area is distributed around a lava lake, bound by the summits of three volcanic cones (Radford-Knoery et al., 1998). Both well-defined active chimneys such as Eiffel Tower belching out very hot fluids and zones where hydrothermal activity is more diffuse, can be found at Lucky Strike (Desbruyères et al., 2001). Eiffel Tower, located at 200 m from the southeastern edge of the circular lava lake, consists of chimneys combined into an edifice rising 7 m above the neighboring seafloor (Radford-Knoery et al., 1998). The structure presents numerous high temperature smokers and cracks emitting lower temperature fluids. It is colonized nearly uniformly by the vent mussel *Bathymodiolus azoricus*, several patches of mussel

47 been covered by dense white filamentous microbial mats (Sarradin et al., 1999;
48 Desbruyères et al., 2001) while other are not at all. The filamentous microbial mats are
49 mainly composed of a species of *Beggiatoa* (Mattison et al., 1998). This species is a
50 colonizer, very abundant in environments characterized by the presence of hydrogen
51 sulfide (Wörner and Zimmermann-Timm, 2000). These chemoautotrophic bacteria gain
52 energy by the oxidation of reduced sulfur compounds (Nelson et al., 1989; Hagen and
53 Nelson, 1997; Erbacher and Nelskamp, 2006). *B. azoricus* hosts “dual symbioses”,
54 involving the stable coexistence of chemoautotrophic (also referred to as thiotrophic) and
55 methanotrophic bacteria harbored within the gill (Fiala-Médioni et al., 2002; Duperron et
56 al., 2005). Dual symbioses provide obvious advantages to host individuals recruiting to
57 environments where the availability of substrates is unpredictable or fluctuating
58 (Cavanaugh et al., 1992; Fiala-Médioni et al., 2002). This dual symbioses has also been
59 described in others mussel species that live in reducing environments with high sulfide
60 and methane concentrations, such as hydrothermal vents (Duperron et al., 2005; Stewart
61 et al., 2005) and cold seeps (Fisher et al., 1993). The physiology and biochemistry of the
62 hydrothermal vent mussel *B. azoricus* must be adapted to the rapid fluctuating
63 composition of its environment composed of a mixture of seawater and hydrothermal
64 fluid (Childress and Fisher, 1992). The presence and absence of sulfur-oxidizing
65 filamentous bacteria on the hydrothermal mussel beds could be an indicator of a
66 fluctuating environment, since these chemolithoautotrophic bacteria proliferate in
67 environments with reduced sulfur compounds (Brinkhoff and Muyzer, 1997) and grow in
68 habitats with oxic-anoxic interfaces (Moyer et al., 1995). These chemical conditions may
69 alter the chemistry of mussel habitats (LeBris et al., 2006) and consequently their

70 physiological and toxicological condition. The aim of our investigation was to study the
71 physiological condition and metal accumulation of *B. azoricus* collected from a mussel
72 bed where the presence and absence of filamentous bacteria were observed.

73 **2. Material and Methods**

74 **2.1. Sampling**

75 Samples were collected during the EXOMAR cruise (with the R/V “Atalante”) in July
76 2005, at 1690 m depth on the Eiffel Tower hydrothermal site. The mussels were sampled
77 by the manipulator arm and brought to the surface using the Remotely Operated Vehicle
78 (ROV) “*Victor 6000*”. Samples were collected in two neighboring areas, where mussel
79 shells were covered by filamentous bacteria (designated hereafter as “mussels + mats”) or
80 not covered (designated hereafter as “mussels”). From each area 25 individuals were
81 collected and measured. Individuals from the group “mussels + mats” presented a mean
82 total length of 4.3 cm (\pm 0.5 SD) and individuals from the group “mussels” presented a
83 mean total length of 3.0 cm (\pm 0.3 SD). For each group, the whole soft tissues of 15
84 mussels were separated from the shells and kept frozen (-80°C) until lyophilization and
85 analysis. Left 10 mussels were dissected into gill, mantle, foot, digestive gland and
86 remaining soft tissues and kept frozen (-80°C) until lyophilization and analysis. A sample
87 of microbial mats, that covered the mussels, was collected using the water pumping
88 device of the ROV “VICTOR 6000”. Water samples (5 litres) were filtered on board and
89 filters were preserved at -80°C for further analysis.

90 **2.2. Lipid analysis**

91 Because of small soft tissue weights, 5 mussels were pooled together for single
92 measurement of total lipids levels at each group. The lipid content was determined

93 according to the modified method of Bligh and Dyer (1959) by extracting lipids from a
94 dry powdered in a water-dichloromethane–methanol mixture and by weighing after
95 evaporation to dryness, the organic layer. Level was expressed as mg g^{-1} of dry weight.

96 **2.3. Carbohydrate analysis**

97 The whole soft tissue of 5 individuals from each group of mussels was used for
98 carbohydrate analysis. The carbohydrate content was determined colorimetrically in a
99 NaCl extract, in the presence of 5% phenol and concentrated H_2SO_4 , as described by
100 Dubois et al. (1956). The concentration was determined in glucose equivalents from a
101 glucose calibration curve using glucose as a standard. The levels of carbohydrate were
102 expressed as mg g^{-1} of dry weight.

103 **2.4. Samples preparation for total protein, metallothionein and metal analyses**

104 The tissues of the 10 dissected mussels from each group, were lyophilized, weighed and
105 homogenized in 6 ml of ice-cold 100 mM Tris buffer, pH 8.1, containing 10 mM β -
106 mercaptoethanol. The homogenates were centrifuged for 30 min at 25 000 g, at 4°C to
107 separate the supernatants (S_1) from the insoluble fraction (pellets), used for the study of
108 intracellular metal distribution. Aliquots (1 ml) of the S_1 were used for metallothioneins
109 determination. Pellets and remaining supernatants were digested in an Ethos Plus
110 microwave oven with 5 ml of HNO_3 (69% v/v) for metal analysis.

111 **2.4.1. Total protein analysis**

112 Total protein levels were determined in supernatants S_1 from whole soft tissues of 5
113 individuals from each group of mussels, following the BioRad protein assay kit for the
114 Bradford method (Bradford, 1976). BSA (Bovine Serum Albumin) was used as reference
115 standard. Results were expressed as mg g^{-1} of dry weight.

116 **2.4.2 Metallothionein and metal analysis**

117 The aliquot of the supernatant S₁, was heat-denatured (90°C, 15 min) and centrifuged (13
118 000 g, 10 min, at 4°C) in order to separate the thermostable metallothioneins (MTs) from
119 thermolabile proteins. The heat stable fractions (S₂) were used for quantification of MTs
120 by Differential Pulse Polarography (DPP) according to Olafson and Sim (1979) improved
121 by Thompson and Cosson (1984). A standard addition calibration curve was obtained
122 using rabbit liver MT-I as reference. Results were expressed as mg g⁻¹ of dry weight.

123 After pellets and remaining supernatant S₁ digestion, solutions were dried at 60°C and
124 diluted by adding 2 ml 0.5N HNO₃. Metal levels (Cu, Fe, Zn) were measured by flame
125 atomic absorption spectrophotometry (AAS) with deuterium background correction. The
126 accuracy and precision of the method used were established by regular analysis of
127 certified reference materials of mussel tissue CE278 (European Reference Materials of
128 Belgium) and lobster hepatopancreas TORT-2 (National Research Council of Canada) (Table 1).
129 Certified reference materials and blanks were taken through the procedure in the same
130 way as the samples. Metal levels were calculated and expressed as mg g⁻¹ of dry weight.

131 **2.5. Microbial mat molecular diversity**

132 A preliminary study of the microbial mat diversity was performed by using 16S rRNA
133 gene sequencing. DNA was extracted from frozen mat sample pellets as described in
134 Alain et al. (2002). Archaeal DNA was amplified using the primer A24F (5'-TTC CGG
135 TTG ATC CTG CCG GA-3') and the reverse primer 1407R (5'-GAC GGG CGG TGW
136 GTR CAA-3'). Bacterial DNA was amplified using the primer E8F (5'-AGA GTT TGA
137 TCA TGG CTC AG-3') and the reverse primer U1492R (5'-GTT ACC TTG TTA CGA
138 CTT-3'). PCR reactions were performed on a Robocycler Gradient 96 (Stratagene) (Wery
139 et al., 2002; Nercessian et al., 2003). PCR products were then checked on a 0.8% (w/v)

140 agarose gel and directly cloned using the TOPO TA Cloning® kit (pCR2.1 vector),
141 according to the manufacturer's instructions (Invitrogen). Sequences were analyzed as
142 previously described by Postec et al. (2005).

143 **2.6. *In situ* temperature measurements, CH₄ and H₂S estimated values**

144 Eight autonomous temperature probes (thermistor, Vemco Minilog 12 TR 64K probes)
145 were deployed on each sampling point for two days. The temperature data were corrected
146 against the bottom seawater temperature (4.4°C). The sampling period was 30 seconds.
147 CH₄ concentrations were estimated using the significant Temperature/CH₄ linear
148 relationship obtained during the ATOS cruise on 16 samples from the Eiffel Tower
149 edifice (Sarradin et al., 2003). Total sulfide (ΣS) concentrations were estimated using the
150 significant Temperature/ΣS linear relationship obtained in 2006 during the MoMARETO
151 cruise with the CHEMINI in situ chemical analyzer (Vuillemin et al., 2009). Results were
152 expressed as μmol l⁻¹.

153 **2.7. Statistical analysis**

154 All the results are given as mean level by individual/tissues dry weight. The statistical
155 calculations were performed with STATISTICA software (6.0 release, StatSoft). Data
156 were checked for normal distribution and homogeneity of variance (Leven's test). Non-
157 parametric tests (Kruskall–Wallis and Mann–Whitney) were performed when data were
158 not normally distributed or when they exhibited heterogeneous variances.

159 **3. Results**

160 **3.1. Biochemical composition**

161 Lipid, carbohydrate and total protein results per group of mussels, are shown in Table 2.
162 No significant difference was found between the two groups of mussels (Mann-Whitney,
163 $p > 0.05$) for lipid, carbohydrate and total protein levels.

164 **3.2. Preliminary microbial mat diversity study**

165 Phylogenetic analysis of bacterial clone library evidenced a very large diversity of
166 uncultured bacteria within the α, δ, γ and ϵ -proteobacteria. Bacteria belonging to
167 Cytophaga/Flavobacteria/bacteroides group, planctonmycetes and actinombacteria were
168 detected. Within the γ -proteobacteria, sequences belonging to order Thiotricales were
169 identified. The order Thiotricales includes the filamentous sulfur-oxidizing bacteria such
170 as *Beggiatoa*, *Thioploca* and *Thiotrix*. The archaeal diversity appeared to be very low. All
171 the sequences were located in the marine Crenarchaeota group I and dominated by one
172 phylum closely related to the newly described (and first cultivated species within this
173 group) ammonia-oxidizing Crenarchaeota "*Nitrosopumilus maritimus*" (Konneke et al.,
174 2005). Microscopic observation showed the presence of large intracellular vacuoles
175 surrounded with sulfur granules in some large bacterial filaments, described earlier in
176 many of sulfur-oxidizing species (Godfroy, personal observation).

177 **3.3. In situ temperature measurements, CH₄ and H₂S estimated values**

178 Table 3 presents the results of the temperature measured, CH₄ and H₂S estimated values
179 at the environment surrounding the two groups of mussels studied. The temperatures
180 measured within the two groups presented significant differences (Mann-Whitney,
181 $p < 0.05$). CH₄ and H₂S estimated values are distinct between the two groups of mussels.
182 The temperature measured as well as both estimated values of CH₄ and H₂S presented
183 higher mean in the group "mussels + mat" than "mussels" group. The temperature

184 standard deviation, minimum, maximum and range were also higher in the environment
185 characterized by the presence of microbial mats. The increase of relative temperature,
186 compared to ambient seawater temperature (4.4°C), was +0.2°C within the "mussels"
187 group and up to +3°C within the "mussels + mat" group. The temperature variations
188 sustained by the "mussels" group were less extensive than those faced by "mussels + mat"
189 group. Assuming that temperature can be used as a semi conservative tracer of the vent
190 fluid dilution (Johnston et al., 1988; LeBris et al., 2006), it is possible to estimate the
191 hydrothermal input in the studied environment using the ambient seawater (4.4°C) and
192 the undiluted hydrothermal fluid (324°C) temperatures (Charlou et al., 2000).
193 Consequently, the input of hydrothermal fluid observed in the sampled sites ranged
194 between 0.2% at a temperature of 4.9°C to 1% at a temperature of 7.4°C.

195 **3.4. Metallothionein and metal levels**

196 Table 4 shows the levels of metals and metallothionein (MT) in the different tissues of
197 both mussel groups. Results are presented by tissue analyzed and by group of mussels.
198 Metal in the vent mussel can be ranked in the following order according to the levels
199 found: Fe>Zn>Cu, for individuals from the group "mussels" and Zn>Fe>Cu, for
200 individuals from the group "mussels + mat". Only at group "mussels" were found
201 significant differences (Kruskal-Wallis, $p<0.05$) between the metal levels.. The digestive
202 gland was the organ which presented the highest levels of metals and MT. In this organ
203 Fe levels were found statistically different between groups (Kruskal-Wallis, $p<0.05$),
204 with higher levels found at "mussels" group. No significant difference (Kruskal-Wallis,
205 $p>0.05$) was found between the distribution (soluble/insoluble fractions) of metals within

206 the analyzed tissues. Metallothionein levels in the digestive gland are not statistically
207 different between groups (Mann-Whitney, $p > 0.05$).

208 **4. Discussion**

209 **4.1. Physiological status**

210 The dual endosymbiosis involving sulfur and methane oxidizers is one of the mechanisms
211 developed by *B. azoricus* to use the chemical energy of hydrothermal environment. There
212 is some empirical evidence that the relative abundance of dual symbiosis in this species
213 can vary in response to environmental parameters (Fiala-Médioni et al., 2002; Duperron
214 et al., 2005). Moreover, studies carried out in Eiffel Tower site demonstrate a higher
215 concentration of sulfide (2.1 mM) than methane (0.68 mM) in end-member fluids
216 (Charlou et al., 2000) and accordingly, a dominance of chemoautotrophic symbionts in
217 the gills of *B. azoricus* from this site (Trask and Van Dover, 1999; Salerno et al., 2005;
218 Duperron et al., 2006). The estimated values of CH_4 and H_2S presented in this study lead
219 us to consider that each group of mussels has different energy sources. The site where
220 “mussels + mats” were collected, presented higher H_2S values than the site “mussels” are
221 living at. H_2S contributes to the proliferation of these sulfur-oxidizing filamentous
222 bacteria that uses the sulfide and the oxygen available (Wörner and Zimmermann-Timm,
223 2000) in their proximal environment. The symbiotic sulfur-oxidizing bacteria use the
224 energy produced by the oxidation of reduced compounds, such as sulfide, as energy
225 source to produce organic compounds, acting as primary producers for their host (Fiala-
226 Médioni et al., 2002). Therefore, it was suggested the possibility of the sulfur-oxidizing
227 filamentous bacteria consume part of the environment sulfide also needed by sulfur-
228 oxidizing symbionts. The consumption of sulfide by free-living filamentous bacteria and

229 consequently depletion of this compound available for chemoautotrophic symbionts
230 utilization may influence the physiological status of the mussels associated with
231 filamentous bacteria. Several biochemical markers, such as lipids, carbohydrates and total
232 proteins, were use to study the possible differences on physiological condition between
233 the group “mussels + mats” and the group “mussels”. The analysis of such biochemical
234 parameters in hydrothermal vent animals has proven to be a rewarding approach to
235 understand their biology (Childress and Fisher, 1992). Furthermore, lipids are an
236 important source of energy that can be used during periods of food shortage and as an
237 energy reserve for the successful larval development (Fraser, 1989). Mussels use
238 carbohydrates as energy reserves that are metabolised into lipids during egg maturation
239 (Kopp et al., 2005). Proteins are of fundamental importance in mussels as they are used in
240 several biological functions for maintenance, growth and reproduction (Olsson et al.,
241 2004). Therefore, the variation of these biochemical parameters could be considered a
242 valuable indicator of organism physiological condition (Lagadic et al., 1997). The
243 “mussels + mats” group and “mussels” group did not show differences between the
244 amounts of lipids, carbohydrates and total proteins. Consequently we can not put forward
245 differences between their physiological conditions by this mean. The preliminary study
246 made in the microbial mat collected, identified the presence of several groups of free-
247 living bacteria including the sulfur-oxidizing filamentous type. However it is unknown,
248 for now, the relative abundance of these bacteria at the microbial mat. The similar
249 physiological status found between the two groups of *B. azoricus* mussels studied may
250 indicate a low relative abundance of sulfur-oxidizing filamentous bacteria and
251 consequently low competition for environmental sulfide. From the temperature

252 measurements made in both collection areas, we can assume that the environment
253 surrounding the studied mussels is situated in the really cold part of the mixing zone
254 between the ambient seawater and the hydrothermal fluid (Sarradin, personal
255 observation). However, the “mussels + mats” group presented relatively high
256 temperatures, high levels of ΣS (i.e. H_2S , HS^- , S^{2-}), low pH and dissolved oxygen,
257 accordingly to the proximity of fluid emissions (Sarradin et al., 1999). It is the same for
258 estimated high values for CH_4 and H_2S at the “mussels + mats” group site allowing us to
259 hypothesize the proximity of fluid emissions. Furthermore, the estimated values of H_2S
260 obtained in the collection area are within the range of 0-62 μM for the H_2S concentration
261 reported for mussel beds in Eiffel Tower (Sarradin et al., 1999). The higher values
262 recorded for temperature, CH_4 and sulfur compounds at the “mussel + mats” group site,
263 besides contributing to free-living filament bacteria proliferation, do not seem to have any
264 observed effect on *B. azoricus* physiological condition. Likely, *B. azoricus* as a
265 mixotrophic organism which obtains energy not only from a dual endosymbiosis but also
266 from suspension-feeding and can potentially regulate the relative contribution of both
267 nutritional pathways according to external conditions (Martins et al., 2008).

268 **4.2. Toxicological status**

269 The interactions between the superheated fluids and cold ocean water create a dynamic
270 system that gives, to vent organisms, periodic access to high levels of metals (Geret et al.,
271 1998). Other studies put forward the high concentrations of metals in Lucky Strike
272 hydrothermal vent fluids, like Cu (2 to 30 μM), Fe (70 to 920 μM) and Zn (2 to 40 μM)
273 (Charlou et al., 2000; Douville et al., 2002) and the bioaccumulation of those metals in
274 vent organisms, such as mussels (Colaço et al., 2006; Cosson, 2008). The pattern

275 Fe>Zn>Cu found for individuals from the “mussels” group is in agreement with earlier
276 studies of *B. azoricus* metal bioaccumulation (Cravo et al., 2007; Kádár et al., 2007) and
277 with Lucky Strike fluids composition (Douville et al., 2002). The “mussels + mats” group
278 showed a different pattern (Zn>Fe>Cu), however it was not found any statistical
279 difference between Zn and Fe levels in this group. Moreover, for both groups, the highest
280 accumulation of these metals was found in the digestive gland. It has been shown that
281 digestive gland of bivalves is a target organ for the bioaccumulation of metals
282 (Domouhtsidou and Dimitriadis, 2000). The ability of *Bathymodiolus* sp. to capture and
283 ingest mineral particles, including Fe, Zn and Cu sulfides (Le Pennec et al., 1985) is one
284 pathway for bioaccumulation of metals in the digestive gland (Rousse et al., 1998).
285 Moreover, this organ has an important role in metabolism of metals and is considered as a
286 long-term storage tissue and thus a good indicator of persisting exposure (Hamza-Chaffai
287 et al., 2000). The high levels of Fe found in digestive gland of “mussels” group may be a
288 reflection of a more direct exposure of individuals to the Fe sulfide particles floating in
289 their surrounding environment. Nevertheless, one factor that can affect metal
290 bioaccumulation is body size (Pan and Wang, 2008). Studies developed by Boyden,
291 (1974) regarding mussel allometry in ecotoxicology, show that highest values of trace
292 elements are often recorded in the smallest individuals. That could be the explanation for
293 the high Fe levels found for the “mussels” group formed by individuals presenting a
294 smaller size than those from the “mussels + mats” group.

295 As metals are rather abundant at Lucky Strike hydrothermal field (Rousse et al., 1998;
296 Douville et al., 2002; Kádár et al., 2005), their intracellular distribution between soluble
297 and insoluble forms can be used to evaluate the toxicological significance of each metal.

298 However we could not show any difference for the distribution of metals between the
299 soluble and insoluble fractions of mussels collected at the sampled locations.
300 Metallothioneins are known to be the major metal detoxification process in mussels
301 (Langston et al., 1998). The amounts of MT found in the tissues of *B. azoricus* from both
302 groups were in relation with the accumulation of the analyzed metals, accordingly the
303 higher amounts of MT were found in the digestive gland. However no significant
304 correlation between MT and soluble metal amounts was established. Although the
305 synthesis of MT can be enhanced by the presence of metals in the organisms there are
306 other factors such as oxidative stress and environmental changes that can also stimulate
307 the neosynthesis of these metalloproteins (Viarengo and Nott, 1993; Bebianno et al.,
308 2005; Company et al., 2006). Further studies are needed regarding the relative abundance
309 of sulfur-oxidizing filamentous bacteria at the microbial mats and the putative importance
310 of suspension-feeding nutritional pathway in dual symbionts-bearing mussel *B. azoricus*.

311 **5. Conclusions**

312 The results presented in this study do not show significant physiological and
313 toxicological evidence that emphasize the influence of associated sulfur-oxidizing
314 filamentous bacteria on *B. azoricus* mussel. The physiological markers show a similar
315 physiological condition between the two groups of mussels. As well, the variations found
316 in the abiotic factors measured seem not to influence the physiological status of the two
317 mussel groups. The presence of sulfur-oxidizing filamentous bacteria regarded as an
318 indicator of elevated sulfide concentrations, point out a lesser diluted hydrothermal fluid
319 at the considered area and accordingly higher levels of metals available to the organisms.
320 However, it seems that metals are equally bioavailable for mussel in the presence and

321 absence of sulfur-oxidizing filamentous bacteria. This work shows that, *B. azoricus*
322 mussel seems to be well adapted to the assorted physico-chemical characteristics from the
323 surrounding environment since it is able to manage the constant fluctuation of physico-
324 chemical compounds.

325 **Acknowledgements**

326 The authors gratefully acknowledge the captain and crew of the R/V L'Atalante and
327 Victor 6000 ROV team, during the EXOMAR cruise (IFREMER), the EU research
328 project EXOCET/D, FP6-GOCE-CT-2003-505342, the Portuguese Science Foundation
329 funded program SEAHMA project (FCT/ PDCTM 1999/MAR/15281), the pluriannual
330 and programmatic funding from FCT and DRCT for research unit #531 and LA #9,
331 Camões Institution- Pessoa program (GRICES/FCT). I. Martins works under a FCT PhD
332 grant (SFRH/BD/19736/2004).

333 **References**

334 Alain, K., Olagnon, M., Desbruyères, D., Page, A., Barbier, G., Juniper, K., Querellou, J.,
335 Cambon-Bonavita, M-A., 2002. Phylogenetic characterization of the bacterial assemblage
336 associated with the hydrothermal vent polychaete *Paralvinella palmiformis*. FEMS
337 Microbiol. Ecol. 42, 1331-1339.

338

339 Bebianno, M.J., Company, R., Serafim, A., Camus, L., Cosson, R.P., Fiala-Médioni, A.,
340 2005. Antioxidant systems and lipid peroxidation in *Bathymodiolus azoricus* from Mid-
341 Atlantic Ridge hydrothermal vent fields. Aquat. Toxicol. 75, 354-373.

342

- 343 Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification.
344 Can. J. Biochem. Physiol. 37, 911-917.
345
- 346 Boyden, C.R., 1974. Trace element content and body size in mollusks. Nature 251, 311-
347 314.
348
- 349 Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram
350 quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72,
351 248-254.
352
- 353 Brinkhoff, T., Muyzer, G., 1997. Increased Species Diversity and Extended Habitat
354 Range of Sulfur-Oxidizing *Thiomicrospira spp.* Appl. Environ. Microbiol. 63, 3789-
355 3796.
356
- 357 Cavanaugh, C.M., Wirsén, C.O., Jannasch, H.W., 1992. Evidence for methylo-trophic
358 symbionts in a hydrothermal vent mussel (Bivalvia: Mytilidae) from the Mid-Atlantic
359 Ridge. Appl. Environ. Microbiol. 58, 3799-3803.
360
- 361 Charlou, J.L., Donval, J.P., Douville, E., Jean-Baptiste, P., Radford-Knoery, J., Fouquet,
362 Y., Dapoigny, A., Stievenard, M., 2000. Compared geochemical signatures and the
363 evolution of Menez Gwen (37°50'N) and Lucky Strike (37°17'N) hydrothermal fluids,
364 south of the Azores triple junction on the Mid-Atlantic Ridge. Chem. Geol. 171, 49-75.
365

- 366 Childress, J.J., Fisher, C.R., 1992. The biology of hydrothermal vent animals:
367 Physiology, biochemistry and autotrophic symbioses. *Oceanogr. Mar. Biol. Annu. Rev.*
368 30, 337-441.
369
- 370 Colaço, A., Bustamante, P., Fouquet, Y., Sarradin, P.M., Santos, R.S., 2006.
371 Bioaccumulation of Hg, Cu, and Zn in the Azores triple junction hydrothermal vent fields
372 food web. *Chemosphere* 65, 2260–2267.
373
- 374 Company, R., Serafim, A., Cosson, R.P., Fiala-Médioni, A., Dixon, D., Bebianno, M.J.,
375 2006. Temporal variation in the antioxidant defense system and lipid peroxidation in the
376 gills and mantle of hydrothermal vent mussel *Bathymodiolus azoricus*. *Deep-Sea Res.*
377 Part I 53, 1101-1116.
378
- 379 Cosson, R.P., Thiébaud, É., Company, R., Castrec-Rouelle, M., Colaço, A., Martins, I.,
380 Sarradin, P.M., Bebianno, M.J., 2008. Spatial variation of metal bioaccumulation in the
381 hydrothermal vent mussel *Bathymodiolus azoricus*. *Mar. Environ. Res.* 65, 405-415.
382
- 383 Cravo, A., Foster, P., Almeida, C., Company, R., Cosson, R.P., Bebianno, M.J., 2007.
384 Metals in the shell of *Bathymodiolus azoricus* from a hydrothermal vent site on the Mid-
385 Atlantic Ridge. *Environ. Int.* 33, 609-615.
386
- 387 Desbruyères, D., Biscoito, M., Caprais, J-C., Colaço, A., Comtet, T., Crassous, P.,
388 Fouquet, Y., Khripounoff, A., Le Bris, N., Olu, K., Riso, R., Sarradin, P.M., Segonzac,

- 389 M., Vangriesheim, A., 2001. Variations in deep-sea hydrothermal vent communities on
390 the Mid-Atlantic ridge near the Azores plateau. *Deep-Sea Res. Part I* 48, 1325-1346.
391
- 392 Domouhtsidou, G.P., Dimitriadis, V.K., 2000. Ultrastructural localization of heavy
393 metals (Hg, Ag, Pb, and Cu) in gills and digestive gland of mussels, *Mytilus*
394 *galloprovincialis* (L.) *Arch. Environ. Contam. Toxicol.* 38, 472–478.
395
- 396 Douville, E., Charlou, J.L, Oelkers, E.H, Biennu, P., Jove Colon, C.F., Donval, J.P.,
397 Fouquet, Y., Prieur, D., Appriou, P., 2002. The Rainbow vent fluids (36°14'N, MAR):
398 the influence of ultramafic rocks and phase separation on trace metal content in Mid-
399 Atlantic Ridge hydrothermal fluids. *Chem. Geol.* 184, 37-48.
400
- 401 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric
402 method for determination of sugars and related substances. *Anal. Biochem.* 28, 350–356.
403
- 404 Duperron, S., Nadalig, T., Caprais, J.C., Sibuet, M., Fiala-Médione, A., Amann, R.,
405 Dubilier, N., 2005. Dual symbiosis in a *Bathymodiolus* sp. mussel from a methane seep
406 on the Gabon continental margin (Southeast Atlantic): 16S rRNA phylogeny and
407 distribution of the symbionts in gills. *Appl. Environ. Microbiol.* 71, 1694-1700.
408
- 409 Duperron, S., Bergin, C., Zielinski, F., Blazejak, A., Pernthaler, A., McKiness, Z.P.,
410 DeChaine, E., Cavanaugh, C.M., Dubilier, N., 2006. A dual symbiosis shared by two
411 mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (Bivalvia:

- 412 Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge. Environ.
413 Microbiol. 8, 1441–1447.
- 414
- 415 Erbacher, J., Nelskamp, S., 2006. Comparison of benthic foraminifera inside and outside
416 a sulphur-oxidizing bacterial mat from the present oxygen-minimum zone off Pakistan
417 (NE Arabian Sea). Deep-Sea Res. Part I 53, 751-775.
- 418
- 419 Fiala-Médioni, A., McKiness, Z.P., Dando, P., Boulegue, J., Mariotti, A., Alayse-Danet,
420 A.M., Robinson, J.J., Cavanaugh, C.M., 2002. Ultrastructural, biochemical, and
421 immunological characterization of two populations of the mytilid mussel *Bathymodiolus*
422 *azoricus* from the Mid-Atlantic Ridge: evidence for a dual symbiosis. Mar. Biol. 141,
423 1035–1043.
- 424
- 425 Fisher, C., Brooks, J.M., Vodenichar, J.S., Zande, J.M., Childress, J.J., Burke, R.A.,
426 1993. The co-occurrence of methanotrophic sulfur-oxidizing bacterial symbionts in a
427 deep-sea mussel. Mar. Ecol. 14, 277-289.
- 428
- 429 Fraser, A.J., 1989. Triacylglycerol content as a condition index for fish, bivalve and
430 crustacean larvae. Can. J. Fish. Aquat. Sci. 46, 1868-1873.
- 431
- 432 Geret, F., Rouse, N., Riso, R., Sarradin, P.M., Cosson, R.P., 1998. Metal
433 compartmentalization and metallothionein isoforms in mussels from the Mid-Atlantic

434 Ridge; preliminary approach to the fluid-organism relationship. *Cah. Biol. Mar.* 39, 291-
435 293.

436

437 Hagen, K.D., Nelson, D.C., 1997. Use of reduced sulfur compounds by *Beggiatoa spp.*:
438 Enzymology and physiology of marine and freshwater strains in homogeneous and
439 gradient cultures. *Appl. Environ. Microbiol.* 63, 3957-3964.

440

441 Hamza-Chaffai, A., Amiard, J.C., Pellerin, J., Joux, L., Berthet, B., 2000. The potential
442 use of metallothionein in the clam *Ruditapes decussatus* as a biomarker of in situ metal
443 exposure. *Comp. Biochem. Physiol.* 127, 185-197.

444

445 Johnson, K.S., Childress, J.J., Hessler, R.R., Sakamoto-Arnold, C.M., Beelher, C.L.,
446 1988. Chemical and biological interactions in the Rose Garden hydrothermal vent field.
447 Galapagos spreading center. *Deep-Sea Res. Part I* 35, 1723– 1744.

448

449 Kádár, E., Costa, V., Martins, I., Santos, R.S., Powell, J.J., 2005. Enrichment in trace
450 metals (Al, Mn, Co, Cu, Mo, Cd, Fe, Zn, Pb and Hg) of macro-invertebrate habitats at
451 hydrothermal vents along the Mid Atlantic Ridge. *Hydrobiologia* 548, 191–205.

452

453 Kádár, E., Costa, V., Segonzac, M., 2007. Trophic influences of metal accumulation in
454 natural pollution laboratories at deep-sea hydrothermal vents of the Mid-Atlantic Ridge.
455 *Sci. Total Environ.* 373, 464–472.

456

- 457 Konneke, M., Bernhard, A.E., De La Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl,
458 D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437,
459 543-546.
460
- 461 Kopp, J., Cornette, F., Simone, C., 2005. A comparison of growth and biochemical
462 composition of *Mytilus galloprovincialis* (Lmk.) and *Mytilus edulis* (L.) on the West
463 coast of Cotentin, Normandy, France 1999–2000. *Aquac. Int.*13, 327–340.
464
- 465 Lagadic, L. Caquet, T., Amiard, J.C., 1997. Biomarqueurs en ecotoxicologie: Principes et
466 definitions. In: Lagadic, L., Caquet, T., Amiard, J.C., Ramade, F., (Eds.), *Biomarqueurs*
467 *en ecotoxicologie. Aspects Fondamentaux*, Paris, pp. 1 –9.
468
- 469 Langston, W.J., Bebianno, M.J., Burt, G.R., 1998. Metal handling strategies in molluscs.
470 In: Langston, W.J. Bebianno, M.J., (Eds.), *Metal metabolism in aquatic environments*.
471 Chapman and Hall, London, pp. 219–283.
472
- 473 Le Bris, N., Govenar, B., Le Gall, C., Fisher, C.R., 2006. Variability of physico-chemical
474 conditions in 9850VN EPR diffuse flow vent habitats. *Mar. Chem.* 98, 167–182.
475
- 476 Le Pennec, M., Prieur, D., Lucas, A. 1985. Studies on the feeding of a hydrothermal-vent
477 mytilid from the East Pacific Rise. In: Gibbs, P.E., (Eds.), *Proceedings of the nineteenth*
478 *European marine biology symposium*, 16-21 September 1984, Plymouth, Devon, U.K.,
479 159-165.

480

481 Martins I., Colaço, A., Dando, P.R., Martins, I., Desbruyères, D., Sarradin, P.M.,
482 Marques, J.C., Santos, R.S, 2008. Size-dependent variations on the nutritional pathway of
483 *Bathymodiolus azoricus* demonstrated by a C-flux model. Ecol. Model. 217: 59-71.

484

485 Mattison R.G., Abbiati, M., Dando, P.R., Fitzsimons, M.F., Pratt, S.M., Southward, A.J.,
486 Southward, E.C., 1998. Chemoautotrophic microbial mats in submarine caves with
487 hydrothermal sulphidic springs at Cape Palinuro, Italy. Microb. Ecol. 35, 58-71.

488

489 Moyer, C., Dobbs, F.C., Karl, D.M., 1995. Phylogenetic diversity of the bacterial
490 community from a microbial mat at an active, hydrothermal vent system, Loihi seamount,
491 Hawaii. Appl. Environ. Microbiol. 61, 1555-1562.

492

493 Nelson D.C., Wirsen, C.O., Jannasch, H.W., 1989. Characterization of large, autotrophic
494 *Beggiatoa spp.* abundant at hydrothermal vents of the Guayamas Basin. Appl. Environ.
495 Microbiol. 55, 2909-2917.

496

497 Nercessian, O., Reysenbach, A.L., Prieur, D., Jeanthon, C., 2003. Archaeal diversity
498 associated with *in situ* samplers deployed on hydrothermal vents on the East Pacific Rise
499 (13°N). Environ. Microbiol. 5, 492-502.

500

501 Olafson, R.W., Sim, R.G., 1979. An electrochemical approach to quantification and
502 characterization of metallothioneins. Anal. Biochem. 100, 343-351.

503

504 Olsson, B., Bradley, B.P., Gilek, M., Reimer, O., Shepard, J.L., Tedengren, M., 2004.
505 Physiological and proteomic responses in *Mytilus edulis* exposed to PCBs and PAHs
506 extracted from Baltic Sea sediments. *Hydrobiologia* 514, 15–27.

507

508 Pan, K., Wang, W-X., 2008. Allometry of cadmium and zinc concentrations and
509 bioaccumulation in the scallop *Chlamys nobilis*. *Mar. Ecol. Prog. Ser.* 365, 115-126.

510

511 Postec, A., Urios, L., Lasongeur, F., Ollivier, B., Querellou, J., Godfroy, A., 2005.
512 Continuous enrichment culture and molecular monitoring to investigate the microbial
513 diversity of thermophiles inhabiting the deep-sea hydrothermal ecosystems. *Curr.*
514 *Microbiol.* 50, 138-144.

515

516 Radford-Knoery, J., Charlou, J.L., Donval, J.P., Aballéa, M., Fouquet, Y., Ondréas, H.,
517 1998. Distribution of dissolved sulfide, methane, and manganese near the seafloor at the
518 Lucky Strike (37°17'N) and Menez Gwen (37°50'N) hydrothermal vent sites on the Mid-
519 Atlantic Ridge. *Deep-Sea Res. Part I* 45, 367-386.

520

521 Rouse, N., Boulegue, J., Cosson, R.P., Fiala-Medioni, A., 1998. Bioaccumulation des
522 métaux chez le mytilidae hydrothermal *Bathymodiulus* sp. de la ride médio-atlantique.
523 *Oceanol Acta* 21, 597-607.

524

- 525 Salerno J. L., Macko, S. A., Hallam, S. J., Bright, M., Won, Y-J., Mckiness Z., Van
526 Dover, C L., 2005. Characterization of symbiont populations in life-history stages of
527 mussels from chemosynthetic environments. Biol. Bull. 208, 145-155.
528
- 529 Sarradin, P.M., Caprais, J.C., Riso, R., Kerouel, R., Aminot, A., 1999. Chemical
530 environment of the hydrothermal mussel communities in the Lucky Strike and Menez
531 Gwen vent fields, Mid Atlantic Ridge. Cah. Biol. Mar. 40, 93-104.
532
- 533 Sarradin P.M., Desbruyères, D., Le Bris, N., Caprais, J.C., Rodier, P., Fabri, M.C., Le
534 Gall, C., Khripounoff, A., Vangriesheim, A., Crassous, P., Briand, P., Segonzac, M.,
535 Fouquet, Y., Cambon, P., Etoubleau, J., Riso, R., 2003. Ventox WP5:
536 Organisms/Fluid/Substrate interactions, final data report, Ifremer, Brest, 28 pp.
537
- 538 Stewart, F., Newton, I., Cavanaugh, C., 2005. Chemosynthetic endosymbiosis:
539 adaptations to oxic–anoxic interfaces. Trends Microb. 13, 439-448.
540
- 541 Trask, J.L., Van Dover, C.L., 1999. Site-specific and ontogenetic variations in nutrition
542 of mussels (*Bathymodiolus sp.*) from the Lucky Strike hydrothermal vent field, Mid-
543 Atlantic Ridge. Limnol. Oceanogr. 44, 334–343.
544
- 545 Thompson, J.J., Cosson, R.P., 1984. An improved electrochemical method for the
546 quantification of metallothioneins in marine organisms. Mar. Environ. Res 11, 137–152.
547

548 Viarengo, A., Nott, J.A., 1993. Mechanisms of heavy metal cation homeostasis in marine
549 invertebrates. *Comp. Biochem. Physiol.* 104, 355-372.

550

551 Vuillemin, R., LeRoux, D., Dorval, P., Bucas, K., Sudreau, J.P., Hamona, M., LeGall, C.,
552 Sarradin, P.M., (2009). CHEMINI: A new in situ CHEmical MINIaturized analyzer.

553 *Deep-Sea Res. Part I*, in press

554

555 Wery, N., Cambon-Bonavita, M.A., Lesongeur, F., Barbier, G., 2002. Diversity of
556 anaerobic heterotrophic thermophiles isolated from deep-sea hydrothermal vents of the
557 Mid-Atlantic Ridge. *FEMS Microbiol. Ecol.* 41, 105-114.

558

559 Wörner, U., Zimmermann-Timm, H., 2000. *Beggiatoa leptomitiformis*- a filamentous
560 sulfur-oxidizing bacterium colonizing laboratory-made aggregates. *Limnologica* 30, 215-
561 221.

562

563

564

565

566

1 Table 1

2 Levels of Cu, Fe and Zn found in certified reference material, mussel tissue CE278 (ERM-Belgium) and lobster hepatopancreas TORT-2 (NRCC-Canada).

3 Results as mean \pm SD, in mg g⁻¹ dry weight.

Certified reference material		Cu (mg g ⁻¹) n= 6	Fe (mg g ⁻¹) n= 8	Zn (mg g ⁻¹) n= 11
CE278	Certified	-	-	0.08 \pm 0.002
	Observed	-	-	0.10 \pm 0.001
TORT-2	Certified	0.11 \pm 0.01	0.11 \pm 0.01	-
	Observed	0.11 \pm 0.002	0.11 \pm 0.01	-

4

5 Table 2

6 Lipids, carbohydrates and total proteins in whole soft tissues for the two groups studied. Results are presented as mean \pm SD, in mg g⁻¹ dry weight. n

7 represents the number of analyzed mussels.

Biochemical	n	mussels	n	mussels + mats
Lipids ^a	1	138.5	1	174.8
Carbohydrates	5	29.1 \pm 4.7	5	36.2 \pm 9.9
Total proteins	5	889.9 \pm 67.4	4	863.3 \pm 60.9

8 ^a pooled sample

9 Table 3

10 In situ temperature measurements (°C) and estimated values of CH₄ and H₂S (μmol l⁻¹) obtained within the sampling sites. For temperatures measurements: n=
11 23389, sampling period 30 sec, results are presented as mean ± SD.

	mussels			mussels + mats		
	Temperature (°C)	CH ₄ estimated (μM)	H ₂ S _T estimated (μM)	Temperature (°C)	CH ₄ estimated (μM)	H ₂ S _T estimated (μM)
mean ± SD	4.91 ± 0.10			5.89 ± 0.52		
minimum	4.64	0.06	<dl*	4.76	0.15	1
maximum	5.25	0.5	5	7.39	2.0	23
range	0.61			2.63		

12 * dl= detection limit

13

14

15

16

17

18

19

20

21 Table 4

22 Metallothionein (MT) and metal (Cu, Fe and Zn) levels in the tissues of the two groups studied. The “Mean per group” represents the mean levels of MT, Cu,

23 Fe and Zn for each group. Results are presented as mean \pm SD, in mg g⁻¹ dry weight

	mussels				mussels + mats			
	MT	Cu	Fe	Zn	MT	Cu	Fe	Zn
Gill	16.7 \pm 5.0	0.14 \pm 0.04	0.28 \pm 0.09	0.23 \pm 0.06	16.9 \pm 2.7	0.20 \pm 0.02	0.20 \pm 0.04	0.21 \pm 0.04
Mantle	22.1 \pm 7.7	0.08 \pm 0.04	0.30 \pm 0.08	0.25 \pm 0.06	21.0 \pm 10.7	0.07 \pm 0.02	0.20 \pm 0.07	0.38 \pm 0.11
Foot	20.1 \pm 29.7	0.10 \pm 0.04	0.28 \pm 0.05	0.50 \pm 0.21	21.6 \pm 9.2	0.05 \pm 0.03	0.20 \pm 0.09	0.30 \pm 0.13
Digestive gland	96.8 \pm 27.8	0.18 \pm 0.05	0.97 \pm 0.25	0.67 \pm 0.14	114.2 \pm 38.2	0.20 \pm 0.09	0.65 \pm 0.18	0.65 \pm 0.26
Remaining	18.0 \pm 1.4	0.07 \pm 0.01	0.48 \pm 0.19	0.34 \pm 0.11	15.9 \pm 2.9	0.07 \pm 0.04	0.20 \pm 0.04	0.19 \pm 0.08
Mean per group	21.5 \pm 0.2	0.13 \pm 0.03	0.32 \pm 0.08	0.28 \pm 0.06	19.1 \pm 0.2	0.11 \pm 0.04	0.19 \pm 0.05	0.21 \pm 0.06

24