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The influence of nutritional conditions on metal uptake by the mixotrophic dual symbiosis harboring vent mussel *Bathymodiolus azoricus*

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Abstract:

The vent mussel *Bathymodiolus azoricus,* host thioautotrophic and methanotrophic bacteria, in their gills and complementary, is able to digest suspended organic matter. But the involvement of nutritional status in metal uptake and storage remains unclear. The influence of *B. azoricus* physiological condition on its response to the exposure of a mixture of metals in solution is addressed. Mussels from the Menez Gwen field were exposed to 50 µg L^{-1} Cd, plus 25 µg L^{-1} Cu and 100 µg L^{-1} Zn for 24 days. Four conditions were tested: (i) mussels harboring both bacteria but not feed, (ii) harboring only methanotrophic bacteria, (iii) without bacteria but fed during exposure and (iv) without bacteria during starvation. Unexposed mussels under the same conditions were used as controls. Eventual seasonal variations were assessed. Metal levels were quantified in subcellular fractions in gills and digestive gland. Metallothionein levels and condition indices were also quantified. Gill sections were used for fluorescence in situ hybridization (FISH) to assess the temporal distribution of symbiotic associations. Starvation damages metal homeostasis mechanisms and increase the intracellular Zn and MT levels function. There is a clear metallic competition for soluble and insoluble intracellular ligands at each condition. Seasonal variations were observed at metal uptake and storage.

Keywords: Vent mussel *B. azoricus*; Metal accumulation; Nutritional condition; Metallothioneins; Condition indices

1. Introduction

The mytilid mussel, Bathymodiolus azoricus, is one of the dominant hydrothermal vent macroorganisms in the Azores Triple Junction region, distributed at depths ranging from 840 m (Menez Gwen vent field) to 2300 m (Rainbow vent field) ([Colaço et al., 1998] and [Desbruyères et al., 2001]). This hydrothermal vent mussel is a mixotrophic organism whose energy is obtained by 1) dual endosymbiosis, based on both thioautotrophic (SOX) and methanotrophic (MOX) bacteria within gill bacteriocytes ([Fiala-Médioni et al., 2002] and Duperron et al., 2006 S. Duperron, C. Bergin, F. Zielinski, A. Blazejak, A. Pernthaler, Z.P. McKiness, E. DeChaine, C.M. Cavanaugh and N. Dubilier, A dual symbiosis shared by two mussel species, Bathymodiolus azoricus and Bathymodiolus puteoserpentis (Bivalvia: Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge, Environ Microbiol 8 (2006), pp. 1441–1447. Full Text via CrossRef | View Record in Scopus | Cited By in Scopus (43)[Duperron et al., 2006]), and 2) suspension-feeding, through well developed labial palps and a functional feeding tract, which allow B. azoricus to filter and digest organic matter particles ([Le Pennec et al., 1990] and [Page et al., 1991]). Both nutritional pathways allow B. azoricus to colonize environments where the availability of substrates is unpredictable or fluctuating (Salerno et al., 2005). B. azoricus relies mostly on the primary production of the symbionts for its nutritional requirements ([Salerno et al., 2005] and [De Busserolles et al., 2009]). Suspension-feeding, a secondary mode of nutrition ([Colaço et al., 2009], [De Busserolles et al., 2009] and [Riou et al., 2010]), works as an additional nutritional strategy according to external concentrations of hydrogen sulfide (H2S) and methane (CH4) (Martins et al., 2008). The hydrothermal fluid provides to the vent fauna not only necessary energy sources and suspension organic particles, but also potentially toxic compounds such as metals ([Desbruyères et al., 2000], [Douville et al., 2002] and [Kádár et al., 2005a]). Nevertheless, hydrothermal vent mussels developed mechanisms enabling the detoxification of metals, like their storage under insoluble granules or their binding to specific metalloproteins, namely metallothioneins (Cosson and Vivier, 1995). Several studies have shown the ability of B. azoricus to accumulate large amounts of metals from their environment ([Kádár et al., 2005b], [Cosson et al., 2008] and [Martins et al., 2009]) but the

role of the trophic pathway in their uptake is still not clear. In the present study, the accumulation of two essential (Cu and Zn) and one non essential (Cd) metals, was followed in the gills and the digestive gland of *B. azoricus*, maintained under various conditions susceptible to modify their physiological status. Our protocols were designed in order to show eventual differences in the uptake and bioaccumulation of these three metals when mussels rely on (i) both symbionts, (ii) only methanotrophic symbionts, (iii) supplied organic food, as energy source or (iv) are starving.

2. Material and Methods

2.1. Sample collection and maintenance

Acoustically retrievable cages were moored during the MoMARETO cruise (with the French R/V "Pourquoi Pas?") in August-September 2006. They were positioned on diffuse venting areas at Menez Gwen hydrothermal field (37º51'N; 31º31'W) and filled with approximately 400 mussels using the Ifremer Remotely Operated Vehicle (ROV) "Victor 6000". Our experiments were performed on mussels from Menez Gwen collected during MoMARETO cruise (brought to the surface using the ROV and designated hereafter as "Summer mussels"), and from two cages recovered by the Portuguese R/V "Arquipélago" in January and May 2007 (designated hereafter as "Winter mussels" and "Spring mussels", respectively). Specimens from Menez Gwen hydrothermal field were chosen for the experiments, because this field is the shallowest one of the Azores Triple Junction (ATJ) and mussels can survive and maintain their endosymbionts active at atmospheric pressure (Kádár et al. 2005a, Bettencourt et al. 2008, Company et al. 2008, Riou et al. 2008). Once the mussels were brought to the surface, they were immediately transferred to fresh cooled seawater and held in plastic cool boxes during transit to a land-based refrigerated laboratory "LabHorta" (environment temperature 8-11ºC (Colaço & Santos 2002)). In the laboratory, 8 groups of 27 mussels of comparable sizes (summer: 45 ± 7 mm, winter: 46 ± 5 mm, spring: 46 ± 5 mm) were cleaned of visible adhering material. Each group was kept in 10 L plastic containers filled with 8-9ºC seawater (warmer water was found to inactivate methane oxidizers in the vent mussel

93 *Bathymodiolus childressi* (Kochevar et al. 1992)) and air-oxygen supply, for laboratory
94 acclimatization before metal exposures. Seawater for the containers was supplied from a
95 reservoir containing sand-filtered seawater from an unpolluted bay in Horta, Azores
96 (38º58'N; 28º78'W) (Cruz et al. 2010). Water conditions (temperature, pH and O_2
97 saturation) were daily monitored (table 1).
98
99 *2.2. Experimental protocols*
100
101 The following procedure was undertaken for each of the 3 type of seasonal mussels.
102 *Protocol (i)*: mussels harboring both symbiont bacteria and not fed
103 Mussels were kept in plain seawater with no gas or food supply, for a concomitant
104 symbiont bacteria mislay, and exposed to the metal mixture after 3 days of laboratory
105 acclimatization.
106 *Protocol (ii)*: mussels harboring only methanotrophic bacteria
107 Mussels were kept in seawater enriched only by bubbling ultra-pure CH_4 (N45, Air-
108 Liquide), during 3 weeks, for thiotrophic bacteria loss prior to their exposure to the metal
109 mixture and during the exposure. Since high methane concentrations, above 300 µM,
110 were found inhibitory for symbionts of the vent mussel *B. childressi* exhibiting high
111 methane consumption rates (Kochevar et al. 1992), we tried to maintain CH_4
112 concentration below this level (20-50 µM). The dissolved CH_4 in water was monitored
113 daily (table 1) by sampling 50mL of seawater in a 500mL bottle which was rolled for 20
114 minutes before measuring the headspace CH_4 with a portable GMI Gasurveyor 500.
115 *Protocol (iii)*: mussels without symbiont bacteria but supplied with food
116 Mussels were kept in methane- and sulfide-free seawater during 3 weeks for both
117 symbiotic bacteria loss prior to their exposure to the metal mixture. Before and during
118 exposure, the mussels were fed with a concentrate solution ($6x10^{10}$cell/liter) of
119 microalgae Nannochloropsis
120 *Protocol (iv)*: mussels without symbiont bacteria and not fed
121 Mussels were kept in methane- and sulfide-free seawater during 3 weeks for both
122 symbiotic bacteria loss prior to their exposure to the metal mixture.
123

124 *2.3. Cd, Cu and Zn exposure*

125

126 The 4 groups from the initial 8 groups were maintained for 24 days in sea water
127 containing 50 µg L^{-1} Cd (Cd $(NO_3)_2$, Merck, CertiPur®), plus 25 µg L^{-1} Cu (Cu $(NO3)_2$,
128 Merck, CertiPur®) and plus 100 µg L^{-1} Zn (Zn $(NO3)_2$, Merck, CertiPur®). The
129 concentrations of added metals were selected according to Company (2005) who handled
130 several experiments with metals on *B. azoricus*. The remained 4 groups of 27 mussels
131 were maintained following similar protocols (i to iv) but without the addition of metal
132 mixture and were used as controls. Water was renewed every 3 days to eliminate organic
133 matter that could result in oxygen depletion, and maintain a pH of 7-8. Food and metals
134 were added to renewed water. No sulfide was added in the water during the exposure to
135 prevent the precipitation of metals. Before the first addition of metals 6 mussels
136 (designated hereafter as "day 0") were sampled from each protocol. Then 6 mussels were
137 sampled from each protocol at days 6, 15 and 24 of the exposure. As well, 6 mussels
138 (designated hereafter as "control mussels") were sampled at days 15 and 24 from each
139 control protocol. From each sampled individual, shell length was recorded and gills and
140 digestive gland tissues were dissected and preserved at -80º C until freeze-drying. Metals
141 and metallothioneins analysis were performed in both tissues.

142

143 *2.4. Metal analyses*

144

145 Approximately 100 mg of dry tissue were homogenized in TRIS buffer (100 mM, 10 mM
146 ß-mercaptoethanol, pH 8.6) and centrifuged (30000g, 30 min, 4º C). For each sample, the
147 pellet containing the insoluble compounds (designated hereafter as "insoluble fraction")
148 and an aliquot (2 ml) of the supernatant, containing the soluble compounds (designated
149 hereafter as "soluble fraction") were digested with nitric acid (65% v/v, Merck, p.a.) at
150 room temperature during 12 hours follow by a 2 hours heat-bath at 60º C. After pellets
151 and supernatant digestion, solutions were dried at 60º C and solubilized by adding 2 ml
152 0.5N HNO_3. Cd, Cu and Zn were determined by flame atomic absorption
153 spectrophotometry (GBC-Avanta Σ), with deuterium background correction, or graphite
154 furnace atomic absorption spectrometry (Perkin–Elmer, Zeeman 4110ZL) depending on

155 metal levels. The accuracy and precision of the method used were established by regular
156 analysis of certified reference materials, mussel tissue CE278 (European Reference
157 Materials of Belgium) and lobster hepatopancreas TORT-2 (National Research Council
158 of Canada). Certified reference materials and blanks were taken through the procedure in
159 the same way as the samples. Our values and the certified values are given in Table 2 as
160 $\mu g\ g^{-1}$ of dry weight.
161
162 *2.5. Metallothioneins (MT)*
163
164 To determine MT level, an aliquot of the supernatant from the centrifugation described
165 above was heat-denatured (90°C, 15 min) and centrifuged at 13000 g, 10 min, at 4ºC. In
166 the heat-denatured supernatant the amount of MT was determined by Differential Pulse
167 Polarography (DPP) according to Olafson and Sim (1979) and Thompson and Cosson
168 (1984). A standard addition calibration curve was obtained using rabbit liver MT-I as
169 reference. Results were expressed as $\mu g\ g^{-1}$ of dry weight. Olafson and Olsson (1991)
170 have confirmed, by quantitative analyses on chromatographic fractions, that the method is
171 specific for MT after removal of contaminating proteins in tissue homogenates by heat
172 denaturation. The contribution of the mercaptoethanol and the low-molar-mass
173 compounds (such as glutathione and free cysteins) during MT quantification is negligible
174 (Olafson & Sim 1979, Geret & Cosson 2002).
175
176 *2.6. Condition Indices*
177
178 Tissue condition index (TCI) and gill index (GI) were used to assess the mussel
179 physiological condition before (day 0) and during the exposure period. The measure of
180 both indices in mussels at day 0 will represent their status after acclimatization and before
181 exposure.
182 The tissue condition index was calculated as follows:
183 TCI = tissues dry weight (g)/shell volume (ml) (Voets et al. 2006)
184 The mussel volume (Mv) was calculated based on the length of the mussel with the
185 formula: $M_v = (\text{length})^a$

This allometric relationship was determined empirically as follows. The shell volume of 127 *B. azoricus* with a length between 25 and 97 mm was measured with graduated cylinders (to the nearest 0.5 ml) using the Archimeds Principle. The length of the mussel was plotted against the shell volume and a power relation was calculated (R^2=0,984; p<0,01) with allometric exponent (a) equal to 2,719 (unpublished data).

The gill index was calculated as follows:

GI = (gill tissue dry weight (g)/shell volume (ml)) x 10

2.7. Fluorescence in situ-hybridization (FISH)

To check the presence or absence of symbionts in *B. azoricus*, according to each experiment protocol, gill tissues of 1 mussel was dissected for FISH analysis at 0 and 24 days. Analyses were performed according to Duperron (2005) with slight modifications. Gill tissues were dissected immediately after removing mussels from the aquaria, preserved in 10% buffered formalin and processed for paraffin embedding according to standard protocol. Transverse sections (7 µm thick) were subjected to deparafinization and re-hydration through successive baths of xylene, ethanol (100%, 95% and 70% respectively) and rinsed with distilled water. Before hybridization, the sections were incubated in a pre-hybridization solution for 5 min at 46ºC. The hybridization reaction was carried out at 46ºC for 4 h with a hybridization solution containing the specific bacteria probes, individualized by a circle of PAP pen. After incubation, the sections were placed 15 min in a washing solution. The Alexa Fluo-488 GCTCCGCCACTAAGCCTA and Alexa Fluo-532 CGAAGGTCCTCCACTTTA fluorescent probes were used to target respectively thiotrophic and methanotrophic symbionts (Duperron et al. 2005, Bettencourt et al. 2008). The fluochromes Alexa 488 and Alexa 532 were from Molecular Probes, Invitrogen. Gill filaments were visualized under fluorescent light and differential interference contrast (DIC) microscopy using a Leica DM6000 digital microscope (Leica Microsystems CMS GmbH, Germany). Results are presented in Figure. 6

2.8. Data analysis

Data were first tested for normality by normal probability plots and the homogeneity of variances was checked using Bartlett's test. Since data did not respect the assumptions of analysis of variance, the reciprocal square-root (sqrt) transformation [1/sqrt (x + 0,5)] was applied. Analyses of covariance (ANCOVA) and one-way ANOVA were used on transformed data to evaluate the variability between groups of samples. Tukey's test was used as post hoc comparison of means. Sperman rank correlations were used to evaluate the relation between the levels of MT and metals associated with soluble compounds measured in both exposed mussels organs. Tests were performed with STATISTICA 6.0 (StatSoft). Differences were considered significant when $p<0,05$. Statistical methods were selected in accordance with Zar (1999).

3. Results

3.1. Water conditions and mortality

The measured water parameters (temperature, pH, O_2 and dissolved CH_4) did not show variations in each protocol between the collection seasons, therefore Table 1 represents the mean values measured at the three collection seasons.

Mortality was recorded at summer season in protocol (ii), with 16% in exposed group and 7% in control group and in protocol (iv), with 4% of mortality record in exposed group. In winter, 7% of mortality was recorded in exposed group of protocol (ii).

3.2. Metal accumulation

Mean metal levels in the gills and the digestive gland of control and exposed mussels, following the protocols at the 3 seasons, are shown in Figures 1 to 3.

3.2.1. Cadmium

Gills (Figures 1, a-b-c)

For the 24 days of exposure, whatever the experimental protocol or season, a similar bioaccumulation pattern was observed, resulting from significant increases of Cd levels (ANOVA, $p<0,05$). On the contrary, the levels in control mussels remained constantly low with the exception of some erratic values noticed at the three seasons for protocol (i). In non-exposed mussels Cd was preferably associated to insoluble compounds (Table 3). Whatever the experimental protocol or season the subcellular distribution of Cd did not change, Cd remained preferably associated to insoluble compounds.

Digestive Gland (Figures 1, d-e-f)

For the 24 days of exposure, whatever the experimental protocol or season, a similar bioaccumulation pattern was observed, resulting from significant increases of Cd levels (ANOVA, $p<0,05$). On the contrary, the levels in control mussels remained constantly low with the exception of non significant values noticed after 24 days in summer for protocols (i) and in spring (iv) (ANOVA, $p>0,05$). In non-exposed mussels Cd was equally distributed between soluble and insoluble fractions whatever the season (Table 3). The ratio of Cd bioaccumulated under soluble forms increased when mussels were submitted to protocol (i) in summer and winter. The opposite was observed with protocol (ii) with Cd preferably bioaccumulated under insoluble forms. For both protocols, in spring, bioaccumulated Cd remained equally distributed.

3.2.2. Copper

Gills (Figures 2, a-b-c)

Cu levels were generally higher in exposed mussels than in control mussels (ANOVA, $p<0,05$) with few exceptions at day 24 for protocol (i) in summer and for protocol (ii) in winter. Cu bioaccumulation patterns varied with season for a same experimental protocol with maximum Cu levels reached by day 24 being lower in winter than in summer or spring (ANCOVA, $p<0.05$). Whatever the experimental protocol or season the subcellular distribution of Cu did not change, Cu remained preferably associated to soluble compounds (Table 3).

Digestive Gland (Figures 2, d-e-f)

Whatever the experimental protocol and season, Cu levels in exposed mussels were not significant different from levels in control mussels (ANOVA, $p>0,05$) with few exceptions at day 24 for protocol (i) at the 3 seasons, at day 24 for protocol (ii) in summer and at day 15 for protocol (ii) in spring (ANOVA, $p<0,05$). Whatever the experimental protocol or season the subcellular distribution of Cu did not change, Cu remained preferably associated to soluble compounds (Table 3). After 24 days of exposure the bioaccumulation of Cu seems to be more intensive in the gills than in the digestive gland, especially following the protocol (iv).

3.2.3. Zinc

Gills (Figures 3, a-b-c)

Whatever the experimental protocol and season, there was no clear evidence of Zn bioaccumulation in the gills of exposed mussels compared to controls. In summer Zn was equally distributed between soluble and insoluble compounds at the subcellular level in non-exposed mussels (Table 3). After exposures using the protocols (iii) and (iv), Zn shifted to the soluble compartment. In winter and spring, Zn was preferably associated to soluble compounds in non-exposed mussels and this distribution did not change except in winter after exposures using the (i) and (ii) protocols leading to an equal distribution of Zn between the two compartments.

Digestive Gland (Figures 3, d-e-f)

Zn levels in control mussels were uniform only for mussels collected in spring, while in summer and winter levels varied whatever the experimental protocol with extreme values reached at day 24 at protocol (iv). A part from the equal distribution of Zn in non-exposed mussels from summer, Zn was preferably associated to soluble compounds whatever the experimental protocol or season (Table 3). In summer and winter the variations of Zn levels were more intense in the digestive gland than in the gills for corresponding experimental protocols and exposure duration. In spring, no obvious differences were observed between the variations of Zn levels in gills and digestive gland.

309 *3.3. Metallothioneins*

310

311 *Gills* (Figures 4, a-b-c)

312 The mean level of MT in controls (whatever the experimental protocol, season or
313 collection day) was 2052 ± 613 ($\mu g g^{-1}$) with exceptional values noticed in protocol (i) in
314 summer and in protocol (iv) in winter. No common trend could be observed for a given
315 experimental protocol regarding the pattern of MT level variations with seasons.
316 Generally, MT levels of exposed mussels were not significantly different from control
317 mussel levels (ANOVA, p>0,05).

318 *Digestive Gland* (Figures 4, d-e-f)

319 MT levels varied a lot in control mussels in summer and winter whatever the
320 experimental protocol. In spring the levels were lower (ANOVA, $p<0.05$) and their
321 variation was less important. Mean levels of MT in mussels exposed in winter under
322 protocols (ii), (iii) and (iv) were higher than the levels reached for the same protocols in
323 summer and spring (ANCOVA, $p<0.05$).

324

325 *3.4. TCI and GI*

326

327 The mean values of tissue condition index (TCI) and gill index (GI) in non-exposed (day
328 0) and exposed mussels collected at the 3 seasons are shown in Figure 5.

329 *TCI* (Figures 5, a-b-c):

330 At day 0 TCI did not showed significant variation between experimental protocols in
331 summer and spring (ANOVA, $p>0.05$). In winter, TCI at day 0 was significantly higher
332 for protocols (i) and (ii) (ANOVA, $p<0.05$). Whatever the experimental protocol and
333 season, there was no significant variation of mean TCI during 24 days for exposed
334 mussels (ANOVA, $p>0.05$). Between seasons, whatever the experimental protocol,
335 mussels collected in summer showed significant lower TCI (ANCOVA, $p<0.05$).
336 Moreover, for protocol (iii) and (iv) mussels collected in spring showed significant higher
337 TCI (ANCOVA, $p<0.05$).

338 *GI* (Figures 5, d-e-f):

339 At day 0 whatever the season, GI did not show significant variation between
340 experimental protocols (ANOVA, $p>0.05$). Whatever the experimental protocol, there
341 was no significant variation of mean GI during 24 days for exposed mussels (ANOVA,
342 $p>0.05$) in summer and spring. In winter, a significant decrease of GI was observed after
343 day 6 for protocol (iii) (ANOVA, $p<0.05$). Whatever the experimental protocol or season,
344 control mussels did not show any variation during the experimental period (ANOVA,
345 $p>0.05$). Between seasons, whatever the experimental protocol, mussels collected in
346 summer showed significant lower GI (ANCOVA, $p<0.05$).
347
348 *3.5. FISH*
349
350 The images of the detection of thioautotrophic and methanotrophic bacteria in the gill
351 tissue are shown in Figure 6. No difference was found in bacterial densities between the
352 mussels collected at the 3 seasons. Therefore, the panels contain the representative
353 images of bacteria densities found at the beginning (day 0) and by the end (day 24) of
354 each exposure protocol.
355 *Protocol (i)*
356 The gill tissues were taken from the fresh mussels immediately after their arrival at the
357 laboratory, therefore bacterial detection at day 0 corresponds to the natural abundance of
358 endosymbionts (A1). The fluorescent probes detected both thioautotrophic (red) and
359 methanotrophic (green) bacteria. After 24 days of exposure (A2), the density of
360 thioautotrophic bacteria was rather low and the gill filaments showed empty
361 bacteriocytes. The methanotrophic bacteria were not detected anymore.
362 *Protocol (ii)*
363 The gill tissues, at day 0, were taken from mussels acclimatized during 3 weeks in plain
364 sea water supplied with CH_4. The fluorescent probes detected the methanotrophic (green)
365 bacteria distributed at the bacteriocytes boundary (B1). After 24 days of exposure (B2),
366 methanotrophic bacteria were rather detected and gill filaments showed empty
367 bacteriocytes.
368 *Protocol (iii) and (iv)*

The gill tissues, at day 0, were taken from mussels acclimatized during 3 weeks in plain sea water. Both bacteria were rather detected or undetected (C1). After 24 days of exposure (C2) no bacteria was detected. Nevertheless, gill filaments showed the presence of hemocytes.

4. Discussion

Several reasons are responsible for the choice of *B. azoricus* as an experimental model for understanding how its physiological functioning manages metal bioaccumulation and storage. (a) The amount of data regarding the relationships of bivalves and metals present in their environment is extensive (Viarengo & Nott 1993, Langston et al. 1998, Company 2005, Wang & Rainbow 2005) (b) Bathymodiolus genus is present at most of the known chemosynthesis-based ecosystems. (c) As other species, *B. azoricus* relies on mixotrophy for its nutrition by ways of dual symbiosis (MOX and SOX bacteria). (d) *B. azoricus* abundance at moderate depth sites on Menez Gwen MAR-ATJ field allows its maintenance at atmospheric pressure in suitable devices (Colaço & Santos 2002). Recently special attention was devoted to study the effects of metals (Cu, Cd) on the antioxidant defense system of *B. azoricus* and concomitant neosynthesis of metallothioneins (Company et al. 2008, Company et al. 2010). At the moment no data are available regarding this species about the combined effects and the bioaccumulation of a mixture of metals. However *in situ B. azoricus* are exposed simultaneously and continuously to several metals under dissolved and particle form (Sarradin et al. 1999, Sarradin et al. 2008). In spite of our current knowledge on uptake of metals by *B. azoricus*, an evaluation is still lacking on the relative importance of the nutritional pathway versus the direct uptake by diffusion into the organs in contact with the surrounding water. Our work was devised in order to meet the exposure to a mixture of metals (Cd, Cu, Zn) during four physiological conditions, in combination with *B. azoricus* food source. They relied as food source, at the beginning of the exposure, on the presence of both SOX and MOX bacteria in the gills (i). They relied as food source on the sole presence of MOX bacteria that were supplied with methane during the exposure (ii). They did not harbor any kind of symbiotic bacteria but were provided with

400 microalgae during the exposure (iii). They did not harbor any kind of symbiotic bacteria
401 and were not given any food supply (iv). It was shown that metal bioaccumulation could
402 be influenced by the season in relation with the reproductive cycle (Langston et al. 1998).
403 To take into account this biotic factor, our experiments were performed on mussels
404 collected during summer, winter and spring that correspond respectively to sexual pause,
405 spawning period and spawning recovery as shown by Colaço et al. (2006).
406
407 *4.1. Mussel physiological status*
408
409 The lower TCI and GI found in mussels collected in summer could be a consequence of a
410 stress linked to animal collection using the ROV. In opposition to a cage recovery in
411 which mussels arrive to the surface in approximately 20 minutes, a submersible recovery
412 may take hours inflicting a decompression physiological stress difficult to recover (Dixon
413 et al. 2004, Pruski & Dixon 2007). Results from FISH showed that even with methane
414 supply the MOX bacteria disappeared from mussel gills by the end of the 24th day of
415 exposure to the metal's mixture. Experimental research developed by Kochevar et al.
416 (1992) on *Bathymodiolus* sp. showed that optimal rates of methane consumption by
417 methanotrophic symbionts bacteria are reached when methane is supplied at
418 concentrations ranging from 150-350 μM. In our study we supplied the mussels with
419 methane concentrations ranging 20-50 μM. Kochevar et al. (1992) showed that at low
420 methane concentrations mussel metabolic functions seem decreasing, including lower
421 rates of oxygen and methane consumption. At similar methane conditions, low rates of
422 carbon incorporation in gills were evidenced by Riou et al. (2008). The mussel low
423 metabolism and a consequent increase of maintenance requirements can result in a low
424 TCI (De Coen & Janssen 2003).
425 Exposed mussels fed with microalgae, collected in spring, showed high TCI. We can
426 assume that feeding exposed *B. azoricus* with microalgae at this time of the year
427 improves a little bit their physiological status or limits its deterioration. Moreover,
428 phytoplanktonic fatty acid biomarkers were detected in *B. azoricus*, from Menez Gwen,
429 collected in May (Pond et al. 1998, Colaço et al. 2009) indicating a potential use of
430 suspension-feeding on particles sedimenting from the sea surface at this season (Riou

2009). Is noteworthy the ability of *B. azoricus* to survive under starvation conditions, metal exposure and endosymbionts loss. Similar to coastal mussels, *B. azoricus* present storage cells in mantle connective tissue and gills (such as the hemocytes observed), enriched with lipids and glycogen that allow the specie to survive if their nutrition is affected by the loss of the symbiotic bacteria (Lobo-da-Cunha et al. 2006, Bettencourt et al. 2008).

4.2. Metal uptake and storage

4.2.1. Gills

In the gills where metals can be uptake directly from the medium Cd and Cu were bioaccumulated between day 0 and day 24 while Zn levels remained stable. The bioaccumulation of Cd and Cu from water in the gills was already shown in coastal mussels by several authors (Carpené & George 1981, Geret 2000, Isani et al. 2000) with the respective involvements of Ca^{++} or Na^{+} channels. Whatever the season, exposed and non exposed mussels harboring both bacteria showed a highly variable Cd and Cu accumulation. That can reflect changes in their physiological system due to mislay of bacteria during the 24 days. Zn is not bioaccumulated when mussels are fed with microalgae while an increase of Zn levels is observed in summer, when they are starving. The observed increases of Zn levels following starvation may likely be explained by the dysfunction of internal metal regulatory mechanisms as a consequence of starvation. A preferential storage of Cd in association with insoluble compounds was observed while Cu was preferably stored in association with soluble forms. Company et al. (2008) and (2010) exposed *B. azoricus* to the same concentrations of Cd and Cu, under similar conditions of maintenance, however not simultaneously. After 24 days of exposure both bioaccumulated Cd and Cu were stored preferably in association with soluble compounds in the gills. We can hypothesize that the competition between Cd and Cu for soluble ligands favored Cu and resulted in Cd binding to insoluble compounds. The important contribution of organelles and lysosomes to Cd detoxication has been mentioned earlier by Viarengo and Nott (1993). Cd binding as insoluble form in the gills, which are in

462 direct contact with the external medium, brings advantages since it could reduce Cd
463 cellular toxicity and may eventually facilitate its excretion by exocytosis (Langston et al.
464 1998). On the other hand, the sequestration of Cd under insoluble forms may result from
465 saturation or damages of the mechanisms involved in Cd storage under soluble forms as
466 mentioned by (Company et al. 2006, Choi et al. 2007). Copper, bioaccumulated in
467 association with soluble compounds, was the unique metal that could induce the
468 neosynthesis of MTs. As almost no increase of MT levels were observed, we can assume
469 that accumulated Cu in the soluble compartment was bound to existing MTs as the result
470 of substitution to MT constitutive Zn or Cd (Langston et al. 1998). Zinc intracellular level
471 is regulated with an equal distribution between soluble and insoluble associated
472 compounds and a tendency to favor the association with soluble compounds. We can
473 assume that in our experimental conditions *B. azoricus* was able to reduce the uptake of
474 zinc from gill surrounding medium and regulate its storage or excretion as shown
475 previous for coastal mussels (Anandraja et al. 2002, Kondoh et al. 2003, Wang &
476 Rainbow 2005). The bioaccumulation of Zn as soluble form in mussels under starvation
477 is likely to participate to MT level increases in summer and winter. But as shown earlier
478 by several authors the increases of MT levels may also be attributed to a global stress
479 reaction to starvation (Cosson 2000, Viarengo et al. 2000, Kondoh et al. 2003).
480
481 *4.2.2. Digestive gland*
482
483 In general Cd was bioaccumulated with an equal distribution between the soluble and the
484 insoluble compartment, or rather preferably associated to soluble compounds. In the
485 coastal mussel *Mytilus galloprovincialis*, collected in a highly Cd contaminated area, a
486 very important accumulation of Cd was observed in the digestive gland (Raspor et al.
487 1999). Whatever the season or protocol Cu was preferably associated to soluble
488 compounds. The same preference was also mentioned by Geret (2000) for the coastal
489 mussel *Mytilus edulis* experimentally exposed to Cu. At all seasons a increase of MT
490 levels was observed in the digestive gland by the end of the exposures. This increase of
491 MT levels by the end of the exposures may be related to the binding of bioaccumulated
492 Cd to soluble compounds. The association of the bioaccumulated Cd to insoluble

493 compounds as an explanation to the lack of increase of MT levels was already pointed out
494 above for the gills. Such increases of MT levels following Cd exposures of coastal
495 mussels were observed previous and attributed to Cd bioaccumulation as soluble forms
496 (Raspor et al. 1999, Geret 2000). Mussels with both bacteria as food source showed a
497 correlation between soluble Cu and MT levels at all seasons (Table 4). Moreover, several
498 correlations were established between soluble Zn and MT levels whatever the protocol
499 used, but significant correspondent increases of MT levels were not noticed underlining
500 the complexity of MT synthesis regulation in *B. azoricus* (Hardivillier et al. 2004,
501 Company et al. 2006, Martins et al. 2009).
502
503 In summary, the use of exposures to a mixture of metals was designed to approach
504 mussel *in situ* conditions. Nevertheless, we can not neglect the fact that during the 24
505 days exposure a modification of the ratio: number of mussels/metal availability occurs,
506 which may occasionally increase the levels of metals by the end of the exposure. Our
507 results regarding the distribution of Cd at the subcellular level were not in accordance
508 with previous results obtained after mono-metallic exposures of *B. azoricus* or coastal
509 mussels. In addition, the observed Cd preferential storage as insoluble forms in the gills
510 comes into conflict with data from *in situ* collected *B. azoricus*. These discrepancies may
511 be explained by several factors (a) the use of metal concentrations higher than that
512 measured in *B. azoricus* surrounding environment (b) in the mixture, metals were in
513 solution while *in situ* they are present under both dissolved and particulate forms (c) in a
514 mixture, metals compete for their uptake and storage by the exposed organisms contrarily
515 to what happens when a mono-metallic exposure is used. However, our results show that
516 *B. azoricus* is able to adapt its metal handling strategies to versatile close environment
517 conditions. It underlines the *B. azoricus* ability to store metals (here Cd) under insoluble
518 forms preventing potential toxic impacts. We put forward that *B. azoricus* metal uptake
519 and storage mechanisms are similar to those already described for coastal mussels which
520 are highly influenced by metallic ions size and electronic affinity for cellular compounds.
521 Comparing our results with the reproductive cycle of *B. azoricus*, described earlier by
522 Colaço et al. (2006), the influence of reproductive status, on metal uptake and storage
523 does not appear clearly despite observed seasonal variations. Further work is needed to

establish the existence of a relationship similar to that observed for coastal bivalves. Regarding the impact of the nutritional status of exposed *B. azoricus* on the handling of added dissolved metals we confirm that starvation results in a dysfunction of metal homeostasis mechanisms with an increase of intracellular Zn and MT levels. We also show that, when *B. azoricus* relies solely on its alimentation through its functional digestive tract (microalgae supply) metal uptake and storage regulation mechanisms are still efficient to prevent the dysfunction of essential metal homeostasis. The ability of *B. azoricus* endosymbionts bacteria to survive in starvation conditions, under experimental conditions, was described earlier (Kádár et al. 2005a, Colaço et al. 2006, Bettencourt et al. 2008). Experiments performed under improved exposure and nutritional conditions are needed to go further in our knowledge of metal uptake routes bioaccumulation and regulation in *B. azoricus*.

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750
751 Fig.1- Mean variation of Cd levels (µg g^{-1}, dry weight) in the gills (a-b-c) and digestive
752 gland (d-e-f) of control (dashed lines) and exposed mussels (solid lines) at each
753 experiment (i, ii, iii, iv) for 24 days. Here and in Fig. 2-5, panels (a) and (d) represent the
754 experiments undertaken in summer, (b) and (e) represent the experiments undertaken in
755 winter and (c) and (f) the experiments undertaken in spring. Vertical bars represent the
756 standard error of the mean. Symbol (*) represents significant differences between day 0
757 and day 24 of exposure. Symbol (**) represents significant differences between day 0
758 and day 24 of control.
759
760
761 Fig. 2- Mean variation of Cu levels (µg g^{-1}, dry weight) in the gills (a-b-c) and digestive
762 gland (d-e-f) of control (dashed lines) and exposed mussels (solid lines) at each
763 experiment (i, ii, iii, iv) for 24 days.
764
765

766 Fig. 3- Mean variation of Zn levels (µg g^{-1}, dry weight) in the gills (a-b-c) and digestive
767 gland (d-e-f) of control (dashed lines) and exposed mussels (solid lines) at each
768 experiment (i, ii, iii, iv) for 24 days.
769
770 Fig. 4- Mean variation of MT levels (µg g^{-1}, dry weight) in the gills (a-b-c) and digestive
771 gland (d-e-f) of control (dashed lines) and exposed mussels (solid lines) at each
772 experiment (i, ii, iii, iv) for 24 days.
773
774 Fig. 5- Mean variation (g ml^{-1}) of TCI (a-b-c) and GCI (d-e-f) of control (dashed lines)
775 and exposed mussels (solid lines) at each experiment (i, ii, iii, iv) for 24 days.
776
777
778 Fig.6. Detection of thiotrophic and methanotrophic bacterial endosymbionts in gills
779 tissue. Fluorescent probes were used to target thiotrophic (red) and methanotrophic
780 (green) bacterial symbionts in fluorescence in-situ hybridization (FISH) experiments.
781 Differential interference contrast (DIC) visualization is shown (right side of panels).
782 Scale bar (10 µm) and original objective magnifications are indicated. Panel A shows the
783 density of both bacteria at day 0 (A1) and day 24 (A2) of exposure for experiment (i).
784 Panel B shows the methanotrophic bacteria density at day 0 (B1) and day 24 (B2) of
785 exposure for experiment (ii). Panel C shows the density of both bacteria at day 0 (C1) and
786 day 24 (C2) of exposure for experiment (iii) and (iv).
787

Table 1

Mean water conditions for the three collection seasons at each experiment (i; ii; iii; iv) for exposed and control mussels. Results as mean ± SD; n = 123.

experiment	Temperature (ºC)	pH	O_2 saturation (%)	Dissolved CH4 (µM)
i	8.7 ± 1.1	8.0 ± 0.2	59.7 ± 7.5	-
control	8.7 ± 1.1	8.1 ± 0.2	60.1 ± 9.7	-
ii	9.1 ± 0.9	8.1 ± 0.3	53.9 ± 11.1	41.2 ± 25.0
control	9.1 ± 0.9	8.2 ± 0.2	55.5 ± 10.3	33.5 ± 17.8
iii	8.9 ± 1.0	8.2 ± 0.2	58.3 ± 9.1	-
control	8.9 ± 1.0	8.0 ± 0.7	55.5 ± 8.4	-
iv	8.9 ± 1.0	8.2 ± 0.2	55.9 ± 6.6	-
control	8.9 ± 0.9	8.1 ± 0.2	59.3 ± 9.6	-

Table 2

Levels of Cd, Cu and Zn found in certified reference material, mussel tissue CE278 (ERM-Belgium) and lobster hepatopancreas TORT-2 (NRCC-Canada). Results as mean ± SD, in µg g^{-1} dry weight.

Certified reference material		Cd (µg g^{-1}) n= 21	Cu (µg g^{-1}) n= 30	Zn (µg g^{-1}) n= 15
CE278	Certified	-	-	83.1 ± 1.7

	Observed	-	-	83.8 ± 2.5
TORT-2	Certified	26.7 ± 0.6	106 ± 10	-
	Observed	25.6 ± 1.2	105 ± 6	-

Table 3

Amounts of metals associated with insoluble compounds (expressed as percentages- mean ± SD) in the gill and digestive gland tissues of non-exposed (ne) (mussels from day 0) and exposed mussels for each experiment (i; ii; iii; iv), collected in summer, winter and spring.

	Summer		Winter		Spring	
	G	GD	G	GD	G	GD
Cd						
ne	86 ± 5	46 ± 14	58 ± 26	59 ± 21	80 ± 10	57 ± 8
i	64 ± 11	30 ± 11	54 ± 12	29 ± 8	77 ± 9	56 ± 8
ii	73 ± 8	65 ± 12	73 ± 8	61 ± 14	77 ± 8	46 ± 9
iii	80 ± 6	53 ± 14	80 ± 8	56 ± 12	73 ± 9	51 ± 6
iv	64 ± 14	58 ± 16	58 ± 15	57 ± 11	84 ± 4	55 ± 8
Cu						
ne	61 ± 11	45 ± 13	35 ± 7	26 ± 8	47 ± 4	21 ± 4

i	37 ± 13	30 ± 9	34 ± 7	30 ± 10	44 ± 7	21 ± 4
ii	31 ± 7	30 ± 8	29 ± 6	26 ± 9	44 ± 9	16 ± 5
iii	34 ± 5	26 ± 12	34 ± 4	19 ± 8	43 ± 6	19 ± 5
iv	40 ± 7	27 ± 8	35 ± 6	21 ± 7	43 ± 6	19 ± 5
Zn						
ne	52 ± 17	53 ± 15	29 ± 18	26 ± 13	28 ± 14	33 ± 9
i	53 ± 7	38 ± 9	51 ± 7	37 ± 9	39 ± 3	41 ± 3
ii	43 ± 6	37 ± 8	46 ± 11	36 ± 11	42 ± 4	32 ± 4
iii	17 ± 5	25 ± 8	18 ± 5	21 ± 7	13 ± 2	23 ± 6
iv	17 ± 6	11 ± 6	16 ± 7	13 ± 8	13 ± 3	25 ± 4

Table 4

Values of Spearman rank *r* correlations between MT and metals associated with soluble compounds in gill (G) and digestive gland (DG) of mussels collected at the three seasons (summer, winter and spring) and exposed to each experiment (i, ii, iii, iv). In superscript allied *p* values are present (n.s.: not significant; *: p<0.05; non superscript: p=0.05; -: metal associated with insoluble compounds)

	G		DG	
Summer	Cu	Zn	Cu	Zn

	i	-0.16$^{n.s.}$	-	0.79*	0.73*
	ii	-0.13$^{n.s.}$	0.18$^{n.s.}$	0.50*	0.61*
	iii	0.25$^{n.s.}$	0.39$^{n.s.}$	0.57*	0.48*
	iv	0.10$^{n.s.}$	0.42*	0.36$^{n.s.}$	0.20$^{n.s.}$
Winter					
	i	-0.26$^{n.s.}$	-	0.70*	0.64*
	ii	-0.10$^{n.s.}$	-0.21$^{n.s.}$	0.28$^{n.s.}$	0.47*
	iii	0.27$^{n.s.}$	0.45*	0.10$^{n.s}$	0.27$^{n.s.}$
	iv	0.18$^{n.s.}$	0.10$^{n.s.}$	0.62*	0.30$^{n.s.}$
Spring					
	i	-0.01$^{n.s.}$	0.12$^{n.s.}$	0.75*	0.63*
	ii	0,50*	0.71*	0.33$^{n.s.}$	0.72*
	iii	0.54*	0.11$^{n.s.}$	0.65*	0.69*
	iv	0.47*	0.43	0.25$^{n.s.}$	0.43*

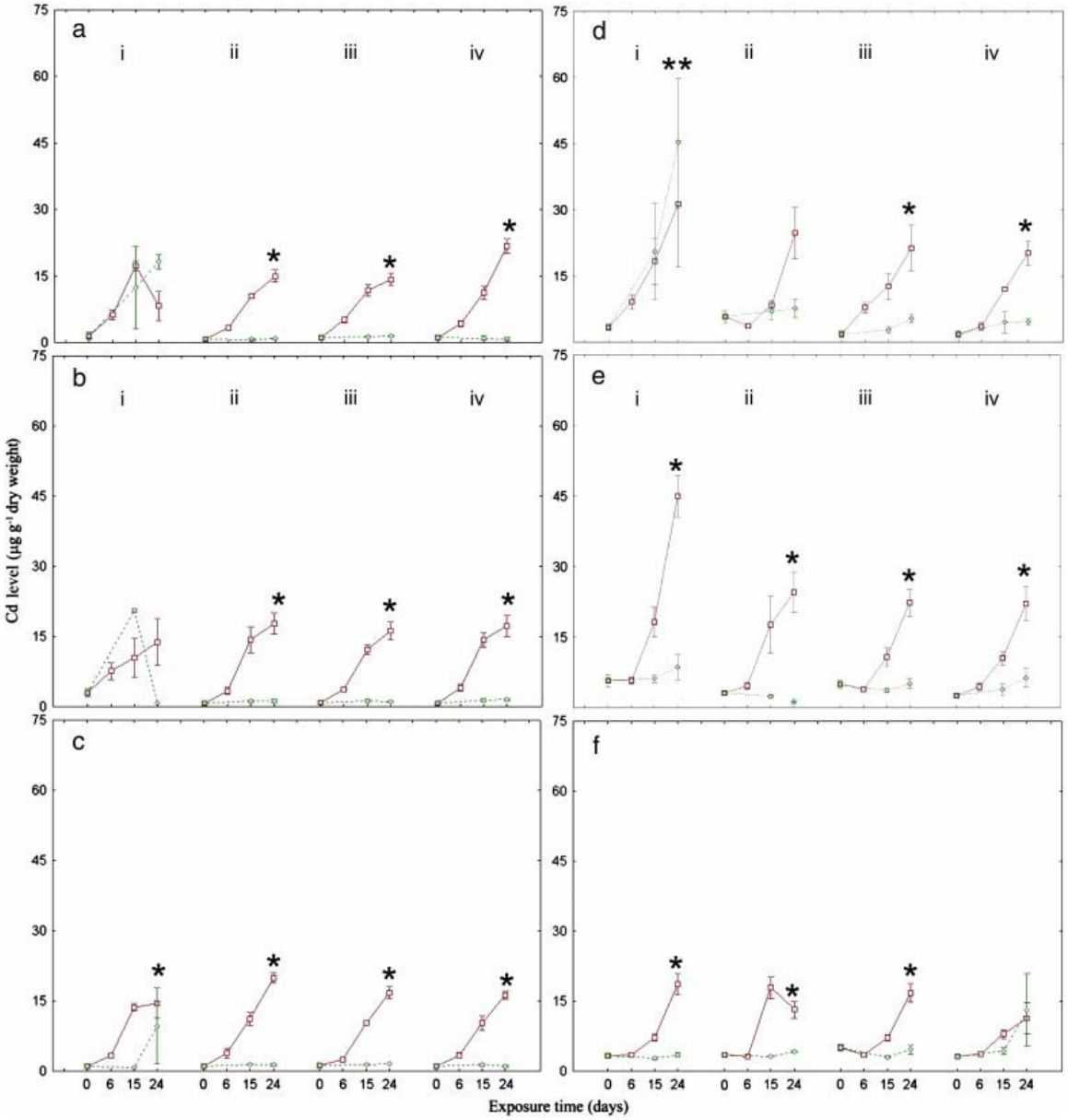

Fig. 1. Mean variation of Cd levels (µg g^{-1}, dry mass) in the gills (a–b–c) and digestive gland (d–e–f) of control (dashed lines) and exposed mussels (solid lines) at each experiment (i, ii, iii, and iv) for 24 days. Here and in [Fig. 2], [Fig. 3], [Fig. 4] and [Fig. 5], panels (a) and (d) represent the experiments undertaken in summer, (b) and (e) represent the experiments undertaken in winter and (c) and (f) the experiments undertaken in spring. Vertical bars represent the standard error of the mean. Symbol (*) represents significant differences between day 0 and day 24 of exposure. Symbol (**) represents significant differences between day 0 and day 24 of control.

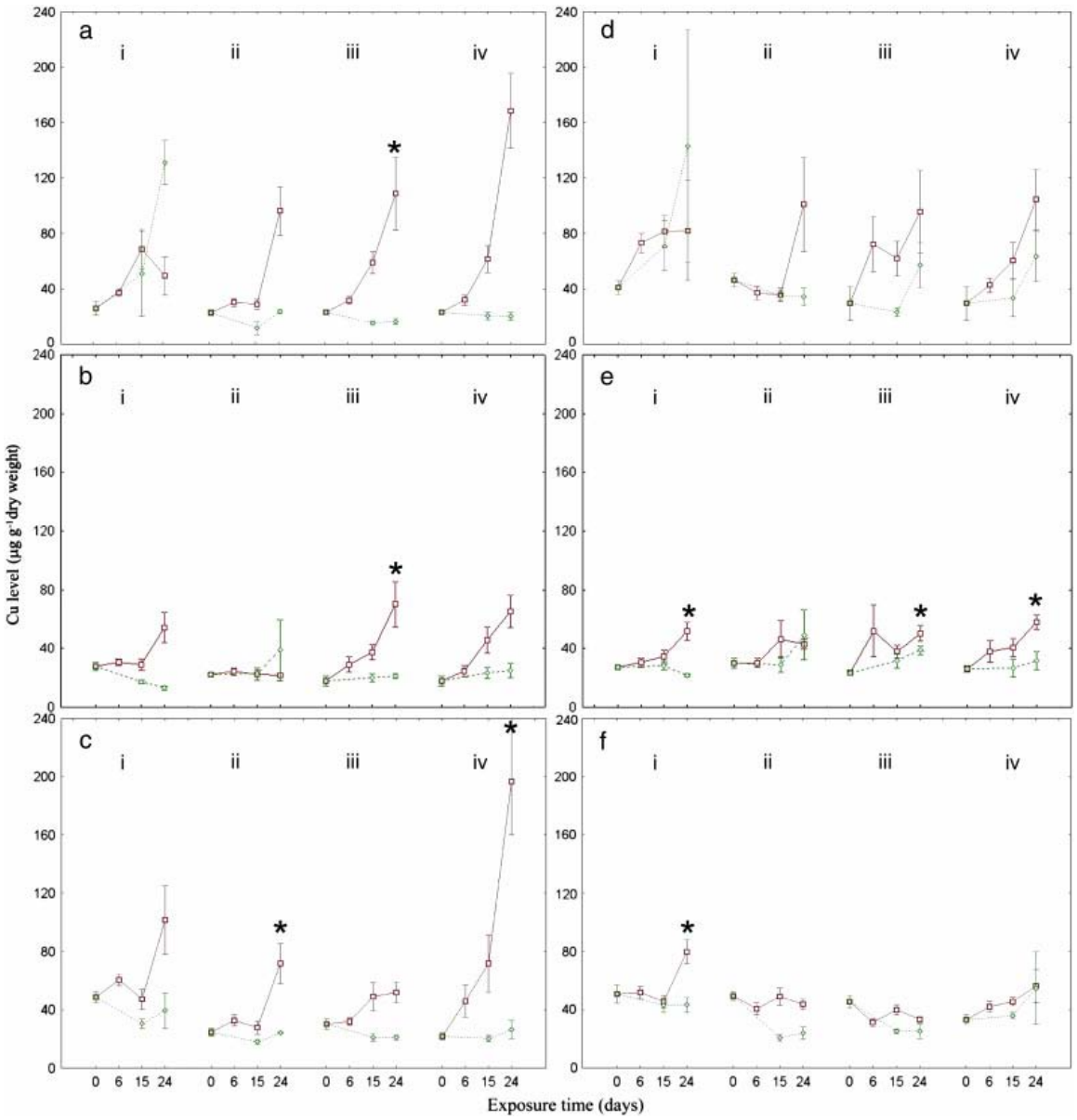

Fig. 2. Mean variation of Cu levels (µg g^{-1}, dry mass) in the gills (a–b–c) and digestive gland (d–e–f) of control (dashed lines) and exposed mussels (solid lines) at each experiment (i, ii, iii, and iv) for 24 days.

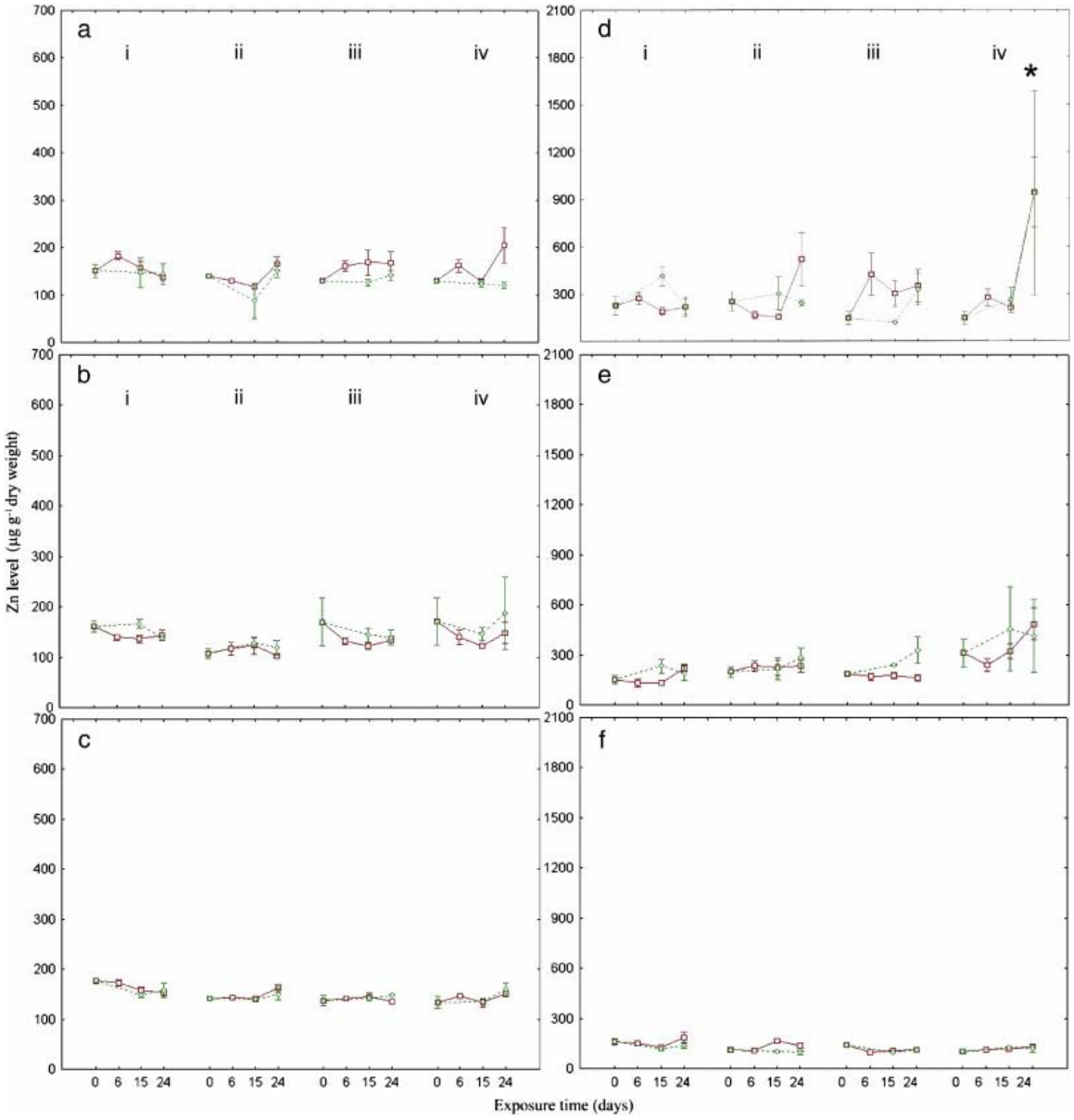

Fig. 3.

Mean variation of Zn levels (µg g^{-1}, dry mass) in the gills (a–b–c) and digestive gland (d–e–f) of control (dashed lines) and exposed mussels (solid lines) at each experiment (i, ii, iii, and iv) for 24 days.

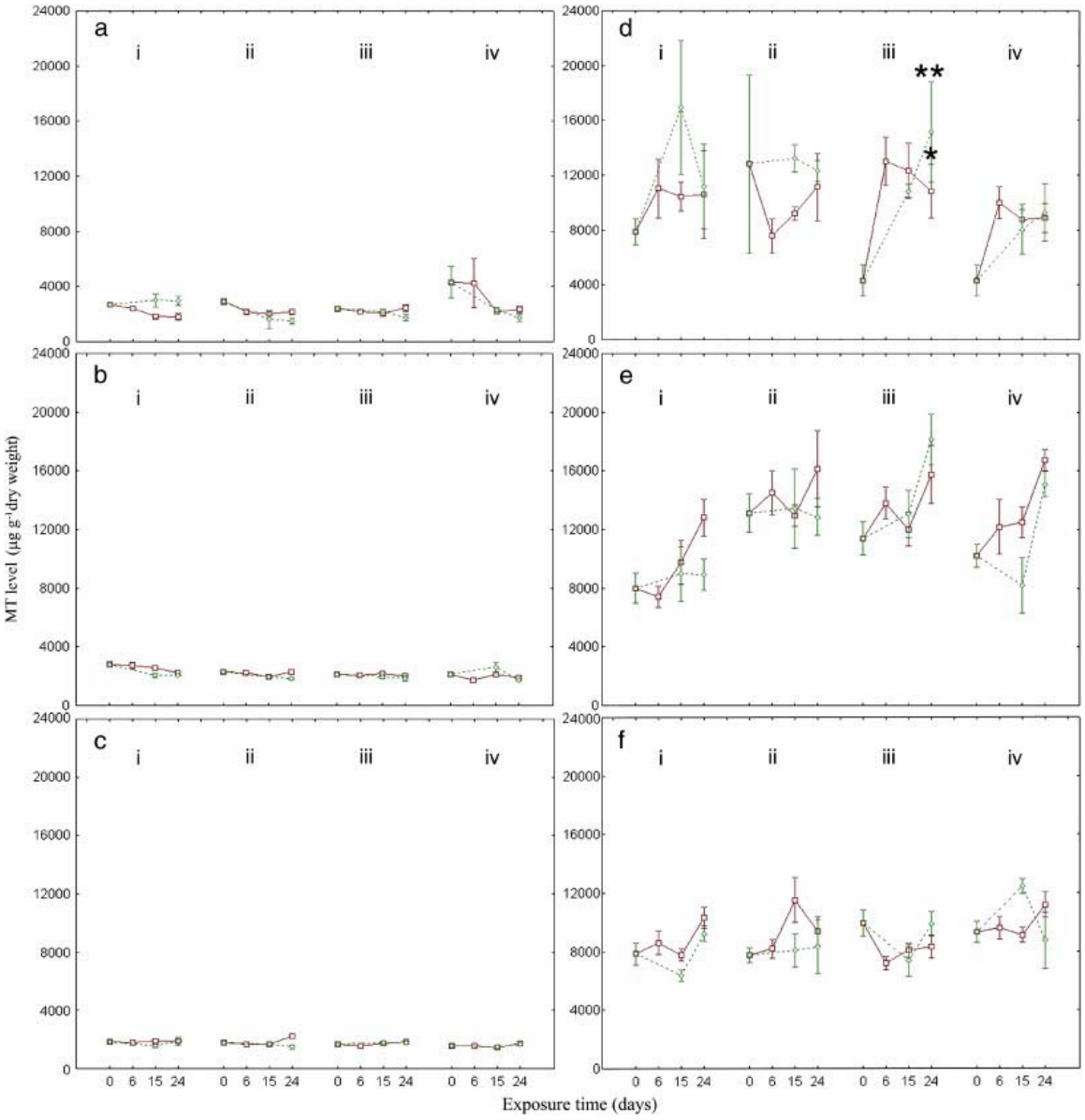

Fig. 4.

Mean variation of MT levels (µg g^{-1}, dry mass) in the gills (a–b–c) and digestive gland (d–e–f) of control (dashed lines) and exposed mussels (solid lines) at each experiment (i, ii, iii, and iv) for 24 days.

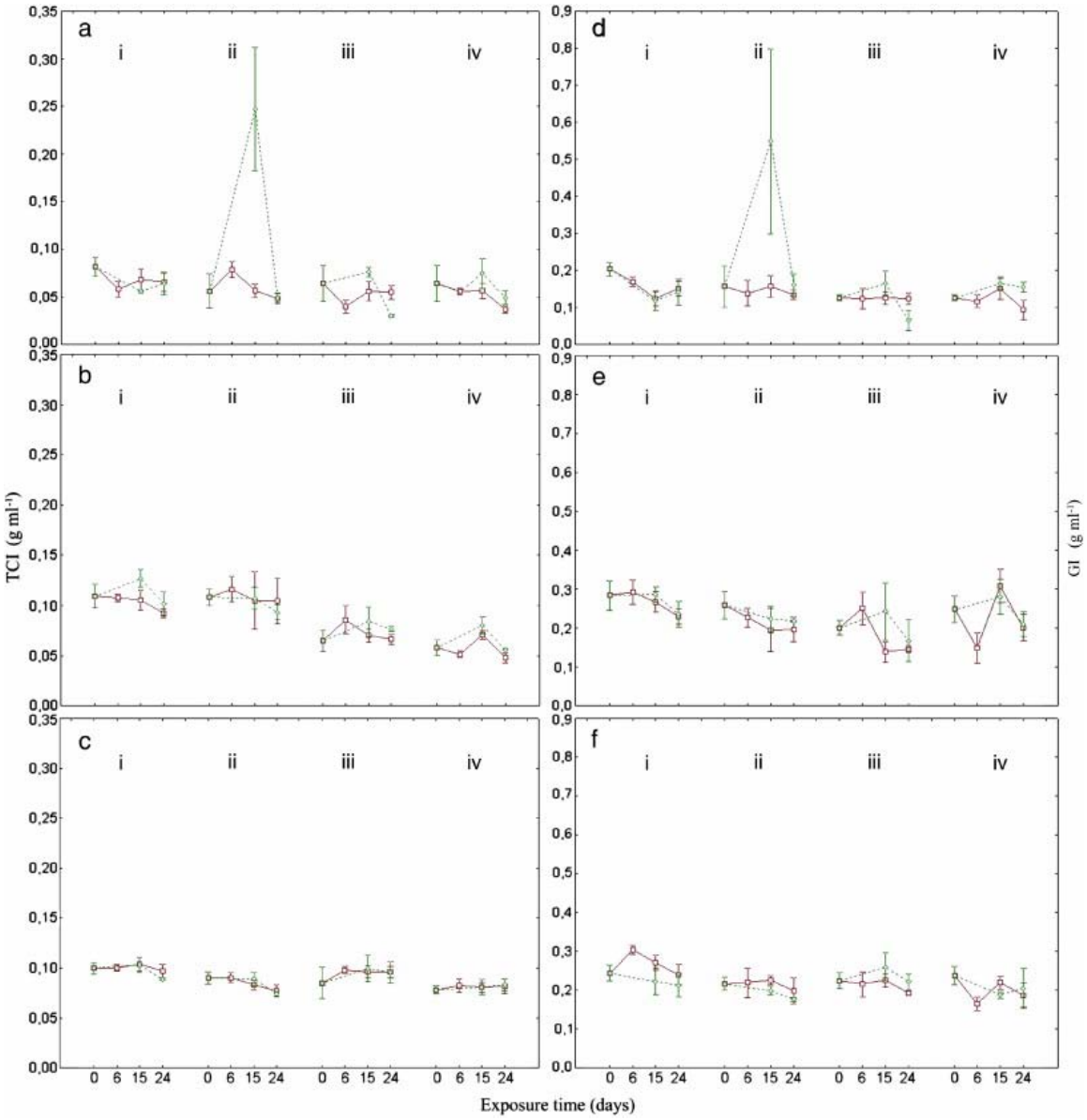

Fig. 5. Mean variation (g mL^{-1}) of TCI (a–b–c) and GCI (d–e–f) of control (dashed lines) and exposed mussels (solid lines) at each experiment (i, ii, iii, and iv) for 24 days.

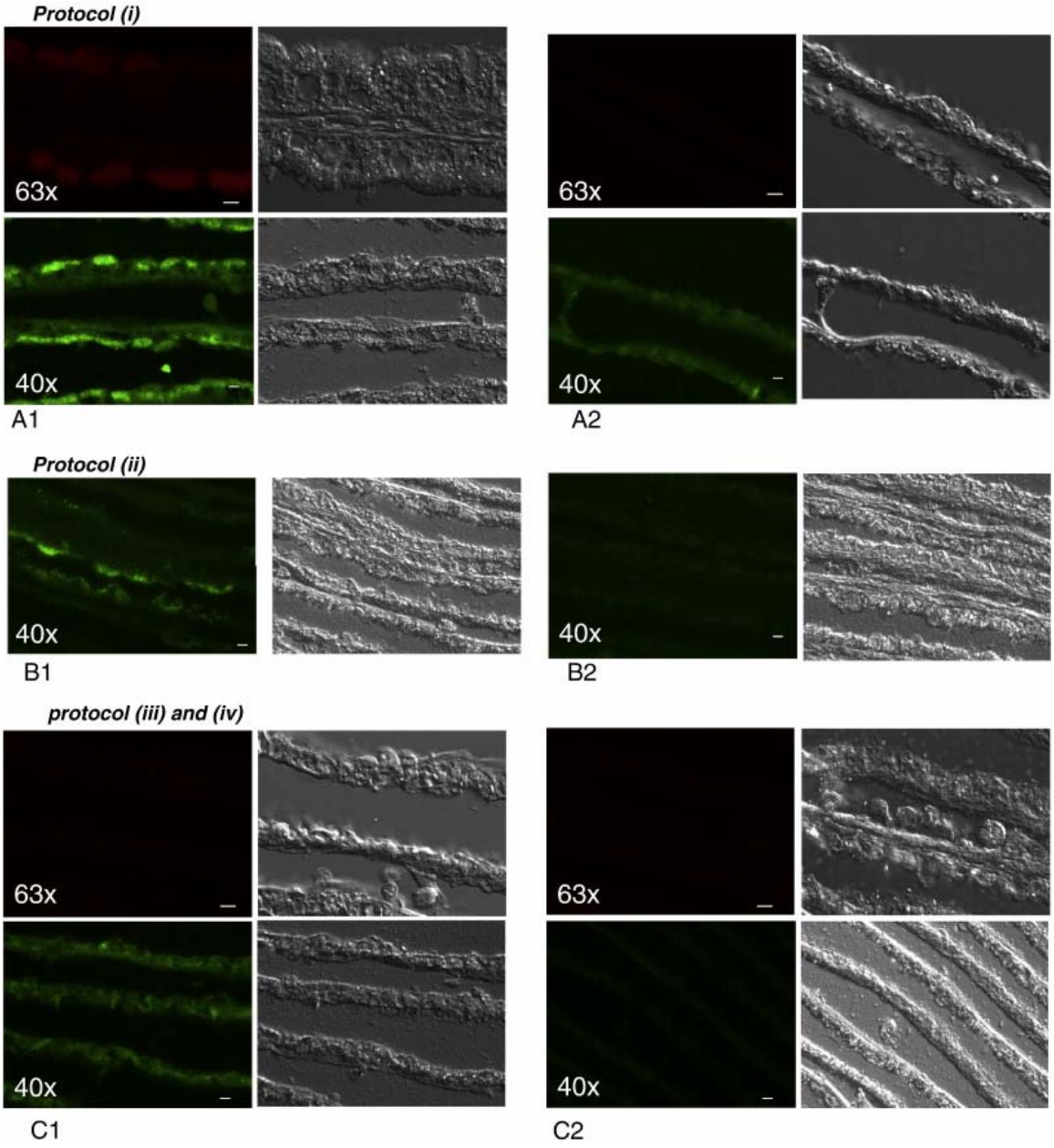

Fig. 6. Detection of thiotrophic and methanotrophic bacterial endosymbionts in gill tissue. Fluorescent probes were used to target thiotrophic (red) and methanotrophic (green) bacterial symbionts in fluorescence in situ hybridization (FISH) experiments. Differential interference contrast (DIC) visualization is shown (right side of panels). Scale bar (10 μm) and original objective magnifications are indicated. Panel A shows the density of both bacteria at day 0 (A1) and day 24 (A2) of exposure for experiment (i). Panel B shows the methanotrophic bacteria density at day 0 (B1) and day 24 (B2) of exposure for experiment (ii). Panel C shows the density of both bacteria at day 0 (C1) and day 24 (C2) of exposure for experiments (iii) and (iv).