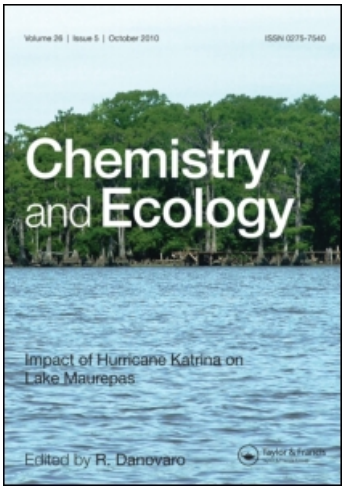

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Deep-sea caging of the mussel *Mytilus galloprovincialis*: Potential application in ecotoxicological studies

F. Galgani[a]; J. F. Chiffoleau[b]; P. LE Gall[a]; Y. Pichot[a]; B. Andral[c]; C. Martin[c]
[a] IFREMER, BP171, Sète cedex, France [b] IFREMER, Nantes, France [c] IFREMER, La Seyne/mer, France

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Deep-sea caging of the mussel *Mytilus galloprovincialis*: Potential application in ecotoxicological studies

F. GALGANI*†, J. F. CHIFFOLEAU‡, P. LE GALL†, Y. PICHOT†, B. ANDRAL§ and C. MARTIN§

†IFREMER, BP171, Ave. J Monnet, 34203, Sète cedex, France
‡IFREMER, rue de l'île d'Yeu, 44311, Nantes, France
§IFREMER, zone de Bregaillon, 83507, La Seyne/mer, France

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We experimented with caging the Mediterranean mussel (*Mytilus galloprovincialis*) at various depths for 69 d to measure basic physiological parameters, histological response and bio-accumulation of contaminants in a deep-sea contaminated area. In preliminary experiments, we demonstrated, under artificial pressure conditions, the ability of mussels *Mytilus galloprovincialis* to tolerate rapid immersion (at a speed of up to 120 m min^{-1}). *In situ* experiments were performed using submerged lines enabling mussels to be maintained at depths ranging of 40–1550 m with survival rates ranging from 80 to 38%, respectively. No significant differences in condition indexes were observed between treated and control specimens. However, histological observations demonstrated a clear reduction in thickness of the digestive epithelium with increasing depth exposure. By determining the contaminants in caged mussels, we found the following values for chromium accumulation: 27.4 μg g^{-1} dry weight at 580 m depth and 9.8 μg g^{-1} dry weight at 1550 m. Selected stations were located downstream of an industrial effluent at 420 m. The biological and environmental consequences of deep-sea contamination demonstrate the suitability of caged mussels for monitoring contaminant accumulation.

Keywords: Mussel; *Mytilus*; Deep sea; Caging; Canyons; Chromium; Contamination

1. Introduction

The adaptation metabolism of marine organisms to high pressure has been extensively investigated [1–6]. These studies are mainly related to vertebrates, whereas limited evidence is available for invertebrates, in hyperbaric chambers or natural deep-sea organisms [7–9], and there is little information on the environmental applications.

Transplantation and caging experiments are largely used for monitoring the quality of the marine environment [10, 11]. However, monitoring programs are currently limited to shallow and coastal waters, and their application to the deep sea requires a different strategy, since the collection of deep-sea organisms remains difficult, uncertain and highly expensive.

*Corresponding author. Email: francois.galgani@ifremer.fr

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The pollutant impact on mussels transplanted at a depth of 650 m for 2 months has been previously reported [12]. In this case, no corrections were considered for the possible effect of pressure on the mussels. For deeper depths, only short immersions (less than a few hours) were carried out with mussels [1].

To our knowledge, the immersion of shallow-water species for longer periods has not yet been described. In this context, we wanted to test the survival capability of the mussel *Mytilus galloprovincialis* for more than 2 months in order to provide a scientific and technical basis for its possible use in deep-sea caging experiments. The more precise aims of the present work were twofold: (1) to investigate the tolerance of mussels to different immersion speeds at different depths (pressure) and exposure times and (2) to assess the physiological response of mussels immersed at different depths down to 1550 m depth. To do this, first we carried out laboratory experiments under artificial pressure conditions in hyperbaric chambers to assess the immersion speed for which animals were not affected by increasing pressure; and second, we carried out *in situ* immersions and evaluated mussel adaptation using physiological parameters. We also measured the accumulation of some chemical contaminants in a deep-sea contaminated area.

2. Materials and methods

In order to assess the optimal speed of immersion, for *in situ* immersion, 20–30 specimens of the mussel *Mytilus galloprovincialis* were maintained in a hyperbaric chamber (diameter 50 cm, 53 l of natural sea water covered with 20 l of shell morlina C10 oil). The chamber was placed in a closed thermostated steel box (10 °C) and subjected to increasing pressure from 3 to 120 m min^{-1} simulating immersions at 2000, 3000 and 4500 m depths for different times (15–120 h, table 1). Animal behaviour was monitored during the experiment using a camera and video records. A control experiment was performed for the long-duration experiment (No. 4) to avoid any interference due to natural mortality, which could be significant after more than 50 h in chamber.

In situ experiments were performed, submerging four lines at four different depths in the canyon of Cassidaigne (north-west Mediterranean sea, France; see figure 1). At each depth,

Table 1. Protocol for high-pressure simulation for mussel *Mytilus galloprovincialis* in hyperbaric conditions and mortality under different simulated hyperbaric conditions[a].

Experiment	1	2	3	4[b]	5
Date	27/11	28/11	30/11	01/12	06/12
Start hour	14:40	15:30	14:25	10:45	14:30
Number of mussels	23	26	23	16	24
Sinking/resurface speed (m min^{-1})	120	30	50	30	30
Depth in metres (bar)	2000 (204)	2000	3000 (308)	2000	4500 (465)
Time at bottom (h)	21	39	16	118	14
Mortality at end of experiment (%)	0	0	20	50 (50[b])	78
Live mussels after 24 h in sea water (%)	100	100	78	50 (50[b])	12
Live mussels after 6 d in sea water	100	100	78.25	100 (50[b])	0

[a]At the end of the experiments, live mussels were further maintained in cages in natural sea water (−1 m depth) for 24 h and 6 d.
[b]For this experiment and because of a long period of time, a control was used under the same conditions at atmospheric pressure to measure mortalities related to the decrease of available oxygen in the chamber. Please not the thermostated temperature of the chamber is in the same range than the temperature from natural conditions.

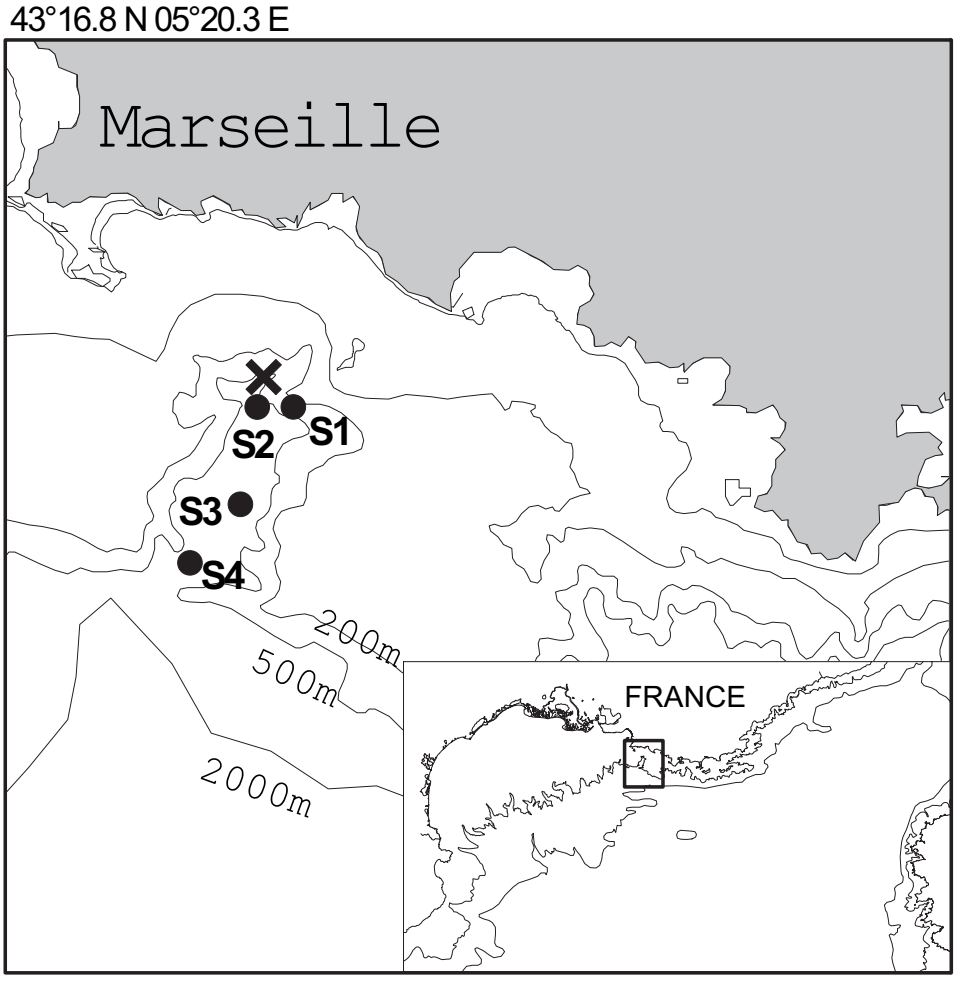

Figure 1. Locations selected for mussel caging (S1–S4) and position of the effluent output from a bauxite treatment plant (X).

two cages were deployed, 1 m above the bottom and at various heights above the bottom, thus enabling survival rates to be monitored at depths varying from 40 to 1550 m (table 2). The Cassidaigne canyon is situated close to the coastline where waste from an aluminium factory is discharged. This submerged outflow, located at a depth of 420 m (figure 1) has a current flow rate of approximately 300 tons/d (20 million tons of waste have been discharged since 1968) with a plume that can extend to more than 20 km at a depth of 2200 m. Prior to experimentation, the mussels (*Mytilus galloprovincialis*) were maintained for 15 d in 60 × 40 cm polyethylene

Table 2. *In situ* immersion scheme for caged mussels.

Station	Position	Depth (m)	Sinking speed (m min^{-1})	Cage number	Cage depth (m)
1	43° 07.9 N–5° 31.1 E	242	13	2	240/40
2	43° 07.8 N–5° 29.4 E	581	16	2	580/430
3	43° 05.2 N–5° 29.1 E	965	28	2	960/710
4	43° 01.5 N–5° 25.3 E	1552	41	2	1552/1300

Table 3. Basic physiological parameters in caged mussels *(Mytilus galloprovincialis)* after 69 d at different depths *in situ* (values given as mean ± S.D.)

Depth	Sample size (number of mussels in cage)	Dry weight/ wet weight	Dry weight/ shell length	Mortality (%)
Control	119	0.169 (0.020)	0.084 (0.012)	13.4
40	116	0.146 (0.027)	0.092 (0.014)	20.6
240	131	0.132 (0.032)	0.089 (0.011)	32.1
430	122	0.139 (0.026)	0.087 (0.014)	32.8
580	109	0.137 (0.023)	0.087 (0.009)	19.4
1300	118	0.142 (0.023)	0.092 (0.017)	38.9
1550	137	0.151 (0.022)	0.072 (0.018)	62.0

cages (109–137 per cage [11]) in a coastal lagoon (13–16 °C, saturated with O_2) in order to allow them to grip, then immersed at sea (starting on 27 May 2001) for 69 d using 8-mm-diameter polyethylene lines with a dead weight (80 kg), stabilized by buoys at mid-depth and subsurface (–50 m) and localized by two buoys (50 l) on the surface.

Immediately after recovery onboard, the samples were dissected and prepared for chemical analysis (21–26 individuals, 30 g of flesh per cage; table 3) or histology, or kept on the surface to measure the survival rates. The analyses mainly concerned the basic physiological parameters such as mortality and condition indexes (dry weight/length). Measurements of dry weight were performed after freeze-drying, and the maximum shell sizes (overall length) were measured with slide callipers. An ANOVA (means comparisons) was performed using Statistica (Statsoft Inc.). Histological observations were carried out on total samples after fixation with Davidson fixative and embedding with paraffin. An anterior transverse section was taken, including the digestive gland, mantle and gills. Histological sections (6 μm thick) were stained with haematoxylin (Herlich's haematoxylin) and eosin. Observations were carried out using a Zeiss optical microscope (Axioskop, ×450) to evaluate the effect of pressure on tissues and epithelia (condition, thickness and shapes).

After recovery, mussels (21–26 specimens per station) were washed with sea water, removed from their shell, ground, homogenized and freeze-dried according to standardized procedures [13]. After acid digestion, total chromium (Cr) and nickel (Ni) were analysed by graphite-furnace atomic absorption spectrometry (VARIAN, Model AA800) following the method described in Chiffoleau and Bonneau [14]. Mercury was determined directly for the freeze-dried sample in an automated mercury analyser (Altec, model AMA 254) according to the method described by Cossa *et al.* [15]. This protocol has been used for years in the context of national networks and enables to avoid any individual variability as standards are used to avoid any interference from the method or technical aspects. Results are expressed as micrograms per gram of dry weight.

3. Results

In order to test the tolerance of mussels to extreme conditions (increasing pressure), we measured, in preliminary experiments, the mortality rate of animals under artificial conditions of high pressure. Mussels were shown to tolerate high pressures (see table 1). All mussels were found alive after 19 h at a simulated depth of 2000 m and an immersion pressure rate of 120 m min^{-1}. Direct observations during the experiment demonstrated clear signs of stress, including emission of gametes. Nevertheless, they all survived and, after having been starved for several days in surface seawater, showed a clear recovery from stress just hours after the experiment, as indicated by their rapid production of byssus. After 6 d, all mussels were

alive and aggregated normally. At simulated depths of 3000 m (16 h) and 4500 m (14 h), 80 and 12%, respectively, of the organisms survived. In longer-duration (118 h) experiments, mortality rates were 50% at a depth of 2000 m. Inside the chamber, mussels showed byssus fixation on the polyethylene support after a few hours, indicating activity. Similar mortality rates were observed for control organisms subjected to the same experimental conditions, with the exception of increased pressure (*i.e.* kept at surface conditions). At the end of the experimentation, however, mussels that had undergone a simulated pressure of 2000 m showed a higher mortality rate after 6 d on the surface than the control mussels, indicating the stress they received from exposure to high pressure.

From these preliminary results, we defined a protocol for *in situ* immersion of mussel-containing cages: eight cages were immersed at 40 m and 1550 m depths from four lines. The lines were lowered at speeds of 13–43 m min^{-1}, enabling us to rule out the immersion-dependent mortalities. After 69 d of experimentation, only three lines were recovered at stations 1, 2 and 4. Mussels were alive in all cages. Mortality rates, although higher than for the controls, remained low down to 580 m depth but cannot be compared with mortalities from lab experiments performed solely to determine the effect of immersion speed. For cages deployed at 1300 and 1550 m depths, the mortality rates were higher (39 and 62%, respectively). From an ANOVA analysis (Fisher index $F = 5.60$), the parameters related to the general physiological state such as the percentage dry matter and the condition index (dry weight/maximum shell length) showed no statistical differences at $P < 0.01$.

The histological analysis performed on whole organisms showed differences only for the digestive gland. Although the data are not reported quantitatively, figure 2 shows

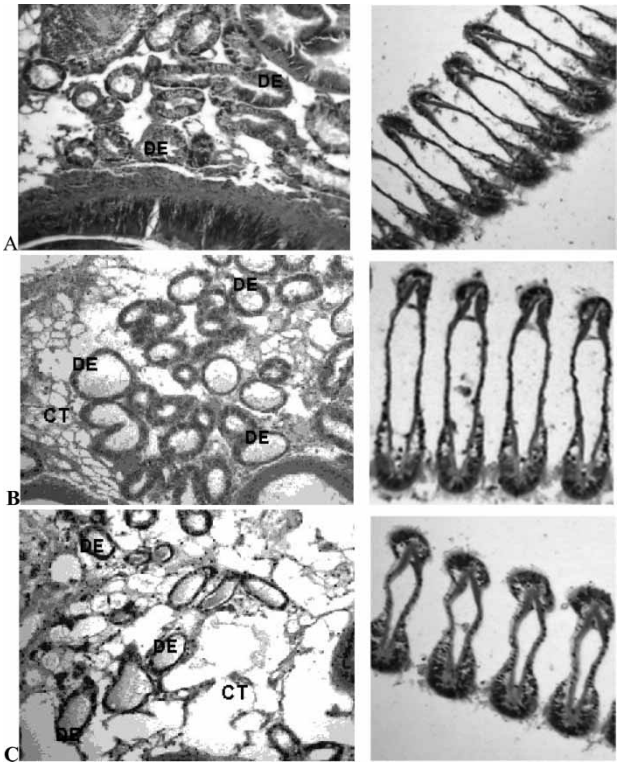

Figure 2. Histological sections (×450) of digestive glands (left) and gill filaments (right) from mussel (*Mytilus galloprovincialis*) caged at depths of (A) 40 m, (B) 430 m and (C) 1550 m. DE: digestive epithelium; CT: connective tissues.

Table 4. Concentrations ($\mu g\, g^{-1}$ dry weight) of chromium (Cr), nickel (Ni) and mercury (Hg) in caged mussels at different depths[a].

Depth	Number of mussels	Station 1			Station 2			Station 4		
		Cr	Ni	Hg	Cr	Ni	Hg	Cr	Ni	Hg
Control[b]	24	0.04	0.76	0.040						
Line 1										
40 (open water)	21	0.6	1.15	0.061						
240 (bottom)	26	0.48	1.10	0.054						
Line 2										
430 (open water)	22				15.8	1.11	0.056			
580 (bottom)	26				27.4	1.41	0.045			
Line 4										
1300 (open water)	26							8.0	1.0	0.052
1550 (bottom)	25							9.8	0.82	0.045

[a]Results are expressed as an average value of contaminants measured in pools of some of the live samples after recovery (column 2). It should be noted that the output of aluminium residues from a factory is located at 420 m (see figure 1).
[b]The control was mussels starved for 15 d at 2 m depth in a coastal lagoon. For each line (each site), two cages were sampled: one on the bottom and one on the same line in open water.

photomicrographs of the digestive gland from mussels immersed at 50, 430 and 1550 m depths. At 1550 m, the connective tissue was locally destroyed. Haemalun-eosin colouring revealed important differences in relation to depth in the thickness of the digestive epithelium (DE) bordering the lumen of the digestive diverticuli. In contrast, no differences were observed in mantle and gills (figure 2, right).

Mercury, which is not a component of bauxite, was found at concentrations varying from 0.040 to 0.061 $\mu g\, g^{-1}$ for controls and mussels immersed at 40 m. The lowest concentrations were observed in the deepest samples. The nickel concentration did not change notably, ranging from 0.76 to 0.82 $\mu g\, g^{-1}$ (control and 1500 m depth, on the bottom), and reaching 1.41 $\mu g\, g^{-1}$ at 500 m depth, on the bottom. However, the analysis of chromium (table 4) was chosen because of its importance as a main component of bauxite residues. Concentrations were low in controls (0.04 weak $\mu g\, g^{-1}$) and still in mussels immersed at 240 m on the bottom (0.48 $\mu g\, g^{-1}$) and 42 m depth (0.6 $\mu g\, g^{-1}$). Close to outflow of bauxite waste effluent (420 m depth) were found the highest concentrations in the mussels (27.4 $\mu g\, g^{-1}$ at 580 m depth on the bottom) and at 430 m in open water (15.8 $mg\, kg^{-1}$). Caged mussels from the deepest station (station 4) had chromium levels of 9.8 $mg\, kg^{-1}$ (1550 m) and 8 $mg\, kg^{-1}$ (1300 m).

4. Discussion and conclusions

We have demonstrated that the common mussel (*Mytilus galloprovincialis)* is able to survive for more than 2 months (69 d) at 1550 m depth. Preliminary experiments in hyperbaric chambers have also demonstrated that these organisms are tolerant to rapid changes in pressure. These results are not surprising. For invertebrates, 50% of living organisms have been observed after 12 h at 520 bar (*Arenicola marina*), 750 bar (*Littorina littorea*) and 800 bar (*Mytilus edulis*) [16]. Moreover, mussels have been kept *in situ* for 60 min at 2227 m depth with 100% survival [17]. However, our experiments provide additional information: high speeds of immersion ($120\, m\, min^{-1}$) were tolerated without increasing the mortality in spite of clear stress signs (emission of gametes); moreover, follow-up experiments have indicated that mussels have an apparently normal activity with the valve opened and byssus production. Survival rates were very good in surface waters after simulated short-duration immersion. Under these conditions, *in situ* immersion of cages at high speed without intermediate steps ($40\, m\, min^{-1}$) is possible.

These operating conditions are possible down to a depth of 1550 m. Under these conditions, because of the survival rate (38%), a significant number of specimens could be recovered. Our results must be viewed in a particular environmental context: the Mediterranean water temperature varied very little from depths of 1550 to 50 m in the area studied [18]. Therefore, any deep-sea caging in Mediterranean waters will be performed only within a temperature range higher than the surface winter averages encountered in the usual natural environment of *Mytilus galloprovincialis.* In addition, under surface conditions, 2 months of caging does not alter the physiological conditions [11]. We think that under these conditions, experimentation over 69 d enables to obtain relatively unstressed organisms from a physiological point of view (as shown by the condition indexes). Observation of the histological structures revealed a few alterations. Using mussel gills in a hyperbaric chamber, the ciliairy activity, measured *in vitro*, did not decrease below a simulated depth of 4000 m [3]. From the depth experiments reported here, mussels have been shown to adapt to the different depths, meaning that the gills can function normally, even though the oxygen concentration decreases with depth. Previous results [6, 19] have shown that the metabolic rates of fish inhabiting shallow depths are much higher and decrease with increasing capture depths and, thus, with decreasing temperatures. However, Roer and Pequeux [20] noted that there might be little or no difference between the metabolic rates of the deep and shallow species in relation to depth when metabolism is corrected for temperature effects. Using crustaceans as model species, results from various authors [21] suggest that the typical pattern for animals living in deeper environments may be that metabolic rates on average vary as a function of depth due primarily to the effects of temperature on locomotion. This is not the case with sessile organisms such as mussels. By using deep-sea or shallow-water fish exposed to high pressure, a variety of evidence, largely based on similar counteracting effects of temperature and pressure on behavioural, cellular and membrane phenomena [4, 22, 23], suggests that the pressure-tolerance limits of organisms are determined by the effects on membrane functions. The results concerning the digestive structures of the mussels immersed at depths of 430 m and 1500 m is for this reason of interest with a clear reduction in thickness of the digestive epithelium with depth. The homogeneity of the condition index values indicates that a good food assimilation is likely. Therefore, mussels are often very slow in showing changes in condition index, and histological alterations might appear earlier than changes in bulk composition; also, a reduction in the thickness of the epitheliums could be linked to increased pressure, rather than to feeding conditions. We hypothesize that *Mytilus galloprovincialis* is able to feed by filtration on dissolved organic matter and aggregates. These aggregates (marine snow) are made up partly of phytoplankton debris, zooplankton bacteria and heterotrophic organisms [24, 25] which are abundant in deep coastal areas. Together with bottom currents transporting large quantities of food, sediments and particulate organic carbon, aggregates may be major transport agents for surface-derived organic production in the deep sea [26, 27], acting as a major food component in the context of our experiment.

All observations indicated the potential use of the caging technique for physiological studies in the deep sea. In particular, contaminant accumulation and biomarkers can be monitored. This objective led Foerlin *et al.* [12] to carry out caging experiments in a Norwegian trench where mussels (*Mytilus edulis*) had been maintained successfully for 2 months at 650 m. We demonstrated that this technique can be used at greater depths. In this context, we were also able to investigate the accumulation of chromium. The accumulation of trace metals, including chromium, has been described in mussels [10, 28, 29]. In our study, the likely source is the effluent outlet located at 420 m depth in the area of experimentation. It comprises mainly arsenic, titanium, aluminium, chromium, iron and manganese [30]. Near the outlet, located slightly upstream of station 2, the concentrations reach 1815 $\mu g\, g^{-1}$ (dry weight) of effluent, and the chromium concentrations in the sediments close to stations 1 and 4 are 106 and

601 $\mu g\, g^{-1}$ of dry sediment, respectively (IFREMER, unpublished data). There is no doubt that these values would explain the concentrations measured in the mussels maintained at the bottom downstream of the outlet (station 2, 580 m and station 4, 1550 m) with higher levels for the station close to the outlet. These mussels are located very close (1–2 m) to neighbouring sediments. In addition, although Station 4 is more than 10 km from the output, the sediments there have been shown to be covered by red mud. The lower concentrations measured on the samples located 150 m above the sediments (−430 m at station 2) and 250 m above the sediments (−1300 m at station 4) also demonstrate the vertical transport of sediments to open water. In addition, the mortality rates observed in the mussels most exposed (station 2, 580 m) to the effluent indicate that there was no link between mortality and chemical contamination of the site.

From a more general point of view, with the method used in our preliminary experiment, physiological adaptation to deep-sea conditions can be studied, using an original approach, and physiological, ecological and ecotoxicological measurements can be made. Original experimentation schemes could also be included, such as three-dimensional (depth, latitude and longitude) screenings that can yield valuable information on natural flows and flows of contaminants, even after accidental disturbance events.

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