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Respiration of bivalves from three different deep-sea areas: cold seeps, hydrothermal vents and organic carbon-rich sediments

Khripounoff Alexis [1, *], Caprais Jean-Claude [1], Decker Carole [1], Le Bruhec J [1], Noel Philippe [1], Husson Berengere [1]

[1] IFREMER/ Centre de Brest, Département REM/EEP/LEP, CS 10070, 29280 Plouzané-France

* Corresponding author : Alexis Khripounoff, email address : alexis.khripounoff@ifremer.fr

Abstract :

We studied bivalves (vesicomyids and mytilids) inhabiting four different areas of high sulfide and methane production: 1) in the Gulf of Guinea, two pockmarks (650 m and 3150 m depth) and one site rich in organic sediments in the deepest zone (4950 m average depth), 2) at the Azores Triple Junction on the Mid-Atlantic Ridge, one hydrothermal site (Lucky Strike vent field, 1700 m depth). Two types of Calmar benthic chambers were deployed, either directly set into the sediment (standard Calmar chamber) or fitted with a tank to isolate organisms from the sediment (modified Calmar chamber), to assess gas and solute exchanges in relation to bivalve bed metabolism. Fluxes of oxygen, total carbon dioxide, ammonium and methane were measured. At the site with organic-rich sediments, oxygen consumption by clams measured in situ with the standard benthic chamber was variable (1.3-6.7 mmol m−2 h−1) as was total carbon dioxide production (1-9.6 mmol m−2 h−1). The observed gas and solute fluxes were attributed primarily to bivalve respiration (vesicomyids or mytilids), but microbial and geochemical processes in the sediment may be also responsible for some of variations in the deepest stations. The respiration rate of isolated vesicomyids (16.1-.25.7 µmol g−1dry weight h−1) was always lower than that of mytilids (33 µmol g−1dry weight h−1). This difference was attributed to the presence of a commensal scaleworm in the mytilids. The respiratory coefficient (QR) ≥1 indicated high levels of anaerobic metabolism. The O:N index ranged from 5 to 25, confirming that vesicomyids and mytilids, living in symbiosis with bacteria, have a protein-based food diet.

Keywords : Deep-sea ; Benthic chamber ; Vesicomyid and Mytilid bivalves ; Respiration rate ; Cold seep ; Hydrothermal vent

Introduction

In hydrothermal sites, cold seeps or areas rich in organic carbon, high fluxes of methane and sulfide support chemoautotrophic free-living and symbiotic bacteria. These fluxes provide the basis for complex microbial and metazoan communities. Many biological studies in these particular habitats have focused on the distribution, structure, nutrition, and food web architecture of faunal communities as well as on their interaction with the geochemistry of their environment. However, the metabolism of these organisms is poorly documented. Bivalves are one of the most abundant chemosynthetic organisms inhabiting deep-sea reducing ecosystems with production of methane and sulfur (Lutz and Kennish, 1993; Sibuet and Olu, 1998; Fiala-Médioni et al., 2002; Duperon et al., 2005; Cosel and Olu; 2009). Vesicomyids and mytilids at these sites feed via symbiotic sulfide-oxidizing bacteria that inhabit bacteriocytes in their gills (Fiala-Médioni and Le Pennec, 1987). The vesicomyids provides its symbionts with sulfides taken up by its foot buried in the sulfide-rich sediments and mytilids directly uptaken via gill-associated symbionts. Oxygen and carbon dioxide are absorbed from the seawater that flows through the bivalve siphons above the sediment (Arp and Childress, 1983; Childress et al, 1984; Roeselers and Newton, 2012).

To study the necessary physiological requirements for deep-sea bivalve life, oxygen consumption and carbon and nitrogen excretion rates represent the gains and losses of energy associated with metabolism. The physiological responses of vesicomyids or mytilids to changes in the environment are extremely variable (Widdows et al., 1984; Tedengren et al., 1990; Navarro and Gonzalez, 1998). Bivalve-bacterial associations can thrive in sulfide-rich environments, surviving on the oxidation of sulfide. Bivalves uptake both sulfide and oxygen which do not normally exist together in water column due to rapid oxidation of sulfide. They are assumed to be involved in numerous interactions with the surrounding environment and organisms due to their size and abundance, (Olu et al, 2007; Marcon et al, 2014).

Research directed at studying the unusual physiological adaptations of these animals is necessary to understand how these bivalves can live in such hostile environments, largely unhospitable to other animals. Studies on oxygen consumption rates have been performed using *in situ* measurements on hydrothermal vent mussels (Smith Jr., 1985) and other deep-sea habitats maintained under *in situ* pressure conditions (Arp et al., 1984; Childress and Mickel, 1982, 1985; Henry et al, 2008). In these experiments, observed oxygen consumption rates are generally as high as those measured in shallow-water mussels.

The objective of our study was to estimate *in situ* the respiratory rate of vesicomyids and mytilids at four different deep-sea sites in the Gulf of Guinea and on the Mid-Atlantic Ridge. Measurements of oxygen, total dissolved inorganic carbon, methane and ammonium were obtained *in situ* using two different benthic chambers and were assessed along with vesicomyids or mytilids density and biomass. The results were examined according to the type of benthic chamber used, explored sites and bivalve species.

Materials and Methods

Study area

The study was carried out in three sites in the Gulf of Guinea explored during the WACS cruise (February 2011), the CongoLobe cruise (December 2011-January 2012) and in one site on the Mid-Atlantic Ridge in the Lucky Strike vent field during the Momarsat 2015 cruise (April 2015) on the R/V *Pourquoi Pas?*. All the deployments of Calmar were realized using the ROV *Victor*.

The first site in the Gulf of Guinea (Fig. 1) is an organic carbon-rich area on the distal lobe complex of the Congo deep-sea fan. This area is characterized by brown sediment with several “black pools” composed of a surface of reduced black sediment. This site likely receives large terrestrial organic inputs from the African continent, transported via the Congo

89 submarine canyon system (Khripounoff et al., 2003; Vangriesheim et al, 2009). Four stations
90 (A, B, F and C) were sampled in this area from 4750 to 5070 m depth (Figure 1). A distinctive
91 ecosystem is associated with black pools and is characterized by a biological community that
92 resembles those observed on pockmarks with microbial mats and vesicomyid bivalves
93 (dominant species: *Christineconcha regab* and *Abyssogena southwardae*, Krylova et al.
94 2010). The three organic-rich stations (Fig. 1) were located in the main deposition zone of the
95 Congo canyon along the track of the micro-channel that funnels the turbidity material to the
96 terminal lobes except Station E, which was out of the turbidity input and was chosen as a
97 deep-sea reference station.
98 The second site (Fig. 1), called Regab, is a giant pockmark 800 m in diameter at 3150 m
99 depth, along the Congo margin (Olu-Le Roy et al. 2007). It is characterized by high habitat
100 heterogeneity, with assemblages of the three major symbiont-bearing taxa encountered at
101 cold-seeps: Vesicomyid bivalves (dominant species: *C. regab*), Mytilid bivalves
102 (*Bathymodiolus* aff. *boomerang*) and Siboglinid polychaetes (*Escarpia southwardae*), as well
103 as microbial mats.
104 The third site, called Guiness (Fig. 1), includes several small, less active pockmarks, from 580
105 to 690 m depth. The major benthic taxon observed was vesicomyids (*Calyptogena valdiviae,*
106 and *Elenaconcha guiness*, Cosel & Olu 2009). This pockmark is characterized by patchy
107 vesicomyid beds associated with microbial mats.
108 The fourth site was the hydrothermal Lucky Strike vent field (1700 m average depth) at the
109 Azores Triple Junction (ATJ) on the Mid-Atlantic Ridge. This area consists of a large central
110 lava lake surrounded by three volcanic cones with several translucent smokers. Mytilid
111 bivalves *Bathymodiolus azoricus* are the dominant megafaunal species and are distributed in
112 patches of thousands of individuals (Desbruyères et al., 2001).
113

114 *Calmar benthic chambers*

115 To assess the *in situ* metabolism community of bivalves, the Calmar benthic chamber
116 (Caprais et al, 2010) was deployed by the ROV *Victor 6000*. Basically, Calmar is a 41 cm
117 diameter cylinder that is open at one end. The Calmar unit weighs 14 kg in water and it is
118 equipped with six 100 ml sampling cells, an oxygen probe (Aadi, Norway) and a stirrer to
119 homogenize the water in the chamber. The position of the sampling cells under the Calmar
120 chamber and their closure mechanism preclude any suction and infiltration of uncontrolled
121 water movement in the sealed Calmar chamber (Caprais et al. 2010). During the standard
122 Calmar experiments (Fig. 2a), the incubation of bivalves directly on the sediment lasted for
123 about 3 h. At each station, a ring of 50 cm in diameter was deployed to guide the Calmar
124 positioning. The standard Calmar measured exchanges between water and bivalves lodged in
125 the sediment was deployed two times during the WACS cruise on mytilid beds and three
126 times during the CongoLobe cruise on vesicomyid beds. Two standard Calmars were also
127 used to measure flux exchanges on sediment without bivalves and represent background
128 references in this study. Measurements on undisturbed *B. azoricus* beds with standard Calmar
129 were not possible because the chimney walls do not allow such measurements.
130 The respiration rate of isolated bivalves was measured *in situ* using a modified Calmar
131 chamber fitted with a specific tank (Fig. 2b) (Khripounoff et al.; 2014). It was deployed by the
132 ROV at all four sites: Lobe, Regab, Guiness and Lucky Strike (Table 1). At the beginning of
133 the experiment, bivalve shells were sampled on the bottom by the ROV with a net or with the
134 ROV arm and dropped into the cylindrical tank. Then, after about 1 h of stabilization, the
135 Calmar unit was placed over this tank, immersing the bivalves in exactly 31 l of bottom water
136 (Fig. 2b). The incubation then started and lasted for 3 h *in situ* on the bottom, close to the
137 sampling area. The modified Calmar chamber fitted with a tank was used to incubate 7 shells
138 of *C. regab* at the Lobe station, 17 shells of *C. regab* at the Regab station, 21 shells of *E.*

guiness at the Guiness station and 24, 19 and 170 shells of *B. azoricus* at the Lucky Strike vent field.

Size, weight, density, biomass and elemental composition of vesicomyids and mytilids

After each experiment with the benthic chamber (standard or modified Calmar), the sampled bivalves were identified, washed and frozen (samples incubated with the modified Calmar) or fixed in 4% buffered formalin (samples incubated with the standard Calmar). In the laboratory, shells were measured and dry weight (dw) of bivalves was determined after the body, excised from the shell, had been dried for 24 h at 60 C. *B. azoricus* mussels generally have a commensal polychaete, *Branchipolynoe seepensis*. The biomass of each species was measured separately in each shell. Total carbon and sulfur concentrations of dried total body were analyzed with a Leco CS 125 (USA) elemental analyzer.

The *in situ* bivalve density under the standard Calmar was estimated with two methods using photos and quantitative core samples (Decker et al., 2012). First, a ring of 50 cm diameter was deployed on a bivalve bed and photos of this circle were taken by the ROV to estimate bivalve density before benthic chamber deployment (Khripounoff et al., 2015). Then, after the recovery of Calmar at the end of incubation, a blade core (0.036 m^2) was taken from within the ringed off area. Mean density was estimated from the average of these two measurements taken at each Calmar deployment. For each standard Calmar deployment, mean of biomass (g dw m^{-2}) were calculated using the mean density, estimated from photos and blade cores, and mean individual tissue dry weight calculated with the bivalves sampled with core. Only the living animals were taken into account to calculate de density and biomass of bivalves sampled with the blade corer. But it is not possible to discriminate dead and living animals in the photos.

Analytical methods and flux calculation

Oxygen was measured *in situ* with an electrochemical sensor optode (Aadi). To calibrate the oxygen optode, oxygen concentration was also analyzed in triplicate aliquots on 10 mL water sampled in each cell using the modified Winkler titration method (Carrit et al. 1996). Total dissolved inorganic carbon (DIC) was determined using an infrared gas analyzer (Kaltin et al. 2005). Methane was analyzed by gas chromatography coupled with a head-space sampler (GC/HSS) (Sarradin and Caprais 1996) in 5 mL of water in 10 mL vials; precision was less than 4%. Each chemical analysis was done in triplicate for each sampling cell. Ammonium was analyzed using a manual fluorimetric technique (Holmes et al., 1999).

The fluxes were calculated for the standard Calmar from the slopes of the linear regressions of oxygen, ammonia, ΣCO_2, concentrations:

Total flux ($\mu mol\ m^{-2}\ h^{-1}$) = S (V/A) where S is the slope of the linear regression of gas or solute analysis, V is the chamber volume and A, the surface area of the chamber.

The respiratory coefficient (RQ) was calculated as $\Sigma CO_2/O_2$ and the O:N index was calculated according to their atomic equivalents.

Bivalve respiration rate using the modified Calmar (in $\mu mol\ g^{-1}\ h^{-1}$) was calculated using the following expression: $B^{-1}[Vt^{-1}\ (C_2-C_1)]$ where B is the bivalve dry weight (g dw); C_1 and C_2, gas or solute concentration in the Calmar with bivalves at time 1 and 2 ($\mu mol\ L^{-1}$); V, the volume of water under the Calmar chamber (l); and t, the time interval between measurements (h).

For the numerical treatment of oxygen consumption and body size data, the relationship between whole animal oxygen consumption and body weight can be described by the following equation (1): oxygen consumption = aW^b (Von Bertalanffy, 1957), where W is the body weight. The relationship between weight-specific oxygen consumption R and body weight can therefore be expressed as $R = aW^{b-1}$ (2). Since the value of (b) in equation 1

189 normally has a value of < 1, the value of (b- 1) in equation 2 will have a negative value (Von
190 Bertalanffy, 1957).
191
192 **Results**
193 *Bivalve characteristics*
194 During the incubation with the standard Calmar on the sediment, bivalves dominated the
195 megafauna biomass at each experiment. A comparison of the two methods used to calculate
196 the bivalve density (except for the mytilids at the Regab station where only photos were used)
197 showed that the results obtained with the corer were higher by factor 2-3 of those obtained by
198 counts based on the photos (Table 2). At the Lobe and Regab sites, colonized by *C. regab*,
199 vesicomyid biomass ranged from 157 to 1066 g dw m^{-2}. The biomass of mytilids at Regab
200 was low (75-90 g dw m^{-2}).
201 The bivalve characteristics in the incubation experiments with the standard Calmar and the
202 modified Calmar-tank are summarized in Fig 3. With the standard Calmar at Lobe, we
203 observed a minor difference in *C. regab* weight between Lobe B and Lobe A. The mytilid *B.*
204 *boomerang* at Regab was the bigger (mean=150 ± 15.4 mm) of the bivalve species used in
205 this experiment. With the modified Calmar-tank at Regab, individual main length and dry
206 weight of *C. regab* was higher than that observed at Lobe, by a factor of two: respectively
207 91.2 ± 7.7 and 54.9 ± 5.9 mm for length and 4.52 ± 0.9 and 1.47 ± 0.4 g dw for mass. The size
208 of *E. guiness* shells at Guinness was more similar to that of *C. regab* at Lobe (54.2 ± 5.9 mm)
209 (Fig. 3). The mussels at the Lucky Strike hydrothermal vent were the smallest bivalves used
210 in this study with individual length ranging from 12 to 72 mm and dry weight mass ranging
211 from 0.005 to 2.2 g dw. *B. azoricus* features the presence of a commensal scaleworm,
212 *B. seepensis*, in almost all living shells. The weight of the polychaete varied from 0.001 to
213 0.15 g dw and represented about 10% of the mussel weight in each shell.

The elemental composition (Table 3) of two vesicomyid species and the two mytilid species showed similar concentrations of carbon (38.5 to 45.6%). However, we observed a large difference in tissue sulfur concentration between *E. guiness* (5.1%) and *C. regab* (12.7 to 17.2 %) and between *C. regab* (Lobe= 12.7 %) and *C. regab* (Regab = 16.0), (Kruskal-Wallis test, $p<0.01$ and non-parametric multiple test). *B. boomerang* and *B. azoricus* mytilids were characterized by a very low concentration of sulfur (1.2-1.5%) (Table 3).

In situ *exchanges of gases and solutes at the water-sediment interface under the standard Calmar chamber*

The total oxygen uptake (TOU), measured with the standard Calmar on vesicomyids or mytilids lodged on sediment, was between 1.29 and 6.71 mmol O_2 m^{-2} h^{-1} (Table 4). The minimum O_2 consumption was observed in the mytilid population and the maximum at the Lobe A station. The two TOU references measured close to the vesicomyid bed at Lobe A and C show a low sediment metabolism of 0.3-0.4 mmol O_2 m^{-2} h^{-1}. The flux of ΣCO_2 ranged from 1.0 (Regab) to 9.6 (Lobe A) mmol CO_2 m^{-2} h^{-1}. During incubation, NH_4^+ concentration increased significantly, indicating measurable ammonium production by organisms living in the sediment. The production rate observed under the Calmar chamber ranged from 0.1 (Regab) to 0.24 (Lobe A) mmol NH_4^+ m^{-2} h^{-1}. CH_4 diffusion was always detected at the Regab and Lobe sites with the standard Calmar, varying from 0.35 (Lobe B) to 4.5 mmol m^{-2} h^{-1} (Lobe A). CH_4 flux can be observed also at the Lobe C reference (0.1 mmol m^{-2} h^{-} 1). No significant sulfide flux was detected under any Calmar chamber; the detection level of our analytical method was >20 mmol/L. All these results are summarized in Table 4.

Respiration rate of vesicomyids and mytilids under the standard and modified Calmar chambers

We first assumed that the metabolism of bivalves (host and symbionts or commensals), in the experiments using the standard Calmar, was responsible for the main exchange at the interface water-sediment. Based on this hypothesis, the bivalve respiration rate was calculated in terms of bivalve dry weight (Table 5) on the estimated biomass under each Calmar chamber (Table 2). Across the different stations, vesicomyid oxygen consumption varied from 6.2 µmol O_2 g^{-1} dw h^{-1} (Lobe A and F) to 11.2 µmol O_2 g^{-1} dw h^{-1} (Lobe B). Mussel oxygen respiration rate was about 16 µmol O_2 g^{-1} dw h^{-1} (Table 5). Assuming that bivalves produced the majority of ΣCO_2, mean production was about 10 µmol CO_2 g^{-1} dw h^{-1} and the RQ ranged from 0.8 to 1.5. During incubation, the bivalve excretion was certainly the main factor contributing to the ammonium production and it was about 0.56 µmol NH_4^+ g^{-1} dw h^{-1}. The maximum ammonium rate was observed in the mussel bed (0.83 µmol NH_4^+ g^{-1} dw h^{-1}) (Table 5). The mean O:N ratio obtained with vesicomyids was 7.4 ± 4.6 and 24.4 with mytilids.

Under the modified Calmar-tank, only the respiration rate of isolated bivalves and their symbionts was measured, i.e. without the interaction of any other organism or any chemical exchanges from the sediment. For vesicomyids, the *E. guiness* respiration rate in the shallower Guiness station was greater (25.7 µmol O_2 g^{-1} dw h^{-1}) than that of *C. regab* sampled at Regab or Lobe (about 17 µmol O_2 g^{-1} dw h^{-1}). The oxygen consumption rate of *C. regab* varied very little between the Regab and Lobe stations. However, the biomass and the mean size of individuals used in the modified Calmar-tank were higher at Regab than at Lobe (Fig. 3). The mytilids at the hydrothermal vent smaller than 0.3 cm in length had the highest respiration rate observed in this study (33.6 µmol O_2 g^{-1} dw h^{-1}). The respiration of larger *B. azoricus* was lower (about 20 µmol O_2 g^{-1} dw h^{-1}) and it was comparable to the respiration of similarly sized vesicomyids. The bivalve ΣCO_2 production ranged from 16 (Regab) to 28 (Guiness) µmol CO_2 g^{-1} dw h^{-1} and the RQ was close to 1. During incubation, NH_4^+

concentration always increased during the experiment with the modified Calmar-tank. The measured excretion rate ranged from 0.3 (*B. azoricus*) to 1.25 (*C. regab*) µmol NH_4^+ g^{-1} dw h^{-1}. The O:N ratio was clearly lower than under the standard Calmar with vesicomyids.

Relationship between oxygen consumption and body weight

In the present study, it was only possible to determine the relationship between oxygen consumption and body weight for small bivalves between 0.03 to 4 g dw. Only clams from Regab exceeded 2 g dw. The relationship between oxygen consumption and body weight of bivalves obtained during the present study is

$R = 21.3\ W^{-0.16}$ for vesicomyids and $R = 21.2\ W^{-0.19}$ for mytilids (Fig. 4).

Discussion

General features of bivalves

Bivalves (vesicomyids and mytilids) are widespread in chemosynthetic ecosystems, and occur at most known cold-seep sites (Krylova and Sahling, 2010) and hydrothermal vents (Desbruyères et al., 2001). Our results show variation in density and mean individual biomass in the Gulf of Guinea and in the ATJ. The hydrothermal mytilids at the Lucky Strike site were small and the distribution around the chimney was patchy. In the Gulf of Guinea, we observed some patterns in vesicomyids sampled from different areas (Fig. 3). The large majority of *C. regab* specimens were particularly small at all Lobe sites compared with the same species at Regab sites. This difference suggests that vesicomyid population at Lobe consisted mostly of young or dwarf individuals. However, the presence of only one or two cohorts at Lobe (personal data) suggests a young population or the stunted growth hypothesis. The elemental composition of vesicomyid tissues was characterized by high differences in sulfur concentrations between *E. guiness* and *C. regab* (Table 3). The symbionts of vesicomyid clam

tend to accumulate elemental sulfur while the mussel symbionts do not. These differences may be due to interspecific variation in sulfide physiology, e.g. divergent patterns in sulfide exploitation and storage, as previously observed in both species in Monterey Bay (Goffredi and Barry 2002). Elemental sulfur is known to be an energy storage product for sulfide-oxidizing symbiotic bacteria (Vetter, 1985). These reserves provide fuel for symbiotic bacteria metabolism, even in the absence of a sulfide supply (Vetter et al. 1985, Fisher et al. 1988, Fiala-Médioni and Lepennec, 1989, Goffredi and Barry, 2002). The higher sulfur storage capacity in *C regab* is also a mechanism that can facilitate the adaptation to the fluctuations in sulfide fluxes that occur in these habitats.

Effects of bivalves on transfers at the water-sediment interface

The role of vesicomyids or mytilids in gas exchanges at the water-sediment interface has not been studied as well as their distribution in cold seeps. However, they were the main contributor to benthic respiration at the different stations studied in the Gulf of Guinea. Our values of TOU varied from 1.3 to 6.7 mmol O_2 m^{-2} h^{-1} and similar measurements have been previously obtained at these cold-seep sites. For example, *in situ* TOU measured at the Regab cold-seep on clam beds varies between 2.1 and 24.6 mmol O_2 m^{-2} h^{-1} (Pop Ristova et al., 2012), 13.8 and 20.5 mmol O_2 m^{-2} h^{-1} (Decker et al., 2012) and 11.3 and 18.0 mmol O_2 m^{-2} h^{-1} (Khripounoff et al., 2015). TOU rates ranging from 1.3 to 6.7 mmol O_2 m^{-2} h^{-1} have been measured in different habitats outside of the Gulf of Guinea, on the Napoli mud volcano in Mediterranean sea (Caprais et al., 2010), on the Hakon Mosby mud volcano (Felden et al., 2010) and at the Hikurangi margin (Sommer et al., 2010). ΣCO_2 fluxes, ranging from 1 to 9.6 mmol m^{-2} h^{-1}, are not only produced by clams, mussels and metazoan respiration, but also by the oxidation of methane by archea (Boetius et al., 2013). Bacterial activity is responsible also for the degradation of organic matter. Conversely, CO_2 can be consumed by methanogenic

bacteria (Whiticar, 1999). The consequence of the -time-lag between oxygen consumption and CO_2 production is high variation in the RQ value. The RQ value is generally equal to 0.75 for various marine invertebrate species (Koopmans (2010), but it ranged from 0.8 to 1.2 in this study (Table 4). In this case, high RQ values may be due to an increase in anaerobic mineralization of organic matter and anaerobic oxidation of methane (AeOM). The benthic chamber, which accumulates carbon during incubation, can *in situ* artificially accelerate the process of carbonate precipitation (Aloisi et al., 2002). Our RQ values were greater than those obtained by Khripounoff et al. (2015) at the same stations (0.6). This discrepancy is not easily explained.

The excretion of ammonium is due to protein breakdown and its flux measured under the Calmar chamber can be attributed almost exclusively to vesicomyids, like O_2 and ΣCO_2 fluxes. The excretion of bivalves was on average to 0.17 mmol $NH4^+$ m^{-2} h^{-1} and this value is lower than the values (0.37 mmol $NH4^+$ m^{-2} h^{-1}) reported by Khripounoff et al. (2015). Energy availability, in the form of methane, sulfide and oxygen fluxes, and the type of seafloor substrate play a central role in the composition and distribution of cold-seep faunal communities at local and regional spatial scales (Olu-Le Roy et al., 2007; Ritt et al., 2011; Decker et al., 2012; Fischer et al., 2012).

Respiratory rate of bivalve beds on sediment

Our approach using the Calmar chamber depicts the metabolic function of vesicomyids or mytilids based on their integrated response at rest without manipulation. The first assumption is to consider that the observed oxygen consumption is essentially due to global clam or mytilid respiration (i.e. host and symbionts). The respiratory rate of vesicomyids and mytilids was estimated at 7.9 and 16 µmol O_2 g^{-1} dw h^{-1}, respectively (Table. 5). However, this estimation requires the exact biomass of bivalves under the standard Calmar, leading to

uncertainty in the estimated respiration values. Regardless of these limitations, our results show vesicomyid oxygen consumption rates that are roughly half those that were measured in Khripounoff et al. (2015) and Decker et al. (2012) using the same equipment at the same stations. But given the uncertainties in estimation of bivalve biomass, that is more or less within the expected error range. The proportion of oxygen, consumed by other reactions, such as the respiration of small benthic organisms (macrofauna, meiofauna, and microfauna), a variety of methanotrophic archea, nitrification or chemical oxidation was estimated using the Calmar reference deployed at Lobe A and C (Table 4). It was about 10 % of the total TOU measured under standard Calmar with clams. But this value is given for information only because the heterogeneity of sediment at a scale of few cm does not ensure the good representation of selected areas as reference. Thus, the intensity of these oxygen consumptions in the sediment represents additional sources of uncertainty. The ratio between macrofauna and symbiotic bivalve biomass is low in cold seeps (Sahling et al. 2002; Decker et al., 2012) and therefore, macrofaunal metabolism is likely negligible for the estimation of respiration in this study. The contribution of AeOM and sulfide oxidation by bacteria to the total oxygen consumption is unknown and may vary with the intensity of fluid flow and related microbial and faunal colonization (Boetius and Wenzhofer, 2013). In our case, there was no bacterial mat under the standard Calmar with the clams and the thickness of the oxidized sediment was less than 1 mm, thereby limiting the possibility of AeOM activity (Sommer et al., 2006). Bivalve communities were an oxygen sink, but not to an extent that would result in insufficient re-oxidation and stimulation of methane, manganese and iron efflux compared with bare sediments. However, we cannot exclude the possibility that bivalve communities had increased respiration and bio-deposition and thus stimulated anaerobic processes (Zaiko et al. 2010). In this study, high CO_2 rates with high RQ values (1.5) are due to anaerobic

363 metabolism, which was greater than during the previous study at the same area (Khripounoff
364 et al., 2015) and demonstrate the heterogeneity and the diversity of sediment at small scales.
365 Atomic O:N ratios are related to the availability of energy stores and the utilization of body
366 proteins (Shumway and Newell, 1984). This ratio produces an index of the relative amounts
367 of protein, compared with carbohydrates and lipids that are catabolized by the organism. O:N
368 ratios provide meaningful data on the metabolic state of an organism. When only protein is
369 metabolized, the O:N ratio is 7 (Ikeda et al. 2000). When O:N values are higher than 24, lipids
370 or carbohydrates are identified as the major components in the catabolism. O:N ratios can
371 vary, rising or falling in response to body weight, type of diet, and starvation, depending on
372 the biochemical composition of the organism (Mayzaud and Conover, 1988). In the present
373 study, the O:N values ranged from 4.9 to 10.6, indicating the dominance of protein catabolism
374 (Ikeda, 1977). Vesicomyids indeed feed on the symbiotic bacteria production likely rich in
375 proteins. However, these O:N values cannot reflect fasting conditions because the duration of
376 the experiment was not long enough to mobilize reserves and alter metabolism, as occurs
377 when animals are exposed to starvation conditions.
378
379 *Respiration rate of isolated vesicomyids and mytilids*
380 The advantage of the Calmar-tank chamber is to eliminate disturbance provided by the-
381 sediment exchanges, to know the exact biomass of bivalves in the tank and to study the
382 respiration of organisms living on hard substrates. However, the bivalves are transferred from
383 their substrate to the benthic chamber tank and they are not in their original sulfur-rich water
384 during the incubation period. This study shows that isolated *C. regab* and *E. guiness* have
385 respiration rates equal to 17 µmol O_2 g^{-1} dw h^{-1} and 26 µmol O_2 g^{-1} dw h^{-1}, respectively. *C.*
386 *regab* was sampled between 3000 and 5000 m depth at a temperature ranging from 2.38 to
387 2.55°C, whereas *E. guiness* lives at 600 m depth and a temperature of 7.1°C. These species

may have distinctive respiratory physiology that can explain the observed variation of respiratory rate. Nevertheless, the difference in size between the studied vesicomyids from 0.7 to 4.3 g dw per individual is the main source of this difference. The figure 5 shows that the ratio respiration rate/ biomass between the two species plots on the same curve. In addition, the incubation experiment on *B. azoricus* of several sizes also showed respiration rates increasing from 19.6 to 33.6 µmol g^{-1} dw h^{-1}, in a pattern inversely proportional to the size of the individuals (Table 5). The comparison between hydrothermal mytilids and vesicomyids living in cold seeps or organic-rich sediment does not show large difference. The power regression lines of vesicomyids and mussels at Figure 4 actually overlap perfectly. The presence of the commensal polychaete *B. seepensis* does not seem increase the individual respiratory rate knowing that the polychaete represents about 10% of the mussel weight.

In the relationship between oxygen consumption and body weight, values of the weight exponent b-1 in the equation $Ro_2 = aW^{b-1}$ show high interspecific variation within different invertebrate groups (Bayne and Newell, 1983). Among bivalves, there are not only interspecific differences in the value of the exponent b-1**,** but also intraspecific differences. *Mytilus edulis* shows typical values: for example -0.226 in winter and -0.298 in summer (Bayne et al., 1973), suggesting that the measured physiological differences, such as respiration, are largely determined by environmental rather than genotypic factors (Widdows et al., 1984). For the vesicomyids or mytilids in this study (Fig. 5), the difference of in the value of the b-1 exponent between vesicomyids and mytilids (-0.16 and -0.19 respectively) was essentially due to differences in size rather than in the metabolism-to-respiration ratio (Taylor and Brand, 1975).

The comparison of vesicomyids or mytilids oxygen consumption rates measured in this study with those of the literature is complicated by the close relationship between food supply and metabolism and the lack of a standard approach. Therefore, comparisons can only be done

between groups of animals that exhibit the same type of metabolism. Although many species of deep-sea bivalves have been described, physiological measurements have been made on very few species not only due to sampling difficulties, but also because deep-sea animals are difficulty to study *in situ* or to maintain alive in the laboratory. Respiration rates have been measured for a few deep-sea mussels (review by Mahaut et al., 1985; Smith Jr, 1985; Jarnegren and Altin, 2006). For example, the *ex situ* experimentally obtained oxygen consumption rate of the vent species *Calyptogena magnifica* (Boss and Turner, 1980) from the Galapagos Rift ranges from 0.4 µmol O_2 g^{-1} wet weight h^{-1} at 2.1 °C to 1.58 µmol.O_2 g^{-1} wet weight h^{-1} at 8 8 °C (Arp et al., 1984). Assuming that the dry weight of clams is equal to 25% of the wet weight (personal data), the respiration rate of *C. magnifica* is 1.6 to 6.3 µmol O_2 g^{-1} dw h^{-1}. Conversely, large numbers of bivalves in shallow depths have been studied in different ways within physically variable environments (see review in Bayne and Newell, 1983). Standard metabolism is represented by negligible feeding activity, and is considered to be a resting state. In the present study, the standard rates of metabolism approached maintenance rates. Deep-sea vesicomyids of the Gulf of Guinea and mytilids of ATJ are at the upper extremes of respiration rate when comparing these values with the measurements made on shallower mussels (Fig. 5) investigated at similar experimental temperatures (from 2.7 to 20 µmol O_2 g^{-1} dw h^{-1}, Jarnegren and Altin, 2006). The shallow suspension-feeding mussels chosen from this selection were generally adults, measuring 20-50 cm in length (Vahl, 1973). The compilation of all the existing data on the respiration rate of deep-sea benthic organisms, measured at comparable ambient temperatures (2-4°C), suggest an allometric relationship, relating individual respiration rate to individual weight of about 10 µmol O_2 g^{-1} dw h^{-1} (Mahaut et al, 1985). Similarly, the respiration rate of the deep-sea hydrothermal mytilid *Bathymodiolus thermophilus* is 10.3 µmol O_2 g^{-1} dw h^{-1} (Smith Jr, 1985) at 2500 m depth on the Galapagos Rift for mussels with a mean dry weight of 10 g. In comparison, *B. azoricus*

(19.6-33.6 µmol O_2 g^{-1} dw h^{-1}) in our experiments were smaller with a mean size from 0.03 to 0.7 g dw. This large difference in size explains the difference in respiration rate observed between the two hydrothermal mytilid species which both harbor commensal polychaetes. The water temperature differences between the Galapagos Rift (1.8° C) and this study (4.6° C) is also a factor that can explain the observed differences in respiration rate between these two species.

Several factors, other than temperature and size, also affect the respiration rate of most bivalves, including food availability, ecological situation, stress, species characteristics and gametogenic stage. This list is not exhaustive. Smith Jr (1985) showed a significant difference in the respiration rate between mytilids close to a vent and mytilids at several meters from this vent due to the modification of the water environment. Furthermore, our experiments were always based on a group of a dozen individuals in the benthic chamber and not a single individual as generally described in the literature. The "group effect" on the respiration rate has never been studied and may also affect the interpretation of our results. The production of CO_2 by the respiration of isolated bivalves (from 16 to 47 µmol CO_2 g^{-1} dw h^{-1}) was higher than that observed in animals living in the sediment. Consequently, RQ was always > 1 and was greater than this theoretical range of 0.75 (see above). When measuring CO_2 production and O_2 consumption rates over less than 6 h, the degree of coupling for these two gases is a function of the differential storage capacity of the animal. The differential diffusion rates of CO_2 and O_2 between the animal and the water, and the differential controls on CO_2 production and O_2 consumption are often determined by the physiological characteristics of the species (Hatcher, 1989). Symbionts, when fueled with reduced chemicals, also consume CO_2. But, in the Calmar-tank, without sulfur and methane, the activity of symbionts should be stopped altogether. In the standard Calmar, symbionts from vesicomyids could rely on elemental sulfur and continue to consume CO_2 because the pathway is activated. The two types of

463 experiments (standard versus tank) could therefore yield different RQ values. Ammonium
464 excretion in mussels was lower than in vesicomyids (Table 5). As with the standard Calmar,
465 the O:N ratio was < 10 for vesicomyids that depend exclusively on symbionts for their
466 nutrition, indicating the dominance of protein in catabolism (Ikeda, 1977). The food sources
467 for mytilids in hydrothermal vents are more diverse, and include particles in the water
468 environment as well as different symbiotic forms. The ratio O:N ≈ 24 may be the result of
469 increases in lipid and carbohydrate catabolism. However, the O:N ratio can vary also in
470 response to several factors such as starvation and the biochemical composition of the
471 organism (Bayne et al., 1973). The low ratio may need another explanation. Several
472 vesicomyid symbionts possess nitrate reductase genes (Newton et al., 2008), suggesting the
473 production of $NH4^+$ and hence a greater than expected proportion of ammonium excretion.
474 This could also explain the difference between the mussels and the vesicomyids in this study.
475
476 *Comparison of standard Calmar chamber and the modified Calmar-tank chamber*
477 The comparison of our data obtained with standard Calmar chamber and the modified
478 Calmar-tank chamber during the same cruise shows that undisturbed clams have lower
479 respiratory metabolism than clams isolated in the tank. One major limitation of the
480 measurements is that the biomass is not estimated the same way. The biomass calculation
481 under the Calmar-tank did not raise any question because all bivalves were sampled and dried.
482 On the contrary, the biomass under the standard Calmar was only estimated by photographs or
483 partial collections and the error in the calculation of the biomass can therefore be high. Stress from
484 ROV handling at the beginning of the experiment appears to lead to oxygen consumption
485 rates that can remain high for several hours, leading to an overestimation of basal rates. Deep-
486 sea species are possibly more sensitive to this handling disturbance. In our case, during the
487 Calmar-tank experiments, bivalves were taken out of their natural environment without

sulfide fluxes that supply energy to the bacterial symbionts. Reactions of the bivalve/symbiotic associations to this new situation are unknown and changes in metabolism cannot be excluded. Oxygen consumed by vesicomyids measured in the Calmar-tank was about 1.5 to 3 times higher than those measured intact on sediment with the standard Calmar (Table 5). Is this difference only the consequence of the bivalve transfer into the tank? Interestingly, the measurements made by Decker et al. (2012) and Khripounoff et al. (2015) with the standard Calmar at the same stations (Table 4) showed results similar to our Calmar-tank values rather than to those obtained here with the standard Calmar (Table 5). In any case, handling affected deep-sea animal metabolism in all experiments, performed *in situ* or *ex situ*.

Conclusion

Advances in our understanding of processes underlying life in extreme deep-sea environments are very likely to contribute to a new model of the limits or main factors that regulate metazoan life. Closer study of the physiological ecology of these very different species has the potential to reveal, for example, the range of adaptations in clams and mussels and can serve as a model for other chemosynthetic taxa. The present study provides new insights based on intact, undisturbed sediments with bivalve communities and on isolated clams and mussels. We demonstrated that the presence of bivalves significantly affects benthic fluxes of O_2, CO_2 and NH_4^+ in deep-sea sediments. However, these data have some limits because interactions occur between vesicomyids or mytilids, small benthic organisms and sediment. In isolated bivalves removed from sediments, allometric differences in bivalve size are the major explanation behind differences in respiration rate. At similar sizes, there were no differences between mussels and clams in terms of oxygen consumption. The CO_2 production rate by deep clams was always higher than the predicted model indicating possible decoupling between oxidation and the storage capacity of the animal for carbon dioxide and oxygen

513 gases. Ammonium excretion rate differs between mussels and clams, suggesting that only
514 protein was metabolized as an energy substrate in vesicomyids, but not in mytilids.
515
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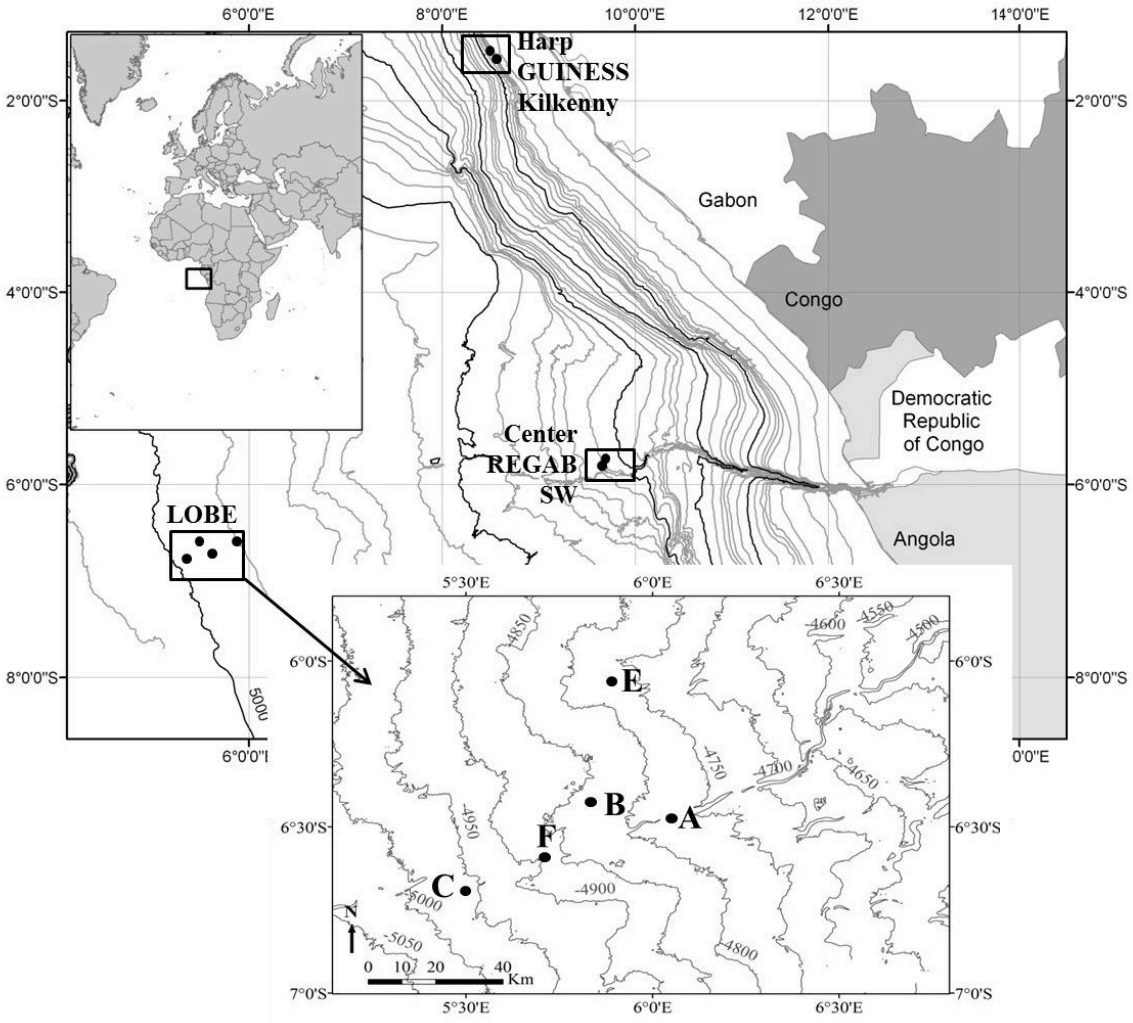
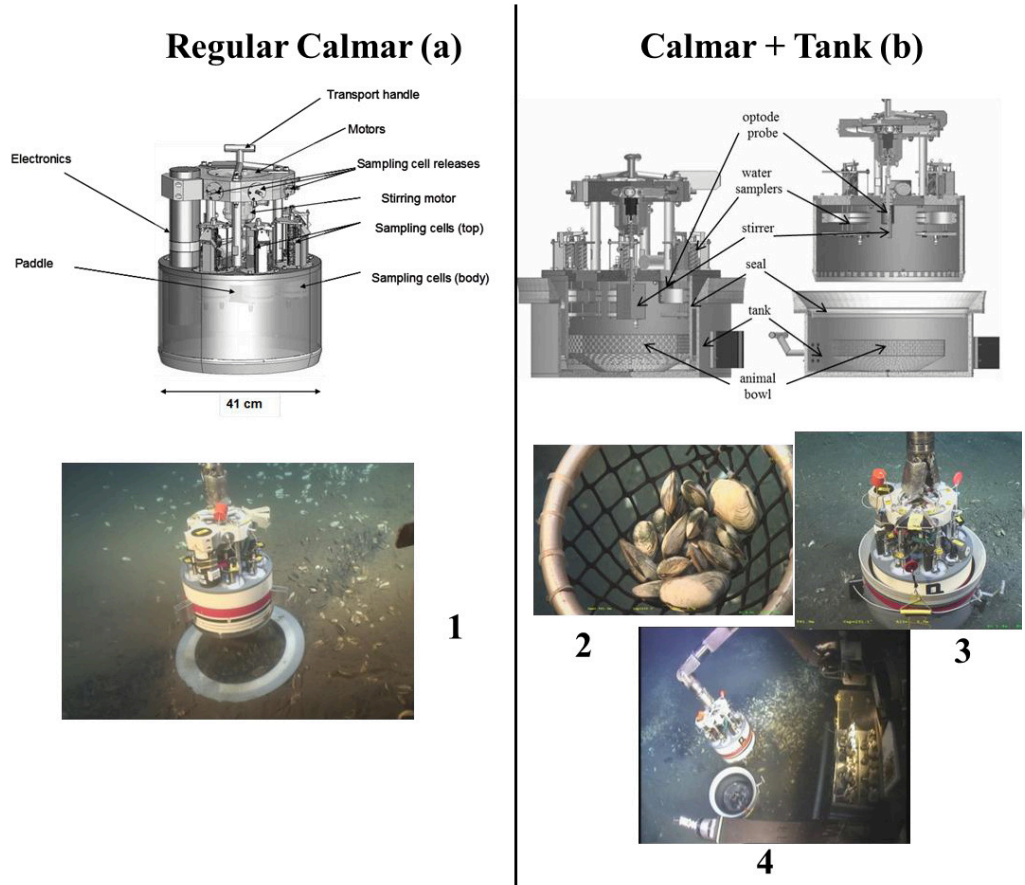


Figure 1 : Location of the study stations in the Gulf of Guinea.



Lobe A Lobe F Lobe B

Regab Center Regab Center

Figure 2: Top: Examples of deployment of Calmar benthic chambers during the Congolobe cruise. Pictures on the left: (a) diagram of the standard Calmar, (1) the ring delimiting the surface area measured and (1) the Calmar chamber placed on the delimited sediment.
Pictures on the right: (b) diagram of the Calmar benthic chamber fitted with a tank, (2) vesicomyids sampled with a net (3) the modified Calmar fitted with a tank and (4) general view of the experiment with use of the ROV arms.

Bottom: Illustrations of different sediments with bivalves before the deployment of the standard Calmar.

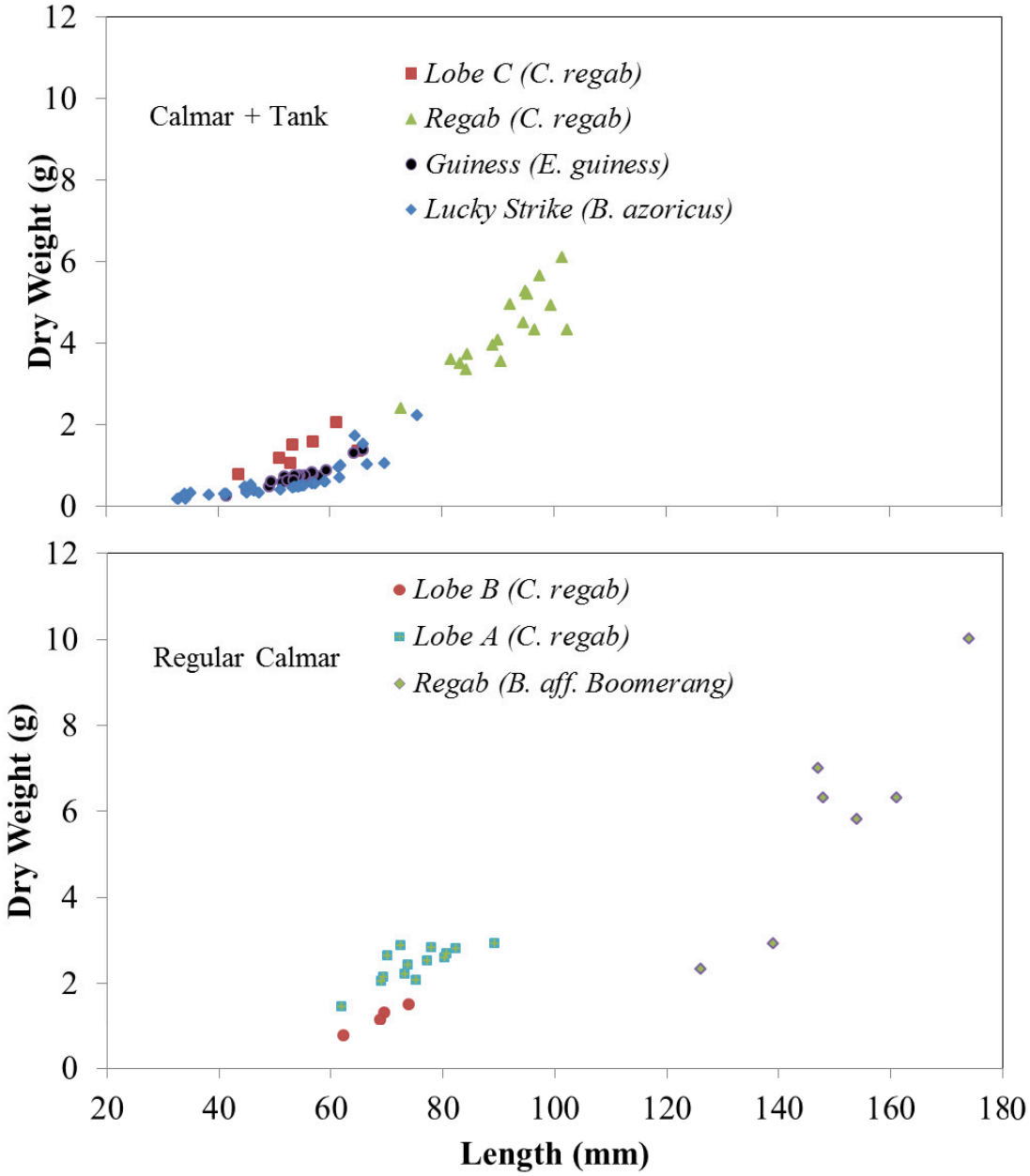


Figure 3: Characteristics of the bivalves used in the incubation experiments with the standard Calmar benthic chamber and the modified Calmar chamber fitted with a tank (The length of bivalves is the antero-posterior size).

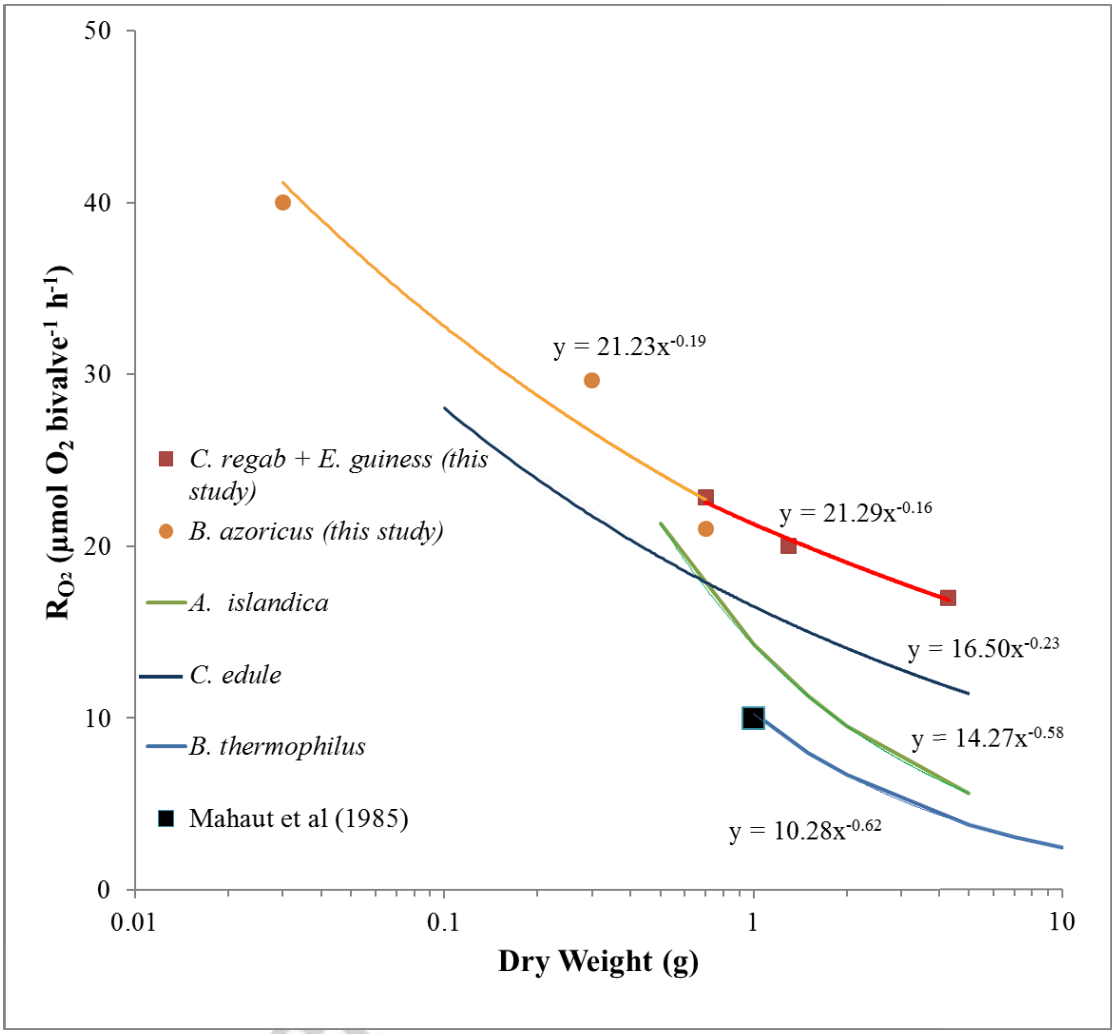


Figure 4: Relationship between individual oxygen consumption (R_{O2}) and dry weight for various bivalve species (This study; *Bathymodiolus thermophilus*, Smith Jr, 1985; *Artica islandica*, Taylor and Brand, 1975; *Cardium edule*, Vahl, 1973, Mahaut et al, 1985)

Table 2: Biomass of bivalves calculated under the standard Calmar.

Station (Marker)	Dominant species under Calmar	Density by photos (ind. m^{-2})	Density by corer (ind. m^{-2})	Mean density (ind. m^{-2})	Biomass (g dry weight m^{-2})
Regab (W06/M3)	*Bathymodiolus.* aff. *boomerang*	15		15	75
Regab (W02/M2)	*B.* aff. *boomerang*	18		18	90
Lobe A (CoL-01)	*Christineconcha regab*	185	485	335	1066
Lobe F (CoL-03)	*Calyptogena southwardae*	50	114	82	329
Lobe B (Col-11)	*C. regab*	71	143	107	157

Table3

Table 3: Elementary composition of bivalve tissues (% total C and S). (*Christineconcha regab= C. regab, Elenaconcha guiness=E. guiness, Bathymodiolus* aff. *Boomerang= B. aff. Boomerang, Bathymodiolus azoricus= B.azoricus)*

Type of chamber used		*C. regab* (Lobe)	*C. regab* (Regab)	*E. guiness* (Guiness)	*B.* aff. *boomerang* (Regab)	*B. azoricus* (ATJ)
Standard Calmar						
	Number	9	11		7	
	C (%)	45.1 ± 5.1	42.3 ± 2.7		45.2 ± 2.7	
	S (%)	15.3 ± 1.3	17.2 ± 3.2		1.2 ± 03	
Calmar with a tank						
	Number	10	21	22		25
	C (%)	40.3 ± 3.2	38.5 ± 2.5	45.6 ± 2.5		39.9±1.1
	S (%)	12.7 ± 2.6	16.0 ± 5.6	5.1 ± 1.2		1.5±0.2

Table4

Table 4: Flux of O_2, ΣCO_2, NH_4^+ and CH_4 measured under the standard Calmar in this study and similar values found in the literature. (*Christineconcha regab= C. regab, Calyptogena valdiviae= C. valdiviae, Bathymodiolus* aff. *Boomerang= B. aff. Boomerang, Abyssogena southwardae = A. southwardae)*

Station	Dominant Species	O_2 (mmol m^{-2} h^{-1})	ΣCO_2 (mmol m^{-2} h^{-1})	RQ	NH_4^+ (mmol m^{-2} h^{-1})	CH_4 (mmol m^{-2} h^{-1})	
Regab Center	*B.* aff. *boomerang*	-1.29	1.04	0.03	0.10	1.80	This study
Regab Center	*B.* aff. *boomerang*	-1.42					
Lobe A	*C. regab*	-6.71	9.58	0.06	0.24	4.52	
Lobe A	Reference	0.3			0.01		
Lobe F	*A. southwardae*	-2.04	3.08	0.06	0.16	0.54	
Lobe C	Reference	0.4	0.45	1.1	0.03	0.1	
Lobe B	*C. regab*	-1.79	3.13	0.07	0.16	0.35	
Regab SW	*C. regab*	-11.33	2.08	0.01	0.37	0.51	Khripounoff et al. (2015)
Regab Center	*C. regab*	-18.04	3.58	0.01	0.43	1.91	
Guiness Kilkenny	*C. valdiviae*	-1.17	3.96	0.14			
Guiness Harp	*C. valdiviae*	-11.29	6.46	0.03			
Lobe A	*C. regab*	-6.54	1.50	0.01	0.31	0.08	
Lobe C	*C. regab*	-14.42	77.38	0.23	2.99	5.79	
Regab Center	*C. regab*	-13.83				0.61	Decker et al. (2012)
Regab SW	*C. regab*	-20.50				0.01	
Regab Center	*C. regab*	-12.96				0.04-3.38	Pop Ristova et al. (2012)

Table 1: Characteristics and origins of vesicomyids and mytilids used in this study (Equipment: C = standard Calmar and CT = Calmar with a tank)

Station (Cruise-Marker)	Position	Species	Depth (m)	Temperature (°C)	Dive number (equipment)
Regab (Wacs 06/M3)	S 5°47.865 E 9°42.691	*Bathymodiolus* aff. *boomerang*	3155	2.55	pl 428 (C)
Regab (Wacs 02/M2)	S 5°47.869 E 9°42.689	*B.* aff. *boomerang*	3154	2.55	pl 429 (C)
Regab (Wacs 03/Marker7)	S 5°47.867 E 9°42.687	*Christineconcha regab*	3072	2.55	pl 481 (CT)
Guiness Harp (Congolobe)	S 1°34.647 E 8°32.911	*Elenaconcha guiness* / *Calyptogena valdiviae*	582	7.0	pl 495 (CT)
Lobe A (Congolobe-01)	S 6°28.281 E 6°02.143	*C. regab*	4751	2.4	pl 483 (C)
Lobe F (Congolobe-03)	S 6°35.426 E 5°41.406	*Abyssogena southwardae* / *C. regab*	4873	2.4	pl 486 (C)
Lobe C (Congolobe-09)	S 6°42.068 E 5°29.273	*C. regab*	5070	2.4	pl 491 (CT)
Lobe B (Congolobe-11)	S 6°25.229 E 5°49.709	*C. regab* / *A. southwardae*	4712	2.4	pl 492 (C)
Lucky Strike (Momarsat 2015)	N 7°17'336 W 32°16.530	*Bathymodiolus azoricus*	1680	4.6	pl 599, 601, 605 (CT)

Table5

Table 5: Calculated rate of O_2, ΣCO_2, NH_4^+ consumed or produced by vesicomyids and mytilids using the standard Calmar chamber and the modified Calmar fitted with a tank (The dry weight of mytilids was measured without the commensal polychaeta). (*Christineconcha regab= C. regab, Elenaconcha guiness=E. guiness,*, *Bathymodiolus* aff. *boomerang= B. aff. boomerang, Abyssogena southwardae= A. southwardae Bathymodiolus azoricus= B.azoricus, Branchipolynoe seepensis= B. seepensis)*

	Cold system										Hydrothermal system		
	Calmar with a tank			Standard Calmar							Calmar with a tank		
	Regab Center	Lobe C	Guiness Harp	Regab Center	Regab Center	*Regab Center*	*Lobe C*	Lobe A	Lobe F	Lobe B	Lucky Strike		
Dominant species	*C. regab*	*C. regab*	*E. guiness*	*B.* aff. *boomer-ang*	*B.* aff. *boomer-ang*	*C. regab (Khripounoff et al. 2015)*	*C. regab (Khripounoff et al. 2015)*	*C. regab*	*A. south-wardae*	*C. regab*	*B. azoricus +B. seepensis*		
Total dry weight per Calmar (g)	64.7	8.1	12.9	12.9	12.6			138.6	42.8	16.5	8.7	14.7	6.0
Number of bivalves	17	7	21	4	5			6	8	8	22	19	170
Mean individual dry weight (g)	4.3 ±1.1	1.3 ±0.4	0.7 ±0.3	4.3 ±1.3	6.3 ±0.9			2.4 ±0.9	4.0 ±0.5	0.8 ±0.2	0.3 ±0.1	0.7 ±0.2	0.03 ±0.01
O_2 rate ($\mu mol\ g^{-1}\ dw\ h^{-1}$)	16.1	17.7	25.7	16.1	15.8	*10.4*	*22.9*	6.2	6.3	11.2	23.3	19.6	33.6
ΣCO_2 rate ($\mu mol\ g^{-1}\ dw\ h^{-1}$)	16.2	22.9	28.3	12.7		*2.0*	*123*	9.2	9.6	12.9	30.0	25.9	58.6
RQ	1.0	1.3	1.1	0.8		*0.2*	*5.3*	1.5	1.5	1.2	1.3	1.3	1.7
NH_4^+ rate ($\mu mol\ g^{-1}\ dw\ h^{-1}$)	0.87	1.25		0.83		*0.24*	*4.8*	0.25	0.51	0.67		0.33	0.53
O:N ratio	7.4	5.7		5.0		*17.3*	*2.0*	10.6	4.9	6.7		23.7	25.4