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Research paper

Novel high-pressure culture experiments on deep-sea benthic foraminifera — Evidence for methane seepage-related $\delta^{13}C$ of *Cibicides wuellerstorfi*

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ABSTRACT

In field studies of active hydrocarbon seeps the carbon isotopic composition of Rose Bengal stained benthic foraminiferal tests ($\delta^{13}C_{test}$) and bottom water DIC ($\delta^{13}C_{DIC}$) deviates from their normal marine ratios. This circumstance led to ongoing discussions on whether aerobic foraminifers like *Cibicides wuellerstorfi* are capable of living at seepage sites and, more importantly, if their tests reflect the low $\delta^{13}C$ values of emanating methane. To evaluate the discrepancy between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$, we conducted methane seepage-emulating culture experiments on undepressurized sediments from the Håkon Mosby Mud Volcano, a modern methane seepage structure that hosts living *C. wuellerstorfi* with distinct negative $\delta^{13}C$ values. The collected sediments were cultured at a site-alike pressure and mean bottom water methane concentration using newly developed high-pressure aquaria. Over an experimental period of 5 months our novel technology enabled a successful reproduction of all calcareous deep-sea benthic foraminiferal species living at that site, notably the first *C. wuellerstorfi* cultured in the laboratory. To show the influence of methane on $\delta^{13}C_{test}$, we ran parallel experiments with >99% ^{12}C- and 99% ^{13}C-methane in the experimental "bottom water". During the experimental running time methanotrophs in the water column obviously converted the experimentally added methane source to $\delta^{13}C$-enriched and -depleted DIC, respectively. Since whole sediment cores were cultured, it was impossible to keep $\delta^{13}C_{DIC}$ constant over the 5-month duration, which is reflected in a variability of $\delta^{13}C_{test}$ in foraminiferal shells. Irrespective of that, the methane source is reflected in $\delta^{13}C_{test}$ of foraminiferal shells, and for the natural seep-conditions simulating ^{12}C-experiment the mean $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$ in *C. wuellerstorfi* were equal. Although for future culturing experiments improvements of the experimental conditions are advisable, our first results are evidence that persistent methane emanation impacts the carbon isotopic composition of deep-sea benthic foraminifera.

1. Introduction

Our understanding of palaeo-deep-water circulation modes and deep-water renewal in the world's oceans is essentially based on isotopic and geochemical ratios recorded in tests of selected calcareous deep-water benthic foraminiferal species (e.g. Hester and Boyle, 1982; Lea and Boyle, 1989). Hereby, it is assumed that the foraminiferal calcite reflects the elemental or isotopic composition of the past ocean at the time of calcification, with constant ratios and offsets (Emiliani, 1955; Shackleton and Opdyke, 1973; Hester and Boyle, 1982; Curry and Lohmann, 1982; Boyle, 1988, 1992; Lea and Boyle, 1989, 1990; Nürnberg et al., 1996; Marchitto et al., 2000; Elderfield and Ganssen, 2000; Lea et al., 1999, 2000; Anand et al., 2003; Bickert and Mackensen, 2004; Curry and Oppo, 2005). Based on field data the $\delta^{13}C_{test}$ of epifaunal deep-sea foraminifers, notably of *Cibicides wuellerstorfi*, reflects the $\delta^{13}C$ of the bottom water's dissolved inorganic carbon (DIC) with a principally constant offset from equilibrium calcite (e.g. Woodruff et al., 1980; Belanger et al., 1981; Graham et al., 1981; Duplessy et al., 1984; Zahn et al., 1986; Curry et al., 1988; Mackensen, 2008).

However, these assumptions are exclusively based on field data and therefore afflicted with several problems. Foraminifers grow in an array of interacting environmental parameters. Thus, it is difficult to assess the influence of individual environmental parameters on foraminiferal calcite and, since at field sites just a selection of environmental parameters is measured, essential influences are easily obscured. Furthermore,

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a foraminiferal shell recovered during an expedition may record the mean of an environmental parameter over its growth period of days to months (e.g. Saraswat et al., 2011; Keul et al., 2013; Hohenegger et al., 2014), whereas the water mass measurement during that cruise and at that particular site is just a snapshot. Especially at high latitudes (e.g. Wollenburg and Kuhnt, 2000) and seepage sites there (e.g. Berndt et al., 2014), environmental parameters vary significantly between seasons, from week to week, and day to day. At high northern latitudes at the majority of sites primary and export production occurs during short periods of time. Since a water mass $\delta^{13}C_{DIC}$ reflects multiple organic matter contributors, at high northern latitudinal seepage sites this value may vary seasonally, disregarding on the consistency of the site's methane emanation. Moreover, in northern polar waters even the water temperature may vary seasonally (Rudels et al., 2014) affecting the decay of gas-hydrates (Berndt et al., 2014). Therefore, we have to be aware that as we nowadays collect increasingly more modern field data, we observe increasingly more sites where water mass and field data deviate from formerly field-based established relationships.

All studies on modern cold seep sites so far show a disequilibrium between stable isotope ratios of Rose Bengal stained foraminifera and DIC (Torres et al., 2003; Herguera et al., 2004; Hill et al, 2003, 2004; Rathburn et al., 2003; Martin et al., 2004; Mackensen et al., 2006; Lobegeier and Sen Gupta, 2008; Bernhard et al., 2010). Naturally occurring methane is impoverished in the ^{13}C isotope and easily oxidized after its release to the bottom water. Therefore, at seep sites both bottom water $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$ values should be significantly depleted (Rathburn et al., 2003). In this context, large negative excursions in the $\delta^{13}C_{test}$ record of benthic foraminifera have been interpreted as indicating mass releases of methane from clathrate deposits (Kennett et al., 2000, 2002, 2003). However, until now such a relationship has not been confirmed in field studies. Opposed to the typical marine environment where $\delta^{13}C_{test}$ of living benthic foraminifers and $\delta^{13}C_{DIC}$ covary, at seep sites the two parameters are decoupled. At some seep sites $\delta^{13}C_{test}$ of Rose Bengal-stained benthic foraminifers resembles that from nearby non-seep sites in spite of extremely low porewater $\delta^{13}C_{DIC}$ (Torres et al., 2003), whereas at others foraminifers show large variability in $\delta^{13}C_{test}$ (Bernhard et al., 2010; Rathburn et al., 2003). Finally, at siboglinoid tubeworm sites of the Håkon Mosby Mud Volcano (HMMV) $\delta^{13}C_{test}$ of living foraminifers, e.g. *C. wuellerstorfi*, is by up to 4.4‰ lower than at the reference sites not affected by methane venting, whereas the bottom water $\delta^{13}C_{DIC}$ of seepage and reference sites were the same at the time of sampling (Mackensen et al., 2006).

The discrepancy between $\delta^{13}C_{test}$ and $\delta^{13}C_{DIC}$ has been interpreted to indicate either that 1) methane-derived CO_2 has no significant influence on bottom water $\delta^{13}C_{DIC}$, and/or low $\delta^{13}C_{test}$ values may reflect carbon oxidation or enhanced photosynthetic carbon rain (Stott et al., 2002), or that 2) bacterial diets or endosymbionts spoil the common relationship of foraminiferal shell to DIC (Hill et al., 2004; Mackensen et al., 2006), or that 3) most/all species do not live, or precipitate only little new shell material if any, at sites/times of venting (Torres et al., 2003; Bernhard et al., 2010). Dead foraminiferal cytoplasm can be stained with Rose Bengal from weeks to months after an individual's death (Jorissen et al., 1995; Bernhard, 1988; Bernhard et al., 2010), and ultrastructural analyses of Monterey Bay seep Rose Bengal stained specimens showed that all *C. wuellerstorfi* (2 specimens) and approx. 2/3 of all Rose Bengal stained specimens had deceased (Bernhard et al., 2010). This prompted the discussion whether the observed carbon isotope offsets between Rose Bengal stained foraminifera and DIC simply originate from a methodological error (Bernhard et al., 2010). The impossibility to establish a relation between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$ at the investigated seep sites thus far questions the credibility of paleoenvironmental records derived from benthic foraminiferal $\delta^{13}C_{test}$ not only at seepage sites but also in general. The majority of paleodeepwater circulation models relies on a constant $\delta^{13}C_{test}$ to $\delta^{13}C_{DIC}$ ratio (in equilibrium for *C. wuellerstorfi*) (e.g. Bickert and Mackensen, 2004; Curry and Oppo, 2005; Murgese and Deckker, 2007; Ahamad et al., 2008). Since observations at modern seeps unhinge this relationship, culture experiments under controlled conditions, aiming to document the capability of benthic foraminiferal $\delta^{13}C_{test}$ to record deep-water $\delta^{13}C_{DIC}$ seem necessary and are addressed in this study.

Growing foraminifers in the laboratory under known and stable environmental conditions is a valuable alternative to empirical field studies. Culturing allows the establishment of reliable calibration curves for proxies influenced by one or more environmental parameters and tests to verify limits and failures of proxies based on field studies. Hereby, shallow-water foraminifera have been investigated in the laboratory over the last four decades (e.g. Arnold, 1974; Lee, 1980; Bender, 1989; Havach et al., 2001; Langezaal et al, 2004; Raitzsch et al., 2010; Dueñas-Bohórquez et al., 2011), whereas deep-sea species are more difficult to maintain in laboratory cultures. However, since the study by Kitazato (1989) an increasing number of successful attempts have been made (e.g. Linke, 1989, 1992; Weinberg, 1990, 1991; Turley et al., 1993; Hemleben and Kitazato, 1995; Gross, 1998; Heinz et al., 2001, 2002; Geslin et al., 2004; Nomaki et al., 2005). To date deep-sea foraminifera are usually cultured at atmospheric pressure, but Turley et al. (1993) and Gross (1998) carried out limited and short-lasting pressure experiments. Most deep-sea foraminifera survive a pressure difference of 120–180 bar without obvious harm. However, many species like notably *C. wuellerstorfi* neither grow nor reproduce when cultured at 1 bar (e.g. Gross, 1998, unpubl. pers. observation). Laboratory studies on deep-sea samples collected and kept under in-situ conditions (retention of pressure, sediment structure and composition, micro- and macrofauna) are missing. In this study, we give a comprehensive functional introduction in our newly developed high-pressure laboratory facilities (Wollenburg and Tiedemann, 2010a,b) that allows culturing of undepressurized sediments and show initial results on our experiments on the incorporation of methane-derived $\delta^{13}C$ in foraminiferal tests (e.g. *C. wuellerstorfi*) from seepage sites.

2. Material and methods

2.1. Equipment

Our equipment is constructed of stainless steel, glass, polyether ether ketone (PEEK), fluorinated ethylene propylene (FEP), polymethylmethacrylat (PMMA), and of gas-proof materials unknown to discharge plasticizers. All materials used were cleaned for a minimum of 2 h in 70% ethanol prior to their use in the systems. Exceptions are the pushcore tube and head both constructed of PMMA. This material embrittles when it is exposed to alcohol.

2.1.1. High-pressure aquarium basic construction (Fig. 1a)

Our high-pressure aquaria are designed for a maximum pressure of 250 bar and consist of a two-piece high-pressure sample container constructed of non-corrosive steel. The aquarium was affixed securely to the drawer of the Remotely Operated Vehicle (ROV) Quest Marum Bremen, which was used to perform in situ sampling. Therefore the aquarium's size and weight is determined by the limitations of the ROV's drawer, that is to a closed height of 430 mm, and an air-weight of 42 kg. Inside the sample container is rounded cylindrical and has a total capacity of 3 L. This is sufficiently large to store a pushcorer with a dimension of 110 mm in diameter and 210 mm in length (Fig. 1b). The concave bottom of the sample container is filled with sterilized gravel (diameter $\geq$2 to <8 mm), followed by a core-deposition ring and pushcore-tube catcher where the sample container merges to the straight part. A pushcore-tube catcher is necessary to lock the sediment core-containing pushcore tube in the aquarium. Each sample container has five ball valve-lockable in-/outlets, three at the bottom of the sample container, and two in the lid (Fig. 1a). A manometer is adjusted in front of one of the lid's ball valves (Fig. 2).

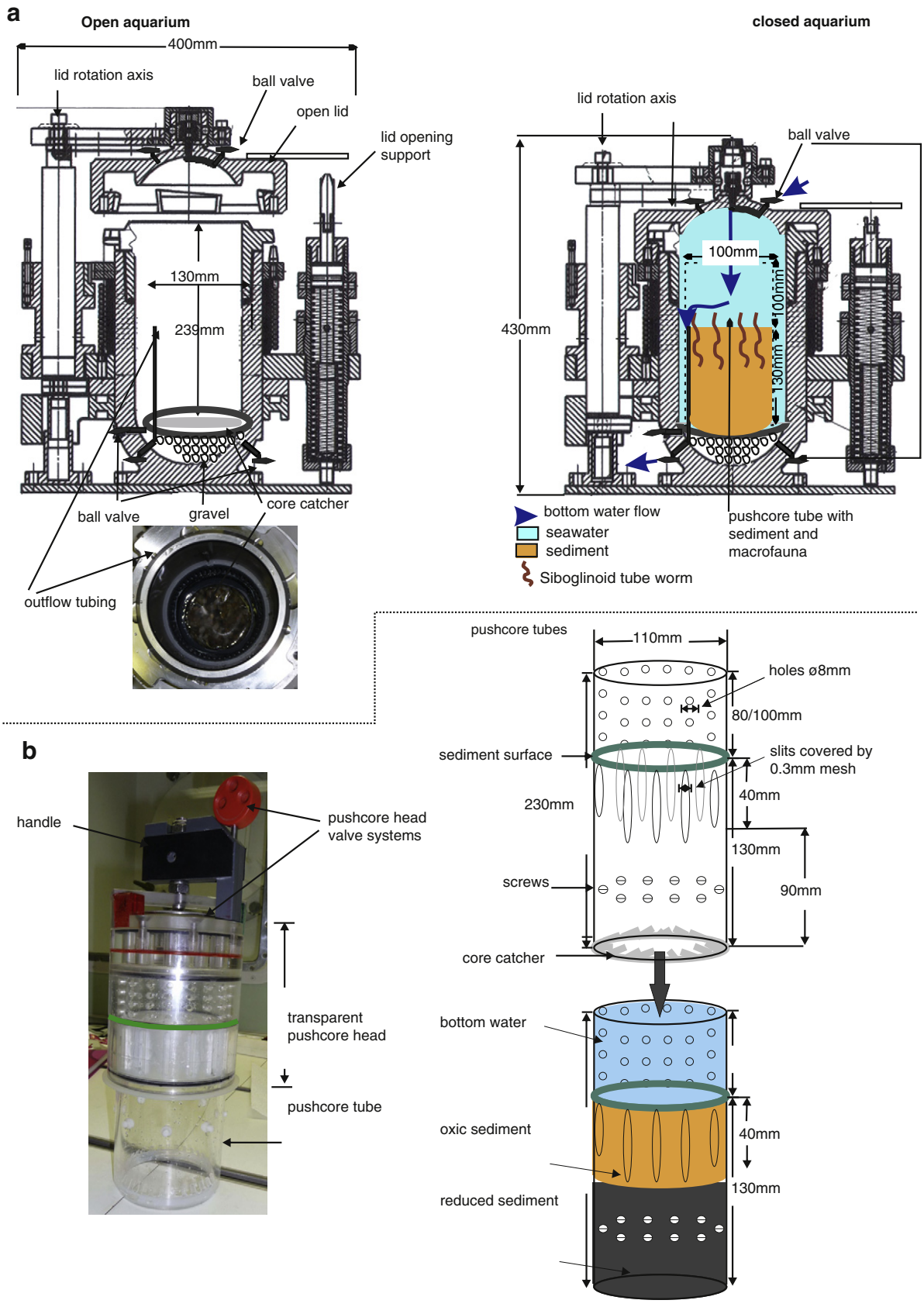

Fig. 1. High-pressure aquarium and pushcore constructional details: 1a) High-pressure aquarium and 1b) Pushcore.

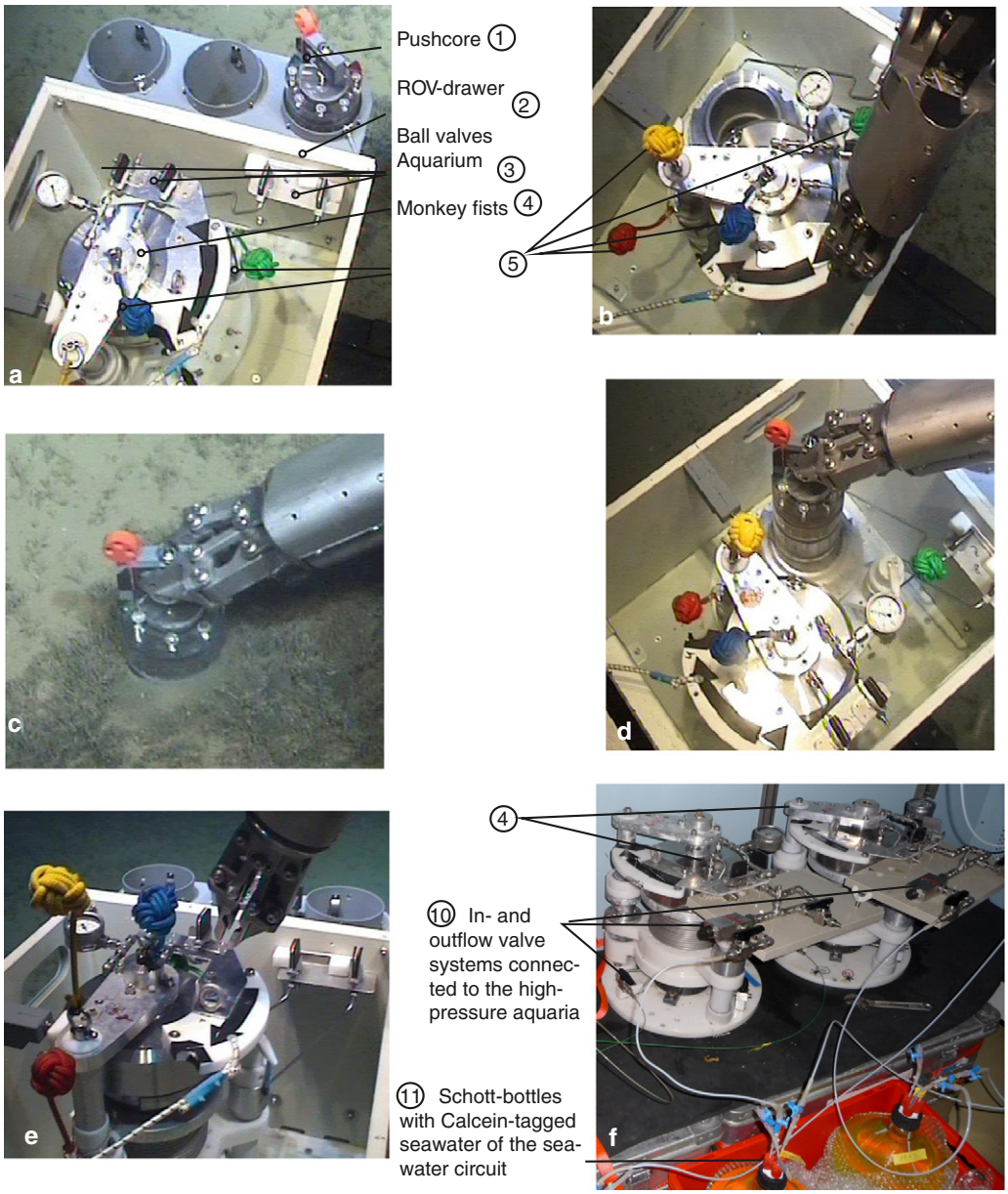

Fig. 2. High-pressure aquarium operation at the HMMV by the ROV Quest during Polarstern expedition ARKXXVI/2 in 2009.

2.1.2. Pushcore basic construction (Fig. 1b)

To allow visual control of the sediment penetration depth during the coring, pushcore tube and head are constructed of transparent PMMA (Fig. 1b). Because the pushcore has to penetrate ductile sediments sticky enough to stay in the tube during the core transfer, the tube's dimension is adapted to the type of sediments at the respective core sites. For unconsolidated sediments at the Håkon Mosby Mud Volcano we used tube lengths of 230 mm and fixed a comb-like core catcher to the lower tube edge to prevent sediment loss (Fig. 1b). The tube body holds multiple holes in the upper part to enable “bottom water” exchange during culturing (Fig. 2). Mesh covered slits in the middle tube part allow limited pore-water exchange within the top-most 40 mm of the sediment core. The remaining tube is imperforated but shows a row of screws that becomes latched onto the pushcore-tube catcher while being transferred (Fig. 1a, b). The pushcore head shows marker lines indicating the desired sediment penetration depth during coring and the depth at which the pushcore tube becomes latched onto the aquarium's pushcore-tube catcher after transfer. The pushcore head is equipped with a valve system that allows water to escape during coring and the pushcore-head removal, whereas it is closed once the corer is removed from the seafloor to prevent sediment loss during the pushcore transfer.

2.1.3. High-pressure aquarium operation (Fig. 2)

In preparation of the descent to the seafloor the spring-operated mechanical construction groups for the opening and closure of the aquarium have to be prepared, these are set into action by pulling respective monkey fists. Then the semi-closed (unlocked) aquarium is fixed with screws in the ROV's drawer and the pushcore rack is mounted in front of the drawer. The aquarium's ball valves are opened and a spring is fixed between the aquarium's lid and drawer. The latter one prevents an unwanted backward pivoting of the opened aquarium's lid during the core transfer.

During the dive the ROV searches for a suitable core site, then stops, opens the drawer and the high-pressure aquarium's lid (Fig. 2). Then the manipulator takes a pushcore handle and starts the coring process. Controlled by the video systems the pushcore penetrates the sediment until the respective marker line contacts the sediment surface (Fig. 2).

Inside the pushcorer the sediment surface is now positioned at the upper border of the mesh-covered slits in the pushcore. Carefully the core is transferred into the high-pressure aquarium where it has reached its optimum position when the red marker line visually contacts the sample containers border. Now the screws on the pushcore tubes exterior (Fig. 1b) got latched onto the aquarium's pushcore tube catcher. This is a prerequisite so that the pushcore head can be removed. Only the sediment-filled pushcore tube remains inside the sample container. After the core transfer the aquarium's lid and ball valves are closed, then the drawer is retracted, and the ROV is ready for resurfacing. On deck the high-pressure aquarium is unscrewed from the drawer and carried into the cold room (Fig. 2).

2.2. Sample material

In 2009, RV POLARSTERN (expedition ARK-XXIV/2) visited the strong negative δ^{13}C-methane-emanating Håkon Mosby Mud volcano (HMMV) located at roughly 1280 m water depth on the continental slope of the SW Barents Sea (72°N, 14°E) (Damm and Budéus, 2003; Klages, 2010) (Fig. 3). The circular structure (diameter 1.5 km) consists of three concentric habitats (Gebruk et al., 2003), the central two of which are nearly devoid of living foraminifera (Wollenburg and Mackensen, 2009). Two endemic siboglinoid tubeworm species characterize the hummocky periphery of the HMMV (Smirnov, 2000; Gebruk et al., 2003). The small (<7 cm) and more abundant (80%) *Sclerolinum contortum* occupies oxic sediments, whereas the large (>20 cm) *Oligobrachia haakonmosbiensis* prefers more reduced surface sediments. From previous expeditions we know that tubes of living *S. contortum* are usually densely covered with living *C. wuellerstorfi*, whereas tubes of *O. haakonmosbiensis* and the sediments are devoid of living *C. wuellerstorfi* (Wollenburg and Mackensen, 2009). Thus, controlled by multiple video systems of the ROV *Quest* (Marum Bremen) we prospected a suitable site, densely covered by *S. contortum* and visually free of *O. haakonmosbiensis*, for our core collection. Here in the north of the HMMV, located at roughly 72°0.38′N and 14°43.5′E, the ROV *Quest* collected three sediment cores plus overlaying water and transferred them into the high-pressure retaining aquaria at the seafloor (Figs. 2–3). Since the total volume of the high-pressure retaining aquarium is 3 L, the aquarium was filled with 1 L sediment and approx. 2 L of the site's bottom water.

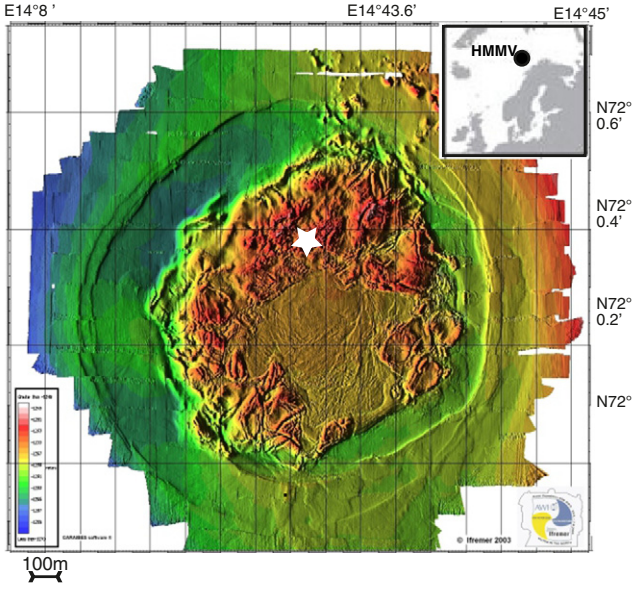

Fig. 3. Coring sites for high-pressure culture experiments at siboglinoid tubeworm sites of the Håkon Mosby mud volcano (HMMV) occupied by R/V Polarstern and ROV Quest Marum Bremen in 2009. Microbathymetric map is based on remotely operated vehicle (ROV)-based echosounding in 2003 (IFREMER). Full image of colors represents water depths between 1249 and 1289 m.

2.3. Experimental setup

After resurfacing the aquaria were immediately transferred into a cold lab, running at a site-alike temperature of 0 °C. Chains of valves were fixed to each high-pressure aquarium's ball valves (Fig. 3). Hereby one chain of valves controlled the seawater outflow, the other chain accomplished the seawater inflow into the aquaria by isocratic and isobaric high-pressure pumps. Each aquarium was connected to its own seawater cycle with a total volume of 25 L split over 4 gas-proof glass bottles (Schott bottles with Bola-connections). Every day 2–4 L of this volume was replenished.

We ran our experiments during the remaining expedition with bottom water collected at the HMMV from a CTD cast. Thereafter, in the home laboratory we used North Sea water adjusted to the respective core site salinity (34.9‰) by addition of Hobby Marine sea salt for the subsequent replenishing of parts of the experimental seawater cycle. Hereby, filtering over a 2 μm mesh ensured the removal of potential North Sea foraminiferal propagules (Alve and Goldstein, 2003; Goldstein and Alve, 2014). The experimental seawater was tagged with 0.01 g/L Calcein (Bernhard et al., 2004). This method allows distinguishing experimentally precipitated shell material by fluorescence microscopy (excitation wavelength 470 nm) from non-fluorescent pre-experimentally precipitated shells or shell parts (e.g. Dissard et al., 2009, 2010). Since the basic work of Bernhard et al. (2004) labeling foraminifers in Calcein has become a common tool to differentiate between pre-experimental shell material and the shell parts that were secreted during the experiments. Because most experiments are carried out on isolated foraminiferal specimens, they are usually incubated in Calcein prior to the experiments. During the experiment no Calcein is added and experimentally precipitated shell material is non-fluorescent. Since we have cultured whole sediment cores with many empty tests we ran our experiments for the entire duration with Calcein-tagged water (see also Bernhard et al., 2004; Dissard et al., 2010).

Two experiments were conducted. High-pressure aquaria 1 and 2 were connected to a circuit enriched in ^{13}C-methane (Campro Scientific, Methane-^{13}C gas, minimum 99 at.% ^{13}C). Otherwise, by using standard >90% ^{12}C-methane with an isotopic composition that approximates the HMMV field methane values (Damm and Budéus, 2003), the carbon isotopic composition of an experimental offspring would have been close to natural values, which might have been misinterpreted as a pure reflection of nutrition on ^{12}C-enriched organic detritus by the foraminifers (Hill et al., 2004; Mackensen et al., 2006). For high-pressure aquarium 3, HMMV-conditions were simulated by an addition of ^{12}C-methane (Campro Scientific, Methane-^{12}C gas, minimum 99.95 at.% ^{12}C). Hereby, an experimental setup was designed to emulate siboglinoid tubeworm-sites mean bottom water methane concentrations of 0 to 129 μmol/L (de Beer et al., 2006; Sauter et al., 2006) and minimum oxygen concentrations of >100 μmol/L to assure a high survival probability for *C. wuellerstorfi*. To achieve such methane concentrations we circulated an adjustable proportion of the aquarium's outflow via peristaltic pumps over a gas-enrichment bottle. In this 5 L bottle a 2 L methane atmosphere, replenished twice per week, topped the seawater (Fig. 2c). The inflowing seawater, via a fixed air stone, passed the atmosphere as small drops adsorbing methane. Through variable flow rates of the respective peristaltic pumps seawater from the aeration and gas-enrichment bottles were mixed to meet the mean marginal HMMV methane and oxygen concentrations (Fig. 2c). With aid of a high-pressure piston pump this marginal HMMV-alike water of the mixing or inflow bottle was then pumped with 7 mL/min through the aquaria. At this exchange rate the pressure inside the high-pressure aquaria remained stable, whereas, despite pulsation dampeners and restriction valves the pressure seemed to vary at higher pumping speeds.

Table 1
High-pressure aquaria 1 & 2: variations in bottom water oxygen (extract from daily measurements), and methane concentrations and $\delta^{13}C_{DIC}$ values. Variability of duplicate oxygen measurements was <1 μmol/L.

Date	O_2(μmol/L)	CH_4(μmol/L)	CH_4 Std.Dev.	$\delta^{13}C$ DIC	$\delta^{13}C$ Std.Dev.
7/23/2009	297.12	1.09	0.04		
7/24/2009	298.50				
7/25/2009	298.32				
7/26/2009	299.58				
7/27/2009	300.12	36.79	1.18		
7/28/2009	300.12				
7/29/2009	300.15				
7/30/2009	300.16				
7/31/2009	300.29				
8/1/2009	300.35				
8/2/2009	309.15	88.71	3.78	3.24	1.12
8/3/2009	308.96				
8/4/2009	312.22				
8/5/2009	318.12				
8/6/2009	318.14	86.68	3.21		
8/7/2009	318.56				
8/8/2009	319.01				
8/9/2009	321.23	80.92	3.45		
8/10/2009	322.45				
8/11/2009	325.61				
8/12/2009	329.68				
8/13/2009	328.76	74.17	3.52	352.81	9.89
8/14/2009	316.89				
8/15/2009	297.76	58.35	2.80	1167.71	195.55
8/16/2009	296.54				
8/17/2009	298.36				
8/18/2009	299.13				
8/19/2009	303.51	56.13	2.84		
8/20/2009	299.88				
8/21/2009	254.44				
8/22/2009	214.06	54.70	1.90		
8/23/2009	234.65				
8/24/2009	239.99				
8/25/2009	246.01	51.91	2.56		
8/26/2009	265.88				
8/27/2009	271.57	50.06	2.49		
8/28/2009	272.83				
8/29/2009	268.54				
8/30/2009	270.32				
8/31/2009	270.15	65.43	2.39		
9/1/2009	269.99				
9/2/2009	268.56				
9/3/2009	268.56				
9/4/2009	267.41	73.69	3.77		
9/5/2009	268.99				
9/6/2009	268.98				
9/7/2009	268.45				
9/8/2009	268.37	81.89	3.87		
9/9/2009	265.44				
9/10/2009	261.98	88.75	4.26		
9/11/2009	268.94				
9/12/2009	271.57	95.40	4.74	1449.10	2.19
9/13/2009	272.34				
9/14/2009	270.64				
9/15/2009	269.99				
9/16/2009	267.69	100.67	4.78		
9/17/2009	259.98				
9/18/2009	258.64				
9/19/2009	258.34				
9/20/2009	257.89	98.65	3.98		
21/09/09	257.64				
22/09/09	257.34				
23/09/09	256.83				
24/09/09	256.63	100.23	5.02		
25/09/09	255.83				
26/09/09	255.23				
27/09/09	254.89				
28/09/09	253.67	89.13	4.89		
29/09/09	253.23				
30/09/09	250.83				
01/10/09	248.74				
02/10/09	247.93				
03/10/09	246.53	102.12	5.69		
04/10/09	246.00				

Table 1 (*continued*)

Date	O_2(μmol/L)	CH_4(μmol/L)	CH_4 Std.Dev.	$\delta^{13}C$ DIC	$\delta^{13}C$ Std.Dev.
05/10/09	244.23				
06/10/09	243.98				
07/10/09	243.19	99.58	3.99		
08/10/09	240.37				
09/10/09	239.29				
10/10/09	238.98				
11/10/09	235.11	96.34	3.76		
12/10/09	233.67				
13/10/09	231.24				
14/10/09	230.21				
15/10/09	228.89	94.34	4.67		
18/10/09	224.66				
19/10/09	218.67				
20/10/09	217.53				
21/10/09	214.06	102.75	4.98	1055.39	3.78
22/10/09	201.01				
25/10/09	198.65				
26/10/09	185.86				
27/10/09	175.72	105.77	5.26		
28/10/09	179.99				
29/10/09	182.11	86.26	4.27		
30/10/09	195.67				
31/10/09	198.73				
01/11/09	199.21				
02/11/09	205.24				
03/11/09	210.86	71.48	3.48	1333.84	43.79
04/11/09	245.32				
05/11/09	261.98	73.63	3.56		
08/11/09	258.91				
09/11/09	243.22				
10/11/09	220.45	83.78	4.01		
11/11/09	200.11				
12/11/09	194.89	105.97	4.77		
13/11/09	193.21				
14/11/09	198.72				
15/11/09	203.49				
16/11/09	234.21				
17/11/09	255.59	111.09	5.60		
18/11/09	228.71				
19/11/09	201.28	108.53	5.20	1346.89	97.45
20/11/09	204.88				
21/11/09	210.58				
22/11/09	230.87				
23/11/09	256.71				
24/11/09	271.57	97.62	4.75		
25/11/09	268.93				
26/11/09	255.59	89.69	4.44		
27/11/09	249.71				
28/11/09	246.59				
29/11/09	232.23				
30/11/09	226.71				
01/12/09	220.45	72.00	3.23	1415.80	317.80
02/12/09	219.21				
03/12/09	217.25	56.64	2.81		
06/12/09	219.63				
07/12/09	220.55				
08/12/09	223.64	72.98	2.83		
09/12/09	221.83				
10/12/09	220.45	88.86	2.83	1651.50	2.15
13/12/09	234.64				
14/12/09	234.53				
15/12/09	230.12				
16/12/09	226.77				
	Mean 253.44	Mean 80.84	Mean 3.73	Mean 1086.25	Mean 74.86

During the experiment we kept the high-pressure aquaria environment at a 'site-alike' pressure of 125–130 bar, constant temperatures (0 °C), constant pH (7.9), and within a certain tolerance, stable oxygen (>100–500 μmol/L) and methane (at the beginning 0, then 40–150 μmol/L) "bottom water" concentrations (de Beer et al., 2006). However, no food was added. With no visual control of the amount of potentially ingested algae, there would have been the risk of decaying algae accumulation on top of the sediments, creating a strong $\delta^{13}C$ gradient from the liberation of $\delta^{13}C$-depleted CO_2 (Mackensen et al., 1993; Sen Gupta, 1999; Jorissen and Wittling, 1999).

Oxygen concentrations were measured daily, pH once per week. Water samples for subsequent methane measurements were taken twice, for $\delta^{13}C$ analyses on DIC once per week (Tables 1, 2; Fig. 4). Hereby, water sampling for geochemical measurements were conducted directly from the aquarium's outflow. DIC samples were poisoned with mercury chloride (0.006 mL mercury chloride solution (3.6 g

Table 2
High-pressure aquarium 3: variations in bottom water oxygen (extract from daily measurements), and methane concentrations and $\delta^{13}C_{DIC}$ values. Variability of duplicate oxygen measurements was <1 μmol/L.

Date	O_2(μmol/L)	CH_4(μmol/L)	CH_4 Std.Dev.	$\delta^{13}C$ DIC	$\delta^{13}C$ Std.Dev.
7/23/2009	325.88	0.00			
7/24/2009	327.90				
7/25/2009	330.21				
7/26/2009	339.20				
7/27/2009	346.80	15.62	0.89		
7/28/2009	359.90				
7/29/2009	367.50				
7/30/2009	365.30				
7/31/2009	372.43				
8/1/2009	378.65				
8/2/2009	380.19	23.43	1.17	−0.38	0
8/3/2009	381.36				
8/4/2009	372.25				
8/5/2009	375.38				
8/6/2009	373.48	45.67	1.53		
8/7/2009	369.85				
8/8/2009	370.54				
8/9/2009	369.71	59.82	2.32		
8/10/2009	365.42				
8/11/2009	368.17				
8/12/2009	365.13				
8/13/2009	364.22	53.50	2.68	−0.46	0.04
8/14/2009	356.71				
8/15/2009	335.46	69.65	3.48		
8/16/2009	338.71				
8/17/2009	339.84				
8/18/2009	336.63				
8/19/2009	335.46	95.40	4.67	−0.78	0.04
8/20/2009	340.82				
8/21/2009	358.72				
8/22/2009	361.02	101.70	5.09	−1.20	0.03
8/23/2009	362.83				
8/24/2009	365.43				
8/25/2009	367.41	66.65	3.33		
8/26/2009	354.23				
8/27/2009	338.66	59.25	2.96		
8/28/2009	341.23				
8/29/2009	342.68				
8/30/2009	346.53				
8/31/2009	343.21	85.93	3.12		
9/1/2009	342.87				
9/2/2009	341.70				
9/3/2009	344.51				
9/4/2009	344.53	79.13	2.89		
9/5/2009	344.54				
9/6/2009	345.08				
9/7/2009	345.05				
9/8/2009	345.05	98.34	4.92	−1.19	0.02
9/9/2009	332.18				
9/10/2009	322.68	103.87	5.19		
9/11/2009	316.72				
9/12/2009	313.10	84.87	4.24		
9/13/2009	318.21				
9/14/2009	321.49				
9/15/2009	319.86				
9/16/2009	318.53	101.34	3.52		
9/17/2009	318.03				
9/18/2009	317.40				
9/19/2009	317.00				
9/20/2009	315.80	101.32	4.98		
21/09/09	313.65				
22/09/09	311.91				
23/09/09	311.63				
24/09/09	311.43	98.64	3.82	−1.22	0.03
25/09/09	308.63				
26/09/09	308.42				
27/09/09	306.97				
28/09/09	304.84	100.56	5.01		
29/09/09	303.29				
30/09/09	300.83				
01/10/09	297.84				
02/10/09	293.42				
03/10/09	290.65	96.72	3.46		
04/10/09	287.69				

Table 2 (*continued*)

Date	O_2(μmol/L)	CH_4(μmol/L)	CH_4 Std.Dev.	$\delta^{13}C$ DIC	$\delta^{13}C$ Std.Dev.
05/10/09	285.94				
06/10/09	284.35				
07/10/09	309.90	95.87	4.05		
08/10/09	319.49				
09/10/09	320.21				
10/10/09	317.64				
11/10/09	313.10	87.86	4.36		
12/10/09	338.66				
13/10/09	345.05				
14/10/09	332.27				
15/10/09	329.07	63.27	3.27		
18/10/09	322.68				
19/10/09	309.90				
20/10/09	293.93				
21/10/09	313.10	46.00	2.30		
22/10/09	293.93				
25/10/09	293.93				
26/10/09	252.40				
27/10/09	277.96	56.25	2.81	−1.25	
28/10/09	268.37				
29/10/09	284.35	56.00	2.80		
30/10/09	284.68				
31/10/09	285.75				
01/11/09	287.54				
02/11/09	271.57				
03/11/09	293.93	53.55	2.68		
04/11/09	261.98				
05/11/09	258.79	98.85	4.94	−1.61	
08/11/09	297.12				
09/11/09	259.43				
10/11/09	236.42	147.50	3.93		
11/11/09	217.25				
12/11/09	261.98	148.90	6.45		
13/11/09	259.71				
14/11/09	253.82				
15/11/09	246.01				
16/11/09	253.35				
17/11/09	239.62	100.90	5.05	−1.39	
18/11/09	233.23				
19/11/09	236.42	105.35	5.27	−1.57	
20/11/09	243.83				
21/11/09	250.78				
22/11/09	255.59				
23/11/09	268.37				
24/11/09	242.81	74.75	3.74	−1.99	
25/11/09	290.73				
26/11/09	276.63	80.75	4.00		
27/11/09	282.45				
28/11/09	265.78				
29/11/09	252.40				
30/11/09	277.96				
01/12/09	281.15	116.85	4.84	−2.24	
02/12/09	287.54				
03/12/09	249.20	123.50	5.38		
06/12/09	226.84				
07/12/09	246.01				
08/12/09	268.37	84.80	4.24		
09/12/09	233.23				
10/12/09	233.23	32.80	1.64	−1.99	
13/12/09	239.62				
14/12/09	210.86				
15/12/09	271.57				
16/12/09	271.57				
	Mean 309.06	Mean 79.88	Mean 3.71	Mean −1.33	Mean 0.03

mercury chloride in 100 mL purified water) per 9994 mL water sample) and stored in waxed 10 mL-borosilicate vials until measurement. Prior to measurements samples were sterile-filtered (0.2 μm) to eliminate bacteria and particulate organic matter. DIC was extracted from seawater with phosphoric acid in an automatic preparation line (Finnegan Gasbench I), coupled online with a Finnigan MAT 252 mass spectrometer to determine its $^{13}C/^{12}C$ ratio (Mackensen, 2013). All samples were run at least twice. For methane measurements water samples were 1:10 diluted with demineralized water, and topped with 2 mL Argon space for 12 h. Thereafter, methane concentrations from the head space were measured with a gas chromatograph (GC) (Chrompack, 9003) with flame ionization detector (FID). The standard error of duplicate measurements including both gas extraction and GC analysis was ~5%.

After 5 months we stopped the experiments, opened the high-pressure aquaria lids and pushed a circular steel sheet inside the pushcore tube to prevent a sediment loss through the drills in the upper pushcore tube during sampling. We fixed the pushcore head on

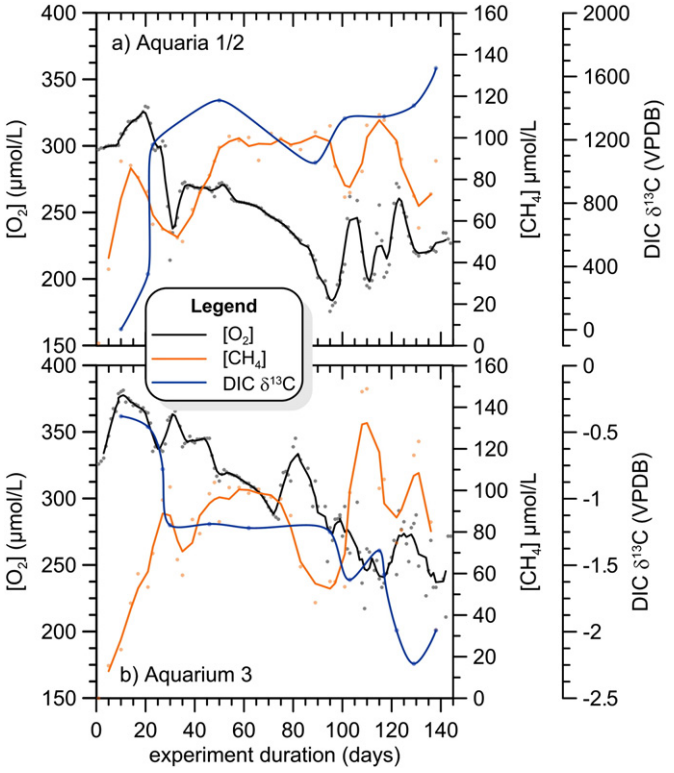

Fig. 4. Monitored concentrations of oxygen, methane and carbon isotopic composition of DIC in a) aquaria 1 and 2 (^{12}C-enriched methane added), and b) aquarium 3 (^{13}C-enriched methane added). Orange and black lines represent 3-pt running averages, and the blue line a smoothed spline through data.

the pushcore tube, and unscrewed the pushcore-tube catcher. This enabled the transfer of the pushcore–pushcore tube catcher unit onto a sediment ejector. The sediment was ejected, sliced in 1 cm thick slices and immediately wet-sieved with tap water using a 63 µm mesh. The residue was oven-dried at 50 °C. From the dried residue all benthic foraminifera from a minimum sample split of 1/8 equivalent to 10 cm^2 surface area × 1 cm sediment depth were selected and analyzed under an Axiovert 200 microscope (Table 3). Using an excitation wavelength of 470 nm experimentally precipitated specimen could be distinguished by their bright yellow-green color (emission wavelength 490 nm) from older non-fluorescent shells (Fig. 5). For stable isotope analyses specimens were selected not only from the split but the total residue.

2.4. Analytical methods

Foraminiferal stable isotope analyses were carried out on pristine fully Calcein-labeled thus fully fluorescent specimens. We have analyzed each shell carefully in fluorescent transmission light. Only when these analyses ensured that a specimen's septa and walls were exclusively built of layered fluorescent calcite, the walls were pristine and the pores perfect circles, this specimen was regarded as belonging to the experimental offspring (see Fig. 5 and Suppl. Plate). High-magnification analyses under fluorescent transmission light ensured that we didn't overlook a potential overgrowth by authigenic carbonates or corrosion. We didn't routinely verify the potential fluorescence of corroded specimens that were obviously already dead before the experiments started. However, when cross-checked for potential fluorescence we noticed that sometimes dull-looking as well as heavily corroded tests show a bright surface fluorescence under the fluorescence microscope (supplementary Plate 1). Apparently, Calcein penetrated to a certain depth into heavily corroded test parts. Yet, in contrast to our experimental offspring Calcein did not label the precipitated calcite crystallites but an amorphous-looking calcite mass on the corroded tests surface. The bright fluorescent layered chamber walls of Calcein-labeled precipitated shell material are lacking (Fig. 5).

According to the standard pretreatment of carbonate samples in isotope laboratories, tests were only ultrasonically cleaned if contaminations from sediments were visible under the microscope. This method was chosen despite the fact that the carbon isotopic composition of organic linings in foraminiferal shells is isotopically lighter than the calcite (e.g., Ravelo and Hillaire-Marcel, 2007) due to metabolic (respired) CO_2 bound in the organic linings (Ní Fhlaithearta et al., 2013.). However, experiments on different cleaning techniques of planktonic foraminifera prior to stable isotope analyses have shown that the difference between uncleaned samples and oxidized (organics removed) samples is statistically not significant (Löwemark et al., 2005). These authors even recommended analyzing untreated samples in order to prevent the loss of carbonate material from the later chambers that are thinner and thus preferentially removed during the oxidation step while the initial chambers from a different ontogenetic stage are preserved. Furthermore, one intension of this study was to support and elucidate the findings of Mackensen et al. (2006) on Rose Bengal stained foraminifers of the HMMV. Therefore, tests were treated in the same way and organics were not removed prior to isotope analyses.

The carbon stable isotopic composition of benthic foraminiferal tests was determined with a Finnigan MAT 251 isotope ratio gas mass spectrometer directly coupled to an automated carbonate preparation device (Kiel II) and calibrated via NIST 19 international standard to the Vienna Pee Dee Belemnite (VPDB) scale. Depending on the specimen size and shell thickness, we used pristine fully fluorescent specimens of *C. wuellerstorfi* (n = 3–5), *Lobatula lobatula* (n = 3–5), *Cassidulina neoteretis* (n = 25), *Epistominella exigua* (n = 25), *Pullenia bulloides* (n = 25), *Melonis zaandami* (n = 25), and *Cassidulina reniforme* (n = 40) for analyses. Due to static charging of their very thin shells, we lost most *Bolivina pseudopunctata* specimen from the gas mass spectrometer carousels hampering isotopic measurements on this at the HMMV abundant taxon. The precision of the measurements at 1σ based on replicate analyses of an internal laboratory standard (Solnhofen limestone) over a 1-year period was better than ±0.08 and ±0.06‰ for oxygen and carbon isotopes, respectively.

3. Results and discussion

3.1. High-pressure culturing of deep-sea foraminifera

After opening the aquaria we first noticed that mobile macrofauna, sea spiders and gastropods were still crawling on the sediment surfaces. When we analyzed the dried and sieved sediments we found that most rotaliid and lageniid species that according to previous Rose Bengal analyses were found living at this site (Rose Bengal analyses of Wollenburg and Mackensen, 2009), did reproduce during the duration of experiment (Table 3; Fig. 5). However, due since Calcein labeling just works for calcareous not agglutinated taxa and Calcein penetration can hardly been differentiated from precipitation in miliolids we could not verify a potential reproduction of miliolids and agglutinated species. The fully fluorescent offspring of rotaliid and lageniid species was 110, 102, and 128 specimens in one eight split of the total surface centimeter (78 cm^2 × 1 cm depth) in high-pressure aquaria 1, 2 and 3, respectively (Table 3). Since the number of fully fluorescent calcareous tests approximates the number of Rose Bengal stained specimens in previous field studies from that area (Wollenburg and Mackensen, 2010a,b), we deduce that Calcein neither affected the survival nor the reproduction of foraminifers in our cultures. Furthermore, despite the exposure to elevated “bottom water” methane concentrations and the cessation of phytodetritus rain specimen(s) of all abundant benthic rotaliid and lageniid species stayed alive and reproduced. As at the previously studied site PS66-02, the rotaliid foraminiferal fauna is strongly dominated by *B. pseudopunctata*, a species that already in the HMMV field study

Table 3
Absolute abundance of fully fluorescent experimental offspring per 10 cm^2, and number of fully fluorescent specimens selected for isotope measurements in high-pressure aquaria 1–3, and absolute number of Rose Bengal stained foraminifers per 10 cm^2 at site PS66-02 (Wollenburg and Mackensen, 2009) close to the ROV push-coring site. X denotes <1 specimen in Wollenburg and Mackensen (2009).

Species	High-pressure		High-pressure		High-pressure aquarium 3		
	No. spec. 10 cm^2	No. spec. selected for isotope measurement	No. spec. 10 cm^2	No. spec. selected for isotope measurement	No. spec. 10 cm^2	No. spec. selected for isotope measurement	PS66-02 no. spec. 10 cm^2 (Wollenburg and Mackensen, 2009)
Astrononion gallowayi	–	–	1	–	–	–	–
Bolivina pseudopunctata	83	328	57	456	80	559	164
Cassidulinoides bradyi	–	–	4	–	–	–	4
Cassidulina reniforme	6	51	4	32	15	127	15
Cassidulina teretis	4	31	4	35	23	91	6
Ceratobulimina arctica	2	–	1	–	2	–	X
Cibicides wuellerstorfi	2	16	3	22	2	16	1
Epistominella arctica	2	–	–	–	–	–	–
Epistominella exigua	2	13	2	18	–	–	–
Epistominella pusilla	–	–	1	–	–	–	–
Fursenkoina fusiformis	3	–	–	–	1	–	–
Guttulina glacialis	–	–	–	–	1	–	–
Ioanella tumidula	–	–	1	–	–	–	–
Islandiella helenae	–	–	1	–	–	–	–
Islandiella norcrossi	–	–	1	–	–	–	–
Lobatula lobatula	2	15	2	16	1	2	–
Marginulina glabra	–	–	–	–	1	–	–
Melonis zaandamae	1	12	1	17	–	0	–
Oridorsalis tener	2	6	1	3	1	2	–
Patellina corrugata	–	–	2	–	–	–	X
Pullenia bulloides	–	–	3	26	–	–	X
Pullenia osloensis	–	–	2	–	–	–	X
Pullenia quinqueloba	–	–	1	–	–	–	–
Robertinoides charlottensis	–	–	1	–	–	–	X
Robertinoides pumillum	–	–	1	–	–	–	–
Rosalina vilardeboana	–	–	1	–	–	–	–
Seabrokia earlandi	1	–	1	–	1	–	1
Scutuloris tegminis	–	–	1	–	–	–	–
Spirillina vivipara	–	–	1	–	–	–	X
Stainforthia spp.	–	–	1	–	–	–	–
Stetsonia horvathi	–	–	1	–	–	–	–
Unilocular spp.	–	–	1	–	–	–	X
Valvulineria arctica	–	–	1	–	–	–	X

was identified as a local methane seepage indicator species with dwindling abundances in the HMMV non-seepage oligotrophic surroundings (Soltwedel et al., 2005; Wollenburg and Mackensen, 2009). Since bacteria and archaea are the only carbon source that is much denser at the HMMV than in its' surroundings it has been suggested that *B. pseudopunctata* feeds on them rather than on phytodetritus. Such a diet may also explain why this species dominates our experimental offspring in similar proportions as at the previously studied seep site. Over the experimental running time the experimentally used methane source became progressively evident in the $\delta^{13}C_{DIC}$ signal. Thus, the bacterial and archaean density in the water column probably not decreased, but rather increased over the 5 months experimental running time. However, since *B. pseudopunctata* is an infaunal species, it should feed on carbon sources in the sediment. The cores we collected should already have had a high microbial density prior the experiments. If, after being cut off from HMMV's methane seepage from depths, the *S. contortum* specimens with their methane seep-dependant endosymbiontic bacteria died during the experiment, this likewise should have increased rather than decreased the bacterial density within the sediment. It is not surprising that the density of all other taxa decreased compared to *B. pseudopunctata* since all these species are presumed to be directly or indirectly (via bacteria-that-diet-on-phytodetritus) linked to an algae flux to the sea floor, which that was not provided during the experiment (Lutze and Thiel, 1989; Linke and Lutze, 1993; Gooday and Lambshead, 1989; Wollenburg and Mackensen, 1998). The fact that these taxa nonetheless reproduced in our aquaria was a pleasant surprise and may indicate that these taxa nourished either on phytodetritus that accumulated before coring or that they facultatively also digest bacteria or archaea (Hill et al., 2004; Mackensen et al., 2006).

Besides the time-consuming effort to isolate the experimental offspring, culture experiments on bulk sediments have been criticized because different sediment cores harbor multiple, varying and in parts unknown interacting environmental parameters (Hintz et al., 2004, 2006). However, it was also mentioned that at least for the reproduction of certain species culturing in original sediments is crucial (Hintz et al., 2004). For instance, none of the dominant species at the HMMV has ever been successfully cultured on artificial sediments. Furthermore, we deliberately ran experiments with sediment cores to verify the applicability of paleoenvironmental proxies in certain oceanic areas. Focus of this study was a verification of findings of field studies at the HMMV (Mackensen et al., 2006; Wollenburg and Mackensen, 2009).

The culturing technique described here allows foraminiferal culturing at ambient deep-sea environmental conditions for the first time. A high-pressure approach is important for the following reasons: (1) High-pressure culturing has allowed the first reproduction and shell growth of the barophilic *C. wuellerstorfi*. Proxies related to the shell composition of this species are the most valuable tools for reconstructions of past ocean deep-water circulation; (2) the solubility of gases in seawater is dependent on pressure. Thus, culture studies on the influence of CO_2 or methane on the geochemistry, isotopic composition or simple survival of calcareous deep-sea foraminifers (Bernhard et al., 2009) require the systems to run at equivalent pressure. (3) In oligotrophic deep-sea areas of e.g. the Arctic Ocean or North Atlantic or at sites with extremely high bacterial standing stocks, foraminifers may feed on bacteria (Gooday and Turley, 1990; Kröncke et al., 2000; Hill et al., 2004; Mackensen et al., 2006). Because many deep-sea bacteria are barophilic (Yayanos, 1986), culturing benthic foraminifera at atmospheric pressure may

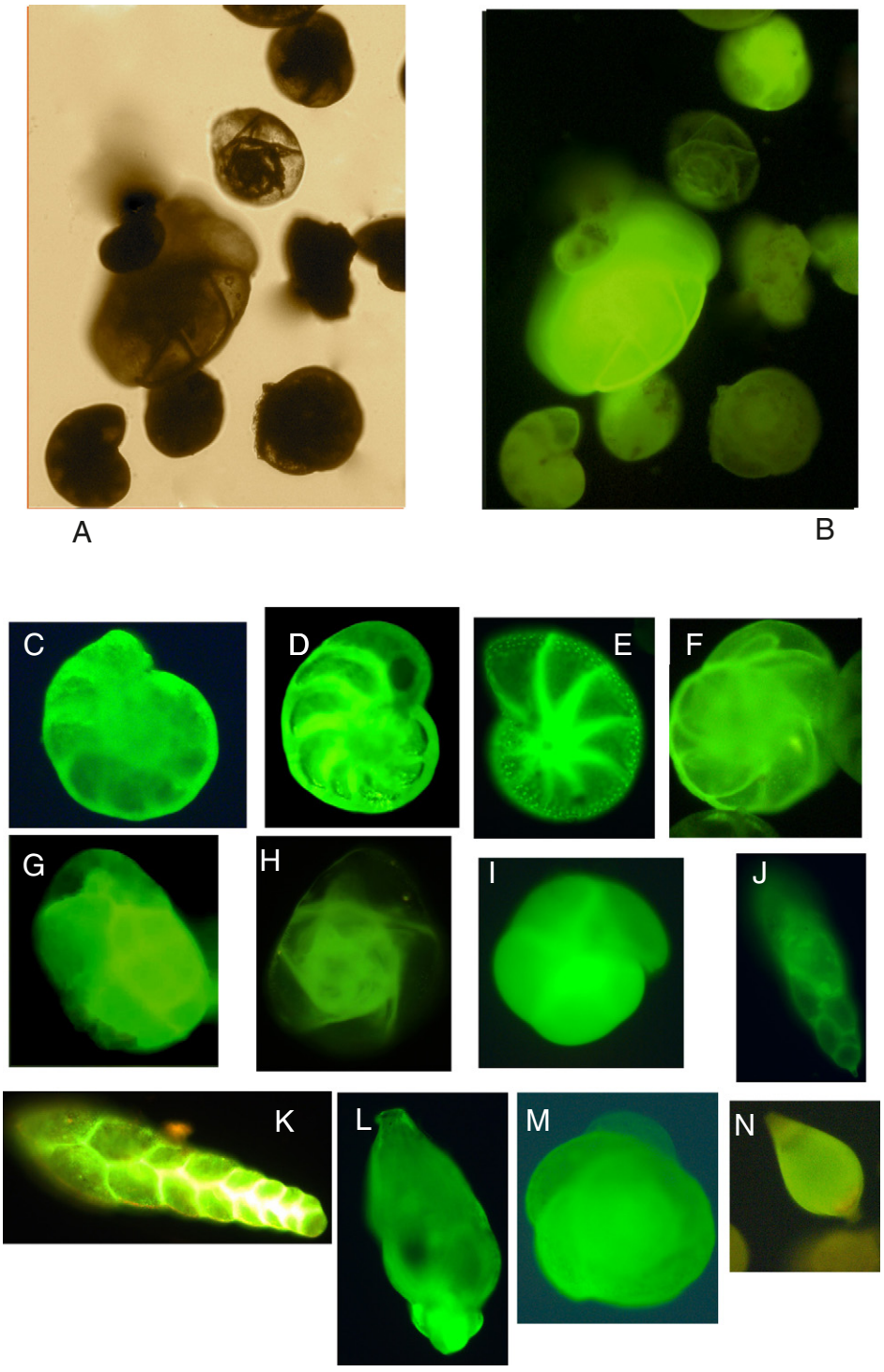

Fig. 5. Fluorescent experimental offspring of HMMV experiments 2007/2009. All photos were taken with an Axiovert microscope and connected Zeiss camera. A) A sample of pristine calcareous foraminifera from high-pressure aquarium 1 in 2009, viewed under normal light. B) Same sample viewed at 470 nm excitation and 490 nm emission. The image shows two bright fluorescent specimens of *Cassidulina teretis* among many non-fluorescent tests. C) Fully fluorescent juvenile *Cibicides wuellerstorfi*. D) Fully fluorescent adult *Cibicides wuellerstorfi*. E) Fully fluorescent *Melonis zaandami*. F) Fully fluorescent *Cassidulina teretis*. G) Fully fluorescent *Cassidulina reniforme*. H) Fully fluorescent *Epistominella exigua*. I) Fully fluorescent *Pullenia bulloides*. J) Fully fluorescent *Stainforthia loeblichi*. K) Fully fluorescent *Bolivina pseudopunctata*. L) Fully fluorescent *Bulimina aculeata*. M) Fully fluorescent *Trifarina angulosa*. N) Fully fluorescent *Seabrookia earlandi*. O) Fully fluorescent *Buccella tenerrima*. G) Fully fluorescent *Lagena stelligera*. Note that the intensity of fluorescence varies with shell thickness. Thin-shelled rotaliids like *E. exigua* show the maximum fluorescence in the older shell parts with increased shell-thickness. Furthermore, light intensity increases exponentially with respect to magnification, thus, brightness of fluorescence decreases when photos are taken at low magnification (e.g. D and L).

provide them with a likely different bacterial nutrition. (4) Cytoplasmic processes like the functioning of vacuoles and membranes differ at different pressures (Michiels et al., 2008). Thus, even if experimental results from deep-sea foraminifera cultured under atmospheric pressure are promising, there is a great chance that at a site-alike pressure, the cultured foraminifera would have behaved

differently or precipitated their shell with different geochemical or isotopic ratios.

3.2. Influence of methane on $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$

With our experimental setup we were able to increase the "bottom water" methane concentrations from approx. zero[3] in the original combined CTD cast (23 L) plus core site (2 L) volume, to maximum ~150 or a mean of ~80 μmol/L (Tables 1–2, Fig. 4).

By sterile-filtering of the "bottom water" bacteria and other particulate organic remains were removed. Carbon isotope analyses carried out on these sterile-filtered "bottom water" samples from our experiments showed a progressive in- and decrease in $\delta^{13}C_{DIC}$. Under the influence of almost pure ^{13}C-methane, "bottom water" samples from the aquaria 1 and 2 experiments showed a rapid increase in $\delta^{13}C_{DIC}$ over the initial three weeks of the experiment. After three weeks $\delta^{13}C_{DIC}$ remained at values that were constantly larger than 1000‰ (1055 to 1651‰, mean 1086‰) (Table 1; Fig. 4a). Within four weeks "bottom water" samples from aquarium 3, under the influence of ^{12}C-methane, dropped to a $\delta^{13}C_{DIC}$ value of −1.2‰. The $\delta^{13}C_{DIC}$ values remained around that value for approx. 2 1/2 months, then a final phase of decreasing $\delta^{13}C_{DIC}$ lead to minimum values of −2 to −2.2‰ at the end of the experiment (Table 2; Fig. 4b). Since methane can only be converted to DIC by methanotrophs we can infer from these results that methanotrophic bacteria stayed alive in our systems and converted the methane to DIC with high and low $\delta^{13}C_{DIC}$ values for the ^{13}C- and ^{12}C-experiment, respectively.

The revealed extremely high $\delta^{13}C_{DIC}$ values of our sterile-filtered and thus bacteria-free water samples from aquaria 1 and 2 were a pleasant surprise. However, our routinely used mass spectrometers use VPDB (for "Vienna PDB") as a standard that is calibrated to the original but yet exhausted *Belemnitella americana* standard (PDB). This cretaceous belemnite had an anomalously high ^{13}C:^{12}C ratio of 0.0112372, and was established as $\delta^{13}C$ value of zero. The use of this standard gives most natural material a negative $\delta^{13}C$. However, more problematic than other factors is the mathematics behind the established carbon-13 work. The delta notation $\delta^{13}C$ is an isotopic signature, a measure of the ratio of stable isotopes ^{13}C: ^{12}C, reported in parts per thousand (per mil, ‰), $\delta^{13}C_{sample} = ((13C_{sample} / 12C_{sample}) / (^{13}C_{PDB} / ^{12}C_{PDB}) - 1) \times 1000$. Therefore, the mean $\delta^{13}C_{DIC}$ value of 1086 in the ^{13}C methane experiment equals a contribution of 2% ^{13}C to the total ^{12} & ^{13}C DIC carbon pool during the experiment. This means that in the ^{13}C methane experiments with 99% ^{13}C in the methane source, the methane source contributed presumably with up to 3% to the total DIC signal (Fig. 4).

We had transferred up to 300 specimen of *B. pseudopunctata* in single vials from the mass spectrometer. However, due to the species extremely thin and light weighted shell we lost most specimens during the preparation and/or measurement. The low carbonate level led to unreliable low intensities, thus values are not shown in this manuscript. The measured $\delta^{13}C_{test}$ values of the experimental offspring reflect the used methane source (Table 4, Fig. 6). For the ^{13}C methane experiment the interspecies and species-specific $\delta^{13}C_{test}$ values are very variable. The $\delta^{13}C_{test}$ values of deep infaunal living foraminifers (*P. bulloides* $\delta^{13}C_{test} = 14.99$‰ and *M. zaandami* $\delta^{13}C_{test} = 15.51$‰) and shallow infaunal *C. neoteretis* ($\delta^{13}C_{test} = 2.38$‰) were lower than the $\delta^{13}C_{test}$ values of epizoic or epifaunal foraminifers (*C. wuellerstorfi* mean $\delta^{13}C_{test} = 59.24$‰, *L. lobatula* mean $\delta^{13}C_{test} = 30.23$‰, *E. exigua* $\delta^{13}C_{test} = 23.18$‰) and shallow infaunal *C. reniforme* mean $\delta^{13}C_{test} = 401.17$‰. *M. zaandami* and *P. bulloides* are seldom found at HMMV sites (Wollenburg and Mackensen, 2010a,b) and could also not be revealed in sediments of high-pressure aquarium 3. Carbon isotope analyses on fully fluorescent specimens from aquarium 3 revealed mean $\delta^{13}C_{test}$ values of −1.44‰ for *C. wuellerstorfi*, −1.85‰ for *C. reniforme*, and −2.24‰ for *C. neoteretis*. Hereby, the mean carbon isotope values of these species approximate values measured on Rose Bengal stained specimen from the coring site (Mackensen et al., 2006; Fig. 6).

Although the external calibration of artificially high ^{13}C concentrations against the low ^{13}C standard may have led to inaccurate isotope ratio values in the ^{13}C methane experiment, carbon isotope analyses on water samples collected during the experiments showed that the methane source was partly converted to CO_2 and contributed to a respective DIC signal and that at least part of this signal is reflected in the carbon isotope signature of the analyzed foraminiferal tests. The observed high variability in $\delta^{13}C_{test}$ values may be due to following reasons (Fig. 6; Table 3): 1) High variability in foraminiferal proxies is also observed when benthic foraminifera are cultured on artificial sediments (Hintz et al., 2006; McCorkle et al., 2008; Dissard et al., 2010; Filipsson et al., 2010) or as isolated specimens (Mewes et al., 2014). 2) Like for the DIC measurements the measured $\delta^{13}C_{test}$ values of the ^{13}C methane experiment are far beyond natural values and may thus be afflicted with large errors. 3) The mathematical formula for the calculation of delta notations is especially sensitive for artificially high ^{13}C values. However, despite the uncertainties regarding the precision of analyzed $\delta^{13}C$ values, the fact that the admixture of ^{13}C-methane in "bottom water" led to significantly enriched $\delta^{13}C$ of both the DIC and calcite tests is an undoubted proof that the carbon isotopic composition of benthic seep foraminiferal shells is affected by methane seepage. At the beginning of the experiments the retrieved sediments should have hosted the same $\delta^{13}C$-depleted bacterial standing stock and sedimentary organic matter inventory as the control experiment of aquarium 3 and the sediments we retrieved from the area during various previous expeditions (Mackensen et al., 2006). Solely the addition of methane with a different isotopic composition altered the stable carbon isotope ratios of epifaunal foraminiferal calcite in these cores to ^{13}C-enriched values.

The conversion of methane to DIC by methanotrophs was slow at the beginning of the experiments, and experimental mean DIC values were only reached within 3–4 weeks after the start of the experiments (Fig. 4a). After this initial phase the measured $\delta^{13}C_{DIC}$ in aquaria 1 and 2 stayed at $\delta^{13}C_{DIC} > 1000$‰, those of aquarium 3 $\delta^{13}C_{DIC} < -1.2$. Since the water was continuously changed it is quite unlikely that $\delta^{13}C_{DIC}$ values of the experimental "bottom water" between the days at which samples were collected were much lower or higher.

Measurements from aquaria 1 and 2 showed a significant discrepancy between the relatively stable high $\delta^{13}C_{DIC}$ and much lower $\delta^{13}C_{test}$ (Fig. 6a). This may be due to following potential reasons:

1) It could be possible that most specimens died very early in the experiment when the $\delta^{13}C_{DIC}$ values were still low. However, sea spiders and gastropods were still crawling on the sediment surface in aquarium 2, being a biological proof that the "bottom water" was constantly well oxygenated. In the present experiments, we achieved our mean experimental methane concentration four days after starting the experiments; meanwhile the $\delta^{13}C_{DIC}$ was still low (3.24‰). Within the next eleven days, the $\delta^{13}C_{DIC}$ increased to >352‰, thus increasing quite steeply at the beginning of the experiment (Fig. 4a). Assuming an absolute match between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$, the reproduction and chamber formation of the majority of analyzed individuals of *C. wuellerstorfi* should have happened during the first week. Regarding the low chamber formation rate of deep-sea foraminifers of 1–3 chambers per 5 month at low temperatures of 4 °C (no observation at 1 °C), this is a relatively unrealistic assumption (Filipsson et al., 2010).
2) The general offset between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$ in aquaria 1 and 2 may reflect a different sensitivity of the mass spectrometers to artificially high $\delta^{13}C$ values. Sensitivity studies are requested to evaluate if and at which artificially high ^{13}C concentrations these

[3] Zero methane concentrations in bottom waters at siboglinoid tubeworm sites are a common phenomenon at the HMMV and result from a rapid dilution of methane in bottom water flow above the HMMV (Damm and Budéus, 2003; Sauter et al., 2006).

Table 4
Carbon and oxygen isotope ratios recorded in calcareous foraminiferal tests of experimental offspring and their standard deviation.

a) Foraminiferal offspring from aquaria 1 & 2

C. wuellerstorfi				*L. lobatula*				*C. reniforme*			
$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.	$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.	$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.
28.95	0.16	3.72	0.17	61.10	0.01	3.58	0.03	235.66	0.01	4.10	0.06
220.51	0.02	3.62	0.02	15.04	0.01	3.81	0.06	566.67	0.13	4.10	0.02
27.82	0.01	3.75	0.03	28.54	0.02	3.46	0.06				
23.39	0.01	3.80	0.01	20.93	0.01	3.64	0.02				
54.96	0.03	3.64	0.08	26.69	0.01	3.72	0.03				
30.16	0.01	3.72	0.04	29.05	0.02	3.59	0.08				
95.93	0.01	3.53	0.03								
27.93	0.21	3.64	0.40								
23.53	0.01	3.68	0.03								
Mean 59.24	Mean 0.05	Mean 3.77	Mean 0.09	Mean 30.23	Mean 0.01	Mean 3.68	Mean 0.05	Mean 401.17	Mean 0.07	Mean 4.10	Mean 0.04

P. bulloides				*E. exigua*				*C. teretis*			
$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.	$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.	$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.
14.99	0.01	4.45	0.02	23.18	0.02	3.68	0.02	2.38	0.00	4.30	0.02

M. zaandami			
$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.
15.51	0.01	3.96	0.01

b) Foraminiferal offspring from aquarium 3

C. wuellerstorfi				*C. reniforme*				*C. teretis*			
$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.	$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.	$\delta_{13}C$	$\delta_{13}C$ Std.Dev.	$\delta_{18}O$	$\delta_{18}O$ Std.Dev.
−1.90	0.01	3.87	0.02	−1.15	0.03	3.92	0.02	−2.73	0.01	4.35	0.02
−1.88	0.01	3.80	0.01	−1.93	0.02	4.02	0.03	−2.58	0.02	4.31	0.02
−1.66	0.02	3.76	0.03	−2.47	0.01	4.03	0.02	−2.73	0.01	4.37	0.04
−0.52	0.01	3.90	0.01								
Mean −1.44	Mean 0.01	Mean 3.83	Mean 0.02	Mean −1.85	Mean 0.02	Mean 3.99	Mean 0.02	Mean −2.24	Mean 0.01	Mean 4.345	Mean 0.02

spectrometers may measure reliable and comparable isotope values, in a potential repetition of experiments carried out in this study.

3) Although each $\delta^{13}C_{test}$ analysis represents an average of 4 to 30 specimens, the variability in $^{13}C_{test}$ from aquaria 1 and 2 was extremely high (Fig. 6a). It has been shown that differences in the $\delta^{13}C_{test}$ values can be attributed to different size classes of the analyzed specimens, with higher $\delta^{13}C_{test}$ and $\delta^{18}C_{test}$ measured in larger specimens (Schmiedl et al., 2004; McCorkle et al., 2008; Schumacher et al., 2010). In this study, most analyzed *C. wuellerstorfi* and *L. lobatula* specimens were small adults (>125 to 200 μm); only two measurements were carried out on larger specimens. Therefore, it is impossible that the large variability in $\delta^{13}C_{test}$ from aquaria 1 and 2 offspring are due to a growth effect (Schumacher et al., 2010), which is confirmed by the fact that it is just observed in the $\delta^{13}C$, and not in the $\delta^{18}O$ values.
4) Specimens of *Amphistegina lessonii* were shown to grow in the same experimental set-up at very variable speeds (Mewes et al., 2014). Therefore, the variable $\delta^{13}C_{test}$ values could be attributed to variable growth rates, thereby reflecting variable DIC records of the early versus the late culturing period.
5) Furthermore, and potentially the most likely cause, the methane source contributed with only a maximum of 3% to the whole $\delta^{13}C_{DIC}$ signal and its contribution may be significantly less at the water sediment interface, on pebbles in depressions, or even in the sediment. This assumption is supported by the observation that the $\delta^{13}C_{test}$ values of infaunal species (*P. bulloides*, *M. zaandami*, *C. neoteretis*) are, with exception of *C. reniforme*, lower than those of epifaunal species (*C. wuellerstorfi*, *L. lobatula*, *E. exigua*) (Fig. 6a). During the experiment ^{13}C-methane was added to the "bottom water" only whereas, no pore water was exchanged. Therefore, from the lower $\delta^{13}C_{test}$ values of endobenthic foraminifers it can be inferred that the ^{13}C-enriched "bottom water" penetrated only little into the sediments.
6) We did not feed our faunas, and the cultured sediments contained abundant pre-experimental $\delta^{13}C$-depleted organic matter. Therefore, the observed strong variability in $\delta^{13}C_{test}$ values of epifaunal species, similar to the discrepancy between DIC and shell measurements, may indicate a contribution of metabolically derived CO_2 to test calcification through a variable consumption rate of sedimentary bacteria that feed on these organic remains or an incorporation of the organic matter itself (Hill et al., 2004; Mackensen et al., 2006). The smallest magnitude of influence of ^{13}C-enriched "bottom water" on $\delta^{13}C_{DIC}$ and infaunal species is reflected in the $\delta^{13}C_{test}$ of *C. neoteretis*. Since it has been suggested that this shallow-infaunal species feeds on bacteria (Gooday and Lambshead, 1989), we assume that either the species' presumed diet on significantly ^{13}C-depleted bacteria outweighs the small "bottom water" DIC influence, or that they secreted their tests at a deeper sediment level where the "bottom water" DIC influence was close to zero. The apparent match between $\delta^{13}C_{test}$ of experimental offspring in experiment 3 and in-situ measurements from Mackensen et al. (2006) corroborates these assumptions (Fig. 6b). Although we continuously changed the "bottom water" at the sediment surface, decaying ^{12}C-depleted sedimentary organic matter may have affected $\delta^{13}C_{test}$ of epi- and infaunal foraminifera with increasing intensity.

The results of this study corroborate all field studies while at methane seepage sites the $\delta^{13}C_{test}$ value of living foraminifers is usually not in equilibrium with the pore or bottom water $\delta^{13}C_{DIC}$ (Torres et al., 2003; Herguera et al., 2004; Hill et al, 2003, 2004; Rathburn et al., 2003; Martin et al., 2004; Mackensen et al., 2006; Lobegeier and Sen Gupta, 2008; Bernhard et al., 2010; Herguera et al., 2014). However, the $\delta^{13}C$-experiment also shows that not only bottom and pore water $\delta^{13}C_{DIC}$ but also the foraminiferal diet contributes to the $\delta^{13}C_{test}$ signal (Hill et al., 2004; Mackensen et al., 2006). Yet, this study first of all

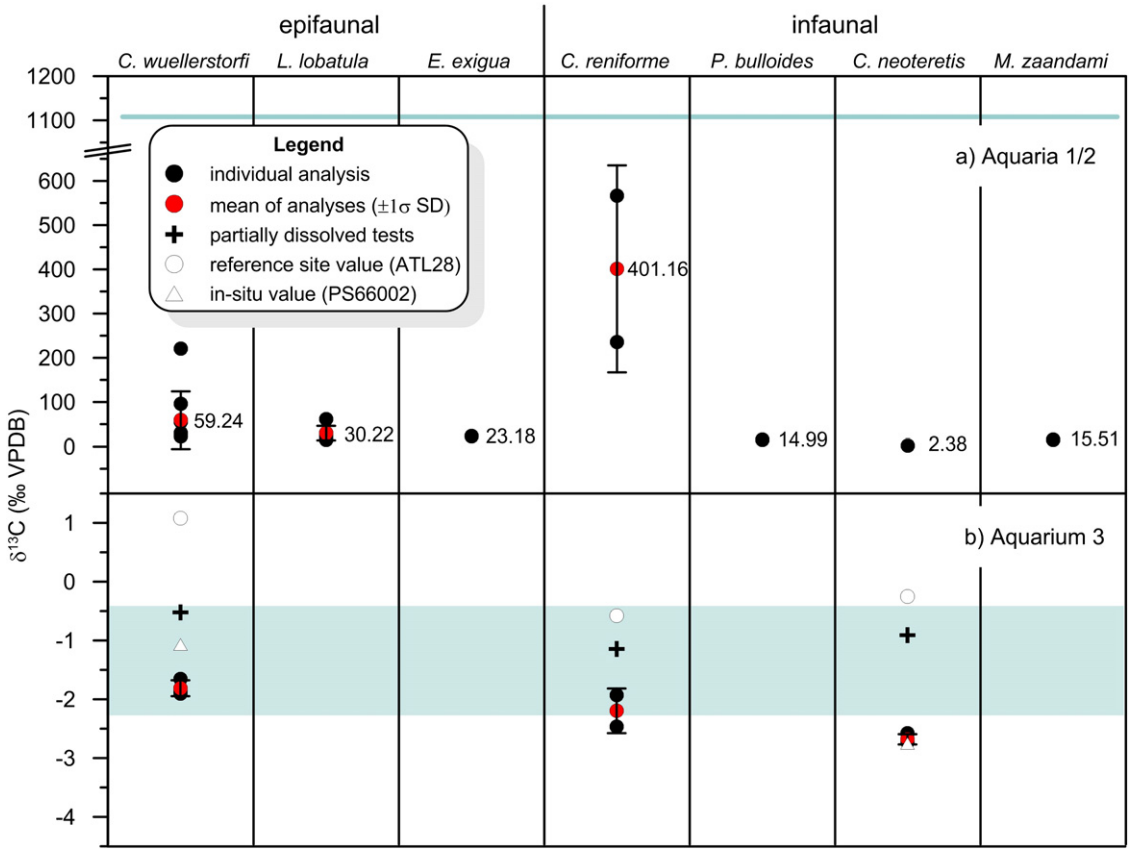

Fig. 6. Carbon isotopic composition of epibenthic and endobenthic foraminifera from a) aquaria 1 and 2 (^{12}C-enriched methane added), and b) aquarium 3 (^{13}C-enriched methane added). Numbers in a) denote the mean $\delta^{13}C$ to emphasize increased values in this experiment. The blueish shaded areas represent the range of $\delta^{13}C_{DIC}$ measured throughout the experiment.

shows that at least at some methane seepage sites benthic foraminifers are able to grow and reproduce, incorporating this mixed isotope signature (compare Torres et al, 2003; Bernhard et al., 2010; Herguera et al., 2014).

It has been shown that Calcein does not affect the uptake of Mg or Sr (Dissard et al., 2009). In our experiments the experimentally used methane sources are reflected in the carbon isotopic composition of the fluorescent offspring. Moreover, for the standard ^{12}C methane experiment the $\delta^{13}C_{test}$ values of *C. wuellerstorfi* match those from the field study by Mackensen et al. (2006). Therefore, we deduce that Calcein had no significant influence on the carbon isotopic composition of analyzed foraminiferal shells.

Corrosion of empty foraminiferal shells in the first centimeter of surface sediments has already been described from HMMV sediments (Wollenburg and Mackensen, 2009). Even in our experimental offspring we could detect fully fluorescent tests that showed dull, thus slightly corroded test portions suggesting that these specimens did not live until the end of the experiments. If we assume that this very initial carbonate corrosion did not cause a significant preferential removal of the light isotope ^{12}C (Skidmore et al., 2004; Schulz and Zabel, 2006), the higher $\delta^{13}C_{test}$ recorded in these tests (Fig. 6b, measurements marked by crosses) may reflect early thus less depleted experimental $\delta^{13}C_{DIC}$ in the aquarium 3 experiment. The mean bottom water $\delta^{13}C_{DIC}$ over the last four months in this aquarium is −1.6‰, with values progressively decreasing towards the end of the experiments (Fig. 4b). Analyses of pristine and fully fluorescent *C. wuellerstorfi* offspring are in accordance with the mean $\delta^{13}C_{DIC}$ and decreasing $\delta^{13}C_{DIC}$ trend (Fig. 6b). Adjusted by a vital effect of ~ −0.9‰ (calculated according to Andrews and Dunhill, 2004; Mackensen et al., 2006), this also applies to *C. neoteretis*. However, since measurements of *C. neoteretis* offspring are in accordance with the in-situ reference site values of Mackensen et al. (2006) and the species $\delta^{13}C$ values from the 13 C methane experiment were also only slightly increased, the species $\delta^{13}C$ may mainly reflect their nutrition on sedimentary bacteria.

3.3. Future perspectives

Our study was challenging in many ways. We have developed and constantly improved a novel time-consuming deep-water culturing technology. At the same time, we have simulated seepage conditions not knowing whether the methanotrophs would stay alive, which methane concentrations our foraminifers would tolerate, or how the supply of pure ^{13}C-methane would be reflected in "bottom water" DIC when the sediments were loaded with significantly ^{13}C-depleted organic matter. Hence, the results of this study show that benthic foraminifera can live at oxic sites of methane seepage and methane seepage can be reflected in the carbon isotopic composition of species living under such conditions. However, this is just the beginning of future multiple experiments involving aquatic microbiota, "bottom water" and methane supply to get an idea of how much methane might be reflected in $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$ values. Additional experiments could then be run on foraminiferal isolates, a culturing approach that the majority of modern studies follow (e.g. Hintz et al., 2004, 2006; McCorkle et al., 2008; Allison and Austin, 2008; Allison et al., 2010; Dissard et al., 2009,

2010) and DIC-preconditioned water could be used to constrain the experimental results. However, compared to the cultivation of whole sediments (Hemleben and Kitazato, 1995) only a small number of species reproduces once detached from the sediments (e.g. Hintz et al., 2004, 2006; McCorkle et al., 2008). Thus, we might determine that it simply needs a combination of various faunal, geochemical and physical influences to both stimulate reproduction in many foraminiferal taxa and to generate reliable $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$ values in a geological sense.

4. Summary

We have developed facilities and techniques that enable culturing deep-water sediment samples and faunas at ambient conditions. They enable reliable culture experiments on pressure-sensible parameters like seawater-gas concentrations or the composition of the seawater and sedimentary microbiota. After the experiments we found fully Calcein fluorescent specimens of all foraminiferal species known to live at the respective core sites in our cultured sediments. Particularly important, it was possible for the first time to successfully culture *C. wuellerstorfi*, the species whose test composition is the most commonly used as recorder of paleo-deep water conditions. In our experiments we verified the influence of methane-induced changes in $\delta^{13}C_{DIC}$ on $\delta^{13}C_{foraminiferal\ test}$ of benthic deep-sea foraminifera from hydrocarbon seeps. For that, we injected ^{13}C-enriched methane to the experimental "bottom water". During the experiment methanotrophs obviously stayed active and converted the experimentally added methane source to $\delta^{13}C$-enriched DIC. Although it was not possible to keep $\delta^{13}C_{DIC}$ constant over the 5-month duration, the used methane source is reflected in $\delta^{13}C_{test}$ of experimental offspring, indicating that methane emanation impacts the carbon isotopic composition of deep-sea benthic foraminifera. Ongoing and future culture experiments under in-situ pressure will enable us to test and verify the bundle of paleoproxies linked to the isotopic and geochemical composition of calcareous tests of benthic deep-sea foraminifera, including barophilic species such as *C. wuellerstorfi*.

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