



UiT The Arctic University of Norway

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# **Assessing the relationship between living benthic foraminifera and methane emission in the Arctic Ocean**

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## Preface

This thesis is the result of a four-year PhD study undertaken at the Centre for Arctic Gas Hydrate, Environment and Climate (CAGE), Department of Geosciences, at the University of Tromsø (UiT the Arctic University of Norway), between October 2015 and August 2021. The project was financed by the Research Council of Norway through its Centre of Excellence funding scheme grant no. 223259, the NORCUST project, The Loeblich and Tappan Student Research Award, and a Travel grant from The Norwegian Research School in Climate Dynamics (ResClim). The candidate wishes to thank the supervisors of the first part of the PhD study and who supervised the study for the first paper: J. Bernhard, G. Panieri and M. Hald. The candidate also thanks supervisors J. Bernhard and M. Hald, who supervised the study for the second paper that included samples provided by G. Panieri. The candidate also thanks the supervisors for the second part of the PhD study: T.L. Rasmussen, T. Treude and M. Zajączkowski, and K. Szybor, who supplied the samples and supervised the project part for the last two papers.

The PhD program at UiT required that 25% (one-year equivalent) of the four-year period be dedicated to undertaking duty work which was fulfilled through the preparation and teaching classes in *Micropaleontology* (GEO-3122) and *Reconstructing Quaternary Marine Climate and Environments* (GEO-3111), as well as assistance in The Stable Isotope Laboratory (SIL) at the Department of Geosciences, UiT. Samples for this project were obtained during a 5-month laboratory experimental period at the Bernhard Lab: Benthic Foraminifera Ecology and Paleoecology, Woods Hole Oceanographic Institution, along with collecting surface sediment samples during several CAGE research expeditions to the Svalbard margin on board the R.V. *Helmer Hanssen* (2015 to 2017). Samples were also made available for the second part of the PhD study from a cruise with R.V. *Poseidon* to Vestnesa Ridge, NV Svalbard margin in 2011.

The doctoral thesis resulted in four first-authored scientific articles, which contribute new knowledge on benthic foraminifera and their geochemistry associated with methane cold seeps. These articles are listed below:

1. **Melaniuk, K., Bernhard, J.M., Hald, M., Panieri, G.** Impact of hypoxia and high  $p\text{CO}_2$  and diet on benthic foraminiferal growth: experiment with propagules. Manuscript in revision.
2. **Melaniuk, K.** Effectiveness of Fluorescent Viability Assays in Studies of Arctic Cold Seep Foraminifera. *Frontiers in Marine Science* **8**, doi:10.3389/fmars.2021.587748 (2021).
3. **Melaniuk, K., Szybor, K., Treude, T., Sommer, S., Rasmussen, T.L.** Evidence for influence of methane seepage on isotopic signatures in living deep-sea foraminifera, 79 °N. Manuscript in revision in Scientific Reports.
4. **Melaniuk, K., Szybor, K., Treude, T., Sommer, S., Zajączkowski, M., Rasmussen, T.L.** Response of benthic foraminifera to ecological succession in cold seeps from Vestnesa Ridge; implications for interpretations of paleo-seepage environments. Manuscript in preparation.

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As Franklin D. Roosevelt said, “a smooth sea never made a skilled sailor”. Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people, but mostly from my main supervisor Tine L. Rasmussen. I could not have completed this dissertation without her support. I want to thank you for the support, encouragement, and all of the opportunities I was given to further my research. I would like to show my appreciation for my current co-supervisors Tina Treude and Marek Zajączkowski, for their strong support and scientific input which greatly improved my work.

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# 1 MOTIVATION AND OBJECTIVES

Methane is a powerful greenhouse gas, produced in marine sediments either by exposing deep complex organic molecules to high temperatures or by microbial transformation of organic and inorganic carbon at more shallow depths (Reeburgh, 2007; Strapoć et al., 2020). At temperatures lower than 25°C and a moderate pressure greater than 3–5 MPa, corresponding to a combined water and sediment depth of 300–400 m, methane forms ice-like structures called methane hydrates (Reeburgh et al., 2007; Ruppel and Kessler, 2017). Deposits of methane hydrates are widespread in marine sediments on continental margins and are known to be sensitive to environmental changes, such as for example temperature increases and/or changes in pressure or sediment movements (e.g., Archer et al., 2009; Maslin et al., 2010). Past massive methane releases from sub-seabed reservoirs have been linked to changes in climatic conditions, with an increase in temperature recorded during Quaternary and the Paleocene (Wefer et al., 1994; Smith et al., 2001), Late Paleocene (Kennett and Stott, 1991; Dickens et al., 1997; Katz et al., 1999), and Cretaceous (Jahren et al., 2001). As large amounts of methane are stored on Arctic continental margins in the form of gas hydrates, concern has increased that ongoing ocean warming will trigger destabilization of the gas hydrate reservoirs and cause further release of methane in the future (IPCC, 2007; Phrampus and Hornbach, 2012).

Several studies have proposed that the negative carbon isotope signature ( $\delta^{13}\text{C}$  up to -40‰) measured in carbonate tests of fossil foraminifera might reflect past methane seepages, and that fossil foraminifera have a high potential as a tool in tracking past methane releases (Millo et al., 2005; Martin et al., 2010; Consolaro et al., 2015; Sztaybor and Rasmussen, 2017; Schneider et al., 2017). The  $\delta^{13}\text{C}$  levels in calcium carbonate of some fossil foraminifera can be lower than -10‰ (Hill et al., 2003; Schneider et al., 2017) and  $\delta^{13}\text{C}$  measured in calcite of 'live' (Rose Bengal stained) foraminifera generally do not exceed -7.5‰ (Mackensen et al., 2006; Wollenburg et al., 2015). Thus, it still remains unclear whether (and to what extent) living foraminifera incorporate methane-derived carbon during their biomineralization, and/or if the isotopic signatures in their shells are mostly a result of authigenic overgrowth from precipitation of carbonates by diagenetic processes. It has also been suggested that the  $^{13}\text{C}$ -depleted carbon from methane might be incorporated by the benthic foraminifera from the dissolved inorganic carbon (DIC) pool from the ambient seawater and porewater. Alternatively, foraminifera might feed on (Panieri, 2006), or live in symbiosis with, methane-oxidizing bacteria, which carry a  $^{13}\text{C}$ -depleted carbon signal as suggested by Hill et al. (2004). Remarkably negative  $\delta^{13}\text{C}$  values (up to -40‰) in foraminiferal tests have been shown to derive mainly from overgrowth by methane-derived authigenic carbonates (MDAC; Torres et al., 2003; Consolaro et al., 2015; Sztaybor and Rasmussen, 2017; Schneider et al., 2017).

At cold seeps, the biogeochemical processes involving methane, such as aerobic and anaerobic methane oxidation, affect the properties of pore water in which the benthic foraminifera live. The venting of methane from sub-seabed deposits supports growth of methane-oxidizing bacteria that may serve as food for foraminifera, but simultaneously, microbial activity causes a decrease in the surrounding oxygen concentration, leading to hypoxia or even anoxia, release of hydrogen sulfide H<sub>2</sub>S, and an increase in the partial pressure of carbon dioxide ( $p\text{CO}_2$ ). For many foraminiferal species, oxygen is crucial for an efficient generation of cellular energy (Heinz and Geslin, 2012) and changes in water chemistry, such as increase in  $p\text{CO}_2$ , might affect the process of calcification (Allison et al., 2010). For these reasons, some studies assert that despite the abundance of food (e.g., methanotrophic bacteria) due to the local environmental conditions, cold seeps are hostile environments for foraminifera, and that foraminifera do not calcify during active methane seepage (Torres et al., 2003; Herguera et al., 2014), and the foraminifera which inhabit cold seeps should be adapted to organic-rich and reducing environments (Rathburn et al., 2000, 2003; Bernhard et al., 2001; Torres et al., 2003; Fontanier et al., 2014). Modern cold seeps provide a good analogue for past methane-rich environments and offer an opportunity to investigate possible effects of methane seepage on isotopic signatures and distribution patterns of living benthic foraminifera, which can be further used as an analogue in interpretation of palaeoceanography and intensity of paleo-methane seepage.

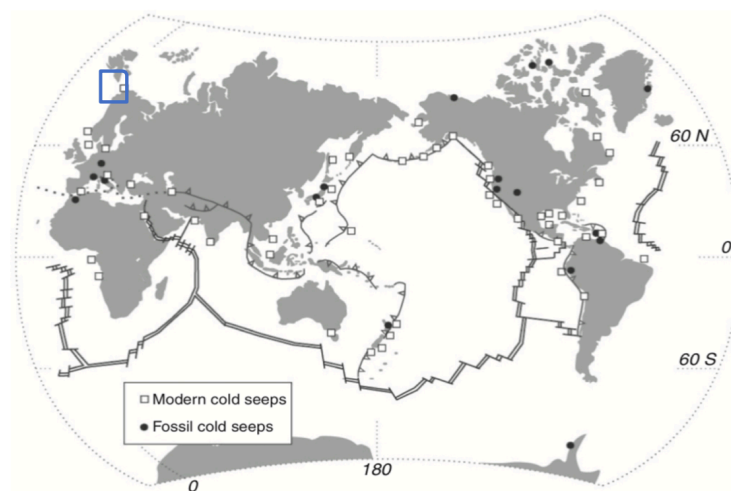
**The main objectives of this doctoral thesis are to:**

- study the ability of foraminifera to survive under low oxygen (hypoxia) conditions, elevated  $p\text{CO}_2$ , and a combined effect of both in conjunction with diet (methanotroph vs algal) by experiments with juvenile benthic foraminifera in Biospherix C-Chambers; Article 1
- testing the effectiveness of fluorescent viability assays in studies of living cold seep foraminifera; Article 2
- compare the carbon isotopic signature ( $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ ) in tests of metabolically active (CellTracker™ Green CMFDA and CellHunt Green labelled) foraminifera (Article 2) and Rose Bengal stained (Article 3) to determine whether methane seepage has any effect on the isotopic signatures of the calcite of living benthic foraminifera.
- investigate modern foraminiferal assemblages from the Arctic cold seeps; Articles 3 and 4
- investigate the impact of methane-related biological processes (MOx and AOM) on the benthic foraminiferal communities; Article 4

## 2 BACKGROUND

### 2.1 Methane cold seeps

Cold seeps are chemosynthetic ecosystems, in which hydrocarbon-rich fluids seep from the sub-seabed gas hydrate deposits or from other petroleum reservoirs providing a carbon and/or energy source (e.g., Sloan 1990; Barry et al., 1997; Olu et al., 1997; Coleman and Ballard 2001; Sahling et al., 2003; Levin, 2005). These ecosystems are commonly found in continental margin environments, both tectonically active and passive, and in terrestrial lake areas (Fig. 1).



**Figure 1.** Distribution of modern and fossil cold seeps, blue square indicate the study area (Figure from Levin, 2005, modified from Campbell et al. 2002).

The hydrocarbon-rich fluids originate from decaying organic matter (e.g., sapropel), from thermogenic degradation of organic matter of marine or terrestrial origin, or biogenic processes (Strąpoć, 2020). Gas seepage can manifest itself in form of gas bubbles escaping from the seabed observable by eye, or evident as acoustical plumes recorded through echo sounding. Other signs are pockmarks (i.e. shallow seabed depressions) or other topographic and geomorphological structures, such as pingos or mud volcanoes. Concentration and strength of flow of methane varies between seeps, and within the same seep site creating different microhabitats. The supply of methane can fluctuate over time, so that the methane flux determines the ephemeral nature of cold seep environments. Heterogeneity in permeability and methane flux results in a spatial heterogeneity in the flux rates and environments. This in turn results in a patchy distribution of biological communities (Tryon and Brown 2001; Levin, 2005). For example, vesicomid clams hosting endosymbiotic chemoautotrophic bacteria are commonly associated with downward directed flows (inflow) and oscillatory flows (Tryon and Brown

2001; Levin, 2005). Bacterial mats are dominated by large, filamentous, sulphide-oxidizing bacteria: *Beggiatoa*, *Thioploca*, *Arcobacter*, and *Thiothrix* that indicate a more consistent and high methane flux (Tryon and Brown 2001; Tryon et al., 2002). Cold-seep biota largely relies on oxidation of sulphur and methane reduced by microorganisms for nutrition, and possibly even on nitrogen fixation (Levin, 2005). Within a cold seep, gas emission from the sub-seabed reservoirs are controlled by aerobic methane oxidation (MOx) or, in a lack of oxygen, by anaerobic methane oxidation (AOM) coupled with sulphate reduction (Knittel and Boetius, 2009; Treude et al., 2007; Orphan et al., 2001). The process of AOM is conducted by anaerobic methanotrophic archaea (ANME; Milucka et al. 2012) or by microbial consortia of ANME and sulphate-reducing bacteria found within the sulphate-methane transition zone (SMTZ; Boetius et al., 2000; Knittel et al., 2005). The carbon isotopic signature ( $\delta^{13}\text{C}$ ) of methane depends on the origin of the methane, with the result that the  $\delta^{13}\text{C}$  from microbial methane have much lighter  $^{13}\text{C}$  signatures (from -110‰ to -60‰) than the thermogenic methane (from -50‰ to -20‰; Whiticar, 1999; Valentine, 2002). As a product of microbial activity, the light  $^{13}\text{C}$  carbon isotope is released in the form of carbon dioxide gas ( $\text{CO}_2$ ) or bicarbonate ions ( $\text{HCO}_3^{2-}$ ) into the sediment and/or ambient water, leading to changes in the water chemistry and isotopic signature of the ambient seawater and pore water (Whiticar, 1999; Treude et al., 2007). Bicarbonate produced during AOM enables carbonate precipitation, which provides a secondary hard-bottom for tubeworms to grow on. Additionally, in anoxic conditions hydrogen sulphide ( $\text{H}_2\text{S}$ ) is produced. The compound is highly toxic for marine organisms; it inhibits ATP (Adenozyno-5'-trifosforan) production by binding to cytochrome c oxidase (CytOx; Somero et al., 1989).

## 2.2 Modern cold-seep benthic foraminifera

Studies on 'live' benthic foraminifera (Rose Bengal-stained) inhabiting hydrocarbon seeps were previously conducted at several locations worldwide, including Oregon Hydrate Ridge (Torres et al., 2003; Hill et al., 2004), Monterey Bay (Rathburn et al., 2003), northern Adriatic Sea (Panieri, 2006), Gulf of Guinea (Fontanier et al., 2014), Blake Ridge (Panieri and Sen Gupta, 2008), Barents Sea (Mackensen et al., 2006; Wollenburg and Mackensen, 2009; Dessandier et al., 2019), and New Zealand (Martin et al., 2010). Several studies have shown that the abundance of foraminifera increases near active fluid discharge spots, indicating that benthic foraminifera may potentially be attracted by the availability of food, for example microbial mats (see e.g., Rathburn et al., 2000; Torres et al., 2003; Heinz et al., 2005; Panieri, 2006; Panieri and Sen Gupta, 2008). However, this may not hold true for all seeps. In Monterey Bay, for example, the abundance of foraminifera is lower at seep sites than at non-seep sites (Bernhard et al., 2001). The distribution of foraminifera might be uneven within one seep,



and is most likely conditioned by variation in microhabitats e.g., presence of bacterial mats or clam beds (e.g., Rathburn et al., 2000; Torres et al., 2003; Panieri and Sen Gupta, 2008; Wollenburg and Mackensen, 2009; Dessandier et al., 2019).

So far, the results of different studies of species compositions of benthic foraminiferal faunas have been consistent and show that there are no endemic species associated with cold seep ecosystems (e.g., Szybor and Rasmussen, 2017 and references therein). Species which are usually present within seep sites have been documented in non-seep marine environments (e.g., Rathburn et al., 2000; Bernhard et al., 2001; Hill et al., 2003; Panieri, 2006; Etiope et al., 2014; Herguera et al., 2014). With a majority of foraminiferal species being aerobic, individuals present at cold seep sites should be able to survive the local geochemical constraints, such as for example low oxygen levels (including temporary anoxia) or presence of toxic hydrogen sulfide (H<sub>2</sub>S; Herguera et al., 2014). The most common species observed at cold seeps belong to several genera, such as *Bolivina*, *Bulimina*, *Nonionella* and *Uvigerina*, which are adapted to organic-rich and reducing environments (e.g., Akimoto et al., 1994, Rathburn et al., 2000, 2003, Bernhard et al., 2001, Torres et al., 2003, Fontanier et al., 2014). Studies from the Gulf of Mexico show that some species, such as *Bolivina albatrossi*, *Cassidulina neocarinata* and *Trifarina bradyi*, are facultative anaerobes able to survive temporary anoxic conditions below bacterial mats (*Beggiatoa* sp.) and show some H<sub>2</sub>S tolerance (Sen Gupta et al., 1997). Epifaunal species, for example *Cibicidoides wuellerstorfi* or *Cibicides lobatulus*, show very specific adaptations, where individuals tend to colonize the outer surface of *Siboglinidae* tubeworms in order to escape H<sub>2</sub>S and/or anoxic conditions (Sen Gupta et al., 2007; Wollenburg et al., 2009).

### **2.3 Stable isotope signatures in modern benthic foraminifera from cold seeps**

Opinions are divided on whether the carbon isotopic signatures ( $\delta^{13}\text{C}$ ) of living calcareous foraminiferal tests from methane seeps reflect any sort of incorporation of methane-derived carbon. Some studies show that the  $\delta^{13}\text{C}$  measured in tests of living foraminifera collected from active seeps are not markedly lower than those from non-seep sites, indicating that living foraminifera might not be able to record the episodes of methane release (e.g., Torres et al., 2003, Rathburn et al., 2003, Etiope et al., 2014; Melaniuk, 2021). A shift of approximately 0–4‰ towards a more negative  $\delta^{13}\text{C}$  was shown to have an origin from a local organic matter degradation (e.g., Torres et al., 2003, Martin et al., 2004). Hostile conditions, such as low oxygen or anoxia combined with high carbon dioxide concentration ( $p\text{CO}_2$ ), most likely inhibit calcification during methane seepage (Herguera et al., 2014). Thus, it has been proposed that foraminifera do not calcify during active methane discharges, but

instead build tests during no or reduced methane flux (Torres et al., 2003). Alternatively, in case of lack of oxygen the foraminifera might migrate to other more oxygenated locations (Bernhard et al., 2010).

Several studies indicate that methane has an effect on isotopic signatures of 'live' benthic foraminifera (i.e. Rose Bengal-stained). For example, the  $\delta^{13}\text{C}$  of tests of *Uvigerina peregrina* was found to be as low as -5.64‰ at cold seeps, while at the control site the value was not lower than -0.81‰ (Hill et al., 2004), and the isotopic signature of *Cassidulina neoteretis* was as low as -7.5‰ (Mackensen et al., 2006), thus indicating that both species were potentially affected by methane. Similarly, the negative  $\delta^{13}\text{C}$  value in tests of epifaunal species, such as *Cibicides* sp., can be explained by the incorporation of light carbon isotope from the ambient seawater (i.e. pore water or bottom water in which the foraminifera calcified) which was transported from deeper sediments by tubeworms inhabiting the methane seeps (Mackensen et al., 2006; Wollenburg et al., 2009). In most cases, the  $\delta^{13}\text{C}$  measured on foraminiferal tests from cold seeps has shown larger degrees of variations when compared to non-seep sites (e.g., Rathburn et al., 2003; Bernhard et al., 2010). Additionally, individuals collected from sites covered with bacterial mats show more negative  $\delta^{13}\text{C}$  both in tests and cytoplasm when compared to individuals from non-seep sites (e.g., Hill et al., 2005; Panieri, 2006). This implies that isotopically lighter food, such as for example methanotrophic bacteria, and/or presence of symbionts contribute to the isotopic signatures of foraminifera from cold seeps (Hill et al., 2004; Bernhard et al., 2010).

## 2.4 Fossil foraminifera from cold seeps

Both planktic and benthic foraminifera preserved in the methane influenced sediments can be affected by precipitation of Methane-Derived Authigenic Carbonates (MDAC) (Torres et al., 2003; Uchida et al., 2004; Martin et al., 2010). Bicarbonate ions ( $\text{HCO}_3^-$ ) are products of anaerobic oxidation of methane (AOM) and enable MDAC formation (see above; chapter 2.1). As a result, the tests of fossil foraminifera from cold seeps are characterized by strongly depleted  $\delta^{13}\text{C}$  values and high Mg content from overgrowth of their shells by MDAC. Eventual primary signals in foraminiferal tests can thus be overprinted by the diagenetic alterations from coatings of both the outside and/or inside of the tests (Schneider et al., 2017) and consist of up to 60% of the total volume of the tests (Torres et al., 2003). Overprinting results in shift in the  $\delta^{13}\text{C}$  of the primary signal of about 15–29‰ and results in  $\delta^{13}\text{C}$  values lower than -10‰ as it was suggested by Schneider et al. (2017) and up to -34.1‰ (Panieri et al., 2017). It is believed that those highly negative values recorded in tests are evidence of increase in methane flux, particularly the past migrations of the sulphate-methane transition zone (SMTZ; Consolaro et al., 2015; Szybor and Rasmussen., 2017; Schneider et al., 2017). Methane seepage events are recorded

in the isotopic signature of foraminiferal tests, but because of the coating of MDAC the foraminifera may date (based on AMS<sup>14</sup>C dating) significantly older than the foraminiferal tests itself. Distinction between the primary and the secondary isotopic signals in fossil foraminifera is a large challenge, thus signals cannot be used to determine a precise stratigraphic history and timing of the seepage (Torres et al., 2003; Martin et al., 2004). Abnormal depletions in  $\delta^{13}\text{C}$  can only provide qualitative information about presence of cold seeps (Martin et al., 2004).

## **3 BACKGROUND**

### **3.1 Laboratory experiment**

#### **Propagule Method**

The Propagule Method is an experimental tool for testing the ecology of benthic foraminifera, and the response of multi-species assemblages to selected environmental parameters (Alve and Goldstein, 2014; Article 1). Propagules are small juvenile foraminifera approximately 10  $\mu\text{m}$  in size, stored in the sediment in form of a “propagule bank”. Propagules are able to delay growth (from months to years) until the environmental conditions become favorable (Alve and Goldstein, 2010). Propagules are isolated from adult foraminifera by sieving the sediment on mesh-size <53  $\mu\text{m}$ .

**Advantages using the Propagule Method:** (from Alve and Goldstein 2014).

- both live and dead individuals harvested at the end of the experiments have responded positively to the treatment,
- focuses on the critical, juvenile developmental stages,
- simple experimental set-up, design to test the effects of changing environmental conditions at assemblage level under controlled conditions (i.e., different assemblages grown from the same propagule bank),
- both small and large foraminiferal species can be studied,
- use the original sediments, which helps to optimally mimic their natural conditions,

#### **Experimental set-up**

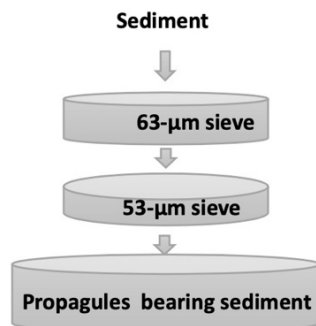
For the purpose of the experiment (Article 1), foraminifera-bearing sediments were collected from the Barents Sea and the Norwegian Sea using a box corer (Fig. 2). Samples were processed according to the propagule method (Goldstein and Alve, 2011). The <53  $\mu\text{m}$ -sediment fraction was divided between four experimental treatments I, II, III, IV, and incubated inside Biospherix C-Chambers

(Parish, New York, USA) for 5 months. Four experimental treatments were designed to simulate different environmental conditions, including oxygen-saturated to hypoxic conditions, modern-day  $p\text{CO}_2$  to elevated  $p\text{CO}_2$ , and dual-stress conditions (hypoxia and elevated  $p\text{CO}_2$ ; Fig. 5; Table 2). To investigate whether diet can affect the growth of foraminifera, once a week the foraminifera were fed by either a mix of algae (*Dunaliella tertiolecta*, Butcher, and *Isochrysis galbana*, Parke) or by a methanotrophic bacterium (*Methyloprofundus sedimenti* PKF-14).

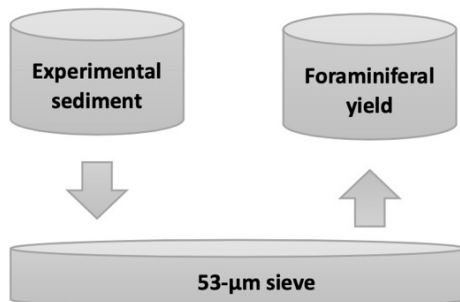
**Table 2.** Experimental treatments (I, II, III, IV),  $\text{O}_2$  (ml/L) and  $\text{CO}_2$  (ppm) concentration, gas sources (400-ppm  $\text{CO}_2$ , 1%  $\text{CO}_2$  /99%  $\text{N}_2$ , and  $\text{N}_2$ ), gas sensors and controllers used for the experiment.

Treatment	$\text{O}_2$ (ml/L)	$p\text{CO}_2$ (ppm)	Gas source	Gas sensors and controllers	Imitated environmental conditions
I	saturated	400	400-ppm $\text{CO}_2$	No controllers	Modern atmospheric conditions
II	0.7	400	1% $\text{CO}_2$ in $\text{N}_2$ ; $\text{N}_2$	Pro $\text{CO}_2$ , ProOx	Hypoxia, modern $p\text{CO}_2$ concentration
III	saturated	2000	1% $\text{CO}_2$ in $\text{N}_2$	Pro $\text{CO}_2$	Oxygenated, elevated- $p\text{CO}_2$ concentration
IV	0.7	2000	1% $\text{CO}_2$ in $\text{N}_2$ ; $\text{N}_2$	Pro $\text{CO}_2$ , ProOx	Dual-stress

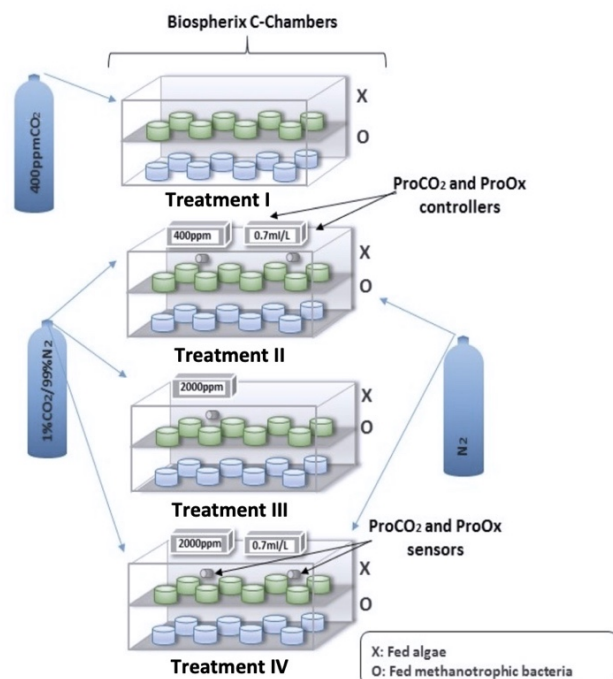
### 1. Preparation before the experiment



### 3. Termination of the experiment



### 2. Experiment



**Figure 5.** Schematic of experiment.

## 3.2 Benthic foraminiferal faunas and isotopic signatures

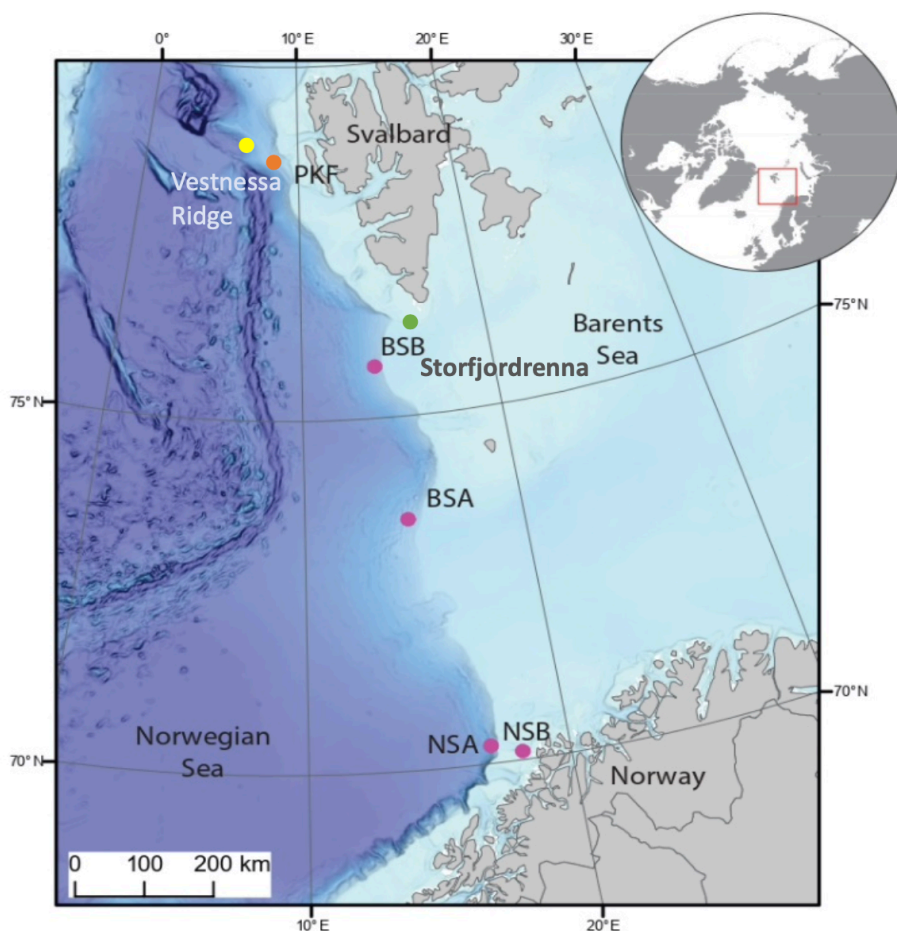
### 3.2.1 Study areas

#### **Vestnesa Ridge:**

Vestnesa Ridge is a deep-sea cold seep area (>1000 m), located in the Fram Strait (~ 1200 m depth), northwest of Svalbard in the Arctic Ocean (79°N, 5–7°E; Fig. 2). The ridge is characterized by a series of perforations called pockmarks (i.e., shallow seabed depressions) where methane-rich fluids are found seeping from gas hydrates and other free-gas reservoirs (Bünz et al., 2012; Plaza Faverola et al., 2015). Of these, the two most active pockmarks have been informally called ‘Lomvi’ and ‘Lunde’ (Bünz et al., 2012). The presence of methane (mostly of thermogenic origin) has been documented both in the sediment and water column and by the recovery of methane hydrates in sediment cores. Sediment core analyses have shown presence of fossil seep-related macrofaunal communities at Vestnesa Ridge (Ambrose et al., 2015; Szybor and Rasmussen, 2017; Hansen et al., 2017; Thomsen et al., 2019) and diagenetic alterations in isotopic signatures ( $\delta^{13}\text{C}$ ) of fossil foraminiferal tests caused by MDAC precipitation (Schneider et al., 2017; Szybor and Rasmussen, 2017). Several seafloor observations revealed presence of megafaunas (Åström et al., 2016, 2017) and carbonate outcrops (Szybor and Rasmussen, 2017; Himmler et al., 2018) associated with methane emission.

#### **Storfjordrenna pingos:**

Storfjordrenna is located at the SW Svalbard continental shelf, in the north-western Barents Sea (76°N, 16°E), at an approximate water depth of 400 m (Fig. 2). The area is characterized by five gas hydrate mounds (pingo-like features) spread within a 2 km<sup>2</sup> area. Gas hydrates pingos (GHP) are known to be 8–12 m high, with diameters ranging from 280–450 m. Georeferenced seabed imagery indicates the presence of chemosynthetic macrofaunas associated with cold seeps (Åström et al., 2016; Sen et al., 2018). Four out of five GHPs show active methane seepage in the form of gas flares around summits, where one is mostly in a “post-active stage” (no visible flare on echo sounder recordings) (Serov et al., 2017; Sen et al., 2018; Hong et al., 2018). Elevated concentrations of methane mostly of thermogenic origin have been detected in both sediments and bottom water and gas hydrates were also discovered from several sediment cores (Hong et al., 2018).



**Figure 2.** Map showing sampling locations for Article 1 indicated by pink dots: BSA-Barents Sea A, BSB- Barents Sea B, NSA- Norwegian Sea A, NSB- Norwegian Sea B. Sampling locations for Articles 2, 3, and 4 are indicated by dots: yellow - Vestnesa Ridge (2, 3, 4); orange - control site (2); green - Storfjordrenna pingo site (2).

### 3.2.2 Sampling and samples treatments

The sediment samples used in Article 2 were collected from Lomvi and Lunde pockmarks at the Vestnesa Ridge during the CAGE 15-2 cruise and from the Storfjordrenna pingos during the CAGE 17-2 cruise (Fig. 2; Table 1), both onboard R.V *Helmer Hanssen* using combined Towed Digital Camera and Multicoring System (TowCam) developed at the Woods Hole Oceanographic Institution's (WHOI) Multidisciplinary Instrumentation in Support of Oceanographic (MISO) Facility. The live-stream feed from TowCam system were used to describe the seafloor conditions and locate active methane vents, authigenic carbonates and bacterial mats, which then enabled an accurate guide of different sampling locations. Cores collected from the Vestnesa Ridge were subsampled onboard into 1-cm thick (10 cm in diameter) horizontal intervals (0–1cm, 1–2 cm, 2–3 cm) using a flat spatula and transferred into plastic containers (HDPE bottles). The sediment was divided on the basis of different treatments:

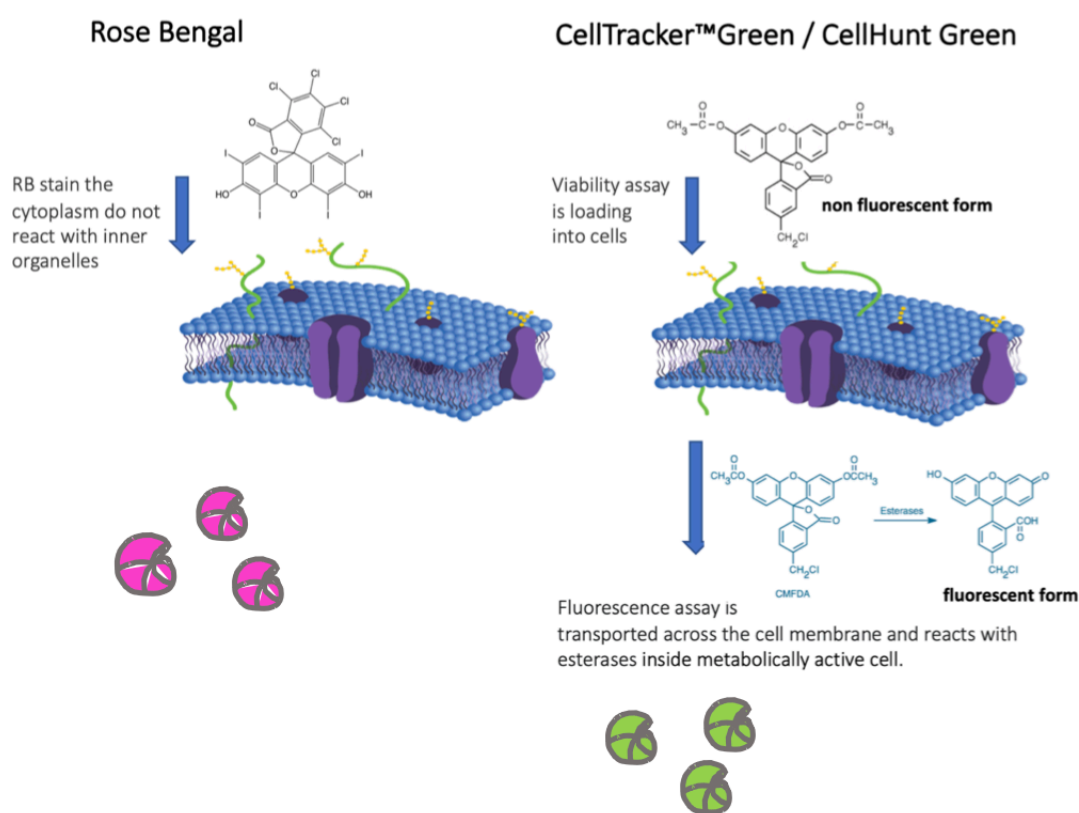
CellTracker™ Green CMFDA (*Thermo Fisher Scientific*) with final concentration of CellTracker™ Green CMFDA 1µM in a sample (Bernhard et al., 2006), and Rose Bengal (2g/L; Schönfeld et al., 2007), preserved with formalin and stored at 4°C until further laboratory processing. Cores collected from Storfjordrenna were processed following a similar protocol, except that the whole sediment was labelled with CellHunt Green and preserved in 96% ethanol.

The sediment used for Article 3 and 4 was collected during the POS419 expedition of the R.V *Poseidon* from the Lunde pockmark (Table 1). Selected multicores were processed on board, and subsampled into 1-cm thick horizontal slices down to 5 cm core depth. The samples were transferred into plastic containers, and stained with Rose Bengal-ethanol solution following the FOBIMO protocol (2 g\L; Schönfeld et al., 2007). Samples were kept onboard in a dark, cool room at +4 °C until further processing. Additional subcores were sampled for sediment pore water analyses, sediment methane analyses and for the determination of methane concentration, methane oxidation, and sulfate reduction. All sediment sampling procedures were conducted at +4 °C inside a cooled laboratory.

**Table 1.** Sampling sites locations, coordinates, water depth, date of sampling, and environmental characteristics at site of multicores used in from Article 2, 3, and 4.

Core number	Location	Coordinates	Water depth (m)	Date	Environmental characteristics
<b>Article 2</b>					
MC 893A MC 893B	Vestnesa Ridge (Lomvi pockmark)	79.18N, 00.44E	1200	20 May 2015	bacterial mats
MC 886	Vestnesa Ridge (Lunde pockmark)	79.38N, 00.04E	1200	20 May 2015	black mud, <i>Siboglinidae</i> tubeworms
MC 880A MC 880B	Site 7808 (Control site)	78.44N, 00.50E	889	19 May 2015	grey homogeneous mud
MC 884	Site 7808 (Control site)	78.30N, 00.82E	900	19 May 2015	grey homogeneous mud
MC 902	Storfjordrenna Pingo (GHP1)	76.91N, 16.08E	377	22 June 2017	strong flares, anemones, <i>Siboglinidae</i> tube worms
MC 917	Storfjordrenna Pingo (GHP1)	76.93N, 16.02E	377	23 June 2017	trawl marks, muddy sediment, anemones, <i>Siboglinidae</i> tubeworms, sea spider, patches of bacterial mats
MC 919	Storfjordrenna Pingo (GHP1)	76.96N, 15.98E	378	23 June 2017	trawl mark, <i>Siboglinidae</i> tubeworms, bacterial mats, anemones, carbonates
MC 920	Storfjordrenna Pingo (GHP5)	76.70N, 16.00E	379	23 June 2017	trawl marks, anemones, hard substrate (carbonates)
MC 921	Storfjordrenna Pingo (GHP5)	76.72N, 16.40E	380	23 June 2017	trawl marks, anemones, hard substrate (carbonates)

MC 922	Storfjordrenna Pingo (GHP5)	76.74N, 16.37E	386	23 June 2017	trawl marks, muddy sediment, Siboglinidae tube worms, anemones, seastars, shrimps
<b>Article 3 and 4</b>					
MUC 10	Vestnesa Ridge (Lunde pockmark)	79.46N 06.27E	1241	25.08.2011	<i>Siboglinidae</i> tubeworms
MUC 8	Vestnesa Ridge (Lunde pockmark)	79. 60N 06.09E	1204	25.08.2011	<i>Siboglinidae</i> tubeworms
MUC 12	Vestnesa Ridge (Lunde pockmark)	79.41N 06.13E	1235	29.08.2011	bacterial mats
MUC 11	Control site	78,77N 06,06E	1191	28.08. 2011	grey homogeneous mud



**Figure 3.** Comparison between Rose Bengal staining method and CellTracker™ Green/ CellHunt Green labelling. Rose Bengal reacts with cytoplasm of foraminiferal tests giving the pink colorization (panel on the left). Fluorescent assays enter a cell and non-fluorescent form is converted into the fluorescent form which further can react with thiols on proteins and peptides, giving green colours in fluorescent light (right panel).

Rose Bengal was designed to detect the presence of cytoplasm; thus, this staining method is known to colour both live and recently dead foraminiferal cytoplasm. The stain can also adhere to the organic lining of foraminiferal tests as well as to the bacteria that can be found attached or located inside these tests (Bernhard et al., 2001, 2006). Rose Bengal staining of recently dead specimens may

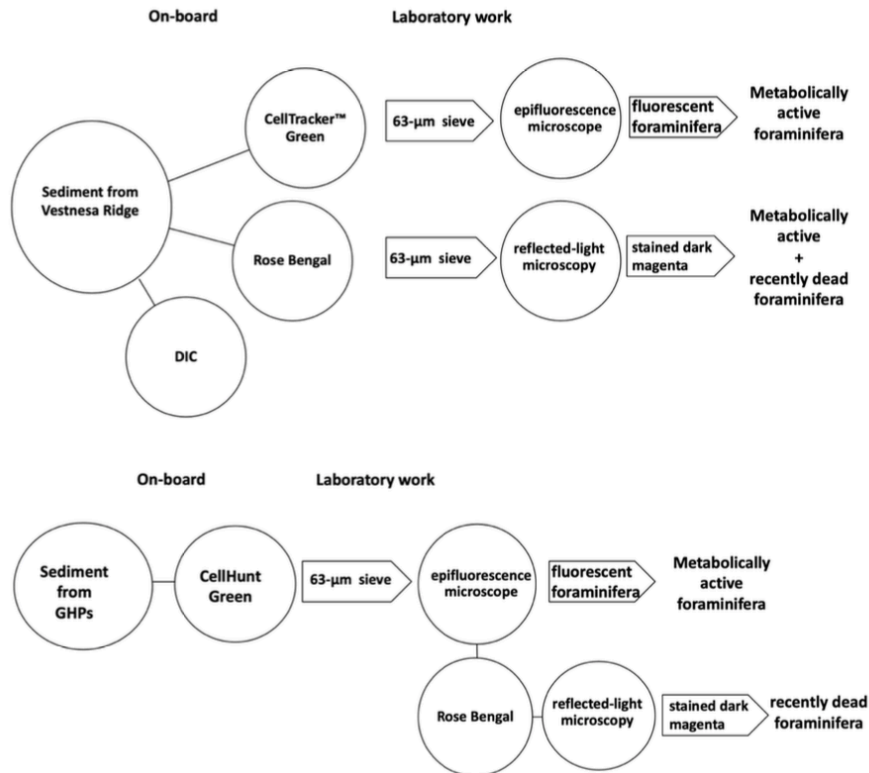


occur from several weeks to months after an individual's death, especially in colder, low oxygenated waters as decomposition of cytoplasm is relatively slower (Jorissen et al., 1995; Bernhard et al., 2001). Consequently, already dead, or recently dead foraminifera appear as live individuals (Bernhard et al., 2006). In contrast, CellTracker™ Green CMFDA (5-chloromethyl fluorescein diacetate; *Thermo Fisher Scientific*) and CellHunt Green (SETAREH biotech, LLC) are vital non-toxic fluorescent dyes (the same compounds) which react with internal cell components, resulting in green-fluorescent adducts (Fig. 3). Probes react with metabolically active cells only.

### **3.2.3 Benthic foraminiferal faunas**

In Article 2, the live benthic foraminifera were identified and quantified in wet samples of material >63µm. Both CellTracker™ Green and CellHunt Green labelled organisms were examined using an epifluorescence-equipped stereomicroscope (485-nm excitation; 520-nm emission). All individuals that fluoresced brightly in at least half of their chambers were considered as live individuals, picked wet and placed on micropalaeontological slides (Fig. 4). Additionally, after selecting all green individuals from CellHunt Green labelled sediments, the residue was subsequently stained with Rose Bengal for approximately 24h. Samples stained with Rose Bengal were examined using reflected-light microscopy (Fig. 4). Foraminifera which stained dark magenta in at least half of their chambers were picked and mounted on micropaleontological slides. All collected foraminifera were identified, counted and sorted by species.

In Article 3 and 4, the Rose-Bengal stained foraminifera from the >100-µm fraction were examined under reflected-light microscopy. All benthic foraminiferal individuals that stained dark magenta and were fully filled with cytoplasm were considered to be 'living' foraminifera i.e., live + recently dead individuals, still containing cytoplasm, and individuals showing no colorization were considered as unstained, empty (dead) individuals. Specimens of the planktic foraminiferal species *Neogloboquadrina pachyderma* from each core were picked and investigated using Scanning Electron Microscopy (SEM) to detect presence of authigenic overgrowth on the outer surface of the test.



**Figure 4.** Onboard and laboratory processing of the sediment collected by multicorer from Vestnesa Ridge and from Storfjordrenna pings.

### 3.2.4 Stable isotope analysis ( $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ )

For stable isotope measurements (Article 2), the most frequently occurring species such as *Melonis barleeanus*, *Cassidulina neoteretis*, *Nonionellina labradorica* and planktic foraminiferal species *Neogloboquadrina pachyderma* were picked, selecting approximately 10 CellTracker™ Green/CellHunt Green labelled, Rose Bengal and empty specimens. Carbon-13 compositions of calcium carbonate tests of benthic foraminifera were determined on a MAT 253 Isotope Ratio Mass Spectrometer (Department of Geoscience, UiT) with analytical precision estimated to be better than 0.07 ‰ for  $\delta^{13}\text{C}$  by measuring a certified standard NBS-19. For Article 3, stable isotope analyses were performed on both Rose Bengal stained and empty specimens of *M. barleeanus*, *C. neoteretis*, *C. wuellerstorfi*, and *N. pachyderma* in separate analyses. When present, approximately 10 specimens of each species were taken from each sample. Isotopic measurements were performed at Woods Hole Oceanographic Institution (WHOI). Data are reported in standard notation ( $\delta^{13}\text{C}$ ,  $\delta^{18}\text{O}$ ), according to the Pee Dee Belemnite (PDB) standard. In both cases isotopic values were expressed as conventional  $\delta$  notation against the Vienna Pee Dee Belemnite (V-PDB) standard (1.96‰, -10.21‰ and -48.95‰ for  $\delta^{13}\text{C}$ ) and reported in parts per thousand (per mil, ‰).

## 4 SUMMARY OF MANUSCRIPTS/ ARTICLES

### Article 1

Katarzyna Melaniuk, Joan M. Bernhard, Morten Hald, Giuliana Panieri. **Impact of hypoxia and high  $p\text{CO}_2$  and diet on benthic foraminiferal growth: experiment with propagules.**

In this manuscript, we present the results of the laboratory experiment. The experimental set-up was designed to investigate the impact of environmental parameters, such as oxygen and  $p\text{CO}_2$ , and diet (methanotrophs vs algal diet) on benthic foraminiferal growth. To establish natural assemblages, sediments were collected from the western Barents Sea and the North Norwegian continental margin and further processed following the propagule method (Goldstein and Alve, 2011). The method is used to treat experimental assemblages in their original sediments, which helps to optimally mimic their natural conditions (Goldstein and Alve, 2011). To test the response of juvenile foraminifera to different environmental conditions, foraminifera-bearing sediment was split into 64 microcosms (translucent plastic containers), and evenly divided between the four experimental treatments, hosted in Biospherix C-Chambers. Each of the treatments represented different environmental parameters, including saturated oxygen to hypoxia (0.7 ml/L), modern-day  $p\text{CO}_2$  (400 ppm) to elevated  $p\text{CO}_2$  (2000 ppm), and dual stressors of hypoxia and elevated  $p\text{CO}_2$ . Once a week, half of each treatment was fed methanotrophic bacterium (*Methyloprofundus sedimenti* PKF-14), while the other half was fed a mix of marine microalgae (*Dunaliella tertiolecta* and *Isochrysis galbana*).

Results of the experiment imply that, at least to some extent, that the benthic foraminifera are able to calcify during exposure to hypoxia and/or elevated  $p\text{CO}_2$  conditions. However, the responses of the foraminifera were different depending on the source of the sediment. Overall, dual-stress treatment (IV) had the strongest effect on the foraminifera. The dietary comparison shows that the methanotrophic bacteria diet did neither promote nor inhibit foraminiferal growth in the experimental conditions. To confidently conclude about an eventual impact of methanotrophs diet on foraminifera further dedicated studies are required.

## Article 2

Katarzyna Melaniuk. **Effectiveness of fluorescent viability assays in studies of cold seep foraminifera.**

Article 2 presents the results of a study on live benthic foraminifera from two active methane seepage sites in the Lomvi pockmark at the Vestnesa Ridge, and from shallow cold-seep sites in Storfjordrenna, SW Svalbard on the western Barents Sea shelf (Fig. 2). Storfjordrenna hosts the so-called 'pingo' sites, where methane is released from pingo-like mounds on the seafloor, many of which contain gas hydrates. One of the investigated gas hydrate pingos (GHPs) is active (GHP1), while the other is presently inactive (GHP5) and considered 'post-active'. In order to distinguish live foraminifera, as an alternative to the commonly used Rose Bengal staining method, we used CellTracker™ Green CMFDA or CellHunt Green green-fluorescent probes, thus indicating only metabolically active foraminifera. To determine whether methane seepage has any effects on the carbon isotopic signatures of primary calcite of live benthic foraminifera, the  $\delta^{13}\text{C}$  measured in CellTracker™ Green or CellHunt Green labelled (metabolically active), Rose Bengal stained (recently dead + dead individuals from Vestnesa Ridge, or recently dead foraminifera from Storfjordrenna), and unstained ('certified' dead) have been compared. The study showed a presence of metabolically active foraminifera in methane affected sediment from both Vestnesa Ridge and Storfjordrenna.

The results confirmed that Rose Bengal overestimated the number of live foraminifera when compared to the numbers obtained with fluorescent probes. The dominant calcareous species were *Melonis barleeanus* and *Cassidulina neoteretis* at Vestnesa Ridge and *M. barleeanus* and *Nonionelina labradorica* at Storfjordrenna. No endemic species were observed in this study. Except, for the foraminifera from the core MC 919 (Storfjordrenna) there is no clear evidence that  $\delta^{13}\text{C}$  in tests of live foraminifera has been significantly affected by methane-derived carbon during biomineralization in any of the investigated sites. The combined use of the fluorogenic probe and the conventional Rose Bengal staining revealed minor shifts in species compositions and differences in ratios between live and recently dead foraminifera from the investigated pingo sites (active versus inactive).

### Article 3

Katarzyna Melaniuk, Kamila Szybor, Tina Treude, Stefan Sommer, Tine L. Rasmussen. **Evidence for influence of methane seepage on isotopic signatures in living deep-sea foraminifera, 79 °N.**

Article 3 reports on a study of isotopic signatures ( $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ ) measured in of benthic foraminifera, together with biogeochemical data from sediments at Arctic seep sites from Vestnesa Ridge (79°N, Fram Strait) at c. 1200 m water depth. Here, we studied the informally named 'Lunde' pockmark focusing on three benthic species: *Melonis barleeanus* (intermediate to deep-infaunal species), *Cassidulina neoteretis* (shallow infaunal species) and *Cibicidoides wuellerstorfi* (epibenthic, suspension-feeding species) and their isotope signals. The upper 5 cm of the sediment of multicores from three different types of seep environments (bacterial mat, *Siboglinidae* worm field and non-seep control sites) were sliced into 1-cm thick samples and stained. Stable isotopes were measured in both tests of Rose Bengal stained specimens and empty tests of the three species. Also, specimens the planktic foraminiferal species *Neogloboquadrina pachyderma* (dead specimens) were measured. Our study confirms that living benthic foraminifera are able to incorporate methane-derived carbon into their shells during their lifespan, most likely via feeding on methanotrophic bacteria. Methane-derived carbon can shift the  $\delta^{13}\text{C}$  signature of living (Rose Bengal-stained) foraminifera towards lower  $\delta^{13}\text{C}$  values. We observed that the  $\delta^{13}\text{C}$  signature of foraminiferal tests is linked to methane-related processes, such as aerobic (MOx) and anaerobic methane oxidation (AOM). The  $\delta^{13}\text{C}$  recorded in tests of RB -stained *M. barleeanus* was as low as -5.21‰ from the *Siboglinidae* field (the site dominated by MOx) indicating methane influence on the signature in the primary calcite of the foraminifera. Under other conditions, at the sediment dominated by AOM and covered by bacterial mats, the  $\delta^{13}\text{C}$  signature of empty tests were influenced by methane-derived authigenic carbonates (MDAC). The  $\delta^{13}\text{C}$  reach values as low as -6.48‰ (*M. barleeanus*), and even -6.17‰ for the epibenthic species *C. wuellerstorfi*. Because, AOM is a strong contributor to authigenic carbonate overgrowth, MDAC precipitation may severely overprint the initial isotopic signature of foraminiferal tests, even at shallow depth such a 3–4 cm (in this study). Additionally, we show a connection between the presence of overgrowth of MDAC and high  $\delta^{18}\text{O}$  values in tests of dead specimens of benthic foraminiferal species *C. neoteretis*.

#### Article 4

Katarzyna Melaniuk, Kamila Sztybor, Tina Treude, Stefan Sommer, Marek Zajączkowski, Tine L. Rasmussen. **Response of benthic foraminifera to ecological succession in cold seeps from Vestnesa Ridge; implications for interpretations of paleo-seepage environments**

Manuscript 4 presents the result of a study on the response of living benthic foraminifera to progressing ecological succession in the development of a cold seep environment. Sediment samples used herein are the same as for Articles 2 and 3, with a greater focus on the samples from Vestnesa Ridge: the 'Lomvi' and 'Lunde' pockmarks. The distribution and species composition of metabolically active (CellTracker™Green labelled) and live (Rose Bengal-stained) foraminifera were analyzed in relation to the geochemical properties of the pore water, presence of bacterial mats, and distribution of macrofaunas (e.g., *Siboglinidae* tubeworms) in comparison to non-seep environments.

Ecological succession is a term used to describe the natural process of change in the faunal structures of an ecological community over time. At cold seeps, ecological succession refers to the duration of methane seepage and is linked to changes in biochemistry of the sediment and benthic faunal communities. Bergquist et al. (2003) suggest a general pattern of ecological successions in the seep environments i.e., stages 1, 2, and 3: from a patchy distribution of bacterial mats and initial seepage at stage 1, to dense microbial mats with H<sub>2</sub>S production at stage 2, to authigenic rock formation and increasing tubeworms aggregations at stage 3, and eventually formation of long-lasting coral reefs after the seepage declines, as an eventual stage 4. The results of the study show that the distribution patterns of benthic foraminifera change accordingly to the progressing ecological succession of the seep environment. For example, at the initial stage (stage 1), oxygen is still available to the foraminifera, and as a result the species composition is similar to control sites, the main species being *Melonis barleeanus* and *Cassidulina neoteretis*. At stage 2 (with maximum seepage), high concentrations of H<sub>2</sub>S create hostile conditions for benthic foraminifera. As a result, the samples were almost barren of foraminifera. At stage 3, moderate methane seepage supports a foraminiferal community of both calcareous and agglutinated species. The presence of chemosynthetic *Siboglinidae* tube worms may potentially support epibenthic *Cibicidoides wuellerstorfi* communities by generating a secondary hard bottom.

Despite the differences between each stage of faunal and environmental successions, none of the faunal characteristics can be used as an exclusive indicator of methane emission or stages of its environments in palaeoceanographic interpretations.

## 5 SYNTHESIS AND FUTURE WORK

The emphasis of this thesis is on improving the understanding of the distribution of living benthic foraminifera and carbon isotopic signatures in their tests in relation to methane seepage in the Fram Strait and Storfjordrenna (Barents Sea). This work consists of four research articles. In Article 1, the results of a laboratory experiment are presented. The second and fourth articles show the results of an analysis of modern foraminifera assemblages from surface sediments collected from two Arctic locations: pockmarks Lunde and Lomvi at Vestnesa Ridge (~1200m depth), and active and post-active gas hydrate pingos at a relatively shallow methane seep in Storfjordrenna (~400m depth; western Barents Sea; Fig. 2). The articles 2 and 3 show the result of isotopic analyses ( $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ ) of cold seep associated foraminifera. The main conclusions based on the results of this work are presented below:

### 5.1 Laboratory experiment

The experiment was an approach to study the response of benthic foraminifera to environmental stress and dual-stress conditions (combined low oxygen and high  $p\text{CO}_2$ ), as well as the first study in which foraminifera were fed with a methanotroph bacterium *Methyloprofundus sedimenti* PKF-14 in controlled laboratory conditions, using natural sediment from the Nordic Seas and Barents Sea. This experiment demonstrates, the ability of benthic foraminifera to both grow and calcify, at least to some extent, under potentially challenging conditions of hypoxia ( $\text{O}_2$ , 0.7ml/L) and elevated- $p\text{CO}_2$  (2000 ppm) showing that foraminifera are very much adaptable to temporary stress conditions.

Depending on the sources of sediment the response of the benthic foraminifera was different. Overall, the dual-stress treatment had the most significant impact on the foraminifera reducing the yield size by about 50% compared to modern-day treatments (I) or inhibiting the calcification completely, which was manifested by barren replicates. The most notable exception was the foraminiferal yield from Norwegian Sea B sediments where individuals seemed to be more resistant to the combined effect of both stressors. Analogously to the experiment, we can expect that the response of a benthic foraminiferal population to the same stressors will depend on the original species composition (pre-seep species composition) within the given methane seep. Some of the species,

particularly those adapted to high organic content (e.g., Akimoto et al., 1994, Rathburn et al., 2000, 2003, Bernhard et al., 2001) are also pre-adapted to cold-seep conditions and have the potential to thrive while other species might completely die out. Overall, environmental conditions seem to impact the growth of foraminifera more than the type of food offered. This means that within cold seeps, even when food is available, geochemical properties of the sediment might put limits on the distribution of the foraminifera. However, further dedicated studies are required to confidently conclude the impact of the methanotroph diet on foraminifera.

The results of the experiment highlight the importance of multi-factor laboratory experiments in studies on foraminiferal ecology. Should the experiment be repeated in the future, I suggest some improvements be made. First of all, the propagule method worked quite well as a set up to study the response of the foraminifera to environmental parameters, but it is not a suitable approach to study the dietary preferences of these organisms. The natural sediments used as a source of propagules (small/juvenile foraminifera) was not sterile and was thus contaminated by pre-experimental organic matter, which could potentially have served as a food source for some of the foraminifera. Therefore, to avoid such contamination, a future feeding experiment should either use pre-labelled microbes to facilitate the recognition of the experimental source of carbon, or the foraminifera should be picked from the sediment and transferred to a sterile environment. In addition, it would be beneficial to use for example Calcein (a cell-permeant dye) in order to mark experimental calcite (i.e., part of the tests built during the experiment).

## **5.2 Foraminiferal faunas**

The study presented in Article 2 and 4 show that the distribution patterns of benthic foraminiferal species are influenced by seepage of methane, and results in uneven distribution of specimens within in the same seep. At Storfjordrenna pingos the density of metabolically active (Cell Tracker Green™ labelled) foraminifera gradually decreases from 12/10cc at the edge of the active gas hydrate pingo 1 (GHP1) with moderate influence of methane to almost barren sediment at the top of the pingo where the gas seeps out (Article 2). Similarly, at Vestnesa Ridge the distribution patterns and the species composition of the benthic foraminiferal faunas change according to the intensity of methane seepage and follow the progressing ecological succession model suggested by Bergquist et al., 2003 (Article 4).

In both cases it seems that foraminifera are indeed attracted to bacterial mats as a potential food source, as suggested earlier (Hill et al., 2005; Bernhard et al., 2010), but only when methane seepage is moderate or low and aerobic methane oxidation (MOx) is the dominant process e.g., ecological succession stage 1 or 3 (Article 4), and as at the edge of the active GHP1 (Storfjordrenna; Article 2). In sediments affected by AOM, with strong methane seepage (stage 2) or top of the GHP1,



even when potential food is available (i.e., bacterial mats are present), geochemistry of the sediment e.g., low oxygen concentration and presence of hydrogen sulfide, as well as sediment movements (in case of the top of GHP1), create unstable and hostile conditions for benthic foraminifera.

Both investigated sites are characterized by a comparable faunal pattern, with no endemic species and the observed species are similar to those from other nearby non-seep locations. No particular species or group of species potentially could indicate methane seepage. The foraminiferal fauna was dominated by species adapted to high organic content and low oxygen conditions. At Storfjordrenna, the main species were *Melonis barleeanus* and *Nonionellina labradorica*, and at Vestnesa Ridge *Melonis barleeanus*, *Cassidulina neoteretis*, and *Reophax* spp., predominated. All of the species are common in the Arctic. Especially, the higher abundance of opportunistic species at Storfjordrenna pingos can reflect both methane seepage and/or the Arctic spring bloom. Interestingly, it seems that at Vestnesa Ridge presence of *Siboglinidae* tube aggregations promotes *Cibicidoides wuellerstorfi* communities by generating secondary hard bottom.

Based on results of this thesis it is difficult if not impossible to find the link between methane seepage and distribution patterns of benthic foraminiferal species that could be further utilised as a template in reconstructions of the strength of past methane emissions. Cold seeps are ephemeral environments that can change rapidly over time, thus more high-resolution studies, preferably by seasonal sampling, in combination with analysis of the geochemistry of the sediment and pore water is recommended in order to obtain a detailed picture of the ecology of modern foraminiferal faunas within methane seeps. Such measures are thus recommended in order to further elucidate the link between methane seepage and foraminiferal distribution patterns.

### **5.3 Fluorescence viability in the study of living foraminiferal assemblages**

This study confirmed that Rose Bengal staining overestimates the number of live benthic foraminifera in a sample. Rose Bengal always indicates a higher number of 'live' foraminifera when compared to CellTracker™ Green or CellHunt Green labelling (Article 2). Studies of benthic foraminiferal assemblages from Vestnesa Ridge show that a) there significantly less live (CellTracker™ Green labelled) foraminifera when compared to live + recently dead (Rose Bengal- stained) individuals; b) in some of the samples, despite the lack of live foraminifera, Rose Bengal still indicated the presence of cytoplasm, which would normally be considered as a 'live' individual; c) there is no significant difference between the  $\delta^{13}\text{C}$  measured in the CellTracker™ Green labelled and the Rose Bengal stained foraminifera.

Samples from the Storfjordrenna pingos site were processed differently. Collected sediment was first labelled with CellHunt Green, live foraminifera were selected, and only afterwards the residue

was stained with a Rose Bengal solution. This approach allowed a) to distinguish metabolically active (CellHunt Green labelled) foraminifera from recently dead individuals (Rose Bengal- stained); b) observe minor changes in foraminiferal populations, which would otherwise be overlooked, c) observe a variation in the ratio between live and recently dead foraminifera. In geochemically active habitats (the active GHP1), approximately 40% of picked foraminifera were actually alive at the time of collection, whereas the other 60% were recently dead individuals. In the post-active GHP5, this percentage is the opposite. This difference in populations might indeed reflect a more unstable and variable habitat, probably associated with methane seepage. Alternatively, because of lower decomposition rates in cold low-oxygen environments, the Rose Bengal stained individuals may have been dead for a relatively longer period of time at the active GHP1 (e.g., Jorissen et al., 1995; Bernhard et al., 2001), which could explain their over-abundance compared to non-seep sites. A notable surprise is the presence of a high abundance of fluorescent labelled *Buccella frigida* in samples from the active GHP1 with lack of Rose Bengal-stained individuals. This implies that the presence of live *B. frigida* might actually reflect a relatively recent appearance of bacterial mats associated with methane seepage.

Our current understanding of ecology of foraminifera from cold seeps is based on studies that have applied the Rose Bengal staining method. As mentioned above, not all Rose Bengal-stained foraminifera are actually alive during the sampling. It was documented that staining of recently dead specimens may occur several weeks after their death (e.g., Jorissen et al., 1995; Bernhard et al., 2001). Thus, it is controversial if, in earlier published papers, foraminifera indicated as living were actually metabolically active or that Rose Bengal indicated dead cytoplasm as well. From the palaeoceanographically perspective, poor understanding of the ecology of foraminifera might result in inaccurate interpretations. For example, individuals that recently died out due to methane seepage could potentially still be stained by Rose Bengal, while simultaneously due to partly decomposed cytoplasm, the exposed surface of the tests have been affected by Methane-Derived Authigenic Carbonates (MDAC) precipitation (Mackensen et al., 2006). As a result, a depleted  $\delta^{13}\text{C}$  signal can be misinterpreted as the incorporation of methane derived carbon during biomineralization, when in fact it was the result of post-mortem deposition in methane-charged sediment. Fossil assemblages that represent a wide time range of foraminifera may thus reflect a mix of several smaller methane seepage events and/or changes in foraminiferal populations due to local environmental variations.

Despite the more time-consuming protocol and higher costs compared to Rose Bengal, both CellHunt Green and CellTracker™ Green are valuable tools in studies of the ecology of benthic foraminiferal species. In order to obtain a better picture of the modern fauna, it is recommended to use fluorescence viability assays in studies of foraminiferal assemblages. CellHunt Green and CellTracker™ Green are equally good indicators, with the former being the more affordable option.

## 5.4 Carbon isotope signatures in tests of live foraminifera

Results reported in Article 2 show no substantial influence of methane-derived carbon on primary calcite in metabolically active foraminifera. This is most likely because the ambient pore water (microhabitat in which foraminifera lives) was not saturated enough in depleted C-13 to influence the isotopic signature of the benthic foraminifera. There were also no signs of methane-derived authigenic carbonates (MDAC) precipitation on empty foraminiferal tests. This supports the hypothesis previously reported from other studies that low  $\delta^{13}\text{C}$  values measured in fossil foraminiferal tests are due to authigenic overgrowth and reflect processes that took place after foraminiferal tests have been deposited in the methane charged sediment.

Nevertheless, since the  $\delta^{13}\text{C}$  was measured on pools of specimens (N=10), it is possible that at least some of the live individuals had more negative  $\delta^{13}\text{C}$  signatures than others, or that some chambers indeed incorporated methane-derived carbon. To obtain more accurate  $\delta^{13}\text{C}$  values and to draw a more robust conclusion, analysis of single CellTracker™ Green or CellHunt Green labelled foraminifera, or more advanced techniques, such as for example secondary-ion mass spectrometry (SIMS), are recommended.

Contrasting results are presented in Article 3, where data shows that, at the *Siboglinidae* field with moderate seepage of methane, dominance of MOx, and low concentrations of sulfide, the live benthic foraminifera (RB-stained) incorporate methane-derived carbon, most likely by feeding on methane-oxidizing bacteria or by direct intake of  $^{12}\text{CO}_2$  produced during MOx. Additionally, primary signals measured in empty foraminiferal tests of benthic and planktic foraminifera from bacterial mats (MUC12) were overprinted by MDAC precipitation. MDAC represents strong methane seepage, and indicate sediment oversaturated in  $\text{HCO}_3^-$  derived from sulfate-reducing and methane-oxidizing microbial consortiums in the sulfate-methane stability zone (SMTZ). Overgrowth starts coating the tests at relatively shallow depth 2–3 cm in the multicores from bacterial mats, causing a  $\delta^{13}\text{C}$  signature shifts of tests towards low values down to -6.48‰ for fossil *Melonis barleeanus*, 6.18‰ for *Cassidulina neoteretis*, and -6.17‰ for *Cibicides wuellerstorfi*.

MDAC overprints seem to affect the  $\delta^{18}\text{O}$  signature of fossil *C. neoteretis*. The  $\delta^{18}\text{O}$  have a relatively heavy signature, and reach up to 5.17‰. It was already suggested that high  $\delta^{18}\text{O}$  measured in fossil records indicated gas hydrate dissociation. Nevertheless, considering that the sediment collected was in a deep-sea Arctic setting and represents modern sediment, the presence of high  $\delta^{18}\text{O}$  is due to gas hydrate dynamics (dissipation, production as well as an AOM activity), it may not result exclusively from gas hydrate dissociation due to the present climate change and warming of deep waters. Which has to be considered while interpreting fossil data.

Similar to the species distribution, the carbon isotopic signature measured in both living and empty foraminiferal tests depend on intensity of MOx and AOM and it changes depending on methane flux (i.e., methane seep intensity). Fossil records reflect the cumulative history of methane seepage which took place during the lifespan of the benthic foraminifera as well as post mortem processes affecting it shell. Therefore, in context of palaeoceanographic studies it seems that depletions in  $\delta^{13}\text{C}$  measured in fossil foraminiferal records can only provide qualitative information about presence of seepage as it was suggested by Martin et al., (2004). Additionally, under certain geochemical conditions, the cold seep environment is hostile for benthic foraminifera i.e., there are no foraminifera for isotopic measurements at all. It is however, promising that the SMTZ-zone is close to sediment surface when methane seepage is strong (Borowski et al., 1996) (2–3 cm at the bacterial mat site); which depending on the sedimentation rates at the time will allow at least timing of when paleo-methane seepage was at its strongest at a given site.

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**Article 1.**

**Impact of hypoxia and high  $p\text{CO}_2$  and diet on benthic foraminiferal growth: experiment  
with propagules.**





# Effectiveness of Fluorescent Viability Assays in Studies of Arctic Cold Seep Foraminifera

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Highly negative  $\delta^{13}\text{C}$  values in fossil foraminifera from methane cold seeps have been proposed to reflect episodes of methane release from gas hydrate dissociation or free gas reservoirs triggered by climatic changes in the past. Because most studies on live foraminifera are based on the presence of Rose Bengal staining, that colors the cytoplasm of both live and recently dead individuals it remains unclear if, and to what extent live foraminifera incorporate methane-derived carbon during biomineralization, or whether the isotopic signature is mostly affected by authigenic overgrowth. In this paper, modern foraminiferal assemblages from a gas hydrate province Vestnesa Ridge (~1,200 m water depth, northeastern Fram Strait) and from Storfjordrenna (~400 m water depth in the western Barents Sea) is presented. By using the fluorescent viability assays CellTracker™ Green (CTG) CMFDA and CellHunt Green (CHG) together with conventional Rose Bengal, it was possible to examine live and recently dead foraminifera separately. Metabolically active foraminifera were shown to inhabit methane-enriched sediments at both investigated locations. The benthic foraminiferal faunas were dominated by common Arctic species such as *Melonis barleeanus*, *Cassidulina neoteretis*, and *Nonionellina labradorica*. The combined usage of the fluorescence probe and Rose Bengal revealed only minor shifts in species compositions and differences in ratios between live and recently dead foraminifera from Storfjordrenna. There was no clear evidence that methane significantly affected the  $\delta^{13}\text{C}$  signature of the calcite of living specimens.

**Keywords:** CellTracker™ Green CMFDA, Rose Bengal, gas hydrate, Vestnesa Ridge, Storfjordrenna, cold seep, Arctic

## INTRODUCTION

Due to the present climate warming, the Arctic region is undergoing remarkably rapid environmental changes, termed the Arctic amplification (IPCC, 2013; Box et al., 2019). The increase in global temperature and atmospheric  $\text{CO}_2$  has severe consequences for the Arctic Ocean, causing among others ocean acidification (Amap Assessment, 2018), loss of sea ice (Stroeve et al., 2012), and increase in primary production (Arrigo and van Dijken, 2011). The ocean warming also impose

a high risk of release of methane from geological reservoirs (IPCC, 2007; Phrampus and Hornbach, 2012) as large amounts of methane are stored on Arctic continental margins in the form of pressure-temperature sensitive gas hydrates (e.g., Maslin et al., 2010; Ruppel and Kessler, 2017). Gas hydrate is a widespread, ice-like substance formed when water and methane or other hydrocarbon gases combine in marine sediments under high pressure (3–5 MPa) and temperatures below  $\sim 25^{\circ}\text{C}$  (e.g., Kvenvolden, 1993). Pressure release and/or increase in temperature can cause destabilization of gas hydrate reservoirs, resulting in a release of free methane gas into the sediment and/or water column (e.g., Archer et al., 2009; Maslin et al., 2010). Several studies have implied a link between the release of methane from geological reservoirs and climate change during the Quaternary and the Paleocene periods (e.g., Wefer et al., 1994; Dickens et al., 1997; Smith et al., 2001). It is feared that ongoing climate change can trigger destabilization of gas hydrate reservoirs and methane release into the water column and eventually to atmosphere (Ruppel and Kessler, 2017). Therefore, it is crucial to understand the fate of methane in marine sediments in order to understand the potential impact of methane release to future climate and Arctic ecosystems.

For the last decades, the carbon isotopic signature  $\delta^{13}\text{C}$  of benthic foraminifera has been commonly used as a proxy in the reconstruction of productivity and origin and ventilation of water masses in the past (e.g., Gooday, 1994, 2003; Smart et al., 1994; Rohling and Cooke, 1999; Murray, 2006; Ravelo and Hillaire-Marcel, 2007). Recent studies have shown that the  $\delta^{13}\text{C}$  incorporated into the calcareous ( $\text{CaCO}_3$ ) tests of benthic foraminifera can record episodes of release of methane in the past (e.g., Torres et al., 2003; Millo et al., 2005; Martin et al., 2010; Schneider et al., 2017; Szybor and Rasmussen, 2017). The  $\delta^{13}\text{C}$  in the shells of some fossil benthic foraminifera can be lower than  $-10\text{‰}$  (e.g., Hill et al., 2004; Schneider et al., 2017; Szybor and Rasmussen, 2017), while the signature of calcite of living foraminifera generally do not exceed  $-7.5\text{‰}$  (Mackensen et al., 2006; Wollenburg et al., 2015), it remains unclear if, and to what extent live benthic foraminifera incorporate methane-derived carbon during biomineralization, or whether the isotopic signature is mostly affected by authigenic overgrowth from carbonate precipitation.

Modern methane cold seeps can provide valuable information about changes in seepage intensity and the possible effects of methane seepage on the distribution patterns of live foraminifera and the isotopic composition of their tests. The  $\delta^{13}\text{C}$  of calcareous benthic foraminifera is determined by species-specific vital effects (i.e., intracellular metabolic processes; e.g., Grossman, 1987; McCorkle et al., 1990; Mackensen et al., 2006) and their microhabitat (e.g., sedimentary organic matter, dissolved inorganic carbon content, temperature, and re-mineralization; e.g., McCorkle et al., 1985; Fontanier et al., 2006). Within cold seeps, the release of methane from the seafloor is partly controlled by sulfate-dependent anaerobic oxidation of methane (AOM) and aerobic methane oxidation (MOx; Treude et al., 2007; Knittel and Boetius, 2009). Thus, as a product of these microbial activities,  $^{13}\text{C}$ -depleted carbon is released in the form of carbon

dioxide ( $\text{CO}_2$ ) or bicarbonate ( $\text{HCO}_3^{2-}$ ), causing changes in the carbon isotopic signature of pore water i.e., changes in the microhabitat (e.g., Whiticar, 1999; Rathburn et al., 2003; Treude et al., 2007).

Since benthic foraminifera construct their tests by incorporating carbon from the surrounding pore water or bottom water and from the intracellular storage of inorganic carbon (e.g., de Nooijer et al., 2009; Toyofuku et al., 2017), the foraminiferal calcite supposedly records the isotopic signal of ambient waters (i.e., pore water or interstitial water in which the foraminifera live) at the time of calcification (e.g., Rathburn et al., 2003; Panieri and Sen Gupta, 2008). Alternatively, foraminifera might absorb the  $^{13}\text{C}$ -depleted carbon *via* the food web (Panieri, 2006) or by feeding on, or living in symbiosis with, methanotrophic bacteria, as suggested by Hill et al. (2004). Some studies show that the  $\delta^{13}\text{C}$  measured in tests of living foraminifera collected from active seeps are not markedly lower than those from non-seep sites, indicating that living foraminifera might not be able to record the episodes of methane release (e.g., Rathburn et al., 2003; Torres et al., 2003; Etiope et al., 2014; Herguera et al., 2014; Dessandier et al., 2020). Simultaneously, numerous other studies indicate that methane has an effect on isotopic signatures of “live” foraminifera (Rose Bengal stained; e.g., Hill et al., 2004; Mackensen et al., 2006; Panieri, 2006; Wollenburg and Mackensen, 2009; Wollenburg et al., 2015).

Studies of live foraminiferal assemblages are commonly based on Rose Bengal staining, presumably marking specimens that were alive at the sampling time. Stained specimens can include both live and recently dead individuals (Bernhard et al., 2006; Figueira et al., 2012) thus, it is still not clear if live foraminifera have recorded the  $^{13}\text{C}$  signal that comes from incorporation of carbon from methane in their shells during calcification. Compared to the conventional Rose Bengal, the CellTracker™ Green (CTG) CMFDA (5-chloromethylfluorescein diacetate; Thermo Fisher Scientific) and CellHunt Green (CHG) (SETAREH biotech, LLC) probes are reactive with internal cell components and gives a green-fluorescent coloring of the cytoplasm, indicating metabolically active foraminiferal specimens. The combined usage of the fluorogenic probes together with the Rose Bengal staining can be used to separate live foraminifera from recently dead individuals, and thus be a useful tool to build up a more detailed picture of benthic foraminiferal distribution patterns and ecology. It might be especially useful in studies of heterogeneous and variable environments such as cold seeps, which depend on the highly variable flux of methane and can evolve and change rapidly over time (Levin, 2005; Cordes et al., 2006; Åström et al., 2020).

This paper presents results of a study of live benthic foraminifera from a gas hydrate province on Vestnesa Ridge ( $\sim 1,200$  m water depth; western Svalbard margin) and from gas hydrate “pingo” structures from Storfjordrenna ( $\sim 400$  m water depth) in the western Barents Sea (Figure 1). The aims of the study are to (1) identify species compositions of the benthic foraminiferal faunas in these Arctic methane seeps and (2) to compare the carbon isotopic signature ( $\delta^{13}\text{C}$ ) in the tests of

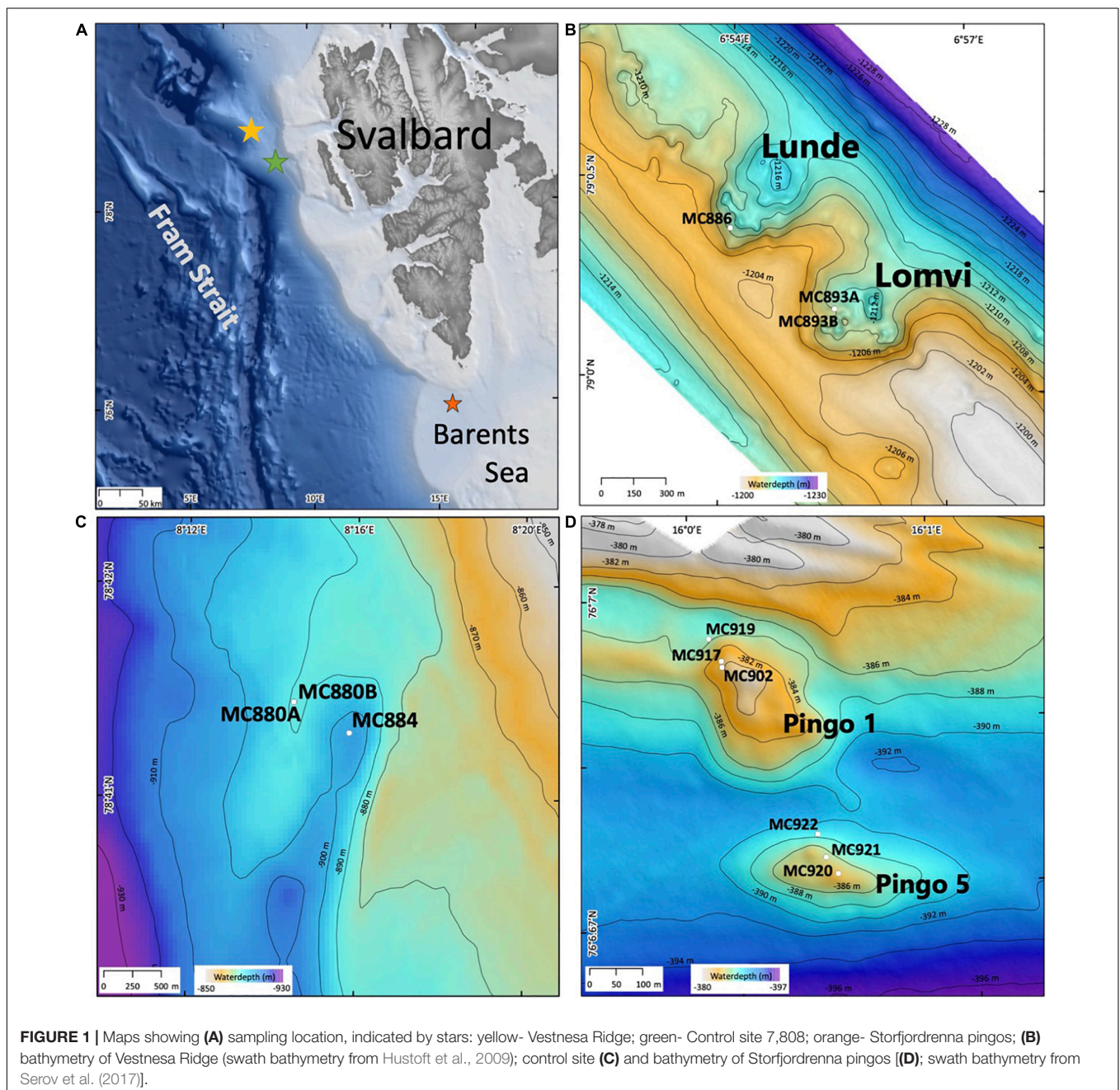
metabolically active (CTG or CHG labeled) foraminifera, with Rose Bengal stained and with unstained tests (empty tests), to determine if methane seepage has any significant effects on the isotopic signatures of calcite of live benthic foraminifera.

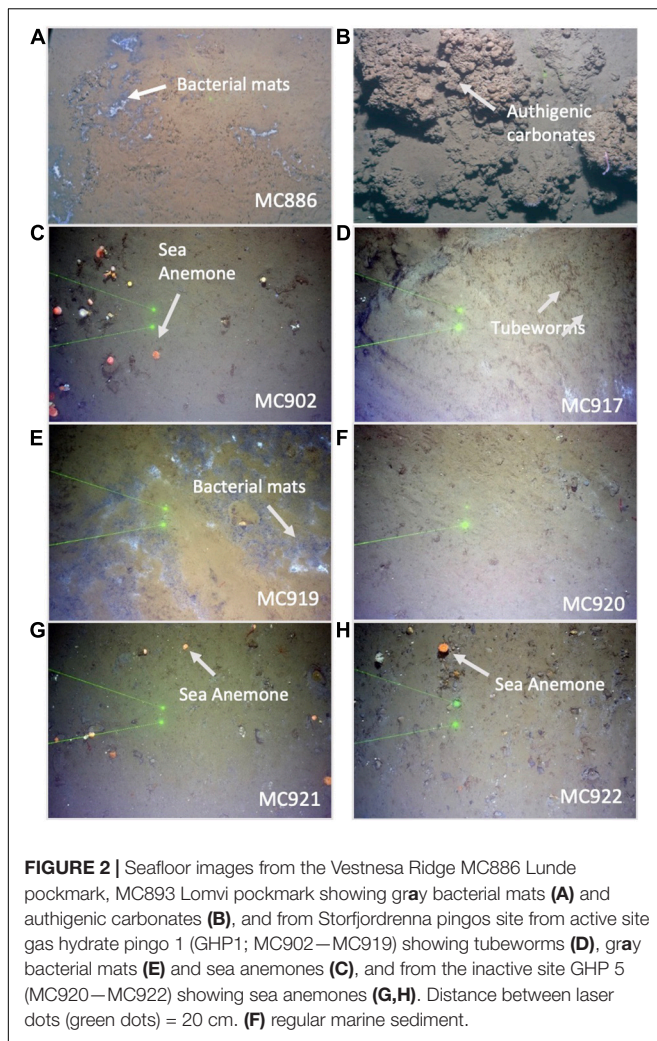
## MATERIALS AND METHODS

### Study Area

Vestnesa Ridge is located northwest of Svalbard in the eastern Fram Strait and is an approximately 100 km long sediment drift at water depths of ~1,200–1,300 m. The Fram Strait forms

the gateway between the North Atlantic Ocean and the Arctic Ocean. This region is characterized by large annual fluctuations in sea-ice cover. Relatively warm (3–6°C), saline ( $S < 35.4$  psu), and nutrient-rich Atlantic water pass through the Fram Strait into the Arctic Ocean carried by the West Spitzbergen Current (WSC) (e.g., Manley, 1995; Rudels et al., 2000; Walczowski et al., 2005). The southwestern part of the Vestnesa Ridge is characterized by the presence of several active pockmarks (i.e., shallow seabed depressions) where methane-rich fluids seep from gas hydrate and free gas reservoirs (Bünz et al., 2012; **Figure 1B**). The most active pockmarks, “Lomvi” and “Lunde,” are approximately 10–15 m deep depressions with diameters





of 400–600 m. The TowCam—guided multicore investigation of the Vestnesa Ridge shows heterogeneity of the site and presence of macrofauna and seafloor structures associated with the occurrence of methane seepage. These include, e.g., bacterial mats and tubeworm fields (*Siboglinidae*) within the Lunde pockmark, and Methane-Derived Authigenic Carbonate (MDAC) outcrops at the seafloor within the Lomvi pockmark (e.g., Sztybor and Rasmussen, 2017; Åström et al., 2018; Figure 2). The  $\delta^{13}C_{DIC}$  values of pore water for the Lomvi pockmark have been reported to range between  $-25.1$  and  $-37.7\text{‰}$  and for the Lunde pockmark  $-22.4\text{‰}$  to  $-39.4\text{‰}$  in surface sediments (Dessandier et al., 2019).

The Storfjordrenna hydrate mound “pingo” area is located  $\sim 400$  m water depth on the Arctic continental shelf, south of the Svalbard archipelago in the north-western Barents Sea (Figure 1D). Similar to the Vestnesa Ridge, Storfjordrenna is under influence of relatively warm Atlantic water (Loeng, 1991). The area is characterized by five gas hydrates mounds (pingo-like features) spread within an area of  $2 \text{ km}^2$ . The gas hydrate pingos (GHPs) are between 8 and 12 m high, with diameters between 280 and 450 m. Four of the five GHPs

are presently active and show active methane seepage in the form of acoustically detected gas/bubble streams (i.e., acoustic flares) around the summits and one is in a “post-active stage” and presently inactive (Hong et al., 2017; Serov et al., 2017). Elevated concentrations of methane (mostly of thermogenic origin) have been detected in both sediments and bottom waters at GHP1, and gas hydrates were recovered in sediment cores (Hong et al., 2017; Carrier et al., 2020). The  $\delta^{13}C_{DIC}$  values of pore water for the top of GHP1 (MC902) reached  $-24.2\text{‰}$  (Dessandier et al., 2020). Seabed images acquired with a Multicorer-TowCam during the CAGE17-2 cruise revealed the presence of white and gray bacterial mats as well as sediments colonized by chemosynthetic *Siboglinidae* tubeworms, biota well known to indicate active hydrocarbon seepage (Niemann et al., 2006; Treude et al., 2007; Figure 2). The megafauna community associated with cold seeps has been previously documented at the Storfjordrenna by Åström et al. (2016) and Sen et al. (2018).

## Sampling

Sediment samples were collected during the CAGE 15-2 cruise in May 2015 to Vestnesa Ridge from the sites of active methane emission, the Lomvi and Lunde pockmarks, and at site 7,808 located south-east from the Vestnesa Ridge as a control site where no methane seepage occurs (Figures 1B,C). During CAGE cruise 17-2 in June 2017 to Storfjordrenna pingo area, several samples were taken from the active gas hydrates pingo (GHP1) along a transect from the top the pingo toward its edge (Figure 1D). For comparison, the inactive GHP5 was sampled in a similar manner (Figure 1D).

The samples from both Vestnesa Ridge and the pingo area in Storfjordrenna (Table 1 and Figure 1) were collected with a multicorer equipped with six tubes (10 cm diameter) and combined with a Towed Digital Camera (TowCam) developed at the Woods Hole Oceanographic Institution’s Multidisciplinary Instrumentation in Support of Oceanographic (MISO) Facility onboard the R/V *Helmer Hanssen*. The live-stream feed from the TowCam system were used to identify the different seafloor environments and to locate active methane vents, authigenic carbonates, and bacterial mats for targeted accurate sampling locations (Figure 2).

After recovery, undisturbed cores were selected for this study. The uppermost core section of each selected core was subsampled using a flat spatula slicing the sediment into 1-cm thick, horizontal intervals (0–1, 1–2, and 2–3 cm). Sediment samples from Vestnesa Ridge were processed as follows: One-third of each slice designated for different treatments, (1) labeling with CTG, (2) staining with Rose Bengal, and (3) extraction of dissolved inorganic carbon (DIC) from pore waters. Each sample was transferred into plastic containers (125-ml HDPE). The CTG solution was prepared beforehand as follows: 1.4 ml of DMSO (dimethyl sulfoxide; not anhydrous) was added to 1 mg CTG, mixed gently, and kept in the original plastic vial from the supplier at  $-20^{\circ}\text{C}$ . The solution was thawed approximately 20 min before the sampling. CTG was added to 20 ml of seawater sampled in the multicore tube and added to the sediment immediately after sampling and giving a final concentration of

**TABLE 1** | Core numbers, location, coordinates, water depths, and dates of sampling.

Core number	Location	Coordinates	Water depth (m)	Sampling date
MC893A and MC893B	Vestnesa Ridge (Lomvi pockmark)	79.18N, 00.44E	1200	20 May 2015
MC886	Vestnesa Ridge (Lunde pockmark)	79.38N, 00.04E	1200	20 May 2015
MC880A and MC880B	Site 7808 (Control site)	78.44N, 00.50E	889	19 May 2015
MC884	Site 7808 (Control site)	78.30N, 00.82E	900	19 May 2015
MC902	Storfjordrenna Active GHP1	76.91N, 16.08E	377	22 June 2017
MC917	Storfjordrenna Active GHP1	76.93N, 16.02E	377	23 June 2017
MC919	Storfjordrenna Active GHP1	76.96N, 15.98E	378	23 June 2017
MC920	Storfjordrenna Inactive GHP5	76.70N, 16.00E	379	23 June 2017
MC921	Storfjordrenna Inactive GHP5	76.72N, 16.40E	380	23 June 2017
MC922	Storfjordrenna Inactive GHP5	76.74N, 16.37E	386	23 June 2017

GHP, gas hydrates pingo.

CTG of 1  $\mu\text{M}$  in seawater (Bernhard et al., 2006). Samples were incubated in a temperature-controlled room at 4°C for approximately 12 h. Rose Bengal solution was made prior to sampling by dissolving Rose Bengal powder in distilled water (2 g/L). The solution was added to the designated sediment samples, agitated gently, and kept in plastic containers (250 ml). Sediment labeled with CTG and stained with Rose Bengal was preserved with 36% formaldehyde (to final concentration 5.5%) and kept at 4°C.

The sediment collected from Storfjordrenna pingo area was treated differently when compared to samples from the Vestnesa Ridge. Each 1-cm slice of sediment taken from GHP multicores was directly transferred into a 125 ml HDPE bottle. The whole sediment was treated with the CHG solution. The CHG solution was prepared beforehand (following the protocol for CTG). Samples were incubated in CHG onboard in a dark, temperature-controlled room at 4°C for approximately 12 h. Hereafter, the samples were preserved in ethanol with final concentration 70%.

## Foraminiferal Fauna Analysis

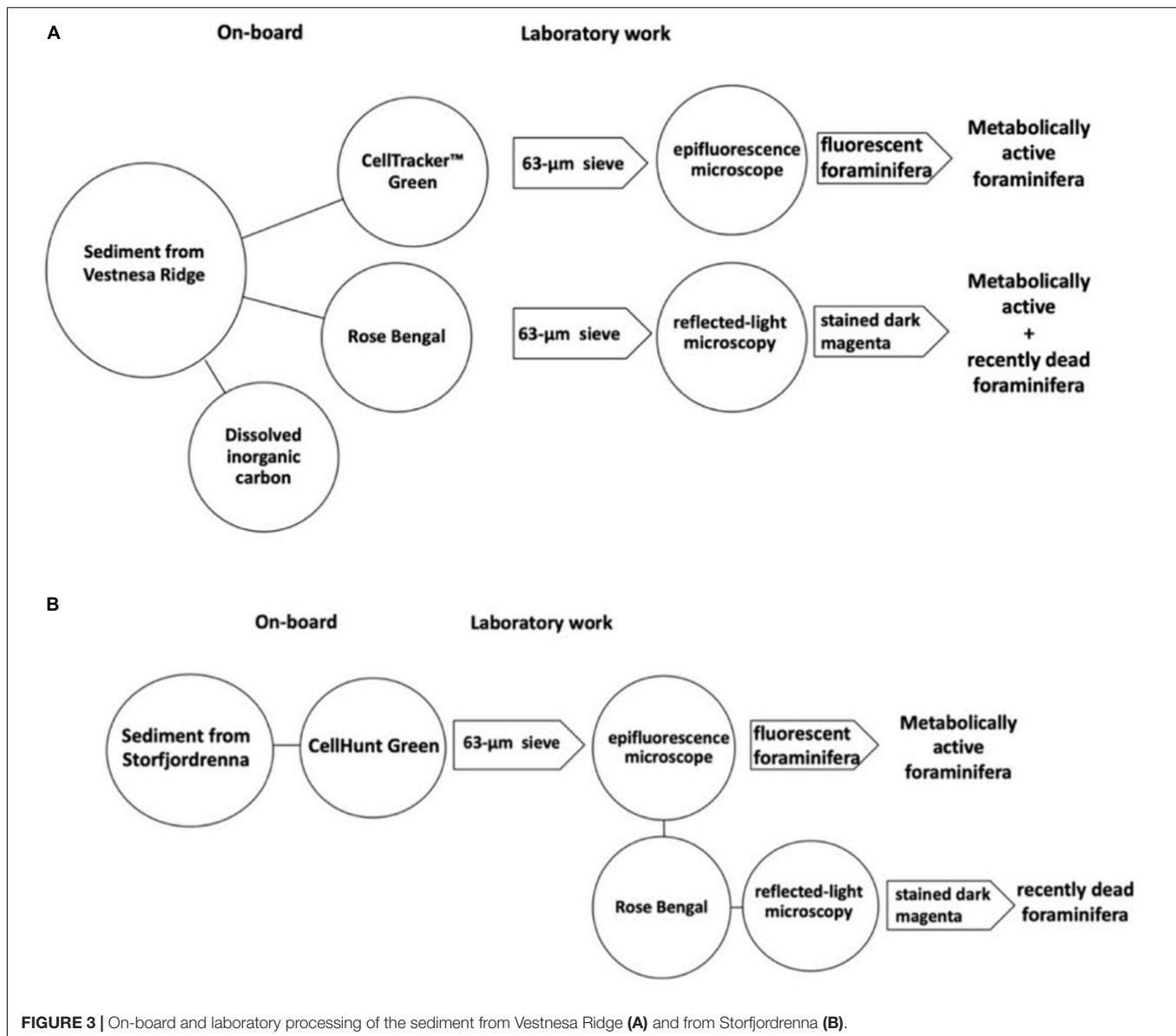
Rose Bengal stained and CTG labeled (i.e., fluorescently labeled) samples from Vestnesa Ridge were washed over a 63- $\mu\text{m}$  sieve using filtered seawater (0.45  $\mu\text{m}$ ); the >63  $\mu\text{m}$  fraction was kept in filtered seawater and further analyzed. The fluorescently labeled samples were examined using an epifluorescence-equipped stereomicroscope (Leica MZ FLIII; 485 nm excitation; 520 nm emission). All individuals that fluoresced brightly in at least half of their chambers were considered as live (Figure 6). They were picked wet and placed on micropaleontology slides. The Rose Bengal stained samples were examined with reflected-light microscopy using a Zeiss Stemi SV6. All foraminifera that stained dark magenta in at

least half of their chambers were picked and mounted on micropaleontology slides (Figure 3). All collected foraminifera were sorted by species and counted and identified to species level (Tables 2, 3).

The CHG labeled (i.e., fluorescently labeled) sediment samples from GHPs were processed in the same manner as CTG labeled, except that individuals that did not show any green coloration were subsequently incubated in a Rose Bengal-ethanol solution (2 g/L). After approximately 24 h, samples were re-sieved over a 63- $\mu\text{m}$  sieve. Obtained Rose Bengal stained foraminifera were wet picked (Figure 3). Unstained tests have been omitted and not counted in this study.

The density of foraminifera was normalized per unit volume at the number of specimens per 10  $\text{cm}^3$ . The Shannon index  $S(H)$  of diversity, Evenness index, and Chao1 index (Tables 2, 3) was calculated for each sample. The number of CTG labeled and Rose Bengal stained foraminifera, as well as the CHG labeled and Rose Bengal stained foraminifera, were compared by chi-square testing. Assuming that CHG foraminifera would have stained with Rose Bengal, the number of Rose Bengal stained foraminifera was determined as a sum of CHG labeled (living) and Rose Bengal stained (recently dead) individuals. For our chi-square test, the Rose Bengal stained individuals were treated as the expected values, whereas the CTG or CHG labeled individuals were treated as the observed values; this approach is adapted from Bernhard et al. (2006). The percentage of CHG labeled (living) faunal assemblages from GHP sites were calculated relative to total foraminiferal abundance (CHG labeled + Rose Bengal stained, i.e., foraminifera containing cytoplasm; Figure 7). Due to similar properties, further in the text CTG and CHG labeled foraminifera are interchangeably referred to as “fluorescently labeled.”





## Stable Isotopes Analyses

For carbon ( $\delta^{13}\text{C}$ ) stable isotope analyses of Vestnesa Ridge samples, the most numerous individuals of species indicated as a metabolically active (CTG labeled; live) and individuals “live + recently dead” (Rose Bengal stained) were selected. Due to the small size of most specimens, between 8 and 10 specimens of *Melonis barleeanus* and *Cassidulina neoteretis* (when present) and 10 unstained tests of the planktonic foraminiferal species *Neogloboquadrina pachyderma* were picked from each sample. In case of foraminifera from GHPs,  $\delta^{13}\text{C}$  measurements were performed on metabolically active (CHG labeled) foraminifera and recently dead (Rose Bengal stained) foraminifera of the two most numerous species *M. barleeanus* and *Nonionellina labradorica* (between eight and 10 specimens). Some “dead” (unstained tests) were picked for isotope analyses for comparison. Whenever possible, replicates were processed and analyzed.

Isotopic measurements were performed on a MAT 253 Isotope Ratio Mass Spectrometer (Department of Geosciences, UiT The Arctic University of Norway). Carbon isotopic compositions are expressed in conventional  $\delta$  notation against the Vienna Pee Dee Belemnite (V-PDB) standard (1.96,  $-10.21$ , and  $-48.95\text{‰}$  for  $\delta^{13}\text{C}$ ) and reported in parts per thousand (per mil,  $\text{‰}$ ). Analytical precision was estimated to be better than  $0.07\text{‰}$  for  $\delta^{13}\text{C}$  by measuring the certified standard NBS-19.

## RESULTS

### Foraminiferal Assemblages Vestnesa Ridge

Fluorescently labeled (living) individuals were present in the sediment from core MC893A (Lomvi pockmark) at 0–1 cm and

**TABLE 2** | Direct counts of CellTracker™ Green labeled foraminifera from all samples.

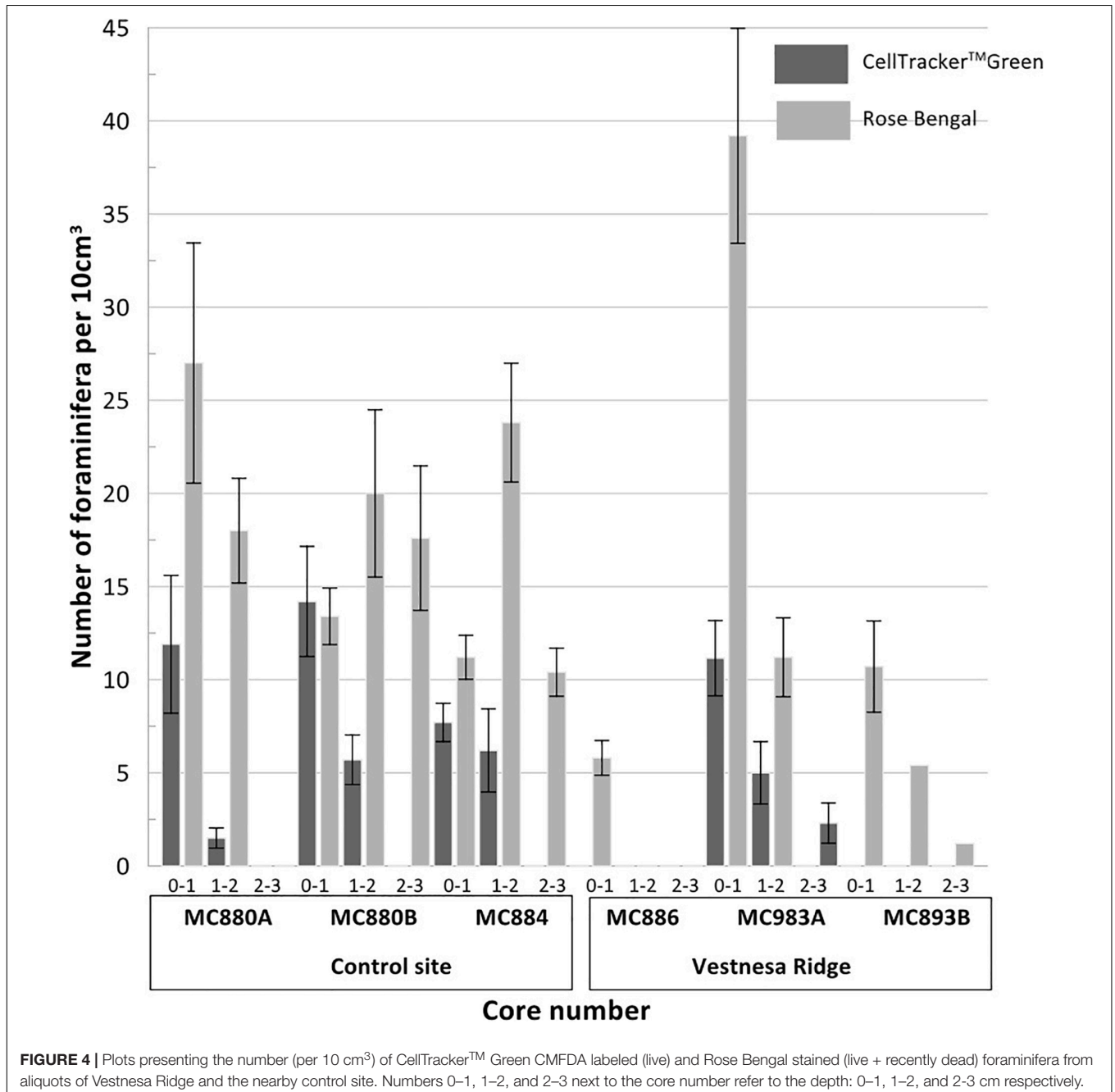
Core number	Control site						Vestnesa Ridge											
	MC880A			MC880B			MC884			MC886			MC893A			MC893B		
Depth (cm)	0–1	1–2	2–3	0–1	1–2	2–3	0–1	1–2	2–3	0–1	1–2	2–3	0–1	1–2	2–3	0–1	1–2	2–3
<i>Cassidulina laevigata</i>	4						1											
<i>Cassidulina neoteretis</i>	22	1		21	7		4	1					13	2				
<i>Cassidulina reniforme</i>				3	1		4						2					
<i>Cibicides lobatulus</i>				3									1	1				
<i>Melonis barleeanus</i>	4	3		6	7		8	7					10	9				
<i>Nonionellina labradorica</i>								3					1					
<i>Pullenia bulloides</i>	1			4			2	5					2					
Total number/sample	31	4		37	15		19	16					29	12				
SD	9,60	1,41		7,7	3,46		2,68	2,584					5,26	4,35				
Number/10 cm <sup>3</sup>	11.9	1.5		14.2	5.7		7.7	6.2					11.15	5				
#Taxa	4	2		5	3		5	4					6	3				
Shannon's H index	0.88	0.56		1.03	1.22		1.41	1.21					1.33	0.72				
Evenness index	0.60	0.87		0.70	0.81		0.82	0.84					0.62	0.58				
Chao1 index	4	2		5	3		5	4					6.3	3				

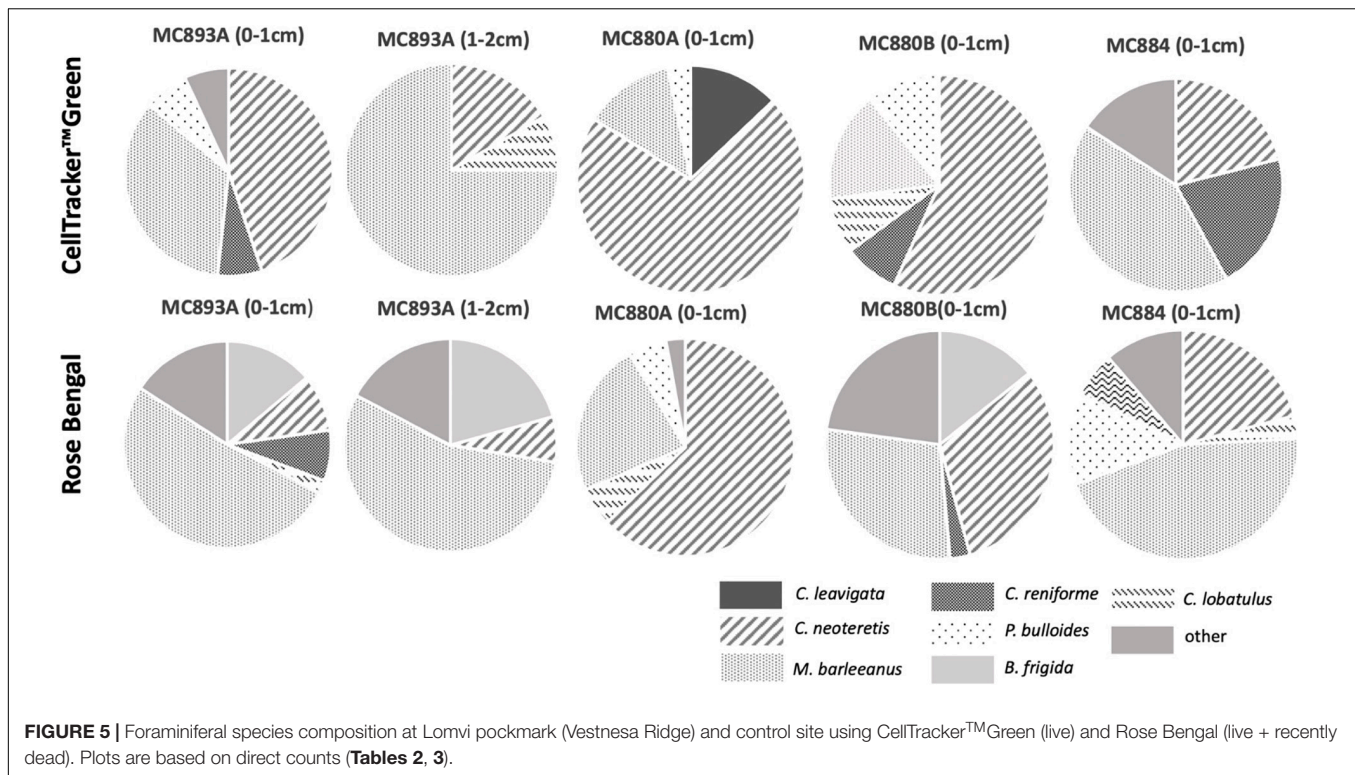
**TABLE 3** | Direct counts of Rose Bengal stained foraminifera from all samples.

Core number	Control site						Vestnesa Ridge											
	MC880A			MC880B			MC884			MC886			MC893A			MC893B		
Depth (cm)	0–1	1–2	2–3	0–1	1–2	2–3	0–1	1–2	2–3	0–1	1–2	2–3	0–1	1–2	2–3	0–1	1–2	2–3
<i>Adercotryma glomeratum</i>		3																
<i>Buccella frigida</i>		7		5				1					14	6				
<i>Cassidulina laevigata</i>	1							1	1	1								
<i>Cassidulina neoteretis</i>	44	24		11	12	17	9	13	3				9	2				
<i>Cassidulina reniforme</i>		3		1	1		3	1					8	1	1			
<i>Cibicides lobatulus</i>	4						2	2					2					
<i>Elphidium excavatum</i>												1						3
<i>Fissurina</i> sp.				2														
<i>Labrospira crassimargo</i>										7								
<i>Lagena</i> sp. 1		1						1										
<i>Lagena</i> sp. 2		1							1				1					
<i>Melonis barleeanus</i>	16			10	31	23	8	28	12	1			52	16	5	16	14	3
<i>Nonionellina labradorica</i>					1	3		1	1				1					
<i>Pullenia bulloides</i>	4	3			2	2	2	8	3	1			1	1		2		
<i>Reophax guttifer</i>		3						4	2					1				
<i>Reophax fusiformis</i>							2						12					
<i>Reophax</i> sp.				2	5	1						1	2			7		
<i>Stainforthia loeblichii</i>		1						2		1								
<i>Spiroplectammina earlandi</i>				1						4			1					
<i>Trifarina angulosa</i>	1			2			2											
<i>Triloculina</i> sp.				1			1		2									
Total number/sample	70	46		35	52	46	29	62	27	15			102	29	6	28	14	3
SD	17.83	7.32		3.95	11.70	10.10	3.06	8.34	3.36	2.5			15	5.52	2.82	6.37	0	0
Number/10 cm <sup>3</sup>	27	18		13 >4	20	17.6	11.2	23.8	10.4	5.8			39.2	11.2	2.3	10.7	5.4	1.2
#Taxa	6	9		9	6	5	8	11	10	6			11	7	2	4	1	1
Shannon's H index	1.08	1.55		1.79	1.15	1.04	1.80	1.62	1.75	1.43			1.6	1.37	0.45	1.09	0	0
Evenness index	0.55	0.54		0.66	0.52	0.60	0.76	0.48	0.63	0.69			0.44	0.56	0.78	0.74	1	1
Chao1 index	5	12		9.75	6.5	5	8	14.33	13.33	12			16	8	2	4	1	1

1–2 cm core depths, but not in the 2–3 cm interval. None of the individuals in cores MC886 and MC893B were metabolically active. At the control site, fluorescently labeled foraminifera were found in all samples from 0–1 cm and 1–2 cm intervals, while again no living foraminifera were observed in the 2–3 cm interval (**Figure 4** and **Table 2**). Rose Bengal stained aliquots indicated presence of foraminifera (live + recently dead) in cores MC893A, MC893B, MC884, MC880A, and MC880B in all sampling intervals. No Rose Bengal stained individuals were observed in the 1–2 cm and 2–3 cm samples at site MC886 (**Figure 4** and **Table 3**). Both the chi-square ( $p = 1$ ,  $\alpha = 0.05$ )

and Student's  $t$ -test ( $p = 0.44$ ,  $\alpha = 0.05$ ) test show significant differences between number of fluorescently labeled and Rose Bengal stained specimens, within any given sediment interval. In general, there was a lower number of foraminifera in the cold-seep samples than in the control samples. Density of fluorescently labeled foraminifera at Vestnesa Ridge ranged from 0 to 11.1 individuals per  $10\text{ m}^3$  in the 0–1 cm intervals, and from zero to five individuals per  $10\text{ cm}^3$  in the 1–2 cm intervals. Density of live foraminifera at control sites ranged from 7.7 to 14.2 individuals per  $10\text{ cm}^3$  in the 0–1 cm intervals, and from 1.5 to 6.1 in the 1–2 cm intervals (**Figure 4**). The number of Rose Bengal stained





foraminifera in samples from Vestnesa Ridge active sites ranged from 5.8 to 39.2 individuals per  $10\text{ cm}^3$  in the 0–1 cm interval, from 0 to 11.2 in the 1–2 cm interval, and from 0 to 2.3 in the 2–3 cm interval (Figure 4). In the sediment from the control site, the abundance of Rose Bengal stained (live + recently dead) foraminifera ranged from 11.3 to 27 specimens per  $10\text{ cm}^3$  in the 0–1 cm interval, from 20 to 46 foraminifera in the 1–2 cm interval and from 0 to 17.2 in the 2–3 cm interval (Figure 4).

CTG and Rose Bengal show that dominant and most common species were the same in the assemblages from active seep sites at Vestnesa Ridge (Tables 2, 3 and Figure 5). The S(H) index in CTG labeled samples from Vestnesa Ridge range from 0 (empty samples) to 1.33, and from 0.56 to 1.41 in samples from the control site. The S(H) index in Rose Bengal stained samples from Vestnesa Ridge range from 0 (empty sample) to 1.33, and from 1.04 to 1.8 in control site. The Pielou evenness index in CTG labeled samples from Vestnesa Ridge range from 0.58 to 0.88 and from 0.60 to 0.87 in samples from the control site (Table 2). In Rose Bengal stained samples the same index varies from 0.44 to 0.78 for Vestnesa Ridge and from 0.48 to 0.76 for the control site (Table 3).

In the fluorescently labeled samples from Vestnesa Ridge the foraminiferal faunas are dominated by *M. barleeanus* (34% of total fauna in the 0–1 cm interval and 69% in the 1–2 cm interval) and *C. neoteretis* (45% of total fauna in the 0–1 cm interval and 15% in the 1–2 cm interval) (Table 2). Similarly, in Rose Bengal-stained samples the most abundant species were *M. barleeanus* (51% of total fauna in the 0–1 cm interval and 55% in the 1–2 cm interval), *C. neoteretis* (9% of total fauna in the 0–1 cm interval and 7% in the 1–2 cm interval) and

*Buccella frigida* (14% of the total fauna in the 0–1 cm interval and 6% in the 1–2 cm interval) (Table 3). No apparent endemic foraminiferal species were observed in the Vestnesa Ridge seep sediment samples (Tables 2, 3).

### Storfjordrenna Pingos

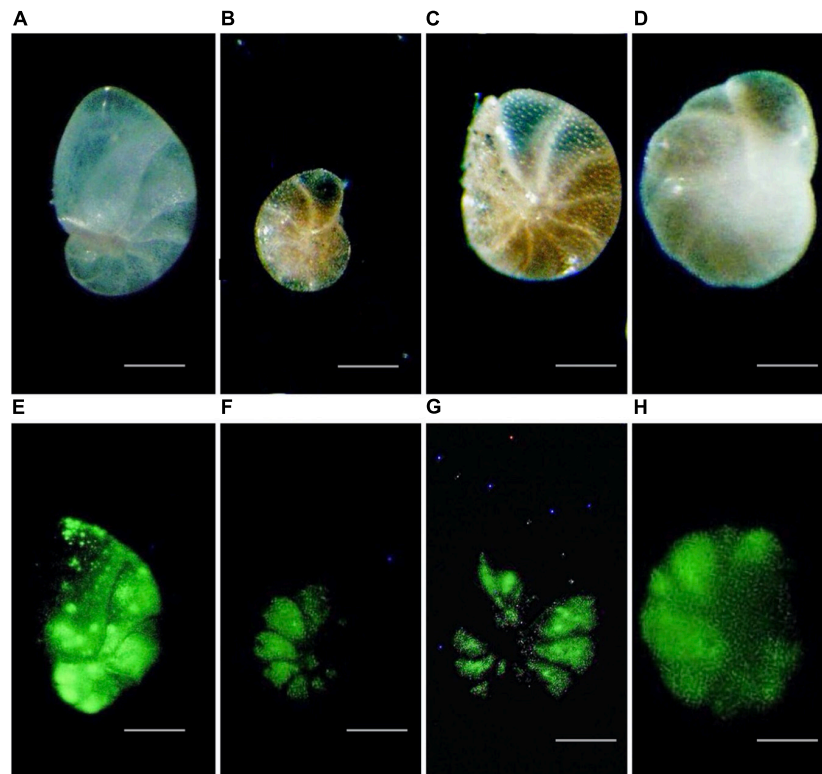
Metabolically active (fluorescently labeled) benthic foraminifera were present in both the active GHP1 and the inactive GHP5, except at site MC902 taken at the top of the active GHP1 (Table 4). In addition to live, metabolically active foraminifera, Rose Bengal staining shows presence of recently dead individuals, i.e., foraminiferal tests that still contain cytoplasm, but were not metabolically active at the time of collection, which could lead to a significant overestimation of the number of live foraminifera ( $p = 0.01$ ,  $\alpha = 0.05$ ; chi-square test) (Figure 7).

The ratio between fluorescently labeled and Rose Bengal-stained foraminifera differed between the active and inactive GHP1 and GHP5. A higher proportion of live to recently dead individuals was found in the inactive GHP5, and in GHP1 (except for the sample MC902, which appeared to be barren; Figure 7). The ratio between live vs. recently dead foraminifera was approximately 2:3 in the active GHP1, and 3:2 in the inactive GHP5 (Figure 7).

At GHP1, the density of live individuals increased along the transect from 0 individuals at the top of GHP1 to 11.84 (per  $10\text{ cm}^3$ ) at the edge of the pingo. At GHP5 (the non-active site), the foraminifera were relatively evenly distributed compared to the active GHP1. Similarly, to GHP1, the lowest density 3.43 (per  $10\text{ cm}^3$ ) of metabolically active foraminifera was observed in the sediment from the summit of GHP5 (Table 4). The S(H) index

**TABLE 4 |** Number per sample of CellHunt Green labeled (CHG) and Rose Bengal stained (RB) foraminifera (direct count), and Shannon diversity index from active GHP1 and inactive GHP5.

	MC902		MC917		MC919		MC920		MC921		MC922	
	CHG	RB	CHG	RB	CHG	RB	CHG	RB	CHG	RB	CHG	RB
<i>Buccella frigida</i>			4		6	14	2		2		3	
<i>Cassidulina laevigata</i>			2	1			1	1			1	
<i>Cassidulina neoteretis</i>					2	37		5		5		9
<i>Cassidulina reniforme</i>				4	1	6						3
<i>Cibicides lobatulus</i>			3	14	1	4	1	13	1	10		
<i>Elphidium excavatum</i>			1		8				3		8	
<i>Globobulimina turgida</i>				2	1	4	4	5	2	1	1	2
<i>Melonis barleeanus</i>			8	8	22	31	9	12	30	22	24	2
<i>Nonionellina labradorica</i>			6	3	18	18	10	5		1	3	
<i>Pullenia bulloides</i>				2		5		1	1	3	2	3
<i>Stainforthia loeblichii</i>						1						
<i>Triloculina</i> sp.				1								1
<i>Uvigerina</i> sp.				1	3	2		1				
Total number/sample			24	36	62	122	27	43	39	42	42	20
#Taxa			6	9	9	10	6	8	6	6	7	6
Shannon index			1.53		1.75		1.3		1.08		1.26	
Evenness index			0.83	0.65	0.59	0.63	0.71	0.70	0.40	0.61	0.54	0.77
Chao1 index			6	10	10.5	10	6.5	11	6.3	7	7.5	6



**FIGURE 6 |** Reflected light (A–D) and corresponding epifluorescence (green coloration from CellTracker™ Green) (E–H) micrographs of foraminifera collected at Vestnesa Ridge (core 893A); (A,E) *Nonionellina labradorica*; (B,C,G,F) *Melonis barleeanus*; (D,H) *Cassidulina neoteretis*. Scales: panels (A–D) = 30  $\mu\text{m}$ ; panels (B,C,F,G) = 25  $\mu\text{m}$ .

in samples from GHP1 ranged from 0 closer to the center to 1.75 at the edge, and in GHP5 from 1.08 to 1.26 (Table 4). The Pielou evenness index in CHG labeled samples from GHP1 varies from 0.59 to 0.83 and between 0.40 and 0.71 in GHP5 (Table 4).

Both the active and the post-active pingo were characterized by presence of the same dominant CHG labeled species: *M. barleeanus* (35% of total living fauna in GHP1, and 52% in GHP5), *N. labradorica* (28% in GHP1, and 11% in GHP5), *Elphidium excavatum* (11% in GHP1, and 9% in GHP5) and, to some extent, *B. frigida* (12% in GHP1; Figure 8). In the Rose Bengal-stained (i.e., recently dead) samples, the dominant species were *M. barleeanus* (25% of the total living fauna in GHP1, and 34% in GHP5), *C. neoteretis* (23% in GHP1, and 18% in GHP5), *C. lobatulus* (22% in GHP5), and to some extent *N. labradorica* (13% in GHP1, and 6% in GHP5) (Figure 8). No endemic species were found in any of the samples from GHP1 or GHP5 (Figure 8).

## Isotopic Signatures

CellTracker™ Green labeled foraminifera tend to have less negative  $\delta^{13}\text{C}$  signatures compared to Rose Bengal stained pools and empty tests of their conspecifics, both at Vestnesa Ridge (core MC893A) and at the control site (core MC880A and MC880B; Figure 9). The difference between  $\delta^{13}\text{C}$  measured in CTG labeled individuals and Rose Bengal-stained specimens is

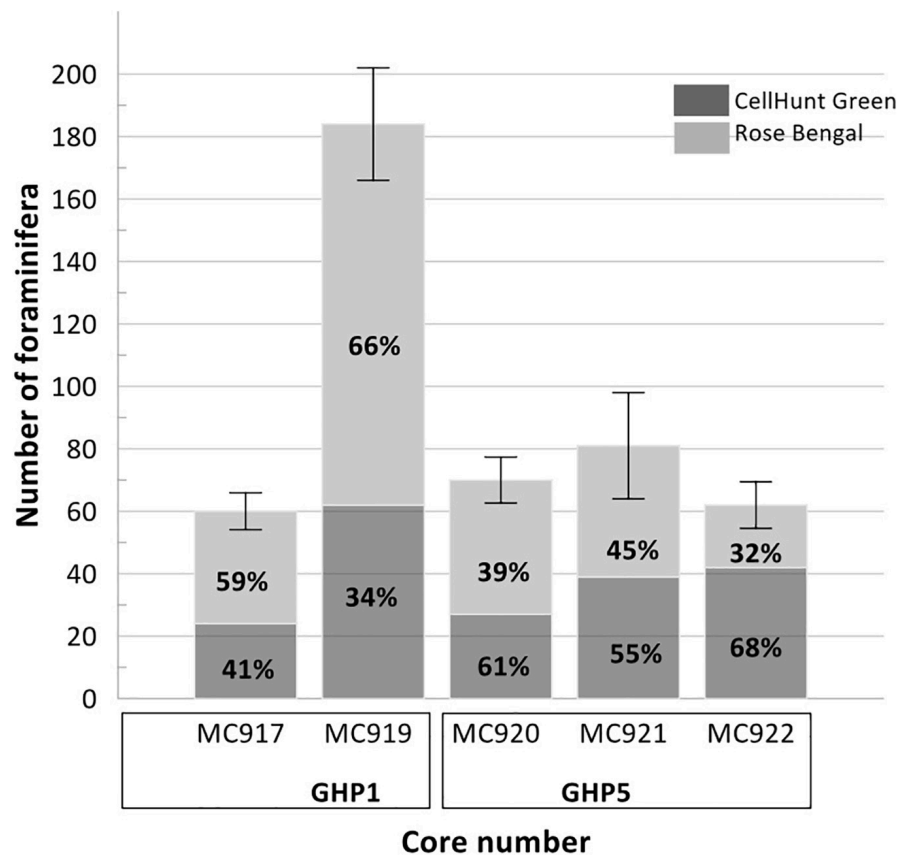
0.22‰ in samples from Vestnesa Ridge, whereas in samples from the control site the difference is 0.41 and 0.15‰. The difference between  $\delta^{13}\text{C}$  measured in CTG labeled and empty tests is 0.29 and 0.22‰ at Vestnesa Ridge, and between 0.04 and 0.27‰ at the control site (Table 5).

In contrast, in both GHP1 and GHP5 the  $\delta^{13}\text{C}$  values measured in CHG labeled pools are always considerably more depleted compared to values measured in Rose Bengal-stained specimens (Figure 8). The difference in  $\delta^{13}\text{C}$  values in CHG labeled foraminifera is 0.08‰ (at GHP1) and 0.20 and 0.49‰ (at GHP5; Table 5). The difference between  $\delta^{13}\text{C}$  values measured in CHG labeled foraminifera and unstained tests is 0.88 and 1.46‰ at GHP1 and range between 0.14 and 0.58‰ at GHP5. The most pronounced difference is found in samples from the active GHP1 (MC919), where the isotopic signature of live *M. barleeanus* is more depleted compared to the signature of dead individuals (about 1.46‰; Table 5 and Figure 8).

## DISCUSSION

### Foraminiferal Fauna

The study shows presence of living foraminifera in sediments from active methane emission sites from pockmarks at Vestnesa Ridge and from hydrate mounds (“pingos”) in Storfjordrenna.



**FIGURE 7** | Percentages of benthic foraminifera labeled with CellHunt Green (CHG) = live, and individuals subsequently stained with Rose Bengal (RB) = recently dead (direct count) from Storfjordrenna pingo. The number of foraminifera represents the sum of CHG (dark gray) and RB (light gray) individuals. Plot based on direct counts.

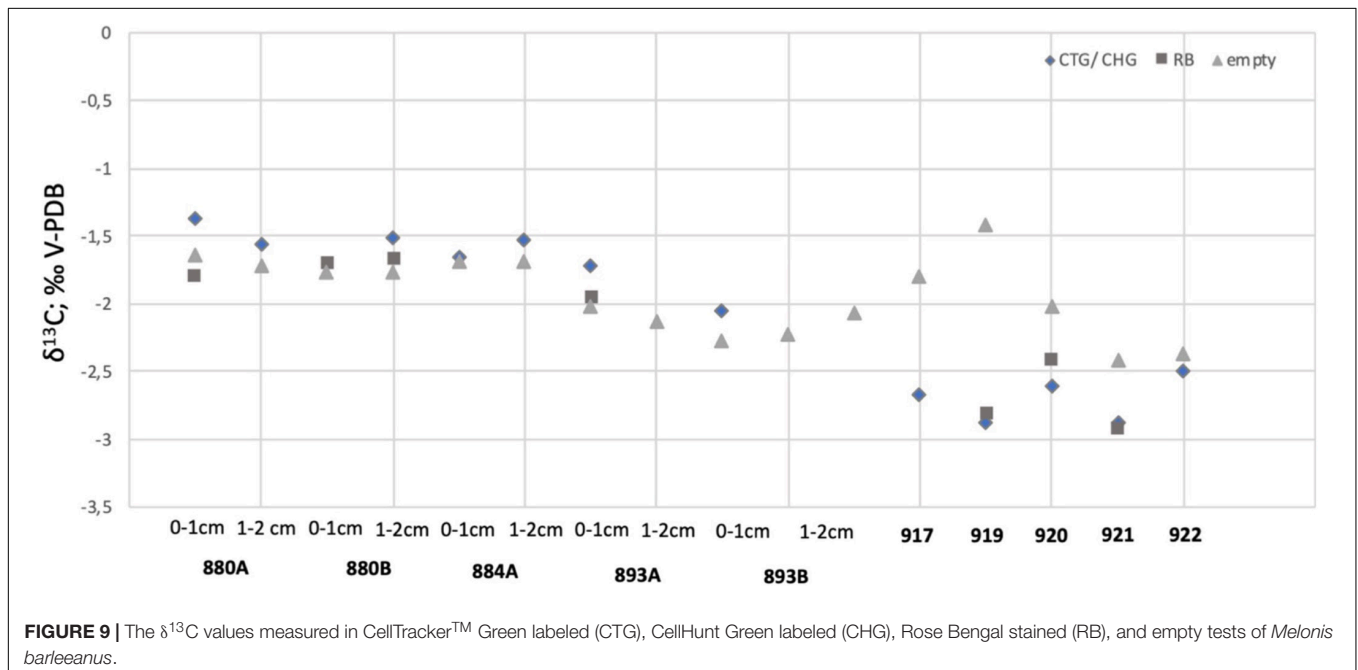
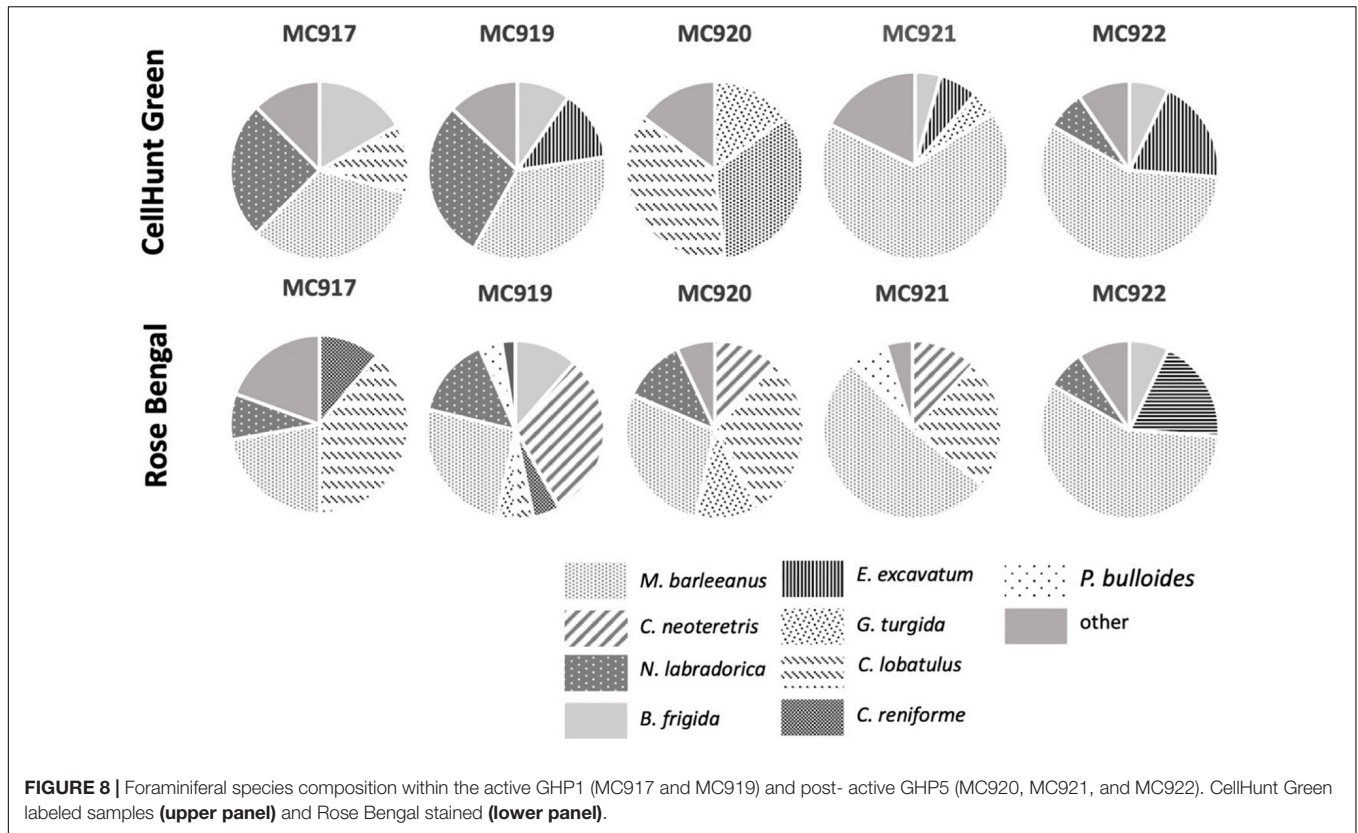
Results imply that, despite the hostile conditions (e.g., low oxygen, high carbon dioxide concentrations and potentially also hydrogen sulfide), which are a result of anaerobic methane oxidation (Herguera et al., 2014), the benthic foraminifera were metabolically active. Furthermore, the results confirmed a previous observation that Rose Bengal staining overestimated the number of live foraminifera (Bernhard et al., 2006). This is manifested for example by the presence of Rose Bengal stained foraminifera in samples with no fluorescently labeled individuals, or by a higher number of Rose Bengal stained foraminifera compared to fluorescently labeled individuals (see samples MC917 and MC919 from Storfjordrenna; **Figure 7**).

Considering the sampling location and previously published studies from Vestnesa Ridge (e.g., Panieri et al., 2017; Szybor and Rasmussen, 2017; Åström et al., 2018; Dessandier et al., 2019, 2020; Yao et al., 2019), the uneven distribution of foraminifera is most likely a result of the horizontal distribution of geochemically diverse microhabitats within the Vestnesa sediments. The TowCam imaging survey during the sampling campaign revealed a patchy distribution of organisms, such as white and gray bacterial mats and tubeworms (**Figure 2**), which correspond to geochemically different microhabitats (e.g.,

Niemann et al., 2006; Treude et al., 2007). For example, gray bacterial mats (*Arcobacter* spp., *Thiomargarita* spp.) are common in unstable environments, whereas white bacterial mats (*Beggiatoa* spp.) and tubeworms fields indicate stable sulfide conditions (e.g., Sahling et al., 2002; Niemann et al., 2006; Treude et al., 2007). It has been previously observed that in response to a heterogeneous distribution of methane-dependent microbial and macrofaunal biota, the foraminiferal species composition and absolute abundance (density) may show great variability within the same seep area (e.g., Rathburn et al., 2000; Wollenburg and Mackensen, 2009; Dessandier et al., 2019).

In this study, CTG shows the lowest number or absence of metabolically active foraminifera in some of the assemblages from Vestnesa Ridge, which indeed can be interpreted as an environment being inhospitable for foraminifera. At the same time, in the samples from the MC886 site with no fluorescently labeled individuals, Rose Bengal stained foraminifera (mostly agglutinated taxa) are still present. This observation suggests that “inhospitable” conditions are temporary variations rather than permanent constraints. Instability/variability of the environment can be related to the ephemeral nature of methane seeps, which are strongly dependent on methane





flux (Levin, 2005; Åström et al., 2020). As shown by Yao et al. (2019), the Lomvi (MC893) and Lunde (MC886) pockmarks are characterized by two different types of methane transport: advective and dominated by methane diffusion (Lomvi and

Lunde, respectively). Additionally, sulfate and methane profiles within the MC886 core indicate non-steady-state conditions (Yao et al., 2019). Those unstable conditions could explain the lack of metabolically active foraminifera with a presence

of Rose Bengal stained specimens in the samples from Lunde pockmark (MC886) at the time of sampling. Similar observations were made by Dessandier et al. (2019): the authors suggested a correlation between low density of foraminifera and methane-diffusive areas due to high sulfide concentration, and decreased or lack of agglutinated foraminifera in cold seep assemblages as a result of an increase in organic matter content due to methane related microbial mass, and stronger competition from calcareous species.

Similarly to Vestnesa Ridge, the benthic foraminiferal distribution pattern within the active GHP1 shows a greater variability along the analyzed transect compared to the transect along the inactive GHP5. The highest density of foraminifera is observed at the edge of GHP1, where white and gray bacterial mats are present, with a small difference in density toward the center of GHP1, and reaching zero individuals approximately at the top, where most of the methane flares are located (Serov et al., 2017; Carrier et al., 2020). The  $\delta^{13}C_{DIC}$  value at the top of the active GHP reached  $-24.2\%$ , which can be linked to methane-related microbial activity (Dessandier et al., 2019). The absence of foraminifera at the summit is thus most likely due to the combined effect of disturbance caused by gas bubbles passing through the sediment and geochemical constraints related to microbial activity (e.g., low oxygen or presence of hydrogen sulfide; Herguera et al., 2014; Carrier et al., 2020). The Shannon index shows that the suite of samples from GHP 5 has less variability compared to the samples from GHP1. The highest density and diversity are observed at the edge of GHP1 (MC919), in bacterial mats. Similarly to other methane cold seeps, the microbial community at the active GHP1 might serve as a food source and support benthic foraminiferal growth (e.g., Rathburn et al., 2000; Panieri, 2006; Fontanier et al., 2014; Herguera et al., 2014).

It is widely accepted that the distribution of benthic foraminiferal faunas at cold seeps is mainly controlled by oxygen levels and organic content, and that species preferring organic-rich environments and reduced oxygen are well adapted to live in the environmental conditions of seep sites (e.g., Akimoto et al., 1994; Rathburn et al., 2000, 2003; Bernhard et al., 2001; Fontanier et al., 2014). In fact, the living foraminiferal fauna at Vestnesa Ridge is dominated by *M. barleeanus* and *C. neoteretis*, and by *M. barleeanus* and *N. labradorica* at the active GHP1. *M. barleeanus* is described as an intermediate to deep infaunal species associated with high-nutrient conditions and resistant to environmental stress due to organic matter degradation (e.g., Wollenburg and Mackensen, 1998; Alve et al., 2016). Both *M. barleeanus* and *C. neoteretis* have been previously observed as the most abundant species in methane-charged sediments at Vestnesa Ridge (Dessandier et al., 2019). Additionally, the TEM (transmission electron microscopy) analyses of *M. barleeanus* from Lomvi pockmark (MC893) at Vestnesa Ridge revealed presence of methanotrophic-like bacteria located outside the test, but very close to their apertural region (Bernhard and Panieri, 2018). Although a possible symbiosis between *M. barleeanus* and methanotrophs remains unconfirmed, the potential influence of seep-related bacteria on *M. barleeanus* cannot be excluded. Similarly, to *M. barleeanus*, *C. neoteretis* (Rose Bengal stained)

was found to be dominant in the top layers of the dysoxic (low oxygen) sediments of the Håkon Mosby Mud Volcano (Wollenburg and Mackensen, 2009) and was one of the most numerous species at Vestnesa Ridge (Dessandier et al., 2019). As in other investigated methane seep sites, to date, there are no endemic species found at Vestnesa Ridge and Storfjordrenna pingos, but only well-known species represented in a wide range of environments (e.g., Rathburn et al., 2000; Bernhard et al., 2001; Herguera et al., 2014; Dessandier et al., 2019).

The combined use of CHG and Rose Bengal allows to distinguish live and recently dead foraminifera from the Storfjordrenna area, which reveals major shifts in species compositions in both the active GHP1 and the inactive GHP5. In live foraminiferal assemblages, the most common species after *M. barleeanus* are *N. labradorica* and *E. excavatum*, whereas in Rose Bengal stained samples *C. neoteretis* and *C. lobatulus* are of high relative abundance. Because species which tolerate high organic concentration and low oxygen conditions are associated both with spring bloom and methane seepage, it is challenging to distinguish precisely to what extent the switch in population is due to methane availability. Particularly, the relatively high number of live *N. labradorica* both in the active GHP1 and inactive GHP5, as well as appearance of *E. excavatum*, might indicate the influence of the seasonal algae bloom. *E. excavatum* is an opportunistic species, with the ability to respond rapidly to deposition of food (pulsed food supply; Corliss, 1991; Altenbach, 1992) and colonize harsh environments (Korsun and Hald, 2000). It almost completely replaces other species, such as *C. lobatulus*, which is an epifaunal species that prefers low food supply and high oxygen concentration (e.g., Hald and Steinsund, 1996; Klitgaard-Kristensen et al., 2002). The significant number of *N. labradorica* and *B. frigida* in samples MC919 from GHP1 is puzzling. Although *N. labradorica* is known to feed on fresh phytodetritus, and is an indicator species of high primary productivity as a result of the retreating summer sea-ice margin or Arctic Front (Cedhagen, 1991; Corliss, 1991), this species also has a potential to thrive at methane seepage sites. *N. labradorica* (Rose Bengal stained) have been found previously in the sediment from the top of the GHP1 (Dessandier et al., 2020). Kleptoplasts present in cell of *N. labradorica* might be involved in ammonium or sulfate assimilation pathways and might potentially support life under adverse conditions (Jauffrais et al., 2019). Alike *N. labradorica*, the distribution of *B. frigida* is related to seasonal sea-ice retreat and appearance of fresh algae (Seidenkrantz, 2013). From all investigated samples from Storfjordrenna, *B. frigida* occurs most frequently in the MC919 samples, where bacterial mats are present. In previous studies from Vestnesa Ridge, it was suggested that the species potentially can feed on microbial food sources, i.e., methane related bacterial mats (Dessandier et al., 2019). Interestingly, in this study *B. frigida* occur in CTG labeled samples, but there were no Rose Bengal stained individuals. This suggests that presence of live *B. frigida* might reflect a relatively recent appearance of bacterial mats associated with methane seepage.

Additionally, the use of both CHG and Rose Bengal reveals a difference in the percentage of living vs. recently dead



foraminifera within each of the investigated GHP types. The active GHP1 is characterized by a greater percentage of recently dead (Rose Bengal stained) individuals, compared to living (CHG labeled) specimens, whereas in the inactive GHP5 pingo this ratio is reversed with more live than dead foraminifera. This difference between the active GHP1 and inactive GHP5 implies more unstable and variable environmental conditions at GHP1, potentially related to methane emissions, rather than general seasonal environmental changes (Carrier et al., 2020). On average, CHG labeling showed that approximately 40% of the benthic foraminifera in GHP1 and approximately 54% in GHP5 were alive at the time of collection.

Interestingly, despite the highest number of living foraminifera in GHP5 the Pielou evenness index in CHG labeled samples shows fairly low values (from 0.40 to 0.71) compared to samples from GHP1 (from 0.59 to 0.83) (Table 4). It indicates the presence of dominant, well-adapted species in the foraminiferal population within the post-active GHP, most likely due to the recent environmental changes. Because, the Pielou evenness index is relatively low in the post-active GHP5 compared to GHP1, we can exclude methane influence. It is possible the evenness index decreased due to the influence of the spring bloom. In the literature methane cold seeps are described as a biological oasis in the high-Arctic deep sea (Åström et al., 2018) due to the presence of microbial communities seeps provide enough food to sustain foraminiferal populations (e.g., Rathburn et al., 2000; Torres et al., 2003; Heinz et al., 2005; Panieri, 2006; Panieri and Sen Gupta, 2008). In contrast, sediments outside the seeps are impoverished in organic substrates for most of the year and depend on benthic-pelagic coupling (Gooday, 1988). Thus, the benthic communities in the Arctic, which experience low food are likely more sensitive to food input from primary production (e.g., Gooday, 1988, 1993; Sander and van der Zwaan, 2004; Nomaki et al., 2005; Schönfeld and Numberger, 2007; Braeckman et al., 2018). After the episode of strong food pulses, a population of specific opportunistic species increased, which can quickly utilize large amounts of detritus (e.g., Gooday, 1988; Nomaki et al., 2005; Braeckman et al., 2018). In fact, samples from GHP5 are dominated by *M. barleeanus*, an opportunistic species well adapted to high organic content (e.g., Wollenburg and Mackensen, 1998; Alve et al., 2016) and shows a relatively high number of *E. excavatum*. A "bloom-feeding" behavior of *E. excavatum* was previously described by Schönfeld and Numberger (2007). In comparison, the foraminiferal fauna from the active GHP consists mainly of species such as *B. frigida* and *N. labradorica*, species that thrive in cold seeps and can feed on bacteria (e.g., Dessandier et al., 2019; Jauffrais et al., 2019).

## The $\delta^{13}\text{C}$ Signature in Foraminiferal Tests

Within methane cold seeps, the geochemistry of pore water is influenced by aerobic and/or anaerobic methane oxidation (Treude et al., 2007). Because methane-derived carbon is characterized by very low carbon isotopic signatures (from  $-50$  to  $-20\text{‰}$  for thermogenic methane, and from  $-110\text{‰}$  to  $-60\text{‰}$  for microbial methane) (Whiticar, 1999), the ambient DIC pool

is enriched in isotopically light carbon in the form of either carbon dioxide ( $\text{CO}_2$ ) or bicarbonate ( $\text{HCO}_3^-$ ) resulting from microbial activity. If foraminifera incorporate methane-derived carbon from the ambient seawater during biomineralization, we would expect to see more negative  $\delta^{13}\text{C}$  values in their tests compared to  $\delta^{13}\text{C}$  values in tests of foraminifera from the non-seep sites. At Lomvi pockmark (Vestnesa Ridge), the  $\delta^{13}\text{C}$  measured on CTG labeled, Rose Bengal stained, and unstained tests of *M. barleeanus* have values within the same range as of its conspecifics in "normal" (non-seep) marine environments, i.e., approximately  $-2\text{‰}$  (e.g., Wollenburg et al., 2001; Dessandier et al., 2020). Likewise, the  $\delta^{13}\text{C}$  measured on live *C. neoteretis* showed values within the expected range for specimens from non-seep environments, i.e., approximately  $-0.3\text{‰}$  to  $-1\text{‰}$  (Wollenburg et al., 2001), and was not as depleted as previously reported values ( $-7.5\text{‰}$   $\delta^{13}\text{C}$ ) measured on Rose Bengal stained *C. neoteretis* from Håkon Mosby Mud Volcano (Mackensen et al., 2006). Therefore, the data provide no clear evidence that *M. barleeanus* and *C. neoteretis* from Vestnesa Ridge incorporate significant amounts of methane-derived carbon during test formation that would markedly affect the isotopic signature of their carbonate tests. The difference between  $\delta^{13}\text{C}$  signatures of *M. barleeanus* and *C. neoteretis* most likely reflects different microhabitat preferences of these species. Infaunal species, such as *M. barleeanus*, tend to have more negative  $\delta^{13}\text{C}$  compared to, for example, epifaunal or shallow infaunal species, such as *C. neoteretis* (e.g., Grossman, 1984; McCorkle et al., 1985; Fontanier et al., 2006).

The  $\delta^{13}\text{C}$  measured in both metabolically active (CHG labeled) and recently dead (Rose Bengal stained) foraminifera from Storfjordrenna pingos is not straightforward to interpret. Although the  $\delta^{13}\text{C}$  in tests of fluorescently labeled foraminifera from the active GHP1 have values slightly more depleted than the values exhibited by the same species in the post-active GHP5, still the  $\delta^{13}\text{C}$  values measure in *M. barleeanus* from both GHPs are not much more depleted compared to Rose Bengal stained conspecific from near non-seep site (i.e., lower than  $-2.1\text{‰}$ ; Dessandier et al., 2019). Overall, the  $\delta^{13}\text{C}$  measured in *M. barleeanus* from Storfjordrenna are not significantly depleted compared to isotopic signatures of other seep-site foraminifera, e.g., *Uvigerina peregrina* with measured  $\delta^{13}\text{C}$  values down to  $-5.64\text{‰}$  (Hill et al., 2004), or *C. neoteretis* with  $\delta^{13}\text{C}$  values of  $-7.5\text{‰}$  (Mackensen et al., 2006). Storfjordrenna is at a relatively shallow water depth ( $\sim 400$  m) and the sediment samples were collected in June. Thus, the negative  $\delta^{13}\text{C}$  signature in foraminiferal tests could originate from a greater flux of particulate organic matter produced during the spring bloom and only potentially partly from methane seepage. A shift of approximately  $0-4\text{‰}$  toward a more negative  $\delta^{13}\text{C}$  is shown to have an origin in local organic matter degradation (e.g., Torres et al., 2003; Martin et al., 2004).

It is generally believed that more negative  $\delta^{13}\text{C}$  signatures in unstained and/or fossil foraminifera compared to those of "living" (Rose Bengal stained) specimens result from an authigenic overgrowth layer covering the tests. Foraminiferal tests deposited in methane-charged sediments might be coated by precipitates from highly  $^{13}\text{C}$ -depleted pore water or

bacterially mediated methane oxidation and associated carbonate precipitation (e.g., Rathburn et al., 2003; Torres et al., 2003; Schneider et al., 2017; Szytybor and Rasmussen, 2017). A similar interpretation can be applied to explain the offset in  $\delta^{13}\text{C}$  values between Rose Bengal stained and fluorescently labeled *M. barleeanus* from Vestnesa Ridge. Since Rose Bengal stained foraminifera represent both live and recently dead individuals, it is possible that in some of the specimens the organic lining was already partially decomposed, and that this surface of the tests had authigenic carbonate overgrowths (Mackensen et al., 2006). Considering the fact that isotopic offset occurred both in samples from Lomvi and the control site, and the isotopic variation is relatively low ( $\sim 0.20\%$  Vestnesa Ridge, 0.15 and 0.41‰ at the control site), the offset could be due to dissolution of biogenic calcite and re-precipitation of inorganic calcite (overgrowth and recrystallization) or other early diagenetic processes that occur in normal non-seep sediments (Ravelo and Hillaire-Marcel, 2007), and as such not necessarily the effect of Methane-Derived Authigenic Carbonates (MDAC) overgrowth. Additionally,  $\delta^{13}\text{C}$  values recorded in unstained tests of the planktonic foraminifera *N. pachyderma* from Vestnesa Ridge are close to the expected  $\delta^{13}\text{C}$  values for normal “Holocene” marine environments ( $-0.5$  to  $0.5\%$ ; Zamelczyk et al., 2014; Werner et al., 2016). Because planktic foraminifera live and calcify in the water column, significantly depleted  $\delta^{13}\text{C}$  signature ( $-7\%$  or higher; Torres et al., 2003) in their unstained tests results from diagenetic overgrowth by authigenic carbonates associated with aerobic methane oxidation (AOM; Torres et al., 2003; Uchida et al., 2004; Martin et al., 2010; Schneider et al., 2017). Values obtained for *N. pachyderma* from Vestnesa Ridge support the inference that benthic foraminiferal assemblages have not been significantly overprinted by MDAC.

Unlike Vestnesa Ridge, in GHP1 and GPH 5 the  $\delta^{13}\text{C}$  values in the fluorescently labeled *M. barleeanus* are always more negative compared to the  $\delta^{13}\text{C}$  in Rose Bengal stained and unstained tests. This could suggest that living foraminifera did incorporate methane-derived carbon during biomineralization. Mackensen et al. (2006) suggested that more depleted isotopic  $\delta^{13}\text{C}$  signatures in living (Rose Bengal stained) foraminifera compared to unstained tests can be interpreted as a result of methane influence. In sample MC919 the difference between  $\delta^{13}\text{C}$  measured in live *M. barleeanum* compared to value in empty tests is pronounced (about  $1.55\%$ ), whereas the difference between  $\delta^{13}\text{C}$  in live foraminifera and empty tests in the post-active GHP5 does not exceed  $0.4\%$ . Most likely, foraminifera absorbed methane-derived carbon *via* the food web by feeding on methanotrophic bacteria (see section “Foraminiferal Fauna”).

Although the  $\delta^{13}\text{C}$  signatures in tests of live foraminifera from the study areas are not significantly depleted to determine the influence of methane, it should be noted that the  $\delta^{13}\text{C}$  are measured on pools of specimens ( $N = \sim 10$ ). It is possible that at least some of the individuals had more negative  $\delta^{13}\text{C}$  signatures than others, or that some chambers indeed incorporated methane-derived carbon, as suggested by Bernhard et al. (2010). However, even if the foraminifera calcified during episodes of high methane flux, it is likely that only parts of the tests were constructed under intense seepage conditions, while

the major part of the tests had a pre-seep or post-seep signatures (i.e., carbon isotopes incorporated before or after a seepage event). Methane is only one of the potential carbon sources at cold seeps. In surface sediments, the biological degradation of marine snow contributes to the local DIC pool and might explain the negative signature of the  $\delta^{13}\text{C}_{\text{DIC}}$  (e.g., Alldredge and Silver, 1988; Bauer and Druffel, 1998; Torres et al., 2003). As an example, a previous study of the  $\delta^{13}\text{C}_{\text{TOC}}$  values for Vestnesa Ridge showed presence of both classical marine  $\delta^{13}\text{C}_{\text{TOC}}$  and depleted  $\delta^{13}\text{C}_{\text{TOC}}$  related to methane seepage (Dessandier et al., 2019). Thus, if the foraminifera use carbon both from ambient water and intracellular storage (i.e., resulting from respiration and diet; de Nooijer et al., 2009; Toyofuku et al., 2017), it seems unlikely that the isotopic signature of foraminifera only reflects the methane-derived carbon; rather, it may be a result of both non-seep and seep carbon. To obtain more accurate  $\delta^{13}\text{C}$  values, analysis of single specimens, or more advanced techniques, e.g., secondary-ion mass spectrometry (SIMS) is recommended.

## CONCLUSION

1. Labeling with fluorescence probes showed that metabolically active foraminifera were present in methane-influenced sediments both at Vestnesa Ridge and Storfjordrenna. Both sites were characterized by comparable faunal patterns, with no endemic species, and the observed species were similar to those from other non-seep locations within the Arctic Ocean. At Vestnesa Ridge, and at the non-seep control site off Vestnesa Ridge, the most abundant calcareous species were *M. barleeanus* and *C. neoteretis*. In Storfjordrenna in both GHP environments, the foraminiferal faunas were dominated by *M. barleeanus* and *N. labradorica*.
2. Methane seepage did not markedly affect the isotopic signature ( $\delta^{13}\text{C}$ ) of primary calcite in metabolically active foraminifera. One exception was sample MC919, where a more negative isotopic signature of *M. barleeanus* could potentially reflect methane influence.
3. The results of this study show the effectiveness of fluorescent probes in ecological studies. At Vestnesa Ridge, Rose Bengal staining overestimated the number of living foraminifera, indicating a higher number of live foraminifera compared to the CTG labeled specimens (23% of foraminifera were live at Vestnesa Ridge and 34% at the control site).
4. There is no significant difference between  $\delta^{13}\text{C}$  measured in fluorescent labeled foraminifera and Rose Bengal stained.
5. At Storfjordrenna, the combined use of CHG and Rose Bengal allowed to distinguish between living and recently dead benthic foraminifera. This demonstrated a marked change in the foraminiferal population from a *C. neoteretis/Cibicides lobatulus* dominated assemblage to an assemblage dominated by *M. barleeanus* and *N. labradorica*, which otherwise would have been overlooked. Despite the more time-consuming protocol compared to Rose Bengal staining, the fluorescent viability

assays such as CHG and CTG CMFDA have a great advantage and it is advised that they be applied more often in studies of the ecology of benthic foraminifera.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

KM collected and processed the samples, analyzed data, and wrote the manuscript.

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**Conflict of Interest:** The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Article 3**

**Evidence for influence of methane seepage on isotopic signatures in living deep-sea foraminifera,**

**79 °N.**

**Manuscript 4**

**Response of benthic foraminifera to ecological succession of cold seeps from Vestnesa Ridge,  
Svalbard: implications for interpretations of paleo-seepage environments.**

