Deposit feeding of a foraminifera from an Arctic methane seep site and possible association with a methanotroph revealed by transmission electron microscopy

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Abstract. Several foraminifera are deposit feeders that consume organic detritus (dead particulate organic material along with entrained bacteria). However, the role of such foraminifera in the benthic food-web remains understudied. As foraminifera may associate with methanotrophic bacteria, which are $^{13}$C-depleted, feeding on them has been suggested to cause negative $\delta^{13}$C values in the foraminiferal cytoplasm and/or calcite. To test whether the foraminiferal diet includes methanotrophs, we performed a short-term (1 d) feeding experiment with Nonionella labradorica from an active Arctic methane-emission site (Storfjordrenna, Barents Sea) using the marine methanotroph Methyloprofundus sedimenti, and analyzed N. labradorica cytology via Transmission Electron microscopy (TEM). We hypothesized that M. sedimenti would be visible, as evidenced by their ultrastructure, in degradation vacuoles after this feeding experiment. Sediment grains (mostly clay) occurred inside one or several degradation vacuoles in all foraminifers. In 24% of the specimens from the feeding experiment degradation vacuoles also contained bacteria, although none could be confirmed to be the offered M. sedimenti. Observations of the area adjacent to the aperture after 20 h incubation revealed three putative methanotrophs, close to clay particles. These methanotrophs were identified based on internal characteristics such as a type I stacked intracytoplasmic membranes (ICM), storage granules (SG) and gram-negative cell walls (GNCW). Furthermore, N. labradorica specimens were examined for specific adaptations to this active Arctic methane-emission site; we noted the absence of bacterial endobionts in all specimens examined but confirmed the presence of kleptoplasts, which were often partially degraded. Based on these observations, we suggest that M. sedimenti can be consumed by N. labradorica via untargeted grazing in seeps and that N. labradorica can be generally classified as a deposit feeder at this Arctic site. These results suggest that if methanotrophs are available to the foraminifera in their habitat, their non-selective uptake could make a substantial contribution to altering $\delta^{13}$C values. This in turn may impact metazoans grazing on benthic foraminifera by altering their $\delta^{13}$C signature.

1. Introduction

In methane seep sites, the upward migration of methane affects the pore-water chemistry of near-surface sediments, where benthic foraminifera inhabiting the sediment interface have been shown to live (e.g., Dessandier et al., 2019). Extremely light isotopic signals of $\delta^{13}C$ have been measured in seep-associated foraminiferal calcite tests (Wefer et al., 1994; Rathburn et al., 2003; Hill et al., 2004b; Panieri et al., 2014). One explanation of low $\delta^{13}C$ signals in foraminifera could be due to the ingestion of $^{13}C$-depleted methanotrophs (McCorkle et al., 1990; Wefer et al., 1994; Rathburn et al., 2003; Panieri, 2006). Recently, specimens of the foraminifer *Melonis barleeanus* (Williamson, 1858) collected from an active methane seep site were closely associated with putative methanotrophs at their apertural region (Bernhard and Panieri, 2018).

The observation by Bernhard and Panieri (2018) brought to light the need to examine feeding habits of foraminifera living on or around methane seeps. The species *M. barleeanus* could feed on aerobic methane-oxidizing bacteria (methanotrophs), which are abundant in the water column around methane seeps (Tavormina et al., 2010). Methanotrophs produce the biomarker diplopterol, which has an extremely light $\delta^{13}C$ signature ($-60$ ‰) and makes methanotrophs isotopically very light themselves (Hinrichs et al., 2003). If foraminifera accidentally or intentionally ingest methanotroph, $\delta^{13}C$ values of foraminiferal cytoplasm should be altered by such phagocytosis. However, experimental evidence was inconclusive whether isotope labelling of food can influence foraminiferal calcite, as no new calcite was produced during experiments using the foraminifera *Haynesina germanica* and *Ammonia beccari* (Mojtahid et al., 2011). Experiments using a novel high-pressure incubator on *Cibicides wuellerstorfi* illustrated the difficulty to measure the relationship between methane exposure, $\delta^{13}C_{\text{DIC}}$ and $\delta^{13}C_{\text{test}}$, as whole cores were incubated, the $\delta^{13}C_{\text{DIC}}$ of the seawater was impossible to keep constant and to compare $\delta^{13}C_{\text{test}}$ formed in the presence of methane to normal marine conditions (Wollenburg et al., 2015).

Several studies found that the lightest isotopic $\delta^{13}C$ values were measured in tests coated by methane-derived authigenic carbonate (MDAC) overgrowth (Torres et al., 2010; Panieri et al., 2014; Consolaro et al., 2015; Panieri et al., 2017; Schneider et al., 2017). MDACs represent a diageneric alteration of the foraminiferal test that alters the $\delta^{13}C$ of the foraminiferal isotope record. It can form high-Mg-calcite coatings contributing to the bulk of foraminiferal carbonate up to 58 wt% MgCO (Schneider et al., 2017). MDACs are formed at the sulfate-methane-transition zone (SMTZ), near the sediment-water interface where the upward flow of methane...
encounters the downward diffusion of sulfate from overlying seawater (Bian et al., 2001; Schneider et al., 2017).

Light $\delta^{13}C$ values of foraminiferal calcite have been explained as being formed in the presence of methane as an active uptake of methane-derived carbon produced by anaerobic oxidation of methane (AOM) (Rathburn et al., 2003; Hill et al., 2004a; Panieri et al., 2014). Within the zone of active AOM, the Dissolved Inorganic Carbon (DIC) exhibits the maximum $^{13}C$-depletion (Whiticar, 1999; Ussler and Paull, 2008). One hypothesis to explain extremely light $\delta^{13}C$ values recorded in benthic foraminiferal calcite is that foraminifera assimilate the carbon as $^{13}C$-depleted methane-derived DIC, which would lead to extremely light $\delta^{13}C$ values. The possibility that $^{13}C$-depleted DIC from the pore water can be assimilated by foraminifera is currently debated. Some studies suggest it is not possible (Herguera et al., 2014) while others assert feasibility if foraminifera calcify close to seeps (Rathburn et al., 2003; Hill et al., 2004a; Panieri et al., 2014).

However, light $\delta^{13}C$ values remain in many tests after MDACs are removed (Panieri et al., 2014) and have been measured also in primary calcite, without MDACs, from tests in methane-rich environments (e.g. Mackensen, 2008; Dessandier et al., 2019). These observations again point to the role of food organisms influencing the cytoplasmatic $\delta^{13}C$ and could be incorporated into the geochemistry of the test.

Foraminifera play an important role in the carbon cycle on the deep seafloor (Nomaki et al., 2005) where feeding behavior and food preference vary with species (Nomaki et al., 2006). Selected species of deep-sea benthic foraminifera have been shown to feed selectively on $^{13}C$-labeled algae from sedimentary organic matter, but unselectively on $^{13}C$-labeled bacteria of the strain Vibrio (Nomaki et al., 2006). A study from the seafloor around Adriatic seeps suggested that $\delta^{13}C$ of foraminiferal cytoplasm could be influenced by feeding on the sulfur-oxidizing bacterium Beggiatoa, whose abundance was also positively correlated with foraminiferal densities (Panieri, 2006). Generally, foraminifera can ingest dissolved organic matter (DOM), some are herbivorous, carnivorous, suspension feeders and most commonly deposit feeders using their pseudopodia. Hence, bacteria are involuntarily part of the “food-mix” (Levinton, 1989). The fact that bacteria are sometimes part of the “food mix” made us investigate if Nonionellina labradorica associated with methanotrophs, e.g. Metyloprofundus sedimenti, in a short-term
feeding experiment. *Nonionellina labradorica* is a benthic foraminifera that can reach substantial sizes, is an abundant species in the North Atlantic, and is the northern-most species of the Nonionellidae (Cedhagen, 1991). It also occurs together with *N. digitata* in Svalbard fjord sediments (Hald and Korsun, 1997; Shetye et al., 2011; Fossile et al., 2020). The genus *Nonionella* is potentially capable to denitrify, which as demonstrated on the species *Nonionella* cf. *stella* (Risgaard-Petersen et al., 2006), and NIS *Nonionella* sp. T1 (Choquel et al., 2021), and has been speculated also for *Nonionellina labradorica* (reviewed Charrieau et al., 2019). Next to its wide distribution, it is an especially interesting experimental species, because it hosts kleptoplasts, *i.e.* sequestered chloroplasts, of diatom origin inside its cytoplasm (Cedhagen, 1991; Jauffrais et al., 2018). SEM images of *N. labradorica*’s aperture show a specific ornamentation, possibly a morphological adaptation to this “predatory” mode of life for obtaining the kleptoplasts (Bernhard and Bowser, 1999). It is speculated that in deep-sea specimens the function of kleptoplasts is rather related to the sulfur cycle rather than with photosynthesis (Jauffrais et al., 2019). Our study does not concentrate on kleptoplasts but rather analyzed feeding preferences and contents of the degradation vacuoles of this species from an active methane-emitting site in the Arctic (Storfjordrenna, Barents Sea).

### 2. Materials and methods

#### 2.1. Site description and sampling living foraminifera

The sampling site was located app. 50 km south of Svalbard at 382 m water depth at the mouth of Storfjordrenna (Serov et al., 2017). The site is characterized by several large gas hydrate pingos (GHP), which actively vent methane spread over an area of 2.5 km². At this site our sample was taken at GHP3 is referred as an underwater gas hydrate-bearing mound (Hong et al., 2017; Hong et al., 2018). GHP3 is a ~500-m diameter, 10-m tall mound that actively vents methane (Fig. 1). Marine sediment samples were collected during CAGE cruise 18-05 supported by the research vessel *Kronprins Haakon* on in October 2018 and sampled from the seafloor by the Remotely Operated Vehicle (ROV) *Ægir*. A blade corer (surface dimensions 27 x 19 cm, Fig. 1c) was used to sample living foraminifera; it was placed directly in the vicinity of bacterial mats. The blade corer containing the sediment sample was opened immediately once onboard. A small aquarium
A hose was used to sample the upper most surface layer (0-1 cm). The wet sediment was collected in petri dishes and wet sieved to a size range of 250-500 μm, which served as source of living (cytoplasm containing) foraminifera. The species *N. labradorica*, which was the visibly abundant, was subsequently used for feeding experiments described in detail below. A previous study on GHP1 in Storfjordrenna showed also *N. labradorica* is also occurring in other sediment cores (MC_902 and MC_919) in the top 2 cm (Carrier et al., 2020).

Figure 1 Description of the sampling site Gas hydrate Pingo 3 (GHP3), a gas-hydrate bearing mount, which actively vents methane, located in Storfjordrenna Barents Sea (a) Map illustrating Svalbard archipelago and the distance towards the sampling site is app. 50 km (b) Map of sampling site GHP3, active gas bubble release is marked on the top of the underwater mount, yellow star indicates location of push corer PUC2 taken for geochemical analysis, black squared box indicates the location of the blade corer BLC18 from which the sediment was derived for the experiment. (c) Underwater image of retrieval of BLC18 taken by camera of ROV (remotely operated vehicle) illustrating the coloration of sediment and the sea-floor visible in background.
2.2. Geochemistry

For geochemical analysis a push corer (PUC2) was used (referred to as geochemistry core) to obtain measurements on $\delta^{13}C_{\text{DIC}}$ and sulfate, as blade corer (BLC18) did not allow those measurements. PUC2 was taken in close vicinity to BLC18, ~5m apart (Fig 1). Pore-water samples were taken from PUC2 using rhizons that were inserted through pre-drilled holes in the core tube at intervals of 1 cm. Acid washed 20-ml syringes were attached to the rhizons for pore water collection. Depending on the amount of pore water collected, the samples were split for $\delta^{13}C_{\text{DIC}}$ and sulfate measurements. To the samples 10 µL of saturated HgCl$_2$ (aq) was added to stop microbial activity, and stored in cold conditions (5°C). $\delta^{13}C_{\text{DIC}}$ was determined using a ThermoScientific Gasbench II coupled to a ThermoScientific MAT 253 IRMS at the Stable Isotope Laboratory (SIL) at CAGE, UiT. Anhydrous phosphoric acid was added to small glass vials (volume 4.5 mL), that were closed and flushed with helium 5.0 gas before the pore water sub-sample was measured. A pore-water sub-sample (volume 0.5 mL) was then added through the septa with a syringe, followed by equilibration for 24 h at 24°C to liberate the CO$_2$ gas. Three solid calcite standards with a range of +2 to -49 ‰ were used for normalization to $\delta^{13}C$ -VPDB. Correction of measured $\delta^{13}C$ by -0.1 ‰, was done to account for fractionation between (g) and (aq) in sample vials. Instrument precision for $\delta^{13}C$ on a MAT253 IRMS was 1σ +/- 0.1 ‰. Sulfate was measured with a Metrohm ion chromatography instrument equipped with column Metrosep A sup 4, and eluted with 1.8 mmol/L Na$_2$CO$_3$ + 1.7 mmol/L NaHCO$_3$ at the University of Bergen.

2.3. Culturing of the marine methanotroph M. sedimenti

*Methyloprofundus sedimenti* PKF-14 had been previously isolated from a water-column sample collected at Prins Karls Forland, Svalbard in the laboratory at UiT in Tromsø. *Methyloprofundus sedimenti* were cultured in 10-ml batches of a 35:65 mix of 1/10 Nitrate Mineral Salt medium (NMS) and sterile filtered sea water using 125-mL Wheaton® serum bottles with butyl septa and aluminum crimp caps (Teknolab®). Methane was injected to give a headspace of 20% methane in air, and the bottles were incubated without shaking at 15°C in darkness. Purity of the cultures and cell integrity was verified by microscopy and by absence of growth on agar plates with a general medium for heterotrophic bacteria (tryptone, yeast extract, glucose and agar).

Transmission Electron Microscopy was performed on culture aliquots to allow morphological comparison to previously published work (Tavormina et al., 2015). *Methyloprofundus sedimenti* strain PKF-14 cells have a gram-negative cell wall, coccoid to slightly elongated shape and
characteristic stacked intracytoplasmic membrane (ISM) and storage granules (SG) (Fig 2c). Additionally, 16S rRNA gene sequencing was performed (data not shown) to confirm it to be similar to the published *Methyloprofundus sedimenti* (Tavormina et al., 2015).

### 2.4. Experimental setup

On the ship, *Nonionellina labradorica* (Fig. 2) specimens showing a dark greenish brown cytoplasm were picked using sable artist brushes under a stereomicroscope immediately after wet sieving the sediment using natural seawater delivered from the ship pump. Living specimens had a partly inorganic covering surrounding the test, which was gently removed using fine artist brushes. Another Nonionellidae, *Nonionella iridea*, was similarly embedded with a cyst / covering in sediment.

Our specimens were subsequently rinsed twice in filtered artificial seawater to remove any sediment before placing them into the experimental petri dishes. Care was taken that those were minimally exposed to light during preparation of the experiment, as kleptoplasts are known to be highly light sensitive in this foraminifer (Jauffrais et al., 2018). The 20-h feeding experiment with *M. sedimenti* started after a short starvation phase where organisms resided in petri dishes with ASW for 2-4 h and were not fed or manipulated during this time. The feeding experiment consisted of several small petri dishes (3.5 cm  Ø, 3 mL) each containing five foraminifera in ASW at ambient salinity 35 (Red Sea Salt). Petri dishes were sealed with Parafilm® and covered with aluminum foil and placed inside the incubator in complete darkness. Temperature inside the chamber was maintained at 2-3°C, which is within the range of the site’s bottom-water temperature (-1.8 – 4.6°C) (Hong et al., 2017). The feeding of *M. sedimenti* was performed once at the beginning of the experiment by adding 100 µL of culture to 3 mL of artificial seawater to produce a final concentration of ~1E10⁶ bacteria / mL in the experiment. Previously conducted feeding studies were used as guides: Muller and Lee (1969) used 1E10⁴ bacteria/mL seawater and Mojtahid et al. (2011) used 4E10⁸ bacteria/mL seawater.

Five foraminifera, which served as initial/field specimens (Table 1), were fixed without *M. sedimenti* incubation. The respective petri dishes, were incubated for 4, 8 and 20 h to determine if incubation duration influenced response of the foraminifera to the methanotroph. One petri dish containing five foraminifera, which were un-fed and fixed at 20 h, served as a negative “control”. After the end of the respective incubation times, each foraminifer was picked with a sterilized fine
artist brush, which was cleaned in 70% ethanol between each specimen, and placed individually into a fixative solution (4% glutaraldehyde and 2% paraformaldehyde dissolved in ASW).

Figure 2 Exemplary illustration of Nonionellina labradorica, utilized in this study. (a) Reflected light microscopy image from a specimen directly after sampling, white arrowhead indicates aperture location (b) Scanning electron image from a specimen before molecular analysis was performed, white arrowhead indicates aperture location. (c) Transmission electron microscopy image of a culture of Metyloprofundus sedimenti, the marine methanotroph used in the feeding experiment. Characteristic features for methanotroph identification include typical type I ICM=intracytoplasmic membranes, SG=storage granules, and GNCW=gram-negative cell wall

### 2.5. Transmission Electron microscopy (TEM) preparation

Samples preserved in fixative solution were transported to the University of Angers, where they were prepared for ultrastructural analysis using established protocols (Lekieffre et al., 2018). Embedded foraminiferal cells were sectioned using an ultramicrotome (Leica® Ultracut S) equipped with a diamond knife (Diatome®, ultra 45°). Grids were stained using UranyLess® EM Stain (EMS, USA). Ultra-thin sections (70 nm) were observed with a JEOL JEM-1400 TEM at the SCIAM facility, University of Angers.

To document the ultrastructure of Metyloprofundus sedimenti, a sub-sample of the culture used for experiments was imaged with TEM (Fig. 2). To do so, an exponentially growing culture was collected, centrifuged, pre-fixed with 2.5 % (w/v) glutaraldehyde in growth medium overnight, washed in PBS (Phosphate Buffered Saline), then post fixed with 1% (w/v) aqueous osmium tetroxide for 1.5 hours at room temperature. After dehydration in an ethanol series, the samples were embedded in an Epon equivalent (Serva) epoxy resin. Ultra-thin sections were cut on a Leica EM UC6 ultramicrotome, and stained with 3 % (w/v) aqueous uranyl acetate followed by staining with lead citrate (Reynolds, 1963) at 20 °C for 4–5 min. The samples were examined with a JEOL
JEM-1010 transmission electron microscope at an accelerating voltage of 80 kV with a Morada 226 camera system at the Advanced Microscopy Core Facility (AMCF), Faculty of Health Science, UiT The Arctic University of Norway.

2.6. Foraminifera ultrastructural observation and image processing

Four specimens per experimental time point (4-20 h) plus one un-fed (control) specimen were examined. From each specimen, a minimum of 50 TEM images was taken, including images detailing the degradation vacuoles (5-27 images of degradation vacuoles per specimen). The ultrastructure was examined at different parts of the images focusing (a) in the cell interior to document vitality, (b) on degradation vacuoles to determine their content, and (c) at the exterior to survey for microbes entrained in remnant “reticulopodial trunk” material, which can be extended outside foraminiferal tests during feeding and locomotion (Anderson and Lee, 1991). Images are deposited at PANGAEA with DOI number XXX. To obtain an overview of the entire specimen and localize putative methanotrophs at the test (shell) aperture, images were compiled automatically using the stitching-feature in Adobe Photoshop CS2.

2.7. Molecular genetics and morphology

DNA metabarcoding and morphological documentation were performed on 13 specimens of *N. labradorica*. Briefly, live specimens were dried on micropaleontological slides and transported in a small container, cooled with ice-pads to the University of Angers. All specimens were imaged for morphological analysis using a Scanning Electron Microscope (SEM; EVOLS10, ZEISS, Fig. S1) followed by individually extracting total DNA in DOC buffer (Pawlowski, 2000). To amplify foraminiferal DNA, a hot start PCR (2 min. at 95°C) was performed in a volume of 25μl with 40 cycles of 30 s at 95°C, 30 s at 50°C and 2 min at 72°C, followed by 10 min at 72°C for final extension. Primers s14F3 and sB were used for the first PCR and 30 cycles at an annealing temperature of 52°C (other parameters unchanged) for the nested PCR with primers s14F1 and J2 (Pawlowski, 2000; Darling et al., 2016). Positive amplifications were sequenced directly with the Sanger method at Eurofins Genomics (Cologne, Germany). For taxonomic identification, DNA sequences were compared first with BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) and then within an alignment comprising other Nonionids implemented in SeaView (Gouy et al., 2010) and corrected manually.
3. Results

3.1. Sample description and geochemistry

The visual observation of the sediments within the blade corer BLC18 immediately after sampling (Fig. 1) indicated that the sediment appears light grey – yellowish in the upper part until app. 13 cm and dark brown from app. 13 cm to the bottom. At approximately 13 cm the sulfate measured in the pore water of the geochemistry core (PUC2) declined from ~2750 ppm at the sediment-water interface to ~706 ppm. A decline in sulfate concentration indicates that the anaerobic oxidation of methane (AOM) occurred at app. 13 cm depth. The SMTZ (Sulfate Methane Transition Zone) characterized by a reduced δ^{13}C-DIC -32‰ at app. 13 cm sediment depth can be considered shallow on the global average (Egger et al., 2018).

3.2. Ultrastructure of methanotroph culture used in the feeding experiment

*Metyloprofundus sedimenti* is characterized by a typical type I intracellular stacked membrane (ISM), storage granules (SG) and typical gram-negative cell wall (GNCW) (Fig. 2). These features were used to identify *M. sedimenti*.

3.3. Foraminiferal ultrastructure from an Arctic seep environment

3.3.1 General ultrastructure

All 17 specimens were considered living at the time of observation (Fig. 3), as the mitochondria had characteristic double membranes and occasionally visible cristae (Nomaki et al., 2016). Cytoplasm exhibited several vacuoles and kleptoplasts concentrated in the youngest chambers (Fig. 3a) and, in some specimens, the nucleus with nucleoli was visible (Fig. 3b). Kleptoplasts were numerous throughout the cytoplasm and occurred in the form of a single chloroplast (Fig. 3a-b), or as double chloroplasts (Fig. S2). Not all kleptoplasts were intact, some showed peripheral degradation of the membranes indicated by an increasing number of white areas between pyrenoid, lamella and thylakoids (Fig. S2). Peroxisomes in *N. labradorica* occurred mostly as pairs (Fig. 3c) or small clusters of 3-4 spherical organelles (Fig. S1a-b). The mitochondria occurred often in small clusters of two to five throughout the cytoplasm and were oval, round or kidney-shaped in cross section (Fig. 3e-f). Sometimes, but not always, peroxisomes were associated with endoplasmic reticulum (Fig. S1c) but could also occur alone. Golgi apparatus (Fig 3d) had intact membranes, often occurring near mitochondria.
Figure 3 Transmission electron micrographs showing cellular ultrastructure of \textit{N. labradorica}. (a) Cytoplasm showing parts of two chambers, with nucleus with nucleoli, vacuoles and several kleptoplasts, (b) nuclear envelope, nucleoli, and kleptoplasts, (c) peroxisomes and electron opaque bodies, (d) Golgi, (e-f) mitochondria. v= vacuole, c=kleptoplast, nu=nucleoli, n=nucleus p=peroxisome, eo=electron opaque body, m=mitochondrion, fv=fibrillar vesicle, li=lipid droplet. Scales: (a) 2 \mu m, (b) 1 \mu m, (c-f) 200 nm
3.3.2 Ultrastructure of aperture-associated bacteria

In total three putative methanotrophs were identified in the vicinity of two foraminifer specimens (sample E39, Fig. 4; E37, Fig. 5). Those were identified next to reticulopodial remains in the cross-section (Fig. 4b). As an aid for identification of *M. sedimenti* we used the characteristics shown in the literature (Tavormina et al. 2015) and our own TEM observation obtained from *M. sedimenti* culture (Fig. 2c). As noted, *Methyloprofundus sedimenti* is characterized by a typical type I intracellular stacked membrane (ISM), storage granules (SG) and typical gram-negative cell wall (GNCW) (Fig. 2). On specimen E39 from the 20 h treatment, we found the methanotroph exhibiting the clearest internal structure, having both typical type I stacked intracytoplasmic membranes (ICM+SG) and a second putative methanotroph showing SG+GNCW (Fig. 4). Specimen E36, from the 20 h treatment, hosted another putative methanotroph showing three large SG (Fig. 5). Storage granules occur throught this putative methanotroph (Fig. 5c).
Figure 4 Transmission electron micrographs of *N. labradorica* from 20 h treatment (sample E39) (a) Stitched cross section of TEM images showing location of methanotroph at the aperture region (black rectangle) (b) Location of two putative methanotrophs next to sediment particles and putative reticulopodial remains. (c) Close up of two putative methanotrophs revealing detailed feature for identification, such as stacked membranes (ISM), storage granules (SG), and gram-negative cell wall (GNCW), scale bars: a: 100 µm, b: 1 µm, c: 200 nm.
3.3 Contents degradation vacuoles

Digestive vacuoles and food vacuoles are often summarized as degradation vacuoles in the literature (Lekieffre et al., 2018) and this makes sense for our study as well. A degradation vacuole is a vacuole where enzymatic activities degrade contents, often making them unidentifiable (Bé et al., 1982; Hemleben et al., 2012). Sediment particles were present in many degradation vacuoles. The sediment grains are easy to recognize in the TEM image as angular grains spiking out of the vacuoles, next to organic debris, which can have many different shapes. Each specimen had at least one degradation vacuole with sediment particles present (Table 1). If a sediment particle was visible, the vacuole was defined as a degradation vacuole (dv), and if it was not then it was defined as a standard vacuole (v) (Fig. 6). Sediment particles are likely the remains of clay grains from the seafloor, and hence show that the vacuole must contain cell foreign objects, around which degradation processes have started. Next to sediment particles, 4 out of 17 specimens examined (23%) had a few bacteria of various sizes inside their degradation vacuoles (Fig 6 b-c).
3.4. Foraminiferal genetics

Six of 13 specimens analyzed for genetics were positively amplified and sequenced (Fig. S3). The sequences are deposited in GenBank under the accession numbers MN514777 to MN514782. When comparing them via BLAST, they were between 98.6% and 99.6% identical to published sequences belonging to foraminifera identified as the morphospecies *N. labradorica*, from the Skagerrak, Svalbard and the White Sea (Holzmann and Pawlowski, 2017; Jauffrais et al., 2018).

Sequences were also included in an alignment comprising other nonionids implemented in Seaview (not shown) and corrected manually to check the BLAST search. This step confirmed the BLAST identification.
4. Discussion

4.1. Sampling site and geochemistry

The sampling site of blade corer BLC18 was in close proximity (~50 m) to an active methane-vent releasing methane bubbles at the gas hydrate Pingo (GHP3) (Serov et al., 2017). At such sites with high methane flux, the SMTZ (sulfate methane transition zone) is shallow, as sulfate from the sediment is readily consumed in the first tens by sulfate-reducing bacteria (SRB) (reviewed in Carrier et al., 2020). Geochemical analysis of PUC2, revealed an SMTZ at app. 13 cm. The depth of 13 cm is rather shallow (Egger et al., 2018), as it can also be several meters deep in other sites (reviewed in Panieri et al., 2017). Geochemical characteristics can be considered similar at the sampling location of living specimens (BLC18) and the core taken for geochemistry (PUC2).

4.2. Association with putative methanotrophs

The association with the three putative methanotrophs could be identified on two foraminifera specimens based on comparing internal bacterial characteristics to published literature (Tavormina et al., 2015). Transmission electron microscopy is a powerful tool to reveal ultrastructural features outside of the foraminiferal cytoplasm. The documentation of this association with putative methanotrophs likely originating from the food given in the experiment, is evidence that methanotrophs can indeed be a food source to *N. labradorica*. The feeding strategy is likely that methanotrophs are ingested via untargeted grazing in seeps, as *N. labradorica* appears to be a non-selective feeder.

After conducting this study and comparing to the result of observations on *Melonis barleeanus* (Bernhard and Panieri, 2018) an association of foraminifera and methanotrophs has been clearly demonstrated. Whether foraminifera feed methanotrophs and under which environmental conditions remains speculative. It has been shown that large scale biofilms of methanotrophs can occur in sediment pockets close to the Sulfate Methane Transition Zone (SMTZ) (Gründger et al., 2019). This is also the location where Anaerobic Oxidation of Methane (AOM) occurs (Boetius et al., 2000). The SMTZ is characterized by sulfate reducing bacteria (SRB), and a consortium of ANME that are driving the AOM (Boetius et al., 2000; Wegener et al., 2015). However, this is not the main habitat for living foraminifera, as the SMTZ can be several meters deep and alters foraminiferal tests with secondary overgrowths of methane-derived authigenic carbonates.
(MDAC) (reviewed in Panieri et al., 2017). It has also been suggested that foraminifera may sometimes be transported into seeps and can also occur at the MTZ, but they likely not live in those sediment layers permanently (Bernhard and Bowser, 1999). Foraminifera in general have several metabolic strategies to cope with anoxic environments (Gomaa et al., 2021) of which many remain to be understudied.

4.3. Feeding on other bacteria and contents of degradation vacuoles

Our results of the feeding experiment and experimental specimens show that only 23% of the examined *N. labradorica* specimens contained bacteria inside their degradation vacuoles. That is not a large quantity compared to sediment particles which occurred in 100% of the examined degradation vacuoles. We infer that *N. labradorica* at this site is a deposit feeder, feeding on organic detritus and associated bacteria. The bacteria observed in the degradation vacuoles resembled those from other deep-sea foraminifera (*Globobulimina pacifica* and *Uvigerina peregrina*) and the shallow-dwelling genus *Ammonia* (Goldstein and Corliss, 1994). Salt-marsh foraminifera also feed on bacteria and detritus, as observed in TEM studies (Frail-Gauthier et al., 2019). Scavenging on bacteria has also been observed by other foraminifera from intertidal environments such as *Ammonia tepida* or *Haynesina germanica* (Pascal et al., 2008) and is a logical consequence from detritus feeding. Certain foraminifera have been shown to selectively ingest algae/bacteria according to strain (Lee et al., 1966; Lee and Muller, 1973). From laboratory cultures we know that several foraminifera cultures require bacteria to reproduce, as antibiotics inhibited reproduction (Muller and Lee, 1969). Future studies will need to employ additionally molecular tools to additionally determine the food contents inside the cytoplasm (e.g. (Salonen et al., 2019). A recent study by used metabarcoding to assess the contribution of bacterial OTUs associated with intertidal foraminifera, and revealed that *Ammonia* sp. T6 can predate on metazoan taxa, whereas *Elphidium* sp. S5 and *Haynesina* sp. S16 are more likely to ingest diatoma (Chronopoulou et al., 2019).

4.4. General ultrastructure of *N. labradorica* collected in a seep environment

Our observations also included the major organelles of the species, as this was essential to conclude vitality after the experiment (Nomaki et al., 2016). Mitochondria were generally homogeneously distributed throughout the cytoplasm confirming previous observations of six *N. labradorica* from
the Gullmar Fjord (Jauffrais et al., 2018; Lekieffre et al., 2018). If mitochondria are concentrated predominately under pore plugs, it can be an indicator that the electron acceptor oxygen is scarce in their environment, as the pores are the direct connection from the cell to the environment. This has been observed in several other studies where mitochondria were accumulated under pores in *N. stella* (Leutenegger and Hansen, 1979) and *Bolivina pacifica* (Bernhard et al., 2010).

For the samples from our particular site, we also observed kleptoplasts abundantly and evenly distributed throughout the cytoplasm, confirming previous TEM studies on the species from fjord sediments (Cedhagen, 1991; Jauffrais et al., 2018). Occasionally, kleptoplasts were degraded, which could happen a) during sampling, b) due to exposure to microscope lights or c) due to the age and condition of kleptoplasts inside host. Kleptoplasts in *N. labradorica* have been studied in detail describing their diatom origin, sensitivity to light and missing photosynthetic functionality (e.g. (Cedhagen, 1991; Jauffrais et al., 2018). It has been suggested that kleptoplasts could function as a seasonal energy reservoir (e.g. in winter) (Jauffrais et al., 2016).

5. Conclusions

Based on the content of degradation vacuoles observed, we conclude that *N. labradorica* is a deposit feeder, as it ingests sediment particles together with bacteria as part of consuming detritus on the sea floor. At the aperture region of two different foraminifera specimens, next to reticulopodial remains and sediment particles, we observed three putative marine methanotrophs after 20 h incubation. One of the putative methanotrophs had characteristic ISM, which resemble the methanotroph *M. sedimenti* in culture. We conclude that it is possible that *N. labradorica* ingests *M. sedimenti* via “untargeted grazing” in seeps. Further studies are needed on feeding strategies of several paleo-oceanographically relevant foraminifera to detangle the relationship between δ^{13}C measured in foraminiferal calcite, cytoplasm and contribution to their diet.

6. Data availability

Data in form of TEM images will be deposited at PANGAEA under doi: Molecular data will be deposited before publication at Genbank.

7. Sample availability

Samples are available upon request and TEM thinsections archived at the University of Angers.
8. Acknowledgments

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Author Contributions

Designed the project and experiment: GP, EG, CS; Collected samples: CS, EG; Performed experiment: CS; Sample preparation: CS, HR; TEM observations and interpretations: CS, JMB, EG, CL; Conducted molecular genetics: MSc; Wrote the paper: CS, GP, JMB; Provided critical review and edits to the manuscript: EG, CL, MSv, MSc, HR; Contributed reagents/materials/analysis tools: MSv, MSc, CL.
Table I. Summary of TEM observations of *Nonionellina labradorica* comparing field specimens and experimental specimens. Field specimens (initials) were not fed, nor was a non-fed control preserved after a 20 h incubation. The only putative methanotrophs were observed and imaged in specimens from the 20 h incubation. Bacteria of unknown origin were described as rod shaped cells in the degradation vacuoles.

<table>
<thead>
<tr>
<th>Duration of experiment (h)/field samples</th>
<th>Food provided (yes/x/no)</th>
<th>Sample ID</th>
<th>Cytoplasm: Degradation vacuole Contents</th>
<th>Aperture region: (putative) Methanotrophs</th>
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<td>Field samples (Initials)</td>
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