Deposit-feeding of *Nonionellina labradorica* (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. FAs foraminifera may associate withfeeding on methanotrophic bacteria, which are ^{13}C-depleted, feeding on them has been suggested tomay cause negative cytoplasmic and/or calcitic δ^{13}C valuesδ^{13}C values in the foraminiferal cytoplasm and/or calcite. To test whether the foraminiferal diet includes methanotrophs, we performed a short-term (20-h1 d) feeding experiment with *Nonionellina 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, post experiment in degradation vacuolesas evidenced by their ultrastructure, in degradation vacuoles after this feeding experiment, as evidenced by their ultrastructure. 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 apertural areae after 20-h incubation revealed three putative methanotrophs, close to clay particles, based on bacterial. These methanotrophs were identified based on internal ultrastructural characteristics. such as a type I stacked intracytoplasmic membranes (ICM), storage granules (SG) and gram-negative cell walls (GNCW). Furthermore, more, *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 *N. labradorica* but confirmed the presence of kleptoplasts, which were often partially degraded. Based on these observationsIn sum, 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 methanothrophs are available to the foraminifera in their habitat, their non-selective uptake could make a substantial contribution to altering $\delta^{13}C_{test}$ values. This in turn may impact metazoans grazing on benthic foraminifera by altering their δ^{13}C signature.

benthic foraminifera – feeding experiment – grazing - marine methanotrophs – Arctic methane seeps– transmission electron microscopy – ultrastructure – kleptoplasts- protist – molecular identification

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). Studies specifically looking at living (bengal rosa stained) foraminiferal tests support the hypothesis that the carbon isotopic composition is strongly influenced by the porewater DIC (McCorkle et al., 1990a). Interspecific $\delta^{13}C$ differences between species with similar depth indicate sometimes taxon-specific "vital" effects (McCorkle et al., 1990a). ~~One~~ Those "vital" effects describe the biology of the different species, which could reflect different feeding patterns. It has been suggested that *Nonionella auris* is an indicator of methane release and possibly ~~explanation of low $\delta^{13}C$ signals in foraminifera could be due to the ingestion~~ingests ~~of~~ ^{13}C-depleted methan~~otrophs~~e oxidizing bacteria ~~—~~(Wefer et al., 1994). ~~.~~ Recently, ~~specimens of the foraminifer~~ *Melonis barlee~~e~~anus* (Williamson, 1858) collected from an active methane seep site ~~were~~ was found to be closely associated with ~~a~~ putative methanotrophs ~~at their apertural region~~~~reasoning the need to examine feeding habits of foraminifera living on or around methane seeps~~ (Bernhard and Panieri, 2018), providing impetus to examine feeding habits of foraminifera living in or around methane seeps.

~~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 (methanothrophs), 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~~ Our hypothesis is that if foraminifera ~~accidentally or intentionally~~ ingest methanotroph~~s~~, $\delta^{13}C$ values of foraminiferal cytoplasm should be altered by ~~such phagocytosis~~their food. ~~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 a novel high-pressure culturing setting incubator on *Cibicides wuellerstorfi* illustrated the difficulty to measure the sensitive relationship between methane exposure and the foraminifera *Cibicides wuellerstorfi*. However, it was shown in onethis experiment using entire cores, that athe methane source was reflected in $\delta^{13}C$test of foraminiferal calcite, $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$, as whole cores were incubated, the $\delta^{13}C_{DIC}$ of the seawater was impossible to keep constant and to compare $\delta^{13}C_{test}$ formed in the presence of methane to normal marine conditions (Wollenburg et al., 2015). It is also not yet conclusive if the food can influence formainiferal calcite, as foraminifera somtimes fail to produce new caclite in experiments (Mojtahid et al., 2011).

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 diagenetic 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 SMTZ, 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).OneAnother 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 the feasibility if that foraminifera calcify close to seeps (Rathburn et al., 2003; Hill et al., 2004a; Panieri et al., 2014). The problem lies in the calcite tests, and the difficulty to asses the time of death of these protists in the sediment. Several studies found that the lightest isotopic $\delta^{13}C$ values were measured in tests coated by methane-derived authigenic carbonate (MDAC) overgrowth, which happens

after the death of the foraminiferprotist (Torres et al., 2010; Panieri et al., 2014; Consolaro et al., 2015; Panieri et al., 2017; Schneider et al., 2017). 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, (Panieri, 2006). Generally, some foraminifera can ingest dissolved organic matter (DOM);, some are herbivorous, carnivorous, suspension feeders and most commonly deposit feeders reviewerd in (reviewed in Lipps, 1983). {Goldstein, 1994 #1903}Deposit feeders are omnivorous, gathering fine-grained sediment (e.g., clay) and associated bacteria, organic detritus (dead particulate organic material) and, if present, diatom cells using their pseudopodia. Hence, bacteria are involuntarily part of the "food-mix" (Levinton, 1989).Based on the ultrastructure of the diet found in vacuoles serveral species of foraminifera from different habitats have already been classified to be deposit feeders (Goldstein and Corliss, 1994).

The fact that bacteria are sometimes part of the "food mix" made usHere we investigate if *Nonionellina labradorica* associated would feed in a short-term feeding experiment on the marine with methanotrophs, e.g. *Metyloprofundus sedimenti*, in a short-term feeding experiment and compare its ultrastructure on experimental specimens and field specimens. .—*Nonionellina labradorica* *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) and occurs. It also occurs together with *N. digitata* in Svalbard fjord sediments (Hald and Korsun, 1997; Shetye et al., 2011; Fossile et al., 2020). (Carrier et al., 2020) NextIn addition to its wide distribution, it is an especially interesting experimental species, for feeding studies because it hosts kleptoplasts, *i.e.* sequestered chloroplasts, of diatom origin inside its

cytoplasm ~~(Cedhagen, 1991; Jauffrais et al., 2018)~~ (Cedhagen, 1991; Jauffrais et al., 2019b). ~~SEM images of *Nonionellina*.~~ *labradorica*'s aperture shows a specific ornamentation, possibly a morphological adaptation to this "predatory" mode of life for obtaining the kleptoplasts (Bernhard and Bowser, 1999). Denitrification has been speculated for *N. labradorica* (reviewed inCharrieau et al., 2019), b~~B~~ecause t~~T~~he foraminiferal genus *Nonionella* ~~is potentially capable to~~ can denitrify, which was demonstrated on two~~the~~ species ~~of *Nonionella* cf. *stella*~~ (Risgaard-Petersen et al., 2006; Choquel et al., 2021), but not yet on *N. labradorica*~~, and NIS *Nonionella* sp. T1 (Choquel et al., 2021), denitrification and~~ has been speculated also for *Nonionellina labradorica* (reviewed ~~(reviewed by 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 (Grzymski et al., 2002).~~ 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) before and after a feeding experiment.~~.~~

2. Materials and methods

2.1. Site description and sampling living foraminifera

The sampling site was located app. 50 km south of Svalbard at 382m 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 km^2. O~~At this site o~~ur samples were~~as~~ taken at GHP3, which is referred to 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 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 visibilly~~ abundant, was subsequently used for a feeding experiment~~s~~ described in detail below. ~~A previous study on GHP1 in Storfjordrenna also showed also *N. labradorica* is also occurreding in other sediment cores (MC_902 and MC_919) in the top 2 cm (Carrier et al., 2020).~~

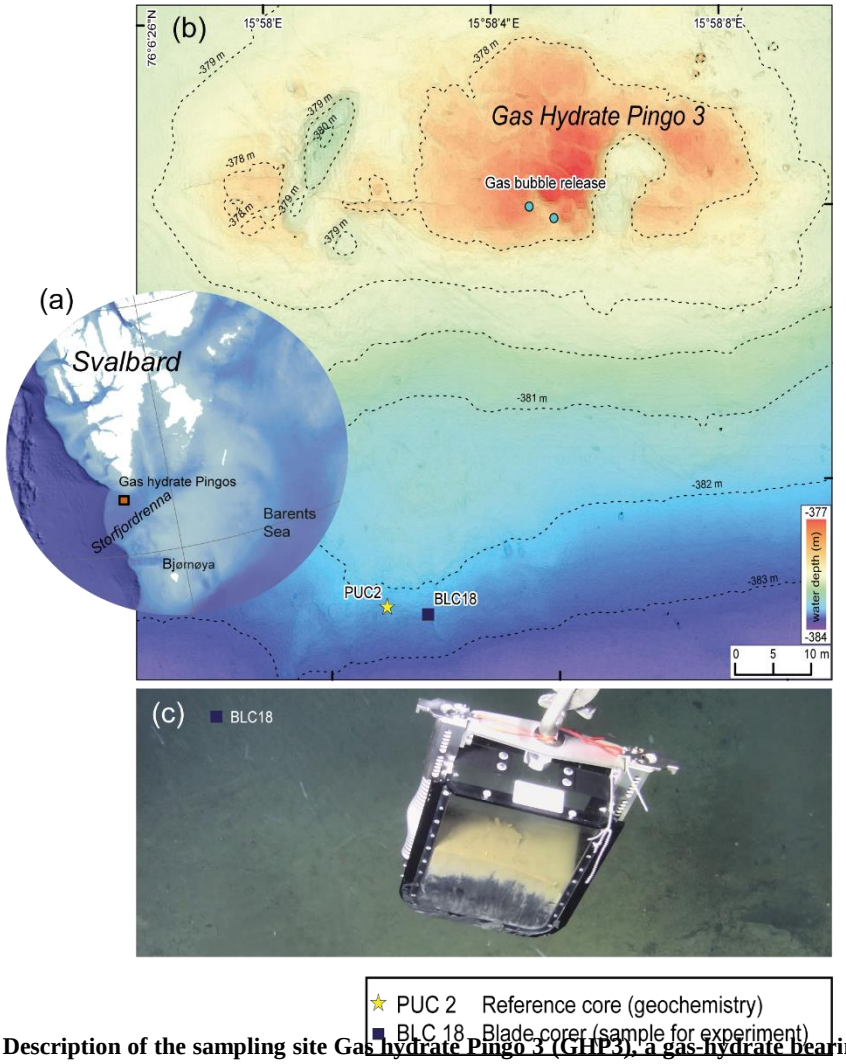

Figure 1. Description of the sampling site Gas hydrate Pingo 3 (GHP3), a gas-hydrate bearing mound~~t~~, ~~which actively vents methane,~~ located in Storfjordrenna Barents Sea. (a) Map illustrating Svalbard Aarchipelago and the ~~distance towards~~ the sampling site, ~~is~~ app. 50 km offshore. (b) 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 square~~d box~~ indicates ~~the~~ location of ~~the blade corer~~ BLC18 (~~from which the~~ sediment ~~was derived~~source for ~~the~~ experiment). (c) Underwater image of retrieval of BLC18 taken by ROV camera ~~of ROV (remotely operated vehicle)~~ illustrating the coloration of sediment with~~and~~ the sea-floor visible in background.

2.2. Geochemistry of the study site

For geochemical analysis of the study site a push corer (PUC2; from now ~~) was used (~~referred to as geochemistry core) was taken to obtain measurements o~~f~~n $\delta^{13}C_{DIC}$ and sulfate, because~~as~~ blade corer (BLC18) did not allow those measurements. PUC2 was taken in close vicinity to BLC18, ~5m apart (see Figure S1~~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 (Table S1). 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 $\delta^{13}C_{DIC}$ and sulfate measurements. To the samples, 10 µL of saturated $HgCl_2$ (aqueous) was added to stop microbial activity~~,~~ and stored in cold conditions (5°C). ~~$\delta^{13}C_{DIC}$ was determined using a~~A ThermoScientific Gasbench II coupled to a ThermoScientific MAT 253 IRMS at the Stable Isotope Laboratory (SIL) at CAGE, UiT was used to determine $\delta^{13}C_{DIC}$ of the pore-water. 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 needle, 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 (gas) and (aqueous~~aq~~) in sample vials. Instrument precision for $\delta^{13}C$ on a MAT253 IRMS was ~~1σ~~ +/- 0.1 ‰ (SD). Sulfate was measured with a Metrohm ion chromatography instrument equipped with column Metrosep A sup 4, and eluted with 1.8 mmol/L Na_2CO_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. 2a,b) 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. Those so-called cysts are nothing unusal with many foraminiferan taxa (Heinz et al., 2005). ~~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., 2019b).

The experiment with *M. sedimenti* was conducted for a~~the~~ total duration of 20-h to resemble previous experiments on *N. labradorica* using~~on~~ transmission electron microscopy and nanometre-scale secondary ion mass spectrometry (TEM-NanoSIMS) isotopic imaging~~u (Jauffrais et al., 2019)~~, and included two more time points at 4 and 8 -h~~, where incubations were terminated~~. A short pre-experimental phase (2-4 h) was included before the ~~initial~~ start of the feeding experiment, to allow specimens to acclimate. During the pre-experimental phase specimens were not fed and resided in the petri dishes to adjust to the experimental conditions. ~~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 ∅, 3 mL) each containing five ~~foraminifera~~ *N. labradorica* 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 ~1E10^6 bacteria / mL in- each petri dish~~the experiment~~. Previously conducted feeding studies were used as guides: Muller and Lee (1969) used 1El0^4 bacteria/mL seawater and Mojtahid et al. (2011) used 4E10^8 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).

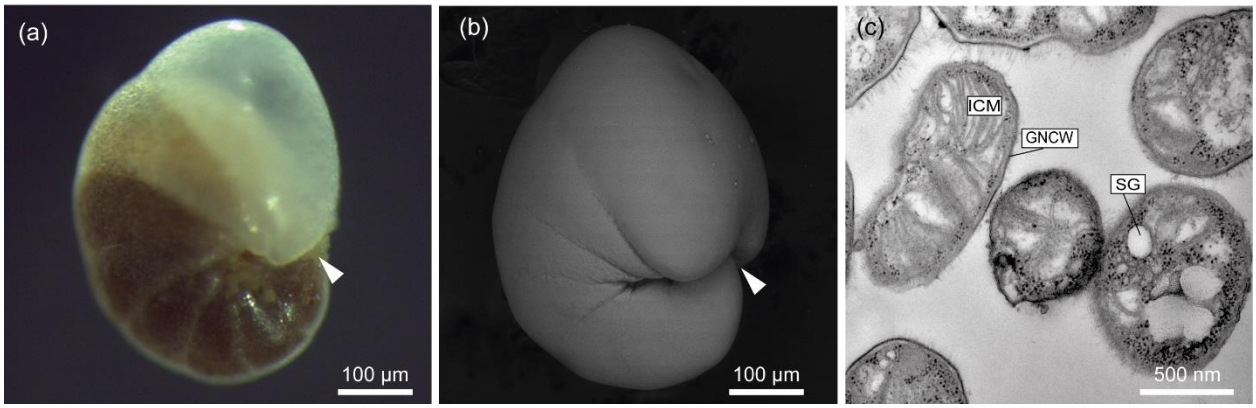

Figure 2 Exemplary illustration of *Nonionellina labradorica*, utilized in this study. (a) Reflected light microscopy image from a specimen directly after sampling, white arrowhead indicates aperture location. (b) Scanning electron image from a specimen before molecular analysis was performed, white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

2.5. Transmission Electron microscopy (TEM) preparation

Samples of *N. labradorica* 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). Four embedded foraminiferal cells per treatment ~~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 *Methyloprofundus sedimenti*, a sub-sample of the culture used for experiments was imaged with TEM (Fig. 2c). 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 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 (initals, 4, 8 and ~~-~~20 h) plus one un-fed (control) specimen were examined with the TEM. 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~~ sections 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)~~. All i~~I~~mages made during the observations at the TEM are deposited at PANGAEA ~~with DOI number XXX.~~(DOI). ~~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 of the study site

The visual observation of the sediments within the blade corer BLC18 immediately after sampling (Fig. 1c) indicated that the sediment appeareds light grey – yellowish in the upper part until app. 13 cm and dark brown from app. 13 cm to the bottom. TAt 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 at approximately 13 cm (see Fig. S1, Table S1). 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

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 appear to have a gram-negative cell wall, coccoid to slightly elongated shape and characteristic intracytoplasmic membrane (ICM) (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). *Metyloprofundus sedimenti* is characterized by a typical type I intracellular stacked membrane (ICSM). Furthermore, it has , storage granules (SG) and a typical gram-negative cell wall (GNCW) (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 examined for ultrastructure 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. S2a-d). 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. S2a-d). 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). Peroxisomes in *N. labradorica* occurred mostly as pairs (Fig. 3c) or small clusters of 3-4 spherical organelles (Fig. S3a1a-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. S31bc) but could also occur alone. Golgi apparatus (Fig 3d) had intact membranes, often occurring near mitochondria.

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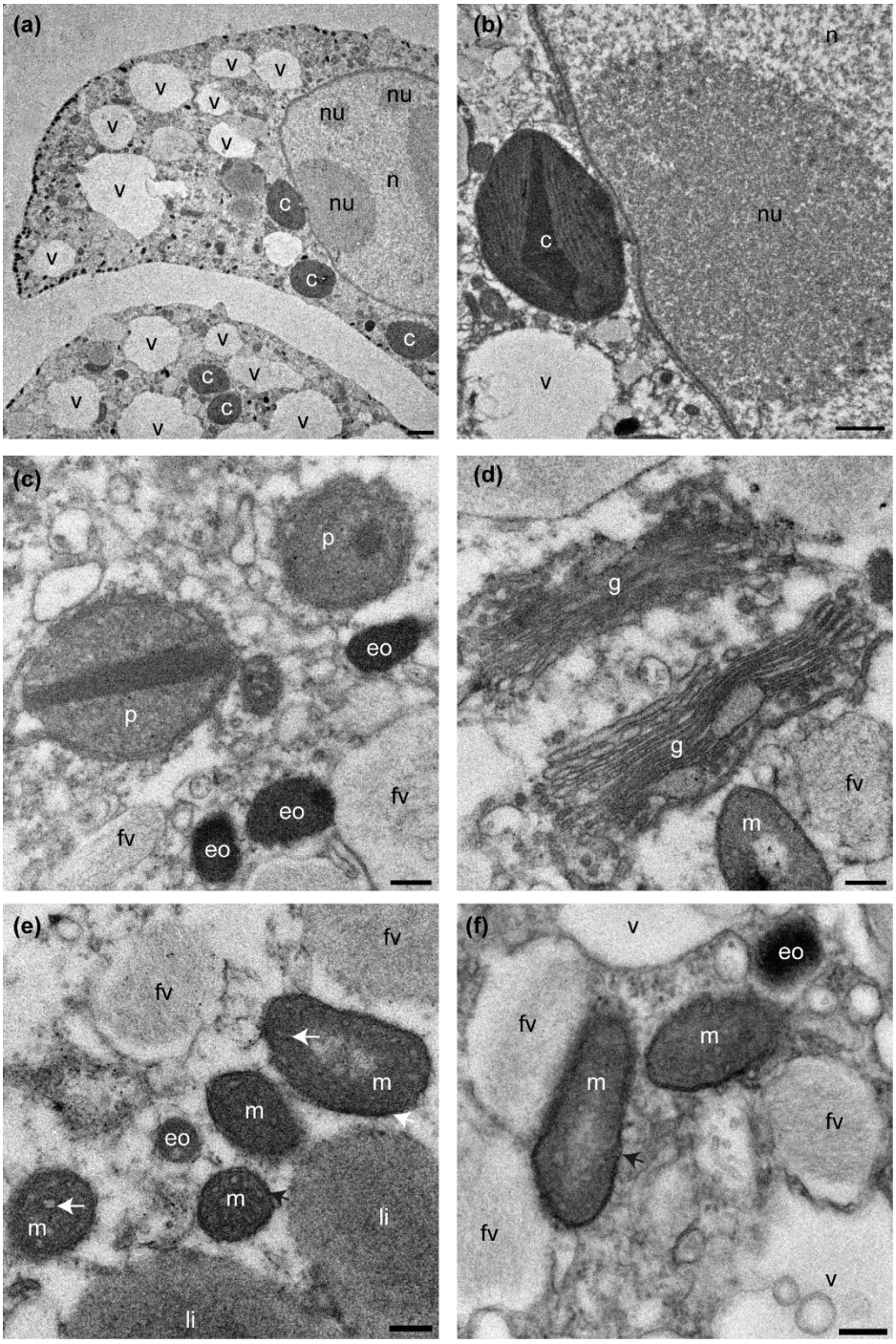

Figure 3 Transmission electron micrographs showing cellular ultrastructure of *N. ll~~l~~abradorica*. (a) Cytoplasm showing parts of two chambers, with nucleus with nucleoli, vacuoles and several kleptoplasts, (b) nuclear envelope, nucleoli, and kleptoplasts, (c) peroxisomes and electron opaque bodies, (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 µm, (b) 1 µm, (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). Th~~eo~~se microbes 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~~ (Tavormina et al., 2015) ~~et al. 2015)~~ and ~~a~~ our own TEM observation obtained from *M. sedimenti* culture (Fig. 2c). As noted, *Methyloprofundus sedimenti* is characterized by a typical type I intrac~~ellular stacked~~ ytoplasmic stacked membrane (IS~~S~~M). Other characteristics, which ~~a~~~~that~~are not specific for methanotrophs~~,~~ ~~were~~included storage granules (SG) and a typical gram-negative cell wall (GNCW) (Fig. 2c). On specimen E39 from the 20 h treatment, we found the methanotroph exhibiting the clearest internal structure, having both typical type I ~~stacked~~ intracytoplasmic stacked membranes (IS~~C~~M) and ~~+~~SG, as well found ~~)~~ and a second putative methanotroph showing ~~SG+~~GNCW and SG (Fig. 4c). ~~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).~~

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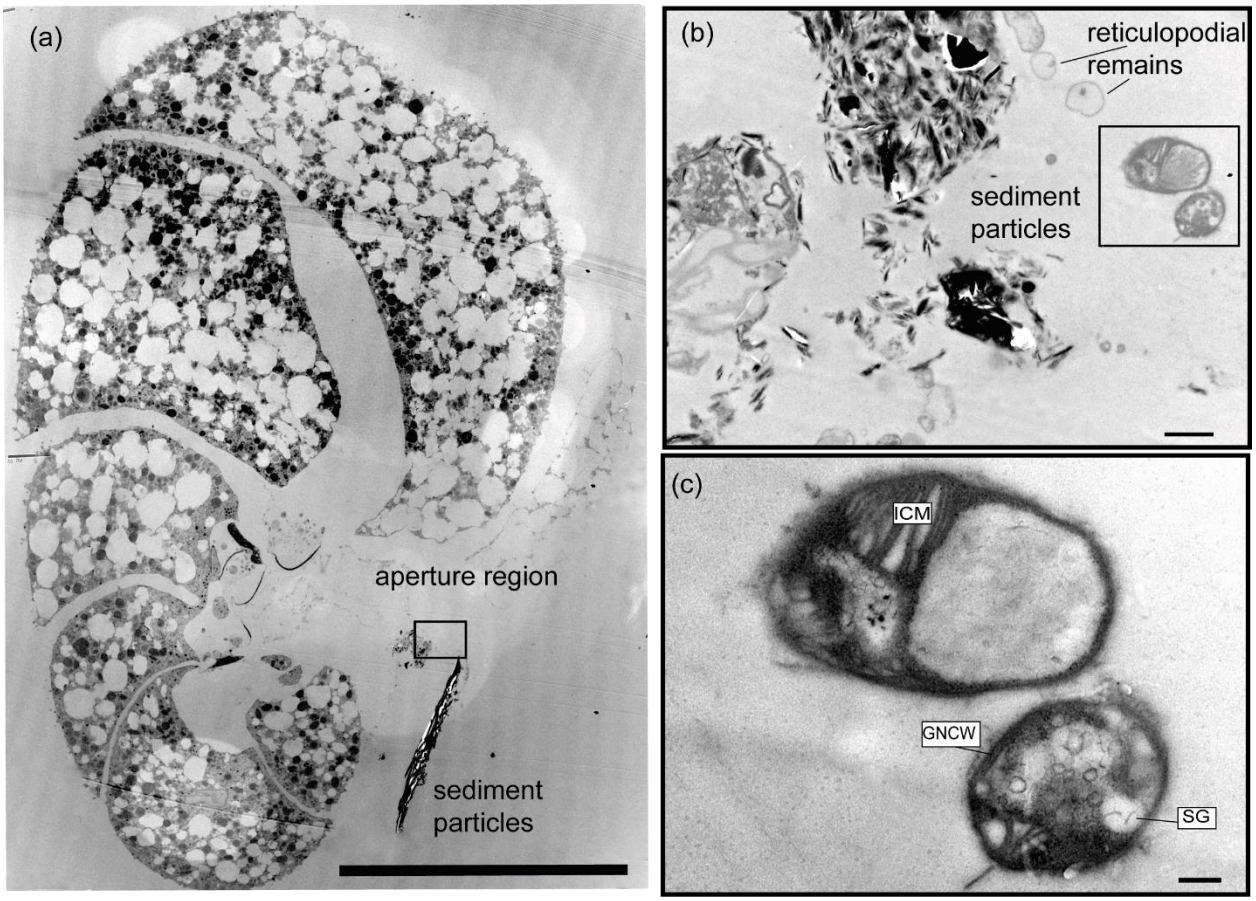

Figure 4 Transmission electron micrographs of *N. labradorica* from 20 h treatment (sample E39) (a) Stitched cross section of TEM images showing location of methanotroph at the aperture region (black rectangle) (b) Location of two putative methanotrophs next to sediment particles and putative reticulopodial remains. (c) Close up of two putative methanotrophs revealing detailed feature for identification, such as type I stacked intracytoplasmic ~~stacked~~ membranes (ISM), and other less-informative characteristics, such as storage granules (SG), and gram-negative cell wall (GNCW), scale bars: a: 100 µm, b: 1 µm, c: 200 nm.

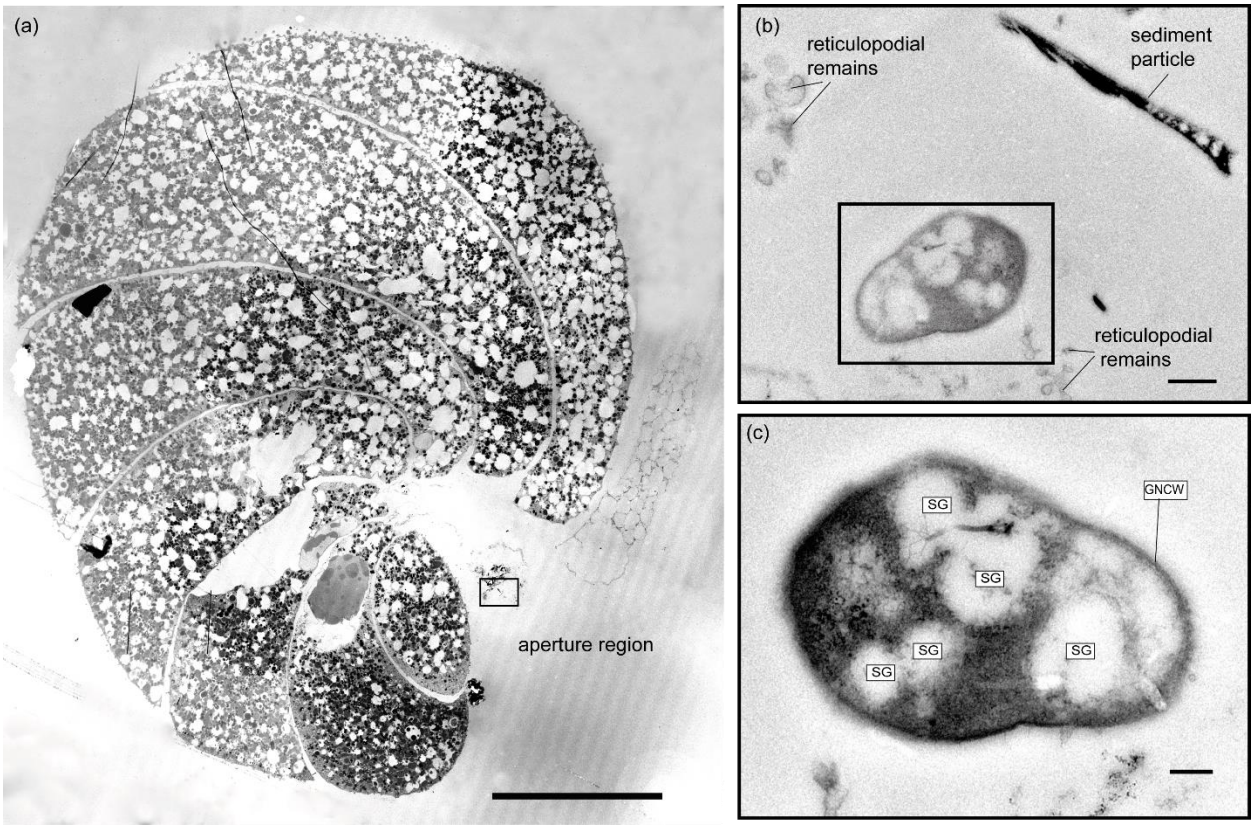

Figure 5 Transmission electron micrographs of *N. labradorica* from 20 h treatment (sample E37) (a) Stitched cross section of TEM images showing location of putative methanotroph (black rectangle) at the aperture region. (b) Location of the putative methanotroph next to sediment particles and sections of the putative reticulopodial remains (c) Close up of putative methanotroph showing several SG throughout its cell, scale bars: a: 100 µm, b: 0.5 µm, c: 200 nm.

3.3.3 Contents of 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 were~~are~~ easy to recognize in the TEM image as angular grains ~~spiking out of~~ inside the vacuoles, next to organic debris, which can have many different shapes. Each specimen had at least one and mostly several ~~-~~degradation vacuoles filled 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 and empty then it was defined as a standard vacuole (v) (Fig. 6). The observed entrained s~~S~~ediment particles were platelets, ~~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,~~ Four~~4 out~~ of 17 specimens examined (23%) had one or more~~a few~~ bacteria of various sizes inside their degradation vacuoles next to sediment particles (Fig 6 c, f~~b–e~~).

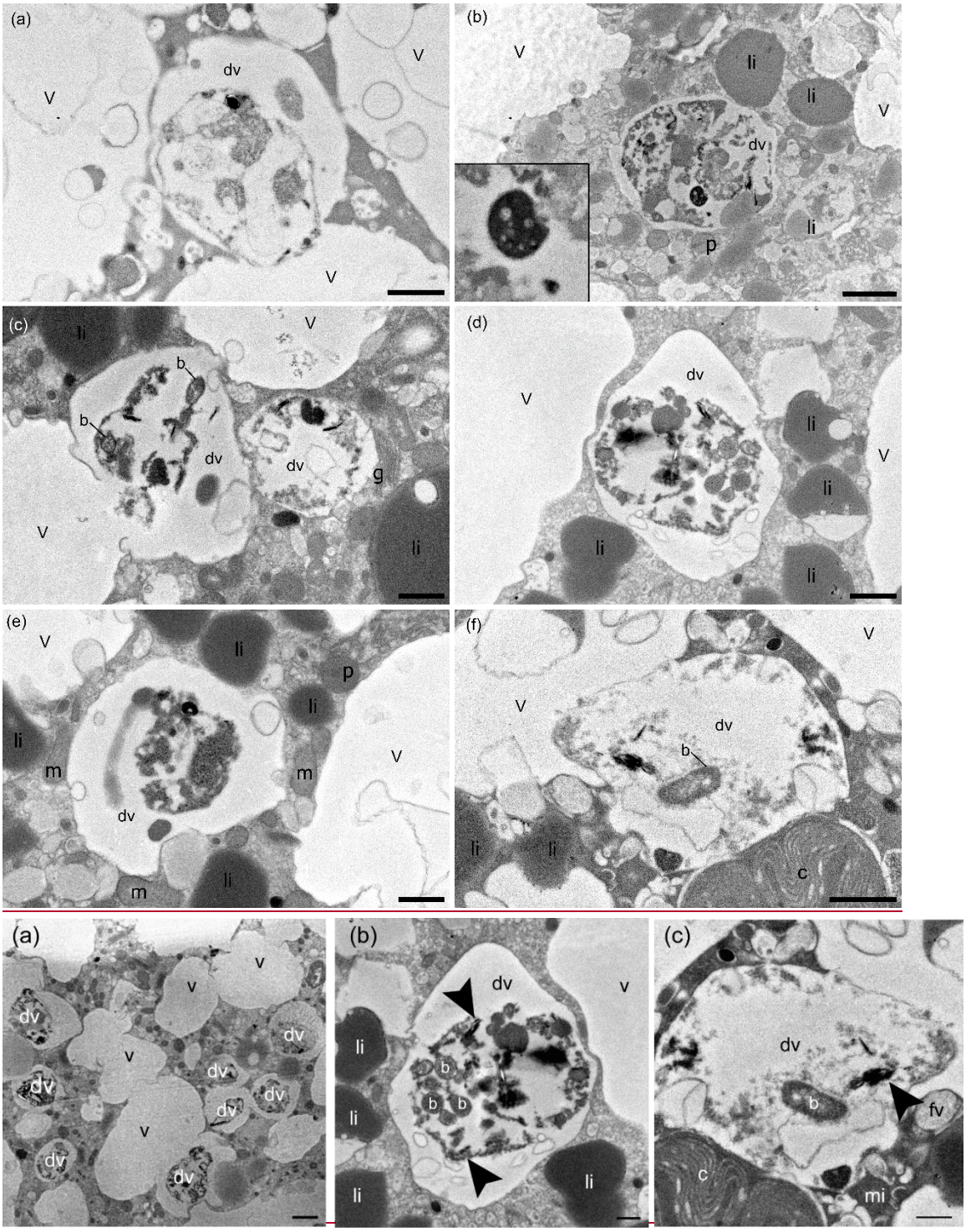

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Figure 6 TEM micrographs of *N. labradorica* showing degradation vacuoles containing miscellaneous items, including bacteria (b), inorganics (clay platelets) and unidentifiable remains after 4h incubation (a,b; specimens E27, E28, respectively); after 8h incubation (c,d; specimen E14), after 20h incubation (e,f; specimens E36, E37, respectively). v=vacuole, dv=degradation vacuole, c=kleptoplast, p=peroxisome, m=mitochondrion, li=lipid, g= Golgi. Scales: (a, c-f) 1 µm, (b) 2 µm.~~TEM micrographs of N. labradorica. (a) Overview of degradation vacuoles (dv) in relation to empty vacuoles (v) in the youngest chambers of specimen E5 (field). (b) Bacteria in degradation vacuoles (white b) next to clay particles (black arrow) in specimen E14 (8 h incubation). (c) Elongated bacterium inside degradation vacuole adjacent to clay particles of specimen E37 (20 h incubation), scale bars: a: 2 µm, b,c: 0.5 µm.~~

3.4. Foraminiferal genetics

Six of 13 specimens analyzed for genetics were positively amplified and sequenced (Fig. S43). 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., 2019b). 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 pPingo (GHP3) (Serov et al., 2017). At such sites with high methane fluxes, the SMTZ (sulfate methane transition zone) is shallow, as sulfate infrom the sediment is readily consumed in the first tens of centimeters (Barnes and Goldberg, 1976; Iversen and Jørgensen, 1993) by sulfate-reducing bacteria (SRB) (reviewed in Carrier et al., 2020). Geochemical analysis of PUC2, revealed an SMTZ at app. 13 cm, which. 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). Similar gGeochemical characteristics can be considered similar at the sampling location of living specimens (BLC18) given the close proximity of the two locationsand the core taken for geochemistry (PUC2).

4.2. Possible aAssociationssociation with putative methanotrophs

The possible association of *N. labradorica* with the three putative methanotrophs could bewas identified documented via presence of two putative methanothrophs, based on microbial ultrastructure foron 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 possible association with putative methanotrophhs likely originating is due to from the foodthe feeding being given in the experiment. However, there is a small possibility that the associated methanotrophssticked on the outside of the test wereand could be remaining field-remains,. to preserve its microbiom OurThe results of our observation matchare similar to the result of observations on field-collected *Melonis barleeanus* (Bernhard and Panieri, 2018), where a putative association of foraminifera andwith methanotrophs has beenwas describedoriginating from the field. is evidence that methanotrophs can indeed be a food source to *N. labradorica*. The However, the non-selective feeding deposit-feeding behavior strategy is likelyingesting large amounts of sedimentof *N. labradorica*, which weas described in this study for this species for the first time, shows that methanotrophs may be 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 tze SMTZ, but they likely not live in those sediment layers permanently(Bernhard and Bowser, 1999). Foraminfiera 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~~ Ddegradation vacuoles show large number of sediment particles and few bacteria

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~~ proportion compared to presence of sediment particles, which occurred in 100% of the examined ~~degradation vacuoles~~foraminifers. From this result, however, we ~~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 additionall~~ly~~ molecular tools to ~~additionally~~ determine the food contents inside the cytoplasm ~~(e.g.~~(e.g. Salonen et al., 2019). A recent study ~~by~~ used metabarcoding to assess the contribution of ~~bacterial~~ eukaryotic OTUs associated with intertidal foraminifera, ~~and~~ reveal~~inged~~ that *Ammonia* sp. T6 preys on metazoans, ~~can predate on metazoan taxa,~~ whereas *Elphidium* sp. S5 and *Haynesina* sp. S16 were~~are~~ more likely to ingest diatom~~sa~~ (Chronopoulou et al., 2019).

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

Our observations also included the intact nature of all major organelle types~~s~~ of thi~~se~~ species, as this was essential to conclude vitality after the experiment (Nomaki et al., 2016). Mitochondria and kleptoplasts were generally homogeneously distributed throughout the cytoplasm confirming previous observations of six *N. labradori~~c~~ia* ~~–~~from the Gullmar Fjord (Lekieffre et al., 2018; Jauffrais et al., 2019b). 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 specimenssamples 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,~~Even though our study did not focus on kleptoplasts, we could observe that kleptoplasts ~~-~~were occasionally degraded, which could have happened; a) during sampling, b) due to exposure to microscope lights or c) due to the age and condition of kleptoplasts inside the host. Kleptoplasts in *N. labradorica* have been studied in detail describing their diatom origin (Cedhagen, 1991), sensitivity to light and missing photosynthetic functionality ~~(e.g.~~ (Jauffrais et al., 2019b). Kleptoplasts in *Elphidium williamsoni* might have an value for providing extra carbon storage and calls the need for more studies on complex feeding strategies developed by kleptoplastidic foraminifera (Jauffrais et al., 2019a).~~It has been suggested that kleptoplasts could function as a seasonal energy reservoir, for example, (e.g. in winter) (Jauffrais et al., 2016).~~

5. Conclusions

Based on the content of degradation vacuoles ~~observed~~, we conclude that *N. labradorica* from ~~GHP3~~our study site, an active methane emmitting site in the Barents Sea, is a deposit~~-~~ feeder~~.~~, ~~as I~~it ingests large amounts of sediment particles together with bacteria ~~as part of consuming detritus detrivorous diet living on the sea floor~~. On two specimens of the feeding experiment, putative methanotrophs were observed near the *N. labradorica* aperture, suggesting ingestion of *M. sedimenti* ~~*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 may ingests M. sedimenti*~~ via "untargeted grazing" ~~in this seep site~~s. Further studies are needed on feeding strategies of ~~several~~ other paleo-oceanographically relevant foraminifera to detangle the relationship between $\delta^{13}C$ ~~measured in~~of foraminiferal calcite, their cytoplasm and dietary composition~~contribution to their diet~~.

6. Data availability

Data in form of TEM images will be deposited at PANGAEA (~~under~~ doi:

Molecular data ~~will be~~ is 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.

Duration of experiment (h)/field samples	Food provided (yes (x)/no)	Sample ID	Cytoplasm: Degradation vacuole Contents		Aperture region: (putative) Methanotrophs
			bacteria	Clay/in-organics	
Field samples (Initials)	No	E1	no	x	no
	No	E3	no	x	no
	No	E5	no	x	no
	No	E6	no	x	no
4	x	E25	no	x	no
	x	E27	x	x	no
	x	E28	no	x	no
	x	E29	no	x	no
8	x	E14	x	x	no
	x	E15	no	x	no
	x	E16	no	x	no
	x	E17	no	x	no
20	x	E36	x	x	1 x
	x	E37	x	x	no
	x	E38	no	x	no
	x	E39	no	x	2 x
Control (20)	no	E44	no	x	no

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