

## Revisions for BG-2021-284

We thank the reviewers for their thorough reading of our manuscript and their constructive comments. Below we have copied each review in full (in black text), and highlighted (main) reviewer comments in **black bold** text. We provide our response to them in orange text. Text quoted from the original manuscript is in grey and proposed changes based on the review are in blue.

Thanks to these requested comments and suggestions, we feel the manuscript has improved considerably and hope that our proposed revision will meet the criteria for publication in **Biogeosciences**.

Sincerely,

Christiane Schmidt (on behalf of all authors)

### Report of Reviewer #2

In this study, living individuals of the calcareous benthic foraminifera *Nonionella labradorica* were collected from active Arctic methane-emission site sediments and used for a one-day feeding experiment to investigate the uptake of the prokaryot methanotroph *Methyloprofundus sedimenti*. Transmission Electron microscopy pictures of the foraminiferal cytoplasm were analyzed to test the hypothesis that *Nonionella labradorica* is a deposit feeder and is able to ingest and feed on *Methyloprofundus sedimenti*. The presented TEM micrographs are of high quality, and show the deposit feeding character of *Nonionella labradorica*. **But the feeding experiment itself demonstrates only weak results.**

We agree with reviewer 2 that the feeding experiment itself did not yield a strong result, as the putative methanotrophs could be also a remain form the field or from the cysts which had been removed before experiments. We have changed the text to reinforce the fact that this species is a deposit feeder and we have put less emphasis on the results related to the experiment. However, the results of the observation with putative methanotroph are clearly visible in Figure 4, and 5, and deserve to be published. We frame the association with the methanotroph to be putative, and do not emphasize in the title that this evidence is due to the feeding experiment. We have included the scientific name in the Title as this was requested by Reviewer 1

Deposit-feeding of *Nonionellina labradorica* (foraminifera) from an Arctic methane seep site and possible association with a methanotroph revealed by transmission electron microscopy

The original scientific question of the manuscript fits into the field of biogeosciences, **but the results presented do not fulfill this claim** and the concept and data can only partly be described as novel. **Therefore, no significant conclusions can be drawn with respect to the initial questions.**

We agree with the reviewer that the manuscript fits into biogeosciences topic, as it is a relevant topic to better understand feeding behavior of benthic foraminifera, which could ultimately have an influence on the isotopic signature of  $\delta^{13}\text{C}$  of their tests.

The concept and the data presented in this manuscript are of novel nature. Very few published papers have shown data from living foraminifera from active methane emission site from the Arctic. On this species no other feeding experiment has been performed to date, and all images presented are original. Furthermore, no ultrastructure of deep water *N. labradorica* has been presented to our knowledge. All other published works used specimens collected shallower from Gullmar Fjord (Sweden) sediment samples were collected from a depth of ~ 70 m using a box-corer (Jauffrais et al. 2019).

The scientific methods and assumptions are valid and clearly outlined, although I want to comment the following points:

- It is not clear for me why the authors put a lot of effort to the **geochemical analyses and molecular genetics** but did not **show these data** in detail and do not really link them to the experiment. **Why they determined sulfate decline and the SMTZ if they collect their foraminifera from the 0-1 cm surface and feed them with methanotrophs from the water column? It would have been better to concentrate on the feeding experiment.**

We note that the methanotrophs were once isolated from a whale fall isolated from sediment, and not methanotrophs from the water column, as Reviewer 2 understood.

We like to keep the geochemical analysis in the manuscript, as they are important to show that the site, where foraminifera were collected, was classified as an active methane emission site. The molecular genetics is used because it is the modern means to identify a species. For foraminifera, this is typically done with both morphology and sequencing. However, the reviewer is correct that we do not show the data enough, and have changed this. We deem it is sufficient to include the data in the Supplementary material.

1. Geochemical analysis: A Table has been added to the supplementary material showing the sulfate, DIC of the pore water of the geochemistry core. This was collected 5 m away from the actual sampling site, which contained the blade corer and the living foraminifera. The blade corer where the living foraminifera originated from was not possible to be analyzed for geochemistry.
2. Molecular data: The molecular sequences uniquely identify the species as *Nonionellina labradorica*. However, as there are cryptic species, and in order to relate the work to future work, the sequence has been published and was referenced already in the old version of the manuscript. Hence, we do not see the need to change or extend discussion of it.

Reviewer 2 asked why we collected foraminifera from the 1-2 cm in the blade corer. That is because this species was abundant at this depth in the blade corer sample. Determining the sulfate decline and DIC of the pore water let us conclude that this is an active site.

- If I understand correctly, the methanotroph food shows a natural labelling (line 63: methanotrophs produce the biomarker diplopterol, which has an extremely light  $\delta^{13}\text{C}$  signature ( $-60\text{‰}$ )). **It would have been of big advantage to use this kind of natural label or any other labelling to track a possible uptake by the foraminifera.**

The reviewer is right about this, our initial plan was also to label the bacteria with a biomarker /label. However, there was a last-minute issue so this was not possible.". The first approach in this study was to investigate whether this foraminifera would feed on bacteria, and this we can clearly answer with the presented data. It turned out that next to the association with bacteria, the

foraminifera are strong deposit feeders. This would have never been noted without close examination via TEM, as presented.

- The **incubation time seems to have been planned too short**, especially at these cold temperatures, because no uptake was observed.

We agree with the reviewer that the incubation time was rather short and longer experiments may have shown uptake. However, based on previous experiments that incubate this species with  $^{13}\text{C}$  and  $^{15}\text{N}$  isotopes at low temperature (Jaufrais et al. 2019) recording metabolism activities after 20 hours, it was logical that we used the same incubation duration. We agree that longer experiments would indeed be good to possibly stimulate feeding uptake by these specific Arctic foraminifera.

(Line 193-195) 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 experiment with *M. sedimenti* was conducted for the total duration of 20-h to resemble previous experiments on *N. labradorica* using transmission electron microscopy and nanometre-scale secondary ion mass spectrometry (TEM-NanoSIMS) isotopic imaging (Jaufrais 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, to allow specimens to recover from the experimental setup. During the pre-experimental phase specimens were not fed and resided in the petri dishes to adjust to the experimental environment.

- The authors mentioned a starvation phase before the experiment of 2-4h. Do they mean 2-4 days? Otherwise, 2-4 h are no starvation time, I would say. Foraminifera probably do not feed 24/7.

The starvation period was chosen to be within 2-4 hours because we wanted to not expose the specimens longer than needed before the actual start of the experiment, and risk their death. A pre-adaptation phase of several days was not feasible in this study. However, as those Arctic specimens are sensitive to both light and temperature we choose to conduct the experiment onboard the vessel. Because of logistics and the entire duration of the cruise, the starvation phase was chosen to be rather short (2-4h). As the results showed that foraminifera did not starve during this time, as they were still having full food vacuoles, we re-name this phase as an pre-experimental phase, where organisms are supposed to adjust to experimental conditions.

(Line 193-195) 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 experiment with *M. sedimenti* was conducted for the total duration of 20-h to resemble previous experiments on *N. labradorica* on transmission electron microscopy and nanometre-scale secondary ion mass spectrometry (TEM-NanoSIMS) isotopic imaging (Jaufrais et al. 2018), 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, to allow specimens to recover from the experimental setup. During the pre-experimental phase specimens were not fed and resided in the petri dishes to adjust to the experimental environment.

- The authors mention that for each experimental time point, 4 out of 5 foraminifera were examined. **Was the selection for these 4 purely random?**

Yes, the selection of samples was purely random. We selected the specimens presenting the better orientation in the resin block to facilitate TEM observation of the chambers of interest. .

Hence, the four samples were chosen which we were able to cut through the aperture region. I have added this in the methods section.

Old Manuskript: 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 UranylLess® EM Stain (EMS, USA).

New Manuscript Lines 189-194: 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** were sectioned using an ultramicrotome (Leica® Ultracut S) equipped with a diamond knife (Diatome®, ultra 45°). Grids were stained using UranylLess® EM Stain (EMS, USA).

**The results are not sufficient to support the interpretations and conclusions, and I want to comment the following points:**

- The TEM analyses showed that all investigated specimens (collected in the field and in the experiment) **contain degradation vacuoles containing clay/inorganics**, which is a strong indicator for sediment uptake and deposit feeding in *Nonionella labradorica*, indeed. **But this does not have something to do with the presented experiment**, because sediment uptake took part before the individuals were placed in the petri dishes and the experiment started.

The reviewer is right that the degradation vacuoles containing clay/ inorganics originate from particle engulfment that took place before the incubation, when specimens were still in sediments. We analyzed 17 samples, 12 fixed after incubation and feeding on bacteria (for 4-20-h), and 4 fixed directly after field collection, and 1 as a non-fed control (fixed after an incubation of 20-hours). In all food vacuoles the sediment was present irrespective of treatment. This is shown in Table 1.

As reviewer 2 pointed out, the degradation vacuoles containing clay particles were formed before the experiment and thus cannot be linked with the ingestion of methanotrophs. However they still demonstrate the deposit-feeding trait of *N. labradorica* species. Modifications have been made in the text to clarify this point.

It has been adjusted in the Title: **Deposit-feeding activity of *Nonionellina labradorica* (foraminifera) at an Arctic methane seep site and putative association with a marine methanotroph**

- The observed bacteria inside vacuoles or at the aperture area of the foraminifera are still few and **not convincing, there seems no significant correlation**. Living foraminiferal tests are surrounded by a sticky cytoplasm cover. Fig 4 shows sediment particles around the aperture. This means that specimens were not cleaned properly before placed in the petri dishes (contradict to the text in line 189). This kind of sediment may also be very sticky, as well as pseudopodia remnants around the aperture. Added methanotrophs may stick here by accident after some time. This seems to be more an **artefact because of the experimental set up and should not be interpreted as an “association of foraminifera and methanotrophs”** as noted in line 354 of the discussion.

We do not agree with reviewer 2 that foraminifera were not cleaned properly before they were placed in the petri dishes. We have cleaned them with several strokes of fine artist brushes and

took great care to not damage their delicate test. Also please note that the association was only observed in the 20-h incubation. Hence, we strongly believe that the foraminifera really approached the bacteria. Furthermore, one of the putative methanotrophs had characteristic ICM (Intracytoplasmic membranes), which resemble the methanotroph *M. sedimenti* in culture. Cleaning the foraminifera with another means, such as ethanol bath would have been too risky to not damage the microbiome around the foraminifera.

However, we agree to the reviewer that this is a valid point, and it needs discussion. The sentence in the discussion has been changed accordingly.

Line 323, 358, 359 The association has been changed to “the possible association”

The paragraph of the discussion “4.2 Possible association with putative methanotrophs” has been shortened from 295 words by 47% to be 168 words, as the discussion on the SMTZ and AOM (Anaerobic oxidation of methane) was removed based on reviewer 1 comments for adjusting introduction and discussion.

#### 4.2. Possible association with putative methanotrophs

The possible association with the three putative methanotrophs was identified on two specimens based on comparing internal characteristics of methanotrophs (Tavormina et al., 2015) to our images. The documentation of this possible association with putative methanotrophs likely is due to the methanotrophs given as food in the experiment. However, there is a small possibility that the associated methanotrophs stuck on the outside of the test and could be remaining from the field, as foraminifera were carefully cleaned but not washed in ethanol before the start of the experiment to preserve its microbiome. The results of our observation match to the result of observations on *Melonis barleeanus* (Bernhard and Panieri, 2018), where a putative association of foraminifera and methanotrophs has been described recently originating from the field. However, the deposit-feeding behavior ingesting large amounts of sediment, which was described in this study for this species for the first time, shows that methanotrophs can be ingested via untargeted grazing in seeps, as *N. labradorica* appears to be a non-selective feeder.

The description of the experiment is very clear and allows reproduction. The authors give proper credit to related work and clearly indicate their own new contribution. The number and quality of the references is appropriate. The language is fine, although there are **some minor mistakes in the text. The title reflects the contents of the paper, and the abstract provides a good summary.**

The introduction is very informative, but in parts **drive off from the topic and raises high expectations in the experiment, e.g. that a direct link can be drawn from methanotrophic bacteria to the extremely light  $\delta^{13}\text{C}$ -signals** in foraminiferal tests.

We agree with the reviewer that the introduction should not focus on  $\delta^{13}\text{C}$  signals alone as those were not measured. However we think it is important to discuss them, as they would be ultimately influenced by different food sources. The text of the introduction has been shortened by 1.5 paragraphs (old manuscript Line 74-87). The sentence about the MDAC, was kept.

Several studies found that the lightest isotopic  $\delta^{13}\text{C}$  values were measured in tests coated by methane-derived authigenic carbonate (MDAC) overgrowth, which happens after the death of

the protist (Torres et al., 2010; Panieri et al., 2014; Consolaro et al., 2015; Panieri et al., 2017; Schneider et al., 2017).

Concerning the supplementary material, I **miss the data of the geochemical measurements.**

We agree with Reviewer 2, that due to revisions of the manuscript the Table showing the geochemistry measurements had been removed. It has been added in the Supplementary Table 1, showing the geochemistry of the PUC2/F7 and an image of this associated core.

**Results:** Line 259 old manuscript: 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 (see Fig. S1 and Table S1).

The overall presentation is well structured and clear described. The TEM micrographs are of high quality, and show the deposit feeding character of *Nonionella labradorica*. **This should be published!** But I recommend publishing the data in a more foraminiferal-focused journal and changing the focus of the manuscript.

We believe that the readership of biogeoscience will find that manuscript interesting as it is an interdisciplinary work between microbiology and foraminiferal research. The study looks to increase our understanding of feeding habits of foraminifera on the sea floor around active methane seepage in a geochemically active site, which is interesting for readers of BG. We hope that Reviewer 2 points were sufficiently addressed, and that if open questions remain they can be resolved to publish this manuscript in the BG journal.

## References

Jauffrais, T., LeKieffre, C., Schweizer, M., Geslin, E., Metzger, E., Bernhard, J.M., Jesus, B., Filipsson, H.L., Maire, O., Meibom, A., 2019. Kleptoplastidic benthic foraminifera from aphotic habitats: insights into assimilation of inorganic C, N and S studied with sub-cellular resolution. *Environmental Microbiology* 21, 125–141. <https://doi.org/10.1111/1462-2920.14433>