Heavy metal uptake of near-shore benthic foraminifera during multi-element culturing experiments

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Response to Reviewer#1 comments

In the following, the Reviewers' comments or questions on the manuscript are given in black italics, and our response is highlighted in blue and indented. We only consider points that provide information and clarifications that are of general interest to the readership. Minor points like " Line 258: add a comma (,) after samples" or "Break this sentence into two" will be performed during the manuscript revision, and hence are not to be discussed here.

For interactive discussions:

1) Higher distribution coefficient values are reported for symbiont-bearing foraminifera species (Line 727). Is it possible or logical to think that lack of food can make the cultured specimens (non-symbiont foraminifera) weak, which can inhibit the incorporation of heavy metals from the surrounding culture medium?

- This is unlikely as a sufficient amount of algal food was added during the experimental period. This was evident by leftovers covering the sediment surfaces in the cavities at the end of each phase. This would have been consumed by the foraminifera if they would have needed more. Furthermore, the foraminifera calcified, which wouldn't be the case if any undersupply occurred (e.g. Lee et al., 1991; Kurtarkar et al., 2019). Therefore, the nutritional status is unlikely to have influenced the metal uptake by the foraminifera.

2) Does the different species of Ammonia show similar rates of incorporation (comparison of this study with published culture-studies with other species of Ammonia?

- We can have a further look for culture studies addressing the incorporation of metals into the tests of other *Ammonia* species and add this to the discussion. Examples are Munsel et al. (2010) addressing Mn, Ni and Cu in the test of *Ammonia tepida* or Marechal-Abrams et al. (2004), who looked at the Cd incorporation into the shell of *Ammonia beccarii*. Furthermore, de Nooijer et al. (2007) cultured *Ammonia tepida* with different Cu concentrations or van Dijk et al. (2017) investigated the Zn uptake of *Ammonia tepida*. These examples are already integrated into the manuscript, but we can go further into detail and point out the species-specific differences. Furthermore, we will add a comparing table or figure.

3) DZn obtained in this study are in good agreement with hyaline species and also a miliolid (Lines 634-636). Some previous work on several miliolid species report elevated concentrations of Zn in their shell compared to the ambient seawater. It would be interesting to discuss about the difference in metal incorporation between miliolid and rotalid species in the discussion section 4.3 Interspecies variability.

- We agree with the reviewer. It is interesting to note that our study compared to both miliolid and rotaliid species. The differences in calcification of miliolid and rotaliid foraminifera and its implication on the heavy metal concentration in the foraminiferal should indeed be discusses further. We will add this to a comparing figure.

Abstract

From the culture experiments, Pb and Ag are seen to incorporate linearly (Figure 4) in the new calcite of all three foraminiferal species. It would value if the distribution coefficients obtained for these metals are added in the abstract.

- The D_{TE} of these elements (apparent partition coefficients for Ag: *A. aomoriensis* = 0.56, *A. batava* = 0.17, *E. excavatum* = 0.47; for Pb: *A. aomoriensis* = 0.39, *A. batava* = 0.52, *E. excavatum* = 0.91) can be added to the abstract.

Introduction

Line 65: another multi-element culture experiments on large benthic foraminifera Amphisorus hemprichii reports the proportional enrichment of Mn, Ni and Cd (Sagar et al., 2021) in the foraminiferal tests from the culture solutions, and the thresholds

- We thank the reviewer for pointing out this recently published study, the results of which will be considered in the revised manuscript. For instance, Sagar et al. (2021a) found that the partition coefficient of *Amphisorus hemprochii* for Mn was 1.3±0.2, which is slightly higher, but in the same order of magnitude than our D_{Mn} values. The presented D_{Ni} of 0.3±0.04 is comparable to our findings and the partition coefficient of Cd (D_{Cd} = 2.6±0.3) reported by Sagar et al., (2021a) is also in the same range. Nevertheless, it should be noted that D_{Cd} is more variable in our data. The applied concentrations of Mn in the culturing medium of our study were higher, while the concentration of Ni was lower and the concentration of Cd was comparable. We will add all this information to the table (or figure) comparing foraminiferal species and culturing experiments.

Material and Methods

Line 105-106, 116-117, and 124: Were the containers pre-cleaned? If yes, mention the pre-cleaning of the EMSA CLIP and Close boxes used for storing samples from Japsand, and the plastic containers used to collect samples from Kiel Fjord

- Yes, all boxes were pre-cleaned with Mucasol soap water and 5% HNO_3 before use. We will add this information to the revises version of the manuscript.

Line 118-119: Does this apply to all the 9 cores collected from Kiel Fjord? If yes, change the sentence and include the term 'all the 9 cores'

- Yes, all sediment cores displayed a very similar sediment succession. This will be specified in the revised version of the manuscript.

Lines 127-128: Any reason why artificial seawater was used for washing and storage of the samples.

- Artificial seawater was used to ensure that no microorganism that could potentially have harmful effects on the foraminifera were introduced, for instance ciliates. If natural seawater were used, it would have to be sterilized before use (e.g., by autoclavation or filtration). Considering the high volume of water that was necessary to process the samples, it was less time consuming to use artificial seawater.

Lines 139-141: Mention the cleaning protocols/steps followed for laboratory ware, which were used to handle the foraminifera specimens

- The following information will be added to the revised manuscript:
- Plastic utensils: First of all, a pre-cleaning with Mucasol-water mixture (soap) was performed to remove oils and other contaminants that could remain from production. Therefore, the laboratory ware was stored in MilliQ water with added Mucasol (soap) overnight in an oven with 35 °C. Secondly, the Mucasol water was rinsed well and 5% HNO_3 was applied for at least 2 days before rinsing.
- Paint brush and other materials made of different material than plastic were rinsed with Ethanol to avoid any biological contamination.

Line 152: cite reference for calcein (16 mg/l)

- This was a mistake. The Calcein concentration used in this study was 10 mg/l as described by Bernhard et al. (2006).

Line 154: Were temperature measurements carried at the sampling locations? If yes, mention it in the section 'Field Sampling'

- Water temperature measurements were performed at the North Sea stations.

Line 218: All salts used were provided in p.a. quality. (does p.a. means pro analysi; please write in full). Please mention the provider of the salts (e.g., SigmaAldrich or CarlRoth or ?)

- Yes, p.a. is an abbreviation of "pro analysi". The providers of the chemicals were Carl Roth ($CrCl_3 \cdot 6\ H_2O$; $SnCl_2 \cdot 2\ H_2O$ and $PbCl_2$), Walter CMP ($CdCl_2$) and Sigma Aldrich ($MnCl_2 \cdot 4\ H_2O$, $NiCl_2 \cdot 6\ H_2O$, $CuCl_2 \cdot 2\ H_2O$, $ZnCl_2$, $AgNO_3$ and $HgCl_2$).

Line 210: Reference 'Frontallini et al., (2018a)" studies the effect of mercury pollution on cultured benthic foraminifera. Frontallini et al., (2018b) studies the ultrastructural alteration in benthic foraminifera induced by heavy metals (e.g., Pb).

- The study of Frontalini et al. (2018b) is already included. We were not aware of the mercury study by Frontalini et al. (2018a), which will be cited in the revised manuscript.

Lines 229-230: Each experimental phase lasted 21 days (three-weeks) and water with heavy metal concentration was fed into the system bi-weekly. Does it mean that in the three-weeks duration, of each phase, the multi-element culture experiment was fed once with the multi-element spike? Please explain clearly.

- The multi element stock solution was added at the beginning of each phase to reach the targeted concentration. Additionally, a smaller aliquot of the same stock solution was introduced twice a week during the three weeks of a phase. The reason was that we expected a loss of metals during the culturing phase (e.g., uptake by foraminifera or algae, adsorption to surfaces of the culturing system).

Line 311: ICP-MS/MS (do the authors mean ICP-MS)

- A tandem ICP-MS/MS instrument (Agilent 8900) was used for analysis.

Results

Line 392-393: In contrast to the text, the figure shows higher Cu/Ca concentrations in the metal experiment of phase 0.

- This sentence was unclearly formulated and the Cu concentration is higher in the metal systems in phase 0, which can be clarified in the revised version of the manuscript.

Line 393: The control system in Mn/Ca shows higher concentration in all phases. Reword lines 391-394.

- Indeed, the Mn/Ca concentration was higher in the control system than in the metal system for the phase 0, 1 and 2, but not for phase 3.

Lines 398-399: For every metal phase experiment, were the samples cultured in the spiked multi-element solution (for that particular phase) for the cultured duration of 21 days. Example: For M2 culture, were the samples spiked with M2-concentration (Table 1) for the whole duration of 21 days? Please see Lines 229-230. Please explain.

- Yes, the culturing water from which the samples were taken, was maintained with M2 concentration for the 21 days. As this concentration was expected to decrease over the 21 days of culturing, a smaller aliquot of the stock solution (Phase 1 = 0.1 ml, Phase 2 = 1 ml, Phase 3 = 10 ml) was added twice a week to keep the concentration stable (see above). This will be clarified in the revised manuscript.

Lines 429-432: In the experiments, there are two systems; Control system (no-spike) and Metal system (spike); Line 374. Each system has 4 phases (0,1,2,3), and phase 0 representing no addition of heavy metals (Lines 200-203). Lines 430-431 says ….when the concentration of these metals in the culturing medium was higher. Please explain.

- Yes, no extra metals were added to the Control system. Nevertheless, there are differences in the metal concentrations, which can be seen in figure 3 and table 4. These changes in concentration can occur due to exchanges with artificial seawater, which contains a certain amount of metals, or by the release of metals like Cu leaching from brass-made system parts.

Line 433: does the phase 3 here belongs to metal system. Add to text.

- Yes.

Discussions

Line 487: Fig. B1; Line 830. The TE/Ca values, for most studied metals, are nearly same for phases 0, 1, and 2 of the metal system, although phase 2 is 10x more than phase 1 (Table 1), and 0 being the control phase. Are there any measurements of spiked-seawater (stock and dilutions) before adding to the culture medium? The culture seawater of phases 1 and 2 (in metal system) should show elevated concentrations (in proportions) compared to phase 0, but is not the case. Please explain why?

- First question: No, the stock solution was not measured prior to the introduction to the system. No dilutions were made and the stock solution was added directly to the system in different amounts, depending on the concentrations required for the specific phase.
- Second question: Yes, the metal concentration in phases 1 and 2 should be elevated, which is the case for some elements (e.g., Sn, Hg for phase 2). However, there is no elevation visible in most cases. Sorption to surfaces or the uptake of metals by the foraminifera and algae are possible reasons, but no explicit cause could be identified. This is already discussed in chapter 4.1 "Experimental Uncertainties" and is why we monitored the metal concentrations so closely.

Lines 493-495: ….are smaller than expected for phases 0, 1, and 2. Phase 0 is mentioned as the control phase with no addition of heavy metals (Lines 200-201). Then why is the metal concentrations of phase 0 smaller than expected (for normal seawater?). is it also because of reasons mentioned in subsequent lines.

- Phase 0 was accidentally mentioned in this context, which needs to be corrected.

Lines 521-522: Is it possible that the low level of food supplies (as inferred from lack of reproduction) might make the cultured foraminifera specimens weaker and relatively lower amount of metal incorporation in them?

- We assume that the food supply was sufficient because there was leftover food after the experiments and furthermore, the foraminifera calcified, which also requires enough food. See comment above.

Lines 615-616: This in turn ….. into the foraminiferal tests. Are the Mn, Zn, and Cu concentrations in the normal seawater (non-polluted) are sufficient as micronutrients considering the fact that these metals are present in the tests of benthic foraminifera recovered from pollution-free environments?

- The artificial salt used for the culturing medium contained all elements or nutrients that are necessary for marine organisms in a sufficient amount, for most elements at concentrations higher than present in seawater naturally.

Lines 634-636: Titelboim et al., (2018) based on their studies report that miliolid shells might have advantage over hyaline as bio archives, since they record higher values than rotaliids from the same ambient seawater. As mentioned, DZn values of this study are in good agreement with results from hyaline as well as miliolid foraminifera. Please discuss the findings of this study with the findings from Titelboim et al. 2018.

- We fully agree with the reviewer and will include Titelboim et al., 2018 in our discussion. The maximum Zn/Ca in our experiments was ~ 68 µmol/mol, which is little lower than reported in Titelboim et al. (2018) (Zn/Ca in *P. calcariformata*= 195 µmol/mol), which may be due to different concentrations in the seawater the foraminifera grew in. It is thinkable, that Zn as a nutrient is in first place used as such and only gets incorporated into the shell after enough Zn was provided to the cell itself. If this was not the case or if the seawater Zn concentrations in our study was not exceeding the necessary nutrient level, this would also explain, why we could not find any correlation between Zn/Ca in seawater and in the foraminiferal calcite. In the revised manuscript version, we will include the findings of Titelboim et al. (2018) into the comparison between species and studies in form of a table or a figure.

Lines 670-676: The mean Pb distribution coefficients obtained from Amphistegina spp. by Titelboim et al., (2021) is 12.9. Please add this to your discussion.

- The information from this study will be displayed in a table (and/ or figure) for direct comparison and discussion. However, it should be noted that *Amphistegina* is a tropical symbiont-bearing species and the symbionts can influence the uptake of certain metals. This could facilitate variations in the incorporation of the metals compared to non-symbiont bearing species like in our study.

Lines 678-679: Please add the results from Sagar et al., 2021b (partition coefficients for Mn, Ni, and Cd from Amphisorus hemprichii).

- See Comment above.

Lines 715-717: Figure 4 shows a positive correlation between the concentrations of Cr in culturing medium and in the foraminiferal calcite of Elphidium excavatum. A distribution coefficient of 2.1 has been calculated by the authors for E. excavatum. These results are also stated in lines 707-710. The variability of incorporation in Ammonia spp. and E. excavatum might be because of individual species response. Lines 716-717 are in contrast of the results obtained in this study.

- We regret this confusion. In the revised manuscript, we will clarify that Cr/Ca values of *E. excavatum* calcite are correlated with the Cr/Ca values in the culturing medium resulting in a D_{Cr} of 2.1. We will also clarify that Cr/Ca values of the two *Ammonia* species are not correlated like this.

Lines 735-736: When growth is slower, is there a possibility of weak E. excavatum specimens and lower incorporation of artificially elevated heavy metals in the culture medium than what they should have done with preferred food source?

- It is possible that a more preferred food source would have stimulated enhanced growth and promoted the incorporation of heavy metal into the shells of *E. excavatum*. For instance, the closely related species *E. clavatum* prefers bacillariophycean diatoms (Schönfeld and Numberger, 2007). It may also be possible that *E. excavatum* is simply a slower growing species than *Ammonia*, which seems not to be necessarily connected to a specific food source (e.g. Haynert et al., 2020). This information will be added to the revised manuscript.

Lines 719-742: Ammonia beccari, Ammonia tepida, have been cultured, by Havach et. al., 2001; Maréchal Abram et al., 2004; De Nooijer et al., 2007; Munsel et al., 2010, for heavy metal partitioning studies. How do their results compare with the findings of this study for Ammonia batava and Ammonia aomoriensis – a comparison table for the common metals *should give a clear picture for Ammonia spp. foraminifera. The same can be done for Elphidum spp.*

- We will add a comparison table (or figure) for *Ammonia*, *Elphidum* and other species from different culturing studies. Furthermore, we can also include a comparison of the heavy metal concentration in the culturing medium that were applied in other studies.

Line 745; Table 5: The table is a nice compilation of heavy metal contamination studies in various parts of the globe. The studies referred to in the table have used various natural archives such as water, sediment, bacteria, microalgae, living organisms, and others including benthic foraminifera. A column mentioning the natural archives used by the various researchers is important for the readers. This will help them to not only know the polluted regions of the globe but also give them a quick idea of the archives used for those studies, which might help some researchers to pursue similar studies in the area they live and the best archives available at that place.

- This is maybe a misunderstanding. The Table 5 presented in this study is only comparing the metal concentrations in the seawater. The studies indeed addressed the metal concentrations in various archives but for the comparison to the metal concentrations in the culturing medium of this study, only the seawater values of the other studies were taken into account. This issue is to be clarified in the table.

Line 749: 'During the past years, many studies were performed to assess the pollution level of seawater.' The natural archives used in the study (for example water, sediments, bacteria, algae, and other should be included in this sentence (See above comment for Table 5).

- This information will be added to the introduction of the revised manuscript as this is the appropriate place.

Line 744: The title of this section is: 'Application of TE/Ca values in foraminiferal shell' – The description in the text talks about the range of concentration used in the culture medium in the current study. The concluding lines of the section (Lines 775-776) says "This means that the concentration range of metals covered by this study is adequate for future research and monitoring of polluted systems". The main point of this research work is to see the incorporation levels of elevated heavy metals in the foraminiferal calcite tests so that they can be used as natural bio archives for monitoring of polluted near-shore marine environments (Abstract Lines 12-13).

- Indeed, the main subject of this study was to address the incorporation of heavy metals into the foraminiferal calcite for using them as natural archives for this environmental signal. Therefore, we decided to shorten or skip this chapter and move the information to an earlier part of the manuscript.

This section lacks the description on the results (TE/Ca values in foraminiferal shell) obtained in this study from culture experiments with A. aomoriensis, A. batava and E. excavatum and their application as potential pollution indicators. This section needs modification.

- We agree with the reviewer. The section will be removed. See Comment above.

Conclusions

It will be helpful for researchers and readers to pick important findings from this study. Those may be written in point format. For example: '1) All three species showed a strong positive correlation between Pb and Ag in the culture water and their calcite.' The authors should mention the distribution coefficient obtained for these metals from their studies. Others important findings be written in point format.

- This is a good idea and will help the reader to pick up take-home messages from this manuscript.

Line 801: 'the presented DTE's' – The DTE's obtained be mentioned here – also which DTE should be chosen, with phase 3 or without, be mentioned in this section.

- We agree and will mention this in the revised manuscript.

New References

Bernhard, J. M., Blanks, J. K., Hintz, C. J., & Chandler, G. T. (2004). Use of the fluorescent calcite marker calcein to label foraminiferal tests. *The Journal of Foraminiferal Research, 34*(2), 96-101.

Haynert, K., Gluderer, F., Pollierer, M. M., Scheu, S., & Wehrmann, A. (2020). Food spectrum and habitat-specific diets of benthic Foraminifera from the Wadden Sea–A fatty acid biomarker approach. *Frontiers in Marine Science, 7*, 815.

Kurtarkar, R. S., Saraswat, R., Kaithwar, A., & Nigam, R. (2019). How will benthic foraminifera respond to warming and changes in productivity?: A Laboratory Culture Study on *Cymbaloporetta plana*. *Acta Geologica Sinica-English Edition*, *93*(1), 175-182.

Lee, J. J., Sang, K., Ter Kuile, B., Strauss, E., Lee, P. J., & Faber, W. W. (1991). Nutritional and related experiments on laboratory maintenance of three species of symbiont-bearing, large foraminifera. *Marine Biology*, *109*(3), 417-425.

Sagar, N., Sadekov, A., Jenner, T., Chapuis, L., Scott, P., Choudhary, M., & McCulloch, M. (2021b). Heavy metal incorporation in foraminiferal calcite under variable environmental and acute level seawater pollution: multi-element culture experiments for *Amphisorus hemprichii*. *Environmental Science and Pollution Research*, 1-14.