Responses to reviewer 2

We would like to thank reviewer 2 for his interest in this work and his careful and expert reading of the manuscript. As this reviewer pointed out, the limited knowledge on poly-P cycling in natural environments makes such a protocol would be very useful. His comments will enable us to improve the manuscript.

Responses to general comments

GC1- Reviewer comment : « *The experimental set-up; using T. elongata as a positive control is excellent and the use of environmental samples is good and necessary since a high-throughput method that is able to detect poly-P in natural samples could potentially give much more insights into the spatial and temporal dynamics of polyphosphate cycling. However, no real negative control has been used. A strain named "RX" was used as a negative control, even though it was shown to accumulate poly-P. Why not use an actual negative control ? The data that has been collected for the "RX" strain could be an excellent addition to show how the protocol functions for species with minor amounts of poly-P, which might be relevant in natural settings.* »

Author's response : PolyP occurs as a ubiquitous biopolymer in representatives of all kingdoms of living organisms and every cell type in nature (*LorenzoOrts et al. 2020, New Phytologist ; Akbari et al. 2021, Microbial Biotechnology*). In all prokaryotic cells, polyPs are found in granules. This storage of polyPs can be exacerbated in certain bacteria, which accumulate these polymers to intracellular concentrations in the millimolar range. PolyP represent up to 20% of cell dry weight (Martin *et al*. 2014). This is the case for *T. elongata*, a bacterium that hyperaccumulates polyP and which we used as a « positive control » in this study. As there is no true negative control, prior to this study we screened our bacterial strain library in the laboratory to identify the strain with the lowest accumulation that we used as a « negative control ». We agree that these terms may be ambiguous. To remove any ambiguity, we propose to use the terms « high accumulator » and « low-accumulator » in the revised manuscript to describe the *T. elongata* and RX strains, respectively.

GC2- Reviewer comment : « *A lot of the data is not shown. Add this data, either to the main results or the supplement.* »

Author's response : In order not to make the manuscript too unwieldy, we have chosen not to present certain figures and data, as they do not provide any additional information to that given in the text. It is true, however, that this decision can be embarrassing for the reader and leave some doubt as to the validity of the results. Therefore, in agreement with reviewer 1, all data cited in the manuscript and not included in the main document will be presented in the form of supplementary figures and/or made available on the Figshare repository.

GC3- Reviewer comment : « *The authors claim that this is a robust protocol whereas the data tells a different story. The observed differences between the proportion of poly-P cells detected by either FCM or Epifluorescence microscopy in sediment samples (Fig 2C, Fig 4BEF, Fig 5C and Fig 6C) shows that this is not a robust protocol and that FCM grossly overestimates the amount of cell accumulating poly-P. Overall, I would conclude that the combination of DAPI staining and FCM would not work and that this method should not be used to detect and quantify polyP. However, since DAPI staining combined with fluorescence microscopy is often used to detect polyP, it is still important to get the knowledge out there to show that it does not work when combining with FCM. The data shown here where the dye FC-D7 was used seems to be more promising but requires more data (i.e., fluorescence microscopy to show that it works in prokaryotes) and a similar validation protocol as presented here for DAPI.* »

Author's response : Yes, you are right to a certain extent. We have clearly demonstrated that DAPI coupled with cytometry is not a method that can be used for complex environmental samples. We should have been more explicit about the fact that we demonstrate in a step-by-step approach the benefits and pitfalls of polyP detection in flow cytometry. In any case, we show that by using the protocol developed in this study, it is possible to couple flow cytometry and DAPI labelling for polyP detection in homogeneous samples (for example during experiments under controlled conditions on strains in microcosms or mesocosms),

The JC-D7 fluorochrome looks very promising. The community should take advantage of the initial results of our article to test this fluorochrome on their samples to ensure its validity. In the same way that DAPI has been the subject of study after study, JC-D7 will also have to enter this "cycle of testing" by different teams on different types of samples before we can get a clear idea of its value for detecting polyP in environmental microbial samples.

We are the first to use JC-D7 for polyP detection in microbial samples and the first to use it in flow cytometry. JC-D7 has never been used to quantify polyP in prokaryotes using epifluorescence microscopy. We cannot validate an approach by using an approach that has not been validated. This is why we chose to validate the cytometric counts after labelling polyP with JC-D7 by epifluorescence counts after labelling polyP with DAPI. Epifluorescence microscopy for the detection of polyP after labelling with DAPI is an approach validated by the scientific community (e.g. Majed *et al.* 2012).

Responses to specific comments

SC1- Reviewer comment *: « I think the introduction could elaborate a bit more or the functions and ubiquitousness of polyphosphate and put it in an environmental context. Almost all bacteria have the enzymatic potential for poly-P built-up and breakdown and besides being used as a storage compounds/energy reserve, many functions are known and none of them are mentioned in the introduction here. The following papers are excellent references:*

1. *Brown, M. R. W., and Kornberg, A. (2004). Inorganic polyphosphate in the origin and survival of species. Proc. Natl. Acad. Sci. 101, 16085–16087. doi: 10.1073/ pnas.0406909101*
2. *Kornberg, A. (1995). Inorganic polyphosphate - toward making a forgotten polymer unforgettable. J. Bacteriol. 177, 491–496. doi: 10.1128/jb.177.3.491496.1995*
3. *Kornberg, A., Rao, N. N., and Ault-riché, D. (1999). Inorganic polyphosphate: a molecule of many functions. Annu. Rev. Biochem. 68, 89–125. doi: 10.1146/ annurev.biochem.68.1.89*
4. *Rao, N. N., Gómez-García, M. R., and Kornberg, A. (2009). Inorganic polyphosphate: essential for growth and survival. Annu. Rev. Biochem. 78, 605–647. doi: 10.1146/annurev.biochem.77.083007.093039 »*

Author's response : Yes, you're right, this information is missing. We suggest introducing the following sentences into the revised manuscript just before talking about hyperaccumulation (p.2 line 43.):

« Likely a key agent in evolution from prebiotic time (Brown and Kornberg 2004; Lorentzo-Orts *et al.* 2020), the functional roles of polyP in the cells of contemporary organisms are many and varied (Konberg *et al.* 1999). PolyP can serve as a source of energy; as a phosphorylating agent for alcohols, including sugars, nucleosides, and proteins; and as a means of activating the precursors of fatty acids, phospholipids, polypeptides, and nucleic acids (Rao *et al.* 2009). »

SC2- Reviewer comment *: « L75: What are "large amounts" of polyP? Is it a large fraction of the cell volume? And if so, how much? Do most cells accumulate poly-P? And if so, how much? »*

Author's response : We will add the following information : up to 30–35% of the total biovolume of bacteria (Nguyen *et al.* 2011)

SC3- Reviewer comment *: « L78: What is "a very small amount of intracellular polyP? Only few cells or does polyP only take up a small amount of the cell volume? »*

Author's response : It's both. We will add this clarification in the revised manuscript

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SC4- Reviewer comment *: « **Figure 1A:** Beautiful TEM image, the A is on a weird spot in the figure. I would like to see false quantitative color scales for the EDX analyses if that is possible ».*

Author's response : We don't really understand the question. There are no false colour scales. A colour corresponds to an element with no differences in shade within the same colour. The relative quantity of the element is determined by the dot density.

SC5- Reviewer comment *: « **Figure 1BC**: Make the figures bigger. »*

Author's response : This will be done in the revised manuscript

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SC6- Reviewer comment *: « **L247:** What is meant by "population structure"? »*

Author's response : As shown in Figure 2A, the dots in the cytograms display a two-cluster structure for the RX strain. These different clusters correspond to cells with different characteristics that affect their position on the cytogram according to side scatter (SSC). The number of cells in the P2 population increases with time, so the structure (in terms of the number of sub-populations) of the RX population is affected when the strain is analysed in TE buffer. If the term "structure" is ambiguous, we can clarify it by simply indicating that the cells are affected.

SC7- Reviewer comment *: « **L254 & 255**: Show the data somewhere. »*

Author's response : We answered this question in the « responses to general comments" section.

SC8- Reviewer comment *: « **L290:** Show the data. »*

Author's response : We answered this question in the « responses to general comments" section.

SC9- Reviewer comment *: « **L292 and Figure 2C:** There are significant differences between the proportion of polyP+ cells observed by labeling with DAPI in PBS using the FCM or epifluorescence microscopy. Why is this? Isn't this already a first insight the DAPI in combination with FCM is not an appropriate method to detect polyP accumulating bacteria because there are too many false positives ? »*

Author's response : Concerning the difference between the counts by flow cytometry and epifluorescence microscopy, for the RX strain, which we have clearly identified and which we have plotted in figure 2C indicating significant differences. These methods are different. The major advantage of the flow cytometry was its counting speed. Microscopy, in turn, yielded better visualization of the research material. However, despite the variation and substantial subjectivity related to both microscopy and flow cytometry, both methods revealed the proportions of polyP+ cells in a rather congruent manner.

SC10- Reviewer comment *: « Also, do you have any idea why the use of HEPES would give you so many polyP+ cells? Please elaborate. »*

Author's response : There is a yet unidentified phenomenon that leads to artefactual labelling of RX cells in HEPES buffer. The experiments replicated several times systematically gave the same result. This artefactual labelling led to an overestimation of the proportion of polyP+ cells within the RX population. What allows us to conclude that these proportions are largely overestimated are the epifluorescence microscopy observations on the same samples. One possible explanation could be that HEPES leads to erroneous measurements of fluorescence parameters assessed by flow cytometry due, for example, to strong alterations of the bacterial membrane due to the composition of this buffer (e.g. lack of divalent cations, Tomasek *et al.* 2018). This buffer is not optimal for diluting the Gram-negative RX strain (leading to artefactual fluorescence detection) and potentially Gram-negative bacteria in general for the detection and quantification of polyP+ cells. This effect was not identified for the Gram positive T. elongata strain suggesting that the artefact observed is due to membrane properties.

Reference : Tomasek K, Bergmiller T, Guet CC. Lack of cations in flow cytometry buffers affect fluorescence signals by reducing membrane stability and viability of Escherichia coli strains. J Biotechnol. 2018 Feb 20;268:40-52. doi: 10.1016/j.jbiotec.2018.01.008.

SC11- Reviewer comment *: « **Figure 3:** Both staining periods, DAPI concentrations, and fixation with formaldehyde seem to significantly affect the detection of polyP+ cells with FCM. How was this with epifluorescence microscopy? Is the detection of polyP+ cells using fluorescence microscopy also affected by the staining period and DAPI concentration? This seems especially relevant since the differences in detection by epifluorescence microscopy when compared to FCM all seem to be significant according to the data presented in figure 1C, and later in Figure 4B, E, F, Figure 5B and Figure 6C. Also, even though there are no significant differences observed between fixed and unfixed polyP+ cells for T. elongata (L307), still show the data. »*

Author's response : We did not perform detection of polyP+ cells after different DAPI concentrations using epifluorescence microscopy because the aim of this study was to establish conditions for cytometry (including a comparison between different conditions for the FCM approach). Epifluorescence microscopy was the validation method.

For detection differences, see response to comment SC9.

For *T. elongata* data, as indicated in the responses to general comments, all data will be provided in the revised manuscript.

SC12- Reviewer comment *: « **L332**: Show the data. Especially because storage is highly relevant for natural samples. »*

Author's response : We answered this question in the « responses to general comments" section.

SC13- Reviewer comment *: « **Figure 4B, 4E, 4F:** Where are the statistics between FCM and Epifluorescence? By eyeballing it, the differences seem significant and FCM appears to overestimate the amount of polyP+ cells before sorting and underestimates the amount op polyP+ cells after sorting. »*

Author's response : We are unable to produce statistics on these data because the sorting was carried out (as is usually done during cell sorting) on a single sample (50:50 mixture) of RX and *T. elongata* strains.

SC14- Reviewer comment : « **L337**: What is significant here? Which values are compared? FCM before sorting to FCM after sorting? »

Author's response : We compared the proportion of polyP+ cells counted by epifluorescence microscopy before and after cell sorting. It is true that the way in which this is presented is not very explicit. This will be corrected in the revised version of the manuscript.

SC15- Reviewer comment : « **L339:** PAB represented less than 10% of the polyP- fraction according to Epifluorescence but the proportion found by FCM is much lower. Why is this? »

Author's response :see response to specific comment SC9

SC16- Reviewer comment : *« **L378:** I do not think that the method has been validated. There are significant differences observed between the FCM and Epifluorescence microscopy in almost every figure shown. The only thing that is validated is that this protocol can be used to enrich polyP+ cells (Figure 4 and Figure 5A). »*

Author's response :see response to general comment GC3

SC17- Reviewer comment : *« **Figure 5B:** This really shows that this is not a robust method to be used for environmental samples. »*

Author's response : See response to specific comment SC16

SC18- Reviewer comment : *« **L399-400**: Show the data. Was this fluorescence intensity measured with FCM or epifluorescence microscopy. »*

Author's response : Fluorescence intensity was measured with the FCM. The data will be presented in the supplementary material of the revised manuscript

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SC19- Reviewer comment : « **Figure 6**: Why was there no (epi)fluorescence microsocopy performed with JC-D7 labeling? And what is the significance between JC-D7 and DAPI-epifluo in the sediment? Again, the enormous difference between DAPI stained poly+ cells observed via FCM and Epifluorescence show that this is not a robust method. »

Author's response : see responses to general comments and to specific comment SC16

SC20- Reviewer comment : *« The focus of the paper lies on the use of DAPI but looking at Figure 6, the dye JC-D7 looks much more promising for the use in natural samples (or at least for sediment samples). I think validating JC-D7 as a polyP specific dye in combination with epifluorescence microscopy that can also be used in prokaryotes would be a big step forward. »*

Author's response : We answered this question in the « responses to general comments" section.

SC21- Reviewer comment *: « **L423**: This line does not seem to be relevant here. »*

Author's response : The sentence will be removed in the revised manuscript.

SC22- Reviewer comment *: « **L458-462:** I think this is an overestimation of what this protocol can do. In almost all the data shown, the observations with FCM and epifluorescence are significant, and especially in the natural samples, the differences are striking (Figure 5B and Figure 6C). The only thing that is shown is that polyP+ cells can be enriched. I would not recommend DAPI staining in combination with FCM to quantify the amount of polyP accumulating bacteria in a natural sample based on this data. »*

Author's response : see response to the reviewer comment GC3

SC23- Reviewer comment : **« L469-472**: This conclusion cannot be made without comparing the different staining periods and DAPI concentrations with *epifluorescence microscopy. »*

Author's response : This conclusion will be removed in the revised manuscript.

SC24- Reviewer comment : *« **L481**: Show the results. The publication of negative results is important so other researchers do not have to try it for themselves. »*

Author's response : There was really nothing conclusive about these experiments. So there's nothing to show for it.

SC24- Reviewer comment : *« **L484-485**: The data obtained shows promising results but it does not show the specific nature of PAB labeling. This would require fluorescence microscopy to visualize the JC-D7 dye in combination with for instance SEM-EDS so co-localization analysis can be performed »*

Author's response : This would be highly relevant, but it needs to be the subject of research work in its own right, given the specific equipment and the huge amount of development required.