Responses to reviewer 1

We would like to thank the anonymous reviewer for his careful reading and very interesting comments. As this reviewer indicates, coupling polyP detection with a high-throughput approach opens up highly interesting avenues of research. This coupling has already been tested in a small number of previous studies, but a general framework leading to a generalisable protocol is currently lacking towards 'routine' use. This is the purpose of the present study. While our results are very encouraging, the community must continue its efforts to ensure the robustness of the PolyP detection by flow cytometry, in particular by using specific polyP dyes. To this end, we will follow the recommendations of reviewer 1 and moderate our assertions in the corrected manuscript by further highlighting that we show that coupling polyP detection with DAPI labelling in cytometry is not relevant for complex natural samples.

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.

Responses to general comments

GC1- Reviewer comment : « *The authors grew a known PolyP-accumulating strain (T. elongata) and used a second strain as a control (their own isolate), that still showed "low" levels of PolyP accumulation. This is one of the first flaws of the experimental approach, as the control strain did in fact also accumulate polyphosphates*. »

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 : « *The authors tested a multitude of conditions for staining and storage of cells, although it does not become clear why all these conditions were tested in the first place*. »

Author's response : The coupling of specific polyP detection to flow cytometry has been used in a very small number of studies that have applied standardised conditions and adapted, for example, to DNA detection by DAPI labelling or polyP detection by epifluorescence microscopy. The problem that we highlighted at the outset of this work is that the basic conditions (buffer, incubation time, etc.) need to be tested because they are not necessarily transposable from *ad hoc* conditions adapted to microscopy. This is why we took care to test these variables. This work will enable the community to avoid pitfalls (for example, McIlvaine, TE or HEPES buffers are not suitable for the detection of polyP in flow cytometry in combination with DAPI staining). A number of other parameters, such as concentration or storage, had to be tested. Although trivial, they are essential for defining future standardised protocols and must be acquired prior to the studies. The work we have done will save the whole community time and effort in carrying out time-consuming analyses.

GC3- Reviewer comment : « *More severely, most of the resulting data is either not shown or not conclusive (see comments Figure 2 and 3).* »

Author's response : We answered this question at the beginning of our responses to reviewer 1. We agree with this comment and will provide the data and graphs.

GC4- Reviewer comment : « *Following their tests, the authors applied their FACS approach to environmental samples and find that the FACS detection highly overestimates the cell numbers/proportions as compared to microscopy (which is the benchmarking tool).* »

Author's response : We do not fully understand the meaning of this comment. Throughout the article, we demonstrate that labelling polyP with DAPI is effective for homogeneous samples. On the other hand, we demonstrated the overestimation of polyP after labelling with DAPI in complex environmental samples, which justifies the use of a specific fluorochrome (JC-D7) when dealing with this type of sample. DAPI is a non-specific polyP dye and, in addition to labelling DNA, it also interacts with lipids, displaying metachromatic properties similar to those of polyP (Serafim *et al*., 2002). Although the DAPI-lipid fluorescence is short-lived, with respect to the speed of flow cytometry analysis, it cannot be ignored (P.12 lines 475-476) whereas in microscopy the longer exposure times for counting make it possible to avoid the artefactual counting of lipids.

GC5- Reviewer comment : « *Surprisingly, the authors conclude that their approach is robust. I strongly disagree with this conclusion.* »

Author's response : We answered this question at the beginning of our responses to reviewer 1. We agree with this comment and will moderate our assertions in the revised manuscript.

GC6- Reviewer comment : *« Also, I fail to understand how the authors classified JC-D7 as a useful PolyP-stain when it failed to show similar numbers to DAPI (which is also has a questionable PolyP-specificity). »*

Author's response : Given its non-specific nature, DAPI leads to an overestimation of polyP detection in flow cytometry (see response to comment GC4). This can be established by comparison with the labelling of polyP with DAPI in epifluorescence microscopy, which was used as a validation method. On the other hand, the counts made with JC-D7 in flow cytometry were not statistically different from those made by epifluorescence microscopy after labelling with DAPI. This leads us to consider that JC-D7 is a useful PolyP-stain for using in flow cytometry. We probably need to explain this aspect further in the revised version of the manuscript for a better understanding.

GC7- Reviewer comment *: « The methods section is not detailed enough to allow for reproduction of the presented approach and tests. »*

Author's response : Could you be more specific about the sections on materials and methods that are not detailed enough ?

GC8- Reviewer comment *: Please also include more literature including (https://enviromicro-journals.onlinelibrary.wiley.com/doi/10.1046/j.1462-2920.2001.00164.x, https://journals.asm.org/doi/10.1128/aem.02592-12, https://doi.org/10.1038/s41396-019-0399-7)*

Author's response : The reference to Liu *et al.* 2001 is relevant to our study as they used DAPI staining procedures combined with FISH to identify directly the polyphosphate accumulating traits of different phylogenetic groups. We will cite this article in the discussion section of the revised manuscript. Regarding the references to the work of Martin and Van Mooy (2013) and Fernando *et al.* (2019), if the editors agree that the manuscript should be longer, we could add, in the introduction section, the following information, referring to the publication of Majed et al. (2012) : « Numerous methodologies to quantify and characterise polyP have been developed, including chemical, biological, molecular and microscopic approaches (Majed *et al.* 2012). Most conventional analytical methods (e.g. electron ionisation mass spectrometry) require extensive sample preparation, pre-treatment and pre-fractionation procedures. Advanced analytical techniques, such as nuclear magnetic resonance, Raman, Raman-FISH (Fernando *et al*. 2019) and X-ray spectromicroscopy require much less pre-treatment and allow polyP to be characterised with high molecular and spatial resolution (< to µm). While the potential of these approaches in environmental and biological research is clear, their use remains limited due to the cost and accessibility of analysis instruments. Photometric approaches offer an interesting alternative to the methods discussed above and, the most relevant to date, are based on the interaction between polyP and the fluorochrome 4', 6-diamidino-2-phenylindole (Martin and Van Mooy 2013). »

Responses to specific comments

SC1- Reviewer comment *: « Please do not use abbreviations in the abstract »*

Author's response : Abbreviations will be removed in the revised manuscript

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SC2- Reviewer comment *: It seems that JC-D7 and DAPI could be applied together as their spectrum is different from each other. This would also allow to see how specific both stains are.*

Author's response :

The spectra of polyP DAPI and JC-D7 overlap particularly well and the acquisition wavelengths are very close indeed (520 nm and 530 nm respectively). Are you referring to the DAPI-DNA spectrum ? If so, the metachromatic effect must be taken into account. It is therefore not possible to carry out a double labelling analysis, DAPI JC-D7. This will probably be possible in the future using deconvolution analysis by spectral cytometry, but it remains impossible to date using conventional cytometry. If you think it is necessary, we can add the fluorescence spectra of DAPI-green, DAPI-blue and JC-D7 in the supplemental material.

SC3- Reviewer comment *: « L65f: add some results please »*

Author's response : We will add the following information (in red) : The assays were performed using *Tetrasphaera elongata*, which represent a large part of the microbial biomass (up to 30–35% of the total biovolume of bacteria, Nguyen *et al.* 2011) in enhanced biological phosphate removal systems for wastewater treatment.

Reference : Nguyen et al. 2011 at https://pubmed.ncbi.nlm.nih.gov/21231938/

SC4- Reviewer comment *: « L77 : I guess it should be MgSO4 x 7 H2O? »*

Author's response : Yes, it's a typo that will be corrected in the revised version of the manuscript.

SC5- Reviewer comment *: « L901. 1500 x g does not sound much? How were the authors sure this sample did not contain particulate organic matter or non-microbial particles that could cause autofluorescence in the FACS ? »*

Author's response : The protocol we are using including the low-speed centrifugation is a classic method used by the scientific community to 'extract' cells from sediments, see for example the publication by Lavergne *et al.* (2014). Of course, there are still organic particles that can lead to aspecific fluorescent labelling in the wavelengths of polyP- DAPI labelling. This is why we use double labelling with SYTO62 to simultaneously label cellular DNA (page 12, lines 452-456).

Reference : Lavergne et al. (2014)- https://www.sciencedirect.com/science/article/pii/S0167701214001870

SC6- Reviewer comment *: « L94: remove "The" at the beginning of the sentence please »*

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

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SC7- Reviewer comment *: « L97f: please remove the '.' Between µg or mg and ml-1. It should be µm ml-1. This appears throughout the manuscript »*

Author's response : It will be corrected throughout the manuscript in the revised version.

SC8- Reviewer comment : *« L100: what was the solvent used for DAPI stocks and why were they stored at -20°C? »*

Author's response

DAPI was prepared according to the supplier's recommendations (Sigma-Aldrich). Solid DAPI (powder) was redissolved (concentration of stock solution = 1mg/ml, i.e. 2.85 mM) in ultrapure water and stored at -20°C.

SC9- Reviewer comment : « *L103 : please choose to use either concentration or molarity throughout the methods section »*

Author's response : We will use molarity throughout the revised manuscript

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SC10- Reviewer comment : *« L113: what does qsp stand for? »*
Author's response : We are sorry, it's a francization. It means « sufficient quantity for ». We will correct this in the revised manuscript by indicating that these quantities are given for 1 litre of buffer.

SC11- Reviewer comment : *« L120: µm not µM* »
Author's response : It will be corrected in the revised manuscript

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SC12- Reviewer comment : *« L125: Darmstadt»*

Author's response : It will be corrected in the revised manuscript

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SC13- Reviewer comment : *« L126: how were the cells stored at these different temperatures? This is critical information missing»*

Author's response : We don't quite understand the meaning of this question. Do you want to know whether cryoprotective agents have been added ?

SC14- Reviewer comment : *« L131: again, please remove the dot '.' between cell and s-1 »*

Author's response : It will be removed in the revised manuscript

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SC15- Reviewer comment : *« L129 and L140: is it correct that two different machines were used for the FACS analysis (counting and sorting)? »*

Author's response :That is right We only have one machine for cell sorting, the BD FACSAria™ Fusion SORP cell sorter, which is an extremely efficient cytometer for this purpose. However, the FACSAria preparation is cumbersome to set up. Cell counting was therefore performed on a BD LSR Fortessa™ X-20™, which is designed for this purpose. Both instruments have the same lasers and filters, making the analysis comparable, and internal quality control using fluorescent microbeads is used.

SC16- Reviewer comment : *« L150f: it sounds as if the cells were filtered onto a black membrane, DAPI-stained, washed and DAPI-stained again. Is this correct? If so, why were the cells stained twice? »*

Author's response : This is the protocol that was applied. Polycarbonate black membrane are typically used for DAPI staining (e.g. https://fcelter.fiu.edu/data/protocols/_assets/bacterial_enumeration_protocol.pdf). Double DAPI labelling was performed at two different concentrations to stain PolyP (10 µg. mL^{-1} final concentration) and DNA (1 µg. mL^{-1} final concentration concentration) with a wash between the 2 steps to remove excess DAPI after the first labelling.

SC17- Reviewer comment : *« L153 : Did the authors not use a mounting medium between filters and coverslips (e.g. Citifluor or Vectashield)? »*

Author's response : Citifluor or Vectashield are generally used for observations after fluorescent in situ hybridisation (FISH). Classically, for DAPI staining, non-fluorescent immersion oil is deposited on top of the filter and covered with a coverslip. This is what we did.

(for example, see : https://fcelter.fiu.edu/data/protocols/_assets/bacterial_enumeration_protocol.pdf).

SC18- Reviewer comment : *« L165 and also before: please indicate what solution was used to prepare the formaldehyde fixative. Water, 1 x PBS, or something else? Same applies for all stains (e.g. JC-D7) and solutions used. Please be more diligent in adding information »*

Author's response We will add the following information in the material and methods section:

JCD7: 10mM in DMSO stock at -20°C then dilution in HEPES buffer

DAPI: Solution in H2O 1milligram ml in water, stored at -20°C then dilution in the chosen buffer

Formaldehyde: 37% commercial solution then diluted directly in sample (stabilized with about 10% methanol, Ref Sigma 8.18708).

SC19- Reviewer comment : *« L181: it does not become clear what independent staining refers to and how that helped to establish thresholds. Please elaborate. »*

Author's response. The minimum threshold was set for FSC only. The settings for morphological (FSC and SSC) and fluorescence (DAPI, Syto, JC-D7) parameters were then set on the basis of samples unstained or independently stained by the different fluorochromes (SYTO, DAPI).

The word "threshold" in cytometry means the signal acquisition threshold ; for the rest, we defined positivity and negativity limits. Each parameter is therefore tested independently to determine where the positivity and negativity limits are for each.

SC20- Reviewer comment : *« L185f: what software was used to perform statistical analyses? »*

Author's response : We used Graph Pad Prism v8. We will add this information in the revised manuscript.

SC21- Reviewer comment : *« Figure 1: Please try to use another micrograph for Figure 1B where the cells are magnified in a similar way as in Figure 1C ».*

Author's response : In the revised manuscript, we will use a micrograph for Figure 1B where the cells are magnified in a similar way as in Figure 1C

SC22- Reviewer comment : *« L246: It does not become clear what the authors mean with "population structure". Why would the staining buffer interfere with population structure in the first place? »*

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.

SC23- Reviewer comment : *« L254: I am convinced that in the era of almost unlimited online space there is no need for "data not shown" anymore. I would like to ask the authors to show these data as well.* »

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

SC24- Reviewer comment : « *L288: why is this reference showing up in the brackets again? It is clear from the methods how the labelling was performed.* »

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

SC25- Reviewer comment : « *L290: Again, it is pretty unacceptable to not show these results. Please add them to the note or to a supplementary file so the readers can judge your conclusions. »*

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

SC26- Reviewer comment : « *L290: How did the authors decide if there was a strong or a weak fluorescent signal. This needs to be clarified in order to judge the results, e.g. the data shown in Figure 2C (comparison of HEPES and PBS).* »

Author's response : In epifluorescence microscopy, the fluorescence signal is the visual intensity. In flow cytometry it is the MFI (mean of fluorescence intensity) in arbitrary units. In the revised manuscript, we will explain that the word "strong" means positive marking with regard to the limits defined by the controls.

SC27- Reviewer comment : *« L294f and Figure 1C: The interpretation of these results is pretty unclear to me. The FCM detection of DAPI-stained PolyP signal in the strain that supposedly did accumulate low values of PolyP in HEPES buffer showed a PolyP signal for 99% of the cells. This is surprising. The authors then state that in PBS, the number of cells with a PolyP signal was around 1%. Given the size of the bar in Figure 1C, this value should be around 5-10%. Please clarify. The authors then state that under PBS and microscopy a "correct" ratio was observed which is why they excluded HEPES buffer from further experimentation. First of all, how do the authors know what the correct value is if their "control" strain accumulated PolyP as well? Also, HEPES buffer without any P should in principle be more suited for PolyP detection than PBS, which introduces P to the cells. In addition, why does the microscopy data and the FCM data not agree more with each other? Microscopy should be the control here so this means that FCM highly overestimates the PolyP-detection? »*

Author's response : There is a yet unidentified phenomenon that leads to artefactual labelling of RX cells in HEPES buffer in flow cytometry. 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.

We will indicate this in the revised manuscript, and show the data for *T. elongata*.

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.

SC28- Reviewer comment : *« Also I would like to ask the authors to also show the data of Figure 2C with TE cells. What are the proportions here? How did HEPES buffer fare in comparison to PBS? »*

Author's response : As indicated in the responses to the general comments, we will provide all the necessary data (which will be deposited in FigShare) and graphics in supplementary data of the revised manuscript. The fluorescence shift of cells labelled with DAPI in HEPES compared to those labelled in PBS buffer was not observed for *T. elongata* cells. See also the response to your comment SC27.

SC29- Reviewer comment : *« L304: The data referred to here is not shown »*

Author's response : The data will be provided as a supplemental figure in the revised manuscript

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SC30- Reviewer comment *:* « *Figure 3B shows the proportion of PolyP detected in cells RX (low-accumulators) for different fixation treatments (8-14%). These proportions are similar to the values shown in Figure 2C where they were referred to as 1%. Again, the data does not really add up and disagrees with the microscopy data shown in Figure 2C. In addition, I want to see the proportion of TE cells according to these tests as they were also counted (see Figure 3A). Why were they not shown in the first place? »*

Author's response : Figure 3C shows the % of polyP+ cells in the RX population after fixation with 2% and 4% formaldehyde. These values are compared with the proportions obtained on 'fresh' samples analysed without fixation. As we carried out the experiments using the same RX culture, it is normal that the data are similar between Figure 2c and 3C for the "fresh" sample.

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.
Concerning the TE strain, the data will be provided as a supplemental figure in the revised manuscript

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SC31- Reviewer comment *:* « *L332: I am highly surprised to see that the storage at -80°C did not affect cell morphology, and thus detectability of PolyP. But again the authors chose to not show these data.* »

Author's response : As indicated in the « responses to general comments" section, we will provide the data (which will be deposited in FigShare) and graphics in supplementary data of the revised manuscript.

SC32- Reviewer comment *:* « *L334: How did the authors prepare the 50/50 mixture relative abundance for these tests? »*

Author's response : We counted the number of RX and TE cells from each culture using flow cytometry. Using the cell densities obtained for each, we mixed them in a 50 : 50 ratio in abundance. This information will be added in the material and methods section of the revised manuscript.

SC33- Reviewer comment : *« L383f: The overestimation was already visible in Figures 2 and 3 which indicates that FCM as established in this study is not very useful for the quantitative detection of PolyP accumulating strains »*

Author's response : See response to comment SC30

SC34- Reviewer comment : *« L424: I strongly disagree with the conclusion that the "present study established the basis of a robust protocol for the detection and enrichment of PAB by flow cytometry". The current study, in my opinion, fails to establish the FACS analysis in a comparable manner to fluorescence microscopy which serves as the benchmark. »*

Author's response : See response given at the beginning of these "Responses to reviewer 1"
