RESPONSES TO REVIEWERS

We thank the reviewers for their careful work. Their comments were constructive and useful, allowing us to incorporate their suggestions into the revised version of the manuscript.

Responses to Reviewer 1 general comments

In his/her general comments, reviewer 1 asked us to :

- **(1)** Provide the taxonomy of the RX strain
- **(2)** Test polyP staining on strains other than RX and *T. elongata*
- **(3)** Extend the polyP labelling tests with JC-D7.

In the revised manuscript, we have addressed all these requests by :

- **(1)** Provide the taxonomy of the RX strain that belongs to the genus *Pseudomonas* (best Blastn match with *Pseudomonas trivialis*) (lines 106-107 of the revised manuscript).

- **(2)** Test of polyP staining with DAPI and JC-D7 on Gram-negative strains belonging to *Acinetobacter lwoffii, Flavobacterium sp., Pseudomonas sp., Stenotrophomonas rhizophila* and the Gram-positive strain *Microbacterium hydrocarbonoxydans* DSM 16089 (lines 109-114 and Figure 6 of the revised manuscript, Table S.24 of the supplemental material).

- **(3)** Extension of polyP-JC-D7 labelling assays to include Gram-negative strains belonging to *Acinetobacter lwoffii, Flavobacterium sp., Pseudomonas sp., Stenotrophomonas rhizophila* and the Gram-positive strain *Microbacterium hydrocarbonoxydans* DSM 16089 (lines 109-114 and Figure 6 of the revised manuscript, Table S.24 of the supplemental material) but also to include six soil samples (lines 129 to 134, Figure 7 of the revised manuscript ; Table S.25 of the supplementary material). In addition, since the publication of our preprint in Biogeosciences Discussions on 2 May 2024, two articles have been published reporting the use of JC-D7 for the quantification of polyP in microbial samples and also showing that JC-D7 is very promising and specific. We have included this information in the discussion of the revised version of the manuscript (lines 666 to 679 of the revised manuscript).

Responses to Reviewer 1 specific questions

Question 1 : *« Why was JC-D7 diluted in HEPES buffer when itw as shown to cause problems with strain RX » ?*

Response: PolyP labelling with JC-D7 was performed in HEPES buffer as recommended by Angelova *et al.* 2014. We tried to perform polyP-JC-D7 labelling in PBS buffer, but the latter is not optimal for this fluorochrome (lines 457 to 459 of the revised manuscript and Supplementary Figure S.4). In addition, no artefact signals were observed for JC-D7-HEPES labelling for different Gram-negative and Gram-postive strains (Figure 6 of the revised manuscript).

Question 2 : *« L204: I still do not understand why there was the need to stain cells twice. First cells were stained with a high concentration (different conc in the manuscript as given in the response to the reviewers), washed and then stained with a low concentration. The authors argue that the second staining was done to stain the DNA of the cells but that was already achieved during the high concentration staining in my opinion. DAPI does not bind specific to a target based on the concentration. It does not create a problem for the analysis, but this could be unnecessary and introduce bias. »*

Response : You are correct, double labelling with DAPI was performed at two different concentrations to stain PolyP (10 µg. mL-1 final concentration) and DNA (1 µg. mL-1 final concentration) with a wash between the two

steps to remove excess DAPI after the first labelling. However, we acknowledge that a single label would have been sufficient.

Question 3 : « *Figure 2: where could the artifact in the McIlvaine buffer come from? The buffer only contains NaH2PO4, citric acid and water. Would it be possible to show the exact same panels for Tris-EDTA for PBS, HEPES, McIlvaine in the supplements? This way one could judge better »*

Response : We do not know the origin of the McIlvaine/DAPI artefact reported in the manuscript (lines 558 to 559 of the revised manuscript). The panels for trisEDTA, PBS and HEPES would not be of interest since no events are detected on the cytograms when these buffers are analysed, without cells, with DAPI or with DAPI/SYTO®62.

Question 4 : *« Figure 3 and other data show that there cannot be a unified protocol for all bacteria when there is such a high difference between a high accumulator and low accumulator strain ? »*

Response : In microbial ecology, we must accept compromises. Microbial communities in natural environments are heterogeneous in terms of physiology, membrane structure, and so on, so it is not possible for one protocol to be optimal for all.

With regard to Figure 3 in particular, the effect of fixation on cytometric analysis of bacteria is well known in ecology (e.g. Kamiya et al., 2007; Troussellier et al., 1995) and a difference in the effect of the fixative between Gram-positive and Gram-negative bacteria has already been observed (Liu et al. 2012). We add this information in the revised manuscript (lines 570 to 572 of the revised manuscript).

Reference : Kamiya, E., Izumiyama, S., Nishimura, M. et al. Effects of fixation and storage on flow cytometric analysis of marine bacteria. J Oceanogr 63, 101–112 (2007).
Troussellier, M., Courties, C. & Vaquer, A. Recent applications of flow cytometry in aquatic microbial ecology. Biology of the Cell 78, 111–121 (1993).
Liu BY, Zhang GM, Li XL, Chen H. Effect of glutaraldehyde fixation on bacterial cells observed by atomic force microscopy. Scanning. 34,6-11 (2012).

Question 5 : « *Figure 4: It is shown in Figures 3 and 5 that almost 100% of the T. Elongata cells are repeatedly counted/detected via FCM and epifluorescence microscopy. For this strain a good congruence between FCM and microscopy extist. After mixing the strains T.elongata and RX in equal cell number (50:50 abundance ratio) both methods only detect 36.5 or 12.6 % of PolyP+ cells when it should be approximately 50%. How do the authors explain this discrepancy? Either the cells were not mixed in equal abundance, or the counting does not work anymore as soon as a second bacterial strain is in the mixture which makes the approach not robust enough for any type of mixed communities*. »

Response : In some of our experiments, *T. elongata* had almost 100% polyP+ cells. However, variation in the polyP content of microorganisms is a common feature of PAB (e.g. 93.7 ± 1.5 % and 17.5 ± 2.4 % of polyp+ cells in the culture *of T. elongata*; Fig. 5 and Fig. 6 of the revised manuscript), and in the experiment you mentioned, *T. elongata* did not have less than 100 % polyP+ cells ; therefore a ratio < 50 % was normal. We have indicated in the revised version of the manuscript that the proportion of polyp+ can vary for the same strain (lines 651-653 of the revised manuscript).

Question 6 : *« Fig. 4: Why was only one sample counted with FCM? This makes it very difficult to judge the result, especially if the goal is to show that FCM is a robust way to count cells after sorting. »*

Response : We sorted one sample and then counted it in the FCM. It did not seem appropriate to do this three times.

Question 7 : *« Fig5: Here JC-D7 looks promising in the sediment, that would actually be something to extend on »*

Response : See the response to the general comments

Responses to Reviewer 2 General Comments

Comment : *« L61: Change to: (< µm) »*

Response : This point has been changed in the revised manuscript (line 66 of the revised manuscript).

Comments : L86: "*carried out out tests on microbial cells extracted from water and lake sediment samples*" and

L90: « *It also points out that..* »

Response : These points have been changed in the revised manuscript (lines 95-99 of the revised manuscript).

Comment : *« L100: There is no natural negative control. However, it is possible to modify species to get a true negative control by knocking out the ppk gene »*

Response : The term « negative control » has been removed in the revised manuscript.

Comment : « *L345 and Figure 2C, L 344-347, L463-465: While I agree with the authors that the HEPES buffer should not be used to analyze DAPI-stained cells with FCM, I would also argue that PBS is not great, at least for the low polyP accumulators and caution should be applied. I do not think that the proportion is in "a simlar range" (L344-L347) (7.2% is very different from 0.9% and accounts for a significant fraction of false positives) and the significant difference observed already shows that this method is problematic for low poly-P accumulating organisms. I would state that the PBS buffer is a better option that the HEPES buffer and therefore, one should not use HEPES but using PBS buffer is already a compromise and also results in artefactual labeling and false positives. »*

Response : This point has been changed in the revised manuscript according to your suggestion : « However, we acknowledge that PBS buffer is already a compromise and also results in artefact labelling and false positives for the 'low polyP accumulation' control strain." (lines 384-385 and lines 565-566 of the revised manuscript).

Comment : « *Figure 3A and 3C: I think it is good that the authors tested the different conditions of fixation, storage time and storage temperature for the RX strain. It is clear that this affects the propotion of polyP cells measured but it is very hard to give any advice on sample preparation if there is no "ground truth" present, i.e, if we do not know the amount of actual poly-P+ cells present (i.e., measured by epifluorescence microscopy). Is it in a similar range as the amount measured in Figure 1C (~0.9% poly-P+ cells)? If so, why does the proportion of polyP+ differ so much from the actual amount? And why does the proportion significantly decrease after fixation with PFA? »*

Response : In some of our experiments, *T. elongata* had almost 100% polyP+ cells. However, variation in the polyP content of microorganisms is a common feature of PAB (e.g. 93.7 ± 1.5 % and 17.5 ± 2.4 % of polyp+ cells in *T. elongata* culture ; Fig. 5 and Fig. 6 of the revised manuscript), and in the experiment you mentioned, *T. elongata* did not have less than 100 % polyP+ cells, therefore a ratio < 50 % was normal. We have indicated in the revised version of the manuscript that the proportion of polyP+ can vary for the same strain (lines 651-653 of the revised manuscript).

The effect of fixation on cytometric analysis of bacteria is well known in ecology (e.g. Kamiya et al., 2007; Troussellier et al., 1995) and a difference in the effect of the fixative between Gram-positive and Gram-negative bacteria has already been observed (Liu et al. 2012). We add this information in the revised manuscript (lines 570 to 572 of the revised manuscript).

Reference : Kamiya, E., Izumiyama, S., Nishimura, M. et al. Effects of fixation and storage on flow cytometric analysis of marine bacteria. J Oceanogr 63, 101–112 (2007).
Troussellier, M., Courties, C. & Vaquer, A. Recent applications of flow cytometry in aquatic microbial ecology. Biology of the Cell 78, 111–121 (1993).
Liu BY, Zhang GM, Li XL, Chen H. Effect of glutaraldehyde fixation on bacterial cells observed by atomic force microscopy. Scanning. 34,6-11 (2012).

Comment : « *Furthermore, RX is a gram-negative strain and it is unclear whether the differences between the treatments are the result of the low polyP accumulation or from the difference in the membrane structure. Overall, it is not possible to draw conclusions about sample preservation on gram negative strains or low polyP accumulators from the data presented here since the testing was done on a low polyP gram-negative strain. To untangle the results, one would have to test the same protocols on a gram-negative strain that accumulates high amounts of poly-P and a gram-positive strain that accumulates very little poly-P. »*

Response :In the revised manuscript, we have tested polyP staining with DAPI and JC-D7 on the Gram-negative strains belonging to *Acinetobacter lwoffii, Flavobacterium sp., Pseudomonas sp., Stenotrophomonas rhizophila* and the Gram-positive strain *Microbacterium hydrocarbonoxydans* DSM 16089 (lines 109-114 and Figure 6 of the revised manuscript, Table S.24 of the supplemental material).

Comment : « *Line 372: There is no figure 3D, it is only figure 3C. »*

Response : This is corrected in the revised manuscript.

Comment : « *L475-480: I agree with what the authors state here but specify that this only works for gram-positive high polyP+ accumulating organisms, it does not work for gram-negative low polyP accumulators and that further testing is needed for gram-positive low polyP accumulators and gram-negative high polyP accumulators. »*

Response :We do not understand the question because we have indicated in the manuscript that fixation induces a bias for the Gram-negative strain, but that this is not enhanced by storage at 4°C (lines 569-572 of the revised manuscript).

Comments : « *L475-480: I agree with what the authors state here but specify that this only works for gram-positive high polyP+ accumulating organisms, it does not work for gram-negative low polyP accumulators and that further testing is needed for gram-positive low polyP accumulators and gram-negative high polyP accumulators. » and « L499-501: This would only work if the polyP accumulators accumulate high amounts of polyP »*

Response :We added the following statement in the revised manuscript : « *Despite the substantial variation and subjectivity associated with microscopy and flow cytometry, both methods revealed, with appropriate labelling (e.g. appropriate staining buffer), fairly congruent proportions of polyP+ cells for high polyP accumulators in homogeneous samples, i.e. strain culture samples.* » (lines 611-613 of the revised manuscript) and « *In light of our data, we acknowledge that this approach will be more effective in the enrichment and isolation of PAB that accumulate large amounts of polyP*. » (lines 624-626 of the revised manuscript).

Comment : « *L510-515: It disagree with the statement that it shows the proportions of polyP+ cells in a congruent manner. To me, it did not show this in a congruent manner for flow cytometry in the RX strain. The differences are significant and a lot of false positives would be measured overestimating the amount of polyP+ cells present. More research would be needed to establish this. For now, the only application is 'simple' matrices in the case of gram-positive high polyP accumulators. »*

Response : We have modified this statement in the revised manuscript to read as follows: *« Despite the substantial variation and subjectivity associated with microscopy and flow cytometry, both methods revealed, with appropriate labelling (e.g. appropriate staining buffer), showed the proportions of polyP+ cells to be fairly congruent in the case of high polyP accumulators in homogeneous samples, i.e. strain culture samples. The method combining FCM and DAPI labelling of polyP can therefore be applied to such 'simple' matrices, for example to screen a heterogeneous pool of cells or a single strain for gene-phenotype linkage analyses. The use of flow cytometry combined with DAPI labelling of polyP to count PAB in complex matrices such as sediment or freshwater samples can be used for comparative purposes, but cannot be recommended as a sole method due to high risk of overestimation. »* (lines 611-618 of the revised manuscript).

Comment : L516: *« remove the comma and the however, that way the sentence is much clearer. »*

Response : This is corrected in the revised manuscript.

Comment : « *L519: remove the very »*

Response : This is corrected in the revised manuscript.

Comment : *L520-521: I would specify that you can effectively enrich and isolate PAB that accumulate large amount of polyP, as this method would not work for low polyP accumulators.*

Response : This is indicated in the revised manuscript: "*In light of our data, we acknowledge that this approach will be more effective in the enrichment and isolation of PAB that accumulate large amounts of polyP* ». (lines 624-626 of the revised manuscript)