Can a toluene-degrading bacteria improve the growth of a bacterial community in case of toluene pollution?

Léonard Jequier, Olivier Bützberger, Evan Bexkens and Victor Amstutz

***Abstract*— In the case of an environmental pollution, the microbial community of the soil will have an important role in the degradation of the pollutant. However, adaptation to the sudden change in the soil composition is difficult for most bacterial communities. Bioaugmentation is a method that could be used to help them overcome the effect of pollution. It is the addition of bacteria able to degrade the pollutant into the contaminated environment. In our experiment, we chose to add *Pseudomonas veronii* 1YdBTEX2 and *Pseudomonas putida F1* (both tagged with fluorescent mCherry protein) to a bacterial community extracted from sand and to grow them either with toluene only or with mixed carbon[1] substrate. As the two *Pseudomonas* strains we added are able to degrade toluene, we wanted to know if the addition of one of these strain to this bacterial community would have an effect on the growth of this community. Our results are encouraging and would deserve some further experimentations. However, unlike usual bioaugmentation experiments in literature, our results indicate that the Sand Community was almost entirely replaced by *Pseudomonas veronii* or *Pseudomonas putida*.**

I. Introduction

Every surface, soil or liquid in our environment is colonized by multiple microorganisms, interacting with each other and forming communities. However, the whole complexity of the interactions is still today not always fully understood. In addition, these communities also interact with other organisms and can be perturbed by the effects of pollution such as spillage of oil or solvent. We decided to use toluene as a pollutant for this experiment.

Toluene is an organic solvent widely used in industry nowadays, as a paint solvent or as extraction solvent in the cosmetic industry. Toluene is a monoaromatic volatile molecule which is toxic and ecotoxic [1]. When such event happens, one way to treat it is the use of bioremediation. There are several different techniques of bioremediation but they all have the same purpose: to use live organisms to get rid of pollutants. The technique that interested us in this experiment was bioaugmentation, the addition of a microorganism able to degrade the targeted pollutant to a native community, allowing it to grow back [2].

The microorganisms we chose for our experiment were *Pseudomonas putida* F1 (PP) and *Pseudomonas veronii* 1YdBTEX2 (PV), both able to metabolize toluene [3] [4]. The bacterial community on which we tested this procedure was extracted from sand (sand community (SC) collected at St-Sulpice beach, Switzerland).

To measure the number of cells in our culture we used flow cytometry[2]. This technique consists of passing cells one by one through a capillary and hit each cell with a laser. It also allows us to differentiate cells by their fluorescent markers.

Our hypothesis for this experiment were that the addition of PP or PV will have an effect on SC growth, especially in a toluene-only media, because we think that these strains might produce metabolites the SC could use as carbon source or reduce the toluene concentration and thus its toxic effect. This would lead to a better growth of SC in presence of one of either PP or PV.

II. Material and method

A. Experimental design

Apart from SC, we used two bacterial strains : *Pseudomonas veronii* 1YdBTEX2 (tagged with mCherry) and *Pseudomonas putida F1*(also tagged with mCherry).

However, keep in mind that SC isn't a single species of bacteria, but rather a community containing potentially many different species. We experimented with two media: a mixed carbon medium[1] and a Minimal Media 1x (MM) with only toluene as carbon source.

We separated the work into two weeks, testing SC and PP during Week 1 and SC and PV during Week 2. In each experiment, we tested the 2 communities and their interaction in mixed carbon and toluene.

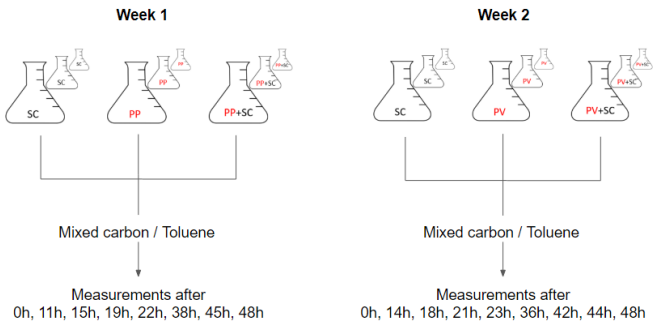


Fig. 1: Experimental design. Experiments were separated into 2 weeks, with triplicates for each bacterial culture. The number of cells was measured at multiple timepoints.

As controls, we used SC alone in both media, to prove that the medium has an effect and compare with the interaction of species. PP(or PV) alone in both media are used to show that the medium has no effect. We therefore had 6 unique treatments, and used 3 replicates

[1]Mixed Carbon media composition : L-Arginine, D-Xylose, Aspartic acid potassium salt, 2-Hydroxybenzoic acid, L-Phenylalanine, L-Serine, Gamma Hydroxy butyric acid, D-cellobiose, alpha-D-Lactose, Putrescine, Itaconic acid, alpha-D-Glucose-1-phosphate, D-Glucosaminic acid, N-acetyl-D-glucosamine, 4-Hydroxy benzoic acid, D-Mannitol, meso-erytritol, Galacturonic acid, Methyl pyruvate, Tween 20. Each component in equal concentration

[2]Flow cytometer, Novocyte : https://www.aceabio.com/products/novocyte-flow-cytometer/, Nov. 2017

for each. We ended up with 18 cultures per experiment. These cultures were then sampled and counted regularly with a flow cytometer at different timepoints. The timespan between two timepoints had to vary to fit our personal schedules.

B. Extraction of Sand Community (SC)

The sand came from St-Sulpice. We took 200g of it and mixed it with 400 mL of 1x Minimal Media (MM) in a flask. We shaked it at 25°C, 115rpm for one hour, the time needed to extract the cells from the sand. The supernatant was collected in 50 mL falcon tubes, then centrifuged at 800rpm for 10 minutes. All the supernatant was then sieved through a 40 µm filter, and centrifuged at 4'000rpm for 30 minutes. The supernatant was discarded and the pellets re-suspended in 5 mL 1x MM. Then centrifuged at 800rpm for 10 minutes. We collected the supernatant and repeated this step until there was no visible pellet.

C. Pseudomonas veronii and Pseudomonas putida

The laboratory's samples were kept at -80°C, taken out at room temperature and streaked on LB plates with antibiotics (Gentamicin GM10). Incubated at 30°C, then one colony was streaked on MM 1x plates without added carbon. All the plates were put in a closed chamber containing 200 µL 100% toluene. The plates were incubated at 30°C. Then took one colony, put it in liquid MM with 5 mM succinate, and incubated it at 30°C.

D. Inoculation

We put 20 mL of mixed carbon media [0.1mM] in nine flasks, and 20 mL of MM 1x in the other nine, where we added 200 µL toluene 50% (100 µL toluene and 100 µL 2,2,4,4,6,8,8-Heptamethylnonane (HMN)[3]) in a tip sealed at the bottom. Toluene being volatile, it will then evaporate and dissolve in the liquid media. It will be the only source of carbon. We quantified the cells using flow cytometry, and calculated to put 10^6 cells/mL in PV/PP alone flasks, SC flasks, and added twice 10^6 cells in PPSC and PVSC flasks. The flasks containing toluene were sealed to avoid toluene leaks (Fig. 15). They were incubated at 25°C and constantly shaken at 110rpm during 48 hours.

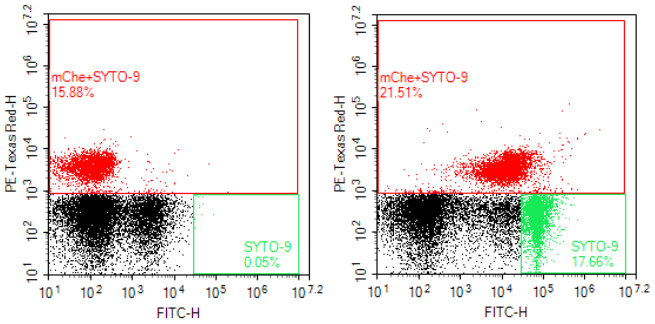
E. Sampling and count of cells

Every few hours (see Fig. 1 for precise times), we took 3 samples from each flask to make technical replicates, and stained them with 4 µL SYTO-9 (50 µM), a green fluorescent nucleic acid stain[4]. We then added 200 µL of each sample to the wells of a 96-well plate.

To measure the growth of our cells we used a flow cytometer. Since *Pseudomonas putida F1* and *Pseudomonas veronii* 1YdBTEX2 were tagged with mCherry (a red fluorescent protein encoded by mCherry gene, inserted downstream of the constitutive promoter of PP strain),

[3]Oil used with volatile solvent to decrease their toxicity by slowing its release in the medium. Ref. https://www.sigmaaldrich.com/catalog/product/aldrich/128511?lang=fr&region=CH, Dec. 2017

[4]ref. https://www.thermofisher.com/order/catalog/product/S34854, Nov. 2017



(a) *(b)*

Fig. 2a: Data of unstained sample (unstained PPSC in toluene, replicate 1, timepoint 8).
Fig. 2b: Data of stained sample (stained PPSC in toluene, replicate 1, timepoint 8). Horizontal axis : intensity of red fluorescence. Vertical axis : intensity of green fluorescence.

and all the cells (both Gram positive and Gram negative cells) were stained with SYTO-9, we could differentiate PP/PV from SC.

The Fig. 2a and 2b are scatterplots of unstained (Fig. 2a) and stained (Fig. 2b) samples. The vertical axis shows the intensity of green fluorescence, while the horizontal axis shows the intensity of red fluorescence.

On Fig. 2a a big black cluster and a red one above it can be observed. The black cluster is composed of SC cells and remaining particles. They show low red and green fluorescence values. The PP cells express mCherry and therefore show strong red fluorescence, which causes an upwards shift on the plot, forming the red cluster above.

On Fig. 2b is a scatterplot of a sample from the same culture at the same timepoint but stained with SYTO-9. The SYTO-9 binds DNA and stains all the cells (PP alike) with a green fluorescence, which causes them to shift to the right side. However, the red fluorescence value still allows for a distinction between PP/PV and SC. The various residual particles (black cluster) are not affected by the SYTO-9.

From the unstained sample, we were able to determine gates (the red and green frames on the scatterplots) to differentiate what we will consider as SC, PP/PV or simply residual sand particles. These gates could then be applied to each sample.

Also, we had to dilute our samples when the concentration of cell became too high. Otherwise we risked to clog the capillaries. In the first week, we diluted our sample 10 fold at timepoint 1 and 100 fold from timepoint 2. In the second week, we diluted our sample 10 fold at timepoints 1 to 4 and 100 fold from timepoint 5. We then adjusted the results to the original concentration during the analysis.

F. Statistical method

Finally, our data was composed of red and green fluorescent cell counts for each treatment, at 4 timepoints every day for two days. We used the R environment for all the analysis [5].
We represented graphically the logarithm of these counts over time for each replica and calculated the area under the curve. It allowed to summarize the data to one

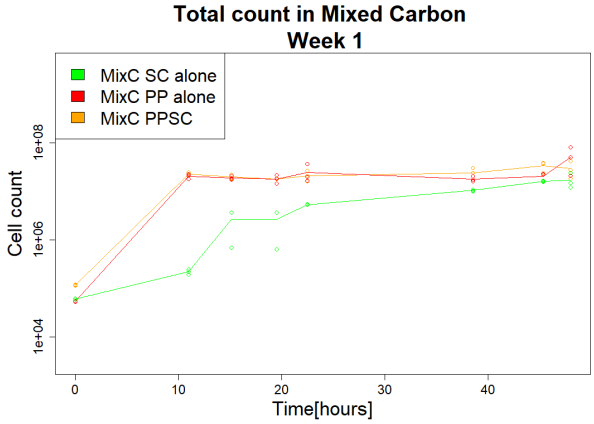


Fig. 3: Total count (SYTO-9 Count + mCherry count) of SC, PP and PPSC over time in a mixed carbon medium during the first week.

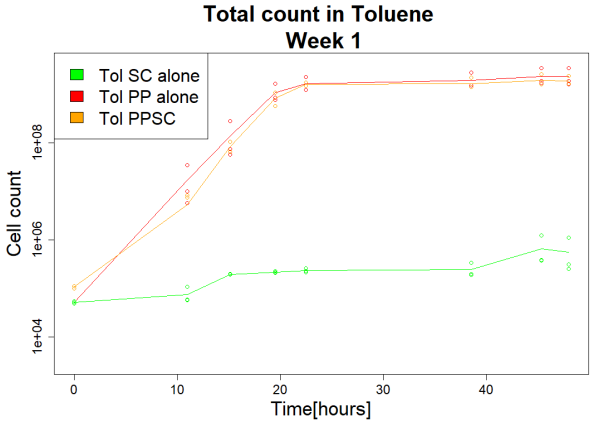


Fig. 4: Total count (SYTO-9 count + mCherry count) of SC, PP and PPSC over time in a toluene only medium during the first week.

value per replica of each treatment (Fig. 5, 6, 9, 10). We performed four two-way crossed Anova to test the difference in growth of each Species separately between the substrate. For example, to test the difference in growth of SC in the first week, we used:
Explicative variables:

- Species in culture: SC or SC + PP
- Media: Toluene or mixed carbon

Response variable:

- Log of the area under the curve

III. Results

A. Week 1 – SC, PP, PPSC

1) Total cell count comparison: First, we are interested in the total cell count of each treatment. We look at the sum of both fluorescence counts. In mixed carbon (Fig. 3), PP and PPSC curves look very similar. SC seems to grow slower but catch up with the other culture after 40 hours. In toluene (Fig. 4), PP and PPSC look again very similar. This time however, we don't observe any growth in the SC culture. This experiment shows no signs that growing PP and SC together influences the growth of the overall population or the total cell count after 48 hours, in a mixed carbon or toluene media (compared to the PP alone control). Since the species are marked with different fluorescence, we can also compare the growth of each population separately in each treatment.

2) Sand Community: The growth of the sand community in each treatment for the first week is represented in Fig. 5 and 16c. There is an important interaction between the explicative variables (F = 26.62, df = 1, $p = 8.64 \cdot 10^{-4}$). In mixed carbon, SC grows similarly in presence or absence of PP (black and blue points). In toluene however, the presence of PP allows for a 1000-fold difference on average (red and yellow points). We conclude that the presence of PP affects SC differently depending on the media. In toluene, it allows for a better growth but has no effect in the mix carbon media. In addition, the effect of the media on SC is important in

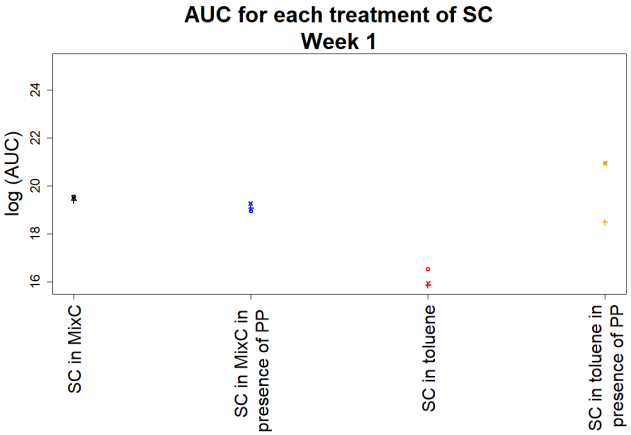


Fig. 5: Week 1 - SC : Each point represents one of the three replicates's AUC for each treatment.

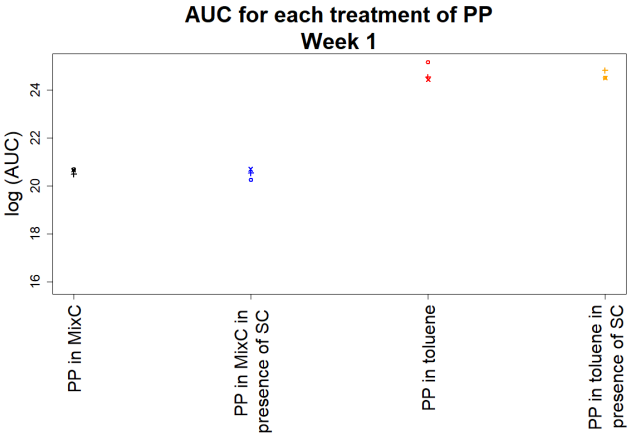


Fig. 6: Week 1 - PP : Each point represents one of the three replicates's AUC for each treatment.

absence of PP. SC alone grows less in toluene than in mixed carbon.

3) Pseudomonas putida: There is an important difference between the media (F =907.30, df = 1, $p = 2.36 \cdot 10^{-6}$). It is clearly shown on the Fig. 6 and 16d (red and yellow curves/points vs black and blue curves/points)). In contrast, our data shows no significant difference due

to the presence or absence of the sand community and no interaction. We conclude that SC has no effect on the growth of PP and that PP grows better in the Toluene medium than in the mix carbon medium. It could be due to the greater amount of carbon in the toluene media.

B. Week 2 – SC, PV, PVSC

1) Total cell count comparison: The total cell count over time is represented on Fig. 7 and 8, the conclusion is the same as in the first week. It shows no signs that growing PV and SC together influences the growth of the overall population nor the total cell count after 48 hours, in a mixed carbon or toluene media.

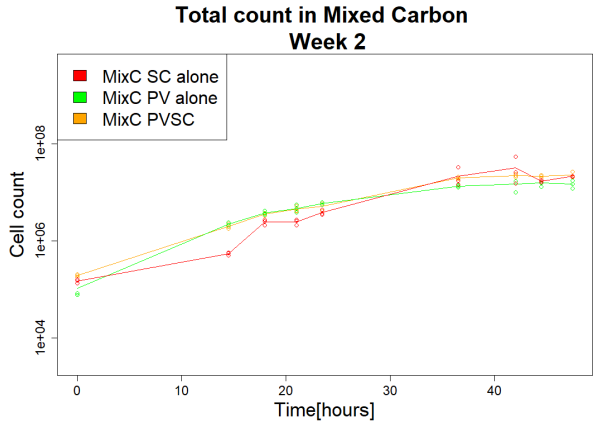


Fig. 7: Total count (SYTO-9 Count + mCherry count) of SC, PV and PVSC over time in a mixed carbon medium during the second week.

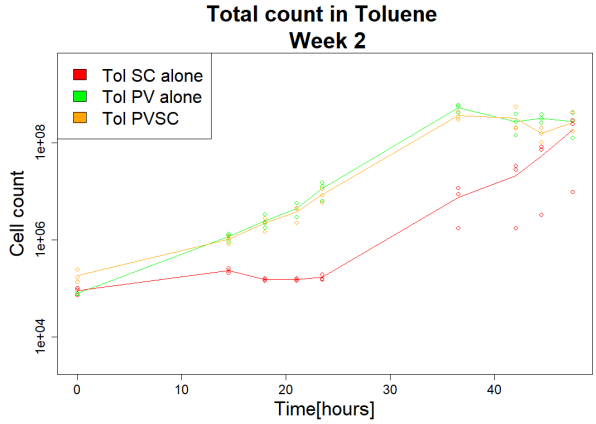


Fig. 8: Total count (SYTO-9 count + mCherry count) of SC, PV and PVSC over time in a toluene only medium during the second week.

2) Sand Community: The growth of the sand community in each treatment for the second week is represented in Fig. 9 and 16g. In contrast with the first week, SC was able to grow in toluene (red curves/points). It is not surprising, as a community extracted from sand is typically composed of many kinds of bacteria. It is likely that at least one of them can degrade a given carbon source. But it shows that SC behaved differently from one week to another and thus cannot be considered identical. Therefore, we won't be able to compare the results from the two weeks. The Anova on the area under the curve shows no significant effect of any explicative variables, but we believe the results could be different if we continued the culture for a day because we can see on the growth graph that SC alone is still in exponential growth at 48 hours in toluene. It is plausible that with a few more timepoints, SC would grow better alone than in presence of PV.

3) Pseudomonas veronii: The growth of PV in each treatment is represented in Fig. 10 and 16h. As in the first week, there is an important difference of growth between the media (F = 784.71, df = 1, $p = 2.85 \cdot 10^{-9}$). PP grew about a hundred-fold better in toluene (red and orange) than in mixed carbon (black and blue curves/points). One can also notice that PP grew slightly less in presence of SC, in both media (blue and yellow compared to black and red curves/points). The difference in area under the curve is significant (F = 5.56, df = 1, $p = 0.046$).While not being as significant as our other results, it could be an indication some competition between them in this type of culture. It seems plausible, knowing from previous experiments that PV grows a bit slower than PP and in this experiment some bacteria from the sand community were able to grow on toluene.

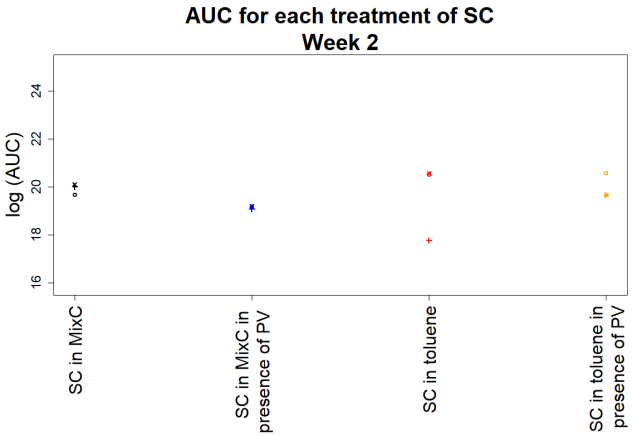


Fig. 9: Week 2 - SC : Each point represents one of the three replicates's AUC for each treatment.

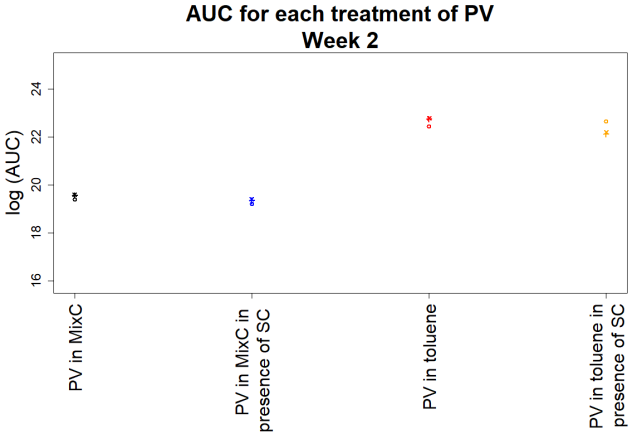


Fig. 10: Week 2 - PV : Each point represents one of the three replicates's AUC for each treatment.

IV. Discussion

A. mCherry issue

Further analysis was carried out to ensure the reliability of our promising results. It brought a slight drawback to light. Some cells were detected in the SYTO-9 only gate in PP alone and PV alone cultures in both media (Fig. 13). Fig. 13a is a representation of PP alone in toluene, and Fig. 13b of PPSC in toluene, both at the same timepoint. These graphs are almost identical, and more importantly, both show an important count in the SYTO-9 only gate (Fig. 11, Fig. 12). We also observe this phenomenon with PV alone. This is quite surprising, knowing that both PP and PV were tagged with mCherry. This protein emits a red fluorescence and should cause an upwards shift in the fluorescence graph (as explained in Fig. 2a and 2b) and bring these cells in the mChe + SYTO-9 gate.

Our hypothesis is that some PP/PV cells died, but weren't lysed. Their DNA is still nicely protected in the cell and can be stained by SYTO-9. However, their metabolic activities stopped, reducing mCherry fluorescence. Without the red tag, these cells would shift downwards and into the SYTO-9 only gate. It renders differentiation between PP (or PV) and SC seemingly impossible because they both appear in the same gate. A multivariate analysis using PCA (Principal Component Analysis) and K-means clustering was performed to try to differentiate the two controls (PP alone, SC alone) with other factors (forward-scatter and side-scatter for example), but to no avail.

This issue makes us question whether the significant increase in AUC in the SYTO-9 only in PPSC compared to SC alone is due to an increase of the number of SC or to the presence of PP cells presenting low red fluorescence values.

To circumvent this problem, another tagging method unaffected by cell death could be used (Fig. 14). For example, a highly specific antibody to *Pseudomonas putida* could be engineered. A secondary antibody, this time linked to a fluorescent protein would bind the first one and therefore mark all *Pseudomonas putida* cells. This would allow a better differentiation of our communities.

B. Bacterial interactions

To summarise, growing PP or PV with SC shows no effect on the total cell count compared to growing PP / PV alone.

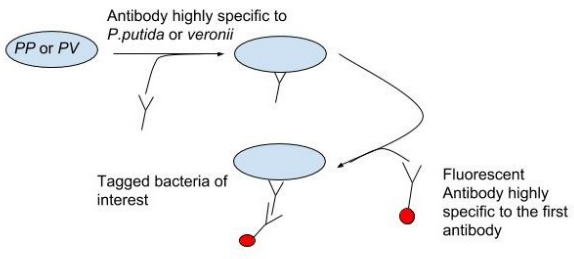


Fig. 14: Fluorescent antibody tagging, as an alternative to mCherry in this experiment

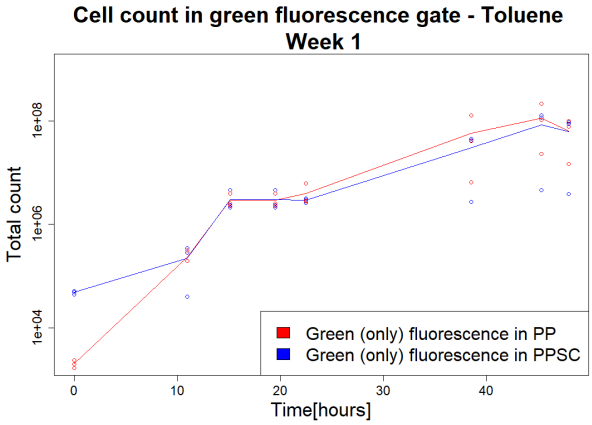


Fig. 11: Total count of green stained cells in toluene for PP alone and PP with SC during week 1. It showed no significant differences. Each point is a replicate, and each line is the mean of the corresponding replicates.

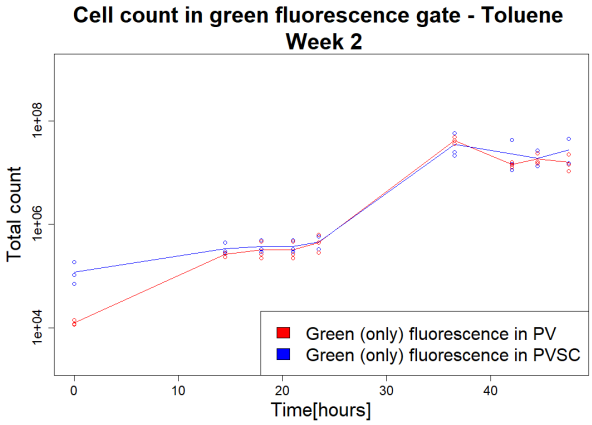
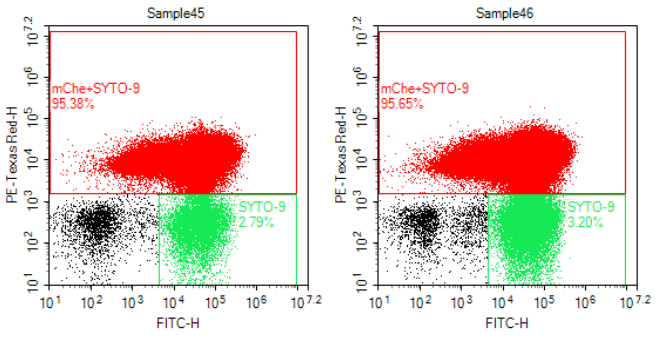


Fig. 12: Total count of green stained cells in toluene for PV alone and PV with SC during week 2. It showed no significant differences. Each point is a replicate, and each line is the mean of the corresponding replicates.



(a) *(b)*

Fig. 13: Scatterplot of a stained PP sample(Fig. 13a) besides a stained PPSC sample (Fig. 13b). They look very similar. A lot of PP seem to have lost mCherry (red) fluorescence and have shifted downwards (Fig. 13a) and look like the supposed SC cells in Fig. 13b.

If we assume this tagging issue is negligible, we can infer on the growth of each population separately within each treatment. In the first week, the data indicates that the presence of PP affects SC differently depending on the media (Interaction: F = 26.62, df = 1, $p = 8,64 \cdot 10^{-4}$). In toluene, it allows a better growth but has no effect in the mix carbon media. There is an important effect of the media in absence of PP: SC alone grows significantly less in toluene than in mixed carbon. Furthermore, PP shows an important difference in AUC between the media (F =907.30, df = 1, $p = 2.36 \cdot 10^{-6}$) and no difference due to the presence of SC.

During the second week, SC behaved differently: it was able to grow in the toluene media, even alone. No significant effect of the media nor of the addition of PV were highlighted by the Anova. Similarly to PP, PV had a better growth in toluene than in mixed carbon (F = 784.71, df = 1, $p = 2.85 \cdot 10^{-9}$). This time however, there was a significant reduction of the number of PV in presence of SC in toluene (F =5.56, df = 1, $p = 0,046$). While not being as significant as our other results, it seems to indicate some competition between them in this type of culture.

It might also be important to notice that even if the AUC of the SYTO-9 gate in PPSC is significantly higher than in SC alone, the proportion of SC in PPSC is very low (about 5%).

To study more deeply these interaction, it might be interesting to change the initial concentration of PP and PV. Indeed we used 10^6 cells of SC and 10^6 cells of PP or PV. Maybe by reducing the initial concentration of PP/PV, we could reduce their competition with SC over resources, and therefore observe a better growth for SC in toluene media.

In addition, we inoculated the media with the same number of PP or PV with SC and haven't considered the fact that SC community is composed of different bacterial species. The high quantity of a single strain (PP or PV) may have made it easier for them to outgrow SC over time.

C. Back to context

In many bioaugmentation experiments, one of the main challenges is the survival of the inoculated strain in presence of a bacterial community [6] [7], particularly in absence of pollutant [8].

In our experiments, PP and PV were able to grow in both media in presence or absence of SC. This led us to think that PP and PV would be interesting candidates for bioaugmentation, especially as they have no problem growing with SC in mixed carbon.

As PV showed a significantly lower growth on average in toluene in presence of SC than PP, it would be tempting to say that PP would be an even better candidate. However, as said in *Results* section *B2*, our data isn't totally reliable to compare the two strains, as the SC extracted the two weeks behaved differently. It would be good to test PP and PV on the same SC.

Further experiments would be required to know if this is also true in a sand medium (and not liquid medium) and especially over a longer period of time. Similar experiments could also be performed on different native communities. Also, it would be interesting to vary the concentration of toluene in these experiments.

D. Conclusion

In the present state, our study shows interesting results. However, the issue with this staining technique might carry some artefacts that should be considered in data interpretation. If time had allowed it, we would have liked to perform the additional experiments proposed above to solve this issue.

Acknowledgments

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APPENDIX A on next page

APPENDIX A

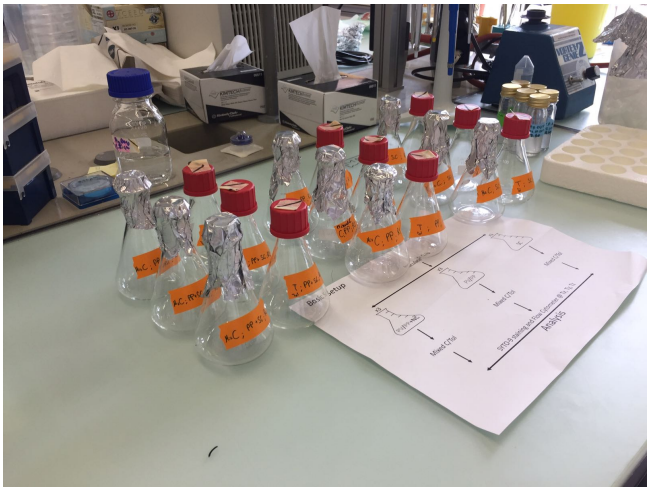

Fig. 15: Flasks of week 1. The ones with the aluminum foil caps contain the mixed carbon medium and are not sealed. The ones with the red cap contain the toluene (see the sealed tip inside) and are sealed to avoid toluene leaks and loss of pollutant.

APPENDIX B on next page

APPENDIX B

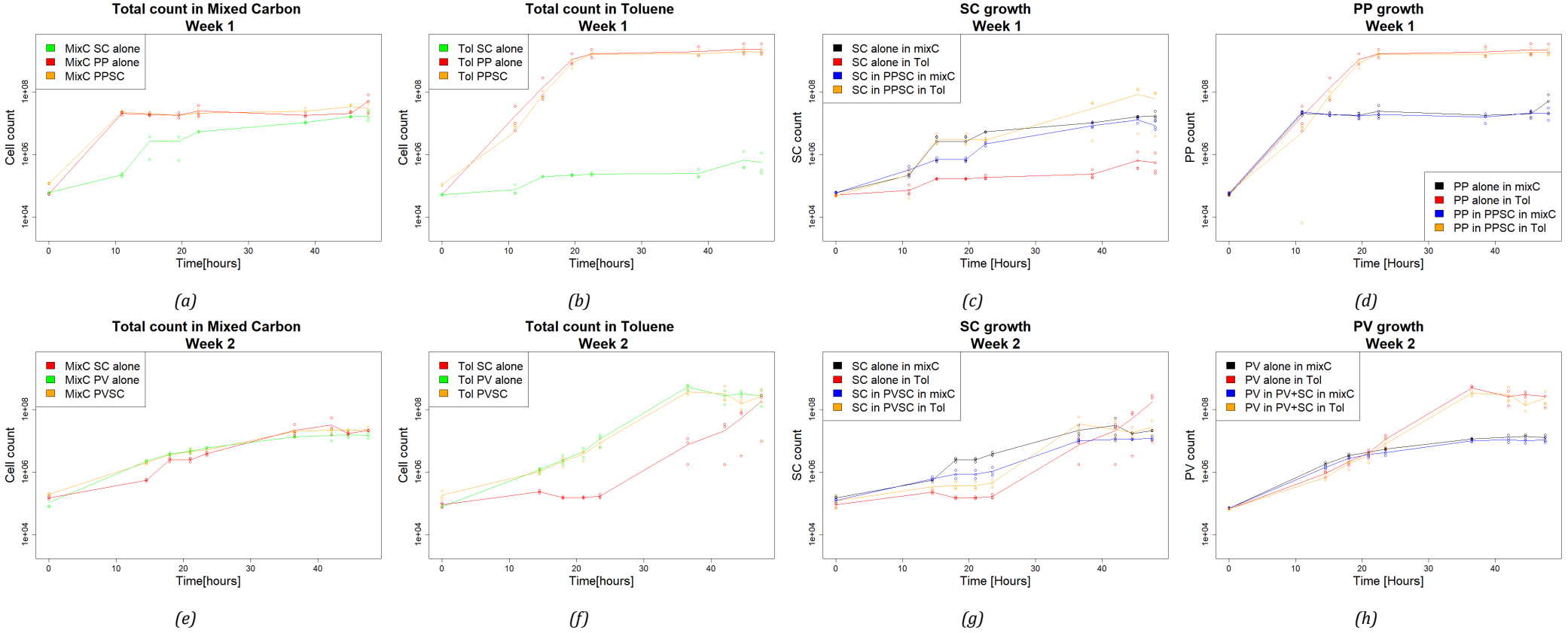

Fig. 16: Growth curves of: Fig. 16a, Total count in Mixed Carbon, Week 1; Fig. 16b, Total count of cells in toluene, Week 2; Fig. 16c, SC growth in different substrates, Week 1; Fig. 16d, SC growth in different substrates, Week 1; Fig. 16e, Total count in Mixed Carbon, Week 2; Fig. 16f, Total count of cells in toluene, Week 2; Fig. 16g, SC growth in different substrates, Week 2; Fig. 16h, SC growth in different substrates, Week 2.