The Effect of Bacterial Interactions on Fitness

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***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 might be difficult for this bacterial community. Bioaugmentation is a method that could be used to help them overcome the effect of pollution. It consists of adding a bacteria able to degrade the pollutant into the native community. In our experiment, we chose to add *Pseudomonas veronii* and *Pseudomonas putida* to a bacterial community extracted from sand and to grow them with toluene as only carbon source. 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, in the experiment conditions, the final amount of *Pseudomonas* cells was way higher than the Sand Community cells, which means that the overall community was quite different from the starting Sand Community.**

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 as spillage of oil or solvent. We decided to use toluene as a pollutant for this experiment.

Toluene is a 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. Being volatile, most of the pollution caused by it will end up in the atmosphere, but small amounts can be found in the ground or water in case of spillage [1]. When such event happens, one way to treat those is the use of bioremediation. There are several different techniques of bioremediation but they all have the same purpose: helping the native community to overcome the effect of pollution and promoting its regrow. 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 choose for our experiment were *Pseudomonas putida* (PP) and *Pseudomonas veronii* (PV), both able to metabolize toluene [3] [4]. The bacterial community on which we tested this bioaugmentation 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 cytometryr[1]. 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 was that the addition of *Pseudomonas putida* or *Pseudomonas veronii* will have an effect on SC growth, especially in a toluene-only media, because we think that these *Pseudomonas* bacteria might produce metabolites the SC bacteria could use as carbon source or reduce the toluene concentration and thus its toxic effect. This would lead to a better growth of the sand community in presence of one of these *Pseudomonas* strains.

II. MATERIAL AND METHOD

A. Experimental design

There were 3 species used for this experiment: *Pseudomonas veronii 1YdBTEX2* (tagged with mCherry, called PV), *Pseudomonas putida F1*(tagged with mCherry, called PP) and a Sand Community, extracted from the beach of St-Sulpice(referred as SC). The 2 *Pseudomonas* strains can degrade toluene. We also used 2 media: a mixed carbon medium[2] and a Minimal Media 1x (MM) with only toluene as carbon source.

We separated the work into 2 weeks, and tested a different *Pseudomonas* strain each time. In each experiment, we tested 2 species and their interaction in both media.

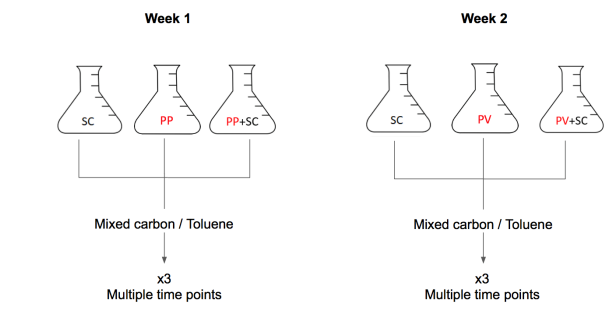

Fig. 1: Design of the 2 experiments. Each species tested on the 2 media. 3 replicates were made.

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

[2]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.

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 conditions, and used 3 replicates for each. In the end, we had 18 cultures per experiment. These cultures were then sampled and counted regularly with a flow cytometer.

B. Extraction of Sand Community (SC)

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

C. Pseudomonas veronii and Pseudomonas putida

Samples were kept at -80°C, taken and streaked on LB plates with antibiotics (Gm10). Incubated at 30°C, then streaked one colony on MM 1x plates without carbon. All the plates were put in a closed chamber containing 200µL 100% toluene. The plates were incubated at 30°C. Then took one colony, put on liquid MM with 5 mM succinate, and incubated at 30°C.

D. Inoculation

We put 20mL of mixed carbon media in half the flasks, and 20mL of MM 1x in the other half, where we added 200µL toluene 100% in a tip (toluene being volatile, it will then evaporate and diffuse in the liquid media, it will then be the only source of carbon). We quantified our cells by using the flow cytometer, then calculated to put 10^6 cells/mL in PV/PP alone flasks, SC flasks, and added twice 10^6 cells in PV (or PP) + SC flasks. The flasks containing toluene were sealed to avoid toluene leaks. They were incubated at 25°C and constantly shaken at 110rpm during 48 hours.

E. Sampling and count of cells

Every few hours (usually every 3 hours during the day, sometimes a bit shifted according to our schedule), we sampled the flasks. We took 200µL from each, and plated it on a 96-well plate. We took 3 samples from each flask, to make technical replicates, and stained them with 4µL SYTO-9 (50µM), a green DNA stain.

To measure the growth of our cells we used a flow cytometer. This technique consists of passing cells one by one through a capillary and hit each individually with a laser. The machine will then measure the scattered light, this way we can measure the size of the cell [time during

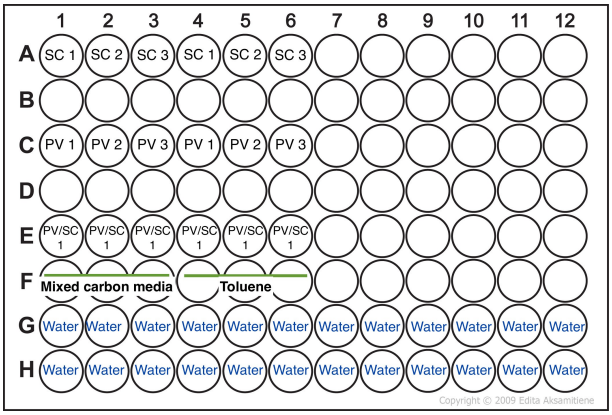

Fig. 2: 96 well plate map used in our experiments. The numbers (1, 2, 3) represent the replicates*.
* http://www.cellsignet.com/media/plates/96.jpg, Nov. 2017

which the light is scattered] and the density of our cell. We can also identify cells if they have a marker. Since the *Pseudomonas* strains were tagged with mCherry, a red fluorescent protein, and all the cells were stained with SYTO-9 (green fluorescent DNA stain), we could differentiate PP/PV from SC. It's important to ensure an accurate distinction between the samples. 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 experiment, we diluted our sample 10 fold at time point 1 and 100 fold from time-point 2. In the second experiment, we diluted our sample 10 fold at time point 1 to 4 and 100 fold from time-point 5. We then adjusted the results to the original concentration during the analysis. The water in the last 2 rows is used to clean the capillary between samples.

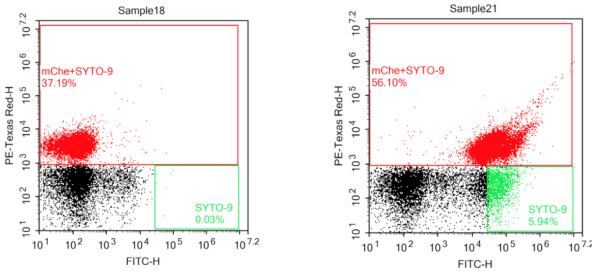

Fig. 3: On the left, the data of unstained sample. On the right, stained samples. Horizontal axis : intensity of red fluorescence. Vertical axis : intensity of green fluorescence.

As the cultures grew, we took less cells (diluted 10x, then 100x), and added MM 1x up to 200µL. We realised in the 2nd experiment that, to be sure that we do not include soil particles (of same mass and size than cells)

in our data, we needed unstained cells. We added to the 96-well plate an exact replica, without SYTO-9, and used this to determine gates, a limitation of what we considered SYTO-9 stained cells and what we considered soil particles. The horizontal axis on figure 3 represents the green fluorescence, and the vertical axis represents the red fluorescence. We can see on figure 3 that the *Pseudomonas* cells are easily recognisable (in red), but the limitation between SC (in green) and sand particles (in black) is a bit messy. We decided to put the gate using the unstained sample, and applied it to all the samples from both experiments.

F. Statistical method

Finally, our data was composed of red and green fluorescent cell counts for each treatment, at 4 time points every day for two days.
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 value per replica of each treatment.

From here on, we will refer to SC, PP and PV as species. However, keep in mind that SC isn't a single species of bacteria, but rather a community containing potentially many different species. In addition, we will refer to each combination of the variables media (tolune or mixed carbon) and Species in culture (SC , PP, PP and SC grown together, PV, PV and SC grown together) by the name treatement. 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 experiment, 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. First experiment, with Pseudomonas putida F1 mCherry

1) Overall population: First, we were interested in the growth of the overall population. We looked at the sum of both fluorescence counts for each treatment. In mixed carbon (figure 7a), PP and PPSC curves look very similar. SC seems to grow slower but catch up with the other culture after 40 hours. In toluene (figure 7b), 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 experiment is represented in figure 7c. 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 curve). In toluene however, the presence of PP allows for a 1000-fold difference on average (red and yellow curves). 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 absence of PP. SC alone grows less in toluene than in mixed carbon. 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 figure 7d (red and yellow curves vs black and blue curves). 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 toluene only than in mixed carbon. It is certainly due the greater amount of carbon in the toluene media.

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 figure 7d (red and yellow curves vs black and blue curves). 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 is certainly due the greater amount of carbon in the toluene media.

B. Second experiment, with Pseudomonas veronii

1) Overall population: Growth of the overall population over time is represented on figure 7e and 7f, the conclusion is the same as in the first experiment. It shows no signs that growing PP 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.

2) Sand Community: The growth of the sand community in each treatment for the second experiment is represented in figure 7g. In contrast with the first experiment, SC was able to grow in toluene. 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 the sand community we extracted behaved differently from one experiment to another and thus cannot be considered similar. Therefore, we won't be able to compare the results from the two experiments. 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 time points, SC would grow higher alone than in presence of PV.

3) Pseudomonas veronii: The growth of the PV in each treatment is represented in figure 7h. As in the first experiment, 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 greater in toluene (red and orange) than in mixed carbon (black and blue curves). 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). 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 seems to indicate some competition between them in this type of culture. It seem 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.

IV. DISCUSSION

While these results are promising, further analysis and plotting were carried out to ensure reliability of our results. The first drawback of the design is the proportion of SC cells compared to PP or PV. We see that the proportion of SC is low compared to the overall population ($\cong 5\%$).

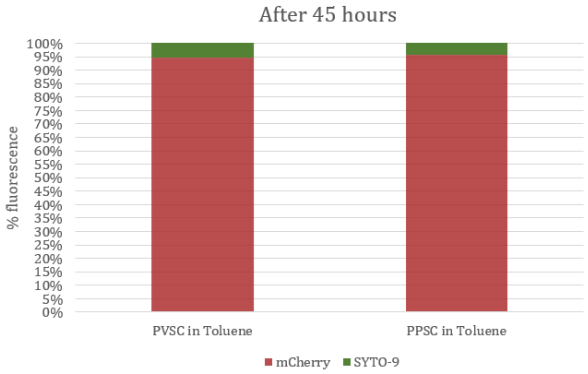

Fig. 4: Proportion of red and green fluorescence for the PV + SC and PP + SC cultures in toluene.

We would like to analyse this further by trying different other culture conditions. Firstly, we would try to have a mixed carbon with toluene substrate. Secondly, it would be great to vary toluene and carbon concentration and observe the effect on fitness. Third and final, we would like our conditions to be as close to reality as possible. For example, by growing the cells in sand,

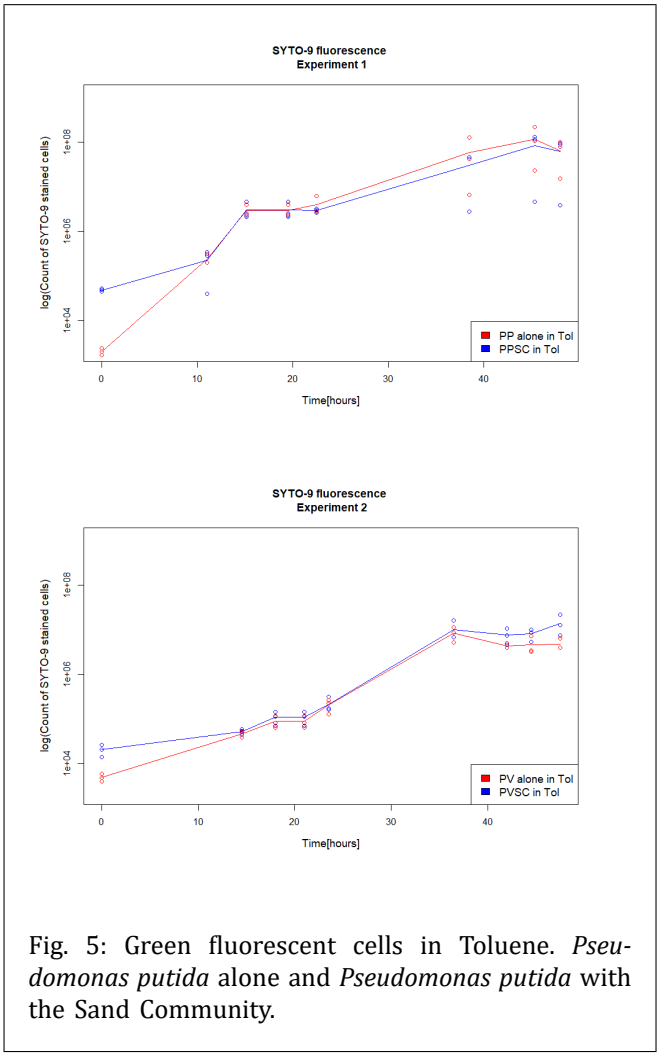

Fig. 5: Green fluorescent cells in Toluene. *Pseudomonas putida* alone and *Pseudomonas putida* with the Sand Community.

which would certainly affect their growth and fitness We noticed the second drawback when we plotted the count of SYTO-9 stained cells over time, for PP alone and PP with the Sand Community in Toluene in figure 5.

Knowing that SYTO-9 was used to identify SC cells, while *Pseudomonas putida* was tagged with mCherry, which emits a red fluorescence. The green cells count in PP alone is surprising. Our hypothesis is that some *Pseudomonas putida* cells died and it stopped their metabolic activities, disabling the mCherry fluorescence. However, if the cells do not lyse, their DNA is still nicely protected and can be stained by SYTO-9. The similarity between the two lines hints us that what we considered as SC cells in PP + SC represented in reality mostly dead *Pseudomonas putida* cells and not SC cells.

Reminder: SYTO-9 was used to identify SC cells, while Pseudomonas putida was marked red with mCherry.

The green cells count in PP alone is surprising. We don't expect any green fluorescence here. Our hypothesis is that some *Pseudomon1as putida* cells died and it stopped their metabolic activities, disabling the mCherry fluorescence. However, if the cells do not lyse, their DNA is still nicely protected and can be stained by SYTO-9.

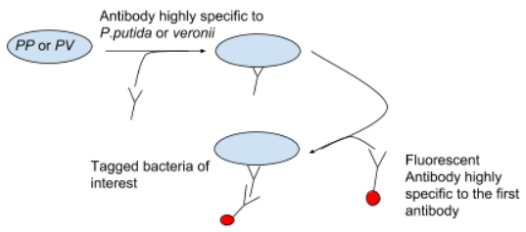

Fig. 6: Fluorescent antibody tagging, as an alternative to mCherry in this experiment

The similarity between the two lines hints us that what we consider as SC cells in PP + SC represents mostly dead *Pseudomonas putida* cells and not SC cells.

A. Improvements

To circumvent this problem, another tagging method unchanged by cell death could be used.

Another point that could be interesting to change is the initial concentration of *Pseudomonas putida* and *Pseudomonas veronii*. 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 observing a better growth for SC in toluene media. In addition, we added in the culture the same number of PP or PV and of SC cells , without considering the fact SC was composed of different species. It was a lot compared to each species present in the community. It is not surprising that PV/PP outgrew SC over time. Finally, as often in the lab, our results seem to unveil more questions than answers. We would have liked, if time allowed it, to perform additional experiments proposed above. Because in the present state, we got interesting results, but due to some unforeseeable events, we cannot be certain that they are not artefacts due to the issue differentiating the cells we mentioned before.

Acknowledgments

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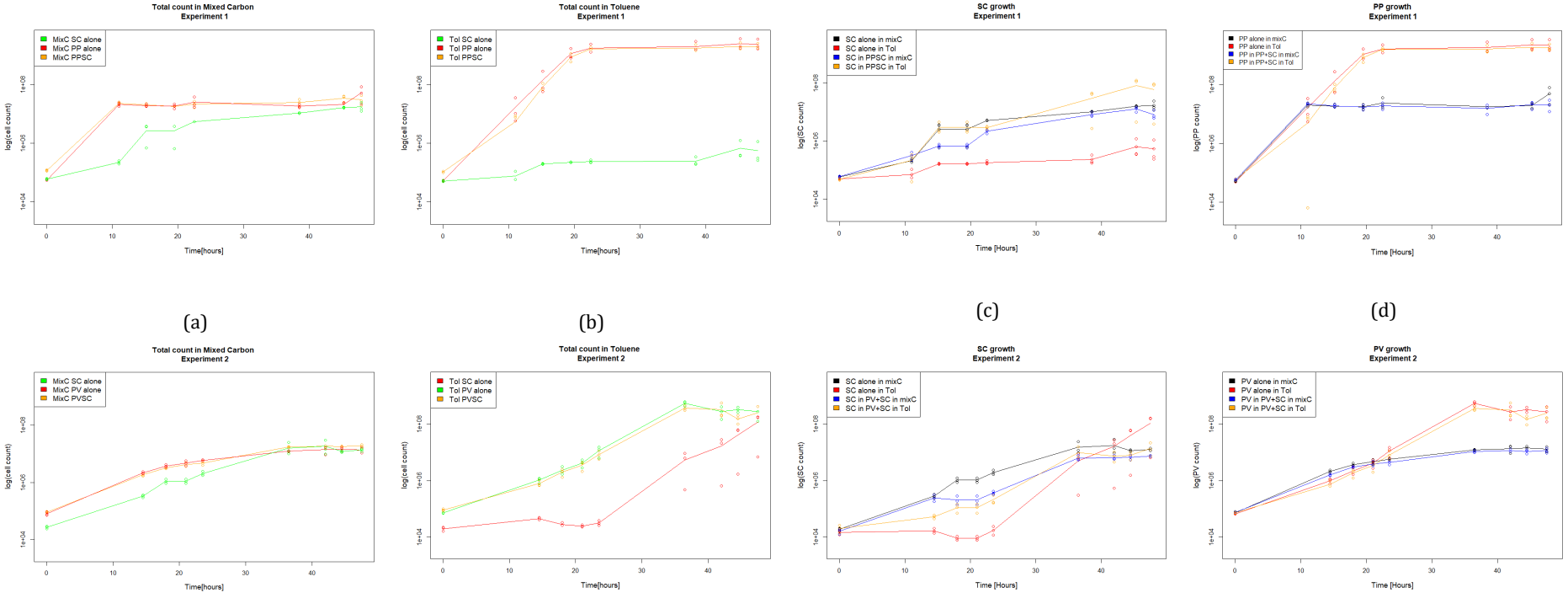
APPENDIX



(a) (b) (c) (d)

(e) (f) (g) (h)

Fig. 7: Growth curves of: 7a, Total count in Mixed Carbon, Experiment 1; 7b, Total count of cells in toluene, Experiment 2; 7c, SC growth in different substrates, Experiment 1; 7d, SC growth in different substrates, Experiment 1;7e, Total count in Mixed Carbon, Experiment 2; 7f, Total count of cells in toluene, Experiment 2; 7g, SC growth in different substrates, Experiment 2; 7h, SC growth in different substrates, Experiment 2.