Appendix

1. Background

The demo site in Delfzijl is roughly 5000 m2, next to and on the same site as a salt factory in an industrial area. From the immediate surrounding of the plant site we took three different soil samples and two water flows at >100 m (see Figure 1). Water site A represents the Zeehavenkanaal Farnsum and water site B is the Oosterhornkanaal, both ultimately connected to the Eems. We sampled the demo site in Delfzijl to investigate the survival rate of wildtype *Synechocystis* PCC6803 (WT) and our modified strain SGP237 under the environmental conditions found at the production site. When investigating the survival rate of the modified strain SGP237m we used a version with a kanamycin resistance cassette (=SGP237). Similarly, for WT, we used a YFP-labelled *Synechocystis*, with a kanamycin resistance marker is used in order to aid selection on plate.

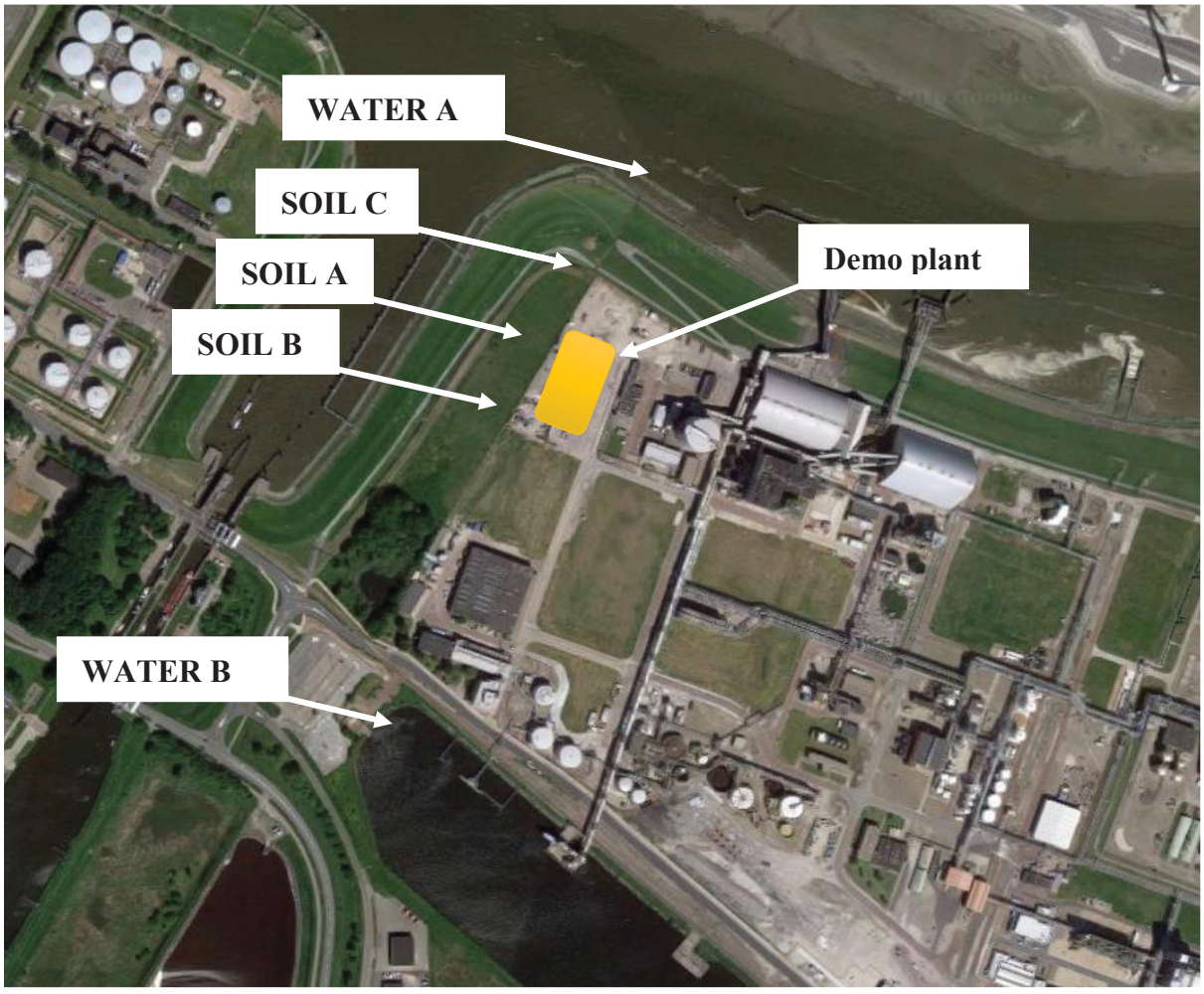

Figure 1. Map of the area where the demo plant will be built with an overview of the sampling points.

This Survival Experiment (SE) started the 26 November 2018 with the sampling of water and soil on site and ended in March 2019. After this first experiment a validation was carried out in order to reinforce the data set collected and to overcome minor technical difficulties. This Survival Experiment Validation (SEV) lasted from the 11 of March 2019 to the 13 of May 2019. Both experiment were carried out following the same protocol as described below.

2. Protocol

a. Goal

Assay the survival of wildtype (WT) Synechocystis, as well as modified strain SGP237, in soil and water samples collected in the vicinity of the future demonstration plant site (Delfzijl).

b. Samples

Samples were collected from the locations highlighted in figure 1: two water samples and three soil samples. Three individual samplings were performed for each location.

c. Strains

The strains used in this experiment are: YFP-labelled Synechocystis (referred as wtYFP), containing a kanamycin resistance marker will be used to assess WT survival, and SGP237, the genetically modified strain, containing a kanamycin resistance marker. YFP and kanamycin will aid selection on plate. Without the presence of kanamycin in the plates, we were unable to count colony forming units (CFU), since there were too many contaminating organisms growing on the plate. The growth rate of wtYFP is very similar to the wt even with the burden of the kanamycin resistance and YFP production (Figure 2-A). Also, growth of the modified strain SGP237 and SGP237m without the kanamycin marker is very similar (Figure 2-B).
Uninoculated flasks of nonsterile soil/water are used as controls at all temperatures.

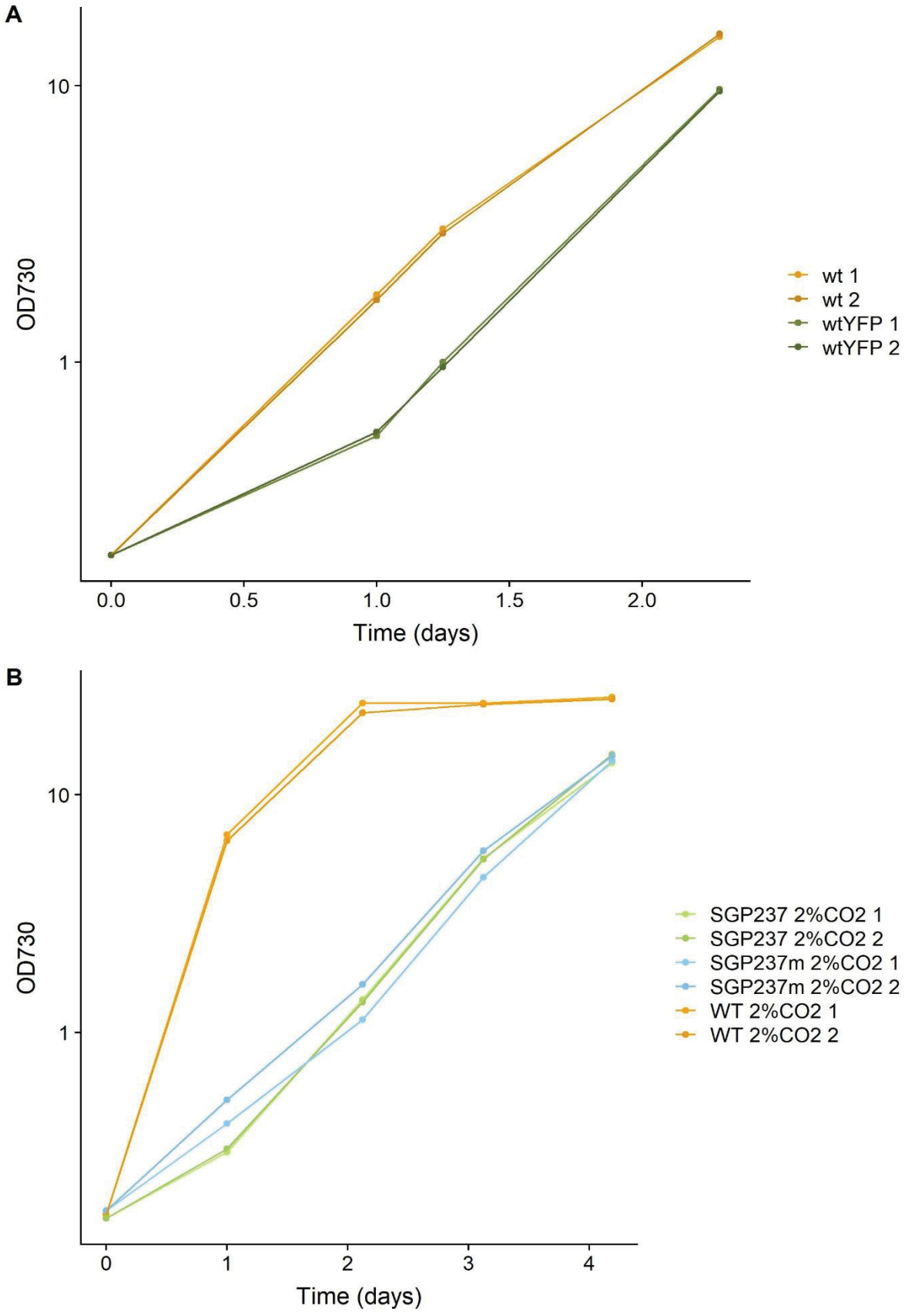

Figure 2. Comparison of growth rate between SGP237 and SGP237m with WT as a reference (A). Comparison of growth rate between wt and wtYFP (B). Growth experiment were performed in BG11 medium at 35°C with 2%CO_2 in the gas phase and 300µE of red light.

d. Treatment of water samples

- Water was collected at the designated locations in sterile bottles and shipped to the laboratory, where it was stored in the dark at 4°C until use.
- Half of the water sample was autoclaved for 45 min (sterile controls) on two successive days.
- 50ml aliquots of water were placed into 250-ml single-use flasks capped with stoppers.
- Flasks were inoculated with a relevant amount of cyanobacteria: fresh precultures were grown in BG11 medium to OD_{730}= 5, and 1mL of inoculum was added to the water samples.
- The non-inoculated flasks were amended with 1ml of BG11
- Flasks were placed in the light at 4°C and room temperature (RT). No shaking was applied.
- Sampling continued every two weeks for ~80 days, or until no viable *Synechocystis* was observed.

e. Treatment of soil samples

- Soil was collected at the designated locations in sterile plastic bags and shipped to the laboratory, where it was stored in the dark at 4°C until use.
- Soil sample was passed through a 2-mm-pore-diameter sieve
- Half of the soil sample was autoclaved for 45 min (sterile controls) on two successive days.
- 15g soil aliquots were weighed in single-use flasks capped with stoppers
- Flasks were inoculated with a relevant amount of cyanobacteria: fresh precultures were grown in BG11 medium to OD_{730}= 5, and 1 ml of inoculum is added to the samples dry soil sample.
- The non-inoculated flasks were amended with 1ml of BG11
- Flasks were placed in the light at 4°C and at room temperature for ~7 days without shaking.
- Samples for counting were withdrawn from the soil microcosms after resuspension in 50 ml of sterile 0.9% saline.
- After sampling, flasks were placed back in the light at 4°C and room temperature. No shaking was applied.
- Sampling continued every two weeks for ~80 days, or until no viable *Synechocystis* was observed.

f. Sampling of flasks

- Flasks were sampled every two weeks.
- Samples were diluted in sterile 0.9% saline (154mM) and a relevant number of subsequent 10X dilutions were plated on BG11 agar plates containing 50 µg/ml kanamycin. The plating was performed by a droplet method (5µL droplet). Plates were incubated at 30°C in a Panasonic MLR-352H-PE incubator with supplied CO_2 (1L/min of 10% CO_2 enriched gas phase) until colony forming unit (CFU) were visible for counting. The results represent CFU/mL in the samples.

3. Practical consideration for inoculation/sampling of survival experiment

The initial cell concentration for inoculation was calculated to mimic a catastrophic event where the total volume at high cell density of the entire facility would be discharged in a short time lapse in the surrounding environment. The samples, once inoculated, were kept in ideal condition with no temperature variation, change in the matrix, dilution or change in light intensity and invasive microorganism. This represent a "worst case scenario" and is still conservative in this extremely unlikely scenario.

For the survival experiment started on 26/11/2018, 180 different conditions were assessed, covering all possible combinations of strain, temperature, sample type and including negative control for all of them. Each condition was inoculated on week one with the precultures as mentioned in table 1. On the inoculation week, only water conditions were sampled. The soil samples inoculum is based on the cell concentration of the inoculum and not an actual measurement.

The inoculation of 180 different flask had to be carried out in several days and required to use different inoculum, creating small variation in the cell concentration at inoculation.

For each condition, a negative control (not-inoculated) was included to make sure that no colonies representing native *Synechocystis* would affect the experiment. To avoid bias in interpretation and ensure a good repeatability, a single operator was responsible for each step of the process, including plating and colony counting. The graphs are the result of ~25.000 droplet plated and a total of ~100.000 CFU counted manually.

The lower limit of detection found in the results was based on the most accurate measurement possible depending the sample origin (water or soil) and was set to 10 CFU in the dilution 10^{-1} and 0 dilution factor for soil and water respectively. It has to be reminded that the autoclaved

conditions are simply controls to be able to observe the impact of local microorganism on the cells survival and do not reflect an actual possible growth condition in a wild sample.

4. Practical consideration for inoculation/ sampling of validation experiment

The validation experiment aimed at again assessing the survival for the GGM during the first 45 days due to some difficulties in the CFU counting in the initial experiment. This should lead to a better insight on GGM behavior in samples from the introduction site. This experiment was carried out only in non-autoclaved samples and with the GGM stain. The rest of the experiment is identical of the initial survival experiment.

The inoculation of the validation experiment was carried out in a single day using a single preculture of the GGM stain and thus avoiding differences of inoculum concentration as in the initial survival experiment. Moreover, sampling was performed every week.

Table 1. Recap of preculture state during inoculation of SE. All preculture were measured for the optical density (OD) at 730nm and the cell number was determined using a CASY counter. The preculture ID allow tracking of cells origin.

Incubation condition and sample origin	Preculture ID	Day of inoculation	OD_{730} of inoculum	Cell concentration $*10^7$ Cells/mL
Soil 20°C site A and B	SGP237-1-231118	03/12/2018	4.5	37.27
	wtYFP-liquid-1-201118	03/12/2018	5.1	9.22
Soil 20°C site A + Soil 4°C site A	SGP237-4-231118	04/12/2018	5.17	42.82
	wtYFP-liquid-1-201118	04/12/2018	5.62	10.16
Soil 4°C site B and C	SGP237-3-231118	05/12/2018	5.23	45
	wtYFP-plate-1-201118	05/12/2018	5.02	9.93
Water 20°C Site A and B	SGP237-5-231118	06/12/2018	5.19	34
	wtYFP-plate-1-201118	06/12/2018	5.19	8.66
Water 4°C site A and B	SGP237-6-231118	07/12/2018	4.83	46.8
	wtYFP-plate-1-201118	07/12/2018	5.16	9.17

5. Results

a. Parent strain

The survival of the parent strain (wt) is shown in figure 3. In soil samples (Figure 3-A), wt showed a constant decrease of CFU in all conditions. At 20°C, only site C in the non-autoclaved samples display a better survival rate. At 4°C, the decrease of CFU is slower but steady in all conditions, with an average of 11% of living cells remaining after 75 days. No outgrowth is observed.

In the water samples (B) at 20°C the strain was able to grow out for 60 days and then stopped, probably due to the lack of nutrient or light access. This shows that without competition the parent strain is capable of survival and even outgrowth in the vicinity of the site of introduction. This is not surprising as it is similar to its natural habitat. This result also indicates that the experimental conditions do not harm the cells. If a strain was capable to grow we would then be able to detect it. In all other water conditions, the cells display a steady death rate (all bellow detection limit after 75 days in the non-autoclaved samples). At 4°C in the autoclaved samples the strain remains viable longer with a slower death rate. This would be the normal death rate when the cells cannot grow due to the low temperature, the faster death rate in non-autoclaved conditions show the effect of competition with other (endogenous) organisms.

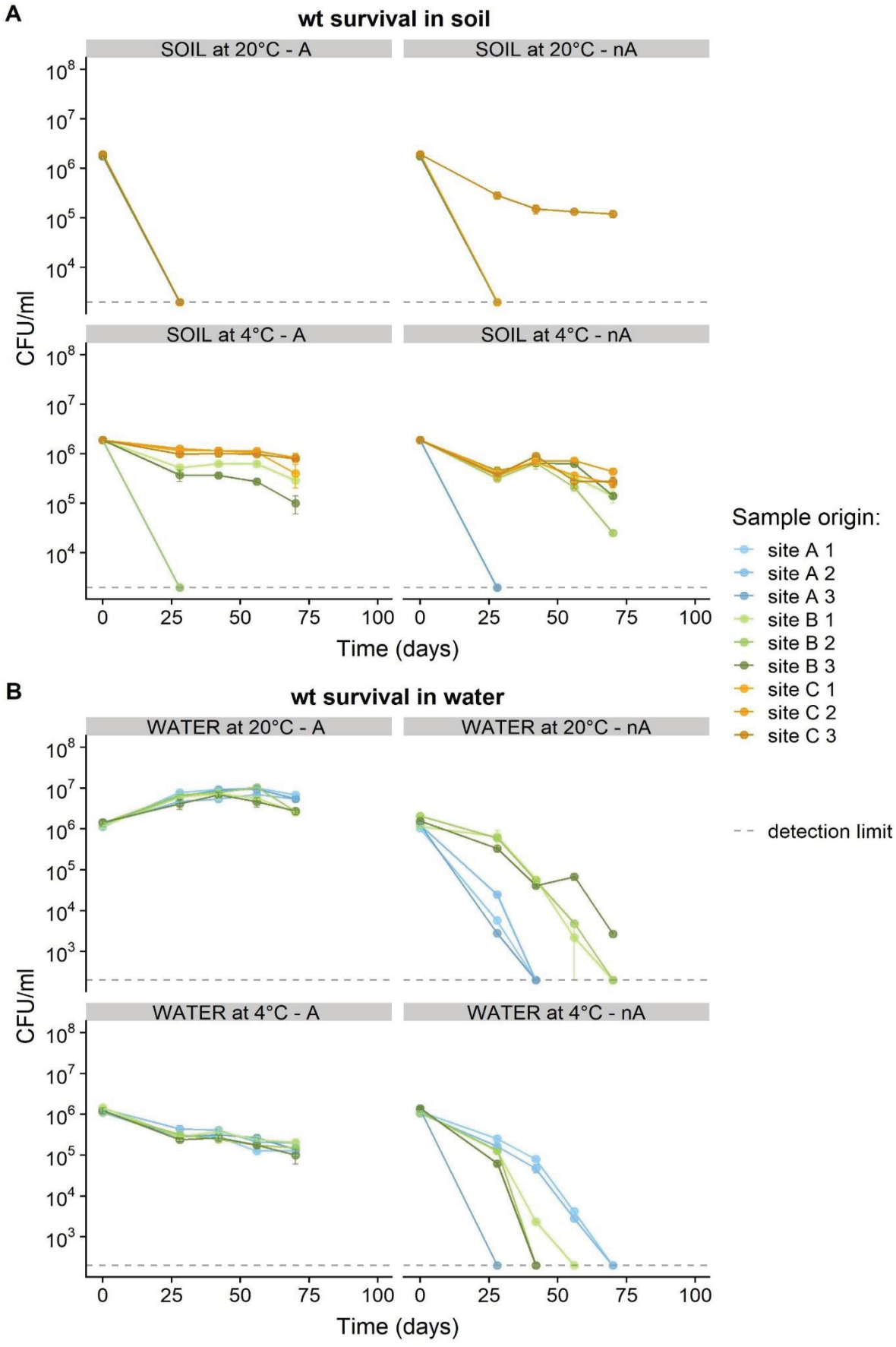

Figure 3. Survival rate of the parent strain in soil (A) and water (B) samples of the site of introduction. A = autoclaved condition. nA = non-autoclaved conditions. Detection limit was 200 CFU/mL in water and 2000 CFU/mL in soil. Each point is the average CFU count of 4 droplets. Error bars are based on standard deviation and account for the variation between each droplet count.

a. GGM strain

The GGM stain survival rate is displayed here in four different graphs. The figure 4 shows the results of the first survival experiment (SE), lasting a total of 100 days. The figure 5 shows the results of the survival experiment validation (SEV), lasting 50 days. The SE is lacking data for the first 45 days due to some difficulties in the CFU counting. In this first period, we underestimated the CO_2 necessary in the plate incubator to allow outgrowth of the colonies on the counting plates. Once this was adjusted to higher concentrations of CO_2, we were able to count the colonies for viable cells. The SEV covers this period of the first 50 days. As shown in Table 1, higher numbers of cells were used for inoculation of the GGM than for the parent strain, due to a larger cell number at the same optical density.

As figure 4-A shows, the survival of GGM in soil depends mainly of the temperature. At 20°C the living cell number decrease of 3 log in less than 75 days. After 75 days, all conditions are below detection level. At 4°C, the cell death rate is slower, as expected. There is not much difference between the autoclaved and non-autoclaved samples. In the non-autoclaved samples, the cells remained slightly longer and showed a steady death rate until day 100 with an average of 1.7% of the initial inoculum remaining.
In the water condition the GGM showed a much faster death rate: after 45 days, the number of CFU is below the detection limit for all conditions except in the non-autoclaved at 4°C. In this last condition, viable cells could be detected until day 70 (Figure 4-B). The validation experiment shows more details on the survival of the GGM during the first few weeks.

The SEV was performed using non-autoclaved samples as these best represent the natural outside conditions. Only two sites were sampled instead of three, but at the exact same location as the SE. The GGM strain shows a behavior similar to what observed in the SE, with the CFU count being below detection limit after 50 days in the soil at 20°C (Figure 5-A). In the soil at 4°C, the cells death rate is slower than observed before, but still display a minimum of 89% death rate after only two weeks for all conditions. After 50 days, only 18% of the cells in site B remain alive and 2.4% of the cells in site C.
In water samples, which is the natural habitat for *Synechocystis*, the death rate of the GGM is quite fast. We observed less than 0.003% of cells remaining alive after only two weeks at 20°C and 3 weeks at 4°C (figure 5-B).

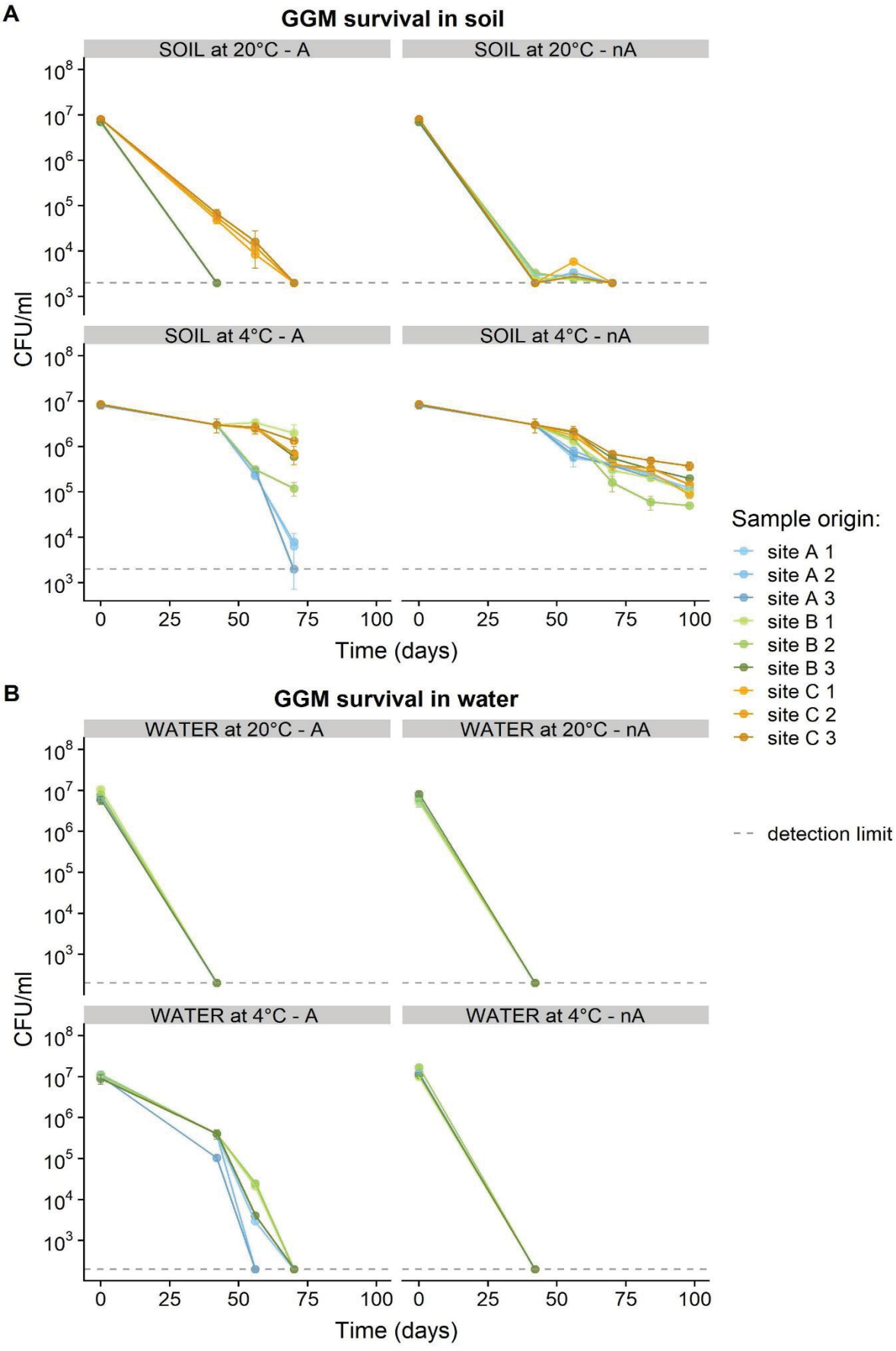

Figure 4. Survival rate of the GGM strain in soil (A) and water (B) samples of the site of introduction. A = autoclaved condition. nA = non-autoclaved conditions. Detection limit was 200 CFU/mL in water and 2000 CFU/mL in soil. Each point is the average CFU count of 4 droplets. Error bars are based on standard deviation and account for the variation between each droplet count.

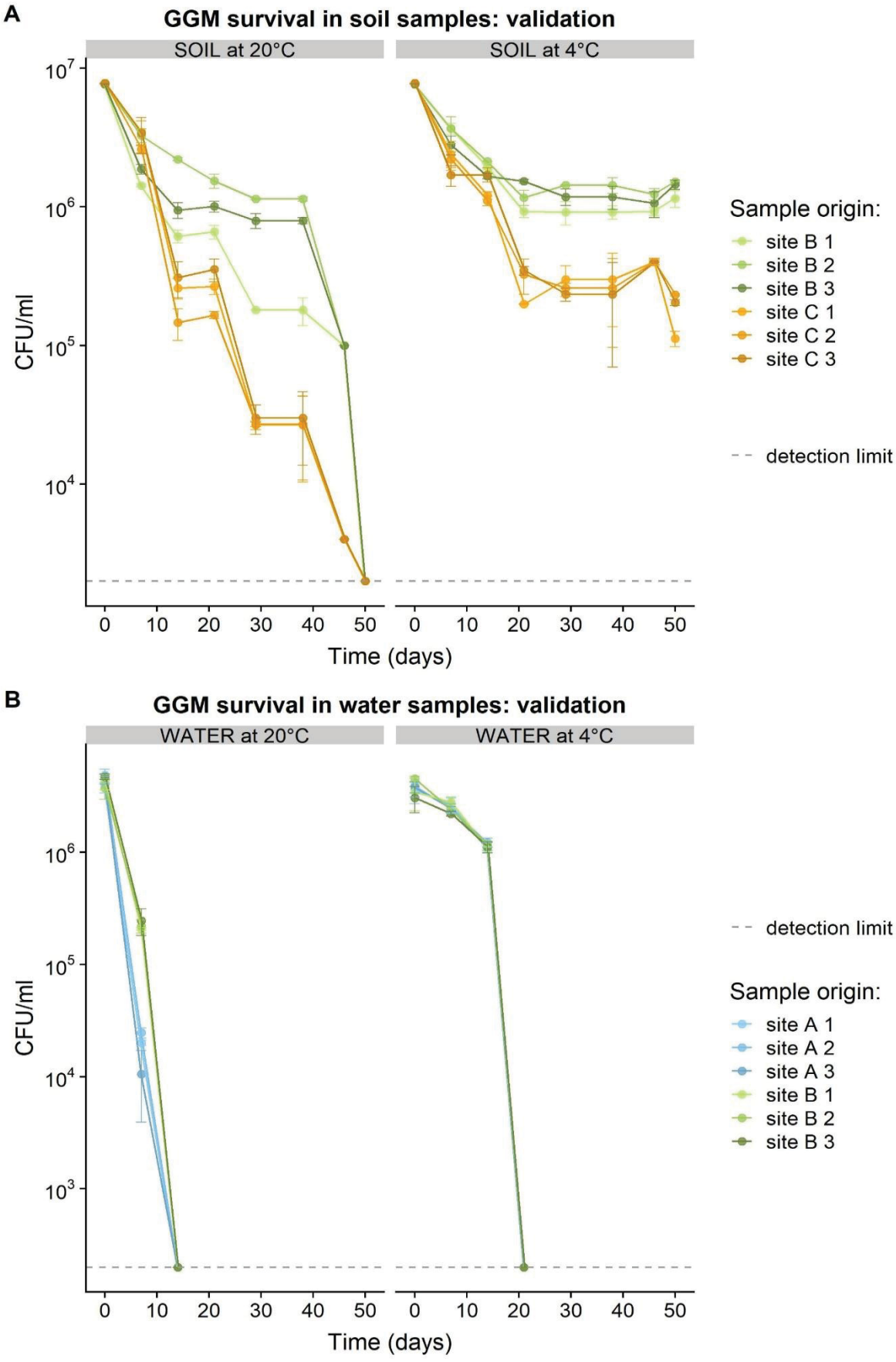

Figure 5. Validation of the survival rate of the GGM strain in soil (A) and water (B) samples of the site of introduction. Detection limit was 200 CFU/mL in water and 2000 CFU/mL in soil. Each point is the average CFU count of 4 droplets. Error bars are based on standard deviation and account for the variation between each droplet count.

6. Conclusion

These experiments were performed to show the potential outgrowth and survival of the modified strain SGP237 at the site of introduction. The data of the survival experiment and its validation show that outgrowth of the GGM is not possible. Although the GGM can persist in the soil for quite a long time, especially at low temperatures, it can never grow out. With the average precipitation of the introduction site, together with the nature of the soil, any microorganism released into the environment will end up in a large water body (underground or open) and be diluted to large proportion. We showed via the SE and SEV that if the GGM stain do end up in water it cannot survive for very long even in the most conservative possible conditions.