Annex 4

Survival study of *Saccharomyces cerevisiae* GMO's versus wild type in surface water and soil [redacted]

1. Summary:

A survival study with 3 different *Saccharomyces cerevisiae* strains in soil and surface water.

The survival of the wild type [redacted] against [redacted] and [redacted] was compared in both sterilized and non-sterilized soil and surface water samples from Delft at two temperatures: 8°C and 25°C.

The final results of the experiment indicates that both genetically engineered strains showed no significant advantage in survival or outgrowth compared to the indigenous flora under any circumstances tested. There is also no development in outgrowth observed of all the yeast strains present in the non-sterile soil samples stored at 8 and 25°C. There is a strong decrease visible of all the yeast strains compared to the indigenous flora examined in the non-sterile water samples. The presence of the yeast strains in the non-sterile soil samples remains stable after inoculation and compared to the indigenous flora no outgrowth is observed.

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2. Introduction:

A newly developed GE-strain has acquired the ability to [redacted] Before approval for use of this new strain can be given, prove needs to be generated that this new strain has no intrinsic advantage over wild type yeast to outgrow and flourish in the environment if containment is breeched. Earlier research with comparable strains showed that the [redacted] indigenous microorganisms.

3. Experiment:

Obtaining Environmental Samples

3.1. Surface Water

Surface water samples were collected directly from the [redacted]. Half of the surface water samples were sterilized for 20 minutes at 121°C (effectively).

3.2. Soil

Soil samples were collected from the [redacted]. The soil was dried at 25°C for one week before sieving (1mm). The soil was divided in two and one half was sterilized for 5 hours at 160°C (effectively).

3.3. Microbial Preparations

- *Saccharomyces cerevisiae* [redacted]
- *Saccharomyces cerevisiae* [redacted]
- *Saccharomyces cerevisiae* [redacted]

3.4. Yeast Inoculum

Fresh strains from BoZ or SCU were streaked for pure culture and one colony was cultured according to the procedure below:

- Pick up one pure colony from the agar plate.
- Dispense into PCB.
- Incubate o/n at the desired temperature, usually 25°C.
- Determine a rough cell count under the microscope.
- Dilute the samples (if needed) to ~1.0e8 cells/ml.
- Inoculums are ready for further use.

3.5. Inoculation of samples

The environmental samples (surface water, soil; sterilized and non-sterilized) were inoculated with the mentioned yeast strains. The inoculations were done using a single strain, as mixing the yeast strains would have made it very difficult to distinguish them, effectively making it impractical to separate them again to see differences in survival rate. As a control, samples without inoculation were also made to be able to compare indigenous flora.

The level of inoculation was targeted at ~106 cfu/ml or g. The exact number of viable cells in the samples was measured during the first t=0 analysis. The samples were stored at two temperatures; 8°C and 25°C.

At every sample point the WT was analyzed in triplicate and the GE organism was analyzed in five fold.

3.6. Microbiological analyses

Throughout the experiment, all samples were cultivated using Oxytetracycline Glucose Yeast extract agar (OGY) with Oxytetracycline to selectively detect the present yeast cells. The plates were incubated at 25°C for ~5 days before counting and recalculation to cfu/ml or gram.

The indigenous population of microorganisms present in the non-sterile samples was done on PCA with and without natamycin (to inhibit growth of the yeast inoculation strains). These plates were incubated for 3 days at 30°C. After incubation, the number of viable cells (formed colonies) were counted and recalculated to cfu/ml or gram using the chosen dilution.

The enumeration method was done by making decimal dilution in stylized and buffered Physiological Salt solution (0.89% NaCL) before testing 1ml dilution using direct pour-plates.

3.7. Analytical time-points

The samples were analyzed at the following time-points see table 1.

Table 1: Analytical time-points:

T=	Timing
t=0	0 days
t=1	1 week
t=2	2 weeks
t=3	3 weeks
t=4	4 weeks

3.8. Analyses and controls:

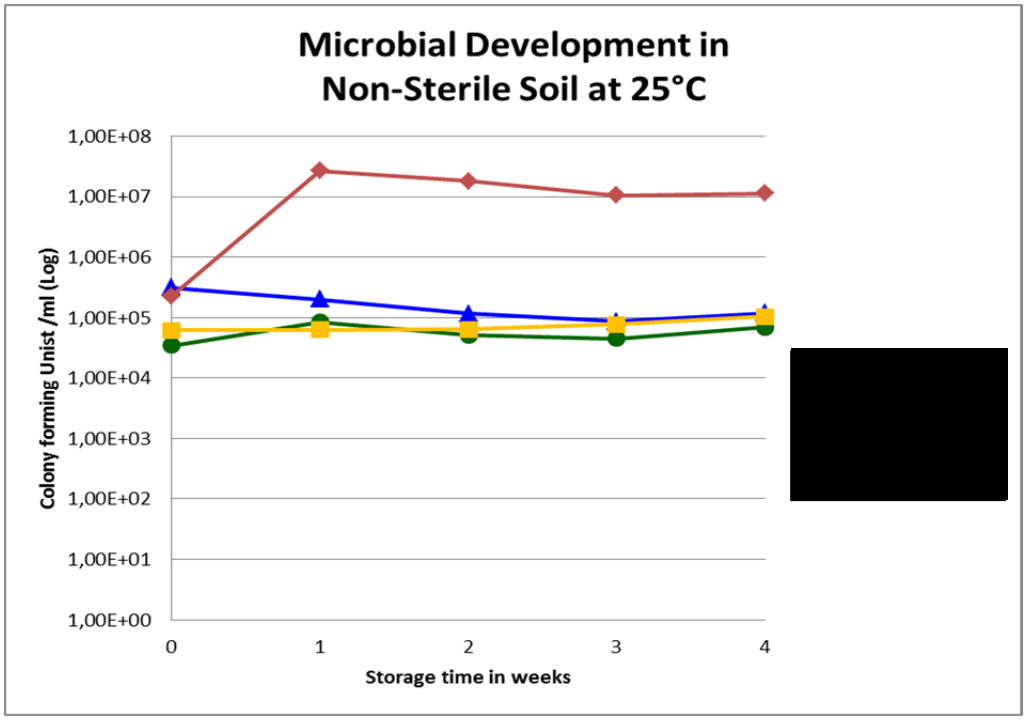
Water Sterile 8°C WT	Water Sterile 8°C GMO	Water Not Sterile 8°C WT	Sterile 8°C GMO	Soil Sterile 8°C WT	Soil Sterile 8°C GMO	Soil Not Sterile 8°C WT	Soil not Sterile 8°C GMO
Water Sterile 25°C WT	Water Sterile 25°C GMO	Water Not Sterile 25°C WT	Sterile 25°C GMO	Soil Sterile 25°C WT	Soil Sterile 25°C GMO	Soil Not Sterile 25°C WT	Soil not Sterile 25°C GMO

The sterile samples were examined at the presence of yeast (GEO or WT) in case there is an yeast inhibiter present in the water or soil.

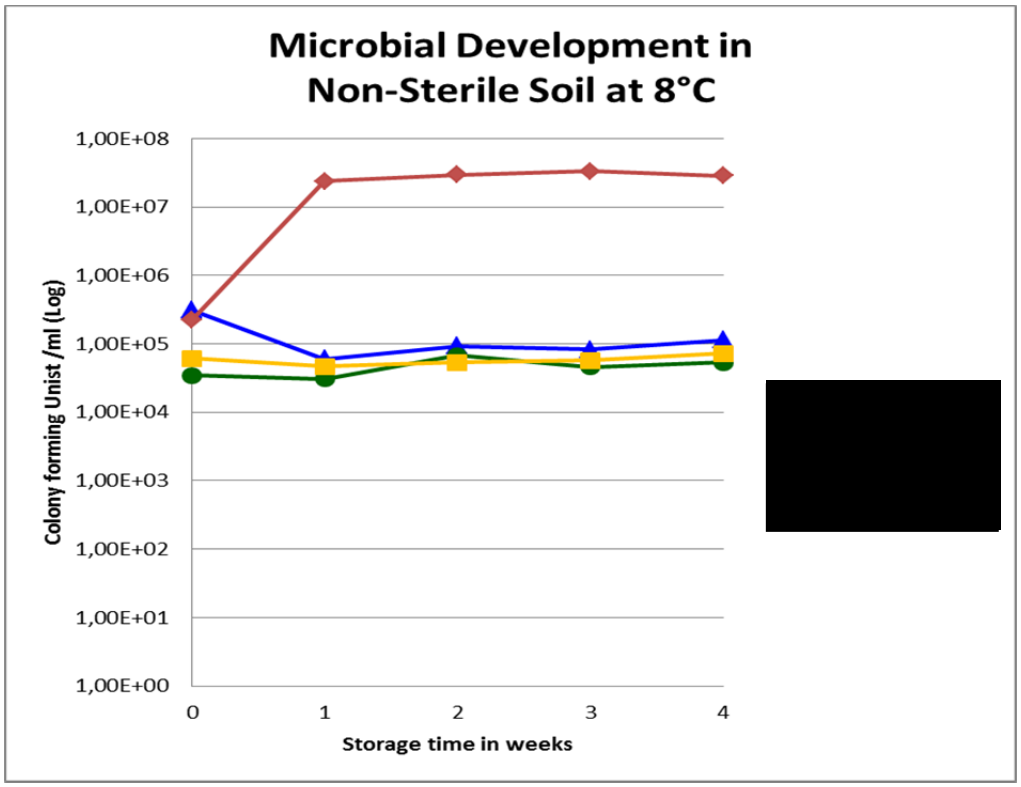
4. Results:

4.1. Graphs:

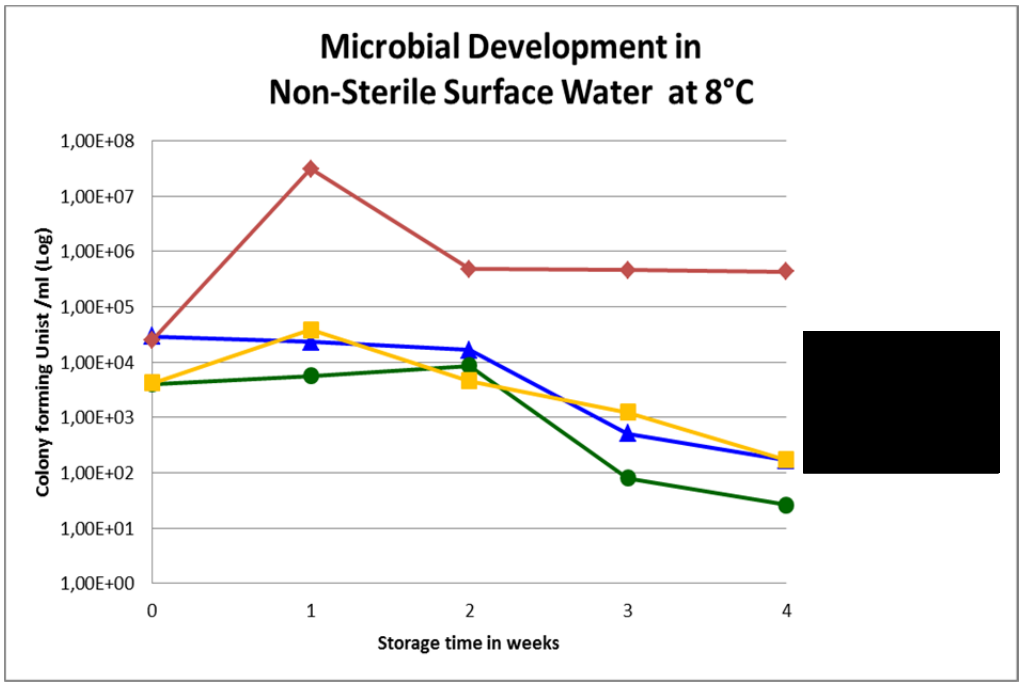
In the graphs the average of the measured cfu's per time point (WT, GMO & indigenous flora) is presented



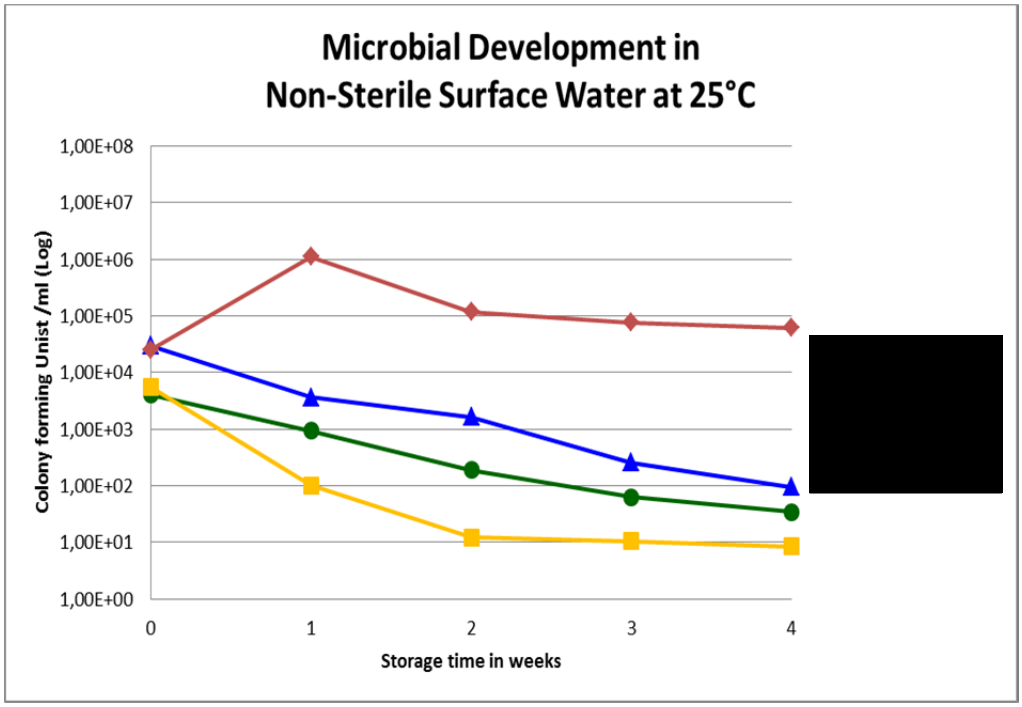
Graph 1



Graph 2



Graph 3



Graph 4

5. Conclusion:

Compared to the indigenous flora all the yeast strains (GEO's and WT) showed no outgrowth during the run time of the experiment. The results confirm that the WT and GE yeasts tested have no competitive advantage when accidently released into the environment.