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Detecting the Toxin Production of the Microcystis species in Hungarian Lakes

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Table of Contents:

Short summary:

Algal bloom causes several damages for lakes all around the word. The goal of this project was to develop an efficient way to follow the toxin production during algal blooming at an early stage.

The goal of the study was to create a biosensor which can detect the starting point of microcystin toxin production of blue-green algae namely the toxic Microcystis sp. population.

Microcystin is synthesized non-ribosomally by 10 genes. Four BioBricks were designed with the promoter of the genes, however instead of the genes green fluorescent protein (GFP) was used to predict the transcription of the genes responsible for microcystin synthesis. The students measured the fluorescence density emitted by the GFP when the toxin production had started.

About the authors:

Alexandra Gyémánt is a 16 years-old pupil, studying in a biology class. Her dream is to pursue a medical career. She is interested in molecular biology and genetics. She found this project highly engaging, because she was able to experience all the steps of a research study while also trying new methods and labor equipments.

László Török a 16 year-old, intrested in cell biology and genetics. His desire is to become a physician or a researcher, which is his immediate goal is to be accepted in medical school. This project, gave him insight into the entire process of a research. He believes this is the first step in understanding research so that he could pursue it in the future.

This project was completed for the IGem (International Genetically Engineered Machine) competition together with our schoolmates and our mentor, Sándor Bán. This is the link to our website: https://2019.igem.org/Team:SZTA_Szeged_HU .

Summary

The focus of the research was to predict the starting point of an algal bloom. Algal bloom is a big and important issue in Hungary, because it affects several hungarian lakes, including Lake Balaton, Lake Neusiedl and Lake Velence. This phenomenon causes many damages for the environment and wildlife, for instance: the lakes become disgusting and toxic. the algae use a lot of oxygen and cover the surface, that is why the light can not reach the deeper water-layers, the toxic environment and oxygen deficiency cause the extinction of the vertebrate faune,

However, if algal bloom is perceived, the damages can be minimalized or prevented. In Hungary, the main cause of algal bloom is the Microcystis aeruginosa which produce several toxins, but the most important is microcystin. The toxin is synthetized in a non-conventional way with the help of ten enzymes. The goal of this project was to develop a biosensor which can detect the starting point of the production of microcystin producing enzymes.

For this purpose we created genetical constructs which contain the promoter sequence of the toxin producing enzymes and a GFP gene. We put the constructs into plasmids. We transformed the plasmids into E.coli.

For the measurements, we grew colonies of Microcystis aeruginosa in order to detect the presence of their microcystin toxin. Samples were added from the algae cultures: the medium and lysed algal cells to the liquid culture of the transformed E.coli. We measured the emitted green fluorescent light.

In summary, if the toxin concentration reach a certain level, the transformed E.coli starts emitting fluorescent light. It means that the production of the toxin producing enzymes would also start in the algal cells. In this way, algal bloom could be detected in an early stage, because the production of the toxin producing enzymes happens earlier than the toxin’s presence in the water.

OUR PROJECT

Inspiration

Algal blooming is a global issue which affects both natural and artificial lakes. In Hungary, lakes and particularly Lake Balaton are essential for tourism. They create huge incomes for the country. But tourism is only one economic aspect of lakes- the other is fishing. Thanks to governmental actions which aim to improve the quality of food, the consumption of freshwater fish has doubled in the past ten years. From an ecological perspective, maintaining balance is crucial to keep fish species in a given ratio in these lakes.

Algal blooming is the excessive increase of algal population in water. This process leads to numerous harmful consequences. As the algae cover the water surface (Fig.1), its transparency decreases significantly and light cannot not reach deep layers, which results the destruction of the aquatic vegetation. This leads to the depletion of dissolved oxygen, further leading to significant high numbers of deaths in fish populations and, on a long term view, to the eutrophication and siltation of lakes. Furthermore, many algae types can secrete harmful toxins. As the time goes by the algae die and they become dark and brown, which is probably even worse, because they can't do photosynthesis, although they keep their other harmful features (for example: cut off the light...). (Fig. 2.)

Fig. 1. The first signs of algal bloom

Fig. 2. The late estate of algal bloom

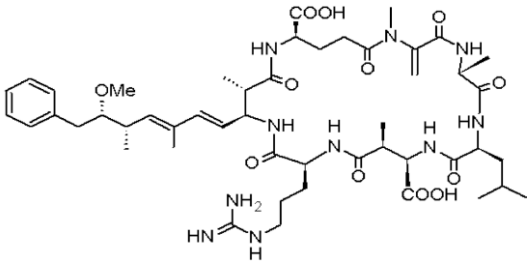
Main goals:

The main objective of this project was to develop an efficient way to follow the toxin production during water blooming at an early stage. Our aim was to detect the starting point of microcystin production of blue-green algae namely the toxic Microcystis sp. population. This goal was to be reached by transforming Escherichia coli. The purpose was to create a biosensor which can detect the starting point of the microcystin toxin production via green fluorescent protein fluorescence.

First steps:

Harmful algal blooming prevention is more succesful if done in early stages. This is why our purpose is to create a system which can detect toxin production in an early stage. We contacted two leading researchers of algal blooming, Prof Judit Padisák who is studying the algae species of the largest Hungarian lake, Balaton, and with Prof. Gábor Vasas who studies the algae species of the lakes in the Great Hungarian Plain. Their contribution and the studying of the study of scientific literature suggested that Microcystis aeruginosa and Microcystis flos-aquae are the main cause of algal blooming in Hungary. Several subspecies of Microcytis aeruginosa are also well known to marine ecologists. The Microcystis species produces several toxins, but the most important is the microcystin, which is mainly a hepatotoxin.

Microcystin:



Microcystin toxin is a cyclic heptapeptide, which has several harmful effects on vertebrates (fish and humans), especially on the liver which is why the toxin is known as *hepatotoxin*. The toxin is synthetized in a non-conventional way with the help of ten enzymes. The genes of the enzymes are in a bidirectional arrangement.(Fig.3.) However, there is no proven regulation pattern yet for this double operon.

Fig. 3. This is the gene cluster of the microcystin producing enzymes.

The putative promoter of the original operon is found between the toxin producing protein genes. In order to retrieve this sequence, we used the Ensemble bacteria, in which we found a huge amount of strains with variable regions containing the putative promoter. In order to reach the strain with the most conservative sequence we imported the sequences into Geneious Prime and create an alignment which was aware of the consensus sequence. This means that the program aligns the sequences and it chooses the base, which appears most often in them. We ran a blast from this and we noticed that the widespread Microcystis aeruginosa NIES-843 is the strain which has the highest percentage of pairwise identity with the consensus. Therefore, it was appropriate to use this during our experiment.

Biobricks

Microcystin is synthesized non-ribosomally by a specific gene cluster comprises 10 genes. The promoter of these genes can start transcription in two directions: mcyABC and mcyDEFGHIJ. These proteins are responsible for toxin production. The putative promoter sequence was necessary for our constructs. When we searched for this particular sequence in a DNA database, we noticed that its putative promoter is variable between the strains of Microcystis aeruginosa. As a result, we created an alignment to see how the consensus sequence look like from the promoter of nine different Microcystis aeruginosa. The result was the most similar to the consensus sequence is the Microcystis aeruginosa NIES-843. Therefore we synthesized our biobricks based on this sequence.

We designed four biobricks each able to predict the transcription of the genes responsible for the microcystin synthesis (a BioBrick contains different Biobrick parts. These parts are standardized DNA sequences, for example: promoters, coding sequences, ribosomal binding sites, etc). Two of them consist of the following parts: at the beginning, there is our promoter sequence where we placed an RBS followed by GFP gene. In addition, there are two types of transcription terminator sequences. Both biobricks contain the same parts, although the putative promoter sequence was reversed in one of them in order to see whether the transcription occurs in both directions or not.

Biobrick A

Biobrick B

The other two biobricks consist of the putative promoter and two GFP gene parts in two directions. This GFP only glows when both proteins are synthesized at the same time and linked together, thus we can measure if the transcription starts in both directions at the same time. We also placed RBSes (ribosomal binding site) between the promoter and GPFs, and the two types of transcription terminator sequences after the GFPs. These two constructs were made using the original promoter sequence, but the order of the two GFPs were changed in the two constructs. The genes from fluorescent proteins will be expressed in the engineered Escherichia coli bacteria.

Biobrick C

Biobrick D

Transformation:

The biobricks were synthesized by Twist Bioscience and IDT (Integrated DNA Technologies), they checked them with sequence analysis. We attached the DNA molecules into plasmids with the Zero Blunt TOPO system of Invitrogen.(Fig. 4.)

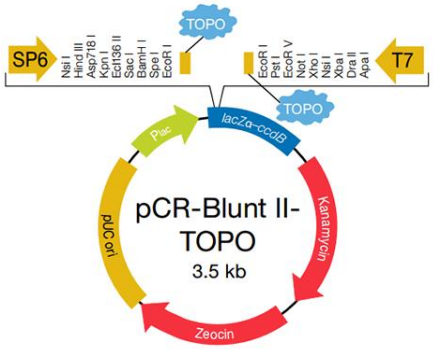


Fig. 4. The Zero Blunt TOPO system

We used gel electrophoresis to inspect the infiltration of the biobricks into the plasmids(Fig. 5). Afterwards we transformed the plasmids into NEB-5 alpha E.coli from the New England Biolabs. Following this step, we amplified the transformed bacteria with selectable markers. We used them for our experiments.

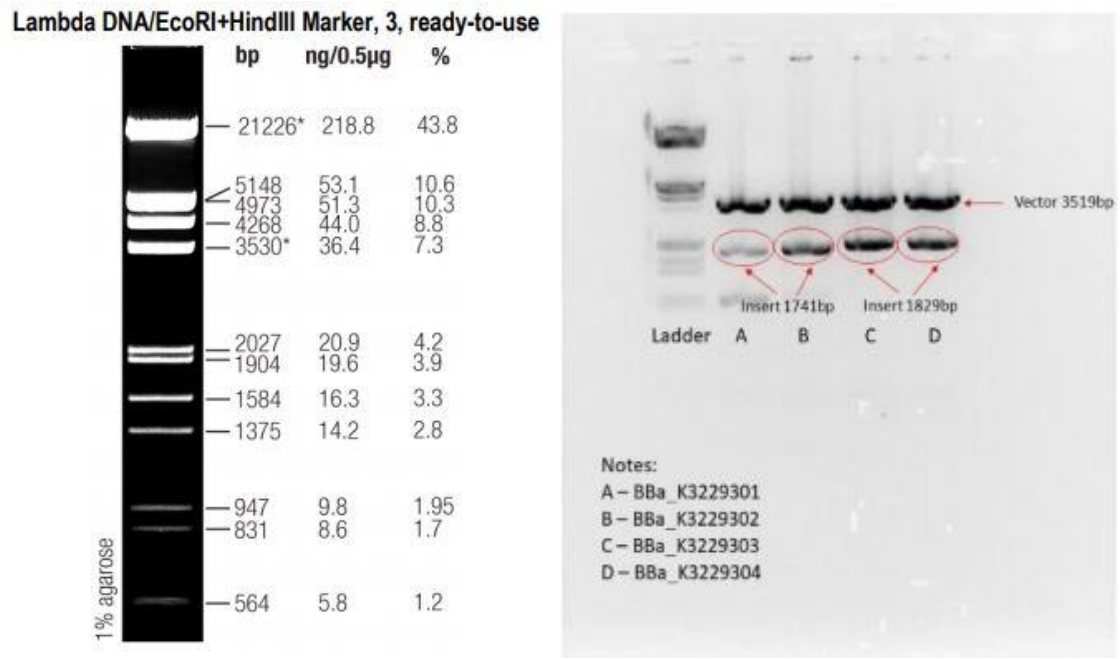


Fig. 5. The result of the gel electrophoresis: in the red circles, there are our BioBricks and the red arrow points to the plasmid backbone

Algae cultures:

We grew colonies of Microcystis aeruginosa (BGSD 243) in order to detect the presence of their microcystin toxin. We chose this algae because of its high level of toxin production. Using this simple growing procedure, we can demonstrate in which development phase of the colony the toxin can be detected. We performed these procedures at the Institute of Plant Biology of the Biological Research Center in Szeged. We cultivated 3-3 colonies at 24 °C (colony 1., 2., 3.) and 30 °C (colony 4., 5., 6.). Except for the temperature, every circumstance was the same. Colonies were kept under 86 micromol photon/m²/sec light intensity constant illumination with 120 rpm shaking in an orbital shaker.

We took a 2-ml sample from each colony every day, for 10 days at the same time. We froze the samples at -20°C.A total of 60 samples were taken for later experimentation.

Fig. 6. Our algae cultures in the first day

In time, the colonies started to show differences in colour, depending on the temperature in which they were grown.(Fig.7.)

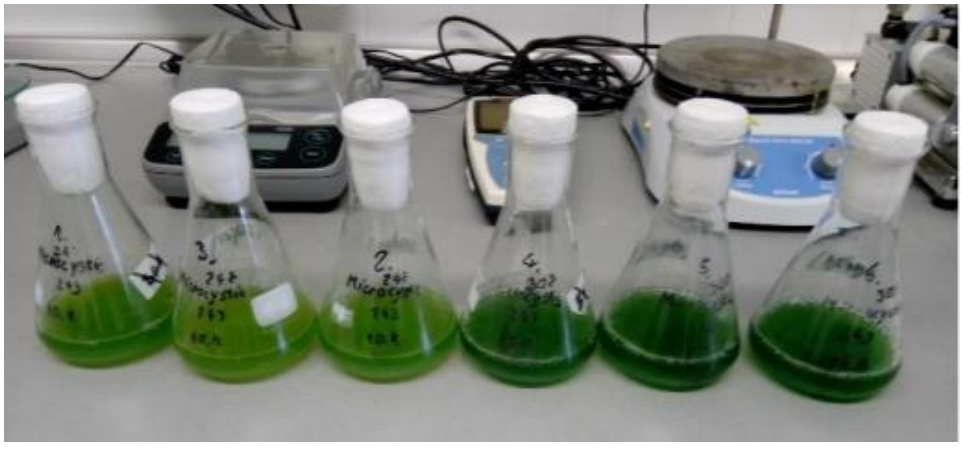


Fig.7. Our algae cultures in the tenth day

Fig.8 shows that the algae grown at 30 °C started blooming in the seventh day. This difference was also shown in the different colors of the cultures at different temperatures. (Fig.7)

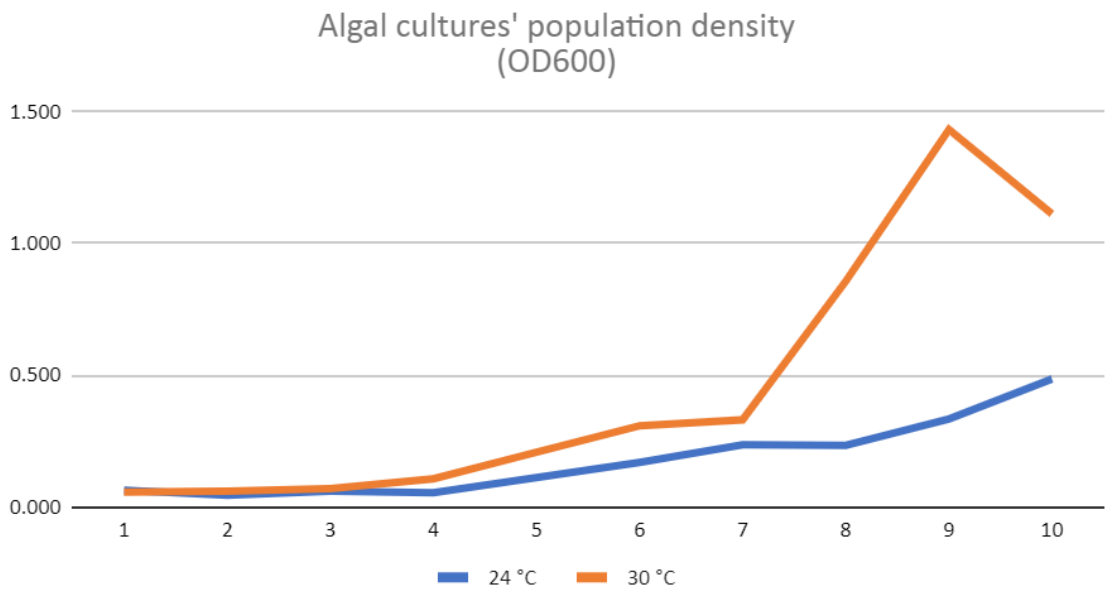


Fig. 8.: The diagram about the algal cultures' population density. You can see the big increase from the seventh day.

Measurement:

Our measurement goals were the following:

- Checking whether our product will be able to predict the starting point of toxin production.
- Clarifying a hypothesis on how the signal of toxin production is generated and provided to the algal cells.

As mentioned previously, the bidirectional promoter is called ‘putative’ in scientific literature. This means that there is no information yet on how it works, so our task was a real scientific challenge. The hypothesis was that the signal switches on promoters can be the result of either an environmental factor (pH, temperature) or a signal-molecule produced by the algal cells, themselves. Some contemporary articles assumes, but not yet proved the second, autoinductive theory.

Our measurement design aimed to primarily check mainly the latter theory. The most probable hypothesis is that the switch-on-molecule-signal is produced by the algal cells themselves. Assuming this, two still remain: 1. the signal molecule is produced on an intracellular level at a particular point of the algal blooming, 2. the signal molecule is exported outside the algal cells in order to trigger other cells to start producing the toxin.

The measurement design aimed to check both of these options. We used samples from our algal cultures: the medium and the lysed algal cells were added to liquid cultures of transformed E. Coli. After one day, and then checked the GFP production of each culture was checked.(Fig. 9.)

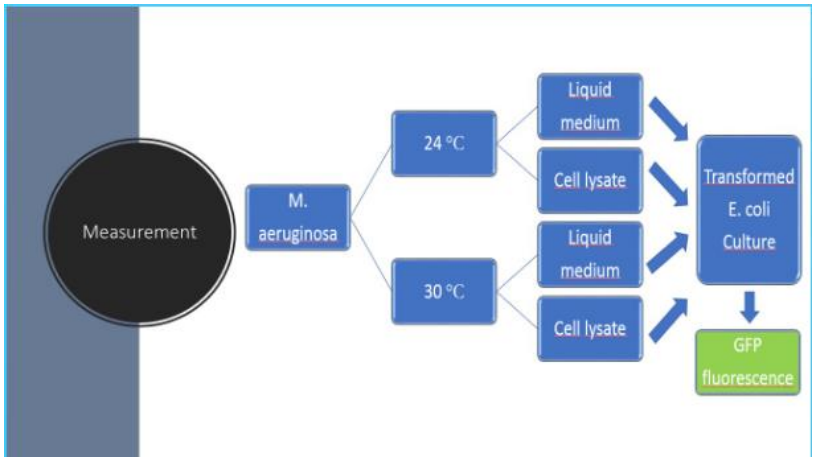


Fig. 9. It shows the arrangement of our measurement. We used both types of algal cultures, but we did the same process with them.

However, measuring the fluorescence level was not simple, as we faced two issues:

Fig. 10. The device

Firstly, our school laboratory had no fluorescence microplate reader appropriate to measure GFP concentration directly. However, we did have the opportunity to use the adequate equipment at the local Biological Research Centre, but we felt, that it would be a great contribution to the iGEM community, especially future iGEM teams, if we demonstrated, that a widespread high school device can still be appropriate for also measuring GFP.(Fig. 10.)+

Secondly, our purpose was to measure not the fluorescence of a pure GFP solution but the fluorescence of the whole bacterial cells themselves. Therefore, browsing the scientific literature, where we discovered that the GFP excitation spectra is rather different if measured in whole bacterial cells, which is called RED SHIFT.

The device we used is a widely-known type of fluoro-spectrophotometer used in high schools. It has two inbuilt excitation wavelengths: 450nm and 500 nm. The scientific literature and the iGEM Registry page of BBa_E0040 suggest using a 504 nm length, but the 500nm was still approximately 90 % efficient, which at high fluorescent levels should produce a satisfactory signal.

Therefore, we used the iGEM GFP calibration package to create the GFP producing bacteria as reference. We recorded the emission spectrum of the bacteria which revealed that it was similar to the spectrum in scientific literature.

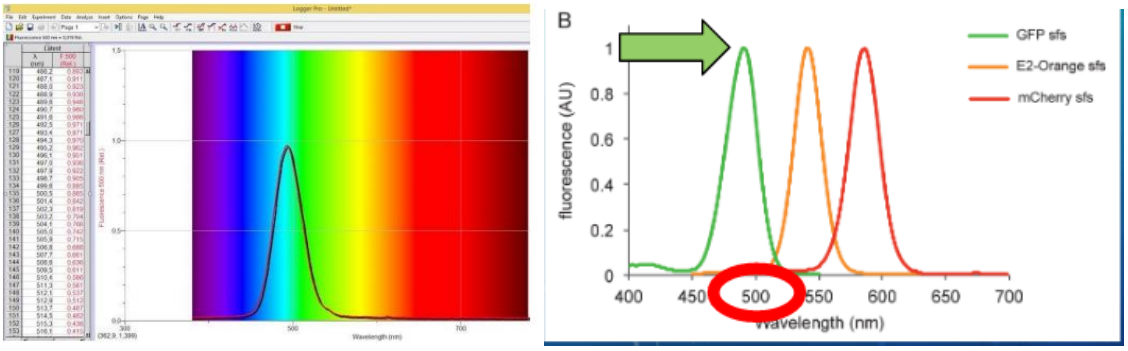
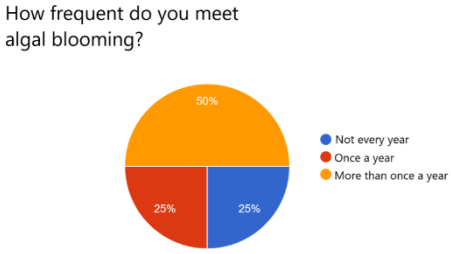


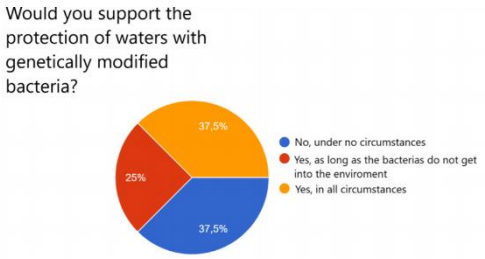
Fig. 11. The wavelength of the emitted fluorescence light

Human practises:

It was beneficial for the study to come in contact with the Hungarian fresh-water-authorities, algal researchers and fishpond-owners who are experiencing the negative effects of water blooming. Directly speaking to these role-players and having other Hungarian and foreign professionals complete our questionnaire, revealed that 10-20% of their annual expenditures is spent on water blooming issues. Furthermore, they all confirmed that it would be crucial to detect algal blooming and its effects at an early stage. Having a system capable of doing this would help them to act rapidly, which could lead tot he usage of a smaller amount of chemicasl for stopping further blooming and thus result in a faster restoration of the ecological balance which normally takesyears.

Fig.12. shows the results of the questionnare:





As a result of the responses received, it became clear that everyone who work in this sector wanted to be able to forecast the eutrophication. Most of them would be willing to use biological cleaning methods to protect the environment. ⅔ of the participants dare to move towards new opportunities including cleaning process with genetically modified bacteria.

Integrated Human Practices:

Wanting to reiterate our experiments using plasmids with the promoter sequence of a local strain, we asked for a **local** Microcystis aeruginosa strain BGSD 243, from Lake Velencei concerned often by algal blooming. The sequence of its genes which we used throughout our project, was still unknown. Thus we decided to sequence the promoter region of the mcy gene promoters of this strain. In order to gain the sequence, we amplified the corresponding DNA from the algal genome using PCR.

Although the mcy promoter region is different among species/strains, the mcyA and mcyD genes are conservative, which is why we used them for designing the primer pair for the PCR. In order to ensure the success of the amplification we used the touchdown PCR method with decreasing annealing temperature. We visualized the product using gel electrophoresis, whose reaction resulted in a major product of ca. 1000 bp. This had met our expectations based on the already known sequences. After having amplified the required DNA we sent it for sequencing at the local Biological Research Centre.

The sequence is reproduced in Fig.13.

TANNNNNAANNNNNNNNTGGATNRCNNNNNNNATTCTGACTTACATTACCAAATAGAG
TAGTTACTGGATCTATTTCTTGANGTTGCCGAATTTGGTAATCTATTGAAACCAGATGTG
CTTCCATTGCTGTTCTAACTTTTTCCCTAAATAGTGGTTGATGGCGGTATTCAGAGTCAT
GGGGTACAATGCTTTTTTCCCCGTTCCCTTAAAGCGATAATTCCTGTCTCTGACGAGTG
GGATTATTGCTATTTGAACTACAGGAAACCCGACTACGGCTAAGGTCAGGGTTTCTGTC
GCTTCTTCAAAATTGTTCTGAGCCTCGACATTGTAGCATACTAACCGACATTCTGCACA
TCTCCATATACTTTTACAAATTTTTACATTCCCTGGGATGTTTTGACAAAAAATCTTCACT
GTAGAAACCACTGAACCCAATAAATTAGTAAAAATTATTAATTGAATATTAAGAAGTGCAG
AAAGTGTGATATTTAAAGATATGATTGCGCGAAACCAGCAGAGCAGAGCTTAGGATGCC
ACACCCCATAAACGTTTATTGCAGATGTCTATTGACAATTACTACCTTGGTCAGGTACGA
TTAGATGTCTGGTCGTAATAGCTGAAGGGGTGACAACGAGGTTGAAATTTAAGTGAGG
TGGGGGAAAAATTTCCTCACTGTGAGCCATCAAATTAATCGACTTACTCGGCAAAAACC
AAATTCCCTCGTTATCTTTTGGCGAAAGTCTCAAGCTTTTTCCCTAAACCCGACACTCT
ACACCCTGTTTCACTTTCCATCTGCTTGTCACCCCGTCAGTCGCAATAATTATTC#TTTT
CCAAGTCGATTCTAACAATATGGACTTTCAAGATAAAAAGAACTTATCCGAAAACGACC
AGTCAATTCAAACTAGAGTTTTTAAGCTCNNASTG#TN##NAAA##AAAAANNAAAAGCT
TGAACTMNNNNNNNNNNNNNNNNNNNM

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Conclusion

The main aim was to create a bioindicator E. Coli which succesfully detects and forecast the starting of the production of microcystin toxin in the freshwater cyanobacteria Microcystis aeruginosa. By the end of the project we achieved and found out several things which can be useful in the future:

1. We started a succesful cooperation with those roleplayers in our country, who are concerned by algal blooming and the toxin production of cyanobacteria.
2. We succesfully designed and created four biobricks for detecting toxin-related enzyme-production and succesfully transformed four E.coli strains with these biobricks.
3. The results confirms that Biobricks 'A' and 'B' are sufficient for detecting the starting point of the toxin production. In the future, the bacteria that contain these BioBricks could be used in our device.
4. The Biobricks A's and B's final scores suggest that the older is the algal culture the smaller is the tempereature's effect on lysate - medium difference. The hypothesis on how the signal of toxin production is generated and provided to the algal cells is still not answered, but The above conclusion seems to strengthen our hypothesis, that the toxin production signal synthesis starts in the cytosol of the algal cells, than it is exported to the environment. The export seems to be accelerated with the increasing population density.
5. We succesfully amplified by PCR the variable sequence of the Hungarian subspecies of Microcystis aeruginosa BGSD 243. Our primer design also confirms the hypothesis, that the toxin-related enzyme coding sequences are conservative. Based on the sequence we found the closest relatives of the Hungarian strain which is Microcystis pannfiromis.

BioBrick C and D didn't provided sufficient difference between the algal medium's and the algal cell lysate effect on their transcription. Compared with the low raw values of fluorescence intensity it is probably mean that these Biobricks are not appropriate to detect microcystin production. In the future, we would like to investigate the expression of A-C and D-J genes. We want to know whether the expression of the A-C and D-J genes happens in the same time or not. For this purpose, we plan to create similar BioBricks to BioBrick C and BioBrick D, but instead of the GFPs, we would use a GFP and an RFP(red fluorescent protein) gene.

Because of the doubt about the signal of toxin production, we plan to transform the biobricks into algae or use a cell-free protein synthesis system, so we could avoid any doubt about the entering of the signal molecules.

Fortunately, we managed to sequence the promoter sequence of a hungarian strain, so we would like to create our biobricks with this promoter and repeat the measurements.

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