Stockholm Junior Water Prize-Hungarian competition, 2020

Detecting the toxin production of the Microcystis species in hungarian lakes

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Short summary:

In our project we wanted to deal with an issue on environmental protection that affects Hungary. Our main objective was to develop an efficient way to follow the toxin production during algal blooming at an early stage.

We would like to detect the starting point of microcystin production of blue-green algae namely the toxic Microcystis sp. population.

Microcystin is synthesized non-ribosomally by 10 genes.We have designed four biobricks. We used the promoter of the genes, but instead of the genes we used GFP to predict the transcription of the genes responsible for microcystin synthesis. We measured the fluorescence density that was emitted by the GFP when the toxin production had started.

Intoduction:

My name is Alexandra Gyémánt and I am 16 years old. I'm studying in a biology oriented class, because in the future I want to work as a doctor. I'm interested in molecular biology and genetics. This project was really interesting for me, because I could see the steps of a research and also try new methods and labor equipments.

My name is László Török.I am 16 years old. I'm intrested in cell biology and genetics. In the future, I want to be doctor or a researcher, that's why I want to study in medical school. In this project, I was able to gain insight into the course of a research. This could help me in the future if I want to start my own research.

We have made this project for the IGem (International Genetically Engineered Machine) competition with our schoolmates and our mentor, Sándor Bán. This is the link of our website: https://2019.igem.org/Team:SZTA_Szeged_HU .

Summary:

In our project we wanted to deal with an issue on environmental protection that affects Hungary. Our main objective was to develop an efficient way to follow the toxin production during algal blooming at an early stage.

We would like to detect the starting point of microcystin production of blue-green algae namely the toxic Microcystis sp. population. We would like to create a biosensor which can detect the starting point of microcystin toxin production via green fluorescent protein fluorescence.

Several researchers told us that the main problem of water blooming is caused mainly by Microcystis aeruginosa, which is a prokaryotic blue green alga species. The algae start blooming when certain circumstances such as light, temperature, nutrients are adequate. In Hungary, the most important factor is the temperature: when water temperature rises above 24 degrees for some days, these bacteria increase their density very aggressively. Meanwhile many of them is going to produce toxins, especially microcystin that is a cyclic hepatotoxin.

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 a necessity to make our constructs. As a result, we searched for this particular sequence in a DNA database, then we noticed that its putative promoter is variable between the strains of Microcystis aeruginosa. Due to this, we made an alignment to see how should the consensus sequence look like from the promoter of nine different Microcystis aeruginosa and we got that the most similar to the consensus sequence is the Microcystis aeruginosa NIES-843

We have designed four biobricks. Two of them consist of the following parts: at the beginning, there is the putative promoter where we placed an RBS followed by GFP gene. Both biobricks contains the same parts, however the putative promoter was reversed in one of them, thus we can see whether the transcription occurs in both directions or not. The other two 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 transcription starts in both directions at the same time. In these two constructs, the order of the two GFPs were changed.

We grew colonies from Microcystis aeruginosa in order to detect the presence of microcystin toxin. We had chosen this algae because it has high toxin production. By this procedure, we can demonstrate in which development phase of the colony the toxin can be detected.

Our measurement goals were to check if any of our biocbrick is able to detect the starting point of microcystin production and answer the hypothesyis of the regulation of toxin production (to see whether it is regulated by a signal molecule or by an environmental condition). For this purpose, we used only the algae liquid or the liquid and the cell lysate of the alga cultures in different experimental groups. We measured the different light density in the experimental groups with a simple fluorometer.

We created four biobrick that could monitor the microcystin production, we cloned and transformed succesfully them into E.coli. Furthermore, we sequenced the promoter region of a hungarian Microcystis strain that is called Microcystis aeruginosa BGSD 243.

In the future, we would like to make further investigation about the transcription of the mcyABC and mcyDEFGHIJ. Plus we want to make biobricks with the hungarian species' promoter that we have senquenced.

OUR PROJECT

Inspiration

In our project we wanted to deal with algal blooming. Algal blooming is a global problem affecting both natural and artificial lakes. These lakes are very important for the tourism because a lot of visitors come every year to enjoy the beauty of Hungarian lakes and a wide range of spa services. It creates a huge income for the country. But there is another economic aspect where natural lakes and artificial fishponds are important and that is fishing. Moreover, the consumption of freshwater fish meat increased twice as it was 10 years ago thanks to some government actions which aim to improve quality of the food basket of the population. Regarding ecological aspects, we can say that maintaining the equilibrium is crucial to keep species in a given ratio in these lake.

Algal blooming is the excessive increase of algal population in marine water and freshwater. This process leads to numerous negative and harmful consequences. As the algae cover the water surface, the water transparency decreases significantly and light could not reach the deeper water-layers, which results the destruction of the aquatic vegetation. This conduces to the depletion of dissolved oxygen, the consequences of which can be considerable fish die-offs and on a longer view the eutrophication and siltation of lakes. Besides there are many algae species which can secrete harmful toxins.

On the photo there's the first signs of algal blooming.The water turns into greenish blue and the algae merge into light green colonies.

In the picture we can see algal blooming's late estate. There's the green area that appears when the bloom starts. 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...).

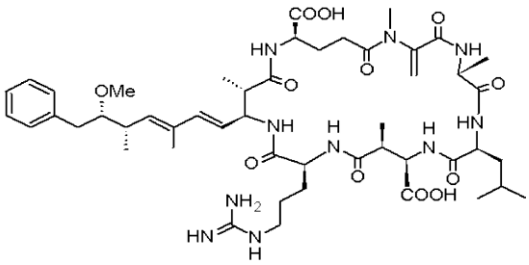
Main goals:

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

First steps:

You have better chances to prevent harmful algal blooming if you perceive it in an early stage. So that's the reason why we would like to make a system which can detect the toxin production in an early stage. We contacted two leading researchers of algal blooming, Ms. Prof Judit Padisák who examine the algae species of the largest lake, Balaton and with Mr. Prof. Gábor Vasas who examine the algae species of the lakes of the Great Hungarian Plain. Their contribution and the studying of the scientific literature also suggested that Microcystis aeruginosa and Microcystis flos-aquae are the main cause of the algal blooming in our country. Several subspecies of Microcytis aeruginosa are well known by marine ecologists, as well. 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 for vertebrates (fishes and humans), especially for the liver that's why the toxin called as a hepatotoxin. This is synthetized in a non-conventional way with the help of ten enzymes. The genes of the enzymes are in a bidirectional arrangement. However, there is no proven regulation pattern yet for this double operon.

As you can see on this picture, the putative promoter of the original operon is found between toxin producing protein genes. For getting this sequence we used Ensemble bacteria, where we could find 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 made an alignment to become aware of the consensus sequence. This means the program aligns the sequences and it chooses the base, which appears most often in them. We ran a blast from this and as a result we noticed that 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 a necessity to make our constructs. As a result, we searched for this particular sequence in a DNA database, then we noticed that its putative promoter is variable between the strains of Microcystis aeruginosa. Due to this, we made an alignment to see how should the consensus sequence look like from the promoter of nine different Microcystis aeruginosa and we got that the most similar to the consensus sequence is the Microcystis aeruginosa NIES-843, therefore we have synthesized our biobricks based on this sequence.

We have designed four biobricks each appropriate to predict the transcription of the genes responsible for microcystin synthesis. 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 contains the same parts, however the putative promoter sequence was reversed in one of them thus we can see whether the transcription occurs in both directions or not.

Biobrick A

Biobrick B

The other two 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 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 sequence after GFPs. These two constructs were made with the original promoter sequence, but the order of

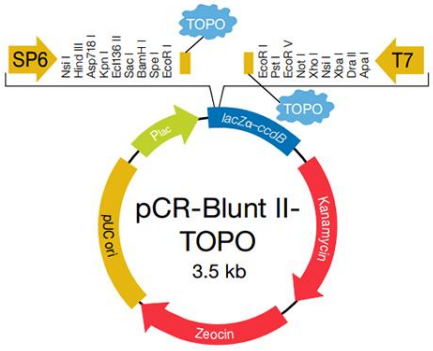
the two GFPs were changed in the two constructs. Genes from fluorescent proteins will be expressed in the engineered Escherichia coli bacteria.

Biobrick C

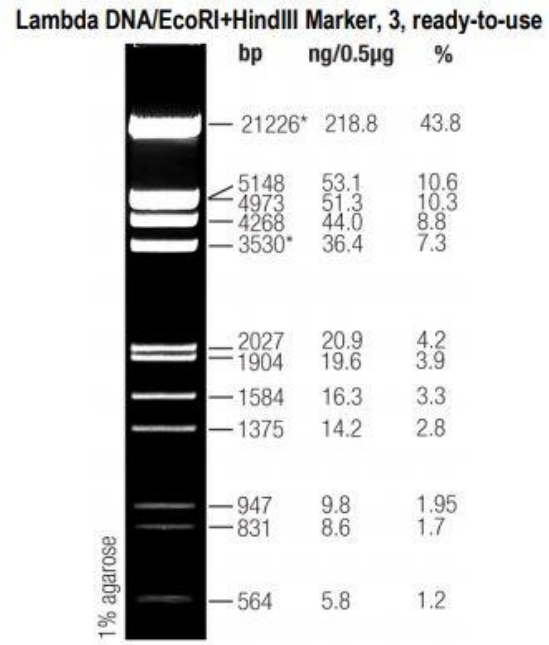
Biobrick D

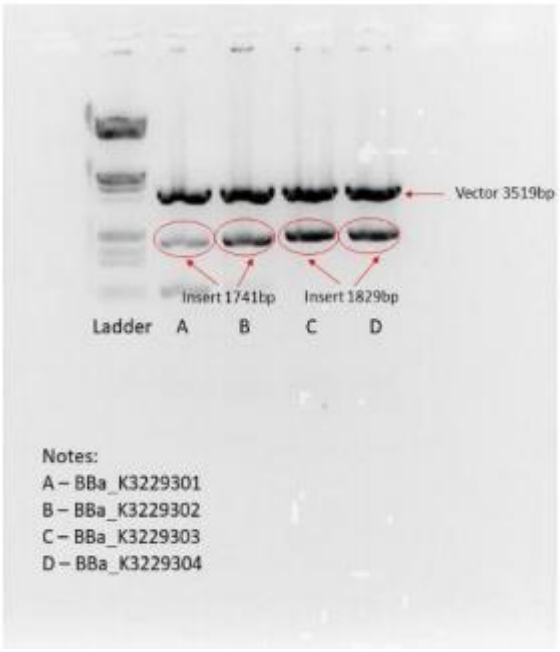
Transformation:

Our biobricks were synthesized by Twist Bioscience and IDT, they checked them with sequence analysis. We attached the DNA molecules into plasmids with the Zero Blunt TOPO system from the Invitrogen company.



We used gel electrophoresis to inspect the infiltarion of the biobricks into the plasmids. After we transformed the plasmids into NEB-5 alpha E.coli from the New England Biolabs. After we amplified the transformed bacteria with selectable markers. We used these for our experiments.

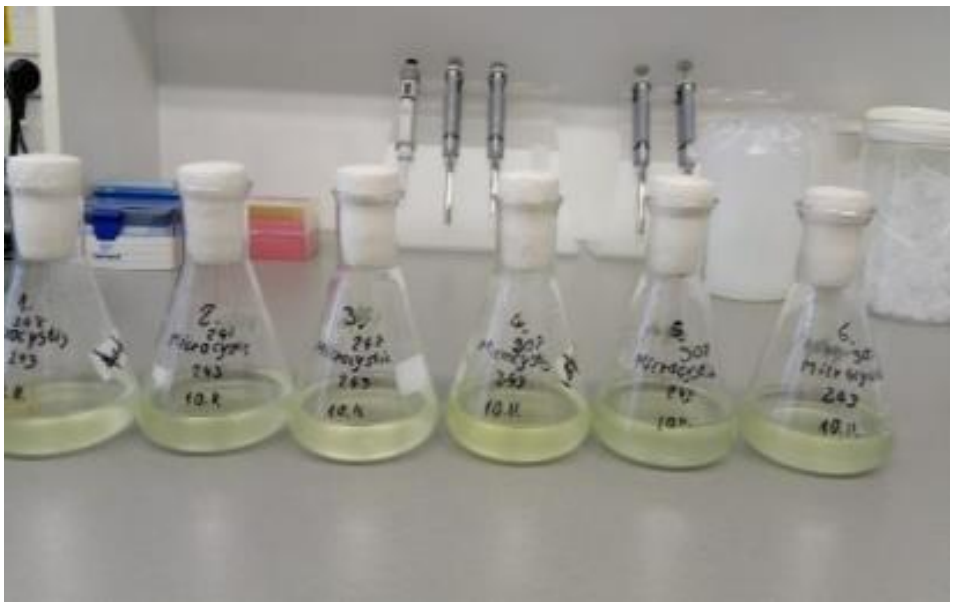




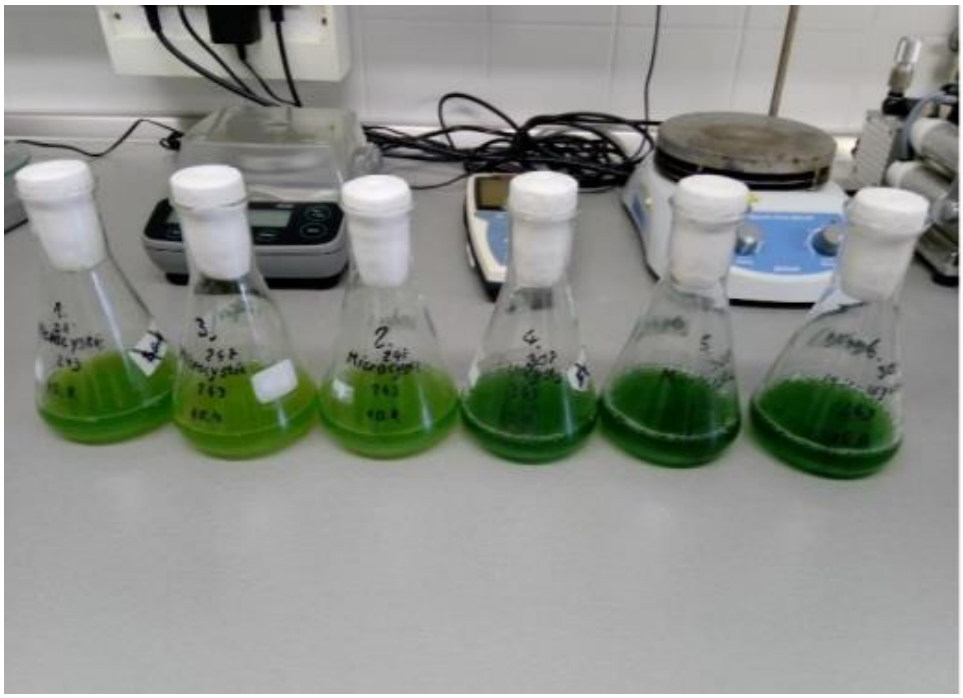
Alga cultures:

We grew colonies from Microcystis aeruginosa (BGSD 243) in order to detect the presence of its microcystin toxin. We had chosen this algae because it has high toxin production. By this procedure, we can demonstrate in which development phase of the colony the toxin can be detected. We have done these procedures in the Institute of Plant Biology of the Biological Research Center in Szeged. We cultivated 3-3 colonies at 24 (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.

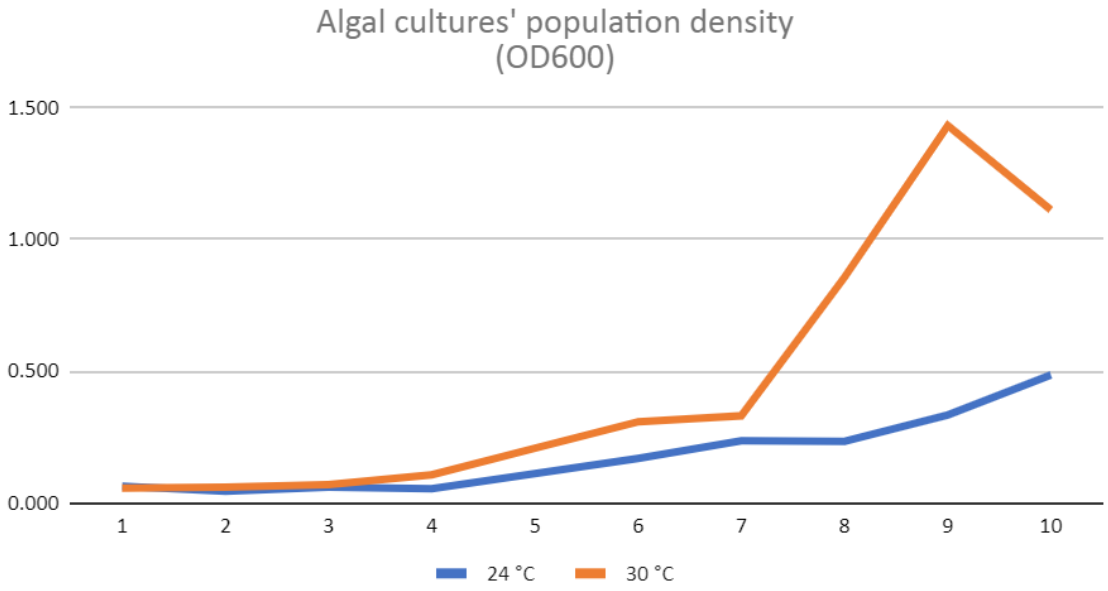
We took a 2-ml sample from each colony every day, for 10 days at the same time.We froze the samples at -20 Celsius.A total of 60 samples were taken for later experimentation.



As the time passed difference could be discovered in the colour of the colonies grown at different temperatures.



In the diagram, you can see that the algae held at 30C started blooming at the 8th day. This was also manifested in different colors of the cultures at the two different temperatures.



Measurement:

Our measurement goals were:

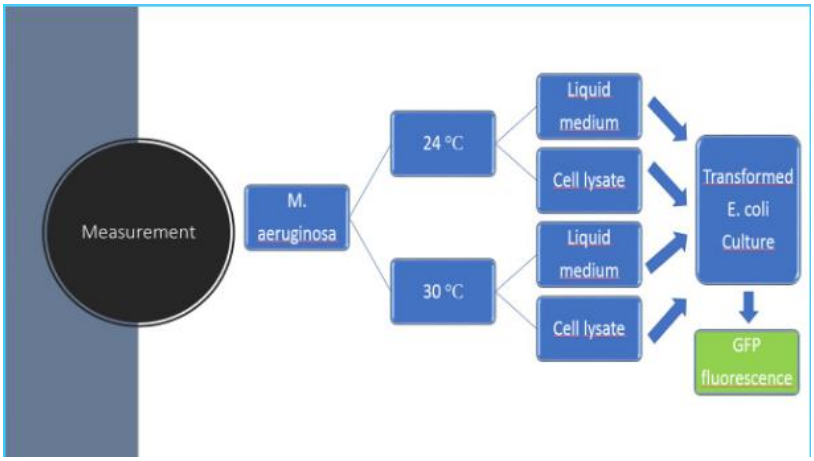
to check whether our product will be able to predict the starting point of toxin production.

to clarify a hypothesis on how the signal of toxin production is generated and provided to the algal cells.

We mentioned previously that the bidirectional promoter is called as ‘putative’ in the 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 these promoters can be 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 check mainly the latter one. The most probable is that the switch-on-molecule-signal is produced by the algal cells themselves. Assuming this, there are still two opportunities remaining: the signal molecule is produced at a certain point of the algal blooming is intracellular, or it is exported outside from the algal cells to trigger other cells to start producing the toxin.

The measurement design aimed to check both opportunities. We used samples from our algal cultures: the medium and the lysed algal cells were added to liquid cultures of transformed E. coli. We waited one day, and then checked the GFP production of each cultures.



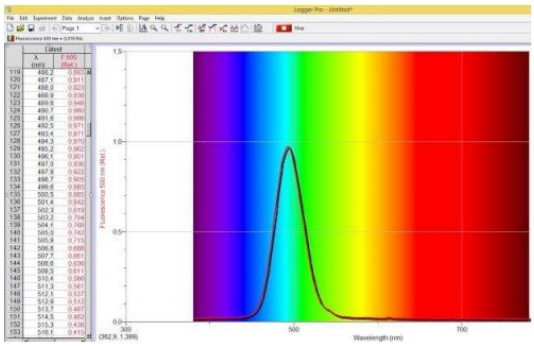
Nevertheless, the fluorescence measurement wasn't so simple, as we face to two problems to be solved:

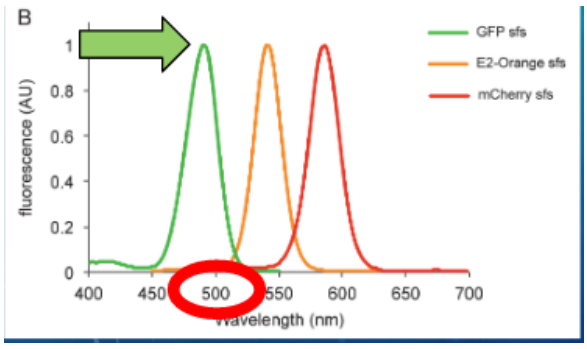
In our school lab there is no fluorescence microplate reader appropriate to measure GFP concentration directly. Of course, we had the opportunity to use sophisticated equipment at the local Biological Research Centre, but we felt, that it would be a great contribution to the iGEM community, especially to future iGEM teams, if we demonstrated, that a widespread high school device can still be appropriate for measuring GFP, as well.

Moreover, we had to measure not the fluorescence of a pure GFP solution but the fluorescence of the whole bacterial cells themselves. Therefore, we looked at the scientific literature, where we found that the GFP excitation spectra is quite different if measured in whole bacterial cells, which is called RED SHIFT.

The device we used is the widely known type of fluoro-spectrophotometer used in high schools. It has two inbuilt excitation wavelength: 450nm and 500 nm. The scientific literature and the iGEM Registry page of BBa_E0040 suggest the 504 nm, but the 500nm still has approximately 90 % efficiency, which at high fluorescent level produces more than satisfactory signal.

Firtsly we used the iGEM GFP calibration package to create GFP producing bacteria as reference. We recorded the emission spectrum of those bacteria and found that it is quite like the spectrum in the article, we mentioned earlier.

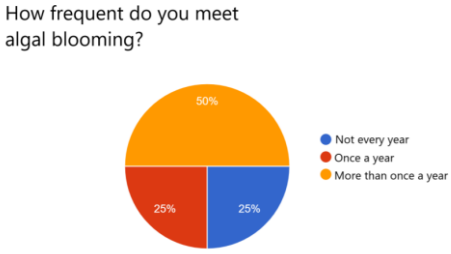


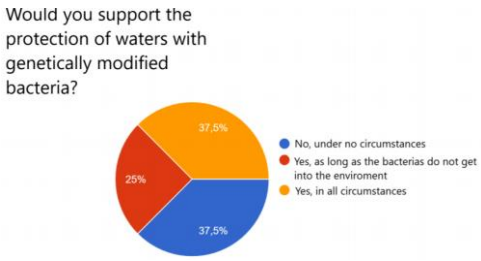


Human practises:

We found it beneficial to contact Hungarian fresh-water-authorities, algal researchers and fishpond-owners who have experienced the negative effects of water blooming. On one hand, by directly speaking to these role-players and sending our questionnaire to other Hungarian and foreign professionals we become aware of 10-20% percentage of their annual expenditures is spent on interfering with water blooming. In addition, they all confirmed that it would be crucial to detect the formation of algal blooming and its consequences at an early stage. Having such a system would help them acting rapidly, which may lead to smaller amount of chemical usage stopping further blooming and a faster restoration of ecological equilibrium which usually needs years.

Here are the results off the questionare:





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 Practises:

As we wanted 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 the Lake Velencei concerned often by algal blooming. The sequence of its genes that we used during our project were still unknown. So we decided to sequence the promoter region of mcy gene promoters of this strain. In order to gain the sequence, we amplified the corresponding DNA from the algal's genome with PCR.

Though the mcy promoter region is very variable in different species/strains, the mcyA and mcyD genes are conservative, which we utilized for designing the primer pair for the PCR. In order to ensure the success of the amplification we used touchdown PCR method with decreasing annealing temperature. We visualized the product using gel electrophoresis. The reaction provided a major product of ca. 1000 bp, which 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. You could see the sequence here:

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

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Acknowledgement:

First and foremost we are grateful for the help of our primary instructor Sandor Ban (Biology teacher, the head of the School District's Science Laboratory) who provided everything we needed to execute our project successfully.

We would like to say special thanks to Szilvia Zita Tóth Ph.D.senior research associate, head of research group institute of Plant Biology and Laboratory of Molecular Photobioenergetics. In addition, Dr. Gábor Vasas head of the Department of Botany in University of Debrecen and the Department of Pharmacognosy. They provided us valuable help during planning and carrying out our experiments. They showed us the way of plasmid designation and also arranged us to be able to use the modular broad-host-range expression vectors, which were baselines in our project. Furthermore, we would like to express our gratefulness to Andrea Borbola, the assistant manager of our school laboratory and her collegue Eniko Gulyas lab assistant who also meant a valuable help for us. They supported our project with their essential pieces of advice and were always there, even on Sunday afternoons, when we needed help. We would also like to say thanks to those who work in Szeged's Biology Research Centre. They highly contributed to our project. Dr. László Kovács and Anna Podmaniczki helped us in sampling, whereas Dr. Meireles André Vidal played part in culturing the needed bacterias.