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Growth of *Synechocystis sp.* for plant biostimulant production

Bachelor's thesis

Biological Systems Engineering – EEABB

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09 June 2023

Abstract

Agriculture is affected by climate change, which directly and negatively affects crops. In addition to this problem, there are more and more people in the world and the world does not have enough resources to support us all. Therefore, we have a duty to try to solve this problem.

Cyanobacteria need nutrients to grow, such as nitrogen and phosphorus. These nutrients can come from secondary effluents from sewage treatment plants. By feeding these effluents to cyanobacteria, we would be using waste as a resource.

In addition, cyanobacteria have a biostimulant activity because they themselves produce phytohormones that help plants grow, so when applied to plants, they will grow faster and increase resources.

The project has three main parts. The first part consisted of growing the *Synechocystis sp.* culture with wastewater in a photobioreactor and measuring its productivity, which was 74 $mg \cdot L^{-1} \cdot d^{-1}$, as well as monitoring nutrients and growth parameters.

In the second part of the project, the best method to lyse the cell of *Synechocysti sp*. to make the phytohormones accessible was investigated. The method chosen was the sonicator, which was subsequently optimised by adding fewer cycles, in total 10 cycles were chosen.

Finally, the last part of the experiment was based on a series of bioassays with seeds and seedlings from *Synechocystis sp*. grown in a BG-11 medium, in order to know the biostimulant effect of these cyanobacteria and to quantify it. On average, *Synechocystis sp*. caused an effect between 1 ppm and 5 ppm of cytokinins.

Resum

L'agricultura es veu afectada pel canvi climàtic, que afecta directament i negativament als cultius. A més d'aquest problema, cada vegada hi ha més gent en el món i aquest no té recursos suficients per a mantenir-nos a tots. Per tant, tenim el deure d'intentar resoldre aquest problema.

Els cianobacteris necessiten nutrients per a créixer, com el nitrogen i el fòsfor. Aquests nutrients poden procedir d'efluents secundaris de plantes de tractament d'aigües residuals. Alimentant als cianobacteris amb aquests efluents, estaríem utilitzant un residu com a recurs.

A més, els cianobacteris tenen una activitat bioestimulant perquè elles mateixes produeixen fitohormones que ajuden a les plantes a créixer, per la qual cosa, en aplicar-les a les plantes, aquestes creixeran més ràpidament i augmentaran els recursos.

El projecte consta de tres parts principals. La primera va consistir a fer créixer el cultiu de *Synechocystis sp.* amb aigua residual en un fotobioreactor i es va mesurar la seva productivitat, que va resultar ser 74 $mg \cdot L^{-1} \cdot d^{-1}$, a part es va fer un control dels nutrients i dels paràmetres de creixement.

A la segona part del projecte es va tractar d'esbrinar quin era el millor mètode per a lisar la cèl·lula de *Synechocysti sp.* per a fer accessibles les fitohormones. El mètode triat va ser el sonicador que posteriorment es va tractar d'optimitzar el procés afegint menys cicles, en total es va optar per fer 10 cicles.

Finalment, l'última part de l'experiment es va basar a realitzar una sèrie de bioassajos amb llavors i plàntules a partir de *Synechocystis sp*. cultivat en un medi BG-11, amb la finalitat de conèixer l'efecte bioestimulant d'aquests cianobacteris i quantificar-lo. De mitjana, el *Synechocystis sp*. va causar un efecte entre 1 ppm i 5 ppm de citoquinines.

Resumen

La agricultura se ve afectada por el cambio climático, que afecta directamente y negativamente a los cultivos. Además de este problema, cada vez hay más gente en el mundo y este no tiene recursos suficientes para mantenernos a todos. Por lo tanto, tenemos el deber de intentar resolver este problema.

Las cianobacterias necesitan nutrientes para crecer, como el nitrógeno y el fósforo. Estos nutrientes pueden proceder de efluentes secundarios de plantas de tratamiento de aguas residuales. Alimentando a las cianobacterias con estos efluentes, estaríamos utilizando un residuo como recurso.

Además, las cianobacterias tienen una actividad bioestimulante porque ellas mismas producen fitohormonas que ayudan a las plantas a crecer, por lo cual, al aplicarlas a las plantas, estas crecerán más rápidamente y aumentarán los recursos.

El proyecto consta de tres partes principales. La primera consistió a hacer crecer el cultivo de *Synechocystis sp.* con agua residual en un fotobioreactor y se mesuró su productividad, que resultó ser 74 $mg \cdot L^{-1} \cdot d^{-1}$, aparte se hizo un control de los nutrientes y de los parámetros de crecimiento.

En la segunda parte del proyecto se trató de averiguar qué era el mejor método para lisar la célula de *Synechocysti sp.* para hacer accesibles las fitohormonas. El método elegido fue el sonicador que posteriormente se trató de optimizar el proceso añadiendo menos ciclos, en total se optó para hacer 10 ciclos.

Finalmente, la última parte del experimento se basó a realizar una serie de bioensayos con semillas y plántulas a partir de *Synechocystis sp*. cultivado en un medio BG-11, con el fin de conocer el efecto bioestimulant de estas cianobacterias y cuantificarlo. De media, el *Synechocystis sp.* causó un efecto entre 1 ppm y 5 ppm de citoquininas.

Summary

List of figures

List of tables

Symbols and acronyms

Abs	Absorbance
AUX	Auxin
BG-11	Prepared medium called BG-11
COD	Chemical Oxygen Demand
d	Day
DO	Dissolved oxygen
DF	Dilution factor
g	Gram
GEMMA	*Environmental Engineering and Microbiology Group*
h	Hours
IAA	Indole-3-acetic acid 98%
KIN	Kinetin
klx	Kilolux
L	Litre
LR	Low range
mg	Milligram
min	Minutes
mL	Millilitre
$N\text{-}NO_3$	Nitrate
$P\text{-}PO_4$	Phosphate
PBR	Photobioreactor
SD	Standard deviation
TIN	Total Inorganic Nitrogen
TSS	Total Suspended Solids
UD	Undiluted
VSS	Volatile Suspended Solids
°C	Degrees Celsius

Acknowledgements

First of all, I would like to thank Teresa Balanyà for agreeing to be my tutor and providing me with the necessary knowledge to carry out the thesis correctly and the effort she has put into making this project a success.

I would also like to thank the GEMMA Group for giving me the opportunity to participate in this project. I would like to thank all the doctoral students for being so kind to me and especially to Ana Álvarez, my external tutor, for all the effort she has made for this project to go ahead and have significant results, as well as for trusting me.

And finally, I would like to thank my family and friends for giving me the emotional support I need to finish this project with the same enthusiasm and energy with which I started.

1. Introduction

The growing world population is a fact that we cannot hide. From the website *Peace, dignity and equality on a healthy planet* of the United Nations it can be read this extract ''*The world's population is expected to increase by 2 billion persons in the next 30 years, from 7.7 billion currently to 9.7 billion in 2050 and could peak at nearly 11 billion around 2100*'' (United Nations, n.d.). In essence, as the world's population grows, less resources will be available. As a global necessity, plant biostimulants could be one way to help in this fight. Cyanobacteria, photosynthetic microorganisms, are a natural source of plant biostimulants (Santini et al., 2021). Secondary metabolites produced by cyanobacteria may or may not be harmful. Phytohormones, siderophores, and different UV-protective substances are examples of non-toxic secondary metabolites (Haque et al., 2017). Phytohormones are significant plant growth and development regulators and signals. (Sergeeva et al., 2002).

Besides, we need to take care of the environment and to be as sustainable as possible. Nitrogen and phosphorous are the main nutrients needed for the cultivation of cyanobacteria. In a circular economy approach, cyanobacteria can be grown in secondary wastewater, reducing the concentration of those nutrients in the waste and obtaining a biomass, which might be further used for the obtention of bioproducts.

Since the project is based in plant biostimulants obtained from the breakage of the cyanobacteria *Synechocystis sp*. I will explain all them in different subsections.

1.1. Plant biostimulants

The definition of a plant biostimulant is: ''Any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content.'' (du Jardin, 2015).

The plant biostimulants can be divided into three groups, humic substances (HS), hormone containing products (HCP) and amino acid containing products (AACP).

HCPs, like seaweed extracts and cyanobacteria, have quantifiable concentrations of active plant growth factors (known as phytohormones) like auxins, cytokinins, or their derivatives (du Jardin, 2015). It is well known that a group of five structurally simple phytohormones may frequently affect plant growth, which are the auxin, the ethylene, the cytokinin, the abscisic acid and the gibberellin (Palme et al., 1991). In this project made, the two hormones studied as a biostimulants are the auxins and the cytokinins commented below.

Auxin was the first growth hormone to be discovered in plants (Taiz, 2010). Auxins are a group of phytohormones, plant hormones, that are active in a variety of plant developmental processes (Tan et al., n.d.). Moreover, auxin regulates a variety of developmental processes, including stem lengthening, apical dominance, root initiation, fruit development, blooming, senescence, abscission and directed, or tropic, growth (Taiz, 2010). In addition, the auxin most frequent and physiologically significant is indole-3-acetic acid (IAA) (Taiz, 2010).

Cytokinins, which is another phytohormone, are related to the stimulation or inhibition of a variety of developmental processes, including root growth and branching, apical dominance regulation in the shoot, chloroplast formation, and leaf senescence (Werner et al., 2001). The synthetic kinetin was the first cytokinin to be found (Taiz, 2010).

1.2. Cyanobacteria

The cyanobacteria are photosynthetic prokaryotes (Whitton & Potts, 2012). The larger part of cyanobacteria are aerobic photoautotrophs capable of photosynthesis that produces oxygen (Chorus & Bartram, 1999). They get energy through photosynthesis so their needs are light, carbon dioxide, inorganic nutrients and water (Chorus & Bartram, 1999). You can find cyanobacteria in a wide range of possibilities: in salty water, brackish or fresh water, cold or hot springs. Their featured habitat is limnic and marine environments (Chorus & Bartram, 1999).

There are some species that has the potential to fix N_2 in an oxygenated environment (Whitton & Potts, 2012). Moreover, the key growth parameters for cyanobacteria tend to be light irradiance, ambient temperature, pH, inorganic carbon concentrations and nutrition levels (Sheng et al., 2011). The various inside components of the cyanobacteria are shown in Figure 1.

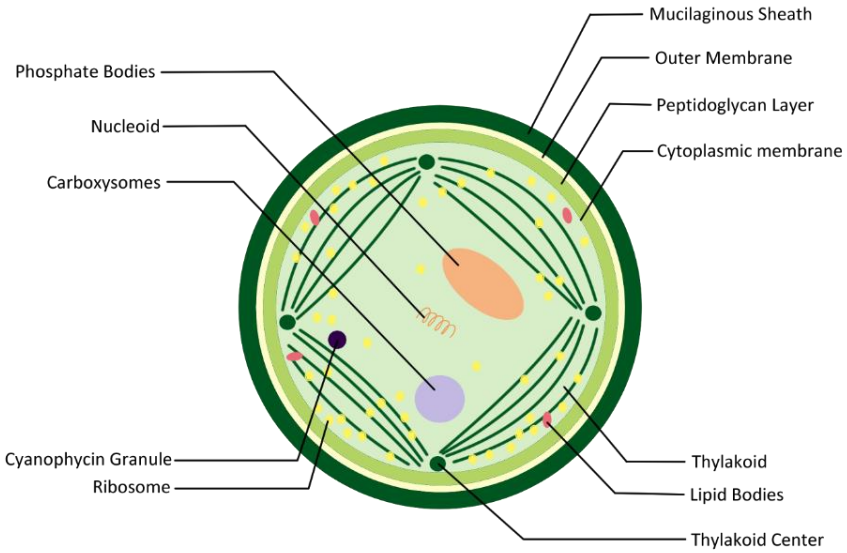


Figure 1. Schematic cyanobacteria parts.

1.3. Synechocystis sp.

The *Synechocystis sp.* (Figure 2) belongs to the cyanobacteria phylum. It has significant potential for large-scale biomass production due to its rapid growth rate, naturally high lipid content, capacity to be genetically modified, and resilience to a variety of environmental conditions (Sheng et al., 2011). The optimal growth temperature of the *Synechocystis sp.* is at 35 °C (Zavřel et al., 2017). The specific growth rate and the lipid concentration were considerably impeded by a higher temperature of 44 °C and lower temperatures of 22 °C and 18 °C. (Sheng et al., 2011).

This species develops under a variety of physiological settings, is small (~1.5 µm in diameter), spontaneously transformable, and integrates foreign DNA into its genome by double-homologous recombination (van de Meene et al., 2006).

Henceforth, this species will be referred to as cyanobacteria, as well as to its own name.

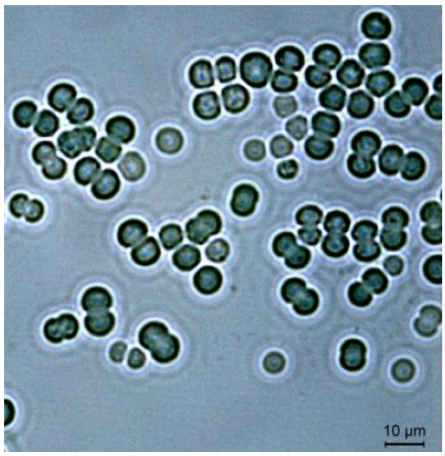


Figure 2. Selim, Khaled. (2018). Microscopic image of cells of the cyanobacterium *Synechocystis sp.* PCC6803. https://uni-tuebingen.de/en/newsfullview-landingpage/article/newly-discovered-protein-operated-in-earliest-organisms/

2. Background

My bachelor's thesis is a small part from a whole amount of researched done in the GEMMA group. I could not have done this part without the previous investigation done by the group.

The aim of group GEMMA is to promote sustainable development. Therefore, water reuse and efficiency, as well as algal-based technologies for wastewater treatment and biomass generation, are some of the key study issues. So, they work with algae and cyanobacteria.

This whole project comes from a research line with cyanobacteria. Moreover, group GEMMA is trying to study different bioproducts that comes from a specific cyanobacteria called *Synechocystis sp.* In addition, previous students have seen that the productivity of the cyanobacterium *Synechocystis sp.* is higher than the other species they have in the laboratory and it is also less susceptible to be contaminated by external wastewater pollutants. This is why *Synechocystis sp.* was the species chosen to carry out this project.

3. Objectives

3.1. General

The purpose of this study is to grow *Synechocystis sp.* using treated wastewater and to study plant biostimulant activity of the biomass obtained.

3.2. Specific

- Succeed in producing and growing the cyanobacterium *Synechocystis sp.* in a photobioreactor (PBR) fed with treated wastewater. The wastewater comes from the secondary effluent of the ''EDAR del Besòs'' and an Activated Sludge Wastewater Treatment located in the UPC in the Campus Nord.
- Identify the best way to extract the plant biostimulants from the cell of cyanobacteria.
- Find the best method and determine plant biostimulant activity.

4. Material and methods

4.1. Study approach

First part: Growth of the *Synechocystis sp.* in secondary treatment wastewater and depuration control

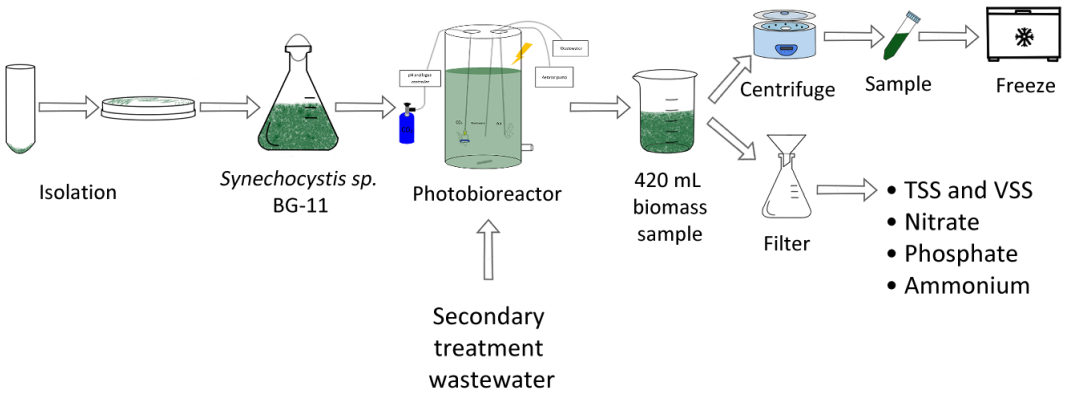


Figure 3. Schematic of the first part of the project. First, isolate *Synechocystis sp.* Second, grow the species in BG-11 (inoculum). Third, grow the species in a photobioreactor by adding treated wastewater, light, oxygen and CO_2 when necessary. Forth, with the 420 mL of sample, filter and measure TSS, VSS, nitrates, phosphates and ammonium and with the reminder, centrifuge and freeze the remaining.

In the first part of the project (Figure 3), different actions have been carried out:

- Maintenance of inoculum on solid synthetic medium, BG-11 (Section 4.3.1.), in Petri dish (isolated by a Group GEMMA PhD student).
- Growth of the inoculum on liquid synthetic medium, BG-11 (Section 4.3.1.), in Erlenmeyer flasks.
- Assembling a photobioreactor (PBR) with all its parts.
- Inoculum cultivation in a PBR with wastewater during 25 days.
- Maintain the continuous photobioreactor at a flow rate of 420 mL/d.
- Analysing biomass through the microscope three times per week.
- Measurement of nitrate, phosphate and ammonium of PBR three times per week. Also, wastewater is analysed but only when a new bottle is started.
- Measure total suspended solids and volatile suspended solids of PBR three times per week. Also, wastewater is measured but only when a new bottle is started.
- Centrifuge the biomass of the PBR and freeze it in order to preserve it.

Second part: Extraction of biostimulants

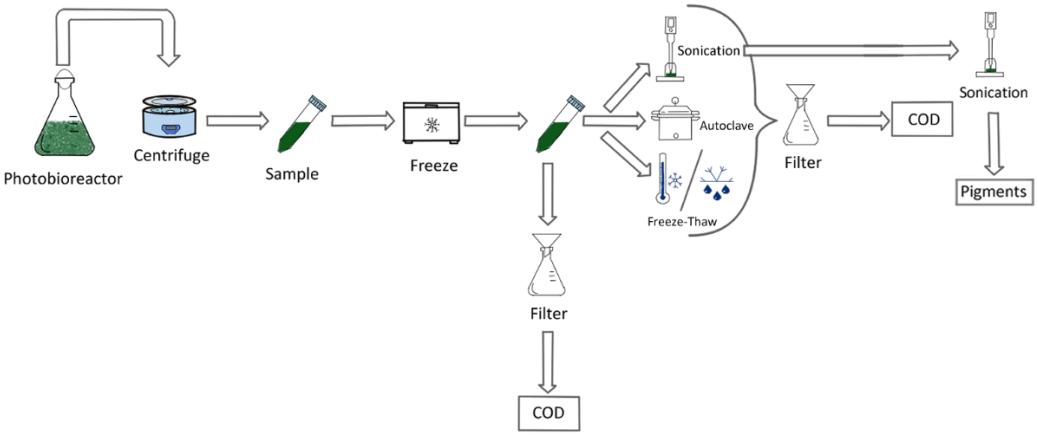


Figure 4. Schematic of the second part of the project (extract biostimulant).

The Figure 4 illustrates the second phase of the project, where several activities have been taken:

- Analyse the best method for extracting plant biostimulants. The COD on the filtrate will be used to assess the degree of cell lysis.
- Sonicate the biomass of the BG-11 (Section 4.3.1.) inoculum in order to get the plant biostimulants. In order to optimize this process and achieve the same COD objectives, the pigments from cell lysis (phycocyanin and allophycocyanin) have been determined.

Third part: Studying biostimulant activity

On this part of the study, firstly a toxicity protocol has been implemented in order to identify if the medium itself is toxic to the plant. Then, the inoculum biomass of *Synechocystis sp.* has been subjected to a variety of bioassays in order to study the auxins and cytokinins effects on plants.

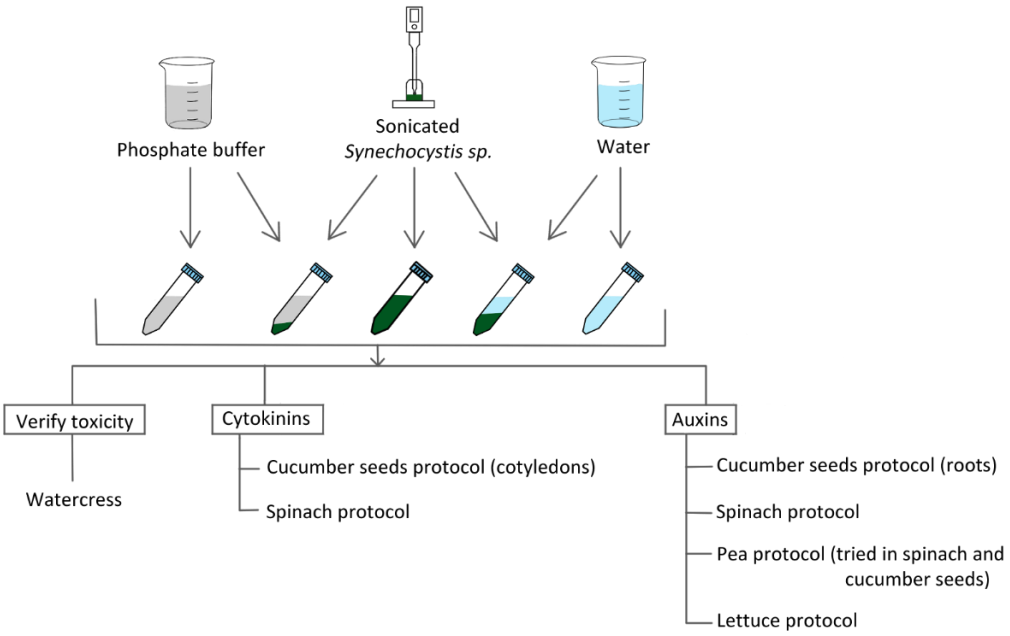


Figure 5. Schematic of all protocols used to study the different phytohormones (cytokinins and auxins) using the different controls (water and phosphate buffer) and extracts (sonicated *Synechocystis sp.* in different concentrations and with phosphate buffer).

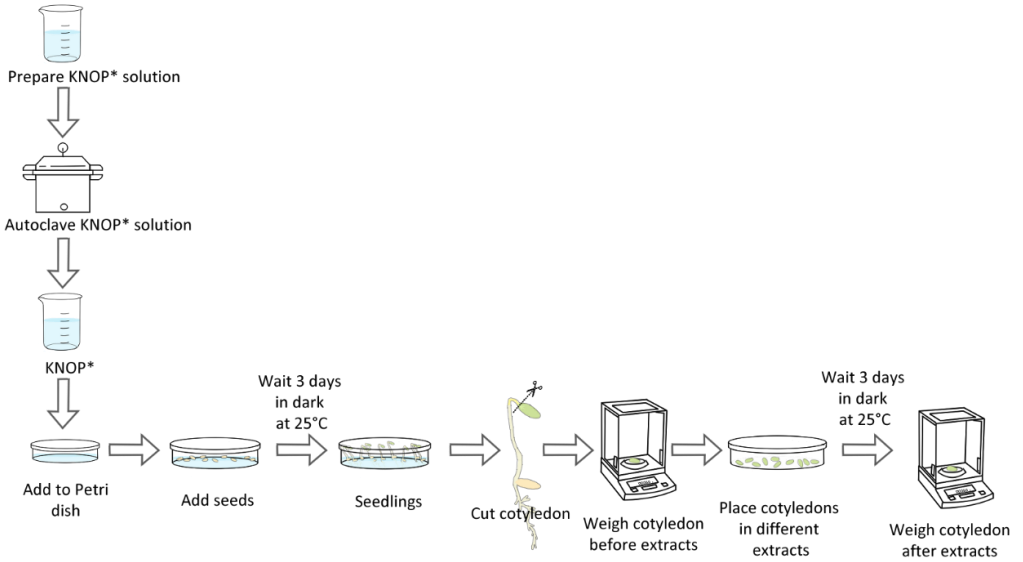


Figure 6. Schematic of the third part of the project (cytokinin activity in cucumber cotyledons).

Several actions have been carried out during the third phase, which is depicted in Figure 5, although Figure 6 shows the final protocol used, to which the results of the study belong:

- Identify if the medium is toxic.
- Measurement of the plant biostimulant activity by different bioassays.
- Use of statistics to analyse the final data.

The main treatments carried out in the two trials were:

Table 1. Treatments used in the project and their nomenclature.

Treatment	Nomenclature
Control with deionized water	Cw
Control with phosphate buffer	Cb
Control with BG-11	Cbg11
Sonicated biomass	SB
Sonicated biomass with phosphate buffer (9:1, buffer:extract)	SBb
Supernatant of SB centrifugated at 7000 rpm for 14 minutes (Figure 7)	SN

Synechocystis sp. biomass was always sonicated at 30 W, 53% amplitude, 100% pulse and 10 cycles and, in addition, it was first frozen and then thawed once.

In these bioassays, different controls in particular deionized water, BG-11 (Section 4.3.1.) because it is the medium where *Synechocystis sp.* is grown, and phosphate buffer to ensure that the pH of the solution is 7, which is a neutral pH. Moreover, different concentrations of sonicated *Synechocytis sp.* and its supernatant (Figure 7) have been used.

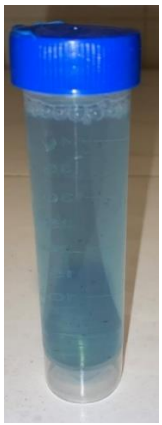


Figure 7. Supernatant after sonicating and centrifuging.

4.2. Problems during the experiment work

First part: Growth of the *Synechocystis sp.* in secondary treatment

In this part of the project, a 3 L photobioreactor was put into operation several times. The first time is the one shown in the results of the project, which lasted 25 days, but as more biomass was needed to carry out the bioassays, three or four more attempts were made, all of which were unsuccessful. In some attempts, the PBR was contaminated with another species of cyanobacteria and, due to resource competition, *Synechocystis sp.* died. For this reason, another attempt was made to isolate another strain of *Synechocystis sp.* but it was not possible. Then, the biomass needed to perform the bioassays was taken from the inoculum of *Synechocystis sp.*

In addition, a 30 L photobioreactor was put into operation. A staggering process was started, after achieving a higher concentration of *Synechocystis sp*., more medium was added. However, it was not possible to reach the desired volume (30 L), as when it reached 20 L, the culture died. The PBR may have been contaminated with other species, and when those species had to fight for resources, they may have prevailed over *Synechocystis sp.*

Third part: Studying biostimulant activity

In the third part of the project, we wanted to look at the plant biostimulant activity of two phytohormones, cytokinin and auxin, obtained of *Synechocystis sp.*

As far as cytokinins are concerned, there were no problems, although the protocol had to be repeated several times. But if we talk about auxins, even though different protocols were tried, none of them was satisfactory and, therefore, no results were obtained.

4.3. Materials

4.3.1. Material

In order to do different experiments, some materials had been needed. Therefore, a briefly description will be made.

- *Synechocystis sp.:* Is the cyanobacteria specie that has been used to do the different experiments. It was isolated by a PhD student in the GEMMA group.

- BG-11 medium: This medium has been used in order to cultivate the inoculum.
  It consists in a mixture of different solid reagents. This recipe comes from Stanier et al., (1971). The amount of the reagents to elaborate 1 L has been 0.40 g of sodium bicarbonate ($NaHCO_3$), 1.50 g of sodium nitrate ($NaNO_3$), 0.04 g of dipotassium phosphate (K_2HPO_4), 0.075 g of magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.036 g of calcium chloride bihydrate ($CaCl_2 \cdot 2H_2O$), 0.006 g of ferric citrate, 0.001 g of disodium EDTA, 0.02 g of sodium carbonate (Na_2CO_3) and 0.006 g of citric acid. Then, all these reagents need to be dissolved in 1 L and after that put it into the autoclave it to sterilize it.

- Wastewater: The wastewater has been used to cultivate the cyanobacteria. It comes from the secondary effluent of the ''EDAR del Besòs'' and also, an Activated Sludge Wastewater Treatment located in the UPC in the Campus Nord (Appendix A).

- Seeds: The seeds used for the experiments were those indicated in the protocol. Specifically, they were cucumber seeds of the Batlle brand called ''Medium long Ashley cucumber'' (Figure 8) and watercress seeds of the Batlle brand called ''Garden watercress'' (Figure 9). Cucumber seeds take 4-7 days to germinate, while watercress seeds take 5 days. In

addition to these, spinach and lettuce seeds have been used for different trials.

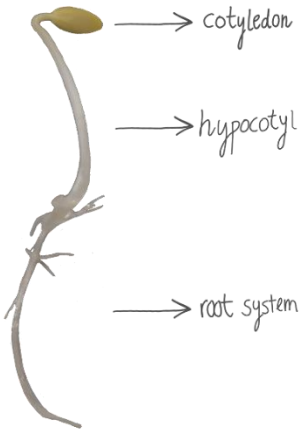


Figure 8. Cucumber seedling parts.

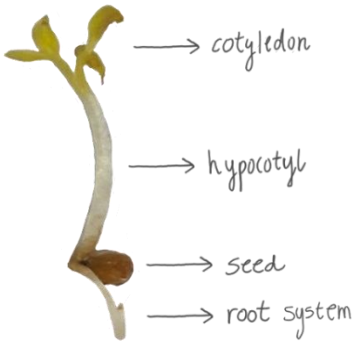


Figure 9. Watercress seedling parts.

- KNOP*: The KNOP* (Morillas-España et al., 2022) is a solution made of different reactive due to make a seed germinate faster. It basically consists of 0.212 g of $CaCO_3$, 0.125 g of KNO_3, 0.125 g of KH_2PO_4, 0.125 g of $MgSO_4 \cdot 7H_2O$ and 0.36 g of $NaNO_3$ in 100 mL of deionized.

- KNOP* Petri dishes: To make the Petri dishes where the seeds will be placed to grow, 25 mL of the KNOP* solution will be taken and mix it with 225 mL of deionized water and 1 g of Agar. Then autoclave it at 121 °C for 20 minutes and then pour it into Petri dishes.

- Stock of auxins: A hormone stock needs to be done where 0.05 g of Indole-3-acetic acid 98 % (IAA), from Fisher brand, is weighed and 1 mL of ethanol is added and then diluting with deionized water to 500 mL.

  Then, to make different concentrations of this hormone, different solutions were made. To make a 0.3 mg/L IAA, 0.3 mL of the stock IAA hormone was taken and diluted in a 100 mL flask with sterile water. To make a 1 mg/L IAA, 1 mL of the stock IAA hormone was taken and diluted in a 100 mL flask with sterile water. And the same for the 3 mg/L IAA and the 10 mg/L IAA.

- Stock of cytokinins: 0.05 g of 6-(Furfurylamino)-purin 99 % (Kinetin), from Fisher brand, is weighed and 2 drops of NaOH 1M is added and then diluting with deionized water to 500 mL.

  Then, to make different concentrations of this hormone, different solutions were made. To make a 0.3 mg/L KIN, 0.15 mL of the stock KIN hormone was taken and diluted in a 50 mL flask with sterile water. To make a 1 mg/L KIN, 0.5 mL of the stock KIN hormone was taken and diluted in a 50 mL flask with sterile water. For the 5 mg/L KIN, 2.5 mL of the stock KIN hormone was taken and for the 10 mg/L KIN, 5 mL was taken, and so the continuation of the procedure is the same as the others.

- Phosphate buffer: a phosphate buffer (pH 7, 0.1 M) its recipe comes from Senatore et al., (2023). To make this buffer, first two different solutions must be done. The solution A consists in diluting 2.78 g of $PO_4H_2Na{\cdot}1H_2O$ with deionized water in a flask of 100 mL. Then, the solution B is done by diluting 7.17 g of $Na_2HPO_4{\cdot}12H_2O$ with deionized water into a flask of 100 mL.

  Then to make it pH 7, an amount of 39 mL of solution A with 61 mL of solution B must be diluted into a 200 mL flask with deionized water.

4.3.2. Equipment

For the purpose of realizing some analyses in an efficient way some equipment had been needed. Hereafter, a short description of the different equipment used in the lab will be made.

- Spectrophotometer: This equipment is used to measure the absorbance of a sample. The Spectronic Genesys 8 is the one that is used. The sample must be introduced into a Hellma High Precision Cell 101-10-40 with a 10 mm light path made of quartz glass where the light goes through it and measures the absorbance (Abs).

- Photobioreactor (PBR): This is the vessel in which the cyanobacteria have been cultured. It has different sizes depending on the amount of cultivar you want at the end. In my case, the bioreactor used was a 3 L PBR and some attempts were made in a 30 L PBR. The material of the photobioreactors is polymethyl methacrylate.

- Centrifuge: The centrifuge used was the Rotanta 460 from Hettich. The usage of this device is to separate the pellet that is the solid part of a sample to the supernatant that is the liquid part of a sample.

- Precision balance: This is the equipment used in order to weigh the different reagents, the filters or other components. The one used was the Entris II BCE Analytical Balance, int. calibration, Sartorius brand.

- Ultrasonicator: This apparatus is in charge of breaking down cells. It has a different amplitude and power depending on the necessities. The one used for the experiment was the Hielscher UP400St.

- DO/T meter: Is a device employed to measure the amount of dissolved oxygen (DO) in a certain place that can be measured in mg/L or in percentage. Furthermore, it also measures the temperature (T) of a medium in °C. The equipment used is the SensoDirect 150 from Lovibond.

- pH analogue controller: This equipment, specifically the Hanna HI-8711, has a pH probe, in particular the one used is the Hanna HI-1001, which is in charge of measuring the pH of the sample. Moreover, this equipment requires a pre-calibration from buffer solutions. In addition, it also has a valve which, depending on the pH value, will open or not and the carbon dioxide coming from a carbon dioxide cylinder will pass through it or not.

- Digester: Is the equipment made to warm up a tube where reagents are poured into, to make them react with the sample. The one used in a time and temperature programmer RAT from the brand JP Selecta and a bloc digest from the same brand.

- Photometer: This device is used in order to measure the concentration of COD in mg/L. The one utilized is the Lovibond MD 600.

- Autoclave: The usage of this machine is to sterilize equipment, liquids or even samples. It works at 121 °C and 103 kPa for 15-20 minutes. The one used was the Selecta Autester 437-G.

4.4. Methods

4.4.1. Phosphate quantification

Following the criteria established under the Standard Methods Protocol the method used is ''4500-P C. Vanadomolybdophosphoric Acid Colorimetric Method'' ("4500-P PHOSPHORUS," n.d.). This method has the purpose to measure the amount of phosphate and it will be explained briefly. Moreover, the range that can be applied is 0 to 20 mg $P\text{-}PO_4^{3-}/L$

The reagent used is Vanadate-molybdate prepared using two different solutions. The solution A is made dissolving 25 g ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$) in 300 mL distilled water. The solution B is made dissolving

1.25 g ammonium metavanadate (NH_4VO_3) in 300 mL distilled hot/boiling water then cooled it down and added 330 mL of concentrated HCl. Cool the solution B to room temperature and pour the solution A into the solution B, mix it and dilute it to 1 L.

In a 50 mL volumetric flask, add 30 mL or less of sample, previously filtrated, 10 mL of Vanadate-molybdate and make up to the mark with distilled water. Then, to do the blank, it is need to take another 50 mL volumetric flask, add 10 mL of Vanadate-molybdate and dilute to the mark with distilled water. After 10 minutes or more, measure the absorbance of the sample versus the blank in a spectrophotometer at a wavelength of 470 nm.

To determine the phosphorus concentration from the absorbance, a calibration curve must first be made (Appendix B). The concentration of the standard solutions used was: 1, 2, 3, 4, 5 and 10 mg P-PO_4^{3-}/L (Figure 10).

Therefore, by the relation between the absorbance and the concentration of phosphorous, the final concentration of P-PO_4^{3-} of the sample will be known (eq. 1). The R2 value while the straight line is done, needs to be more than 0.99. In addition, this process needs to be done every time a reactive is done.

$$P - PO_4^{3-}\ (mg/L) = DF \cdot \frac{(Abs(470) - ordinate)}{slope\ of\ the\ line} \qquad \text{(eq. 1)}$$

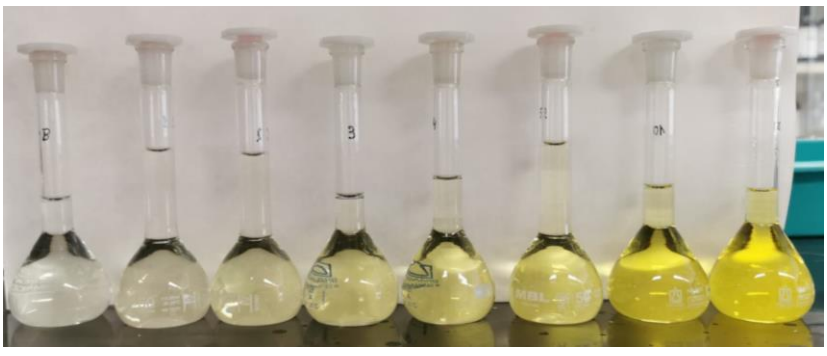


Figure 10. Phosphate calibration curve volumetric flasks.

4.4.2. Nitrate quantification

The "4500-NO_3^- NITROGEN (NITRATE)" technique taken at the Standard Methods ("4500-NO3− NITROGEN (NITRATE)," n.d.) has the intention to measure the quantity of nitrate. The range that can be applicable is 0 to 8 mg N-NO_3^-/L.

0.001 to 10 mg NO3 --N/L for automated cadmium reduction procedures (F and I). Increase the dilution to the range of the chosen technique for larger NO3 --N concentrations.

A 50 mL volumetric flask is needed to dilute the sample, previously filtrated, with different dilution factor depending on the sample and then flatten it with deionized water.

To measure the sample by the spectrophotometer, it is important to first measure it at a 220 nm wavelength, introducing first the blank that is distilled water and then do the same process at a 275 nm wavelength.

To determine the N-nitrate concentration from the absorbance, a calibration curve must first be made (Appendix B). The concentration of the standard solutions used was: 1, 2, 3, 4, 5, 6, 7 and 8 mg N-NO_3^-/L.

Then, from these values, a calibration line is made which will give an equation with which we can relate the concentration of N-nitrate to the absorbance. The R2 value while the straight line is done, needs to be more than 0.99. This process needs to be done every time a reactive is done. So, from this relation, the concentration of N-nitrates of the samples will be calculated (eq. 2). The calibration line for the nitrate will be made at 220 nm and at 275 nm.

$$N - NO_3\ (mg/L) = DF \cdot \frac{((Abs(220) - 2 \cdot Abs(275)) - ordinate)}{slope\ of\ the\ line} \quad \text{(eq. 2)}$$

4.4.3. Ammonium quantification

To measure the ammonium, the protocol described by SOLÓRZANO, (1969) has been used. The range that can be applicable is 0 to 1 mg NH_4^+/L.

In short, one 100 mL Erlenmeyer flask is taken for the blank and one for each sample. For the blank, 50 mL of deionised water is added and for the others, 50 mL of homogenised sample is pipetted into the flask with a volumetric pipette. To each conical flask add 2 mL of sodium nitroprusside (0.50 %), 2 mL of alcoholic phenol solution (10 %) and 5 mL of oxidising solution (freshly prepared).

After 90 minutes, the absorbance is measured at a wavelength of 640 nm. If the measured absorbance is higher than the maximum value obtained doing the calibration curve, a dilution of the sample is necessary.

To measure the amount of ammonium, it is necessary to do a calibration curve before (Appendix B). The absorbance is measured at different known concentration of ammonium: 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg NH_4^+/L and with the absorbance values and the concentration of ammonium at each point, the calibration line is made. Therefore, when measuring the ammonium, knowing the absorbance it gives, the concentration of ammonium will be known (eq. 3). The R2 value while the straight line is done, needs to be more than 0.99. This process needs to be done every time a reactive is done. The Erlenmeyer flask used to create the ammonium calibration line is seen in Figure 11.

$$N - NH_4\ (mg/L) = DF \cdot \frac{(Abs(640) - ordinate)}{slope\ of\ the\ line} \qquad \text{(eq. 3)}$$

Figure 11. Ammonium calibration curve volumetric flasks.

4.4.4. Solid measurement

In order to calculate the concentration of solids in a sample, a filtration process is necessary.

For the filtration process, two different types of filters are used depending on the product that is needed to filtrate. To filtrate the *Synechocystis sp.* a glass microfibre filter (MFV5) from the brand Filter-Lab of 47 mm diameter and a capacity of retention of 0.7 µm is used. Therefore, to filtrate the wastewater a glass fibre filters (product number 10370019) from the brand Whatman, Cytvia with a diameter of 47 mm is used and a retention of 1 µm.

Before any filtration, the filters are cleaned. Basically, this consists of writing a number on each filter to identify them, filtering deionised water through them and then placing them in the muffle at 550 °C for 20 minutes. After this time, they must be transferred to the desiccator before being weighed so that they are at room temperature. To preserve them, put them in the stove.

In the Figure 12 and Figure 13, there is a photo and a schematic about the filtration tool. A vacuum flask is connected to another vacuum flask which is connected to the pump. The pump reaches 400 mmHg. In the first vacuum flask there is a device copied to it through a rubber plug where a filter holder is inserted. A previously cleaned and weighed filter is placed on it. Once the filter is well positioned and without any holds in it, the disposable funnel is placed on top of it. The pump is then activated and the sample, noting in advance the volume being introduced, is poured into the disposable funnel, where it will pass through the filter and fall into the vacuum flask. Afterwards, the pump is stopped and the filter is removed with tweezers and placed on a piece of aluminium foil, which will remain in the stove for 24 hours.

Figure 12. Filtering device.

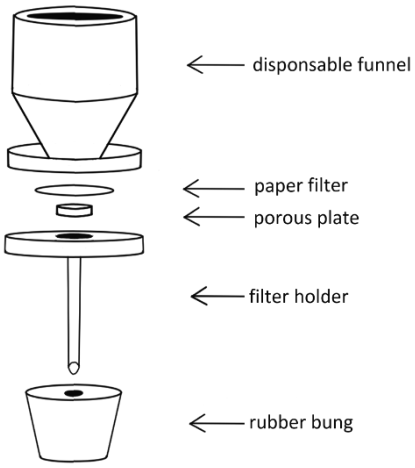


Figure 13. Schematic of the parts of the filtration apparatus.

After 24 hours, the filters must be weighed. Once the weights are known, they are put into the muffle in different heating elements at 550 °C for 20 minutes.

Therefore, three different weights shall be noted. The weight after they have been cleaned (P1), the weight after the sample has been filtered and they have been in the oven for 24 hours (P2) and, finally, the weight after they have been introduced into the muffle (P3). The volume (V) is the sample volume before filtrating. Equation 4 describes how to measure total suspended solids, while equation 5 describes how to measure volatile suspended solids.

$$TSS\ (mg/L) = \frac{(P2\ (g) - P1(g))}{V(L)} \cdot 1000 \quad \text{(eq. 4)}$$

$$VSS\ (mg/L) = \frac{\left(P2(g) - P3(g)\right)}{V(L)} \cdot 1000 \quad \text{(eq. 5)}$$

4.4.5. Chemical Oxygen Demand (COD)

The Chemical Oxygen Demand is a parameter to measure the quantity of organic pollution in water. It measures the quantity of oxygen equivalent to the quantity of organic matter of a sample that can be oxidized by a strong chemical oxidant (Boyles, n.d.).

The method used to measure the COD was taken from the Standard Methods, in particular it has been used the "5220 CHEMICAL OXYGEN DEMAND (COD)," (n.d.). The application range of this method is 5 to 50 mg/L O_2.

The process it consists in take a digestion tube for the blank and another one for each sample (making 3 replicates in the end). Briefly, 2.5 mL of sample is pipetted into the tube. Also, 1.5 mL of digester solution and 3.5 mL of sulphuric acid. Then these tubes are placed in the digester at 150 °C for 2 hours.

After the waiting period, the tubes are cooled down to room temperature and placed in the photometric machine which measures COD in mg/L.

4.4.6. Centrifugation

Centrifugation consists of placing the sample in different containers that will go into the centrifuge (Figure 14) and then, after the process, the pellet will be separated from the supernatant. In the Figure 15 there is a picture where the two phases are perfectly visible. An important thing to know before doing it is that the containers must be placed in the opposite hole to the other container and must have the same weight, otherwise the centrifuge could break.

Figure 14. Centrifuge with containers inside.

Figure 15. Centrifuge container after centrifugation with supernatant and pellet.

The values at which the centrifuge usually operates are 7000 rpm and 14 minutes.

In addition, one of the properties of *Synechocystis sp.* is that they can accumulate pigments, which is why they belong to the cyanobacterial domain. In fact, one of the pigments they can accumulate is phycocyanin. In the Figure 16, this property is visible because the supernatant has a very strong blue colour and this means that it is full of pigments.

Figure 16. Left container with the supernatant with accumulated pigments and right container with pellet of *Synechocystis sp*. after centrifugating for 14 min at 7000 rpm.

4.4.7. Lysis methods

4.4.7.1. Sonication

The Ultrasonication is a machine (Figure 17) that when an electric current passes through a mechanical system, its energy is transformed into intense vibrations that produce ultrasound waves. The target material begins to vibrate as a result of the ultrasound. Millions of small bubbles will form if there are any liquids present, and these bubbles can transfer energy to other materials by rapidly expanding and collapsing (Suslick, 1990).

To sonicate a sample, it is necessary to place it in a container. The one used in this experiment was a falcon tube that was settled in ice water. The amount of sample that was placed was between 15 mL and 25 mL. The apparatus (Figure 17) was programmed to cycle for 1 minute at an amplitude of 53 % and a pulse of 100 % and approximately 30 W was the operating power. In total, 10 cycles were made.

Figure 17. Ultrasonicator device.

4.4.7.2. Freeze-Thaw

This technique involves thoroughly freezing a sample before beginning the thawing procedure. This approach is used to break the cell.

Regarding the Freeze-Thaw method, three cycles of freezing were used in this project. As a result, three thawing cycles were produced. The freezing temperature was -20 °C for 1 hour.

4.4.7.3. Autoclave

The autoclave is an apparatus whose function is to sterilise other equipment. However, when talking about lysis methods, it is used to break the cell. The autoclave works at a certain condition, in particular, at 103 kPa and 121°C for 20 minutes.

4.4.8. Staggering and preparing the culture

Inoculums of *Synechocystis sp.* were maintained in BG-11 (Section 4.3.1.). The experiments were performed in 3 L Photobioreactors (PBR) (Figure 18). First, 2.5 L of wastewater was poured into the PBR. Then, a portion of the inoculum was centrifuged (7000 rpm, 14 min) in order to guarantee a concentration in the PBR of 100 mg/L SSV. The following equation (eq. 6) was used for the calculation of the volume of inoculum needed:

$$Volume_{initial}\ (L) = \frac{Volume_{final}\ (L) \cdot Concentration_{final}(g/L)}{Concentration_{initial}(g/L)} \quad \textbf{(eq. 6)}$$

Due to the fact that the *Synechocystis sp*. is a cyanobacteria and they need a N:P ratio of 20:1 (Rosales-Loaiza et al., 2008), more or less phosphate was added. It was controlled by measuring the nitrogen and the phosphate with the methods previously mentioned and calculating with the next equations.

$$Conversion\ factor = \frac{Molecular\ weight\ NO_3^-}{Atomic\ weight} \quad \text{(eq. 7)}$$

$$[N - NO_3^-] = \frac{[NO_3^-]}{Conversion\ factor} \quad \text{(eq. 8)}$$

$$TIN = [N - NH_4^+] + [N - NO_3^-] + [N - NO_2^-] \quad \text{(eq. 9)}$$

$$N{:}P = \frac{TIN}{[P - PO_4^{3-}]} \quad \text{(eq. 10)}$$

The culture itself needs to be highly homogenised to ensure adequate light exposure of the cyanobacteria, as well as to deliver the same amount of nutrients to all cyanobacteria cells.

The luminosity was constant with a cycle of 15 h light:9 h dark and 5 klx, which is similar to the one used in Rueda et al., (2022). Also, the approximate temperature was 26.17 °C ± 0.68 °C (Appendix C).

The pH of the culture needs to be between a range of 6.5 and 8.5 (Touloupakis et al., 2016) to ensure a high microbial activity. It is measured with the pH meter and controlled by the pH analogue controller. The pH of the PBR was approximately 8.17 ± 0.95 (Appendix C).

To ensure the oxygen demands of the *Synechocysitis sp*. the suggested range is between 1 and 3 mg O_2/L (Kihara et al., 2014). The device used for the measurement is the 'SensoDirect 150'. The dissolved oxygen in the PBR was 5.54 mg/L ± 0.69 mg/L (Appendix C).

Samples were taken three days per week at the end of the dark phase.

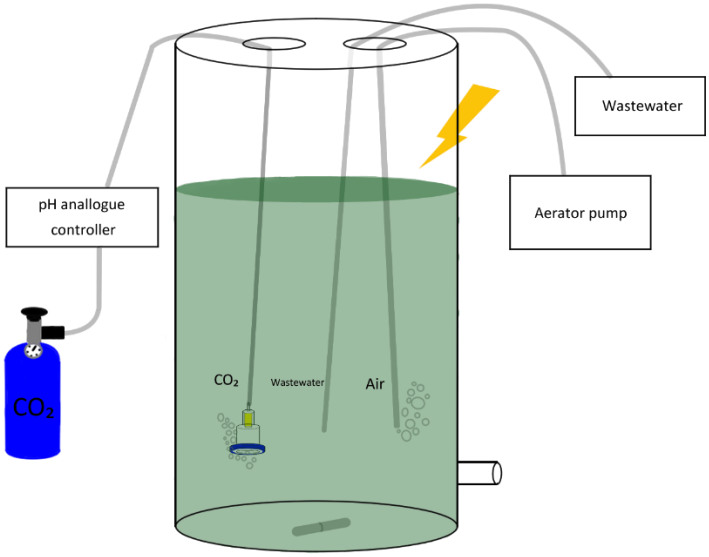


Figure 18. Schematic of the bioreactor with the CO_2 diffusor, the oxygen, the agitation and the light.

The productivity was also calculated with the following equation (eq. 11) where the flow (Q) is 420 L·d^{-1} and the volume (V) is 2.5 L:

$$Productivity\ (mg \cdot L^{-1} \cdot d^{-1}) = \\ = (VSS_{PBR}(mg \cdot L^{-1}) - VSS_{AR}(mg \cdot L^{-1})) \cdot \frac{Q\ (L \cdot d^{-1})}{V\ (L)} \qquad \text{(eq. 11)}$$

4.4.9. Quantify the pigments

To quantify the pigments, the protocol from Senatore et al., (2023) has been used. This process was used to optimise the sonication cycles and to know which is the maximum that should be performed. This method and the COD have the same function.

In essence, the *Synechocystis sp.* was first centrifuged, then only the pellet was taken and frozen. After being thawed, it was sonicated and after that, it was taken and measured the absorbance at different wavelengths, in particular, at 615 nm and at 652 nm. These different wavelengths coincide with the absorption of phycocyanin and allophycocyanin, respectively. The both pigments can be measured with the next equations.

$$Phycocyanin\ (mg/L) = \frac{(Abs_{615nm} - (0.474 \cdot Abs_{652nm})}{5.34} \qquad \text{(eq. 12)}$$

$$Allophycocyanin\ (mg/L) = \frac{(Abs_{652nm} - (0.208 \cdot Abs_{615nm})}{5.09} \qquad \text{(eq. 13)}$$

4.4.10. Plant biostimulant activity

First of all, a protocol called ''Watercress protocol'' was used to identify if the medium used for the experiments result to be toxic or not. Then, different protocols have been used or attempted to quantify the activity of plant biostimulants separated between the two phytohormones studied: cytokinins and auxins.

4.4.10.1. Watercress protocol

This protocol from Morillas-España et al., (2022) is useful to know whether the medium used is toxic or not for the plants.

To prepare the watercress seeds, take them and soak them in 30 % v/v H_2O_2 for 5 minutes. After this time, add filter paper to an 85 mm Petri dish. Then, 2 mL of

each extract, as well as the controls, should be placed on the filter paper. In total, five Petri dishes shall be used. The treatments performed are: Cw, Cb, SB, SBb and SN.

After applying 2 mL of each treatment on the filter paper, place 25 watercress seeds on top of the paper (Figure 19). Then incubate them in the dark for 3 days at 25 °C.

After this time, measure the number of germinated seeds and the length of the hypocotyl of each sprout with a ruler and compare them with the Cw.

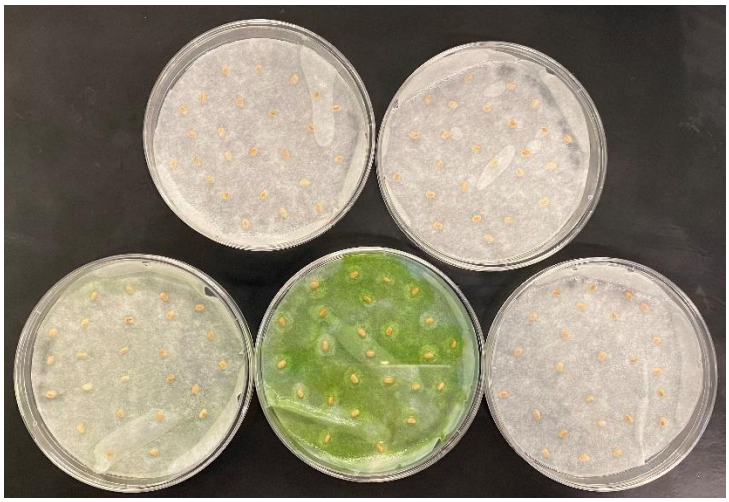


Figure 19. Watercress seeds after having applied the treatment in the Petri dish.

4.4.10.2. Cytokinins

4.4.10.2.1. Quantify cytokinins with cucumber seeds

This protocol is from Morillas-España et al., (2022). To prepare the cucumber seeds, the first thing to do is to soak them overnight in deionised water. Then we have to place the cucumber seeds in the KNOP* Petri dishes and leave it 3 days in dark at 25 °C (Figure 20). At this time, whenever cucumber seedlings are handled, it should be in the dark or under a green light without any other light hitting them.

To find the effects of cytokinins on the expansion of the cucumber cotyledons, 85 mm diameter Petri dishes with filter papers inside had been taken and 3 mL of each extract as well as the controls had been poured into each of them.

The same protocol was repeated thrice, but with some differences. The list of those used in the three replicates is given in table 2.

Table 2. List of solutions used in the cytokinin protocol with and without phosphate buffer.

1st assay	Treatments	Cw, Cb, SB, SBb and SN
	Cytokinin calibration assays with solutions of KIN in the follow concentration:	0.3, 1, 5, 10, 30 and 100 mg/L
2nd assay	Treatments	Cw
	Cytokinin calibration assays with solutions of KIN in the follow concentration:	1, 5 and 10 mg/L
3rd assay	Treatments	Cw, SB and SB diluted by half
	Cytokinin calibration assays with solutions of KIN in the follow concentration:	Ø

At that time, the previously grown cucumber seedlings should be cut at the cotyledon without leaving any hypocotyl. In total, each seedling has two cotyledons. Those cotyledons should be weighed before placing them in the Petri dish, then the weight should be noted and placed in a known place in the Petri dish, in total there should be 10 cotyledons without hypocotyl in each Petri dish (Figure 21). Afterwards, all Petri dishes should be placed in the oven and left there at 25 °C for 3 days.

After the time has passed, the cotyledons must be dried into paper and then weigh them again. The weight of each extract should be compared with the negative control and the kinetin calibration line.

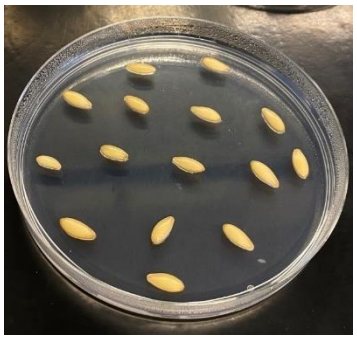


Figure 20. Cucumber seeds into KNOP* medium to grow up.

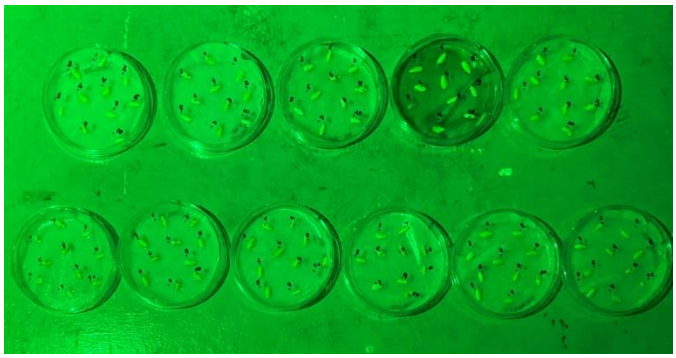


Figure 21. Cucumber cotyledons without hypocotyl after having applied the treatment to measure the cytokinins. 1st assay.

4.4.10.2.2. Spinach protocol

To run the spinach protocol (Rupawalla et al., 2022), the seeds were first surface sterilised. All seeds were soaked in 10 % bleach and left for 12 minutes. After this time, they were washed five times with sterile water.

To prepare the cyanobacterial solutions, the previously thawed pellet was sonicated. Afterwards, some of this material was left and some was centrifuged. Then, the supernatant was saved and the solids were calculated from the pellet generated.

Thus, the extracts used for the experiment were: Cw, Cbg11, SB, SB diluted by half, SN and the different concentrations of auxins and cytokinins: 0.3 mg IAA/L, 1 mg IAA/L, 10 mg IAA/L, 1 mg KIN/L, 5 mg KIN/L, 10 mg KIN/L, and a mixture of 50 % AUX 1 ppm and 50 % KIN 1 ppm.

Therefore, 15 spinach seeds were placed in each Petri dish, then 5 mL of each extract or control was poured into each Petri dish (Figure 22). They were left for 2 days at 4 °C in the dark before sowing.

After these 2 days, these spinach seeds were grown in Petri dishes with 1 % agar. They were incubated for 7 days at a light/dark cycle of 18/6 h (4.5-5.8 klx) and 18.24 °C ± 1.03 °C. From the third day of the experiment until the ninth day, the number of germinated seeds and the emergence of the green cotyledon were recorded. The biomass dry weight of the seedlings was observed on the ninth day.

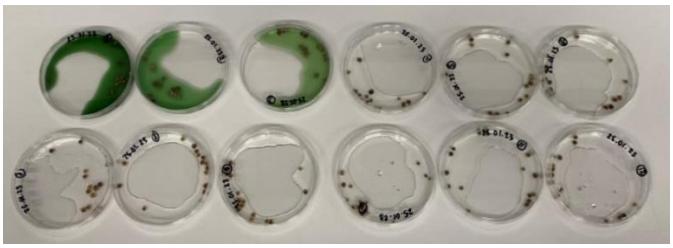


Figure 22. Spinach seeds soaked into different extracts.

4.4.10.3. Auxins

4.4.10.3.1. Quantify auxins with cucumber seeds

This protocol comes from Morillas-España et al., (2022). To prepare the cucumber seeds, the first thing to do is to soak them overnight in deionised water. Then we have to place the cucumber seeds in the KNOP* Petri dishes and leave it 3 days in dark at 25 °C. At this time, whenever cucumber seedlings are handled, it should be in the dark or under a green light without any other light hitting them.

To find out the effects of auxins on root growth of cucumbers, 85 mm diameter Petri dishes with filter papers inside had been taken and 3 mL of each extract as well as the controls had been poured into each of them. The treatments used have been: Cw, Cb, SB, SBb and SN. Then, as well as the extract and the control,

a different concentration of auxins must be done, in total six different ones, a 0.3 mg/L IAA, a 1 mg/L IAA, a 10 mg/L IAA, 30 mg/L IAA and 100 mg/L IAA. At that time, previously grown cucumber seedlings should be cut at the cotyledon leaving 1-2 mm of hypocotyl. So, each seedling has two cotyledons, they must be separated. Then, a total of 10 cotyledons with hypocotyl must be placed in each Petri dish (Figure 23). Subsequent to it, all the Petri dishes must go to the stove and left them there at 25 °C for 4 days.

After the time has elapsed, count the number of main roots larger than 1 mm formed on the 10 cotyledons in each Petri dish and compare with the negative control and the calibration line.

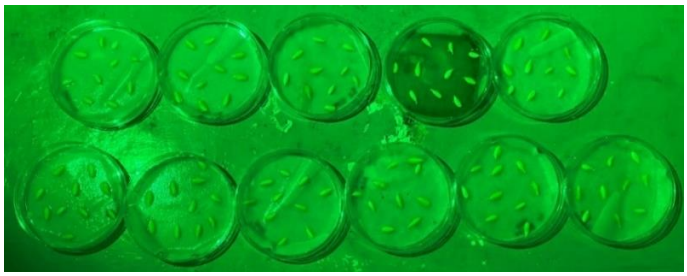


Figure 23. Cucumber cotyledons with hypocotyl after having applied the treatment to measure the auxins.

4.4.10.3.2. Spinach protocol

This process is described in the preceding section 4.4.10.2.2.

4.4.10.3.3. Pea protocol

The third protocol was to know the bioactivity of the auxins on the root growth of pea seeds (Ahmed et al., 2010). This protocol was tried into spinach seeds (Figure 24) and cucumber seeds. It consisted sterilizing the seeds into 10 % bleach for 12 minutes and then washing them with sterile deionized water. After that, 7-8 seeds were placed in Petri dishes containing two layers of filter paper. Then 10 mL of each extract was placed into that Petri dishes, in particular the extracts

were Cw and the following concentrations of auxins: 0.3 mg IAA/L, 1 mg IAA/L and 10 mg IAA/L.

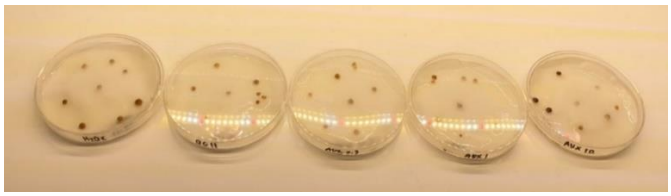


Figure 24. Spinach seeds into Petri dishes with the negative controls (deionized water and BG-11) and the different IAA concentrations (0.3 mg IAA/L, 1 mg IAA/L and 10 mg IAA/L).

4.4.10.3.4. Lettuce protocol

The fourth protocol tried was to know the activity of auxins in the root growth of lettuce seeds. It consisted into place 10 seeds in Petri dishes containing a filter paper and then placing 1.4 mL of sterile Cw, 0.3 mg IAA/L, 1 mg IAA/L and 10 mg IAA/L in different Petri dishes (Figure 25). Then, them were placed in the dark at 25 °C. After 48h the seeds were removed and the root lengths were measured.

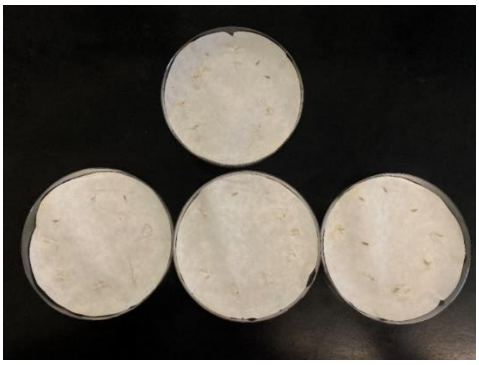


Figure 25. Lettuce seeds into Petri dishes with with the negative control (deionized water) and the different IAA concentrations (0.3 mg IAA/L, 1 mg IAA/L and 10 mg IAA/L).

4.4.11. Microalgae isolation from wastewater

Due to the problems mentioned above (Section 4.2), a new attempt was made to isolate *Synechocystis sp.*

To obtain pure stains of microalgae and cyanobacteria, it is needed to grow them and isolate them at first.

In my case strains were isolated from secondary effluent wastewater. To isolate the microalgae and cyanobacteria from the wastewater, a number of test tubes were needed. First, about 3 mL of wastewater was put into four different tubes. Also, a nutrient-enriched wastewater mixture was put into four different tubes and 3 mL of this water was put into four different tubes. After having the eight tubes, these were put in the light and every day or every other day, these tubes were shaken. After 5 days, it could be observed that at the bottom of the tube there was a green solid (Figure 26.a), which was the microalgae. Only the tubes with nutrient-enriched wastewater produced these algae (Figure 26.b).

After having the tubes with the microalgae, they were collected and plated in petri dishes (having previously placed the medium with the agar) and placed in the light (Figure 26.c).

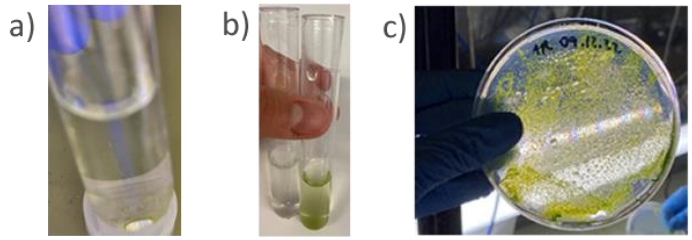


Figure 26. a) Test tube with microalgae in the bottom. b) Test tube with wastewater and test tube with wastewater nutrients and microalgae grown. c) Petri dish with microalgae from the wastewater.

Once the microalgae have grown, it is necessary to replant the culture to have separate colonies (Figure 27).

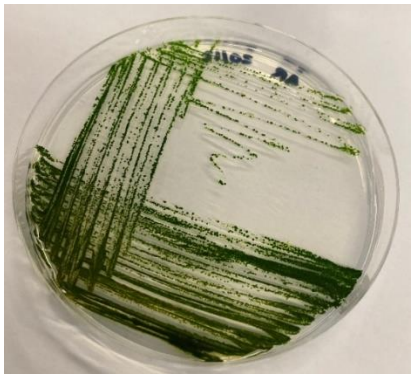


Figure 27. Petri dish with microalgae from the wastewater.

So, one way to have different colonies in the same Petri dish is to divide the dish into eight parts (Figure 28) and then take a single colony, a single spot, and rub it on one of the eight parts. At that point, after growing, you will get eight different strains. From here, it is needed to identify the species on each strain. As well as the species that has the origin form the wastewater, in the past the same procedure was done by Ana Álvarez (May, 2022) but with water from the lagoon. Then the species from the wastewater and from the lagoon become identified.

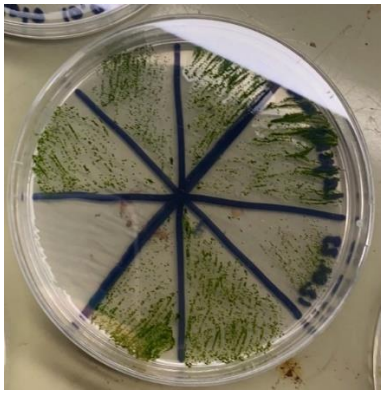


Figure 28. Petri dish with algae from the wastewater divided in eight parts.

4.5. Statistical methods

The statistical methods applied in the study are univariate descriptive statistics, and a normality test was also performed to check whether the data followed a normal distribution. In addition, inferential statistical methods such as hypothesis testing by t-test and ANOVA have been used to study the differences in means and variances of the extracts. Graphs were also used. All these methods were used to present the different data obtained throughout the project.

5. Results and discussion

5.1. *Synechocystis sp.* cultivation in wastewater

5.1.1. Growth rates and productivity

The growth rates and plots are calculated from different analyses made with the cultivar *Synechocystis sp.* with a wastewater medium. This species was grown in a 3 L bioreactor during 24 days.

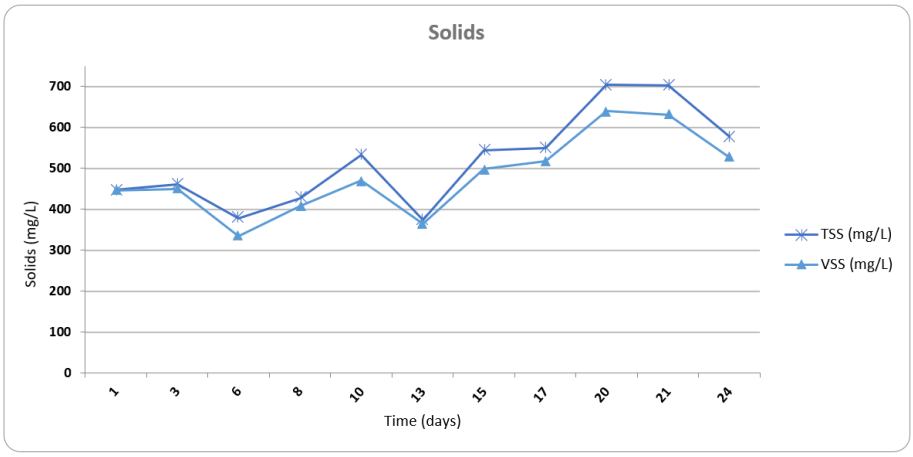


Figure 29. Total and volatile suspended solids growth plot in mg/L from the *Synechocystis sp.* PBR.

The growth graph can be seen in Figure 29 where the values (Appendix C) keep more or less increasing during the 24 days.

On the day 13, a filamentous species appeared in the culture. Since day 20, this species has completely controlled the culture.

Table 3. Productivity of *Synechocystis sp*. growth with VSS.

Day	Productivity (mg VSS·L^{-1}·d^{-1})
1	70.983
3	71.549
6	52.172
8	64.693
10	75.092
13	51.274
15	73.528
17	76.923
20	100.158
21	98.508
24	81.143
Mean	**74.184**

The productivity value is 74 mg·L-1·d-1. In general, the productivities obtained in Table 3 were lower than the one obtained by Senatore et al., (2023) which is 173 mg·L−1·day−1. At first glance, it is easy to think that light could be a possible explanation for this result, as the article states that a higher light intensity is applied, namely 7 klx. Another article from Kim et al., (2010) says that the productivity is 170 mg· L−1·day−1 but in this one a lower light intensity is applied than the one used in the project (5 klx), specifically 2 klx. Therefore, light is excluded as a possible cause of the low productivity. This suggests another possibility for this low productivity. As *Synechocystis sp.* is extracted from sewage and once in the PBR it is constantly contaminated, we can say that the PBR does not contain pure *Synechocystis sp.* but that, to a greater or lesser extent, other species coexist with it, even if they are not visible under the microscope. Then, as it is constantly competing with other species, productivity may decrease.

5.1.2. Nutrient control

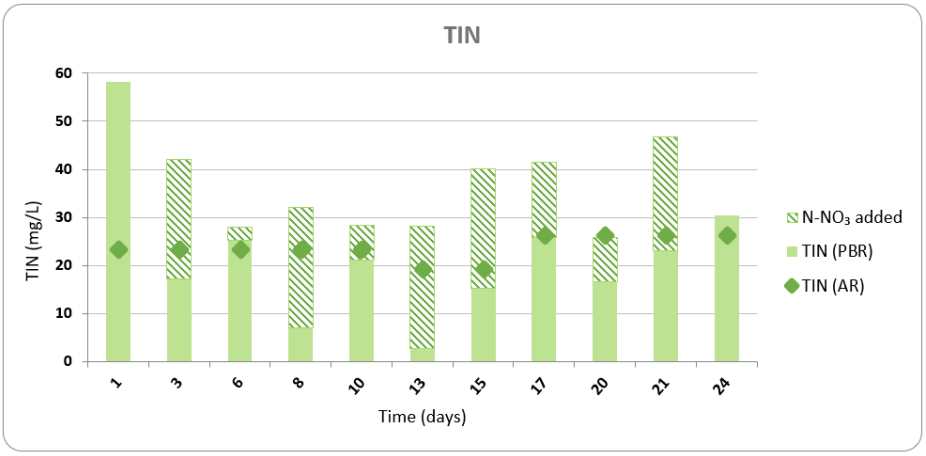


Figure 30. Total inorganic nitrogen *Synechocystis sp.* growth rate in mg/L from the PBR.

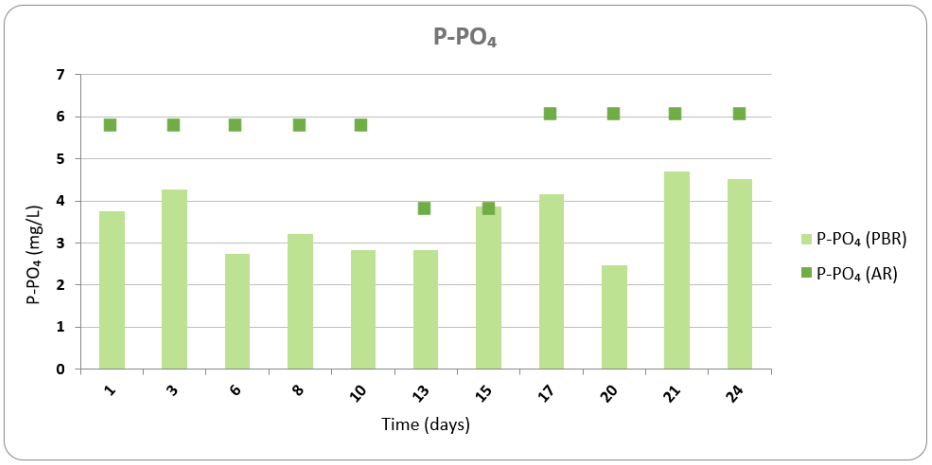


Figure 31. Phosphate *Synechocystis sp.* growth rate in mg/L from the PBR.

On one hand, Figure 30 shows the Total Inorganic Nitrogen (Appendix C) from biomass and wastewater as well as nitrate added to the PBR. It can be seen that nitrate tends to decrease because it is part of the diet of *Synehcocystis sp.* Although each day part of the biomass is removed to add more nitrate-rich medium (wastewater), the amount is not sufficient and from day 3, the addition of nitrate is almost constant.

On the other hand, in the Figure 31, it is showed the phosphate from the PBR and from the wastewater (Appendix C). The amount of phosphate tends to decrease gradually because the consumption is not as high as that of nitrogen for

cyanobacteria. It is true that there are some peaks where phosphate increases and this could be due to the wastewater medium. In addition, on days 21 and 24, phosphate increases a lot due to the reduction of the *Synechocystis sp.* population.

5.1.3. Microscopic analysis

The *Synechocystis sp.* (Figure 32) has a green rounded shape. This specie can be seen alone, forming a group of two cells or even forming a group of four cells.

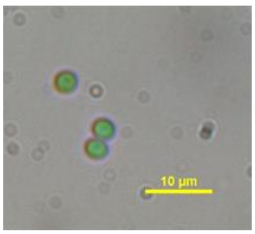
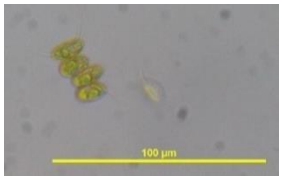
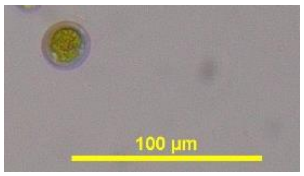
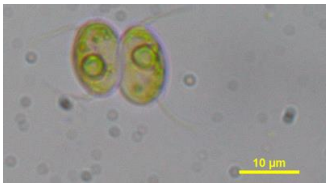
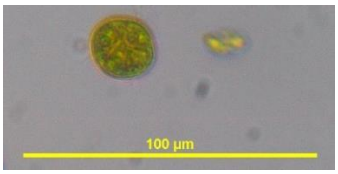
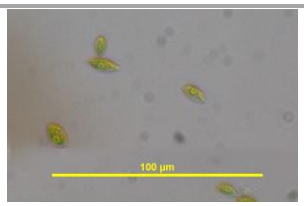


Figure 32.. *Synechocystis sp.* x100 (Compiled by author).

5.1.5. Microalgae isolation from wastewater

The following table (table 4) shows the different species found both in the lagoon and in the wastewater, as well as a brief description of the Petri dish and the microscope. The main intention was to re-isolate *Synechocystis sp.* to redo the first part of the experiment because the cyanobacteria died, but this was not possible. However, the identified species have been used in subsequent experiments.

Table 4. Species found in wastewater isolation.

Origin	Description colony on plate	Microscope description	Microscope photo (Microscope magnification)	Specie
Lagoon	This specie was taken from the liquid medium culture, the colour of the flask was grass green.	This species forms a cluster of four elliptical cells with a transparent spine at each apex of the complex. In each cell, the nucleus is easily visible.	 (40x)	*Scenedesmus* sp.
Lagoon	This specie was taken from the liquid medium culture, the colour of the flask was grass green.	This species has a rounded shape and some organelles can be seen inside.	 (20x)	*Chlorella* sp.
Lagoon	This specie was taken from the Petri dish where it has a light lime green.	This species has an oval shape with four spines. Also, each cell of the complex has a visible nucleus and some organelles inside.	 (100x)	*Scenedesmus* sp.
Wastewater	This specie was taken from the liquid medium culture, the colour of the flask was grass green.	This species has a rounded shape and some organelles can be seen inside.	 (40x)	*Chlorella* sp.
Wastewater	This specie was taken from the liquid medium culture, the colour of the flask was grass green.	This species consists of individual elliptical cells in which the nucleus can be easily seen.	 (40x)	*Scenedesmus* sp.

5.2. Biostimulant extraction

In the Figure 4, there is the schematic where the process to extract biostimulant is illustrated. First of all, COD was measured after freezing the sample (because this is the way to preserve the sample for a long period of time) to obtain a control. Then, the COD of the sample was measured by sonicating it 10 cycles (Section 4.4.7.1.), autoclaving it (Section 4.4.7.3.) and doing a 3 cycle freeze-thaw (Section 4.4.7.2.). All this procedure has been done with 3 replicates.

Table 5. Results of the quantified biostimulant extraction using the COD with different undiluted sample methods.

	LYSIS METHODS			
	After 1 freezing cycle (mg COD/L)	**Sonicate (mg COD/L)**	**Autoclave (mg COD/L)**	**Freeze-Thaw (mg COD/L)**
Replicate 1	<7	36.1	71.5	<7
Replicate 2	<7	21.6	79.8	<7
Replicate 3	<7	39.3	75.6	<7
Mean	<7	32.3	75.6	<7
SD	0	7.7	3.4	0

Results from Table 5 shown that Freeze-Thaw method (Section 4.4.7.2.) did not increase the content of COD comparing with the control. This result suggest that this method was inefficient for lysing the wall of the cells. Then, the autoclave (Section 4.4.7.3.) showed the higher results of COD, indicating it was the best method to lyse the cell.

However, this method was not chosen for further experiments due to the fact that the pressure and temperature conditions, 103 kPa and 121 °C, can damage protein's structure and can be affecting to biostimulants. For instance, the colour of the pigments, turn from blue to brown (Figure 33 and 34).

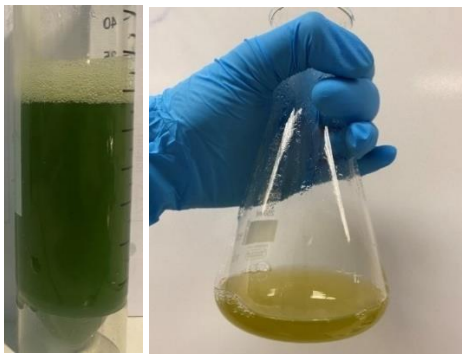


Figure 33. In the left there is the sample after sonicating and in the right the sample after autoclaving it.

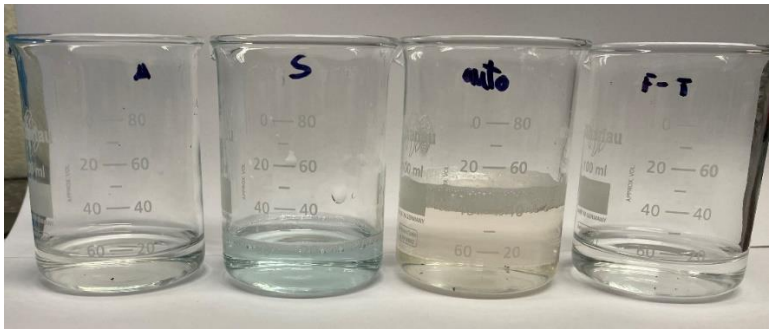


Figure 34. Vessels after filtering the solution from after one-cycle freezing, sonication, autoclaving and Freeze-Thaw methods, respectively, and before doing COD.

Therefore, the sonicating method was selected, another experiment needed to be made to know the cycles that must be done to optimize the process and to know if it really works. To do it, the pigment quantification method was used (Section 4.4.9.).

They are two different pigments that come from phycobiliprotein family (Wikipedia contributors, 2023). In essence, they allow light energy to be captured and are characteristic of cyanobacteria and micro and macroalgae (Dagnino-Leone et al., 2022).

The Figure 37 shows that the amount of pigment increases with the number of cycles (Appendix D). This means that in order to obtain the highest amount of bioproducts, we need to apply 10 cycles. This result is also represented in Figure 35, where it is shown that the colour gets more intense by increasing the number of cycles. Although Figure 36 shows that after 10 cycles the slope continues to increase, this

maximum value of cycles is decided due to the flattening of the slope and time constraints.

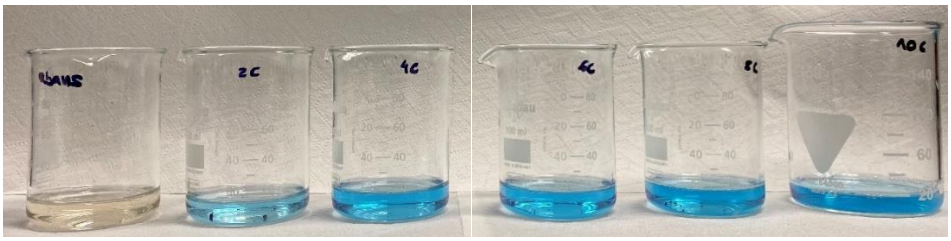


Figure 35. Vessels after sonicating and filtering in each cycle where phycocyanin and allophycocyanin pigments are visible.

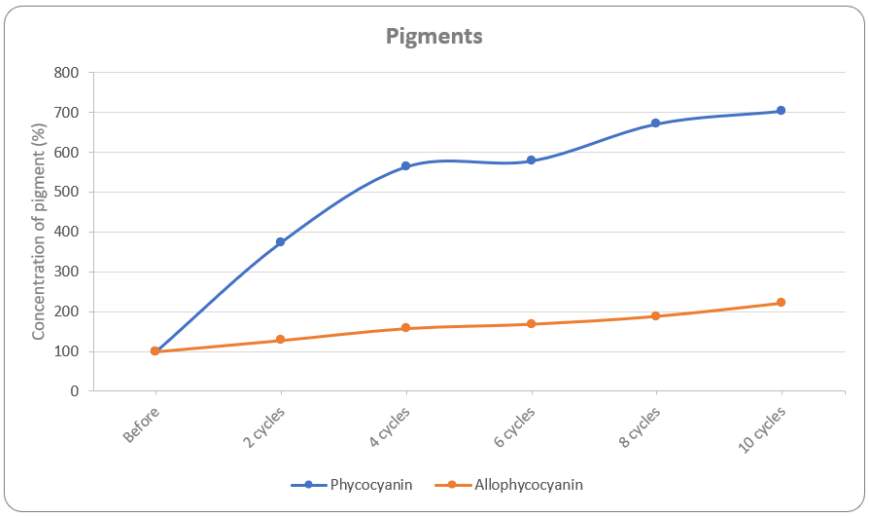


Figure 36. Plot of the phycocyanin and allophycocyanin pigments concentration in % after different sonicate cycles.

5.3. Biostimulant activity

The *Synechocystis sp.* used for the various protocols on the biostimulant activity section was the inoculum that was grown in BG-11 (Section 4.3.1.).

5.3.1. Preliminary results

First of all, simultaneously to the first trials of auxins and cytokinins protocols, the watercress protocol has been carried out. It has been implemented with Cw, Cb, SBb, SB and SN solutions. Figure 37 shows that all solutions containing phosphate buffer do not grow (Cb, SBb and SN), which means that it is toxic for plants. All the data used to do the Figure 38 graphic is in Appendix D.

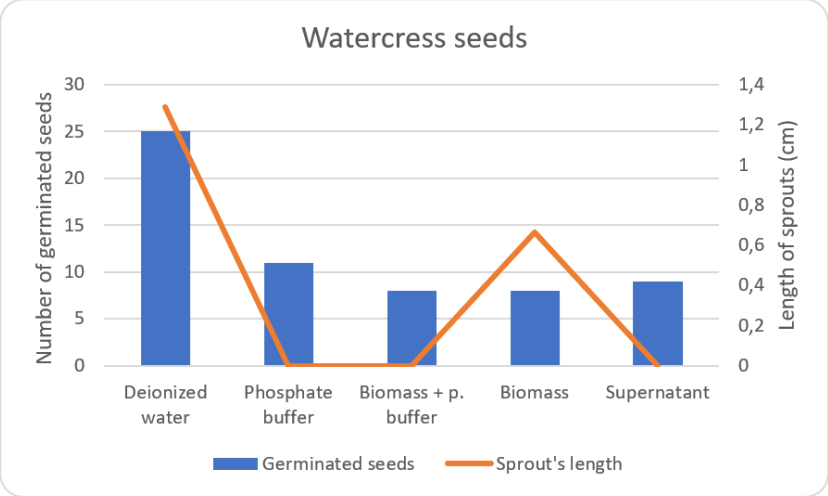


Figure 37. Watercress plot with different solutions.

Looking at Figure 38, which is the results of the pea protocol done with cucumber seeds, it can be seen that the higher the auxin concentration, the less the plant seemed to grow.

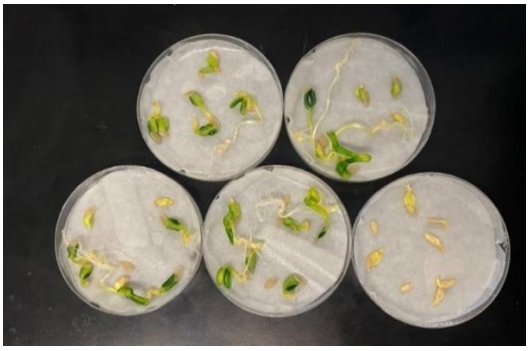


Figure 38. Cucumber seeds into Petri diches with IAA concentrations and the negative control, pea protocol.

Regarding the spinach protocol, in the first and last attempt (Figure 39), it could be seen that it was an easy and interesting method, but due to lack of time it could not be repeated.

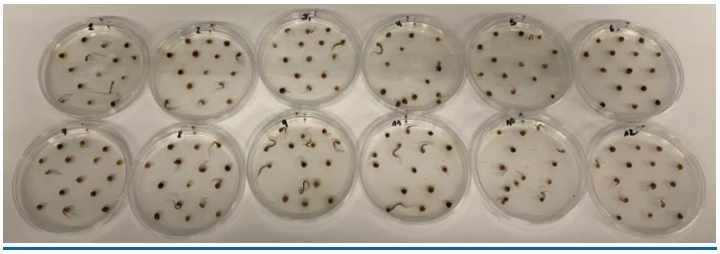


Figure 39. Spinach seeds grew after 7 days.

Therefore, after these preliminary results, no phosphate buffer was used in any of the solutions and the protocol of the cucumber cotyledons was used for the final results.

5.3.2. Results of auxins protocols

Different protocols have been tested for identifying auxin-like activity, which is a phytohormone that contributes to biostimulant activity.

None of the protocols employed provided results that were adequate. The last attempt was the preliminary result in 5.3.1. Figure 38. In this, the higher the concentration of IAA, the less the seedling grew, so the pH of each auxin solution was tested. The auxin hormone stock had a pH of 2, the 10 mg IAA/L auxin solution has a

pH of 3, the 1 mg IAA/L solution has a pH of 4.4 and then the 0.3 mg IAA/L has a pH of 5. It is therefore possible that the pH of the different auxin extracts interferes with the result.

5.3.3. Results of cytokinin protocols

The identification of cytokinin-like activity was performed by methods 4.4.10.2.1. Firstly, a phosphate buffer was tested and proved to be toxic to the plants because it did not allow them to grow properly and caused them to die.

Then, the same protocol was tied but without the phosphate buffer. So, the same experiment was repeated on two different days with different solutions. The experiment on the first day (2nd assay) was designed with a single replicate of 10 cotyledons with four treatments: sterile deionised water (negative control), 1 mg/L KIN, 5 mg/L KIN and 10 mg/L KIN (which were the positive controls). The second day experiment (3rd assay) consisted of three replicates of 10 cotyledons each and with the treatments: sterile deionised water (negative control), *Synechocystis sp.* sonicated and *Synechocystis sp.* sonicated diluted by half (Figure 40).

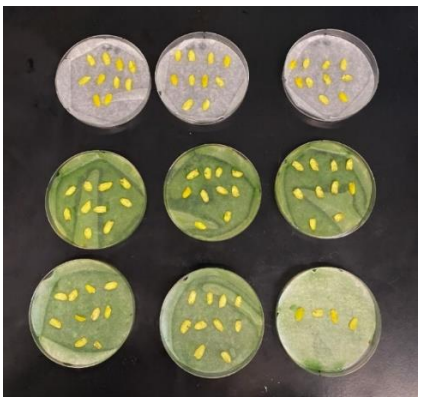


Figure 40. Cucumber cotyledons at the end of the process.

Because both experiments are from different days and there are external conditions that can affect the results, first a significance test was done to check that there is no difference between the two experiments and that they can be grouped together to have more observations and compare all results (Appendix F). The solution that is the

negative control in both experiments is sterile deionised water so this was the one used to do the test.

Table 6. Descriptive statistics of the comparison test of the two sterile deionized waters from different days.

Sample	Number of samples	Mean	Standard deviation	Standard error
H_2O sterile 1	30	11.19	2.42	0.44
H_2O sterile 2	10	11.09	2.70	0.85

The null hypothesis is H_0: $\mu_1 - \mu_2 = 0$ while the alternative hypothesis is H_1: $\mu_1 - \mu_2 \neq 0$.

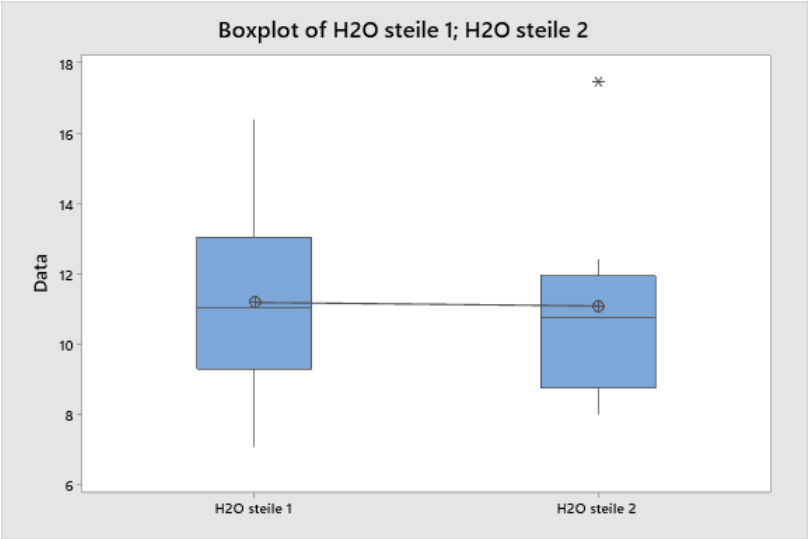


Figure 41. Boxplot of the two sterile deionized waters (negative controls) of the two days.

The null hypothesis of this statistical study is if the sterile deionized water from the first experiment it is significantly the same as the one in the second experiment.

At first glance it can be seen that the means are really similar, it can be observed both in the Table 6 and in the Figure 41. The 95 % Confidence Interval for difference, while the difference is 0.100, is (-1.959; 2.159). Looking at this interval, it can be seen that takes the value of zero and therefore the null hypothesis cannot be disproved, that the null hypothesis is that the difference of the two halves of the groups is zero.

To finish the test, being 0.10 the T-value and having 14 degrees of freedom, the P-value of 0.919 confirms that the null hypothesis is not refuted and that it is therefore statistically significant to hold that there is no difference between the two groups.

Once it is argued that the two sets of data measured on different days are significantly the same, the rest of the data can be compared to each other.

Then, a second statistical study, and the main one, have been done to know if there are significantly differences between the solutions. In the ANOVA, the means of the different extracts have been compared in order to observe and contrast whether there is a statistically significant difference when using the cellular extracts in comparison with the other groups (negative and positive controls). The null hypothesis of the test is that the averages are equal and therefore there is no difference, and the alternative hypothesis is that at least one group has a different average from the others. Before carrying out the ANOVA, we first studied the normality of the observations (Appendix G) and also tested whether the variances were equal or not (Appendix H). The results showed that the data did not follow a normal distribution and that the variances were not equal. As a consequence, the ANOVA was designed to respect the aforementioned characteristics and the Games-Howell method was used when grouping the factors as it is the most appropriate for data that do not have the same variance.

In the one-way ANOVA, the null hypothesis is that all means are equal while the alternative hypothesis is that not all means are equal. The significance level (α) is 0.05. Moreover, equal variances are not assumed for the analysis.

Table 7. Descriptive statistics and confidence Interval of 95% (CI) of the different negative controls and solutions.

Factor	Number of samples	Mean (mg)	SD	95 % CI
H_2O sterile	40	11.17	2.46	(10.38; 11.95)
Sonicated	30	16.90	3.59	(15.56; 18.24)
Sonicated DF=2	24	13.66	3.22	(12.30; 15.02)
KIN 1 ppm	10	11.25	1.97	(9.84; 12.66)
KIN 5 ppm	10	25.57	6.17	(21.16; 29.98)
KIN 10 ppm	10	28.51	6.11	(24.14; 32.88)

Table 8. Games-Howell Pairwise Comparisons of the different negative controls and solutions.

Factor	Grouping			
KIN 10 ppm	A			
KIN 5 ppm	A			
Sonicated		B		
Sonicated DF=2			C	
KIN 1 ppm			C	D
H_2O sterile				D

Means that do not share a letter are significantly different.

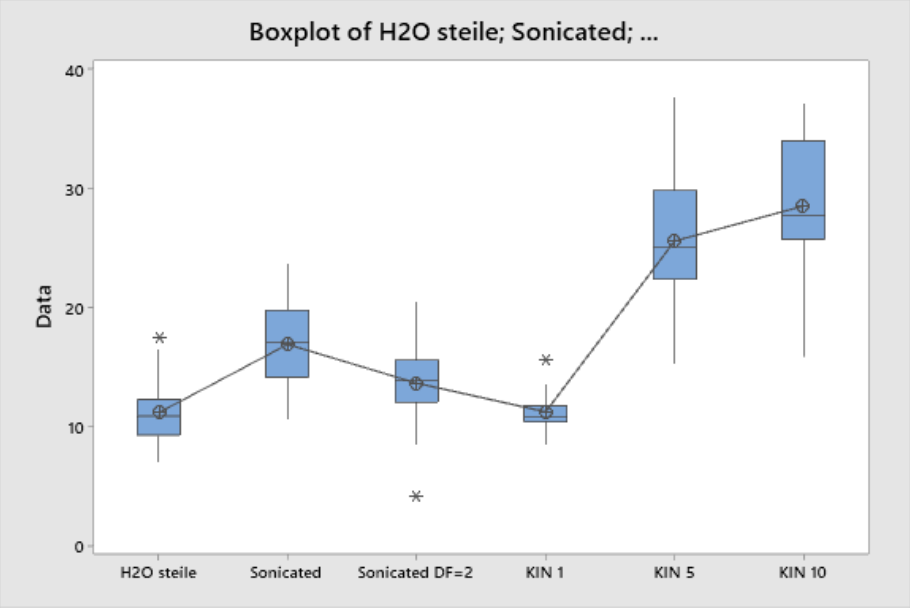


Figure 42. Boxplot of the different negative controls and solutions.

The null hypothesis can be refuted since the confidence intervals do not contemplate at any time the value 0 and the P-value is less than 0.05. In this way, the alternative hypothesis is tested, since in 95 % of the cases there will be differences between the

means of the groups. Analysing the grouping made by the Games-Howell method (Appendix I), the sonicated is different from the rest and it can be observed that on average it has an effect between cytokinin of 1 ppm and 5 ppm (1 mg/L KIN and 5 mg/L KIN, respectively) (Figure 42).

6. Conclusion

6.1. *Synechocystis sp*. cultivation in wastewater

- Species have been growing since day one, except for the last three days where the culture was dominated entirely by another species, then, the solids started to decrease.
- Nitrate tends to decline as part of *Synechocystis sp.*'s diet. As a result, it is regularly added to the PBR.
- Since cyanobacteria intake less phosphorus than they do nitrogen, phosphorus levels tend to decrease more slowly than those of nitrogen.
- Productivity is low, specifically is 74 $mg \cdot L^{-1} \cdot d^{-1}$. Therefore, constant contaminations to the PBR may explain it.

6.2. Biostimulant extraction

- Freeze-thawing is not a good method to lyse the cell.
- Autoclaving produces better results than sonication, but it can also affect biostimulants since it denatures pigments. So, in the end, sonication was the preferred technique.
- To optimise the process of sonication was found that the more cycles, the more pigment is produced and therefore the more the cell breaks down. So, we can conclude that the 10 cycles are necessary and allows the process not to be prolonged.

6.3. Biostimulant activity

- For auxin trials, it can be concluded that more trials and different protocols should be carried out.
- The sonicated biomass has a cytokinin effect between 1 ppm and 5 ppm in cucumber cotyledons and there is a significant difference ($\alpha < 0.05$ %) compared to the control.

7. Further improvement

- Try to grow another type of cyanobacteria that is more resistant to possible contamination.
- Re-try the spinach protocol (Section 4.4.10.2.2.) as a bioassay.
- Continue to test other protocols to measure auxin concentration in *Synechocystis sp.*
- To measure the concentration of *Synechocystis sp.* hormones, a sample should be extracted, purified and then passed through a chromatography called HPLC. In this way, the result will be more precise

8. References

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Appendix

Appendix A: Activated Sludge Wastewater Treatment

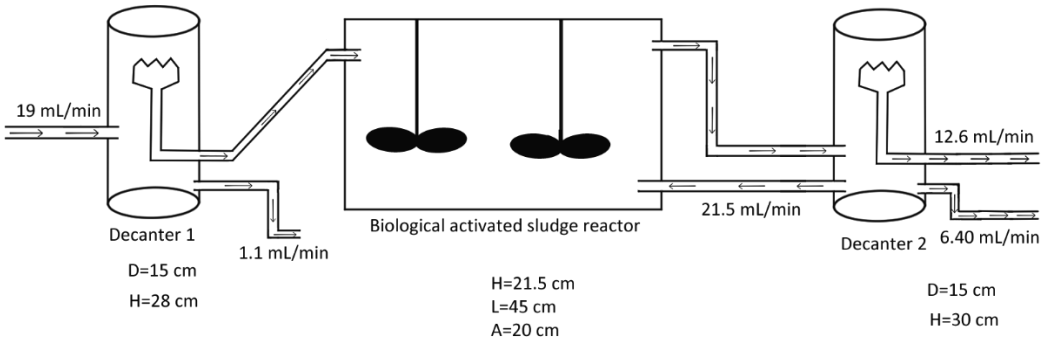

Image 1. **Activated Sludge Wastewater Treatment from UPC Campus Nord.**

Appendix B: Calibration lines

N-NH_4 (mg/L)	Abs
0.1	0.20
0.2	0.33
0.4	0.58
0.6	0.77
0.8	1.03
1.0	1.25

Tables 1. Ammonium absorbance values to do the calibration line.

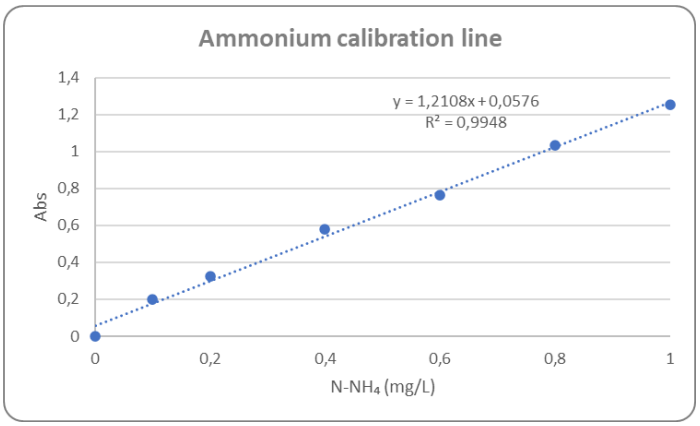

Image 2. Ammonium calibration line.

P-PO_4 (ppm)	Abs (470 nm)
1	0.02
2	0.04
3	0.05
4	0.07
5	0.09
10	0.18
20	0.35

Tables 2. Phosphate absorbance values to do the calibration line.

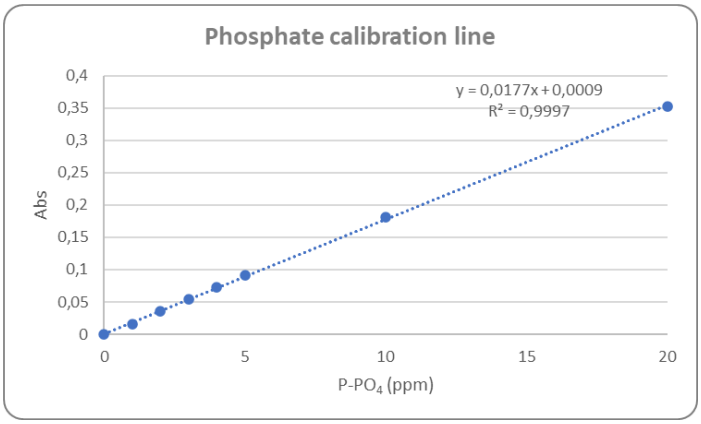

Image 3. Phosphate calibration line.

Days	TIN (PBR) (mg/L)	TIN (AR) (mg/L)	N-NO_3 added (mg/L)
1	58.17	23.30	0.00
3	17.12	23.30	25.04
6	25.31	23.30	2.64
8	7.01	23.30	25.04
10	21.17	23.30	7.18
13	2.72	19.22	25.50
15	15.28	19.22	24.77
17	25.90	26.18	15.61
20	16.69	26.18	9.09
21	23.04	26.18	23.78
24	30.50	26.18	0.00

Tables 3. Nitrate absorbance to do the calibration line.

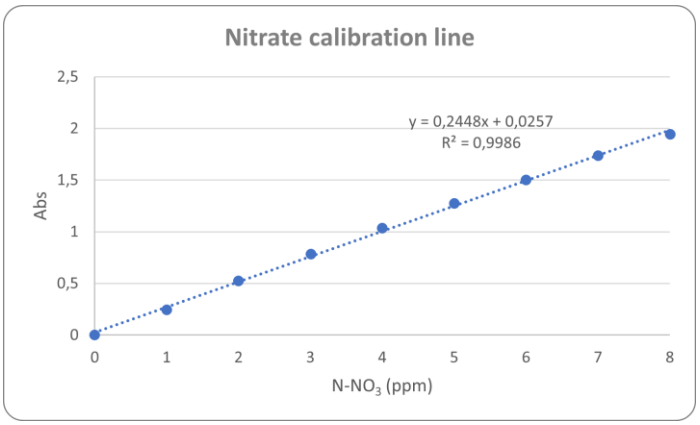

Image 4. Nitrate calibration line.

Appendix C: Data extracted from the PBR cultivation in wastewater

Days	pH	DO (mg/L)	Temperature (°C)
1	8.00	7.10	27.0
3	8.00	5.40	27.0
8	8.00	5.60	25.7
10	8.00	5.60	25.2
13	7.70	5.90	26.7
15	8.20	4.90	26.3
17	7.80	5.10	26.3
20	7.70	5.30	25.6
21	7.60	6.10	25.7
24	7.90	5.80	25.4
Mean	7.89	5.68	26.09
SD	2.39	1.81	7.89

Tables 4. Measured PBR parameters.

Days	TSS (PBR) (mg/L)	VSS (PBR) (mg/L)	TSS (AR) (mg/L)	VSS (AR) (mg/L)
1	447.16	446.33	23.81	23.81
3	461.62	449.70	23.81	23.81
6	378.59	334.36	23.81	23.81
8	427.77	408.88	23.81	23.81
10	531.90	470.78	23.81	23.81
13	374.62	365.37	68.29	60.16
15	543.48	497.83	68.29	60.16
17	548.61	518.04	68.29	60.16
20	703.12	640.68	48.01	44.50
21	703.10	630.85	48.01	44.50
24	578.21	527.49	48.01	44.50
Mean	518.02	480.94	42.54	39.37

Tables 5. Solids data from the PBR cultivation.

Days	TIN (PBR) (mg/L)	TIN (AR) (mg/L)	N-NO_3 added (mg/L)
1	58.17	23.30	0.00
3	17.12	23.30	25.04
6	25.31	23.30	2.64
8	7.01	23.30	25.04
10	21.17	23.30	7.18
13	2.72	19.22	25.50
15	15.28	19.22	24.77
17	25.90	26.18	15.61
20	16.69	26.18	9.09
21	23.04	26.18	23.78
24	30.50	26.18	0.00

Tables 6. TIN data from the PBR cultivation.

Days	P-PO4 (PBR) (mg/L)	P-PO_4 (AR) (mg/L)
1	3.76	5.80
3	4.26	5.80
6	2.75	5.80
8	3.21	5.80
10	2.84	5.80
13	2.84	3.84
15	3.87	3.84
17	4.15	6.05
20	2.47	6.05
21	4.71	6.05
24	4.52	6.05

Tables 7. Phosphate data from the PBR cultivation.

Appendix D: Biostimulant extraction data

Parameters	Before	2 cycles	4 cycles	6 cycles	8 cycles	10 cycles
Abs 615 nm	0.059	0.181	0.267	0.275	0.318	0.337
Abs 652 nm	0.043	0.077	0.104	0.109	0.124	0.138
Phycocyanin (mg/L)	0.007	0.027	0.041	0.042	0.049	0.051
Allophycocyanin (mg/L)	0.006	0.008	0.010	0.010	0.011	0.013
Phycocyanin (%)	0.72	2.71	4.08	4.18	4.85	5.09
Allophycocyanin (%)	0.60	0.77	0.95	1.02	1.14	1.33
Δ Phycocyanin	100.00	374.18	563.74	578.32	671.25	703.27
Δ Allophycocyanin	100.00	128.07	157.72	168.58	188.28	220.98

Tables 8. Pigments data to optimize the sonication process.

Appendix E: Data from the preliminary results

Deionized water		Phosphate buffer		Biomass + buffer		Biomass		Supernatant	
Germinated seeds	Length (cm)	Germinated seeds	Length (cm)	Germinated seeds	Length (cm)	Germinated seeds	Length (cm)	Germinated seeds	Length (cm)
1	1.25	1	0.00	1	0.00	1	0.25	1	0.00
2	1.00	2	0.00	2	0.00	2	0.80	2	0.00
3	1.35	3	0.00	3	0.00	3	0.95	3	0.00
4	1.05	4	0.00	4	0.00	4	0.00	4	0.00
5	1.30	5	0.00	5	0.00	5	0.00	5	0.00
6	1.70	6	0.00	6	0.00	6	0.00	6	0.00
7	1.55	7	0.00	7	0.00	7	0.00	7	0.00
8	1.75	8	0.00	8	0.00	8	0.00	8	0.00
9	1.45	9	0.00					9	0.00
10	0.95	10	0.00						
11	2.10	11	0.00						
12	1.30								
13	1.55								
14	1.25								
15	1.65								
16	2.40								
17	0.80								
18	3.00								
19	1.35								
20	1.55								
21	0.80								
22	0.30								
23	0.65								
24	0.10								

Tables 9. Data from the watercress protocol.

Appendix F: Data from the final experiment of cucumber cotyledon protocol

Cotyledon	Cotyledon weight before (g)	Cotyledon weight after (g)	Cotyledon weight difference (g)	Cotyledon weight difference (mg)	Replicate	Solutions
1	0.017	0.025	0.008	8.00	1	H2O steile
2	0.022	0.033	0.011	11.10	1	H2O steile
3	0.023	0.035	0.011	11.40	1	H2O steile
4	0.020	0.029	0.009	9.40	1	H2O steile
5	0.020	0.031	0.011	11.00	1	H2O steile
6	0.015	0.026	0.012	11.50	1	H2O steile
7	0.018	0.028	0.010	10.10	1	H2O steile
8	0.019	0.034	0.016	15.50	1	H2O steile
9	0.021	0.034	0.013	13.30	1	H2O steile
10	0.015	0.028	0.013	13.20	1	H2O steile
11	0.014	0.021	0.007	7.10	2	H2O steile
12	0.015	0.029	0.014	13.90	2	H2O steile
13	0.018	0.026	0.008	7.70	2	H2O steile
14	0.014	0.026	0.012	12.00	2	H2O steile
15	0.017	0.026	0.009	9.30	2	H2O steile
16	0.014	0.023	0.009	9.30	2	H2O steile
17	0.015	0.024	0.009	8.90	2	H2O steile
18	0.013	0.023	0.010	9.80	2	H2O steile
19	0.016	0,026	0.010	10.40	2	H2O steile
20	0.015	0.025	0.010	10.20	2	H2O steile
21	0.013	0.025	0.011	11.40	2	H2O steile
22	0.017	0.032	0.015	15.00	3	H2O steile
23	0.013	0.026	0.013	13.00	3	H2O steile
24	0.017	0.028	0.012	11.90	3	H2O steile
25	0.015	0.024	0.009	8.90	3	H2O steile
26	0.015	0.024	0.008	8.40	3	H2O steile
27	0.019	0.035	0.015	15.20	3	H2O steile
28	0.017	0.029	0.012	11.80	3	H2O steile
29	0.017	0.033	0.016	16.40	3	H2O steile
30	0.016	0.026	0.011	10.60	3	H2O steile
1	0.014	0.027	0.013	12.60	1	sonicated
2	0.015	0.039	0.024	23.60	1	sonicated
3	0.024	0.045	0.021	20.80	1	sonicated
4	0.022	0.040	0.018	17.90	1	sonicated
5	0.015	0.030	0.015	14.70	1	sonicated
6	0.020	0.041	0.021	20.60	1	sonicated
7	0.018	0.034	0.016	15.90	1	sonicated
8	0.015	0.029	0.014	14.00	1	sonicated
9	0.015	0.029	0.014	14.20	1	sonicated
10	0.018	0.038	0.020	19.50	1	sonicated
11	0.016	0.037	0.021	21.20	2	sonicated
12	0.016	0.034	0.018	18.30	2	sonicated
13	0.014	0.025	0.011	11.20	2	sonicated
14	0.014	0.033	0.019	18.60	2	sonicated
15	0.016	0.026	0.011	10.70	2	sonicated
16	0.013	0.032	0.019	18.80	2	sonicated
17	0.017	0.035	0.017	17.40	2	sonicated
18	0.015	0.027	0.012	12.00	2	sonicated
19	0.011	0.026	0.015	14.50	2	sonicated
20	0.013	0.028	0.015	14.60	2	sonicated

Cotyledon	Cotyledon weight before (g)	Cotyledon weight after (g)	Cotyledon weight difference (g)	Cotyledon weight difference (mg)	Replicate	Solutions
21	0.015	0.032	0.017	16.70	3	sonicated
22	0.015	0.038	0.023	23.00	3	sonicated
23	0.011	0.026	0.016	15.50	3	sonicated
24	0.015	0.031	0.016	15.80	3	sonicated
25	0.015	0.033	0.018	18.00	3	sonicated
26	0.015	0.036	0.021	21.30	3	sonicated
27	0.012	0.034	0.022	21.60	3	sonicated
28	0.014	0.026	0.012	11.90	3	sonicated
29	0.011	0.025	0.014	13.90	3	sonicated
30	0.011	0.029	0.018	18.20	3	sonicated
1	0.016	0.032	0.016	16.00	1	sonicated FD=2
2	0.017	0.033	0.016	16.40	1	sonicated FD=2
3	0.011	0.024	0.013	12.70	1	sonicated FD=2
4	0.015	0.032	0.018	17.60	1	sonicated FD=2
5	0.010	0.024	0.014	14.00	1	sonicated FD=2
6	0.012	0.023	0.012	11.90	1	sonicated FD=2
7	0.012	0.025	0.014	13.80	1	sonicated FD=2
8	0.014	0.023	0.009	8.50	1	sonicated FD=2
9	0.011	0.024	0.013	13.30	1	sonicated FD=2
10	0.005	0.009	0.004	4.10	1	sonicated FD=2
11	0.015	0.031	0.015	15.30	2	sonicated FD=2
12	0.011	0.027	0.016	15.50	2	sonicated FD=2
13	0.014	0.028	0.014	13.90	2	sonicated FD=2
14	0.011	0.023	0.012	12.30	2	sonicated FD=2
15	0.014	0.035	0.020	20.40	2	sonicated FD=2
16	0.014	0.030	0.016	15.60	2	sonicated FD=2
17	0.014	0.029	0.015	15.40	2	sonicated FD=2
18	0.011	0.022	0.012	11.70	2	sonicated FD=2
19	0.017	0.032	0.015	15.40	2	sonicated FD=2
20	0.014	0.030	0.016	16.20	2	sonicated FD=2
21	0.019	0.031	0.012	12.00	3	sonicated FD=2
22	0.014	0.027	0.012	12.40	3	sonicated FD=2
23	0.012	0.025	0.013	12.60	3	sonicated FD=2
24	0.012	0.023	0.011	10.80	3	sonicated FD=2
1	0.020	0.031	0.011	10.80	1	H2O steile
2	0.014	0.022	0.008	8.00	1	H2O steile
3	0.016	0.026	0.011	10.50	1	H2O steile
4	0.012	0.024	0.012	12.40	1	H2O steile
5	0.016	0.025	0.009	8.80	1	H2O steile
6	0.021	0.030	0.009	8.60	1	H2O steile
7	0.018	0.030	0.012	11.80	1	H2O steile
8	0.034	0.046	0.012	11.80	1	H2O steile
9	0.016	0.026	0.011	10.70	1	H2O steile
10	0.013	0.030	0.018	17.50	1	H2O steile
1	0.021	0.029	0.009	8.50	1	KIN 1
2	0.015	0.025	0.011	10.60	1	KIN 1
3	0.014	0.024	0.010	9.80	1	KIN 1
4	0.014	0.025	0.011	11.20	1	KIN 1
5	0.017	0.027	0.011	10.60	1	KIN 1
6	0.017	0.028	0.011	11.00	1	KIN 1
7	0.022	0.035	0.014	13.50	1	KIN 1
8	0.022	0.037	0.016	15.60	1	KIN 1
9	0.018	0.029	0.011	10.60	1	KIN 1

Cotyledon	Cotyledon weight before (g)	Cotyledon weight after (g)	Cotyledon weight difference (g)	Cotyledon weight difference (mg)	Replicate	Solutions
10	0.019	0.030	0.011	11.10	1	KIN 1
1	0.013	0.037	0.024	23.50	1	KIN 5
2	0.015	0.053	0.038	37.60	1	KIN 5
3	0.015	0.041	0.026	26.00	1	KIN 5
4	0.016	0.046	0.030	29.70	1	KIN 5
5	0.012	0.037	0.024	24.20	1	KIN 5
6	0.015	0.045	0.030	30.10	1	KIN 5
7	0.014	0.029	0.015	15.30	1	KIN 5
8	0,016	0.042	0.027	26.50	1	KIN 5
9	0.013	0.032	0.019	18.90	1	KIN 5
10	0.014	0.038	0.024	23.90	1	KIN 5
1	0.019	0.055	0.036	35.90	1	KIN 10
2	0.014	0.040	0.026	25.80	1	KIN 10
3	0.018	0.052	0.033	33.30	1	KIN 10
4	0.018	0.044	0.026	25.90	1	KIN 10
5	0.016	0.046	0.030	30.30	1	KIN 10
6	0.012	0.040	0.028	28.00	1	KIN 10
7	0.011	0.037	0.026	25.50	1	KIN 10
8	0.015	0.043	0.028	27.50	1	KIN 10
9	0.019	0.056	0.037	37.00	1	KIN 10
10	0.014	0.030	0.016	15.90	1	KIN 10

Tables 10. Data from the cucumber cotyledon protocol.

Appendix G: Tests of normality of the final experiment of cucumber cotyledon protocol

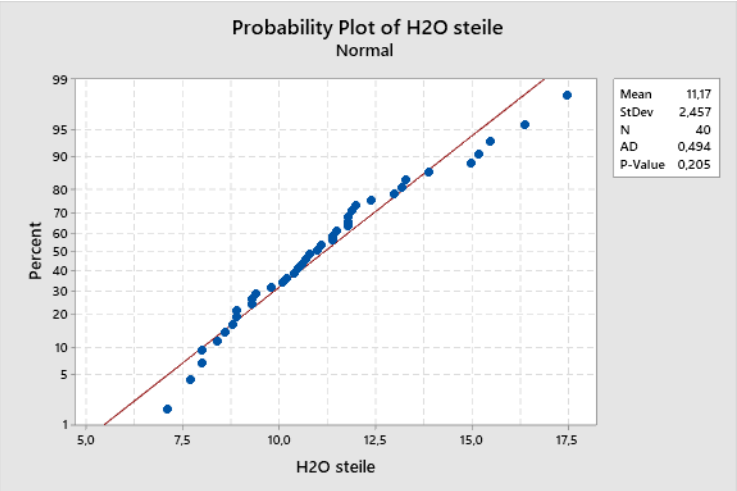

Image 5. Normality test sterile water.

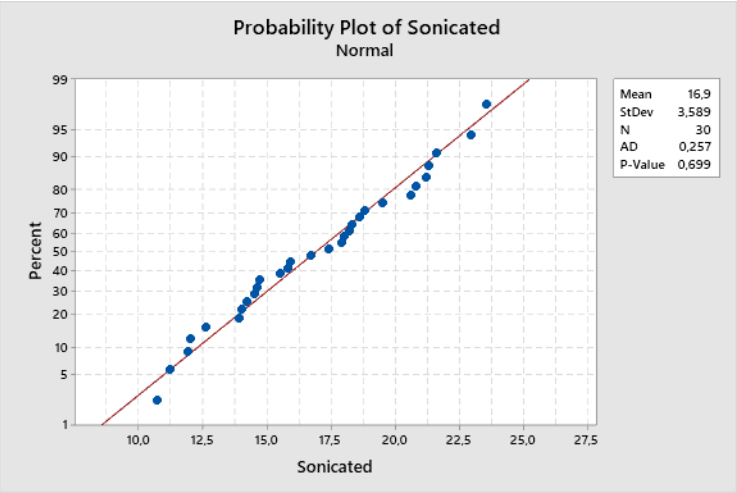

Image 6. Normality test sonicated extract.

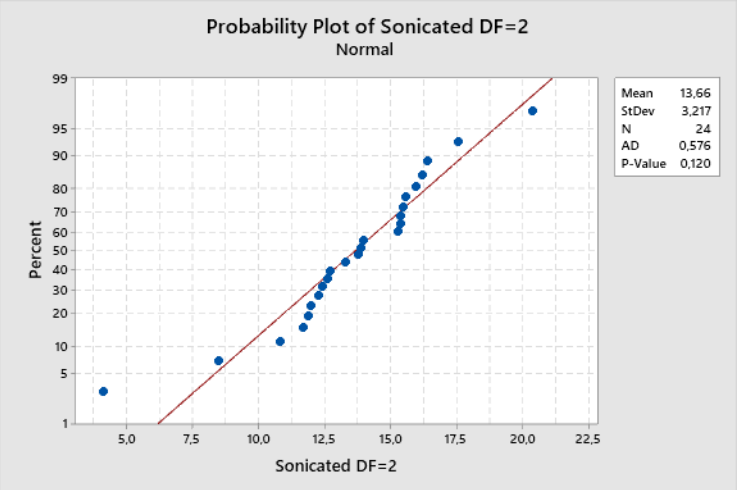

Image 7. Normality test sonicated extract diluted by half.

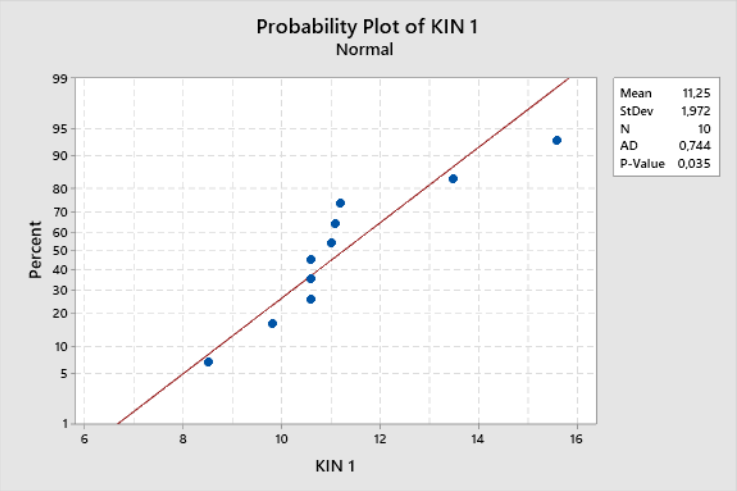

Image 8. Normality test kinetin 1 ppm.

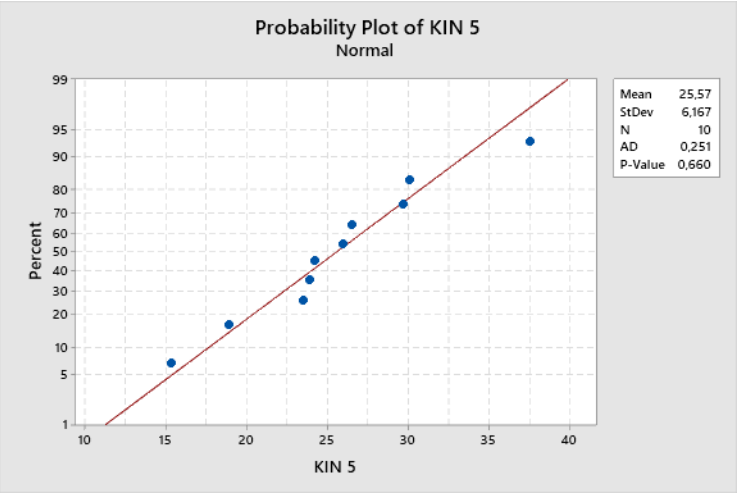

Image 9. Normality test kinetin 5 ppm.

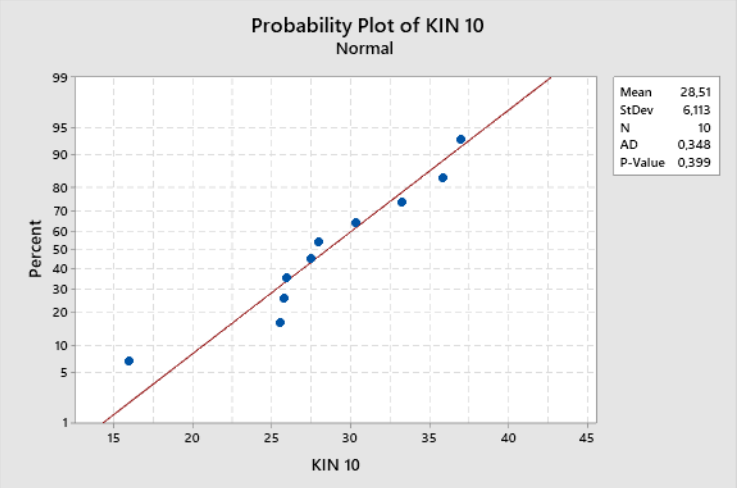

Image 10. Normality test kinetin 10 ppm.

Appendix H: Tests for equal variances of the final experiment of cucumber cotyledon protocol

Test for Equal Variances

Null hypothesis: All variances are equal

Alternative hypothesis: At least one variance is different

Significance level: 0.05

Sample	Number of samples	Standard deviation	CI
H2O sterile	40	2.45697	(1.80720; 3.5762)
Sonicated	30	3.58897	(2.82159; 5.0052)
Sonicated DF=2	24	3.21666	(1.79371; 6.4809)
KIN 1	10	1.97217	(0.68395; 7.7247)
KIN 5	10	6.16677	(2.92724; 17.6472)
KIN 10	10	6.11327	(2.87839; 17.6367)

Tables 11. 95% Bonferroni Confidence Intervals for Standard Deviations. Individual confidence level = 99.1667%.

Method	Test Statistic	P-Value
Multiple comparisons	-	0.041
Levene	4.17	0.002

Tables 12. Tests.

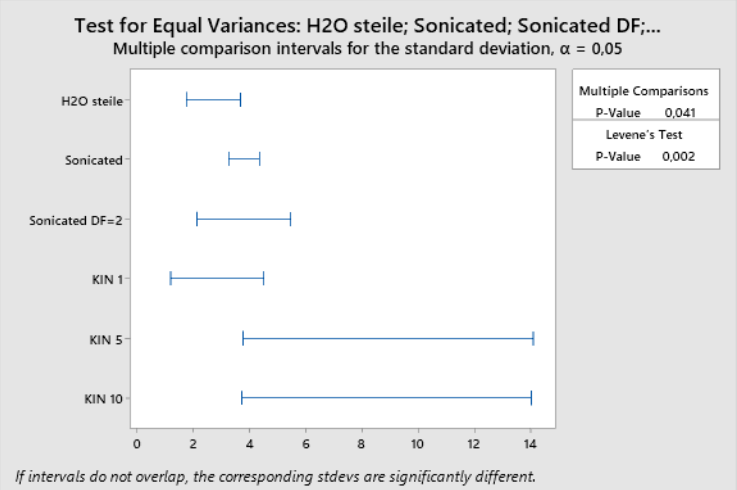

Image 11. Test of equal variances between the different solutions.

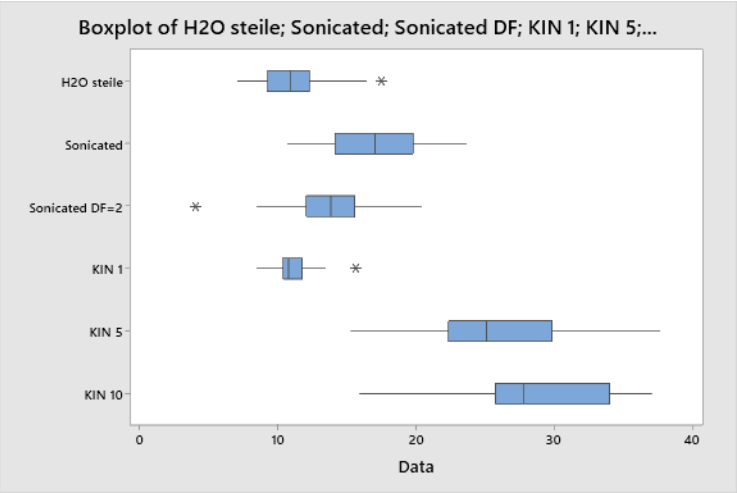

Image 12. Boxplot between the different solutions.

Appendix I: Games-Howell test for the final experiment of cucumber cotyledon protocol

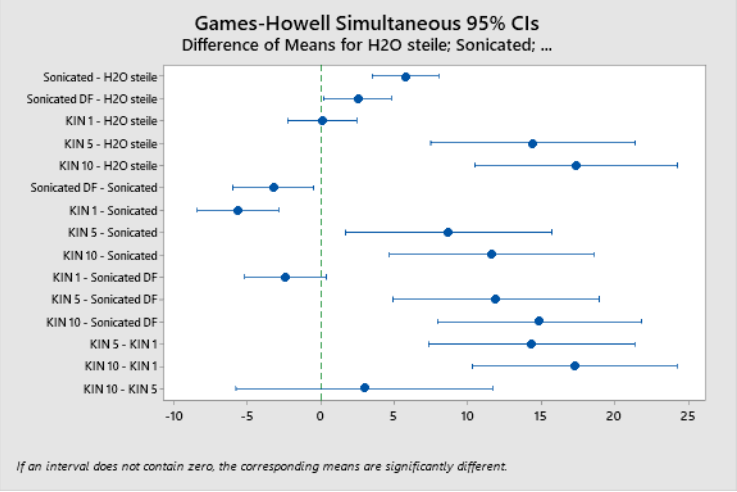

Image 13. Games-Howell test between the different solutions.