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Faculty of Science

Seasonal occurrence of picoplanktonic cyanobacteria in WTP Milence and their application in Nanobiotechnology

Bachelor thesis

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Annotation

This Bachelor thesis' objective was to determine the penetration of the living picocyanobacteria occurring in the Nýrsko water reservoir into the technological stages of the Water treatment plant Milence. Subsequently, the work also included the first experiments with the use of picocyanobacteria in the nanotechnologies used for the sorption of the pollutants occurring in the water, in this case, dyes. For the separation, the microwaved magnetite was experimented with.

Key words: Picocyanobacteria, water treatment, drinking water, magnetite, biosorption, dye, malachite green, safranine O

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

Student's signature

České Budějovice, 11.8.2023

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List of abbreviations

1b% – percentage of individual picocyanobacteria in the total amount
AA – after accumulation
AOC – assimilated organic carbon
AG – aggregation
a.s. – Public Limited Company
AU – absorbance unit
BA – before accumulation
COD_{Mn} – chemical oxygen demand by the permanganate
Col% – percentage of picocyanobacterial colonies in the total amount
Coll. – collection of laws
ČSN – protected marking of Czech technical standards
ČSN EN ISO – Czech version of the international standard
DAF – dissolved air flotation
MASL – metres above sea level
MG – malachite green
mil. – million
MW Mag – Microwave synthesized magnetite particles
NA – numerical aperture
NCBI – National Center for Biotechnology Information
n.d. – no date
p.a. – per analysis
Pcy – picocyanobacteria
PVL – Povodí Vltavy
RCF – relative centrifugation force
RS – Reservoir Surface
RW – Raw Water
S – supplemented material
s.p. – state enterprise
s.r.o. – private limited company
UB VŠCHT – Department of Biotechnology, University of Chemistry and Technology, Prague
UF – ultrafiltration
UV – ultraviolet
VIS – visible light
WHO – World Health Organization
WTJ – Water Tank Janovice
WTP – Water Treatment Plant

1 Introduction

The water treatment for drinking water is a very discussed and crucial topic. The requirements for treated water are developing every day according to new findings, to achieve the best quality without any harmful or problematic substances and items. In the first period of treatment, the view was pointed only to the chemical and physical qualities. Subsequently, with the new developments and new data, the hydrobiological requirements were taken into account. This work deals with the issue of picocyanobacteria in water treatment, showing how deep they can penetrate into the water treatment technology and how these organisms can be eliminated.

However, these organisms can also be used as the biosorbents for the removal of pollutants such as dyes or heavy metals. Nowadays environmental contamination is more probable due to ineffective dyeing techniques and widespread application. In this way, the work was also focused on the use of picocyanobacteria in the direction of nanobiotechnology, in which they are used together with microwaved magnetite for the biosorption of hazardous dyes from the water.

2 Picoplanktonic organisms

Picoplankton is a hydrobiological term for organisms ranging in size from 0,2 μm to 2 μm. Until the 1970s, picoplanktonic microorganisms were not mentioned or considered in scientific literature. The explanation for the situation was due to the size of these aquatic organisms. In some publications, the word 'ultrananoplankton' is also seen and used (Říhová Ambrožová, 2007).

2.1 Picoplanktonic cyanobacteria

2.1.1 General information

Picoplanktonic cyanobacteria (aka picocyanobacteria, Pcy) are autotrophic prokaryotes that live in both aquatic water environments, freshwater and marine, ranging in size from 0.2 to 2 μm (Sandoval Valencia et al., 2022). Picoplankton belongs to a group of ubiquitous organisms, in other words, these organisms can be found in almost every biotope on Earth. These unicellular organisms can occur in aquatic environments not only as individuals, but also in colonies (Figure 1).

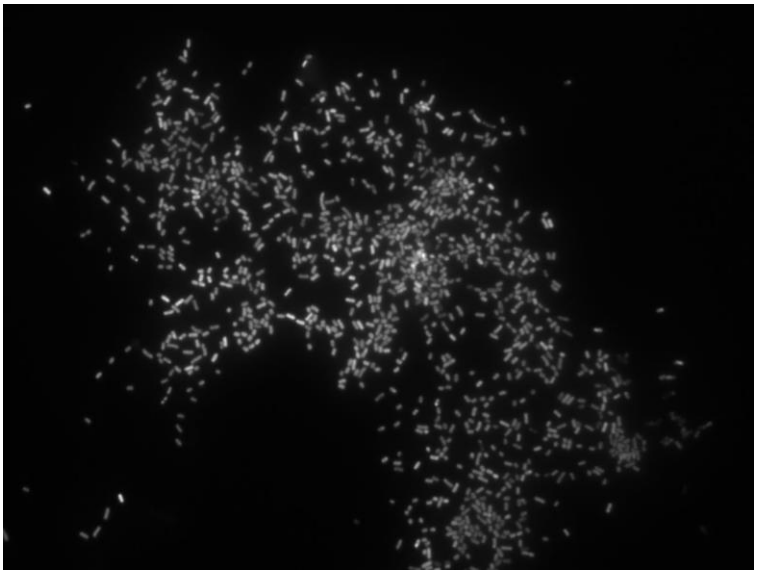

Figure 1. Colony of picocyanobacteria from reservoir Nýrsko captured by fluorescence microscope (author´s archive)

The classification of picocyanobacteria is divided into 5 genera – *Synechococcus*, *Cyanobium*, *Synechocystis*, *Cyanothece*, and *Cyanobacterium*. The most common freshwater groups of Pcy are *Aphanocapsa*, *Aphanothece*, *Chroococcus*, *Coelosphaerium*, *Cyanobium*, *Cyanodictyon*, *Merismopedia*, *Romeria*, *Snowella*, and *Tetracercus* (Jasser & Callieri, 2016; Callieri et al., 2013). These species do not form algal bloom structures but are capable of developing colonies (micro- or large).

There are two shapes, in which Pcy can occur - spherical and rod-shaped. To distinguish the shape, literature suggest the use of epifluorescence methods, which can analyze not the only shape but also colour of the auto-fluorescence of picoplanktonic organisms (Komárková, 2002; Švecová, 2019). The picocyanobacterial cell walls contain both gram-negative and gram-positive elements. Part of this wall structure is peptidoglycans, and in many cases, there is an S-layer (paracrystalline protein thin layer) on the surface that contains a group of substances called glycoproteins. This layer is used to maintain cell integrity (Jasser & Callieri, 2016).

The thylakoids are parietally arranged in the cell and contain photosynthesis pigments. The pigments, specifically phycocyanin (blue-green pigment), phycoerythrin (red pigment), and allophycocyanin, belong to the structure of phycobilisomes. The main function of these structures is to capture the energy and transfer it to the membrane photosystems containing the green photosynthetic pigment chlorophyll *a*. These light-collecting antennas facilitate the use of wavelengths that chlorophyll cannot catch under normal conditions (Haverkamp, 2008).

2.1.2 Ecology and its role in the environment

Pcy survive in most water conditions – they can be found in oligotrophic, mesotrophic, eutrophic, or even in hypertrophic types of waters. Due to their body structure and other features, these organisms are able to survive in conditions with low nutrient concentrations and under reduced light. Their small body size and ratio of surface to volume give them an advantage in the catching and absorption of the nutrients present in water (Agawin et al., 2000). Body size helps to reduce the minimum energy needed for the processes within the cell (Callieri & Stockner, 2002). These and other qualities such as smart and adapted usage of nutrients help Pcy with the ability to grow and survive in deeper section of the water column (Jakubowska & Szeląg-Wasielewska, 2015). Individual cells are usually represented in the deeper layers of the water columns, in contrast to the colonies of Pcy, which can be found mostly on the surface (Psohlavec, 2022).

Pcy have two peaks of their maximum growth number, firstly at the point of spring/summer, and secondly at the point of summer and early autumn of the year's vegetative season. This type of development is called bimodal (Jasser & Callieri, 2016; Psohlavec, 2022).

Picocyanobacteria are a key component of primary production and play a major role in the global carbon cycle and in other biogeochemical cycles (Jürgens & Matz, 2002; Jezberová,

2023). Also, these organisms act as prey (mainly protozooplankton and metazooplankton). Another important ecological role of Pcy is in the development of eukaryotic photosynthesis (Callieri et al., 2022).

Unlike cyanobacteria, the toxicity caused by Pcy is reported very rarely. This is usually because the sampling and methods used are not adapted for this type of organism. However, in several cases, the production of bioactive compounds was indicated by several laboratory experiments. One of the most abundant toxins, which is generated by the cyanobacteria is the toxin from the microcystins family (Jasser & Callieri, 2016). Its production by picocyanobacteria was determined by Bláha & Maršálek (1999). However, the concentrations were much lower than the ones in the cyanobacterial blooms. The production of microcystin was then confirmed by other studies like Domingos et al. (1999) or Vareli et al. (2013). Cox et al. (2005) claims that different groups of cyanobacteria, as well as Pcy, are also able to produce harmful neurotoxins. This study was supported, among others, by Cervantes Cianca et al. (2012). Not only neurotoxins but also endotoxins, like LPS, can be found in the Pcy species (Schmidt et al., 1980). Other cases of toxins produced by the Pcy which were described in recent years are for example by Leland et al. (2023) or Gin et al. (2021).

2.1.3 Presence of picocyanobacteria in water treatment technologies

For the best quality of treated water used for drinking, it is important to monitor not only physical-chemical properties of the water, but also hydrobiological properties, which can cause technological issues throughout the process of water treatment (Ambrožová & Říha, 1998). After the penetration of cells from raw water to the water treatment, there are several risks which may arise. Large cyanobacterial cells can clog the filters - this can also happen with the small cells of Pcy if the amount is sufficient. This may result in the shortening of filtration cycles. However, the picocyanobacteria can break through the filters and influence the quality of the treated water, especially colour and turbidity (WHO, 2015).

One of the water treatment technologies, coagulation complemented by filtration and sedimentation, is highly effective for the elimination of cyanobacteria and cell-bound cyanotoxins from the raw water. Nevertheless, this elimination method is not effective for smaller species of cyanobacteria and for the not cell-bound toxins, continuously degrading water quality. The ineffectiveness can cause the exceeding of hydrobiological requirements of treated water, which is set within the microscopic image of 50 dead individuals per millilitre and zero live organisms as amended by later regulations. These boundaries are set in the Decree No. 252/2004 Coll. with the name “Vyhláška, kterou se stanoví hygienické požadavky

na pitnou a teplou vodu a četnost a rozsah kontroly pitné vody" ("Decree establishing hygienic requirements for drinking and hot water and the frequency and scope of drinking water control"). One of the options for higher effectiveness is using pre-oxidation via chlorination or ozonisation. However, this approach increases the risk of releasing cyanotoxins into the water after the cells burst. For reducing the number of Pcy, post-coagulation is used (WHO, 2015).

Cyanotoxins produced by the cyanobacteria in algal blooms are well-known. However, the production of toxins by picocyanobacteria has also been reported. For example, the strains of *Synechococcus* spp. are able to generate the neurotoxic β-N-methylamino-L-alanine and microcystin (Gin et al., 2021; Bláha & Maršálek, 1999).

Because of the water treatment technology and utilization of disinfection substances, such as chlorine dioxide, the formation of assimilated organic carbon is caused (AOC), which can then be used by living organisms, such as bacteria, which can potentially develop microbiological growth in the distribution system and subsequently generate the biofilms (Müller et al., 2003).

3 Elimination methods of hydrobiological contamination of water

The hydrobiological contamination of drinking water is usually solved using two methods – membrane separation methods and dissolved air flotation.

Membrane separation methods are classified as a physicochemical process. The technique is based on a semi-permeable membrane – a barrier with the ability to let through only a defined group of particles. This membrane divides the solution into two parts – the retentate stream (one with the compounds that the membrane will not let through) and the permeate stream (one that passes through the membrane). The force that powers each membrane process is called the transmembrane gradient. If the process falls into the category of pressure types, the main force used is the pressure gradient (UB VŠCHT, 2014; Honzajková et al., 2010).

The membrane processes are further subdivided according to the pore sizes into microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. However, currently, the division is not very precise and differs in different literatures (UB VŠCHT, 2014; Honzajková et al., 2010). In Figure 2, the categories of membrane separation processes in water treatment and their efficiency are shown.

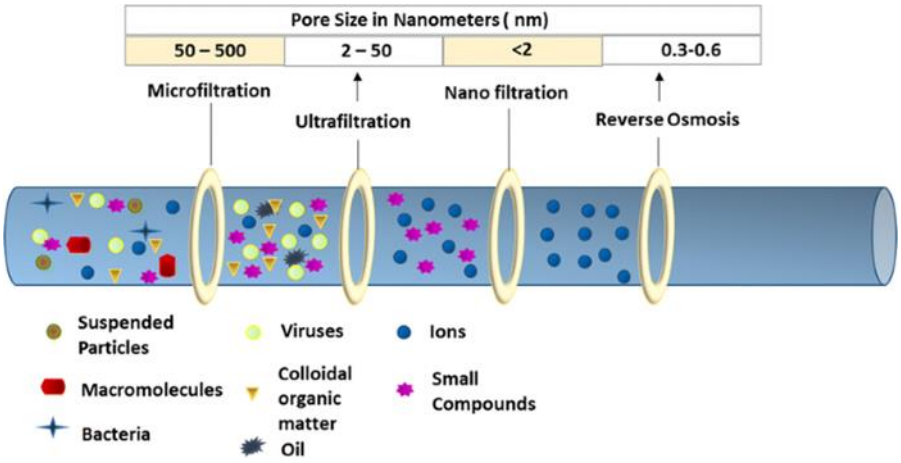

Figure 2. Categories of membrane separation methods and their efficiency of molecules (Rezakazemi et al., 2018)

3.1.1 Ultrafiltration

Ultrafiltration (UF) is a membrane filtration process that is used to separate bacteria, viruses, or parasites and molecules with a size between 10-100 nm (Kožíšek, 2010). Particles that are larger than the pores of the membrane are blocked on the outer side of the membrane. Compared to e.g. reverse osmosis, minerals are still present in water even after this step of the water treatment (Woodard, 2022). The main advantages of UF are compact usage, separation

by molecule size, easy automation, stable water quality free of pollution, less sludge creation, or a lesser need of chemicals (Xia et al., 2004; Nakatsuka et al., 1996).

The mechanism is based on the difference between the pressures of the sides of the UF membrane. The pure water and small molecules in the liquid pass through the pores of the membrane, which means from the side with a higher pressure to the side with a lower one. The molecules that are bigger than 2 nm are stuck in front of the membrane, such as solids, proteins, and microorganisms. The ultrafiltration process is shown in Figure 3 (Li et al., 2018).

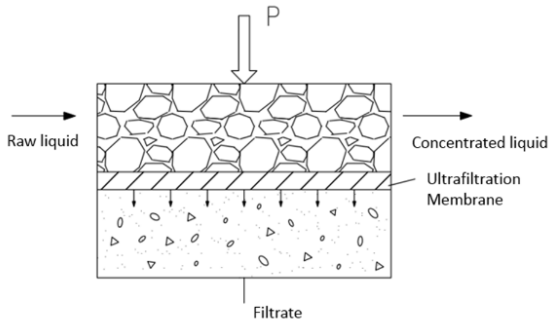

Figure 3. Scheme of the ultrafiltration process (Li et al., 2018)

3.1.2 Dissolved air flotation

Dissolved air flotation (DAF) is a method most frequently used to remove various contaminants, including colloids, precipitates, ions, microorganisms, proteins, and dispersed and emulsified oils from water (Rodrigues & Rubio, 2007). This separation process is classified as the first separation step in drinking water treatment or purification of various types of wastewaters. Substances suspended in the water are precipitated into flakes using the coagulation process. These flakes are subsequently carried away by a stream of air microbubbles (Figure 4). The layer of sludge is removed by hydraulic or mechanical mechanisms (ENVI-PUR, s.r.o., 2023).

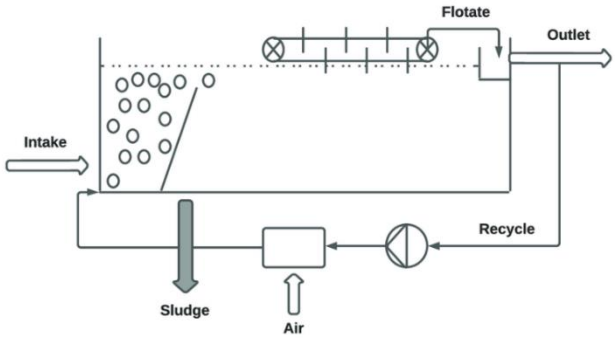

Figure 4. The scheme of the dissolved air flotation system (Philipp et al., 2021)

4 The Water treatment plant Milence

4.1 Basic information and history

The plant was built between 1980 and 1986. The water treatment plant Milence (hereinafter referred to as WTP) began its operation in 1988. This treatment plant is located in the village of Milence next to the water reservoir Nýrsko, from which the raw water for treatment is pumped. WTP Milence currently supplies the city of Klatovy, Domažlice and other towns along the route of the Klatovy drinking water pipeline (water intended for human consumption). The approximate number of individuals supplied by this WTP is 50,000. The maximal original performance of the treatment plant was 440 L/s. In 2005, the WTP was reconstructed with an output of 230 L/s (Vodohospodářský podnik a.s., 2020; Sochor, 2017).

4.2 The Water reservoir Nýrsko

The water reservoir Nýrsko (Figure 5) is located in Western Bohemia on the upper reaches of the Úhlava River, a few kilometers away from the town of Nýrsko. This reservoir is located in the 3rd zone of the National Park Šumava. Toward the village of Hojsova Stráž, above the reservoir, there is a nature reserve called Úhlavský luh.

The main reason for the establishment of this reservoir was the improvement of the river flow for the water treatment plant on Homolka Hill, which is located in the city of Pilsen. Construction of the reservoir ended in July 1969. Now, the water reservoir is managed by the company “Povodí Vltavy, s. p.” (Sochor, 2017).

Figure 5. Water reservoir Nýrsko (Znachor, 2023)

The reservoir itself has a total volume of 20.5 mil. m^3 and a total flood area of 148 ha. It has the capacity to collect water from 80 km^2. The length of the water rise is 2.5 km, and the dam is loose, rocky, and approximately 320 m long. The crown of the dam is 525.11 m above sea level and 36.2 m above the bottom and is located 93.690 km from the estuary of the Úslava River. The scheme of the Nýrsko water reservoir can be seen in Figure 6 (VD Nýrsko, n.d.).

Figure 6. Scheme of the water reservoir Nýrsko (depths in the individual sections marked) (Retour, 1998)

The drainage and communication tunnels stretch along the left bank. The circular tower was built at one end of the communication tunnel. It contains water intake points, an overflow shaft (Figure 7), and two lower outlets. There are three water collection points (sampling profiles) at three different depths: 515 MASL (10.5 m from the overflow level), 506.5 MASL (16.5 m from the overflow level; the only sampling point used), and 498 MASL (25 m from the overflow level) (Vodohospodářský podnik a.s., 2020; Sochor, 2017). The raw water in the plant comes all year only from the 16.5 m deep collection point.

Figure 7. The dam and the overflow shaft of the water reservoir Nýrsko (author´s archive)

4.3 Quality of raw water

4.3.1 Chemical properties

Raw water from the Nýrsko reservoir can be characterised as water with neutral pH (slight changes around pH 7 are tolerated), low hardness (low amount of calcium and magnesium compounds), minimal mineralisation, and minimal inorganic and organic specific contamination (Table 1). This water also contains organic substances of the humic type – such as fulvic acids. Microbiological pollution is in range for this type of water body.

The chemical quality of the reservoir water is described as long-term stable. If there are any fluctuations, they are usually due to exceptional climatic conditions – such as floods, torrential rains, or water circulations in the reservoir (Vodohospodářský podnik a.s., 2020; Sochor, 2018).

Table 1. The parameters of raw water from reservoir Nýrsko measured in the period 01/2022-12/2022

Monitored parameter	Unit	Minimum value for the year 2022	Maximum value for the year 2022	Average value for the year 2022
pH		6.580	7.500	
Alkalinity	mmol/l	0.200	0.300	0.270
Nitrite NO_2^-	mg/l	0.027	0.104	0.056
Nitrate NO_3^-	mg/l	1.600	2.900	2.310
COD_{Mn}	mg /l	1.440	3.200	2.030
Hardness Ca + Mg	mmol/l	0.110	0.180	0.150
Iron Fe	mg/l	0.040	0.260	0.080
Manganese Mn	mg/l	0.015	0.192	0.070
Chloride Cl^-	mg/l	2.000	3.100	2.360
Turbidity	ZF	0.700	1.300	1.030
Colour	mg /l	2.700	27.300	11.850
Calcium Ca	mg/l	3.570	6.430	4.090
Magnesium Mg	mg/l	0.430	1.730	1.220
Conductivity χ	mS/m	3.600	4.000	3.800
Aluminium Al	mg/l	0.010	0.067	0.033
Sulphate SO_4^{2-}	mg/l	3.600	5.800	4.560
Humic substances	mg/l	0.612	2.780	1.730
Phosphate	mg/l	0.010	0.030	0.016

4.3.2 Hydrobiological conditions

The first mention of the higher number of organisms in the reservoir water was in 2014. The definition of the term “organism” and “picocyanobacteria” is defined by the standard ČSN 75 7712 (“Kvalita vod - Biologický rozbor - Stanovení biosestonu”) and ČSN 75 7717 (“Kvalita vod - Stanovení planktonních sinic”) – “organism” is defined as every living animal inside of

the sample, “picocyanobacteria” is defined as those creatures fulfilling the characterisation of this group of organisms (see chapter 2.1). The found organisms were determined as nonspecified picocyanobacteria. Further analyses in 2015 and 2016 confirmed a higher number of picocyanobacteria in the water, especially in the summer season – 10 to 20 thousand in 1 mL of water, which can be seen in Figure 8. In 2015, the experiment showed that they were evenly distributed throughout the water column. For research in those years, the zonal sampling technique was used (Sochorová, 2019).

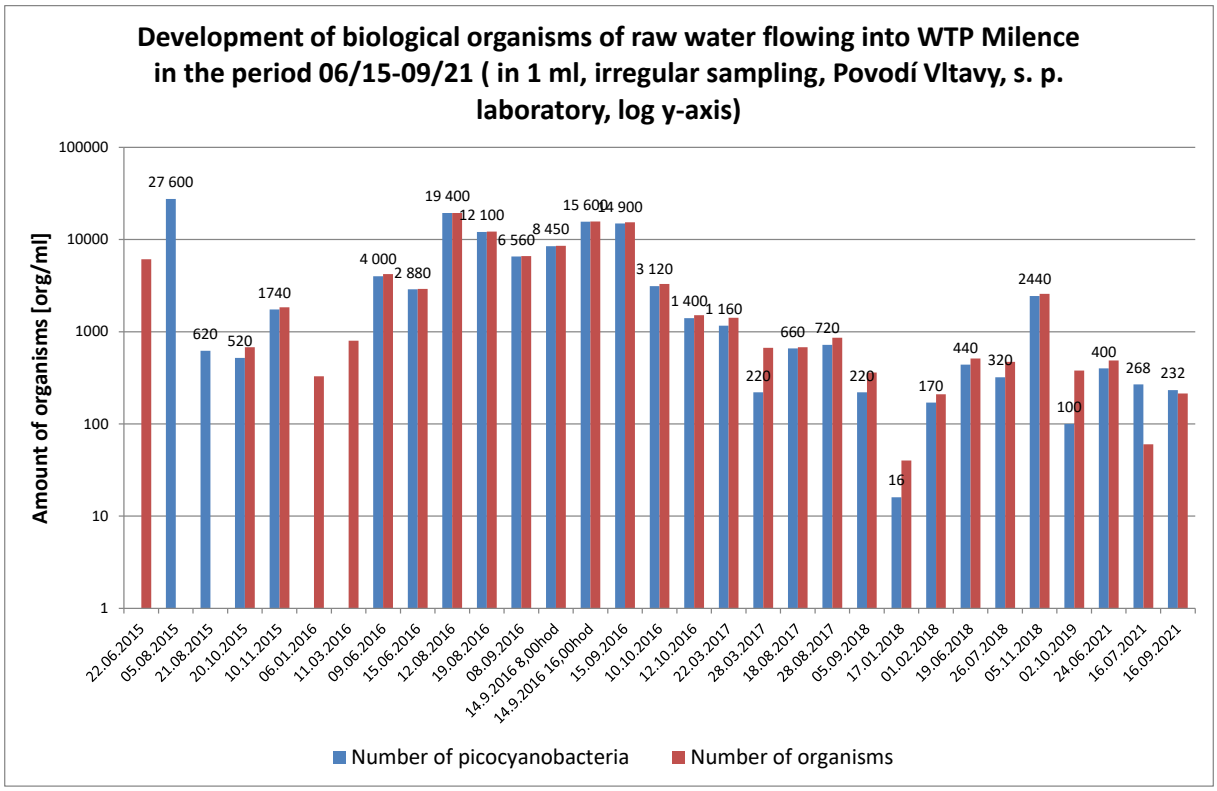

Figure 8. Development of organisms in raw water from reservoir Nýrsko in period 06/15-10/22 (in 1 mL, irregular sampling, laboratory PVL, log axis) (adapted from (Sochorová, 2019))

The value of the hydrobiological parameters fluctuates depending on the season and environmental conditions. The highest values are recognised at the peak of the vegetative period, which means in the summer season. During the monitoring of the Nýrsko reservoir and its qualities, it was confirmed that the number of overall organisms in the water varies throughout the day. This is due to the sunlight and the temperature of the water. Depending on these conditions, also Pcy migrate during the day toward the water's surface (Sochor, 2018; VODOSPOL s. r. o., 2018)

In Figure 9, the picocyanobacteria from the reservoir Nýrsko can be seen. They were determined by the Associate Professor Jan "Hanys" Kaštovský (Department of Botany, Faculty of Science University of South Bohemia) as *Synechococcus sp.*

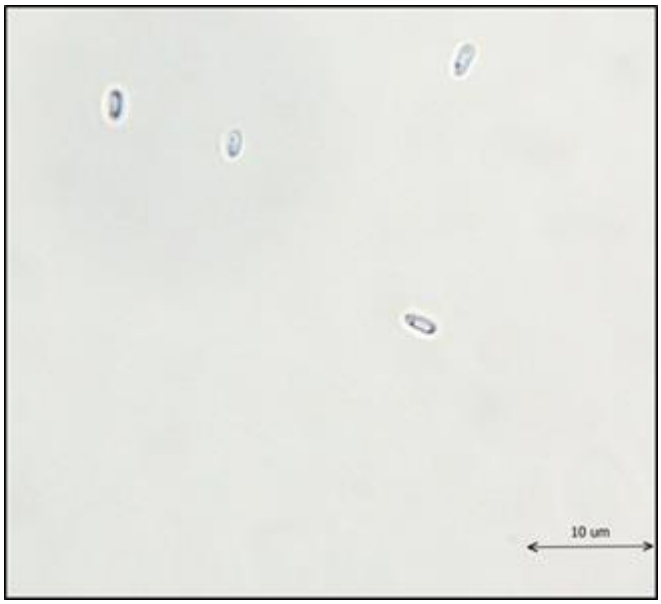

Figure 9. Synechococcus sp. from the Nýrsko reservoir (Source: Povodí Vltavy, Dr. Hess, 2016)

4.4 Technology of water treatment

The first step of the treatment is the transport of raw water from the water reservoir to the WTP. For this purpose, the line from the raw water pumping station is used (inner diameter 600 mm). This mechanism is situated under the dam of the reservoir. The pipe ends up in the dosing and filtration building in the intermittent tank. The regulation valve was installed to maintain a constant level of water in the tank. In this tank, the local water turbulence mechanism is placed, to which the coagulant compound is added. As a coagulant, the pre-polymerized aluminium chloride (PAX-18) is used. The dosage is managed by COD_{Mn} of raw water which is in the interval from 0.72 to 2.00 mg/L Al. The pre-alkalization is not used.

Subsequently, water is transferred through lines with two valves: the first is used to keep the water level in the intermittent tank constant and the second is applied as the homogenising flap with a fixed setting. In the aggregation tank, the water stream then divides into three separate branches – there the aggregation of flakes happens. In this step of the water treatment, the water volume is stirred by perforated walls. Then the water with the products of aggregation - commonly called flakes - is filtrated using sand-type filters.

The WTP has 8 sand-type filters. In 2005, 4 of them were constructed – these can be active in the process of water treatment. Four parallel filters are active at the WTP – three of them are

always in operation, and the fourth one is regenerated. The filters are sand-type and contain the Leopold drainage system. In the WTP Milence, for this type of filtration, the FP2 size sand is used. This type of filter consists of silica sand of a grain size 1.0 – 1.6 mm. The filtration cycle of one filter is approximately 96 hours (sometimes the fluctuation can be seen). After this period, the filter must be upstream regenerated by treated water and air to prevent clogging of the filter and contamination of treated water. The need for washing is detected by the increase in the water level. This aspect is measured by the ultrasound level gauge. From the outgoing water quality and observations by trained staff, the filtration cycle can be adjusted.

The filtered water is then poured into the individual tank, from which the water flows through pipes to the water accumulation section. According to the flow meter, which is installed there, the dosage of batch treatment chemicals is subsequently modified. These compounds are lime water ($Ca(OH)_2$), carbon dioxide (CO_2), and chlorine dioxide (ClO_2). Part of the water is taken before the flow measurement to prepare the lime water solution and as a drift medium for chlorine dioxide. Lime water and carbon dioxide work in water treatment as water stabilators. The objective is to achieve the calcium-carbonate balance of the treated water (Vodohospodářský podnik a.s., 2020; Sochor, 2018). The treated water is collected in the accumulation tank (2×2 500 m^3) and is pumped in the direction of Domažlice. In the direction of Nýrsko, Janovice nad Úhlavou and Klatovy, water is forced to flow by gravitation. The scheme of the current technology of water treatment at WTP Milence is shown in Figure 10.

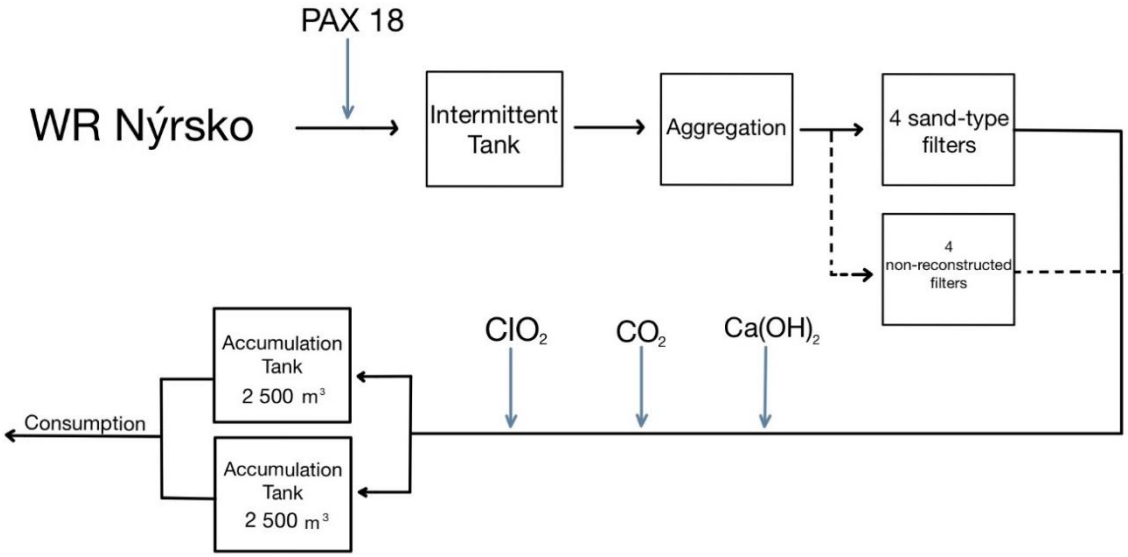

Figure 10. The scheme of the WTP Milence (adapted from Sochor (2017))

4.5 Quality of treated water

The quality of treated water is shown in Table 2.

Table 2. Treated water quality parameters in the WTP Milence in the period 01/2022-12/2022

Monitored parameter	Unit	Minimum value for the year 2022	Maximum value for the year 2022	Average value for the year 2022
pH		7.530	8.560	
Alkalinity	mmol/l	0.200	2.100	1.540
Nitrite NO_2^-	mg/l	0.014	0.100	0.047
Nitrate NO_3^-	mg/l	1.900	2.490	3.000
COD_{Mn}	mg /l	0.560	0.750	1.760
Hardness Ca + Mg	mmol/l	0.630	1.880	1.190
Iron Fe	mg/l	0.010	0.250	0.100
Manganese Mn	mg/l	0.010	0.307	0.093
Chloride Cl^-	mg/l	3.500	5.400	4.270
Turbidity	ZF	0.300	2.400	0.780
Colour	mg /l	0.900	20.000	1.320
Calcium Ca	mg/l	26.500	42.900	34.510
Magnesium Mg	mg/l	0.000	4.340	1.530
Conductivity χ	mS/m	14.000	22.300	18.080
Aluminium Al	mg/l	0.005	0.195	0.041
Sulphate SO_4^{2-}	mg/l	0.010	4.340	3.290
Humic substances	mg/l	0.070	0.680	0.170
Phosphate	mg/l	0.050	0.610	0.190
Chlorine dioxide	mg/l	0.045	0.613	0.194

5 Magnetically modified cells

The water technologists are trying to remove not only hydrobiological material but also harmful substances such as dyes. In this thesis, I discuss and experiment with the idea of the application of the magnetized Pcy as a sorbent of pollutants, in this particular case, dyes.

For the majority of cells, diamagnetic behaviour occurs. The main property of these magnetized cells is the interaction with external magnetic fields (Safarik et al., 2014; 2016). Magnetic conversion is usually achieved by the attachment of the particles showing magnetic properties. This process is called “magnetic labelling”. However, the labelling of the cell is categorized in this field as “magnetic decoration.” The term “decorated cells” usually refers to cells with magnetic micro- or nanoparticles on the surface (Safarik et al., 2014). The iron oxides are used as these magnetic labels – particularly these two compounds – magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3). The most frequent method of preparing these magnetic compounds (from their precursors) is precipitation in alkalic conditions of ferrous and ferric salts (Safarik et al., 2016). However, many micro- and nano- particles are also available commercially (Safarik et al., 2012). The increased utilization of these particles nowadays, is caused by high biocompatibility, easy regeneration and manipulation using basic tools and a non-damaging magnetic field (Prochazkova et al., 2013).

6 Biosorption and biosorbents

The definition of biosorption is the removal of target substances from solutions by biological materials. Biosorption is a physical-chemical process that is independent of metabolism and involves different mechanisms. These can contain absorption, adsorption, and precipitation, but also ion exchange or surface complexity. The mechanisms behind the applications are based on the interactions of the targeted molecules with specific functional groups (Safarik et al., 2021). All different types of biomaterials – such as bacteria and algae but also industrial waste, have generally demonstrated strong biosorption abilities for different kinds of metal ions (Vijayaraghavan & Yun, 2008).

6.1.1 Biosorbents of dyes

Currently, waste containing synthetic dyes causes serious water pollution, especially in industrial wastewater. It has become a significant environmental issue (Diaz-Uribe et al., 2021). Numerous industries are especially implicated in water pollution as a result of various municipal, agricultural, and industrial activities. These represent significant threats to both the environment and human health at the same time (Elgarahy et al., 2021). These pollutants are also chemically, biologically, and photolytically stable and tend to be carcinogenic and toxic (Nidheesh et al., 2018).

In recent years, research has been published on the applications of various organisms in the matter of pollution absorption. Yeast, fungal, bacterial and algae biomass, or spent coffee grounds have been part of research as biosorbents to remove various pollutants (such as dyes) (Safarik et al., 2021).

7 Dyes

7.1 Malachite green

Malachite green (furthermore as MG) is a green-coloured organic compound with the formula $C_{23}H_{25}ClN_2$ belonging to triarylmethane dyes (NCBI, 2023)(Figure 11).

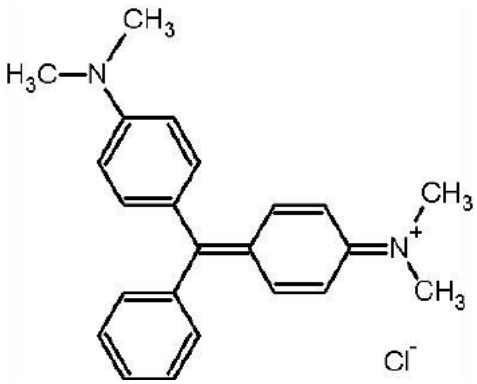

Figure 11. Structure of Malachite green (Prabakaran & Arivoli, 2012)

MG itself is used for various purposes. This substance is used in aquaculture as a biocide, especially due to its ability to eliminate fungi, different types of parasites, and protozoans. This dye is also used in the textile industry for colouring different types of materials, such as cotton, leather, wool, or silk (Srivastava et al., 2004; Safarik & Safarikova, 2002).

Concerns regarding malachite green are mentioned in many sources of literature. The cytotoxicity of mammalians and its effect as an enhancement factor and its ability to cause defects in a developing fetus have been proven for this particular compound. At present, MG is banned or regulated in countries of the European Union (Li et al., 2019; Srivastava et al., 2004; Safarik & Safarikova, 2002).

7.2 Safranine O

This water-soluble red-brown powder is categorized as the cationic azine dye with the empirical formula $C_{20}H_{19}ClN_4$ (Figure 12). Applications for this dye are wide, from colouring in the food industry to the textile industry, especially for wool, leather, or silk. It is not only used for industrial purposes, but also for staining of proteoglycans in cell chondrogenesis or mucin (Kaur et al., 2015; Mullerova et al., 2019).

Figure 12. Structure of Safranine O (Moawed & Abulkibash, 2016)

Although safranine O is widely used, this dye is also highly carcinogenic and has various impacts on the human body, e.g., skin irritation, gastrointestinal tract irritation, or other injuries depending on the location of the affected site (Gun et al., 2022; Arya et al., 2022).

8 Work objectives

There are several aims of this bachelor´s thesis:

- Observe the amount and development of picoplanktonic cyanobacteria in the Nýrsko water reservoir and individual technological stages of water treatment at the Milence plant.
- Determine the depth of penetration of picoplanktonic cyanobacteria in water treatment.
- Determine the dependence of separation efficiency of the filters on the filtration time.
- Analyse elimination options of the picoplanktonic organisms from the treated water.
- Test magnetically modified picocyanobacterial cells for use as a biosorbent of dyes.

9 Methods

9.1 Water sampling of water

The water samples for the experiment were collected according to method "ČSN EN ISO 5667 (75 7051) Kvalita vod – odběr vzorků" by 2 instruments – a sampling rod and a tap. The list of samples and their sampling method are shown in Table 3 (the individual photographs of sampling profiles can be seen in S13-S21).

Table 3. Sampling profiles and their sampling method

Sampling profile	Collection method
Water Reservoir Surface	Sampler
WTP Raw Water	Tap
WTP Agregation	Sampler
WTP Filters (3x)	Tap
WTP Before accumulation	Tap
WTP After accumulation	Tap
Water tank Janovice	Tap

The water was stored in containers marked with the location and date of collection. In the case of filters, the filtration time was also added to the data. The volume taken was 60 mL. To this amount, 3 mL of formaldehyde (36-38% p.a.; PENTA) were added to the final 2% formol concentration, causing the preservation of the organisms present. The samples were kept in a cold environment.

9.2 Counting of picocyanobacteria

9.2.1 DAPI staining

DAPI is a dye with the chemical formula 4′,6-diamidino-2-phenylindole. This substance is used to stain DNA by binding itself to A-T clusters in DNA (Porter & Feig, 1980).

First, the staining apparatus was prepared in the sterile fume hood. The black filter (placed with the shiny part above) was placed on the column covered with a glass cylinder and secured by a clamp. The specified water volume depending on the expected number of Pcy – 1 mL in the summer samples, 5 mL in the autumn ones – was poured into the filter. In the case of the autumn samples, a small volume of liquid was let through the apparatus until approximately 1 mL was left. Then the 60 μL of DAPI stain was pipetted into the sample and allowed to sit for 60 seconds. Then, suction filtration was done. The glass slide was labelled, and the filter was transferred, placed on the glass with the shiny side up. The oil was added on

the filter, and the surface was covered with a glass square. The apparatus used for DAPI staining is shown in Figure 13.

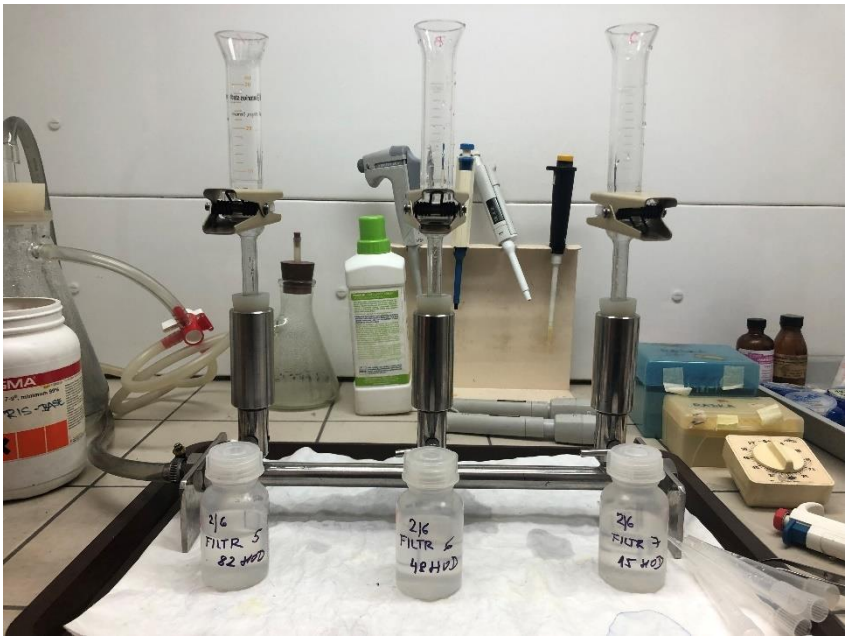

Figure 13. The apparatus used for the purpose of DAPI staining of picocyanobacterial cells and counting filter preparation (author´s archive)

9.2.2 Fluorescence microscopy

This type of microscopy is used to observe small size organisms and their specific body characteristics. For the imaging, higher energy of light is used, but the specimen emits longer wavelengths, which means lower energy (Rice, n.d.). The principle of this technique is as follows. Fluorescence, as a phenomenon, is described as the emission of light concomitant decay of excited molecules, which occurs after the absorption of light (Nelson & Cox, 2008). This process is used to return the molecule to its ground state after electron excitation (Skoog, 2019). The substance always emits a longer wavelength, which means lower energy. The important term in this technique is the Stokes shift, and is the name given to the emission of radiation that has a longer wavelength than the radiation that caused the excitation of the electron (Skoog, 2019). This feature allows one to separate excitation light from the emitted one using suitable optical gear. The shift can be expressed in both wavelength and wavenumber (Edinburgh Instruments Ltd., 2023). The graphical representation and formulas for the calculations are shown in Figure 14. By filtering out the exciting light, but the emitted energy cannot be blocked, it is possible to see the objects that are fluorescent (Lichtman & Conchello, 2005).

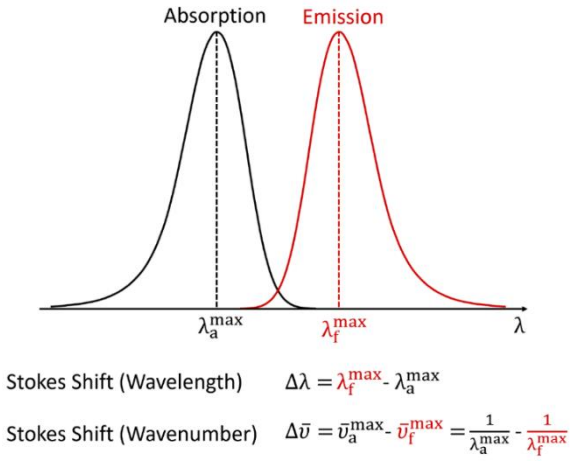

Figure 14. The Stokes shift (Edinburgh Instruments Ltd., 2023)

The microscope which uses this fundamental has to reach the following functions - deliver excitation light to the sample, separate the excitation light from the emitted one, collect the emitted light from the sample, and allow observation of the specimen. Light is distributed to the sample through the objective of the microscope. The dichroic beam-splitting mirror is used to separate excitation and emitted light. In addition, it can reflect light below a specific wavelength, resulting in light above this specific wavelength being able to pass through the mirror unobstructed. Objective lenses, which are used for this type, are developed to collect the emitted light from the substance and to measure and resolution of the specimen. The fluorescence (determined by NA = numerical aperture) is collected by the objective and then passes through the specialised mirror to the detector or ocular (Herman, 1998).

The microscope (shown in Figure 15) used for the purposes of counting picocyanobacteria in the case of my bachelor thesis was Olympus BX60 with ocular Olympus WH10X/22.

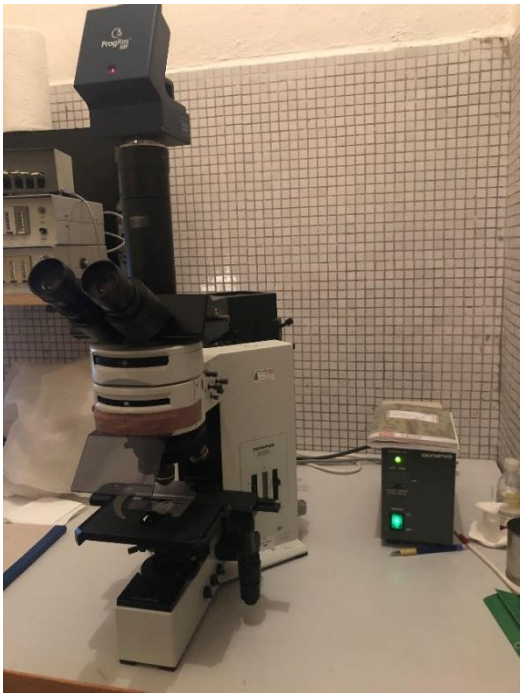

Figure 15. Fluorescence microscope Olympus BX60 used for the purpose of the thesis (author´s archive)

9.2.3 Counting of the individual cells

The counting of the present picocyanobacteria was carried out in a programme called "Advanced Click counter 0.7" made by M. Zeder (http://www.technobiology.ch/). Firstly, the cells were focused with the fluorescent microscope (Figure 16). The programme was set as follows: First, the experimental name was entered. Subsequently, the name of the sample (location and date) was written in the reservoir. Sample volume, dilution factor (1), fit. area (314), counting grid (0.386), and grid factor (1) were specified. After that, using fluorescence microscopy and keyboard, picocyanobacteria in individual samples were counted. The data were then copied to the prepared excel spreadsheet (Microsoft Office) where they were further processed.

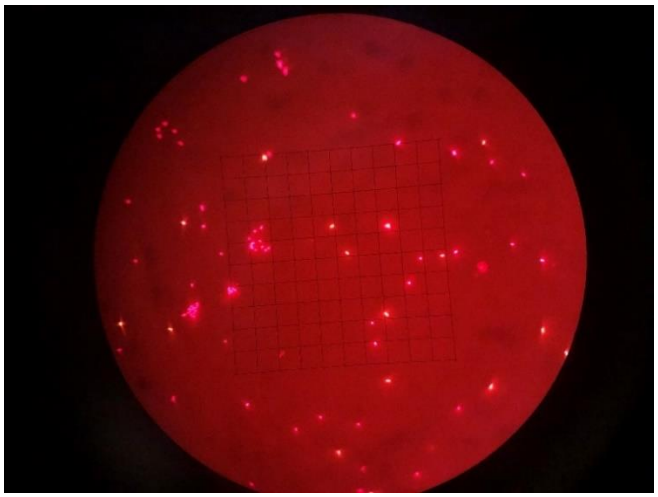

Figure 16. Image of the counted Pcy sample in the fluorescence microscope (author´s archive)

9.3 Inoculation of picocyanobacteria

The entire inoculation process was carried out in a sterile environment (sterile chamber shown in Figure S22) to avoid contamination with other microorganisms from the environment.

Firstly, the Erlenmeyer flasks were labelled with the type of medium – WC (Stanier et al., 1971) or BG (Guillard & Lorenzen, 1972), name of the culture and the date of inoculation (Figure 17). Subsequently, the flasks and the container with the prepared medium were transferred to sterilised conditions in the fume hood. The sides of the flask and container were burnt over the flame to remove external microorganisms from the surface. The cotton plug was removed, 200 mL of medium was poured into the flask, and the 1 ml of the pre-cultured Pcy were pipetted inside. Finally, the glassware was closed with the sterile cotton plug sterilized with burner.

Cultures of picocyanobacteria were grown in a culture room with a constant temperature of 19°C and a light cycle of 16h/8h (light and dark ratio). Light intensity was on average 22.23 μmol photons/m^2/s.

Figure 17. Erlenmeyer flasks inoculated with Pcy RD51 and Vír (two of the cultures used for the biosorption experiments (author´s archive)

9.4 Magnetized cells used as biosorbent

9.4.1 Preparation of non-stoichiometric magnetite

In one litre beaker 1 g of iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) was dissolved in 250 mL of distilled water. While mixing continuously, the 1M solution of sodium hydroxide (NaOH) was added until complete precipitation and pH of 12 (the estimation of the pH was carried out with pH paper). After this step, the entire volume was placed in a domestic microwave oven for 10 minutes at the maximum output of 800 W. After heating, the magnetite was separated using a NdFeB magnet and washed with water to a neutral pH (Figure 18). Subsequently, the supernatant was decanted until the ratio of magnetite to supernatant was 1:4.

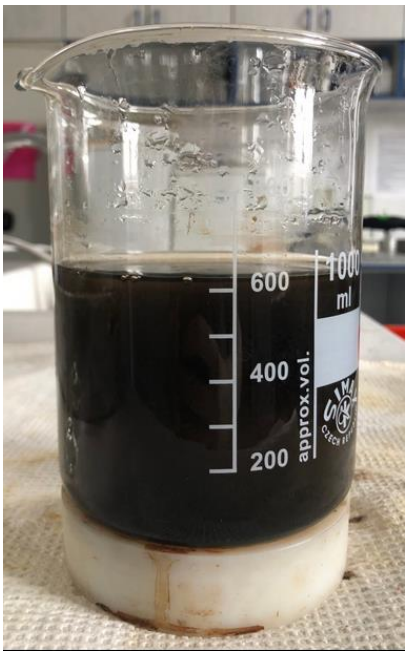

Figure 18. Microwave synthesized magnetite on the NdFeB magnet (author´s archive)

9.4.2 Magnetization of the cells

For experiments with magnetised cells, dense picocyanobacterial cultures inoculated for 5 months were used. The inoculation and growth of picocyanobacteria are described in chapter 9.4. Before performing the experiments, 150 ml of the Pcy culture was centrifuged for 30 minutes on RCF 4220, after that the supernatant was discarded. 9 mL of the cultivated strain of Pcy was transferred to the labelled container and 1 mL of the magnetite suspension was added. The solution was left to mix on the rotator (Stuart) for 20 hours at 20 rpm. After this period, the magnetised cells were fixed with formaldehyde in a 1: 20 ratio.

9.5 UV/VIS spectroscopy

The technique of UV/VIS spectroscopy is based on the absorption of electromagnetic radiation by substances in the ultraviolet and visible parts of the spectrum. The principle lies in the absorption of photons by the compound. Subsequently, the energy of an electron is increased, and the substance is placed in an excited state. The energy absorbed is generally used to transfer electrons to form chemical bonds, increase the vibrational energy of chemical bonds, or increase the rotational energy of molecules. UV-VIS wavelength ranges between 190 and 800 nm (Hejsková, 2016).

The result of UV-VIS spectrometry is the absorption spectra, which graphically represent the dependence of absorbance (A) on the wavelength (λ), but absorption, in general, can be

represented in transmittance or absorbance. The correlation between transmittance and absorbance is:

$$A = -logT \qquad 1$$

The fundamental mathematical description of the dependence of absorbance on the concentration and thickness of the cuvette is described by the Lambert-Beer law:

$$A = \varepsilon \cdot c \cdot d, \qquad 2$$

where A is absorbance [AU], ε represents absorptivity [L.mol^{-1}.cm^{-1}], c is molar concentration in mol/L and d is optic path length (cuvette length) in cm (Hejsková, 2016) (Dept. of Chemistry, Michigan State University, n.d.).

The scheme of the used apparatus of UV-VIS spectroscopy is displayed in the following diagram (Figure 19).

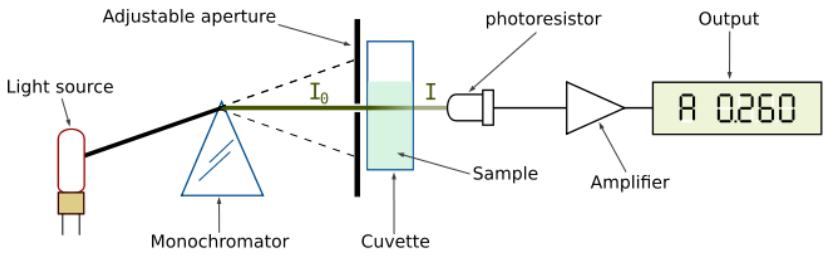

Figure 19. Scheme of single beam spectroscopy apparatus (MRC ltd., n.d.)

For the purpose of this thesis, the VWR Spectrophotometer V-1200 VIS SING was used.

9.5.1 Adsorption experiments

The volume of magnetized Pcy (depending on the experiment – 100 µL, 500 µL, 1000 µL) was pipetted into a resealable tube and 5 mL of the dye, with a known concentration, was added. This mixture was left to rotate for 24 hours at 20 rpm (Figure 20).

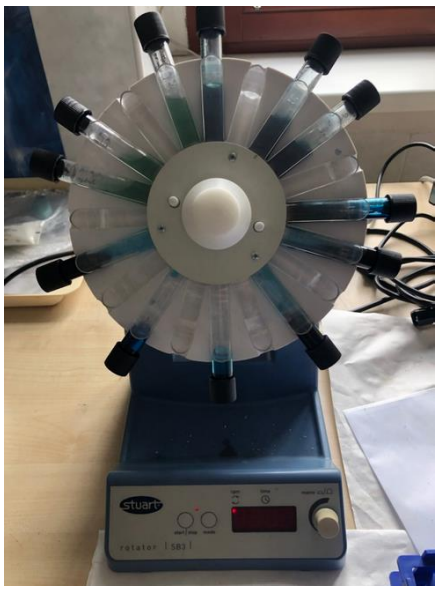

Figure 20. Solution of magnetised picocyanobacteria and dye left for the incubation on the rotator (author´s archive)

For the absorbance measurement of the individual sample, the volume of 1 mL of solution was pipetted into a plastic cuvette (overall volume of the cuvette was 2.5 mL). If the absorbance of the initial concentration was greater than 1.300 A, the solution was diluted (10×, 100×, or 1000×). As a blank, distilled water was used. The experiments were performed in triplication for the most precise measurements. The light wavelength for the MG is 618 nm and for Safranin O 520 nm.

10 Results

10.1 Development of the picocyanobacteria in the Nýrsko reservoir and WTP Milence

The following graphs in Figure 21 show the trend of picocyanobacteria at the sampling points on the individual sampling dates of the vegetative season from May to October 2022. The tables summarizing the data on the individual sampling dates can be found in the appendix in Tables S1-S9.

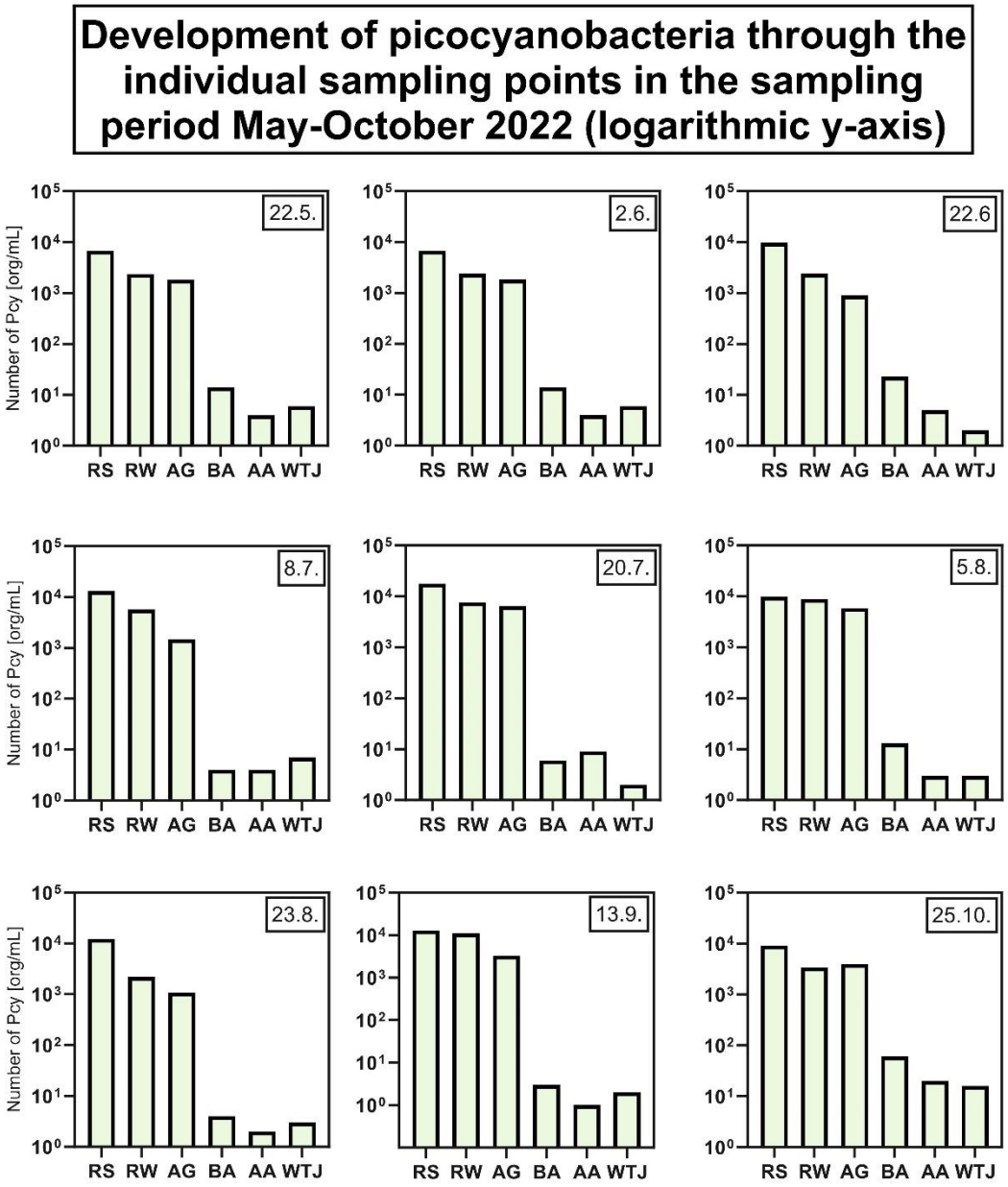

Figure 21. Development of picocyanobacteria through the individual sampling points in the sampling period May to October 2022. RS- Reservoir Surface, RW – Raw Water, AG – aggregation, BA – Before Accumulation, AA – After Accumulation, WTJ – Water Tank Janovice

The largest amounts of Pcy can be seen in the samples from the Nýrsko water reservoir, a slightly lower number of Pcy are detected in the "Raw Water" sampling profile. Most of the Pcy are eliminated in the technological stage of filtration and then by adding the batch treatment chemicals. However, all organisms have not been removed.

The trend of the Pcy in the Nýrsko reservoir indicates the peak of the vegetative season on 20. 7. 2022 – 17 864 org/mL, the minimal amount was determined on 25. 5. 2022 – 3 333 org/mL. The Pcy data at the sampling point "Raw water" showed the maximum Pcy on 13. 9. 2022 – 11 071 org/mL, the minimum was also indicated on 25. 5. 2022 – 606 org/mL.

In Figure 22, the development throughout the sampling period at the individual sampling points is shown.

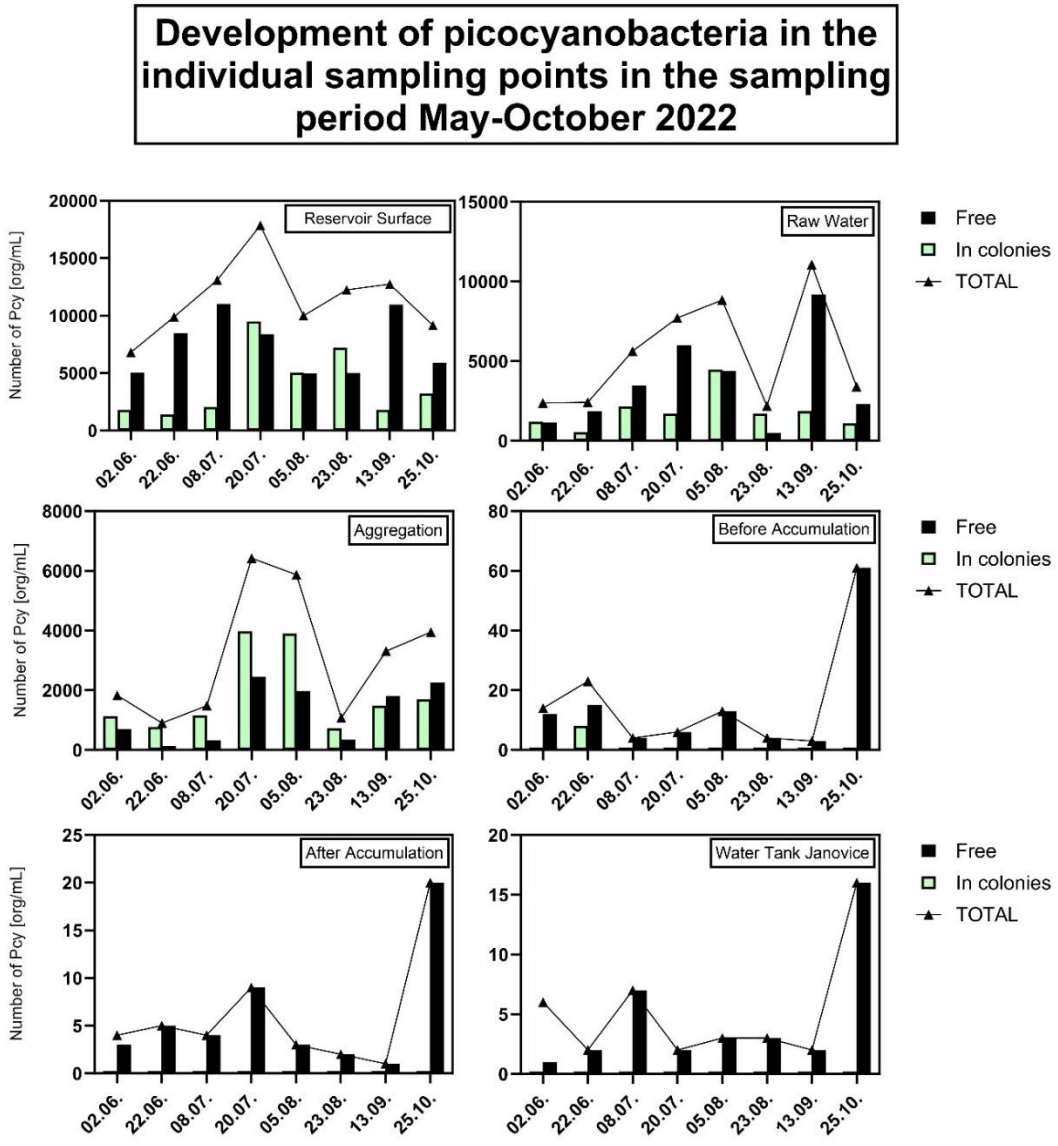

Figure 22. Development of picocyanobacteria in the individual sampling points in the sampling period May to October 2022

In Figure 22, the largest amount of the Pcy can be detected in the water reservoir, subsequently in raw water and in the aggregation sample. Throughout the individual technological stages, the number of Pcy gradually decreases. In the Water Tank Janovice, just a few cells are observed usually up to 10 cells per millilitre. One exception can be seen on the 25.10. sampling date, where the Pcy are 16 cells per millilitre.

The 1b(%) showing the percentage of the Pcy as individual and the col(%) the percentage of the Pcy in colonies are calculated and shown for the individual sampling points in tables S1-S9 attached in the appendix. The ratio of the colonies and individual Pcy in the "Reservoir Nýrsko" and "WTP Raw Water" varies. At the beginning of the vegetative season, mostly individual cells were observed. In the sample "Reservoir Surface", the predominance of colonies occurred during three samples all in the summer period - 20. 7., 5. 8. and 23. 8. The sample "WTP Raw water" showed a predominance of colonies occurred in the samples from 2. 6., 5. 8., and 23. 8.

From the graphic representation of the individual results, it can be seen, that the most diverse ratios of the individual cells and colonies are in the first three technological stages – the samples "Reservoir Surface", "Raw Water" and "Aggregation". The other stages show mostly only individual cells in the samples. The amounts of Pcy determined through the sampling points reveal a continuously decreasing cell number. The reduction during water treatment is shown in Figure 23.

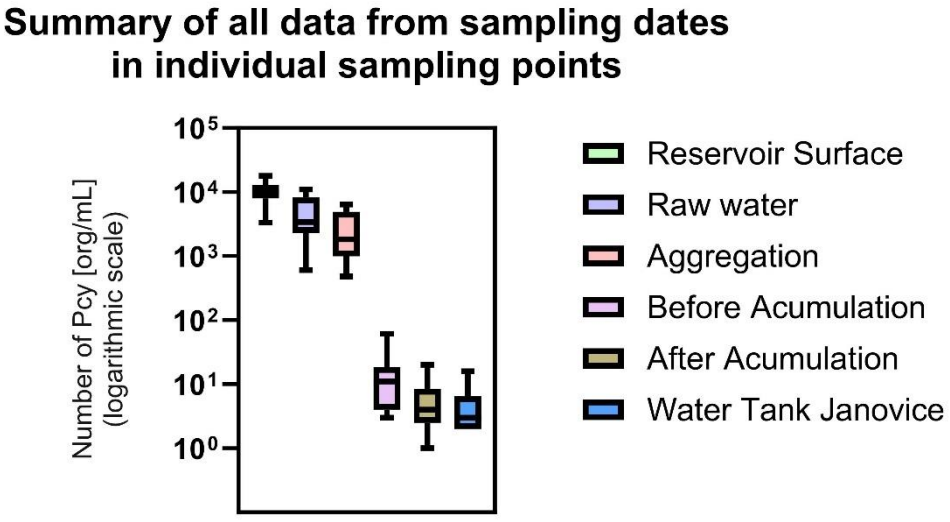

Figure 23. Summary of all picocyanobacterial numbers from the sampling period May-October 2022

To statistically prove that there are significant differences between stages, a one-way ANOVA was chosen to be performed based on the literature by Ostertagova & Ostertag

(2013). The summary of this test can be seen in Table 4. The conclusion of this test is that there are significant differences between the individual technological stages concluded from p value and evidence that the technology of WTP is sufficient to meet hydrobiological requirements.

Table 4. Results of the statistical test – one-way ANOVA, showing significant differences between individual technological stages of WTP Milence

ANOVA Summary	
F	27.31
P value	<0.0001
Significant diff. Among means (P < 0.05)	Yes
R squared	0.7399

10.2 Penetration through the filters

The complete table with the whole data set for the filters is placed in appendages (Table S10).

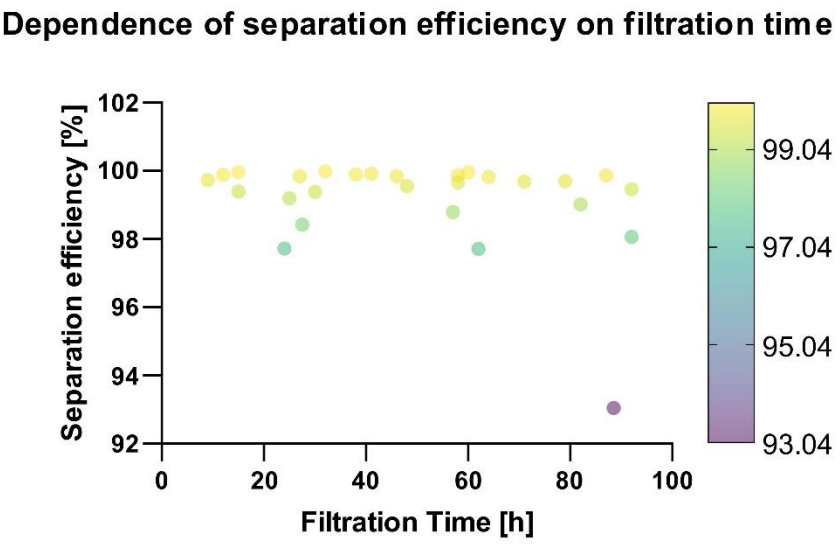

Figure 24. Graphical representation of filters´ separation efficiency dependence on filtration time

To determine the dependency of these two variables, the Spearman correlation statistical test was selected and was resulted with the value of -0.2501. The interpretation of this based on the Schober et al. (2018), suggests that there is very weak correlation of the separation efficiency on time filtration (Figure 24).

10.3 Pre-test for nanobiotechnological experiments

10.3.1 Pre-test of the magnetised cells

The five cultures of Pcy were tested for magnetization. Cultures were chosen in cooperation with previous unpublished research from RNDr. Jitka Jezberová, Ph.D. and Prof. Ivo Šafařík, PhD., DrSc. These can be seen in Table 5 with the results of the pre-test, which Pcy culture will be the best for magnetization. The tubes with cultures can be seen in Figure S23.

Table 5. Chosen picocyanobacteria cultures for magnetisation pre-test with numbers and percentage of magnetised cultures. ("Name of the culture" MAG – cultures with magnetite particles)

Culture	Initial Pcy amount in 100 µl	non-magnetised cells in 100 µl	% not magnetized	% magnetized
RDKV18	6.96E+07		97.77	2.23
RDKV18 MAG		6.803E+07		
Vir	6.88E+07		76.65	23.35
Vir MAG		1.48E+07		
RD51	6.88E+07		13.75	86.25
RD51MAG		9.46E+06		
STRS29	4.28E+07		89.14	10.86
STRS29 MAG		3.82E+07		
D1	6.90E+08		97.19	2.81
D1 MAG		6.71E+08		

The best suitability for the other experiments showed the culture RD51, which is shown with microwave synthesized magnetite particles in Figure 25 taken by light microscope and Figure 26 taken by fluorescence microscope. For further description, in Figure 27 the cells of the initial culture RD51 imaged by a scanning electron microscope and in Figure 28 the cells of RD51 with attached particles of microwave synthesized magnetite. Both figures from the SEM were taken at the Palacký University in Olomouc and were provided by Prof. Ing. Ivo Šafařík, DSc.

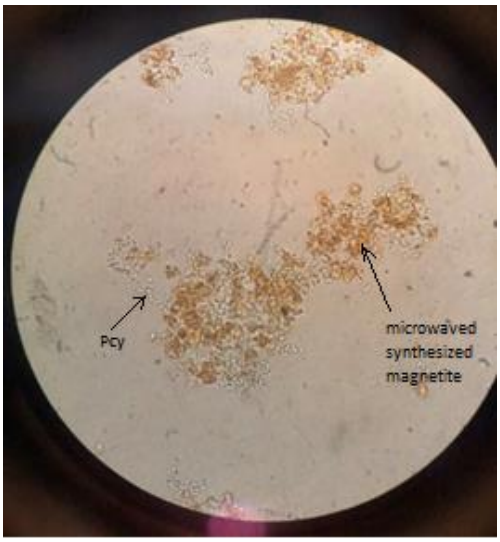

Figure 25. Light microscope image of the picocyanobacterial culture RD51 with particles of microwave synthesized magnetite (author´s archive)

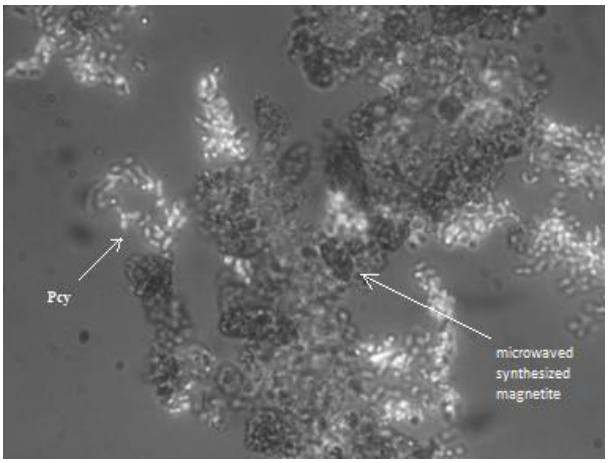

Figure 26. Fluorescence microscope image of the picocyanobacterial culture RD51 with particles of microwave synthesized magnetite (author´s archive)

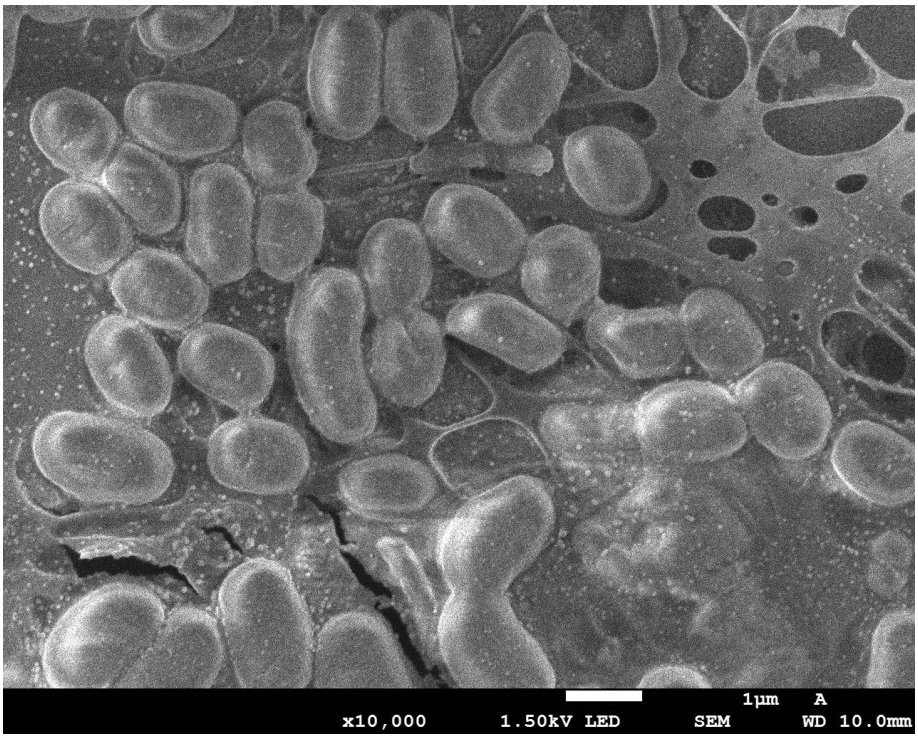

Figure 27. Cells of the picocyanobacterial culture RD51 imaged by the scanning electron microscope

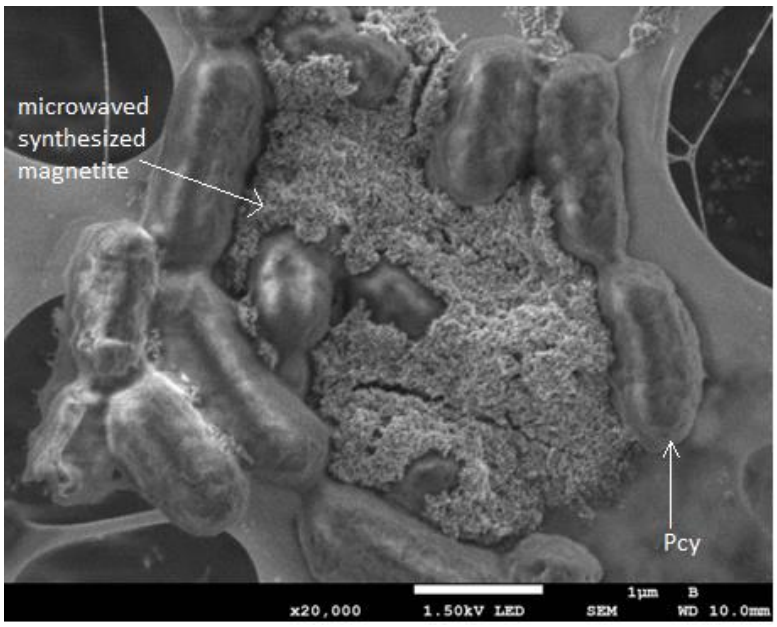

Figure 28. Cells of the picocyanobacterial culture RD51 combined with microwave synthesized magnetite imaged by the scanning electron microscope

10.3.2 Pre-test of the volumes and dyes

A pretest for the best volume of magnetised Pcy and the change in absorbance were performed. Table 6 summarises all dyes used (always with concentrations 0.1 mg/mL) and the

volumes of Pcy. For the volumes three different amounts were tested – 100 µL, 500 µL, 1000 µL with three different dyes – safranine, malachite green (Figure S24) and crystal violet (all dyes with a known concentration of 0.1 mg/mL).

Table 6. Pretest summary for three different volumes of Pcy (100 µL, 500 µL, 1000 µL) with 5 mL of dye - safranin, malachite green and crystal violet (which was not used for the further experiments due to insufficient difference in absorbance)

Safranin (λ = 520 nm)			
A (Initial dye)	0.725	A(dyes after 24 h)	0.784
Volume of MAG Pcy [µL]	Absorbance	Volume of Fe_3O_4 [µL]	Absorbance
100	0.660	10	0.667
500	0.429	50	0.688
1000	0.355	100	0.680
Malachite green (λ = 618 nm)			
A (Initial dye)	0.152	A(dyes after 24 h)	0.116
Volume of MAG Pcy [µL]	Absorbance	Volume of Fe_3O_4 [µL]	Absorbance
100	0.162	10	0.152
500	0.109	50	0.137
1000	0.081	100	0.095
Crystal violet (λ = 595 nm)			
A (Initial dye)	1.293	A(dyes after 24 h)	1.055
Volume of MAG Pcy [µL]	Absorbance	Volume of Fe_3O_4 [µL]	Absorbance
100	0.650	10	0.941
500	0.552	50	0.837
1000	0.404	100	0.888

After the preliminary experiments, the malachite green was shown to be the best dye for differences between the absorbances. The dye chosen for the experiments was malachite green. However, as a control dye the safranine O dye was used. The 500 µL magnetised Pcy was selected as the best concentration for the experiments. The Figure 29 and Figure 30 shows two samples from the preliminary experiments in comparison with the initial dye.

Figure 29. Difference between the sample of dye (5 ml 0.1 mg/mL) with 1000 µL magnetized Pcy and 5 mL of 0.1 mg/mL MG after 24 hours of incubation at laboratory temperature (author´s archive)

Figure 30. Colour difference in the sample with 500 µL MW Mag Pcy with 5 mL of 0.1 mg/mL MG dye (on the left - dye without the MW Mag Pcy; on the right dye with MW Mag Pcy after 24 hours of incubation) (author´s archive)

To prove that the sorption is predominately done by the picocyanobacterial mass, the control tubes only with microwave synthesized magnetite and pure Pcy mass were set with the same conditions (5 mL of 0,1 mg/mL of MG dye, incubated for 24 hours). Data can be seen in Table 7.

Table 7. The measured values for the pure Microwave synthesized magnetite and pure Pcy mass

Malachite green A = 1.520			
Picocyanobacteria mass		Microwaved synthesized magnetite	
Volume [µL]	Absorbance [AU]	Volume [µL]	Absorbance [AU]
100	1.139	10	1.52
500	1.122	50	1.37
1000	1.169	100	0.95

The performed experiments show that the sorption of the dye analysed in the following experiments happened mainly due to Pcy.

10.4 Biosorption of dye by magnetically modified picocyanobacteria

The calibration curves for the two dyes used were prepared with the use of linear regression using Prism programme. Figure 31 and Figure 32 show the measured calibration curves for MG and Safranine O (the data can be seen in Tables S11 and S12).

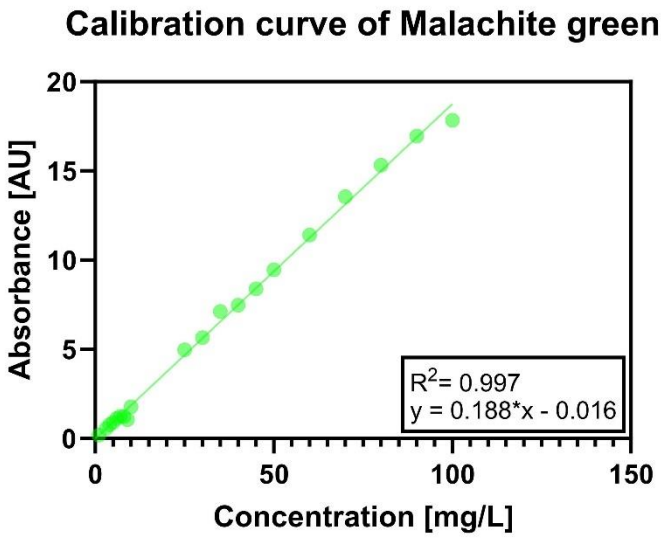

Figure 31. Calibration curve of Malachite green dye

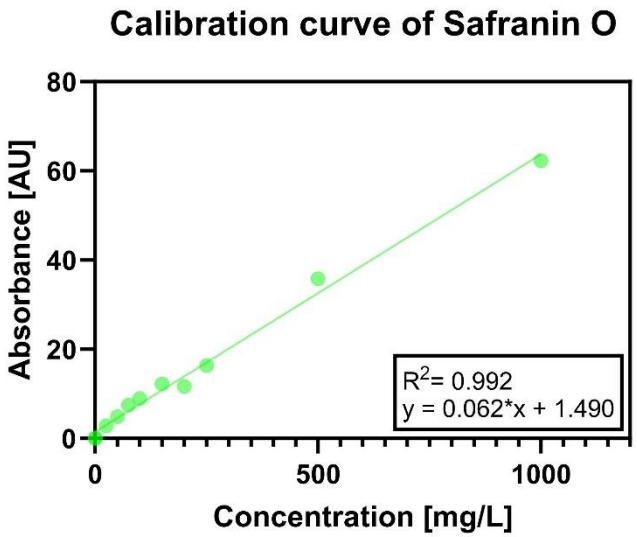

Figure 32. Calibration curve of Safranine O dye

10.4.1 Biosorption by MG

After 24 hours the solution of Pcy and dye was measured using UV spectroscopy. The suspension was placed on the magnet to separate the magnetised Pcy with absorbed dye from the residue of unabsorbed dye. The absorbances of individual triplications were used in the calculation for the *ct* concentration of free dye (Equation 3) and the *qt* value (Equation 4) for the amount of dye bound per unit of sorbent. The measured data and calculations can be seen in Table 8.

$$ct = \frac{A}{k}, \qquad 3$$

where *A* is the absorbance without the blank in absorbance unit and *k* is the slope calculated from the calibration curve.

$$qt = \frac{(0.005 \cdot c_0 - 0.005 \cdot ct)}{m}, \qquad 4$$

where c_0 is initial concentration of the dye in mg/L, *ct* is concentration of the free dye in the solution in mg/L and *m* is the mass of the Pcy in the solution (m = 0.720 g).

Table 8. Measured data and calculation of the individual concentration experiments with the malachite green dye. (Blank – distilled water)

Dye Concentration [mg/L]	A1 [AU]	A2 [AU]	A3 [AU]
10	0.233	0.233	0.376
100	0.526	0.460	0.497
1000	0.594	0.509	0.623
2500	0.570	0.390	0.401
5000	0.672	0.776	0.777
A(arithmetic mean) [AU]	Dilution	A [AU]	Blank
0.280	1	0.280	0.202
0.494	10	4.942	0.202
0.575	100	57.533	0.202
0.454	1000	453.667	0.202
0.742	1000	741.667	0.202
A (without blank)	Ct concetration of free dye [mg/L]	Amount of absorbed colour from 5 mL [mg/L]	qt (mg adsorbed dye/mL sorbent)
0.078	0.418	0.048	0.067
4.740	25.281	0.374	0.519
57.331	305.767	3.471	4.821
453.465	2418.478	0.408	0.566
741.465	3954.478	5.228	7.261

Subsequently, from the two characteristic aspects, ct and qt, the graph was constructed. This graph can be seen in Figures 33 and 34. The statistical parameters are shown in Table 9.

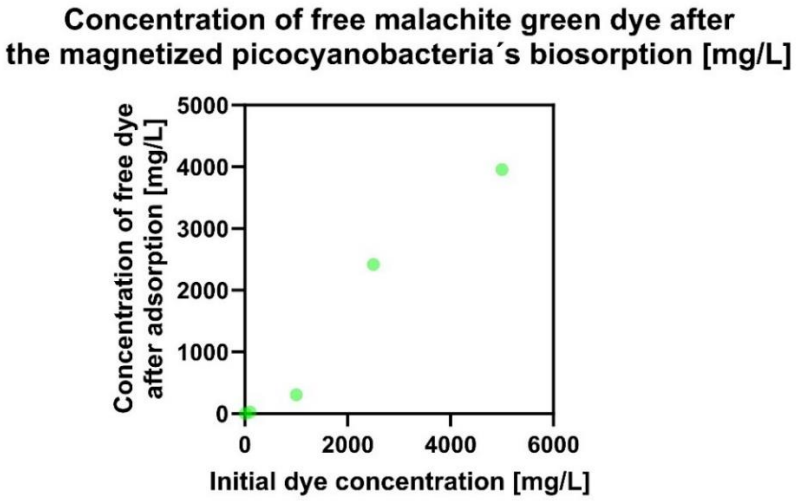

Figure 33. Ct concentration of MG dye after the magnetized picocyanobacteria´s biosorption

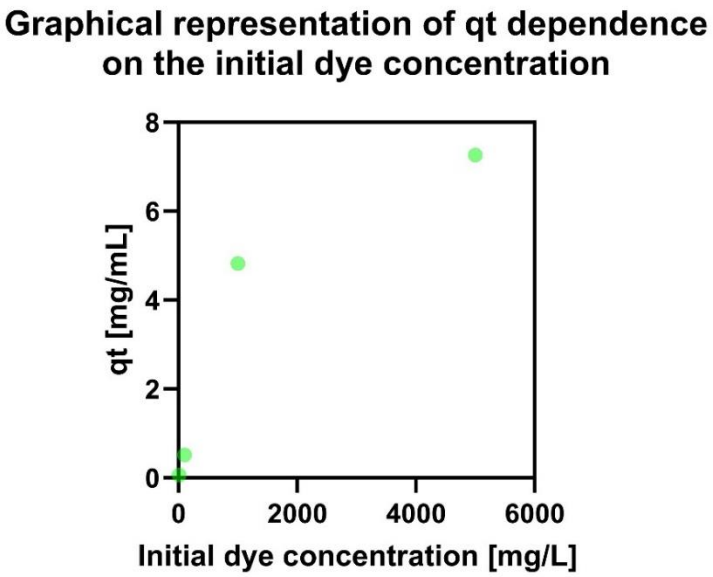

Figure 34. Graphical representation of qt dependency on the initial MG concentration

Table 9. Calculated statistical values by excel spreadsheet (Microsoft Office) for the absorbance triplication of the experiments with the MG

Concentration [mg/L]	Average	Standard deviation	Relative standard deviation
10	0.280	0.082	0.294
100	0.494	0.033	0.067
1000	0.575	0.060	0.104
2500	0.454	0.101	0.222
5000	0.742	0.060	0.081

10.4.2 Malachite green equilibrium adsorption isotherm

The equilibrium adsorption isotherm for malachite green (data provided in Table 10) using picocyanobacteria biomass treated with magnetic iron oxide particles is presented in Figure 35. Neither Langmuir nor Freundlich isotherms could describe the adsorption process with sufficient precision, using specialized software. Anyway, it is clearly visible that the adsorption process is going to reach a limit, which can be equivalent to the maximum adsorption capacity obtained from the Langmuir isotherm. Using SigmaPlot software, the experimental data were evaluated using a simple equation:

$$y = \frac{ax}{(b+x)}, \qquad 5$$

where *a* represents the limit of the equation (analogue of maximum adsorption capacity) and *b* represents equilibrium liquid-phase concentration of the free dye, where 50% of dye is adsorbed. The corresponding model constants and the coefficient of determination (R^2) are a = 7.6863 mg/g, b = 198.2830 mg/L and R^2 = 0.997.

Table 10. Data of the malachite green equilibrium adsorption isotherm

Initial MG concentration [mg/L]	Equilibrium MG concentration [mg/L]	Equilibrium solid-phase concentration of adsorbed MG [mg/g]
0	0	0
10	0.418	0.066
100	25.280	0.518
1000	305.770	4.821
5000	3954.500	7.260

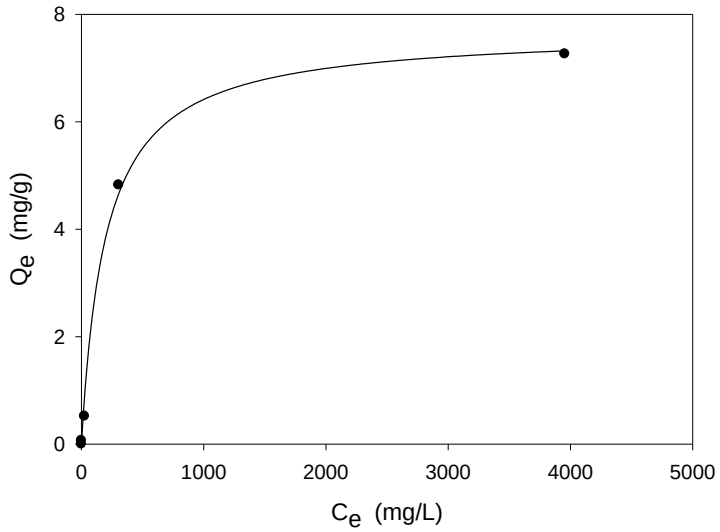

Figure 35. Equilibrium adsorption isotherm of malachite green using the biomass of picocyanobacteria treated with magnetic iron oxide particles. Ce – equilibrium liquid-phase concentration of the unabsorbed dye (mg/L); Qe – equilibrium solid-phase concentration of

The equilibrium solid-phase concentration [mg/g] of the absorbed dye was calculated using equation:

$$Q_e = \frac{V(C_0 - C_e)}{m}, \qquad 6$$

where V is volume of the dye in L, C_0 is initial concentration of the dye in mg/L, C_e represents the equilibrium liquid-phase concentration of the unabsorbed dye in mg/L and m is the mass of the adsorbent in g (Mullerova et al., 2019).

10.4.3 Biosorption of Safranin O

500 µL of Pcy with the 5 different concentrations of the dye was left on the rotator for 24 hours at laboratory conditions. The concentrations and absorbances of the samples can be seen in Table 11 (picture of triplication is placed in appendages – Figure S25).

Table 11. Measured data and calculation of the individual concentration experiments with the Safranine O dye

Dye Concentration [mg/l]	A1	A2	A3
10	0.430	0.450	0.441
100	0.627	0.621	0.576
1000	0.545	0.455	0.508
2500	0.561	0.559	0.572
5000	0.69	0.708	0.7
A(arithmetic mean)	Diluted	A	Blank
0.440	1	0.440	0.129
0.608	10	6.080	0.129
0.503	100	50.267	0.129
0.564	1000	564.000	0.129
0.699	1000	699.333	0.129
A (without A_p)	Ct concetration of free dye [mg/ml]	Amount of absorbed colour from 5 ml [mg/mL]	qt (ml adsorbed dye/mg sorbent)
0.311	5.005359057	0.025	0.03469
5.951	95.67524116	0.022	0.03003
0.439	7.057877814	4.965	6.89579
563.871	9065.450161	-32.827	-45.59578
699.204	11241.22722	-31.206	-43.34411

11 Discussion

11.1 Picocyanobacteria´s development in water reservoir Nýrsko and WTP Milence

The results of the bachelor thesis are difficult to discuss, mainly due to the specificity of the topic and the unavailability of materials dealing with this issue of Pcy and water treatment. Since this is a neglected issue, Bláha & Maršálek (1999) stated that the occurrence of picoplanktonic cyanobacteria during water treatment can cause problems in different treatment areas. The discussion is therefore based on personal observation, discussion with the supervisor and especially with the water treatment plant main technologist and other WTP employees.

Within the period May-October 2022 took place the sampling campaign with sampling from different sampling profiles. The graphs showing the development of the Pcy number through individual sampling points on individual sampling dates lack “filter” sampling points. This is caused by the circumstances of the collection. There is limited access to merging all three streams of water that come from the filters. The sampling point is placed after each filter.

The finding that the Pcy are penetrating into the stages of the water treatment corresponds with the literature written by Jasser & Callieri (2016), which also suggest that these organisms can than cause other issues such as bad odour or odd taste. The diversity in the presence of mostly only individual cells in the technological stages after aggregation is caused by the flow through the filters, which means the separation of large colonies from the water and thus the passage of only single cells. The penetration of the Pcy through the sand filters has been already confirmed by Ambrožová (1999).

The number of picocyanobacteria behind the filters, namely for the "Before accumulation" sample and samples behind this technological step, tends to be biased. The reason is that between the filters and the accumulation tank, batch treatment chemicals are added to the treated water (see Section 5.4). These substances (namely ClO_2) are able to cause cell bursting, and subsequently fluctuation in the data of the real amount of the Pcy behind the filters. ClO_2 (chlorine dioxide) is in water treatment plants as a strong oxidizing agent – to kill bacteria and other microorganisms Haida Nadia Mohamed Jefri et al. (2022).

In Figure 22, the breakthrough into further technological stages in higher level on 25. 10. is shown. This can be caused by several reasons. Excess amounts of Pcy may have passed through the filtration systems as a result of the enormous amounts of impurities trapped in the

filters or as a result of a defect that may have occurred. By all means, the human factor, and the possibility of human error in the collection or preparation of microscopic slides cannot be overlooked.

After all, the efficiency of the WTP Milence was concluded sufficient in removing Pcy from the raw water to the range of the legislative document. However, the problematic separation of these small organisms is also pointed by Ambrožová & Říha (1998). To increase the performance of the WTP, application of one of the two separation methods, described in Chapter 4, is advised. From communication with the WTP staff, information about the installation of ultrafiltration was revealed. Due to the presence of Pcy in the raw water, preparatory work was initiated to ensure the safety of the water produced by the treatment plant. DAF and ultrafiltration semi-operational tests were carried out in 2017 and 2018 by Sochor (2017, 2018). The ultrafiltration option has been selected in preliminary semioperational tests at the treatment plant and will be put into operation in the upcoming years. This arrangement will ensure that all safety requirements are met using a multibarrier water treatment approach as a total barrier Lopes et al. (2013). The multibarrier path with the different methods, such as ozonisation and granulated activated carbon filtration, is described as an efficient option also for the toxins produced by cyanobacteria – this is reported in the article by He et al. (2016). This path would be also helpful in the case of 25. 10., when the breakthrough in the Pcy amount occurs in the technological stages after filtration. If the reason for the increase is the filter defect, the filter cascade with not only a sand filter but also a nanofilter would be the right solution to achieve the highest efficiency.

The null hypothesis was stated to be that the separation efficiency depends on the filter´s filtration time. This hypothesis was disproved. The four filters, used in the WTP Milence technology, did not show any dependence. For confirmation, repetition of the experiment in a longer period with more frequent sampling dates is required.

11.2 Biosorption of the dyes by magnetized Pcy

The biosorption by picocyanobacteria are new direction of applications of biomass in nanobiotechnology. Therefore, there are few sources available on this issue. On the other hand, the use of cells for adsorption has been intensively investigated for some time already. The discussion of this part of the thesis is likewise mainly constructed from the personal observations, discussions with consultants and studies with other biomass types. The adsorption using different species was studied for example with cyanobacteria *Gloeocapsa sp.*

by Pokrovsky et al. (2008) (also showing its application in wastewater treatment plants) or by different types of fungi and *Saccharomyces cerevisiae* (yeast) according to research such as from Kumari & Abraham (2007).

Before the starting of the experiments, the pre-test analysis to find the best usable dye was performed. In these tests, the malachite green as the experiment dye was chosen from the three possibly harmful dyes present in the environment – MG, Safranin O, and crystal violet. The choice was made according to the observing and measuring conditions. The MG shows the sufficient difference in MW Mag Pcy adsorption absorbance from the initial dye absorbance value. The preliminary test shows the higher absorbance in the sample with 100 μL of MW Mag Pcy, which can be caused by the bursting of the Pcy, which subsequently releasing the chlorophyl. This compound increases the absorbance in the green spectrum, which is used for the measurement of the MG.

To prove that the adsorption is predominately performed by the Pcy, the experiment with the pure Pcy mass and pure microwave synthesized magnetite were put through the same experiment as in the pretest with the MG. The magnetite was in the same concentration in all analogous samples due to the same suspension used. It is also important to mention, that the adsorption of the dye is also happening with the glass of the tube, but this process is negligible. The results showed, based on the absorbances, that the adsorption is made predominantly by the Pcy not by microwave synthesized magnetite particles.

The experiment consisting of all parts was performed only with the malachite green dye due to best observation conditions. Analyzing the results of the MG, the conclusion that the picocyanobacteria have the potential to be used as the biosorbents for the pollutants was drawn. The differences in absorbances between the initial dye and the solution of dye with the Pcy were significant enough to prove that these organisms can be used for this purpose. However, further experiments and calibrations have to be performed not only with MG and other dyes but also with the other pollutants, such as heavy metals. However, the microwave synthesized magnetite was determined as an efficient tool for the separation of the picocyanobacteria with adsorbed dye with the sufficient ability to attached to the cells and with this enable the separation from the solution. This claim was in accordance with research of magnetite and is effectivity from Prochazkova et al. (2012). Overall, the Pcy have shown sufficient biosorption capability to conduct further experiments with them and the potential of

putting the mechanism into practice, not only in wastewater treatment plants but also in water treatment plants for treated (drinking) water production.

After the performance of the experiments containing MG, the controlling dye was picked, Safranin O (Table 11). From the evaluated and calculated results of ct and qt, it is shown that experiments with this dye need more adjusting and calibration. There can be several issues that provide wrong or odd results. The poorly mixed dye solution at the initial concentration. This inaccuracy could be transferred to the subsequent concentration and cause fluctuations in the absorbance measurement – not only for the measurement of each sample of the triplications but also for the calibration curve, which was used for the calculations of the concentrations after biosorption. In the measurement itself, the beam may have been intersected with the released particles of the microwave synthesized magnetite present freely in the solution of MW Mag Pcy and dye – this can add another error to the experiment. Generally, the error of the results is brought about by the dilutions of the samples to fit under the 1.300 Abs. This arrangement was taken for the most accurate measurement possible on the instrument used.

For the further research, the experiments should be replicated with the Safranin O, Crystal violet and other alarming dyes present in the water. Not only dyes are pollutants with which should be experiments performed. The Pcy can be also investigated for biosorption of heavy metals, such as antimony, which has been already investigated in the field of cyanobacteria biosorption for example in the study by Sun et al. (2011). Other substances which were already investigated but not with the cyanobacteria are for example pesticides like in research by Hussein et al. (2017), which also represent large environmental danger or radioactive uranium like described in article from Smječanin et al. (2023).

12 Conclusion

The picocyanobacteria, determined to be present in the water reservoir Nýrsko, were detected in each of the technological steps located in the WTP Milence. Despite these findings, the hydrobiological requirements are met by the WTP.

From the data of the individual sampling dates and sampling profiles, a determination of the depth of penetration of picocyanobacteria in water treatment was performed. Picocyanobacterial cells appear in all technological stages of the technological treatment of water.

Through the analysis of the experiments, the separation efficiency of individual samples on the sampling dates was calculated, showing that the separation of the Pcy on the filters was not dependent on the filtration time. The null hypothesis that there is dependency is disproved.

The analysis of the elimination options of the picoplanktonic organisms from treated water, and the ultrafiltration option has been selected also in the semioperational tests at the investigated WTP Milence and will be put into operation in the upcoming years. This system will ensure that all safety requirements are met using a multibarrier water treatment approach.

Despite the small amounts of mass, Pcy showed a satisfactory biosorption capacity of dyes and the ability to attach the particles of MW magnetised magnetite, which can lead to better separation of them not only in the biosorption of pollutants but also in the case of water treatment technologies. However, further experiments are needed to determine the best conditions for the biosorption of not only dyes, but also other pollutants present in water.

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15 Appendages

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Table S1. Results of counted picocyanobacteria at the sampling date 25. 5. 2022

Date	Sampling point	Free	In colonies	TOTAL	1b(%)	col(%)
25.05.2022	WR Surface	3276	57	3333	98.30	1.70
25.05.2022	Raw water	574	32	606	94.64	5.36
25.05.2022	Aggregation	476	8	484	98.32	1.68
25.05.2022	F5 (24 hod)	14	0	14	100.00	0.00
25.05.2022	F7 (88.5 hod)	42	0	42	100.00	0.00
25.05.2022	F8 (57 hod)	7	0	7	100.00	0.00
25.05.2022	Before Acumulation	11	0	11	100.00	0.00
25.05.2022	After Acumulation	8	0	8	100.00	0.00
25.05.2022	WT Janovice	4	0	4	100.00	0.00

Table S2. Results of counted picocyanobacteria at the sampling date 2. 6. 2022

Date	Sampling point	Free	In colonies	TOTAL	1b(%)	col(%)
02.06.2022	WR Surface	5021	1786	6807	73.77	26.23
02.06.2022	Raw water	1166	1217	2383	48.92	51.08
02.06.2022	Aggregation	695	1136	1832	37.96	62.04
02.06.2022	F5 (82 hod)	20	0	24	86.16	0.00
02.06.2022	F6 (48 hod)	9	0	11	86.13	0.00
02.06.2022	F7 (15 hod)	12	0	14	86.18	0.00
02.06.2022	Before Acumulation	12	0	14	86.13	0.00
02.06.2022	After Acumulation	3	0	4	86.11	0.00
02.06.2022	WT Janovice	1	0	6	8.61	0.00

Table S3. Results of counted picocyanobacteria at the sampling date 22. 6. 2022

Date	Sampling point	Free	In colonies	TOTAL	1b(%)	col(%)
22.06.2022	WR Surface	8479	1407	9886	85.77	14.23
22.06.2022	Raw water	1869	546	2416	77.38	22.62
22.06.2022	Aggregation	134	771	905	14.77	85.23
22.06.2022	F5 (25 hod)	19	0	19	100.00	0.00
22.06.2022	F7 (92 hod)	13	0	13	100.00	0.00
22.06.2022	F8 (58 hod)	8	0	8	100.00	0.00
22.06.2022	Before Acumulation	15	8	23	65.48	34.52
22.06.2022	After Acumulation	5	0	5	100.00	0.00
22.06.2022	WT Janovice	2	0	2	100.00	0.00

Table S412. Results of counted picocyanobacteria at the sampling date 8. 7. 2022

Date	Sampling point	Free	In colonies	TOTAL	1b(%)	col(%)
08.07.2022	WR Surface	11030	2059	13089	84.27	15.73
08.07.2022	Raw water	3463	2170	5633	61.48	38.52
08.07.2022	Aggregation	317	1163	1481	21.43	78.57
08.07.2022	F5 (9 hod)	11	4	15	75.00	25.00
08.07.2022	F6 (58 hod)	7	0	7	100.00	0.00
08.07.2022	F8 (41 hod)	5	0	5	100.00	0.00
08.07.2022	Before Acumulation	4	0	4	100.00	0.00
08.07.2022	After Acumulation	4	0	4	100.00	0.00
08.07.2022	WT Janovice	7	0	7	100.00	0.00

Table S5. Results of counted picocyanobacteria at the sampling date 20. 7. 2022

Date	Sampling point	Free	In colonies	TOTAL	1b(%)	col(%)
20.07.2022	WR Surface	8368	9496	17864	46.84	53.16
20.07.2022	Raw water	6009	1704	7713	77.90	22.10
20.07.2022	Aggregation	2448	3977	6425	38.11	61.89
20.07.2022	F5 (30 hod)	48	0	48	100.000	0.00
20.07.2022	F6 (64 hod)	14	0	14	100.000	0.00
20.07.2022	F7 (87 hod)	11	0	11	100.000	0.00
20.07.2022	Before Acumulation	6	0	6	100.000	0.00
20.07.2022	After Acumulation	9	0	9	100.000	0.00
20.07.2022	WT Janovice	2	0	2	100.000	0.00

Table S613. Results of counted picocyanobacteria at the sampling date 5. 8. 2022

Date	Sampling point	Free	In colonies	TOTAL	1b(%)	col(%)
05.08.2022	WR Surface	4956	5032	9988	49.62	50.38
05.08.2022	Raw water	4380	4464	8844	49.53	50.47
05.08.2022	Aggregation	1978	3896	5873	33.67	66.33
05.08.2022	F5 (27 hod)	14	0	14	100.00	0.00
05.08.2022	F6 (15 hod)	4	0	4	100.00	0.00
05.08.2022	F7 (60 hod)	5	0	5	100.00	0.00
05.08.2022	Before Acumulation	13	0	13	100.00	0.00
05.08.2022	After Acumulation	3	0	3	100.00	0.00
05.08.2022	WT Janovice	3	0	3	100.00	0.00

Table S7. Results of counted picocyanobacteria at the sampling date 23. 8. 2022

Date	Sampling point	Free	In colonies	TOTAL	1b(%)	col(%)
23.08.2022	WR Surface	5010	7223	12234	40.96	59.04
23.08.2022	Raw water	495	1704	2199	22.51	77.49
23.08.2022	Aggregation	346	730	1077	32.16	67.84
23.08.2022	F5 (79 hod)	7	0	7	100.00	0.00
23.08.2022	F6 (46 hod)	4	0	4	100.00	0.00
23.08.2022	F7 (12 hod)	3	0	3	100.00	0.00
23.08.2022	Before Acumulation	4	0	4	100.00	0.00
23.08.2022	After Acumulation	2	0	2	100.00	0.00
23.08.2022	WT Janovice	3	0	3	100.00	0.00

Table S8. Results of counted picocyanobacteria at the sampling date 13. 9. 2022

Date	Sampling point	Free	In colonies	TOTAL	1b(%)	col(%)
13.09.2022	WR Surface	10957	1786	12742	85.99	14.01
13.09.2022	Raw water	9188	1883	11071	82.99	17.01
13.09.2022	Aggregation	1818	1493	3311	54.90	45.10
13.09.2022	F5 (38 hod)	13	0	13	100.00	0.00
13.09.2022	F6 (71 hod)	3	16	19	16.67	83.33
13.09.2022	F8 (32 hod)	2	0	2	100.00	0.00
13.09.2022	Before Acumulation	3	0	3	100.00	0.00
13.09.2022	After Acumulation	1	0	1	100.00	0.00
13.09.2022	WT Janovice	2	0	2	100.00	0.00

Table S9. Results of counted picocyanobacteria at the sampling date 25. 10. 2022

Date	Sampling point	Free	In colonies	TOTAL	1b(%)	col(%)
25.10.2022	WR Surface	5909	3246	9155	64.54	35.46
25.10.2022	Raw water	2305	1104	3409	67.62	32.38
25.10.2022	Aggregation	2256	1696	3953	57.08	42.92
25.10.2022	F5 (92 hod)	45	0	45	100.00	0.00
25.10.2022	F7 (27.5 hod)	37	0	37	100.00	0.00
25.10.2022	F8 (62 hod)	53	0	53	100.00	0.00
25.10.2022	Before Acumulation	61	0	61	100.00	0.00
25.10.2022	After Acumulation	20	0	20	100.00	0.00
25.10.2022	WT Janovice	16	0	16	100.00	0.00

Table S10. Filters and their filtration times at the individual sampling dates with calculated separation efficiencies

Date	Filter	Filtration time [hod]	Total	Pcy Sur Total	Separation efficiency	% of going through Pcy	Max of the day	Min of the day
25.05.2022	F5	24	14	606	97.72	2.28		
25.05.2022	F7	88.5	42	606	93.04	6.96	6.96	1.21
25.05.2022	F8	57	7	606	98.79	1.21		
02.06.2022	F5	82	24	2383	99.01	0.99		
02.06.2022	F6	48	11	2383	99.55	0.45	0.99	0.45
02.06.2022	F7	15	14	2383	99.39	0.61		
22.06.2022	F5	25	19	2416	99.19	0.81		
22.06.2022	F7	92	13	2416	99.46	0.54	0.81	0.34
22.06.2022	F8	58	8	2416	99.66	0.34		
08.07.2022	F5	9	15	5633	99.73	0.27		
08.07.2022	F6	58	7	5633	99.87	0.13	0.27	0.09
08.07.2022	F8	41	5	5633	99.91	0.09		
20.07.2022	F5	30	48	7713	99.38	0.62		
20.07.2022	F6	64	14	7713	99.82	0.18	0.62	0.14
20.07.2022	F7	87	11	7713	99.86	0.14		
05.08.2022	F5	27	14	8844	99.84	0.16		
05.08.2022	F6	15	4	8844	99.96	0.04	0.16	0.04
05.08.2022	F7	60	5	8844	99.95	0.05		
23.08.2022	F5	79	7	2199	99.69	0.31		
23.08.2022	F6	46	4	2199	99.84	0.16	0.31	0.12
23.08.2022	F7	12	3	2199	99.88	0.12		
13.09.2022	F5	38	13	11071	99.89	0.11		
13.09.2022	F6	71	36	11071	99.68	0.32	0.32	0.02
13.09.2022	F8	32	2	11071	99.98	0.02		
25.10.2022	F5	92	45	2305	98.06	1.94		
25.10.2022	F7	27.5	37	2305	98.42	1.58	2.29	1.58
25.10.2022	F8	62	53	2305	97.71	2.29		

Table S11. Calibration curve data of the MG

Concentration [mg/L]	A [Abs]
1	0.198
3	0.553
4	0.770
5	0.911
6	1.121
7	1.236
8	1.219
9	1.050
10	1.769
25	4.980
30	5.665
35	7.120
40	7.467
45	8.395
50	9.458
60	11.415
70	13.550
80	15.317
90	16.957
100	17.847

Table S12. Calibration curve data of Safranin O

Concentration [mg/L]	A [Abs]
0.000	0
1.000	0.109
25.000	2.800
50.000	4.920
75.000	7.470
100.000	8.970
150.000	12.210
200.000	11.700
250.000	16.400
500.000	35.800
1000.000	62.300

Figure S13. Sampling profile "Water Reservoir Surface"

Figure S14. Sampling profile "Raw Water"

Figure S15. Sampling profile "Aggregation"

Figure S16. Sampling profile "Filter 5"

Figure S17. Sampling profile "Filter 6"

Figure S18. Sampling profile "Filter 7"

Figure S19. Sampling profile "Filter 8"

Figure S20. Sampling profile "Before Accumulation"

Figure S21. Sampling profile "After Accumulation"

Figure S22. Sterile fume hood used for the inoculation of picocyanobacteria cultures

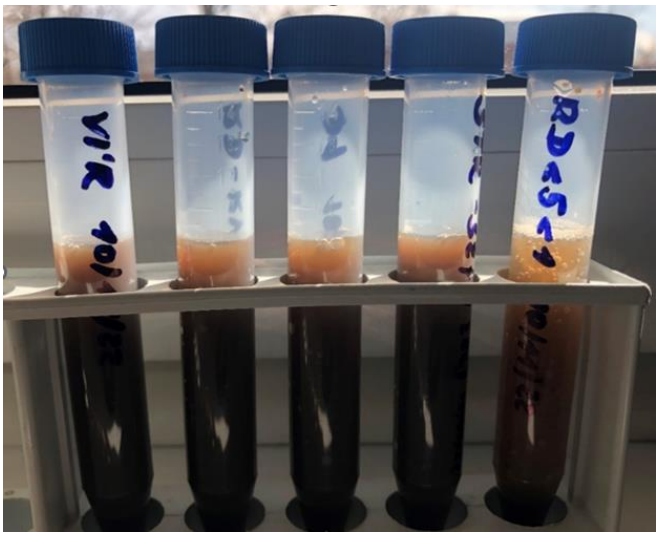

Figure S23. Pretest solutions of picocyanobacteria with microwave synthesized magnetite

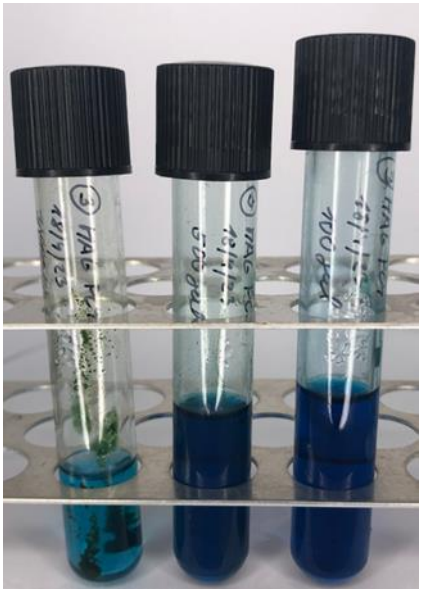

Figure S24. Three samples of three different concentrations of the MW Mag Pcy with 5 mL of 0.1 mg/L MG after 24 hours of incubation at laboratory temperature (from the left 1000 µL, 500 µL and 100 µL) (author´s archive)

Figure S25. Triplication of the experiment with the Safranin O (0.1 mg/mL)