Utjecaj mikroplastike na rast i proces fotosinteze u vodenoj leći (Lemna minor)

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The effects of microplastics on the growth and photosynthesis of *Lemna minor*

Master thesis

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Master thesis

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Microplastic particles (MPs), as waste and degradation products of larger plastics, are widespread pollutants with potential environmental impact. Polystyrene (PS) and poly(methyl methacrylate) (PMMA) plastics are widely used in industry and can accumulate in the environment, so it is important to investigate their possible effects on plants. In this thesis, I investigated the effects of different concentrations (10, 50 and 100 mg L^{-1}) of PS- and PMMA-MPs on the growth and photosynthesis of common duckweed (*Lemna minor* L.), a model organism used for ecotoxicological research and phytoremediation. PS-MPs caused an increase in the number of fronds and total area, while PMMA-MPs, particularly at the highest concentration, caused a decrease. However, these changes were not statistically significant. Neither type of MPs had a significant effect on the maximum quantum yield of PSII (F_v/F_m), although the photosynthetic performance index (PI_{ABS}) was slightly higher for the PS-MP treatments than for PMMA-MPs. PMMA-MPs also led to a greater reduction in pigment content, especially chlorophyll *a*, while lower concentrations of PS-MP appeared to have a positive effect on the content of chlorophyll *a*, chlorophyll *b* and carotenoids compared to the control. Both types of MPs reduced the expression of RuBisCO protein, with PMMA-MPs having a more pronounced impact. In conclusion, although both MPs affected plant physiology, PMMA-MPs appear to be more harmful to *L. minor* than PS-MPs, and it is advisable to continue research into its effects and possible plant defense mechanisms.

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Utjecaj mikroplastike na rast i proces fotosinteze u vodenoj leći (*Lemna minor*)

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Čestice mikroplastike (MP), kao otpad i produkti razgradnje veće plastike, rašireni su zagađivači s potencijalnim učinkom na okoliš. Polistiren (PS) i poli(metil metakrilat) (PMMA) dvije su vrste plastike koje se naširoko koriste u industriji i mogu se nakupljati u okolišu, stoga je važno istražiti njihove moguće učinke na biljke. U ovom radu istraživala sam učinke različitih koncentracija (10, 50 i 100 mg L^{-1}) PS- i PMMA-MP na rast i fotosintezu vodene leće (*Lemna minor* L.), modelnog organizma koji se koristi za ekotoksikološka istraživanja i fitoremedijaciju. Izlaganje biljaka PS-MP je uzrokovalo povećanje površine i broja frondova, dok je izlaganje PMMA-MP, osobito u najvišoj koncentraciji, uzrokovalo smanjenje. Međutim, te promjene nisu bile statistički značajne. Nijedna vrsta MP nije imala značajan učinak na maksimalni kvantni prinos PSII (F_v/F_m), iako je indeks fotosintetske učinkovitosti (PI_{ABS}) bio malo viši u biljaka tretiranih s PS-MP, u odnosu na PMMA-MP. Tretman s PMMA-MP je doveo i do većeg smanjenja udjela pigmenta, posebice klorofila *a*, dok su niže koncentracije PS-MP imale pozitivan učinak na sadržaj klorofila *a*, klorofila *b* i karotenoida u usporedbi s kontrolom. Obje vrste MP smanjile su ekspresiju proteina RuBisCO, pri čemu je PMMA-MP imala izraženiji učinak. Zaključno, iako obje vrste mikroplastike utječu na fiziologiju biljaka, čini se da je PMMA-MP štetnija za vodenu leću, te je preporučljivo nastaviti s istraživanjem učinaka i mogućih obrambenih mehanizama biljaka.

Ključne riječi: PMMA, PS, RuBisCO, pigmenti, JIP-test, broj i površina frondova
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List of abbreviations

AA/Bis – Acrylamide/Bis-acrylamide mixture

ADP – Adenosine diphosphate

App – Mobile application

APS – Ammonium persulfate

ATP – Adenosine triphosphate

BSA – Bovine serum albumin

CAB – Chlorophyll *a*/*b*-binding protein

CRTISO – Carotene isomerase

DCMU – Dichlorophenyldimethylurea

DNA – Deoxyribonucleic acid

IUPAC – International Union of Pure and Applied Chemistry

LBS – Laemmli Sample Buffer

LH1/2 – Light-harvesting complex one/ Light-harvesting complex two

MPs – Microplastics, plastic particles ranging from 1 to 1000 nm in size

$NADP^+$ – Nicotinamide adenine dinucleotide phosphate

NADPH – Nicotinamide adenine dinucleotide phosphate, reduced form with an additional hydrogen atom (H)

OJIP – A method for measuring polyphase increase of chlorophyll *a* fluorescence, name derived from graph peaks/phase names

PBAT – poly(butylene adipate-co-terephthalate)

PDS – Phytoene synthase

PE – Polyethylene

PGA – D-phosphoglyceric acid

pH – Negative logarithm of the concentration of hydrogen ions, it specifies the acidity (<7) and alkalinity (>7) of a solution

Pi – Inorganic phosphate

PMMA – Poly(methyl methacrylate)

PS – Polystyrene

PsbA – Photosystem II reaction centre protein D1

PsbH – Photosystem II reaction centre protein H

PSI/II – Photosystem one/Photosystem two

PSM – Plant Screen Mobile, mobile application used for determining total frond area

PSY – Phytoene desaturase

PVPP – Poly(vinylpolypyrrolidone)

RAF2 – Raf-like kinase 2

rbcL – Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit

RCA – Rubisco activase

ROS – Reactive oxygen species

RuBisCO – Ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBP – Ribulose 1,5-bisphosphate

SDS – Sodium duodecyl sulfate/sodium lauryl sulfate

TBS buffer – Tris-buffered saline buffer

TBS-T – Tris-buffered saline-Tween 20

TEMED – N,N,N′,N′-tetramethylethylenediamine

Tris – Tris(hydroxymethyl)aminomethane

UV – Ultraviolet radiation/light

1. Introduction

1.1. Microplastic pollution

The current estimate is that the human population uses more than 240 million tons of plastic every year, mostly in the form of single use plastic items (Rillig, 2012). Given the slow degradation rate, the sheer amount of discarded plastic and insufficient removal efforts (Rillig, 2012), plastic waste continues to accumulate in various ecosystems. So far it has been found in various terrestrial, freshwater and marine environments (Lambert and Wagner, 2018; Rillig, 2012). In recent years, researchers have focused on a new type of plastic pollutants – microplastics (MPs). These microscopically sized fragments of various types of plastic materials seem to be as ubiquitous as their larger counterparts and may present an even bigger problem (Lambert and Wagner, 2018; Rillig, 2012). For the purpose of this research, MPs will be defined as plastic particles whose size ranges from 1 to 1000 nm (Rozman and Kalčíková, 2022), though other sources may define them with different size ranges.

Though a majority of research focuses on marine environments when it comes to the presence and effects of MPs (Rillig, 2012), recent studies have proven that MPs are also found in soil and freshwater (Lambert and Wagner, 2018; Rillig, 2012), including rivers, lakes, sediment, beaches and even reservoirs, potentially contaminating sources of potable water (Lambert and Wagner, 2018). Of further concern for human health is the bioaccumulation and transfer of MPs through trophic levels (Farrell and Nelson, 2013), meaning that environmentally present MPs could end up being ingested by humans.

We can divide MPs into two categories based on their origin – primary and secondary MPs. Primary MPs are products specifically manufactured to be microscopic in size. This includes products like cosmetic facial scrub microbeads and abrasive particles for paint and lacquer removal (Farrell and Nelson, 2013). Secondary MPs are those that form in the environment through degradation of larger plastic waste (Farrell and Nelson, 2013). Which types of MPs are more present and the rout through which they end up in the ecosystem depend on several factors, such as geographical location, local waste management laws and regulation, which industries are present, climate conditions and societal structure (Cierjacks et al., 2012). For example, poorer and more

industrial countries will likely have less primary MPs in the form of cosmetic microbeads but more abrasive particles or secondary MPs from industrial packaging. Areas with less strict laws regarding recycling or landfill management will have more MPs in general. The formation of MPs in the environment itself means that MP accumulation in the ecosystems cannot just be controlled by limiting new MP production or waste filtration, but must also include the removal of macroplastic waste, i.e. MP clean up cannot be an isolated effort.

1.2. PMMA and PS properties, uses and disposal

Poly(methyl methacrylate) is also known as PMMA, acrylic glass or acrylate, however its full official IUPAC name is poly(methyl 2-methylpropenoate) (The Essential Chemical Industry (ECI) – online, 2019). It is a rigid transparent synthetic polymer with many industrial and medical uses. Annual global production is around 2.9 million tons (ECI – online, 2019), and is predicted to increase to 5.7 million by 2028 (De Tommaso and Dubois, 2021). It is easy to cast and manipulate, biocompatible with human tissue, and the toxicity for humans is relatively low (Frazer et al., 2005). In the transparent sheet form, it is used as a sturdier substitute for glass, e.g. aquarium glass and aircraft windows, and as a powder, it is melted and cast into medical prosthesis, such as dentures and false eyes (ECI – online, 2019), and various medical equipment like incubators and medicine cabinets (De Tommaso and Dubois, 2021). Additional medical uses include antibiotic spacer, filler and delivery vehicle (Jaeblon, 2010), bone cement, surgical screw fixation, stabilization of spinal vertebrae, contact lenses, intraocular lenses, filler for bone deformations and cavities (Frazer et al., 2005; Jaeblon, 2010). Though the medical implants seem to be safe (Frazer et al., 2005), PMMA nanoparticles can enter human cells through endocytosis and cause cell death via apoptosis. They also increase reactive oxygen species (ROS) production and decrease cell vitality (Mahadevan and Valiyaveettil, 2021).

Since PMMA is a polymer, most recycling efforts focus on its de-polymerization into methyl methacrylate monomers, which can then be used as components of paints, coating and adhesives (ECI-online, 2019) or re-polymerized into new PMMA (Esmizadeh et al., 2018). Recycled PMMA is used in manufacture of polymer blends and nanocomposite materials (Esmizadeh et al., 2018). Even though PMMA recycling

is ecologically preferable and economically viable (Esmizadeh et al., 2018), less than 2.7% is actually recycled (De Tommaso and Dubois, 2021).

Polystyrene (PS) is an easily malleable and low-cost synthetic polymer primarily used in the form of a synthetic plastic foam, commonly known as Styrofoam. It is used in the production of disposable cutlery, food and drink containers, compact disks, and insulation and packaging materials (Gautam et al., 2007). In 2021, the global production of PS was 15.61 million tons and it is estimated to increase to 16.75 million tons by 2026 (Statista, 2023). Take-out and food delivery have become more popular in recent years. This trend has largely been popularized by pandemic induced lockdowns and restrictions and the development of food delivery applications (apps), such as Uber Eats (Meena and Kumar, 2022) and shows an increased demand for single use Styrofoam containers and cutlery, while popularity of online shopping increases the use of PS packaging and insulation (Shaw et al., 2022). Recent research also suggests PS foams could be used as an efficient drug delivery method (Canal et al., 2012), which would serve to further incentivize PS production.

Globally, only 10% of PS waste is recycled each year (Gautam et al., 2007), though some countries, such as the USA, have increased that to 28% (Ho et al., 2018). Foam PS is the least recycled, due to its large bulk, which increases shipping costs, and unprofitability, as the value of recycled usable foam PS is much lower than the cost of recycling (Gautam et al., 2007; Ho et al., 2018).

Since PMMA and PS have different industrial and medical uses, have few viable replacement options and the recycling efforts are minimal, we can expect them to continue being a major source of MP pollution for the foreseeable future. Therefore, it is important to determine what impact they may have on ecosystems and living organisms, they enter.

1.3. Effects of PMMA- and PS-MPs on plants

Plants are a key element in almost all ecosystems – they produce oxygen, play a role in biogeochemical cycles of nutrients, serve as a food source for many species, offer hiding places and habitats and prevent soil erosion. Humans use them for food, medicine, materials (wood, textiles), pest control and decoration (Keddy, 2007). Considering the importance of plants for nature and humans, it is important that we

understand how MPs, and other pollutants, affect them and how we may mitigate that effect. Previous research has shown that many MPs can damage plants, affecting their growth and biochemistry and influencing the effects of other pollutants (Biba et al., 2023; Dong et al., 2022; Huang et al, 2021).

Dong et al. (2022) showed that exposing field mustard (*Brassica campestris* L.) to PMMA-MPs has negative effects on plant cell structure and biochemistry. Roots and leaves showed ultrastructural damage and PMMA-MP accumulation, total chlorophyll content decreased and there was an increase in enzymes related to ROS production and oxidative stress including superoxide dismutase, catalase, glutathione reductase and ascorbate peroxidase. The extent of damage correlated with the dosage used, and exposure through roots caused greater damage than leaf exposure. PMMA-MPs also increased the absorption and accumulation of arsenic in the cell wall.

Huang et al. (2021) exposed colonies of *Gymnodinium aeruginosum* Stein to both, PMMA- and PS-MPs, using different concentrations and sizes of MPs. Both MPs caused cell damage and oxidative stress. The MPs partially inhibited the activity of enzymes superoxide dismutase and catalase, which play a role in ROS degradation. The damage to cells and colonies depended on the size and concentration of MPs. For both types of plastics, the largest particles of 100 μm caused the least damage, while the greatest damage was caused by medium-sized particles of 1.0 μm. In addition, for both PS- and PMMA-MPs, higher concentrations and longer exposure time had a greater effect.

On the other hand, some studies suggest that plants may be capable of dealing with MP pollution while sustaining little or no damage. Biba et al. (2023) proved PMMA- and PS-MPs were indeed absorbed by onion (*Allium cepa* L.) roots but they did not decrease root length or cause noticeable cytotoxicity. PMMA-MPs even enhanced root growth. In addition, even though MPs induced oxidative stress, there was no DNA damage detected.

1.4. Photosynthesis

Photosynthesis, specifically oxygenic photosynthesis, is a biological process performed by plants, cyanobacteria and algae in which light energy is converted into chemical energy and oxygen is released at the same time. Photosynthesis is

necessary for the existence of all eukaryotic and some prokaryotic lifeforms, both as a source of oxygen and as a direct or indirect source of food – plants produce their "food" through photosynthesis and other organisms feed on living or decomposing plants (Taiz and Zeiger, 2002).

Photosynthesis is generally divided into light (or light dependent) reactions and carbon fixation (or light-independent) reactions. Light reactions (Figure 1) occur on the thylakoid membranes of chloroplasts. Chloroplasts are plant organelles with two lipid membranes and a stroma – an enclosed internal section of the cell cytoplasm with a specific pH value, enzymes and biochemical reactions. Thylakoids are specialized internal membranes of chloroplasts that contain photosystems – specialized functional units made up of several proteins. All plants have two slightly different photosystems – PSI and PSII, and each photosystem consists of a reaction center and an antenna complex (LH1 for PSI and LH2 for PSII). Antennae consist of several different proteins, many chlorophyll *b* and chlorophyll *a* molecules and various accessory pigments (carotenes and xanthophylls). PSI/LH1 preferentially absorbs light of wavelengths greater than 680 nm (far-red) and PSII/LH2 wavelengths of 680 nm (near-red). Antennae complexes have the function of capturing light energy and transferring it to the reaction center – a complex of several proteins, pigments, and other co-factors. A pigment molecule captures light energy by moving from a ground state (low energy) to an excited state (higher energy) after coming into contact with a photon (light molecule). This excited pigment then drops back down to ground state by transferring energy to the pigment molecule next to it in the antennae, or if it is the chlorophyll *a* pair in the reaction center, the excited electron passes to the primary electron acceptor. The lost electron must be replaced. In PSII, the electron comes from the oxidation of water, while in PSI it comes from the electron transport chain. Whenever chlorophyll molecules return to ground state, they can emit a photon of a higher wavelength (lower energy) than the one they absorbed. This light emission is called fluorescence. The ratio of emitted and absorbed photons is called the quantum yield of fluorescence, and the ratio of synthesized photochemical products and absorbed photons is called the quantum yield of photosynthesis (Taiz and Zeiger, 2002).

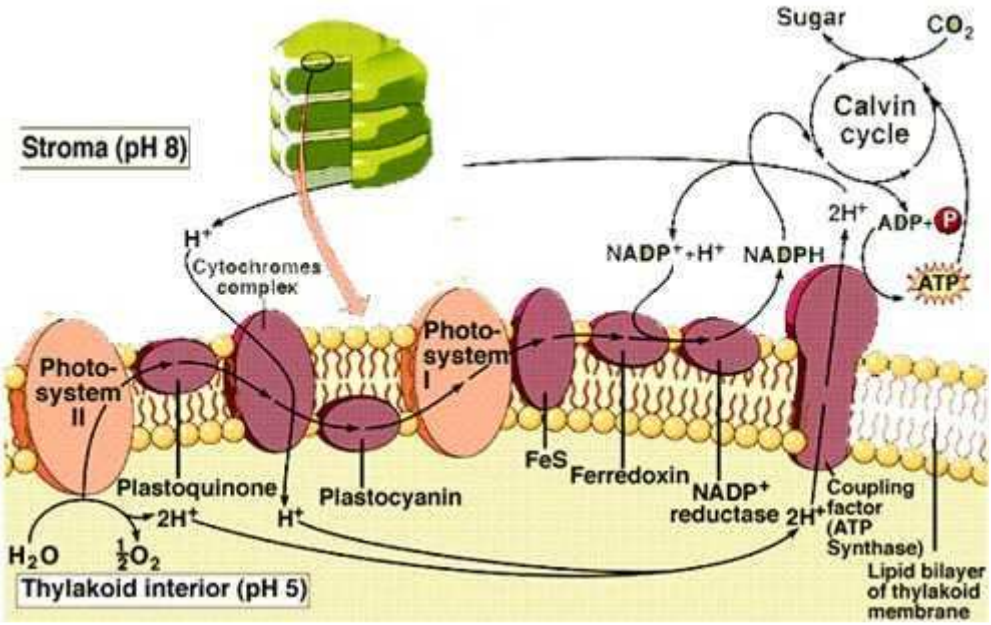

Figure 1. Light-dependent (primary) photosynthesis reactions of thylakoid membranes. The membrane-bound proteins; photosystem II, plastoquinone, cytochrome complex *b*$_6$*f*, plastocyanin, photosystem I, ferredoxin and ferredoxin NADP$^+$ reductase, form the electron transport chain, which creates the proton gradient between the stroma and thylakoid interior (lumen). The proton gradient powers ATP synthesis at ATP synthase. Light-dependent reactions also produce NADPH, which is, together with ATP, used in light-independent (secondary) reactions, also known as Calvin-Benson-Basshamov (CBB) cycle. Image taken from https://shorturl.at/rs07b.

The first step of photosynthesis is the absorption of light at PSII (Figure 1). This energy powers the oxidation of a water molecule (H_2O) into a dioxygen molecule (O_2), a hydrogen ion or proton (H^+), and a free electron (e^-). The proton is released into the lumen (space enclosed by thylakoid membranes). The electron is transferred to electron carrier proteins; first to the primary electron acceptor pheophytin, then to plastoquinone Q_A, then to plastoquinone Q_B, then to the cytochrome *b*$_6$*f* protein complex. Plastoquinone Q_A needs to accept two electrons and two protons before it can continue the transfer. Cytochrome *b*$_6$*f* helps transfer electrons from plastoquinone to plastocyanin and transfer the protons from plastoquinone into the thylakoid lumen, creating a proton gradient and a pH difference between the stroma and lumen. Plastocyanin carries electrons to the PSI. In PSI, the absorbed light and excited electrons power ferredoxin and ferredoxin–NADP$^+$ reductase to reduce NADP$^+$ to NADPH in chloroplast stroma. ATP synthase is a thylakoid membrane-bound protein complex with a channel connecting the lumen to the stroma. When protons diffuse through the channel, due to the gradient produced by electron transfer and cytochrome

b_6f, it powers the ATP synthase to produce ATP from ADP and P_i (Taiz and Zeiger, 2002).

ATP and NADPH produced in light reactions are later used to facilitate light independent reactions and produce organic compounds used as an energy source by the plant. These reactions are also known as the Calvin–Benson–Bassham or Calvin cycle, which is divided into three stages (Figure 2). Stage one is carboxylation – three molecules each of carbon dioxide (CO_2) and H_2O, absorbed from the environment, are combined with three molecules of ribulose-1,5-bisphosphate to form six molecules of 3-phosphoglycerate (PGA). This is done by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase commonly known as RuBisCO. Stage two is reduction – 3-phosphoglycerate molecules are reduced to glyceraldehyde 3-phosphate with the use of ATP and NADPH produced in the light reactions. One of the glyceraldehyde-3-phosphate molecules is used for starch synthesis in the chloroplasts or sucrose synthesis in the cell cytosol while fives are used to recover the CO_2 acceptor ribulose-1,5-bisphosphate in stage three called regeneration, where ATP is spent (Taiz and Zeiger, 2002).

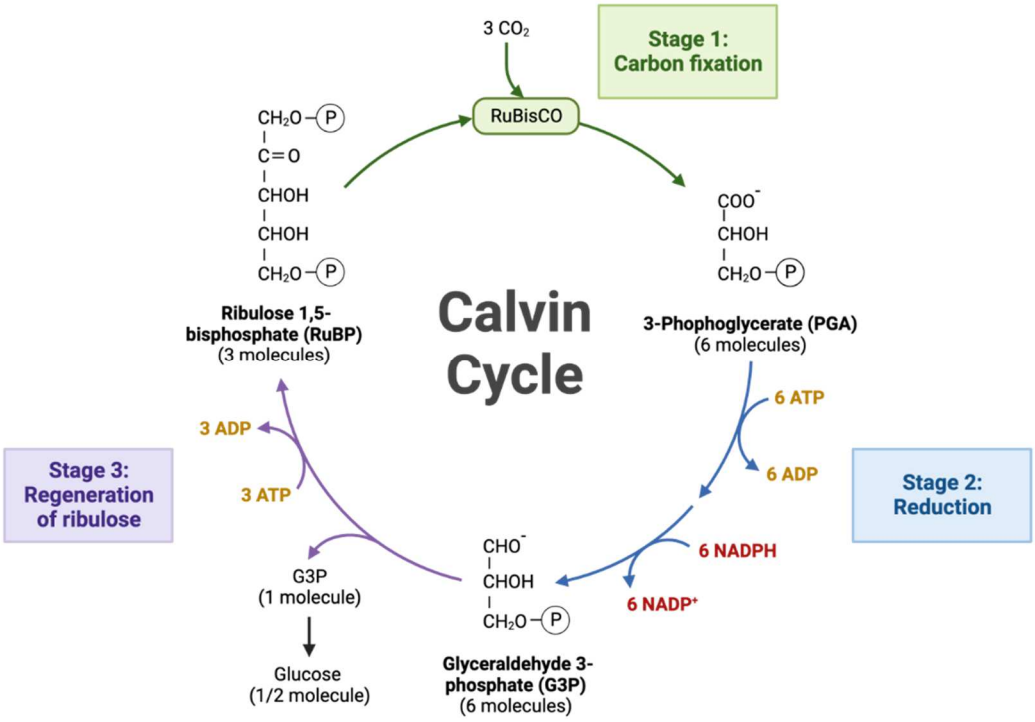

Figure 2. The three stages and reactions of the Calvin-Benson-Basshamov (CBB) cycle – light-independent (secondary) photosynthesis reactions. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) plays the main role by fixating CO_2. ATP and NADPH are produced in light-dependent (primary reactions). Image taken from https://shorturl.at/AgNGI.

1.5. Photosynthesis under stress

Photosynthesis is one of the most important metabolic processes, and exposure of plants to various abiotic stress factors such as drought, salinity and high temperatures or biotic stress can severely affect the mechanism of photosynthesis. Stress modulates the chemical reactions mediated by PSI and PSII and leads to changes in pigment composition as well as changes in the photosynthetic CO_2 assimilation (Chauhan et al., 2023).

1.5.1. Fluorescence of chlorophyll *a*

Antennae can absorb too much light energy to power photochemical reactions and this excess energy can lead to the production of damaging ROS molecules, such as superoxide, singlet oxygen and peroxide. To prevent this, excess energy is safely removed through heat and light emission. With heat emission, energy is transferred from excited chlorophyll molecules to carotenoid molecules. Carotenoids cannot power the formation of ROS and thus emit heat energy when they drop back down to ground state. With light emission, chlorophylls emit more fluorescence then they would if the energy was transferred to power photochemical reactions. In other words, the quantum yield of fluorescence is increased (Taiz and Zeiger, 2002).

By measuring fluorescence of chlorophyll *a* we can determine the efficiency of light dependent photosynthesis reactions (Strasser et al., 2004). Increased fluorescence means that less of the absorbed light energy is used to produce carbohydrates. This indicates less efficient photosynthesis, which can be a sign that the plant is under some form of stress or is exposed to damaging compounds. Namely, exposure of plants to various stress conditions can inhibit the electron transport rate or cause photoinhibition (Chauhan et al., 2023). Measurement of chlorophyll *a* fluorescence is a fast, highly sensitive, non-destructive and reliable method for assessing photosynthetic efficiency (Strasser et al., 2004) and I used this method to detect potential damaging effects of PMMA- and PS-MPs on photosynthesis.

1.5.2. Fast chlorophyll *a* fluorescence induction

Fast chlorophyll *a* fluorescence induction or JIP-test is a method based on measuring the polyphase increase in chlorophyll *a* fluorescence after the start of actinic light which

initiates photosynthesis. Plants are first acclimated to darkness, which ensures that all electron acceptors are oxidized, and then exposed to high light intensity. The emitted increase in chlorophyll fluorescence has a typical curve, known as Kautsky curve, which is usually displayed on a logarithmic time scale: from a minimal fluorescence level (F_0) at the initial O step (which stands for open/origin), when all plastoquinones are completely oxidized and "open", it increases to a maximum fluorescence (F_m) at the P step (which stands for peak), when all plastoquinones are reduced. Two inflections occur between O and P, step J – the point at which the majority of plastoquinones Q_A have been reduced and step I – the point between J and P at which the majority of plastoquinones Q_B have been reduced (Küpper et al., 2019). Various fluorescence parameters can be obtained and derived from the curve using the JIP-test (Strasser et al., 2000, 2004) (Table 1).

Table 1. Parameters obtained and derived from measuring the polyphasic growth of chlorophyll *a* fluorescence using the JIP-test (Strasser et al., 2000).

Parameter	Description
F_0	Fluorescence intensity after 50 μs (step O)
F_J	Fluorescence intensity after 2 ms (step J)
F_I	Fluorescence intensity after 30 ms (step I)
F_m	Maximum fluorescence intensity (step P)
F_v	Maximum variable fluorescence
F_v/F_m	Maximum quantum yield of PSII
V_j	Variable fluorescence between steps O and J
M_0	Overall rate of reaction center closure
$ABS/RC = M_0 \times (1/V_J) \times [1/(F_v/F_m)]$	Photon absorption per active reaction center
$TR_0/RC = M_0 \times (1/V_J)$	Photon capture rate per active reaction center
$ET_0/RC = M_0 \times (1/V_J) \times (1-V_J)$	Electron transfer per active reaction center
$DI_0/RC = (ABS/RC) - (TR_0/RC)$	Energy dissipation per active reaction center
$PI_{ABS} = (RC/ABS) \times (TR_0/DI_0) \times [ET_0/(TR_0-ET_0)]$	Photosynthetic performance index

1.5.3. Photosynthetic pigments

Light-absorbing pigments are necessary components for photosynthesis. The photosynthetic pigments in higher plants are chlorophyll *a*, chlorophyll *b* and carotenoids (Taiz and Zeiger, 2002). Chlorophyll *a* is the primary pigment in green plants that is responsible for photochemistry, while chlorophyll *b* is an accessory pigment that complements chlorophyll *a* by capturing light at slightly different wavelengths and transferring it to chlorophyll *a*, thus expanding the range of light which can be utilized for photosynthesis. Carotenoids are a diverse group of terpenoid pigments that are essential for the normal functioning of plant organisms, as they play an important role in photoprotection and structural integrity of the photosynthetic system (Taiz and Zeiger, 2002). Additionally, they absorb light in the wavelength range of 450-570 nm, partially covering the part of the spectrum that chlorophylls do not absorb, thus reducing the "green hole" in the absorption spectrum of chlorophylls.

A decrease in chlorophyll content negatively affects photosynthesis efficiency and can be linked to various stress conditions, including high light, drought, and exposure to pollutants such as heavy metals (Agathokleous et al., 2020). Mlinaric et al. (2017) determined that the amount of available photosynthetic pigments can affect the development of the D1 protein, an important part of the PSII reaction center. This means that pigments can also indirectly affect photosynthesis efficiency, by interacting with PSI/II proteins. Therefore, by measuring the pigment content of a plant, it can be determined whether MP exposure causes stress or damage to the plant.

Spectrophotometry is a method used to quantitatively measure light-absorbing molecules in a solution or mixture. The amount of light absorbed at a specific wavelength correlates with the amount of molecules that absorb that specific wavelength (Thrane et al., 2015). Chlorophylls *a* and *b*, as well as carotenoids, have maximum absorption at different wavelengths, so their amounts in plant extracts can be determined using spectrophotometry (Wellburn, 1994).

1.5.4. RuBisCO protein

RuBisCo (EC 4.1.1.39) is an enzyme that is required for the first major step of carbon fixation in photosynthesis and is considered the most abundant enzyme on earth (Raven, 2013). Plant RuBisCO consists of eight identical large subunits encoded by

chloroplast genes, which also contain the active site of catalysis, and eight identical small subunits encoded by genes from the nucleus. RuBisCO is crucial for the production of biomass from CO_2, but has a very low turnover rate, which means that at 25 °C only three CO_2 molecules per second are fixed by one enzyme molecule. In order to achieve a higher rate of CO_2 fixation, plants synthesize large amounts of this enzyme, so that it accounts for almost 50% of the total soluble chloroplast proteins (Taiz and Zeiger, 2002).

RuBisCO preforms CO_2 fixation and assimilation by catalyzing a carboxylation reaction between CO_2 and ribulose 1,5-bisphosphate (RuBP), forming two D-phosphoglyceric acid (PGA) molecules (Parry et al., 2003). Alternatively, it can assimilate oxygen, catalyzing the oxygenase reaction between O_2 and RuBP, creating one molecule each of phosphoglycolate and PGA and initiating photorespiration as opposed to photosynthesis. Photorespiration is a less desirable process as it results in loss of carbon and energy (Parry et al., 2003). In normal conditions, RuBisCO is activated by the enzyme rubisco activase (RCA), which uses ATP hydrolysis derived energy to induce conformational changes in RuBisCO. These conformational changes open up RuBisCO catalytic sites and promote the removal of RuBisCO inhibitors (Parry et al., 2003). The activity of RCA, and therefore RuBisCO, is promoted by the presence of ATP and RuBP and is inhibited by heat stress (Portis, 2003). RuBisCO is also directly activated by active-site lysine carbamylation and carbamate stabilization by a Mg^{2+} ion and is directly inhibited by the binding of various inhibitors (Parry et al., 2008). Environmental factors do not just affect RuBisCO activity, but can also influence its abundance, e.g. heavy metal pollution can lower RuBisCO content in plants (Son et al., 2014).

Western blotting or Western blot is a method that detects the presence of a target protein or proteins using antibodies that bind specifically to the target protein. Proteins are first extracted from plant tissue or cells and then separated using gel-electrophoresis. Proteins are transferred from the gel to a membrane, usually nitrocellulose or polyvinylidene difluoride. The membranes are then incubated with an antibody. The antibody is linked to a reporter molecule, such as a fluorescent protein or an enzyme that can generate a light signal or cause a color change in a substrate. The signaling molecule can be linked to the primary antibody (binds the target protein)

or a secondary antibody (binds the primary antibody). The use of a secondary antibody increases signal strength and binding specificity (Hnasko and Hnasko, 2015).

1.6. *Lemna minor* L.

Lemna minor L., also known as common duckweed, is a small floating monocotyledonous freshwater plant. Each plant usually has 1-4 small oval fronds, which are actually a combination of leaf and stem, and a hanging root. It rarely flowers and reproduces mainly by growing new fronds and splitting into two daughter plants once the mother plant has 4-5 fronds of sufficient size (Ekperusi et al., 2019). This means that all daughters are clones of the original mother plant. Individual plants tend to aggregate into colonies that eventually form large blankets across the water surface (Ekperusi et al., 2019). *L. minor* is a subcosmopolitan species, meaning that it inhabits appropriate habitats over a wide geographical range. Its native habitat includes brackish and freshwater areas all over Africa, Asia, Europe and North America. It is an invasive species in Australia and South America (Ekperusi et al., 2019).

Due to its small size, relative ease of maintenance and propagation and its ability to reproduce by cloning, the duckweed is a suitable model organism for research. Another advantage is the long history of its use in research, ecology and economy (Ekperusi et al., 2019; Rozman and Kalčíková, 2022). It has been used to study effects of different growth conditions and phytotoxicity of various compounds (Rozman and Kalčíková, 2022; Vidaković-Cifrek et al., 2015). This history means there is plenty of previous research to compare with and explain new research and results. It has also been used as animal feed, bioindicator, for phytoremediation of organic and floating pollutants, and for remediation and nutrient recovery from wastewater (Ekperusi et al., 2019; Rozman and Kalčíková, 2022).

In the majority of previous studies, the number of fronds and/or the increase in fresh mass were used as growth parameters and, more rarely, the area occupied by the fronds (Mazur et al., 2018; Mendonça et al., 2007). In my thesis I used Plant Screen Mobile, a mobile application (app) available online for free (Müller-Linow et al., 2019) that allows you to precisely measure various visible parameters of plant growth using only a mobile phone. The analysis can be done immediately by selecting the desired method and parameters and taking the picture of the test subject, or it can be done later with saved photographs (Müller-Linow et al., 2019). The analysis can be done

with one picture at a time or as a batch analysis of several photos. In order to properly compare the different photographs, the lighting conditions – light intensity, direction from which the light is coming and distance from the light source, as well as the camera conditions – resolution and distance between camera and subject, should be the same every time a photo is taken. The app can calculate various metric values such as total leaf area and circumference, but it is necessary to first calibrate the camera using a checkerboard pattern with a known square size and number (Müller-Linow, 2019).

In terms of MPs, previous research shows various negative and positive effects on *L. minor* growth, roots and physiology. Rozman and Kalčíková (2022) showed that certain MPs like tire particles, polyethylene microbeads and polyethylene terephthalate fibers adhere to the roots of duckweed, but have no effect on the specific growth rate or chlorophyll *a* content. Tire particles and polyethylene caused reduced root length, probably due to their shape and abrasiveness. Rozman et al. (2022) further proved that *L. minor* tolerates and adheres polyethylene MPs at high environmentally relevant concentrations. *L. minor* also showed some resistance to certain concentrations of PS-MPs (Xiao et al., 2022). Higher concentrations, 1 to 50 $\mu g\, mL^{-1}$, induced plant death and physiological changes. Lower, environmentally relevant, concentrations (lower than 0.1 $\mu g\, mL^{-1}$), had no obvious physiological effect, but could increase expression of genes related to oxidative and osmotic stress (Xiao et al., 2022).

This shows that *L. minor* may have some resistance to certain types of MP pollution and could be used for biomonitoring or bioremediation of MPs. It also shows that research should probably focus on physiological processes and molecular markers rather than morphology. Given the ecological and economical importance of *L. minor* and its suitability as a model organism, I used it to test the effects of PS- and PMMA-MPs with a focus on how they may affect photosynthesis.

2. Aims of the thesis

The goal of this experiment was to see if and how PMMA- and PS-MPs influence growth and photosynthesis of *Lemna minor* under laboratory conditions and to compare the results with previous research. I compared the two different types of microplastics of the same concentration and the different concentrations – 10, 50 and 100 mg L^{-1}, of the same plastic, to see whether one type of plastic or a higher concentration, has a different effect.

Based on previous studies, the MPs probably will not affect plant growth, except perhaps at the highest concentrations, but I hypothesize that the MPs may impair the efficiency of photosynthesis. I also hypothesize that higher concentrations of MPs will have a more significant effect than lower concentrations.

3. Materials and methods

3.1. Materials

The chemicals I used in my experiment are listed in Table 2, while all equipment and appliances are listed in Table 3, along with all relevant information. Aside from those, I used axenic culture of *Lemna minor* L. from the culture established at Faculty of Science, Department of Biology.

Table 2. A list of all chemicals, and their relevant information, used in this experiment.

Chemical – full name	Manufacturer
2-Mercaptoethanol/β-mercaptoethanol	Merck
3′,3″,5′,5″-Tetrabrom-phenolsulfonphthalein/Bromophenol Blue	Sigma-Aldrich
Acetic acid	GRAM-MOL, Croatia
Acetone (100%)	GRAM-MOL, Croatia
Acrylamide	Sigma-Aldrich
Ammonium persulfate/APS	Merck
Anti-rabbit IgG antibody	Merck Millipore, USA
Anti-Rubisco large subunit (rbcL) polyclonal antibody (rabbit); AS03 037	Agrisera, Sweden
Asparagine	Sigma-Aldrich
Bis-acrylamide	Sigma-Aldrich
Boric acid/Hydrogen borate	KEMIKA, Croatia
Bovine serum albuminum/BSA	Sigma-Aldrich
Calcium carbonate	GRAM-MOL, Croatia
Calcium chloride dihydrate	EMSURE
Calcium nitrate tetrahydrate	Honeywell Fluka
Coomiassie Briliant Blue G-250/CBB G-250	Sigma-Aldrich
Dipotassium phosphate	Acros Organics
Disodium molybdate dihydrate	KEMIKA, Croatia
Disodium ethylenediaminetetraacetate dihydrate	Lach:Ner
Dithiothreitol/DTT	Sigma-Aldrich
Ethanol 96%	LabExpert, Slovenia
Ferrous citrate/Iron (II) citrate	Merck
Glycerine/Glycerol	GRAM-MOL, Croatia
Glycine	CARLO ERBA Reagents, Germany
High-performance liquid chromatography water/HPLC water	Honeywell Fluka

Table 2. continued

Hydrochloric acid (5 M)	Sigma-Aldrich
Immobilon Forte Western Horse Radish Peroxidase Substrate	Merck Millipore, USA
Iron (III) chloride hexahydrate	Honeywell Fluka
L-Cysteine	Sigma-Aldrich
Magnesium sulfate heptahydrate	Lach:Ner
Manganese (II) chloride tetrahydrate	KEMIKA, Croatia
Methanol	J.T.Baker, USA
Milli-Q®	Merck Milipore, USA
Monopotassium phosphate	GRAM-MOL, Croatia
N,N,N′,N′ - Tetramethylethylenediamine/Temed	Sigma-Aldrich
N,N`-Methylenebisacrylamide/Bis-acrylamide	Sigma-Aldrich
Non-fat powdered milk	Dukat, Croatia
PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	Thermo Fisher Scientific™
Petroleum jelly/Vaseline	Unilever, USA
Phosphoric acid 88%	CARLO ERBA Reagents, Germany
Poly(methyl methacrylate)/PMMA (0,105 μm), 5% w/v	microParticles, Germany
Poly(vinylpolypyrrolidone)/PVPP	Honeywell Fluka
Polystyrene/PS (0,147 μm), 5% w/v	microParticles, Germany
Ponceaus S	Sigma-Aldrich
Potassium nitrate	GRAM-MOL, Croatia
Protease Inhibitor Tablets S8830-TAB	Sigma-Aldrich
Saccharose	Lach:Ner
Sodium chloride	GRAM-MOL, Croatia
Sodium dodecyl sulfate/SDS	Cytiva Life Sciences
Tris(hydroxymethyl)aminomethane/Tris	Sigma-Aldrich
Tween 20	AppliChem, Germany
Zinc sulfate heptahydrate	Sigma-Aldrich

Table 3. A list of all appliances and equipment used in this experiment, with relevant information.

Equipment	Model	Manufacturer
Analytical balance	Excellence E2000D	Sartorius AG, Croatia
Autoclave	AMB240N	Astell Scientific, UK
Centrifuge	SIGMA 3-18KH	SciQuip, UK
Color image scanner	Canon LiDE 120	Canon, Japan
Fluorimeter	FluorPen FP 100	Photon Systems Instruments, Czech Republic
Image scanner	C-DiGit Chemiluminescence Western Blot Scanner	LI-COR Biosciences – GmbH, USA
Laboratory shaker	Vibromix 204 EV	Tehtnica, Slovenia
Light meter	Quantitherm Chloroview 1 System	Hansatech Instruments, UK
Magnetic stirrer	Rotamix 550 MH	Tehtnica, Slovenia
Nitrocellulose membrane	Amersham™ Protran™ (0.45 µm)	Avantor, USA
pH meter	Basic Meter PB-11	Sartorius AG
Protein membrane transfer cell kit	Trans-Blot Cell	Bio-Rad, USA
Rotating shaker	Roto-Shake Genie	Scientific Industries, USA
Sealing tape	Parafilm	Bemis Company, USA
Small electrophoresis cell kit	Mini-Protean 3 Cell	Bio-Rad, USA
Spectrophotometer	SPECORD 40	Analytik Jena, Germany

3.2. Methods

3.2.1. Medium preparation, plant propagation and acclimation

In order to have enough plant material for the experiment I first prepared a modified Pirson-Seidel growth medium (Pirson and Seidel, 1950), the composition of which is described in Table 4, to propagate plants taken from the established axenic culture. I weighed the macroelements and the organic supplements (Table 4) on an analytical balance and added them one by one in the glass filled with Milli-Q® water on a magnetic stirrer. I previously prepared the microelement stock solutions A and B as two separate mixtures (100× more concentrated then in Table 4). After all the chemicals fully dissolved, I calibrated the pH meter and measured the pH of the medium. I adjusted the pH to 4.55 and filled it up fully with Milli-Q® water. I poured the medium into six large, clean, sterile glass Erlenmeyer flasks. I closed the flasks with a cotton plug and aluminium foil. I sterilized the flasks in the autoclave for 18 min at 121 °C and 1.5 bars. I left them to cool overnight.

Table 4. Chemicals and their final concentrations needed to prepare the modified version of the Pirson-Seidel growth medium (Pirson and Seidel, 1950) for *Lemna minor* propagation.

Macroelements	**Concentration / mmol dm^{-3}**
KNO_3	3.95
KH_2PO_4	1.47
$MgSO_4$ x 7 H_2O	1.21
$CaCl_2$ x 2 H_2O	5.46
Microelements (A)	
$MnCl_2$ x 4 H_2O	0.0015
H_3BO_3	0.0081
Microelements (B)	
Na_2-EDTA x H_2O	0.0049
Iron (II) citrate	0.02
Organic additives	
Saccharose	29.2
Asparagine	0.66

The following day I sterilized the laminar flow cabinet under UV-light for 30 min and wiped it down with 70% ethanol. I used autoclaved, sterile inoculating loops to transfer a few *L. minor* colonies from the axenic culture into each of the prepared propagation

flasks. I sterilized the loops in between each transfer via flambéing. I dipped them into 70% ethanol, lit them with the Bunsen burner and let them cool. I placed the filled propagation flasks in the growth chamber with temperature 28 ± 3 °C, 16:8 day:night cycle, light intensity 130.5 µmol m^{-2} s^{-1} for a week.

Since I used the Steinberg medium (ISO, 2005) for exposing *L. minor* to MPs, I first needed to acclimate the plants so I prepared the medium according to Table 5.

For the microelement stock solution A, I first prepared each of the ingredients as a separate solution, 100× more concentrated than in Table 5. I weighed each of ingredients on an analytical balance and added them to Milli-Q® water in a glass beaker on a magnetic stirrer. In the same way, I prepared the microelement stock solution B, 100× time more concentrated than in Table 5. I added the macroelements in the same manner as for the modified Pirson-Seidel growth medium (pg. 18) and then added the appropriate amount of stock solutions A and B to achieve desired concentrations (Table 5).

Table 5. Chemicals and their final concentrations needed to prepare the Steinberg growth medium (ISO, 2005) for experiment with *Lemna minor*.

Macroelements	**Concentration / mmol dm^{-3}**
KNO_3	3.46
$Ca(NO_3)_2$ x 4 H_2O	1.25
KH_2PO_4	0.66
K_2HPO_4	0.072
$MgSO_4$ x 7 H_2O	0.41
Microelements A	**Concentration / µmol dm^{-3}**
H_3BO_3	1.94
$ZnSO_4$ x 7 H_2O	0.63
Na_2MoO_4 x 2 H_2O	0.18
$MnCl_2$ x 4 H_2O	0.91
Microelements B	
$FeCl_3$ x 6 H_2O	2.81
Na_2-EDTA x H_2O	4.03

I calibrated the pH meter and adjusted the pH to 5.5. I poured the medium into six large, clean, sterile glass Erlenmeyer flasks. I sterilized the flasks and planted them in

the same manner as described for the modified the Pirson-Seidel growth medium (pg. 18), this time using my own propagated colonies. I placed the flasks in the growth chamber to acclimate plants to a new medium for a week in the same growth conditions (temperature 28 ± 3 °C, 16:8 day:night cycle, light intensity 130.5 µmol m^{-2} s^{-1}).

3.2.2. Plant treatment

After a week of acclimating, I prepared a fresh batch of Steinberg growth medium (Table 5). In a sterilized laminar flow cabinet, I prepared 45 small, plastic sterile Petri dishes (6 cm in diameter) for determination of growth rate and 42 small, glass, sterile Erlenmeyer flasks (100 mL) for determination of JIP, photosynthetic pigments content and RuBisCO expression.

I separated the Petri dishes into 6 groups of 6 containers each and 1 group of 9 containers for the control with no MPs, and the flasks into 7 groups with 6 flasks each. For the experiment, I used commercially available suspensions of MPs particles: 5% (w/v) PS (147 µm in diameter) and 5% (w/v) PMMA (105 µm in diameter) (MicroParticles GmbH, Germany). To achieve the desired concentrations of 0, 10, 50 and 100 mg L^{-1}, I filled each Petri dish or Erlenmeyer flask with Steinberg medium and added volumes of PS- or PMMA-MPs according to Table 6.

Table 6. Concentrations of polystyrene (PS) and poly(methyl methacrylate) (PMMA) microplastics (MPs) used for the treatment of *Lemna minor* plants. Three different concentrations were used for each MPs – 10, 50 and 100 mg L^{-1}. Control plants were grown on Steinberg medium without the addition of MPs. The volume of stock solution depended on whether MPs were prepared for the Erlenmeyer flask (50 mL) or Petri dish (15 mL).

Treatment	Concentration (mg L^{-1})	Flask (final volume 50 mL)	Petri dish (final volume 15 mL)
		Volume of MPs added to each replica (µL)	
Control	0	0	0
PS-MPs	10	10	3
	50	50	15
	100	100	30
PMMA-MPs	10	10	3
	50	50	15
	100	100	30

In a sterilized laminar flow cabinet, I transferred the *L. minor* colonies into the flasks and Petri dishes with sterile equipment, flambéed in between every transfer. I

transferred one colony into each Petri dish and several into every Erlenmeyer flask. I closed the flasks with cotton plugs and aluminum foil, and I sealed the Petri dishes with Parafilm. I placed the flasks and Petri dishes in the growth chamber with temperature 28 ± 3 °C, 16:8 day:night cycle, light intensity 130.5 µmol m^{-2} s^{-1} for a week.

3.2.3. Monitoring plant growth

For monitoring plant growth, I used the colonies planted into Petri dishes. Prior to sealing them, I photographed each Petri dish with my phone (Samsung Galaxy J7 Pro); 13 megapixel camera, f/1.9 aperture, 28mm (wide) focal length, 8.2 mm sensor size, 1.12 µm pixel size, 1x zoom type (Samsung Electronics Co., Ltd., Suwon, South Korea).

Every day I counted the number of visible fronds. I photographed them again on the last day of treatment. To calculate the relative frond number, I used the following formula:

$$\text{Relative frond number} = \frac{N_t - N_0}{N_0}$$

where N_t represents the number of fronds on day t (day 1 to day 7), and N_0 represents the number of fronds at the start of the experiment (day 0).

I calculated the average frond number and standard errors for every group based on at least 5 biological replicas, after removing outliers, and performed statistical analyzes as described in Chapter 3.2.7.

For frond area, I measured the total frond area in each dish using the Plant Screen Mobile, a mobile application available for free online (Müller-Linow et al., 2019). I took each photograph under the same conditions; in the growth chamber, on the same shelf and in the same corner with the Petri dish set on the shelf and the phone set on two stacked boxes above the dish, and with the same settings: 1280×720 camera resolution, Green Chromatic Coordinate set to 0.5. I used those same settings and conditions when calibrating the camera with a black and white printed checkerboard pattern with 5 mm squares (9 elements in X direction and 9 in Y direction). In the application, I did a batch analysis of all photos; two per sample, one taken on the day I planted the replicas and one taken on the last day of treatment. I selected the

following parameters: Method – Greenness with threshold: 0.50, min. hole size 1, min. object size 1, and select yes for “Calculate Metric Values”. To account for initial differences in colony sizes at the beginning of the experiment, I calculated the change in area by subtracting the Day 0 values from the Final day values. Finally, I calculated the average growth rate of frond area and standard errors for every group based on at least 5 biological replicas, after removing outliers, and performed statistical analyzes as described in Chapter 3.2.7.

3.2.4. JIP method

For this, and all subsequent methods, I used the plants from the Erlenmeyer flasks. I washed all plants with distilled water and transferred them into large, glass Petri dishes filled with distilled water, making sure to keep the various treatment groups and replicas separated. I covered a plastic tray with moistened filter paper. I prepared one tray for each of the seven groups. On each paper, I sketched six coin-sized circles. On each circle, I placed enough *L. minor* colonies to completely cover the filter paper within the circle; one circle per flask/replica within the group. Then I covered the trays with lids and took them to the dark room for analysis. I kept the plants in the dark at 24 °C for 30 min before the measurement, to ensure that all molecules in the reaction centers (RCs) of PSII are in the oxidized state and that fluorescence is minimal. Using the fluorimeter FluorPen FP100 (Photon Systems Instruments, Czechia), I illuminated the plants with a pulse of blue light ($\lambda = 455$ nm, PFD = 3000 µmol m^{-2} s^{-1}), which triggered photochemical processes in the RCs, including an increase in fluorescence. I used fluorimeter to measure the changes in chlorophyll *a* fluorescence intensity over 1 second, recording data on fluorescence intensity after 50 µs (F_0), 2 ms (F_J), 30 ms (F_I) and maximum fluorescence intensity (F_m). Based on these measured parameters, additional metrics (Table 1) were calculated using the software included with the fluorimeter, of which I used F_v/F_m and PI_{ABS}.

From the data obtained, I calculated the average F_v/F_m and PI_{ABS} and the standard errors for every group based on at least 5 biological replicas, after removing outliers, and performed statistical analyzes as described in Chapter 3.2.7.

I used the rest of plant material from flasks for further analyzes (pigment content and RuBisCO expression). First, I weighed 30 mg of the plant material for the pigment analysis and about 50 mg for the RuBisCO expression analysis from each replica/flask.

I wrapped the plant material separately in aluminum foil, perforated it and froze it in liquid nitrogen. I kept all samples at -80 °C until needed.

3.2.5. Determination of pigment content

I first prepared 80% acetone by mixing 80 mL of 100% acetone with 20 mL of distilled water. I placed the acetone in the fridge, at 4 °C, overnight. Then I put plant material from the foil that was kept at -80 °C into a cold mortar and I added small amount of calcium carbonate (cca 10 mg) and 500 µL of cold 80% acetone. I ground the sample with a cold pestle until I could not see any visible plant fragments. I transferred the extract into an amber microcentrifuge tube. I washed the mortar and pestle with an additional 500 µL of cold 80% acetone and added that to the tube. I repeated the extraction with all 42 samples. I vortexed the samples for 10 s and then put them in the centrifuge for 10 min at 5000 g and 4 °C. I saved the supernatant in a clean amber microcentrifuge tube. I added 500 µL (per tube) of cold 80% acetone to the sediment, vortexed the samples for 10 s and placed them in the centrifuge for 10 min at 5000 g and 4 °C to rinse the sediment of leftover pigments. I repeated the rinsing once more. I pooled together the first supernatant and the two rinses for each sample. Finally, I filled all samples with the cold acetone to a total volume of 1.5 mL. I kept the sample tubes on ice whenever not in use or in the centrifuge.

For the spectrophotometric analysis, I transferred the sample into a cuvette and measured the absorbances at 470, 645 and 663 nm. To calculate the concentration of each pigment (chlorophyll *a*, chlorophyll *b*, and total carotenoids) I used the formulae from Wellburn, 1994:

$$c_a = 12{,}21\ A_{663} - 2{,}81\ A_{645}$$

$$c_b = 20{,}13\ A_{645} - 5{,}03\ A_{663}$$

$$c_{cars} = \frac{(1000\ A_{470} - 3{,}27\ c_a - 104\ c_b)}{198}$$

where c_a, c_b and c_{cars} represent the concentration of chlorophyll *a*, chlorophyll *b*, and carotenoids, respectively. A_{470}, A_{645}, and A_{663} represent the absorbances measured at wavelengths of 470, 645, or 663 nm.

From the concentrations obtained, I calculated the content of the pigments, expressed as micrograms of pigment per gram of fresh tissue (μg mg^{-1} fresh weight), using the formula:

$$\text{Pigment content} = \frac{c_{pigment} \times V_{ex.}}{m}$$

where $c_{pigment}$ represents the previously calculated concentration of chlorophyll *a*, chlorophyll *b* or carotenoids (μg mL^{-1}), $V_{ex.}$ represents the volume of the extraction solvent (1.5 mL), and m is the mass of the fresh tissue (mg).

I calculated the average pigment content and the standard errors for every group based on at least 5 biological replicas, after removing outliers, and performed statistical analyzes as described in Chapter 3.2.7.

3.2.6. Western blot

For this method, I used plants from the Erlenmeyer flasks that were kept at -80 °C. The day prior to extraction of plant material, I prepared the buffers and the acrylamide/bis-acrylamide solution.

3.2.6.1. Buffer and gel preparation

To separate the proteins, I used discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE method), which consists of two gels – a stacking gel (4%) and a separating gel (12%), buffered at different pH values. I prepared the gels using a small gel electrophoresis system.

To prepare gels, I first had to prepare stock solutions. For the 1.5 M, pH = 8.8 Tris-HCl buffer I dissolved 18.2 g of Tris in 100 mL of HPLC water at room temperature and on a magnetic stirrer. When Tris completely dissolved, I adjusted the pH to 8.8 with 5.0 M HCl. For the 0.5 M, pH = 6.8 Tris-HCl buffer I dissolved 6 g of Tris in 100 mL of HPLC water at room temperature and on a magnetic stirrer. Then I adjusted the pH to 6.8 with 5.0 M HCl. I filtered both buffers and stored them in the fridge at 4 °C overnight. For the acrylamide/bis-acrylamide mixture, I dissolved 29.2 g of acrylamide and 0.8 g of bis-acrylamide in 100 mL of HPLC water. I added them one by one at room temperature and on a magnetic stirrer, waiting for the first to fully dissolve before adding the second. I filtered the mixture and stored it in the fridge at 4 °C overnight. I

also had to prepare 10% (w/v) SDS by mixing 10 g of SDS in 100 mL of HPLC water at room temperature. Then, I prepared the 10% (w/v) APS by dissolving 500 mg of APS in 5 mL of HPLC water at room temperature and on a magnetic stirrer. I separated a 500 µL aliquot into a microcentrifuge tube and stored it overnight in the fridge at 4 °C in a dark container. I froze the rest and stored it at -20 °C for long-term use.

For the polyacrylamide gels, I first assembled the gel casting kit and added a thin layer of Vaseline to both foam parts. Then I mixed the chemicals for the 12%-polyacrylamide separating gel as per Table 7, in a clean glass beaker and in the fume hood. I added TEMED last, stirred the mixture and filled the casting kit using an automatic micropipette. I covered the gel with distilled water and left it 45 min to polymerize. Once the first gel polymerized, I prepared the 4%-polyacrylamide stacking gel (Table 7), in the same manner as the previous layer.

Table 7. All chemicals and their final volumes needed to prepare the separating and stacking gel layers for SDS-PAGE electrophoresis.

Separating gel	
Chemical	**Volume**
HPLC water	3.35 mL
1.5 mL Tris. pH 8.8	2.50 mL
AA/Bis	4.00 mL
10% SDS	100 µL
10% APS	100 µL
TEMED	10 µL
Stacking gel	
Chemical	**Volume**
HPLC water	3.05 mL
1.5 mL Tris. pH 8.8	1.25 mL
AA/Bis	665 µL
10% SDS	50 µL
10% APS	50 µL
TEMED	10 µL

3.2.6.2. Protein extraction

To extract the total soluble proteins, I prepared the extraction buffer Tris-HCl, pH = 8.0 (Staples and Stahmann, 1964). The buffer contained 1.12 g of Tris, 17.1 g of saccharose, 100 mg of DTT, and 100 mg of cysteine-HCl. I added and dissolved the chemicals one by one on a magnetic stirrer. When chemicals dissolved completely, I

adjusted the pH to 8.0 with 5.0 M HCl and filled it up to 100 mL with distilled water. I choose one random sample per group, removed the foil packet from the liquid nitrogen and emptied the sample into a cold mortar. I added a bit of PVPP and 300 µL of Tris-HCl buffer. I ground the sample with a cold pestle and transferred the extract into a microcentrifuge tube. I washed the mortar and pestle with an additional 300 µL of Tris-HCl buffer. I repeated this step for all samples. I vortexed the extracts for 10 s and then put them in the centrifuge for 30 min at 20 000 g and 4 °C. I transferred the supernatant in a clean microcentrifuge tube. I kept the samples on ice whenever not in use.

3.2.6.3. Bradford assay

I used the Bradford method to determine the total protein concentration (Bradford, 1976). First, I prepared the Bradford stock solution by dissolving 350 mg of the dye CBB G-250 in 100 mL of 96% ethanol and then adding 200 mL of 88% phosphoric acid. Next, I prepared the working solution, which consisted of 425 mL of distilled water, 15 mL of 96% ethanol, 30 mL of 88% phosphoric acid, and 30 mL of the stock solution.

Then, I prepared a set of labeled microcentrifuge tubes, one for each sample. To them I added 60 µL of supernatant and 60 µL of Tris-HCl buffer. I vortexed them for 10 s. To measure the protein concentration, I prepared two new sets of labeled microcentrifuge tubes for each sample, which were representing technical replicas. To each replica, I added 50 µL of supernatant and 1000 µL of Bradford working solution. For the blank, I used 50 µL of Tris-HCl buffer. I vortexed the tubes for 10 s and then let them incubate at room temperature for 10 min. After 10 min, I transferred the samples one by one into a cuvette and measured the absorbance at 595 nm. Since I had two technical replicas for each sample, I calculated the average absorbance for each sample.

To calculate the total protein concentration in my samples, I created a standard concentration curve using BSA solutions. Using the 1 mg mL^{-1} stock solution, I prepared a range of 0.1 to 0.8 mg mL^{-1} diluted solutions and measured their absorbance at 595 nm. Using the concentrations of the diluted solutions and the measured values of their absorbance, I calculated the standard curve and derived the formula that I used to calculate the protein concentration of every sample.

3.2.6.4. Denaturation of proteins

Once I had determined the protein concentration in my samples, I had to denature the proteins. To do this, I first had to determine the sample with the lowest concentration. Then I diluted all the samples to this lowest concentration using the Tris-HCl buffer. To denature the proteins, I prepared the LBS buffer (Table 8). Before mixing all the necessary chemicals, I first prepared the 0.25% bromophenol blue by dissolving 25 mg of bromophenol blue in 10 mL of distilled water. I added the chemicals one by one in the fume hood and vortexed the buffer for 10 s. I then transferred 25 µL of the diluted protein samples to a new set of labeled microcentrifuge tubes, added 25 µL of the LSB buffer, vortexed the mixture for 10 s, and did a spin-down. Next, I incubated the samples at 95 °C for 5 min and kept them on ice until needed.

Table 8. All chemicals and their volumes needed to prepare the Laemmli Sample Buffer (LBS) for protein extraction from *Lemna minor* plant tissue.

Chemical	Volume
0.5 M Tris-HCl, pH 6.8	175 µL
10% (w/v) SDS	200 µL
Glycerol	450 µL
2-mercapthoethanol	125 µL
0.25% (w/v) Bromophenol blue	50 µL

3.2.6.5. Electrophoresis

Before starting the electrophoresis, I prepared the electrode buffer (Table 9). I weighed the chemicals on an analytic balance and added them one by one to a clean glass beaker filled partially with Milli-Q® water, which was placed on a magnetic stirrer. Then I measured the pH of the buffer, adjusted the pH to 8.3 with 5.0 M HCl and added the rest of the Milli-Q® water.

Table 9. All chemicals and their volumes needed to prepare the electrode buffer for SDS-PAGE electrophoresis.

Chemical	Volume
Tris	3 g
Glycine	14.4 g
SDS	1 g
Milli-Q® water	Up to 1 L

Next, I removed the plastic combs from the previously prepared gels and washed the wells first with HPLC water and then with the electrode buffer. Then I prepared the electrophoresis kit and added the electrode buffer.
In the first well to the left, I added 5 µL of PageRuler™ Plus Prestained Protein Ladder (10 – 250 kDa), which I first vortexed for 10 s. Moving left to right I added 7.91 µL of sample per well, which makes for 2.5 µg of total proteins per well. As soon as I had finished loading the samples, I started the electrophoresis and ran it at 80 V for 27 min and then at 200 V for 28 min.

3.2.6.6. Transfer

During electrophoresis, I prepared the transfer buffer (Table 10) and placed it in the fridge at 4 °C until use. After completing the electrophoresis, I removed the gels from the electrophoresis kit and assembled the transfer kit. To do this, I filled a large Petri dish with transfer buffer. In this buffer-filled dish, I assembled a multi-layer 'sandwich' in the transfer cassette, consisting in sequence of the cassette base, a sponge, filter paper, the gel, nitrocellulose membrane (0.45 µm, Amersham™ Protran™), filter paper, sponge, and the cassette top. Before closing the cassette, I rolled a glass tube over the top sponge to expel any trapped air bubbles and enhance the contact between the membrane and the gel, thus facilitating protein transfer. I then transferred the cassette to the transfer electrode assembly and placed it in a tank with an ice container for additional cooling and filled with cold transfer buffer. The transfer was conducted for 60 minutes at 60 V.

Table 10. All chemicals and their volumes needed to prepare the transfer buffer for Western blot.

Chemical	Volume
Tris	3.35 g
Glycine	14.4 g
Methanol	100 mL
Milli-Q® water	Up to 1 L

To verify that transfer was successful, I stained the membrane with a dye Ponceau S. I prepared the dye by dissolving 25 mg of Ponceau S dye in 50 mL of acetic acid. After seeing the proteins, I marked the molecular weight marker at the expected position of

the RuBisCO protein (~56 kDa) on the membrane with a graphite pencil. I then scanned the stained membrane and removed the excess dye by washing it several times in distilled water and finally washing it in 1× TBS-T. To prepare the TBS-T buffer, I first dissolved 1.2 g Tris and 4.4 g NaCl, one at the time, in distilled water. I then measured the pH of the buffer and adjusted it to a value of 7.5 and made up the volume of the mixture to 500 mL. Then I added 5 mL of Tween 20 and mixed the buffer on a magnetic stirrer.

3.2.6.7. Blocking and incubation with primary and secondary antibodies

After removing all the dye, I incubated the membrane in 10 mL of blocking solution prepared by dissolving 0.5 g of non-fat milk powder in 10 mL of 1× TBS-T buffer. The incubation lasted 1 h at room temperature with constant mixing. After 1 h, I washed the membrane twice for 5 min each time in 1× TBS-T buffer and then incubated it overnight at 4 °C with the primary antibody. I prepared the primary antibody by adding 1 µL of the anti-rbcL antibody to 4 mL of freshly prepared blocking solution. The next day, I washed the membrane again twice for 5 min each time in 1× TBS-T buffer. Then I incubated the membrane in 10 mL of blocking solution to which I added 1 µL of the secondary antibody conjugated with horseradish peroxidase (HRP). The incubation lasted 1 h at room temperature with constant mixing.

3.2.6.8. Detection and analysis of RuBisCO protein

After incubation with the secondary antibody, I washed the membrane twice for 5 min each time in 1× TBS-T buffer. Then I discarded the TBS-T buffer completely and added 1 mL of chemiluminescent substrate to the membrane and incubated it for 5 min.

To detect the chemiluminescent signal, I placed the membrane in a detection device C-DiGit Blot Scanner. In the next step, I quantified the intensity of the detected protein bands using image analysis software (Image Studio™ Lite 5.2, LI-COR Biosciences – GmbH, USA).

3.2.7. Statistical analysis

I preformed all calculations in Excel 2016 (Microsoft Office), using either the base program or with the freely available Real Statistics Resource Pack add on (Zaiontz, 2023). Using the Real Statistics Resource Pack add on, I tested the relative frond number, growth rate of frond area, photosynthetic performance parameters and pigment content for outliers using nonparametric test Tukey Fences with outlier multiplier set as 1.5. After detection of outliers, I tested normal distribution of samples using Shapiro-Wilk's test and variance using Levine's test. At $p > 0.05$, I considered the data to be normally distributed and the variances to be equal. For outlier and distribution testing, I tested each group separately. For variance testing, I compared the groups as I would for the statistical analysis; all 3 concentrations of a single plastic compared to the control and each concentration of one plastic compared to that same concentration of the other plastic; e.g. 10 mg L^{-1} PS-MPs treatment compared to 10 mg L^{-1} PMMA-MPs. To compare the single plastic (all concentrations) to the control I used one-way ANOVA followed by post hoc test Tukey HSD, and Student's t-test to compare the same concentrations of different plastics. All differences were considered significant at $p \leq 0.05$. For the analysis of RuBisCO expression, I normalized the detected bands in the treated samples to the control sample, which I set to 1. I considered values that were 1.5 times larger or smaller than the corresponding control value as a significant difference.

2 Results

1. Effect of microplastics on *Lemna minor* growth

2.1.1Frond number

The treatment of *Lemna minor* plants with polystyrene (PS) MPs for seven days showed no significant effect on the number of fronds between the control group and various PS-MP concentrations (Figure 3). The lowest concentration resulted in a decrease in the number of fronds, which decreased by 27%, 25% and 21% on days 1, 2 and 3, respectively, compared to the control. At higher concentrations, however, there was a positive trend, with the average frond number increasing in correlation with the duration of treatment. The PS-MP concentration of 50 mg L^{-1} showed an increase of 19%, 22%, 35%, 49%, 72% and 96% on days 1, 2, 4, 5, 6 and 7, respectively, compared to the control. The PS-MP concentration of 100 mg L^{-1} showed an increase of 19%, 52%, 80% and 88% on days 4, 5, 6, and 7, respectively, and a decrease of 28% on day 1 compared to the control.

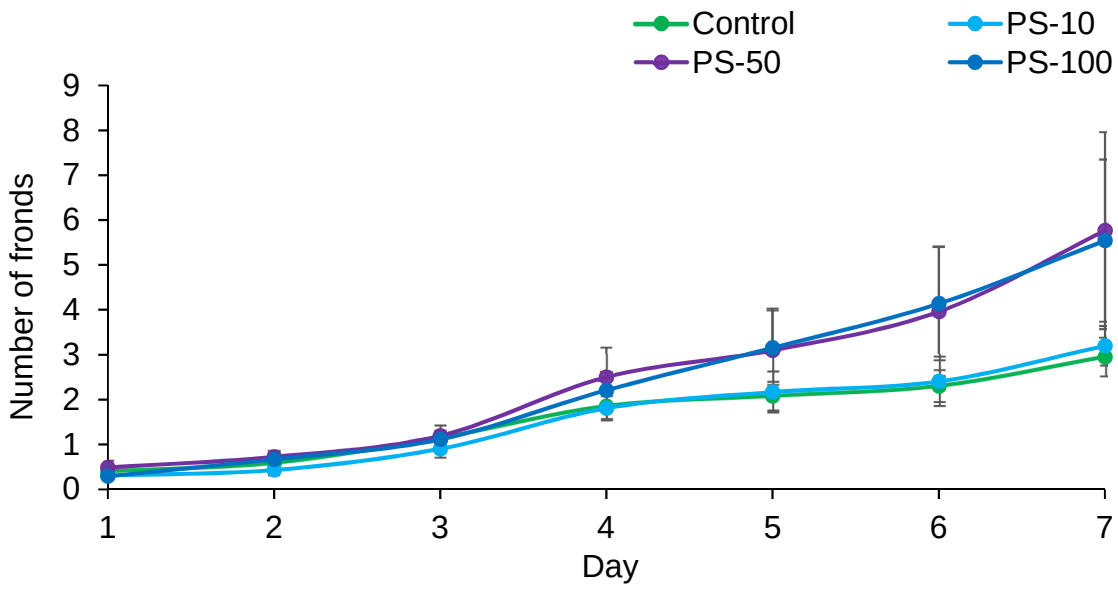

Figure 3. Relative frond number of *Lemna minor* plant colonies exposed to polystyrene (PS) microplastic (MP). Plants were grown for seven days in Steinberg medium supplemented with 10, 50, or 100 mg L^{-1} PS-MPs. Control plants were grown in a medium without MPs. Results are shown as the average value of at least 5 replicates ± standard error. There was no significant difference between control and treated plants on any of the days (one-way ANOVA, followed by post hoc Tukey HSD test, $p \leq 0.05$).

The 7-day treatment with poly(methyl methacrylate) (PMMA) MPs also had no significant effect on the relative frond number of *L. minor* colonies (Figure 4). Statistical analysis showed no significant difference between the control group and various PMMA-MP concentrations. Although there appeared to be some negative effects, these were somewhat inconsistent. The PMMA-MP concentration of 10 mg L^{-1} resulted in a decrease of 49%, 29% and 24% on days 1, 3 and 4, respectively, compared to the control. In contrast, the PMMA-MP concentration of 50 mg L^{-1} showed positive effects with an increase of 17%, 26% and 17% on days 5, 6 and 7, respectively, compared to the control. The PMMA-MP concentration of 100 mg L^{-1}, on the other hand, again only showed decreases, with a decrease of 23%, 32%, 20%, 20% and 25% on days 3, 4, 5, 6 and 7, respectively, compared to the control.

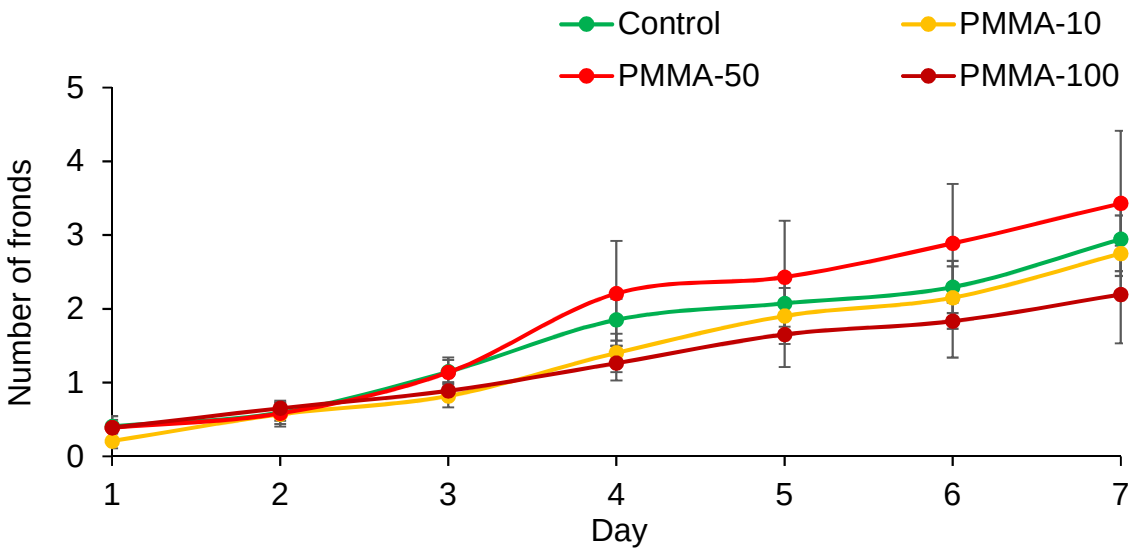

Figure 4. Relative frond number of *Lemna minor* plant colonies exposed to poly(methyl methacrylate) (PMMA) microplastic (MP). Plants were grown for seven days in Steinberg medium supplemented with 10, 50, or 100 mg L^{-1} PMMA-MPs. Control plants were grown in a medium without PMMA-MPs. Results are shown as the average value of at least 5 ± standard error. There was no significant difference between control and treated plants on any of the days (one-way ANOVA, followed by post hoc Tukey HSD test, $p \leq 0.05$).

When comparing the same concentrations of the two types of MPs, the statistical analysis (Student's t-test, $p \leq 0.05$) showed no significant difference. However, PS-MP-treated plants tended to have a greater number of fronds than PMMA-MP-treated ones. Treatment with 10 mg L^{-1} PMMA-MPs resulted in a 32% and 22% lower frond number on days 1 and 4, respectively, and a 32% higher number on day 2 compared to the same concentration of PS-MPs. Treatment with 50 mg L^{-1} PMMA-MPs resulted

in 20%, 19%, 22%, 27% and 40% fewer fronds on days 1, 2, 5, 6 and 7, respectively, compared to the same concentration of PS-MPs. Finally, treatment with 100 mg L^{-1} PMMA-MPs resulted in 20%, 43%, 48%, 56% and 60% fewer fronds on days 3, 4, 5, 6 and 7, respectively, and 33% higher number on day 1, compared to the same concentration of PS-MPs.

2.1.2 Growth rate of frond area

The addition of PS- and PMMA-MPs to the Steinberg nutrient medium did not affect the size of *Lemna minor* colonies, as measured through the growth rate of frond area, after 7 days of treatment (Figure 5).

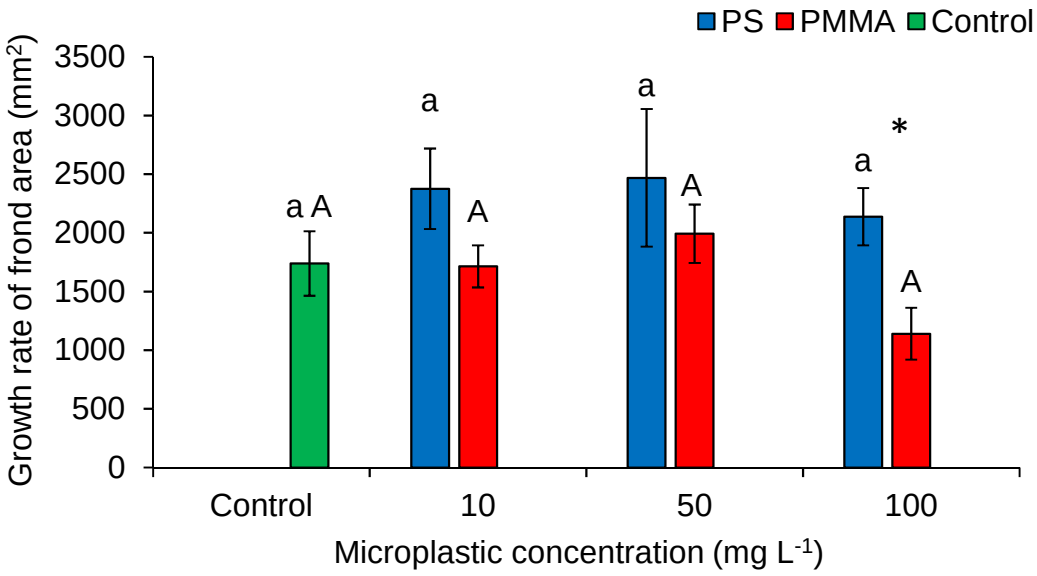

Figure 5. Growth rate of frond area of *Lemna minor* plants exposed to polystyrene (PS) or poly(methyl methacrylate) (PMMA) microplastics. Plants were grown for seven days in Steinberg medium supplemented with 10, 50, or 100 mg L^{-1} PS- or PMMA-MPs. Control plants were grown in a medium without MPs. Results are shown as the average value of at least 5 replicates ± standard error. Different lowercase letters indicate a significant difference between control and PS-MP-treated plants, while different uppercase letters indicate a significant difference between control and PMMA-MP-treated plants (one-way ANOVA, followed by post hoc Tukey HSD test, $p \leq 0.05$). Asterisks indicate a significant difference between two different MPs at the same concentration (Student's t-test, $p \leq 0.05$).

Statistical analysis showed no significant difference between the control group and various concentrations of a single microplastic, PMMA or PS. However, PS-MPs did seem to have some positive effect, showing an increase in frond area growth rate of

37%, 42% and 23% for concentrations of 10, 50, and 100 mg L^{-1}, respectively, compared to control plants. On the contrary, a PMMA-MP concentration of 100 mg L^{-1} showed a decrease of 34% compared to the control. There was no significant difference when comparing the same concentration of two MPs to each other, except for the concentration of 100 mg L^{-1}. Even though, treatments with 10 and 50 mg L^{-1} PS-MPs showed a 39% and 24% higher frond area growth rate than treatments with 10 and 50 mg L^{-1} PMMA-MPs, respectively.

2. Effect of microplastics on photosynthesis

2.2.1 Maximum quantum yield of PSII (F_v/F_m)

The addition of PS- or PMMA-MPs had no significant effect on photosynthesis of *L. minor* as measured by F_v/F_m when comparing the treatments to the control (Figure 4). However, the treatment with PS-MP at 10 mg L^{-1} showed a significantly higher F_v/F_m value than PMMA-MP at 10 mg L^{-1} (Figure 6).

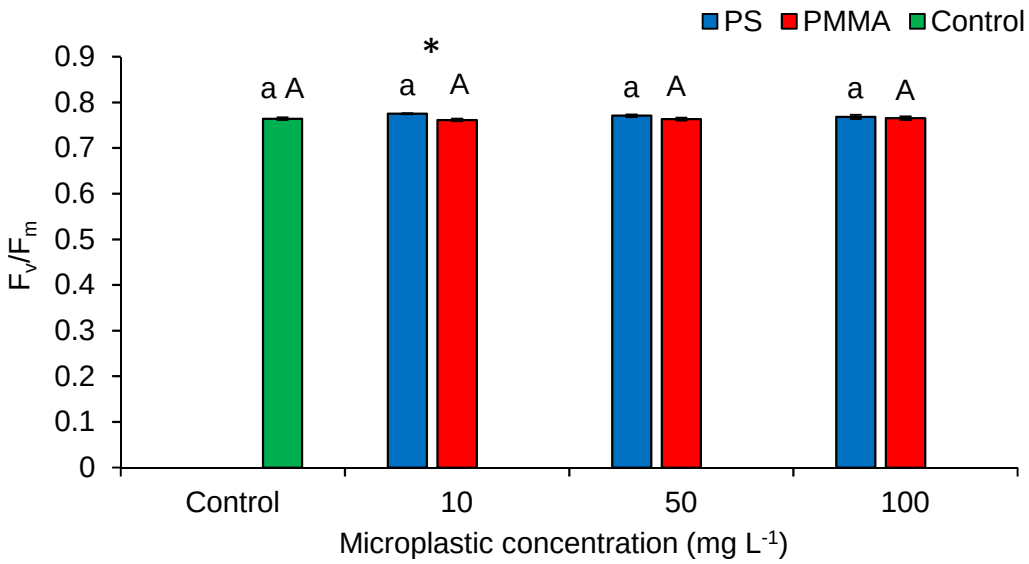

Figure 6. Maximum quantum yield of PSII (F_v/F_m) of *Lemna minor* plants exposed to polystyrene (PS) or poly(methyl methacrylate) (PMMA) microplastics (MP). Plants were grown for seven days in Steinberg medium supplemented with 10, 50, or 100 mg L^{-1} PS- or PMMA-MPs. Control plants were grown in a medium without MPs. Results are shown as the average value of at least 5 ± standard error. Different lowercase letters indicate a significant difference between control and PS-MP-treated plants, while different uppercase letters indicate a significant difference between control and PMMA-MP-treated plants (one-way ANOVA,

followed by post hoc Tukey HSD test, $p \leq 0.05$). Asterisks indicate a significant difference between two different MPs at the same concentration (Student's t-test, $p \leq 0.05$).

2.2.2 Photosynthetic performance index (PI_{ABS})

The addition of PS- and PMMA-MPs had no significant effect on PI_{ABS} of *L. minor* plants when comparing the treatments to the control (Figure 7). Still, treatment with PS-MPs appeared to have some positive effects, showing increases of 28%, 30%, and 20% for the 10, 50, and 100 mg L^{-1} concentrations, respectively, compared to the control plants. In contrast, treatment with PMMA-MPs only showed an increase of 21% for the 50 mg L^{-1} concentration, compared to the control. In addition, the treatments with 10 and 50 mg L^{-1} of PS-MPs showed significantly higher PI_{ABS} values than the treatments with 10 and 50 mg L^{-1} of PMMA-MPs, respectively (Figure 7).

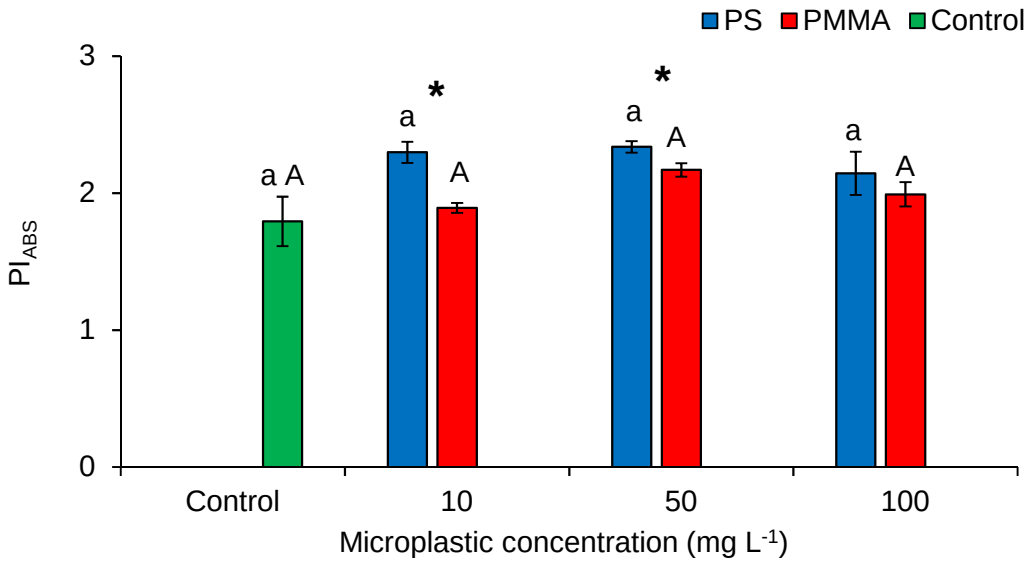

Figure 7. Photosynthetic performance index (PI_{ABS}) of *Lemna minor* plants exposed to polystyrene (PS) or poly(methyl methacrylate) (PMMA) microplastics (MP). Plants were grown for seven days in Steinberg medium supplemented with 10, 50, or 100 mg L^{-1} PS- or PMMA-MPs. Control plants were grown in a medium without MPs. Results are shown as the average value of at least 5 biological replicates ± standard error. Different lowercase letters indicate a significant difference between control and PS-MP-treated plants, while different uppercase letters indicate a significant difference between control and PMMA-MP-treated plants (one-way ANOVA, followed by post hoc Tukey HSD test, $p \leq 0.05$). Asterisks indicate a significant difference between two different MPs at the same concentration (Student's t-test, $p \leq 0.05$).

3. Effect of microplastics on pigment content

2.3.1 Chlorophyll *a*

L. minor plants grown at a PS-MP concentration of 100 mg L^{-1} showed a significantly lower chrolophyll *a* content than those exposed to the other two PS-MP concentrations, but values did not differ significantly from the control plants (Figure 8). Lower PS-MP concentrations appeared to be beneficial, as the plants treated with 10 and 50 mg L^{-1} PS-MPs had 21% and 29% higher chlorophyll *a* content than the control. PMMA-MP-treated plants did not show significant decrease in chlorophyll *a* content, but a negative trend was observed, i.e. 17% and 18% decrease compared to the control, after treatment with 10 and 50 mg L^{-1} PMMA-MPs, respectively. When comparing the two types of MPs of the same concentration, the plants treated with PMMA-MPs at 10 and 50 mg L^{-1} had a significantly lower chrolophyll *a* content than the corresponding PS-MP-treated plants (Figure 8). The plants treated with PMMA-MP at 100 mg L^{-1} had a 17% higher chlorophyll *a* content than those treated with 100 mg L^{-1} PS-MP.

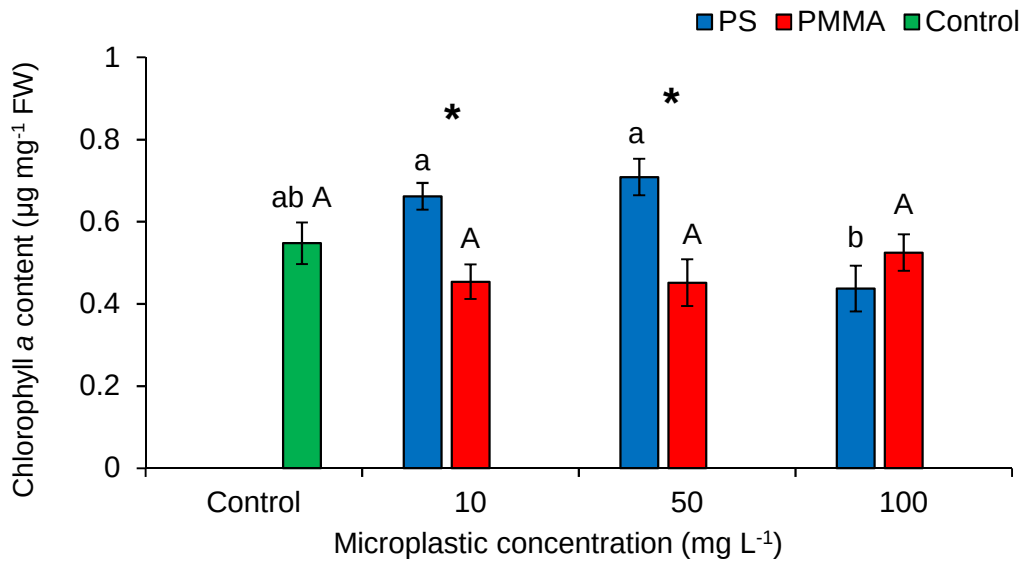

Figure 8. Chrolophyll *a* content of *Lemna minor* plants exposed to polystyrene (PS) or poly(methyl methacrylate) (PMMA) microplastics (MP). Plants were grown for seven days in Steinberg medium supplemented with 10, 50, or 100 mg L^{-1} PS- or PMMA-MPs. Control plants were grown in a medium without MPs. Results are shown as the average value of at least 5 biological replicates ± standard error. Different lowercase letters indicate a significant difference between control and PS-MP-treated plants, while different uppercase letters indicate a significant difference between control and PMMA-MP-treated plants (one-way ANOVA, followed by post hoc Tukey HSD test, $p \leq 0.05$). Asterisks indicate a significant difference between two different MPs at the same concentration (Student's t-test, $p \leq 0.05$).

2.3.2 Chlorophyll *b*

L. minor plants grown at a PS-MP concentration of 100 mg L^{-1} had a significantly lower chrolophyll *b* content than those grown at 50 mg L^{-1} PS-MP (Figure 9). Both concentrations showed no significant differences compared to the control and to 10 mg L^{-1} PS-MP. However, a lower PS -MP concentration had some benefit, as treatment of the plants with a concentration of 50 mg L^{-1} resulted in a 30% increase in chrolophyll *b* content, while the 100 mg L^{-1} concentration resulted in a 15% decrease compared to the control. Again, the plants treated with PMMA-MPs showed only adverse effects, with a decrease in chlorophyll *b* content: 22%, 19% and 16% for 10, 50 and 100 mg L^{-1}, respectively, compared to the control. Also, the PMMA-MP at 10 and 50 mg L^{-1} had significantly lower chrolophyll *b* content than the PS-MP at 10 and 50 mg L^{-1}, respectively (Figure 9).

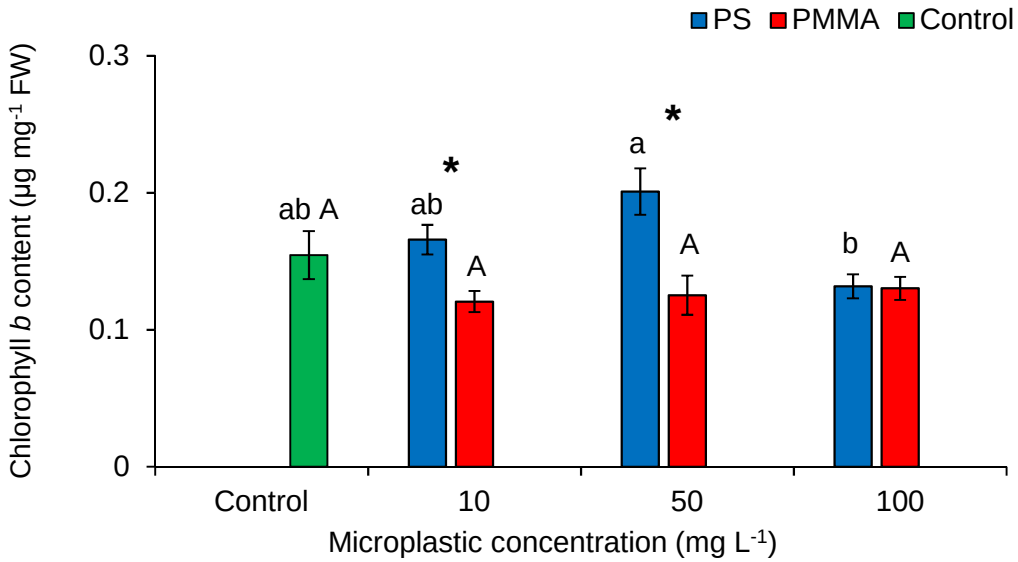

Figure 9. Chrolophyll *b* content of *Lemna minor* plants exposed to polystyrene (PS) or poly(methyl methacrylate) (PMMA) microplastics (MPs). Plants were grown for seven days in Steinberg medium supplemented with 10, 50, or 100 mg L^{-1} PS- or PMMA-MPs. Control plants were grown in a medium without MPs. Results are shown as the average value of at least 5 biological replicates ± standard error. Different lowercase letters indicate a significant difference between control and PS-MP-treated plants, while different uppercase letters indicate a significant difference between control and PMMA-MP-treated plants (one-way ANOVA, followed by post hoc Tukey HSD test, $p \leq 0.05$). Asterisks indicate a significant difference between two different MPs at the same concentration (Student's t-test, $p \leq 0.05$).

2.3.3 Total carotenoids

L. minor plants grown at a PS-MP concentration of 50 mg L^{-1} showed a significantly higher total carotenoid content than the control plants and the plants grown at 100 mg L^{-1} PS-MP (Figure 10). Lower PS-MP concentrations also appeared to be beneficial, as the 10 mg L^{-1} treatment had a 24% higher total carotenoid content than the control, while the 100 mg L^{-1} again showed a decrease in pigment content, with the total carotenoid content decreasing by 20%. In contrast, the PMMA-MP concentrations tested had no effect on the carotenoid content of the *L. minor* plants. The comparison of MPs of the same concentration showed that the treatments with PMMA-MP at 50 and 100 mg L^{-1} led to a significantly lower total carotenoid content of the plants than the treatments with PS-MP at 50 and 100 mg L^{-1} (Figure 10).

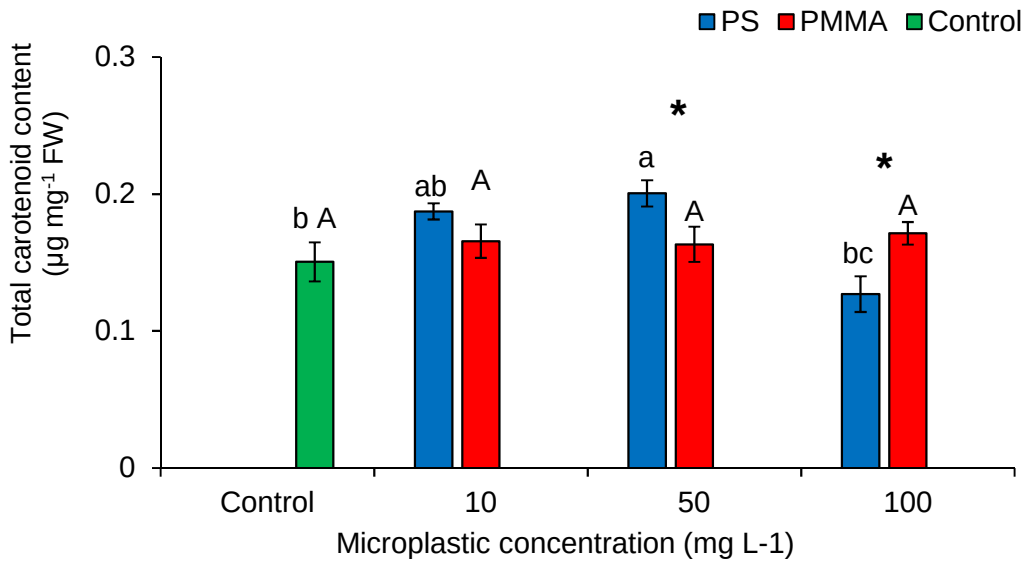

Figure 10. Total carotenoid content of *Lemna minor* plants exposed to polystyrene (PS) or poly(methyl methacrylate) (PMMA) microplastics (MPs). Plants were grown for seven days in Steinberg medium supplemented with 10, 50, or 100 mg L^{-1} PS- or PMMA-MPs. Control plants were grown in a medium without MPs. Results are shown as the average value of at least 5 biological replicates ± standard error. Different lowercase letters indicate a significant difference between control and PS-MP--treated plants, while different uppercase letters indicate a significant difference between control and PMMA-MP-treated plants (one-way ANOVA, followed by post hoc Tukey HSD test, $p \leq 0.05$). Asterisks indicate a significant difference between two different MPs at the same concentration (Student's t-test, $p \leq 0.05$).

4. Effect of microplastics on RuBisCO expression

After the 7-day MP treatment, all treated plants showed at least some decrease in RuBisCO expression (Figure 11). Since I only had one replica per group, I could not perform a statistical analysis. Therefore, I considered a change in expression to be significant if it changed by 1.5-fold or more compared to the control. This corresponds to values of 0.67 or less for a decrease and 1.5 or more for an increase, compared to the control value of 1. Under these conditions, the PS-MP concentration of 100 mg L^{-1} and all PMMA-MP concentrations caused a significant decrease in protein expression, with changes of 0.66 for PS-MPs at 100 mg L^{-1} and 0.28, 0.44 and 0.56 for PMMA-MPs at 10, 50 and 100 mg L^{-1}, respectively (Figure 11). Interestingly, although PS-MP-treated plants showed an overall lower decrease in RuBisCO expression, higher PS-MP concentrations resulted in a greater decrease. In contrast, PMMA-MP-treated plants showed the opposite trend, with the highest concentration causing the smallest decrease and the lowest concentration causing the largest decrease (Figure 11).

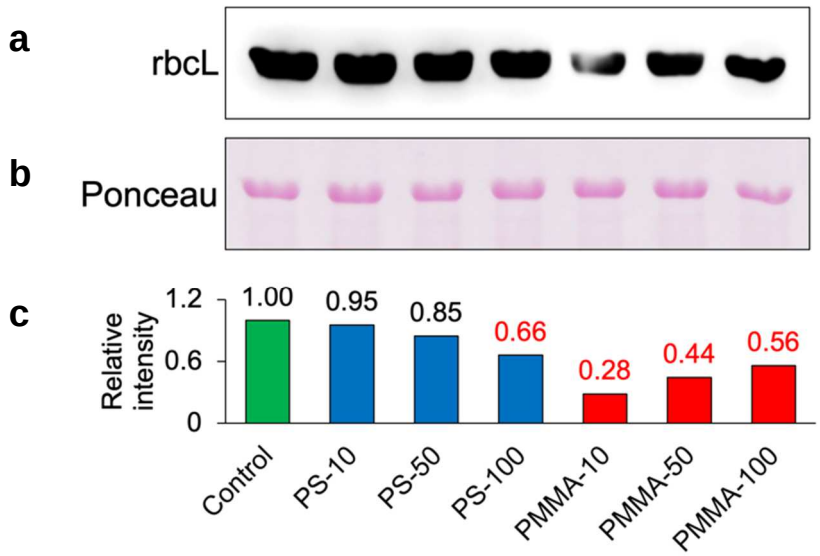

Figure 11. (**a**) Western blot analysis of the abundance of the RuBisCO large subunit in *Lemna minor* plants exposed to polystyrene (PS) or poly(methyl methacrylate) (PMMA) microplastics (MPs). Plants were grown for seven days in Steinberg medium supplemented with 10, 50, or 100 mg L^{-1} PS- or PMMA-MPs. Control plants were grown in a medium without MPs. Results show one randomly chosen replica per group. (**b**) Ponceau staining was used to check the correct loading of the samples on the membrane. (**c**) The membrane was analysed using ImageJ program. Results were normalised to the control, which was set as 1. Red numbers indicate a significant 1.5-fold change compared to the control (0.67 or less).

5. Discussion

Microplastics are an increasingly large and present environmental pollutant. Although the phytotoxicity of MPs is lower than that of some other nanoparticles such as metal nanoparticles, their extremely slow degradation contributes to an increase in their concentration in the environment, and there is a growing concern regarding the possible long-term consequences of chronic exposure (Komazec, 2024). Previous works show that MPs can have negative effects on plants and algae (Dong et al., 2022; Huang et al., 2021; Rozman and Kalčíková, 2022) but there is a lack of data on the effect of MPs on photosynthesis, a process necessary for life on Earth. In this thesis I investigated the influence of two types of microplastics – PS (105 nm in size) and PMMA (147 nm in size), in different concentrations (10, 50 and 100 mg L^{-1}) on growth and photosynthesis *of L. minor*.

Growth analysis of *Lemna* plants, in terms of frond number, showed no significant difference between different treatment types and concentrations or between treated and untreated plants (Figure 3, 4), while the growth rate of frond area was significantly higher for plants treated with 100 mg L^{-1} PS-MPs, when compared to the PMMA-MP equivalent (Figure 5). However, direct comparison of results does show some difference. For example, PS-MP-treated plants, showed some increase in frond number when compared to control and equivalent PMMA-MPs concentrations, on specific treatment days, (pg. 31, 32). It is curious why these seemingly large differences were not deemed statistically significant by the test used. The reason for that could be due to the rather large standard error values, as seen in Figures 3, 4 and 5, and the exclusion of outliers from these test groups. Since the plants used to monitor MP-induced effects on growth were grown in Petri dishes rather than flasks it is possible that small volume of medium influenced higher variability of results. Also, the initial number of plants planted on the medium was not identical, but varied from 2 to 4 fronds, which could affect growth and response of *L. minor* to MPs.

MPs not affecting *L. minor* growth, agrees with several previous studies investigating effects of various MPs with similar concentrations. Mateos-Cárdenas et al. (2019) found no effects of 10-45 μm polyethylene microspheres. Kalčíková et al. (2017) observed minor effects of 71 μm polyethylene fragments. Effects of 149 μm polyethylene fragments and 47 μm tyre wear particles on root length reported by

Rozman et al. (2021) and Rozman et al. (2022), respectively, were mainly related to mechanical abrasion by MPs with sharp edges. Contrary, Xiao et al. (2022) showed that PS particles at similar concentrations caused growth inhibition of *L. minor*. Also, in another aquatic plant *Utricularia vulgaris* L. fluorescent PS-MPs negatively impacted the growth at the highest concentration of 140 mg L^{-1} (Yu et al., 2020). These discrepancies in results could be due to the specific types of MPs used because different MPs have various physico-chemical properties that affect their ecotoxicity (Rozman et al., 2021). Xiao et al. (2022) used positively (PS-NH2) and negatively (PS-SO_3H) charged PS particles, while those used in this work were not so significantly charged. Furthermore, they used 50 nm PS particles, while mine were bigger i.e. 147 nm (0.147 µm) so it is possible that they were too big to enter inside the plants. Bosker et al. (2019) investigated effects of different sizes of MP particles on germination rate of watercress seeds and found that MPs with the largest diameter had the smallest effect. Rozman and Kalčíková (2022) who also did not find negative effects on *L. minor* growth suggested that MPS they used i.e. polyethylene microbeads, tire wear particles, and polyethylene terephthalate fibers, are too large to pass through the cell wall. This is also in agreement with the findings in *Spirodela polyrhiz*a Schleiden where MPs adhered only externally and were not found in the roots (Dovidat et al., 2020).

The difference in results implies that MPs charge plays a significant role in the effect MPs have on plants living in polluted areas or that smaller particles, charged or not, may have a greater effect on the plant. It has already been established that when PS nanoparticles are exposed to different environmental conditions such as UV and high temperatures, various functional groups can be attached to their surface changing their properties and stability, resulting in altered phytotoxicity (Yu et al., 2019). Stability analysis of MPs that I used showed that PS-MPs were relatively stable in Steinberg medium, while PMMA-MPs showed increase in hydrodynamic diameter implying agglomeration of particles which is probably due to the interaction of MPs with the components of medium (Kobelščak, 2024). Therefore, experiments done under laboratory conditions are not sufficient to predict the true danger of MPs pollution. In laboratory conditions, MPs can be kept electrically inert using pH neutral growth mediums and preventing exposure to UV radiation, chemical pollutants and plastic-degrading bacteria. However, in natural and industrial settings, such conditions are unlikely. Another variable is time. It is possible that even inert MPs could have

detrimental effects on plant growth given longer exposure time or in combination with other pollutants and/or environmental stressors. In the related species, *Lemna minuta* Kunth, exposure to 50 and 100 mg L^{-1} of poly(styrene-co-methyl methacrylate) MPs induced a negative effect on plant growth (fresh and dry mass and relative growth rate), but after a prolonged period of exposure. Since SEM showed microspheres adsorbed on roots and frond surfaces authors suggested that this adsorption prevented the passage of light and oxygen and the uptake of nutrients, thus limiting the growth.

Regarding photosynthesis, the results corroborate growth results as neither type of MPs negatively affected photosynthetic performance nor photosynthetic pigment content. It is in agreement with results on chlorophyll content in *L. minor* (Rozman and Kalčíková, 2022; Rozman et al., 2021). Contrary, Yu et al. (2020) found reduced chlorophyll *a* content in *U. vulgaris* treated with PS-MPs even at the lowest concentration (15 mg L^{-1}), probably due to the adsorption of MPs on the leaves, which prevented a normal composition of pigments. Also, in most terrestrial plants studied so far (lettuce – *Lactuca sativa* L., cucumber – *Cucumis sativus* L., tomato – *Solanum lycopersicum* L., cabbage – *Brassica oleracea* L., etc.), treatment with various MPs including PS decreased carotenoids and chlorophylls resulting in reduced photosynthesis and consequently reducing their growth (Jia et al., 2023). Investigation of OJIP transient in those MP-treated plants mostly showed that the structure and function of the PSII were unaltered (Xu et al., 2022; Zha et al., 2021). MP-induced reduction in chlorophyll content was explained by converting the chlorophyll into phytol (Xu et al., 2022). Thus, the mechanism how MPs induce alterations in photosynthesis remains elusive.

Nevertheless, in my work some differences between PS- and PMMA-MPs effects on photosynthesis were observed. Both maximum quantum yield of PSII, F_v/F_m (Figure 6) and photosynthetic performance index, PI_{ABS} (Figure 7) showed significantly higher values in plants treated with 10 mg L^{-1} PS-MPs and 50 mg L^{-1} PS-MPs, than the equivalent PMMA-MPs concentrations. Moreover, these plants also had a higher chlorophyll *a* and *b* content than those treated with PMMA equivalents (Figure 8, 9). However, 100 mg L^{-1} PS-MPs group had a significantly lower chlorophyll *a* content than 10 mg L^{-1} PS-MPs and 50 mg L^{-1} PS-MPs (Figure 8), and lower chlorophyll *b* content than 50 mg L^{-1} PS-MPs (Figure 9). This could indicate that PMMA-MPs has an overall more significant negative effect on photosynthesis than PS-MPs, which is noticeable

even at lower concentrations, while the detrimental effect of PS-MPs only shows at higher doses. It would be expected that reduced chlorophyll content is accompanied with a reduction in photosynthetic efficiency. For example, metallic nanoparticles decrease the level of photosynthetic pigments and hamper photosynthetic efficiency (Tighe-Neira et al., 2018). In this experiment lower chlorophyll amounts did not always corroborate with lower maximum quantum yield and photosynthetic performance, such as when comparing 100 mg L^{-1} PS-MPs to 50 mg L^{-1} PS-MPs and 10 mg L^{-1} PS-MPs. Perhaps the negative effects of 100 mg L^{-1} PS-MPs on chlorophyll levels take longer than seven days to affect the overall photosynthesis process or the effect, at these concentrations and conditions, is not so significant as to be noticeable with this method. Furthermore, increased photosynthetic efficiency and higher chlorophyll content could be associated with slight but insignificant growth enhancement observed in plants treated with lower concentrations of PS-MPs (10 and 50 mg L^{-1}). It has been established that the enhancement in photosynthetic attributes can increase plant growth, plant biomass, yield and productivity (Sherin et al., 2022).

Regarding carotenoids, there was a slight difference compared to chlorophylls, i.e. 50 mg L^{-1} PS-MP group showed a higher total carotenoid content than the control and 50 mg L^{-1} PMMA-MPs (Figure 10), while at 100 mg L^{-1} plants treated with PMMA-MPs had higher carotenoid content than plants treated with PS-MPs of the same concentration (Figure 10). Also, the values were more similar to control ones than for chlorophylls, especially for PMMA-MPs treatments. Carotenoids act both as accessory photosynthesis pigments (Taiz and Zeiger, 2002) and as non-enzymatic antioxidants (Rudenko et al., 2023). Higher amounts of accessory pigments could help compensate for lower chlorophyll content and thus reduce or mitigate the negative effects on overall photosynthesis performance. In other words, this could be why the PMMA-MP-treated plants did not show decreased maximum PSII quantum yield and photosynthetic performance, when compared to PS-MP-treated plants, even though they had lower chlorophyll *a* content.

Furthermore, the antioxidant properties of carotenoids protect both the photosynthesis pigments and various other proteins and enzymes involved in the process. Kobelščak (2024) showed that PMMA- and PS-MPs can induce oxidative stress response mechanisms in *L. minor* plants, i.e. PS-MPs induced enzyme activation, while PMMA-MPs increased a non-enzymatic antioxidant proline. Activation of antioxidant

machinery implies that MPs induced oxidative stress that could damage biomolecules like chlorophylls. Interestingly, Kobelščak (2024) showed that PMMA-MPs, not PS-MPs, had a more significant effect on the activation of non-enzymatic oxidative stress responses. However, his focus was more on proline as antioxidant, and not pigments. Therefore, it is possible that PMMA-MPs more strongly activates non-enzymatic proline which can act as a ROS scavenger, as well as an activator of ROS detoxification pathways and a stabilizer of cellular structures and membranes (Hossain et al., 2014), while PS-MPs more strongly activates non-enzymatic carotenoids as essential for photoprotection of photosynthetic apparatus. Elucidating these potentially conflicting findings would require further experiments specifically focused on the activation and efficiency on non-enzymatic oxidative stress mechanism as a response to PS- and PMMA-MPs pollution. Higher levels of chlorophyll *a* and *b* in PS-MP treatment groups and higher photosynthetic efficiency can therefore be explained by their higher carotenoid levels. Carotenoids neutralize ROS molecules, preventing them from causing damage to chlorophyll molecules and photosynthetic proteins, which would result in lower chlorophyll content and overall decrease in the efficiency of photosynthesis. Although content of chlorophylls and carotenoids in PMMA treated plants were lower than in PS treated plants, the reduction was not significant compared to control plants. This agrees with no observable oxidative damage of lipids or proteins as indicated with no change in lipid peroxidation and protein carbonyl content in these plants (Kobelščak, 2024). On the other hand, in polyethylene (PE)-MP-treated tobacco decreased chlorophyll content was associated with the increased ROS content and inhibited primary photochemistry (measured by OJIP transients), resulting in decreased quantum yields. Moreover, reduced RuBisCO activity was also recorded due to inhibited ribulose 1,5-bisphosphate carboxylation activation and regeneration (Teng et al., 2022). In my work, PMMA-MP groups showed a decrease in relative RuBisCO content compared to PS-MP groups (Figure 11). This corresponds with lower photosynthetic response in PMMA-MPs treated plants and corroborates the assumption of carotenoids playing a key role in protection of photosynthetic apparatus from ROS. Excess ROS levels cause significant damage to RuBisCO and photosynthetic pigments (Tominaga et al., 2020).

Changes in content of photosynthetic pigments and performance observed in this work could also be related to regulation of genes involved in chlorophyll biosynthesis,

metabolism of carbohydrate, and ATP production. For example, in *Arabidopsis thaliana* exposure to polybutylene adipate terephthalate (PBAT) MPs disturbed photosynthesis by downregulating the gene expression of genes encoding light-harvesting chlorophyll *a*/*b* binding proteins (Liu et al., 2022). In tobacco, treatment with PE-MPs resulted in the downregulation of expression of light harvesting-, electron transport- and photosystem-related genes (Teng et al., 2022). This should be further researched using qPCR methods to track possible expression in selected genes, such as those involved in chlorophyll synthesis and degradation and photosynthesis; raf-like kinase 2 (RAF2), chlorophyll a/b-binding protein (CAB), photosystem II reaction centre protein D1 (PsbA) or photosystem II reaction centre protein H (PsbH) (Zhang, et al., 2020).

6. Conclusion

This study investigated the effects of polystyrene (PS) and poly(methyl methacrylate) (PMMA) microplastics on the growth and photosynthetic efficiency of *Lemna minor*. The main results are as follows:

- PMMA-MPs appeared to inhibit, while PS-MPs appeared to promote frond growth, however, these effects were not statistically significant. PS-MP-treated plants showed a larger frond area than those treated with PMMA-MPs, although this was only significant for the 100 mg L^{-1} concentration.
- The maximum quantum yield of PSII (F_v/F_m) differed significantly only between the two 10 mg L^{-1} treatments. The photosynthetic performance index (PI_{ABS}) was slightly higher in PS-MP-treated plants for all treatments compared to both PMMA-MP-treated plants and the control.
- PMMA-MP slightly reduced chlorophyll *a* and *b* content across all concentrations, while PS-MP resulted in a slight increase, except for the highest concentration (100 mg L^{-1}). Carotenoid content was slightly higher in PMMA-MP-treated plants than in the control, though still significantly lower than in PS-MP-treated plants at 50 and 100 mg L^{-1}. The 100 mg L^{-1} PS-MP group had a significantly lower carotenoid content compared to the 50 mg L^{-1} PS-MP and the 100 mg L^{-1} PMMA-MP.
- All PMMA-MP concentrations caused a prominent decrease in RuBisCO abundance, with the greatest effect at 10 mg L^{-1}. In contrast, for the PS-MP treatments, the decrease in abundance correlated with the increase of concentration, with a prominent reduction only at 100 mg L^{-1}.

In conclusion, while the thesis hypotheses were only partially proven, the study revealed interesting trends. *L. minor* growth was affected but not significantly, and neither MP type significantly impaired photosynthesis according to the JIP-test and pigment content, with PS-MP treatment even showing improvement. Most results showed a greater negative effect at higher concentration, but PMMA-MP had the greatest effect on RuBisCO abundance at the lowest concentration. Further research is needed to better understand how *L. minor* responds to MPs and whether the changes in protein abundance and pigment content are caused by depletion (oxidative damage) or by the impact MPs may have on the expression of genes related to their biosynthesis and maintenance.

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Biography

I was born in 1997. In Nova Gradiška, Croatia. At two years old, I moved to Zadar where I attended the Šime Budinić primary school until 2011. The same year I enrolled in the IV Gymnasium in Zagreb, as part of the bilingual study program, having some of my classes in Croatian and some in English. In 2012, I transferred to Zadar's Vladimir Nazor language gymnasium, from which I graduated in 2016. While there, I won first place in the Biology competition at the country level, which granted me direct enrolment to University of Zagreb's Molecular Biology undergraduate program. While attending, I participated in the University's open door event "Day and Night at PMF" (*cro*. "Dan i noć na PMF-u") in 2017 and 2019, as part of the Biology department's public education and demonstration program. In 2018 I helped organise and conduct the Symposium for students of biology and natural sciences (SISB) and helped younger students with their laboratory lessons as a student demonstrator for the course Biology of the Cell (*cro*. Bologija stanice). I graduated from the undergraduate program in 2020, and enrolled in the Molecular Biology graduate program the same year. In 2021, I assisted with the organisation and running of the third Work In Science (WISE) carrier day. I also participated in the BOLDer project in 2021, as part of my Laboratory-practical work course (*cro*. Laboratorijska stručna praksa), which included both fieldwork and laboratory experiments on skink behaviour (*Podarcis situla* and *P. melisellensis*).