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Phytoremediation of Methylene Blue and Congo Red by duckweed (*Lemna minor*)

Flor Y. Ramírez-Castillo [a], Diana E. Guillén-Padilla [a], Cristian I. Méndez-Sandate [a], Alma L. Guerrero-Barrera [b*] & Francisco J. Avelar-González [c]

[a] *Laboratorio de Biología Celular y Tisular, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Aguascalientes, México. flor.ramirez@edu.uaa.mx, guillendiana2018@gmail.com, cristianivmesa@gmail.com*
[b] *Laboratorio de Biología Celular y Tisular, Departamento de Morfología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes. Aguascalientes, México. alguerre@correo.uaa.mx, lilian.guerrero@edu.uaa.mx, *Correspondence author*
[c] *Laboratorio de Estudios Ambientales, Departamento de Fisiología y Farmacología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes. Aguascalientes, México. javier.avelar@edu.uaa.mx*

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Abstract
Synthetic colorants are widely used globally by different industries for the dyeing process. However, these chemicals pollute the environment and affect human health by causing allergies, hives, dermatitis, and cancer. This study aims to compare the effectiveness of duckweed (*Lemna minor*) in the removal of the Methylene Blue (MB) and Congo red (CR) dyes at different concentrations (1 mg/L, 5 mg/L, 10 mg/L, and 15 mg/L). Absorbance values were determined at 665 nm for MB and 497 nm for CR after 96 hours. The results show higher removal of MB compared to CR for all concentrations (95.49 % vs. 59.32%, 1 mg/L; 97.24% vs. 39.43%, 5mg/L; 91.30% vs 28.47%, 10mg/L; y 85.42% vs 20.27%, 15mg/L). The removal of MB was observed after 30 min of contact with duckweed, while the removal of CR was observed after 24 hours in all concentrations.

Keywords: Methylene blue; Congo Red; phytoremediation*; Lemna minor*; duckweed; textile dyes.

Fitorremediación de Azul de Metileno y Rojo Congo por lenteja de agua (Lemna minor)

Resumen
Los colorantes sintéticos son altamente utilizados a nivel global por distintas industrias para el proceso de tinción. Sin embargo, estos químicos son altamente contaminantes para el ambiente y afectan la salud humana provocando alergias, urticaria, dermatitis y cáncer. Este estudio tiene como objetivo comparar la efectividad de la lenteja de agua (*Lemna minor*) en la remoción de los colorantes azul de metileno (AM) y rojo Congo (RC), a distintas concentraciones (1mg/L, 5mg/L, 10 mg/L y 15 mg/L). Los valores de absorbancia fueron determinados a 665 nm para AM y 497 nm para RC. Los resultados muestran que el porcentaje de remoción de AM fue mayor que el de CR (95.49 % vs 59.32%, 1 mg/L; 97.24% vs 39.43%, 5mg/L; 91.30% vs 28.47%, 10mg/L; y 85.42% vs 20.27%, 15mg/L). La remoción del AM se observó a los 30 minutos de contacto, mientras que el RC redujo la absorbancia a partir de las 24 horas.

Palabras clave: Azul de metileno; Rojo Congo; fitorremediación; *Lemna minor*; lenteja de agua; colorantes textiles.

1 Introduction

Dyes are compounds widely used for coloring products in several industries, including textiles, cosmetics, leather tanning, and pigmentation [1]. In the textile industry, the synthetic dyes have greater use. These chemicals are generally discharged before treatment as wastewater effluent into water bodies such as stream water, lakes, ponds, and rivers, thus creating a significant water pollution problem [1-3].

Due to their chemical structure, dyes have high stability

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and resistance to bio-, photo-, and thermo-degradation [4]. Therefore, they tend to persist in the environment, creating problems for the water bodies by colored water, increasing biochemical and chemical oxygen demand, increasing suspended solids, impairing photosynthesis, and inhibiting plant growth [3]. In addition, microorganisms that inhabit aquatic environments could cause direct destruction or inhibit their catalyzing capacity [5,6]. Likewise, synthetic dyes can also cause human health problems such as allergy, rhinitis, asthma, dermatitis, skin and eye irritation, problems with the central nervous system, cancer, and mutation [7,3].

Several physicochemical methods for removing dyes from water sources include adsorption, photocatalysis, advanced oxidation, precipitation, ion exchange, membrane filtration, coagulation, and solvent extraction [8-10].

Phytoremediation is an alternative technology for removing contaminants in water, soil, sediments, and air [11]. These techniques are considered low-impact, environmentally friendly, cost-effective, and easy-to-use methods since they are driven by solar energy and use the capacity of plants, their roots, and microorganisms associated with them to absorb, filter, precipitate, metabolize, and compartmentalize or sequester organic and inorganic pollutants [10-13].

Lemna minor, from the *Lemnaceae* family, commonly known as duckweeds, is one of the most used aquatic plants for bioremediation of aquatic environments with multiple contaminants, such as nitrogen, ammonia, phosphates, total solids, organic compounds, heavy metals, drugs, and synthetic dyes [14]. *L. minor* is a free-floating macrophyte found in freshwater bodies and sewage waters. Due to their rapid vegetative propagation, fastest growth rates, ability to accumulate large amounts of biomass in a short period, easy of harvestable, and great adaptability to diverse environmental factors such as different range of temperature and pH, as well as their tolerance to several pollutants, these aquatic plants have a great potential for phytoremediation [14,15].

Several studies have reported the ability of duckweed for phytoremediation of methylene blue dye [16-18]. However, little is known about the phytoremediation potential for more diverse dyes, such as Congo Red [2,7,19]. This study aims to investigate the removal potential of duckweed (*L. minor*) for the removal of two dyes with different chemical structures, methylene blue (triaryl methane dye, ionic dye) and Congo Red (diazo dye, anionic dye) in aqueous solution. The effect of contact time was also studied to optimize the phytoremediation process.

2 Methods

2.1 Plant cultivation

L. minor was collected from ponds at San Francisco de los Romo, Aguascalientes, Mexico, during June 2023. The plants were gently washed with tap water to remove unwanted impurities. Subsequently, the plants were kept in distilled water for 2 h. Before the experimentation, the plants were maintained using Hoagland´s modified nutrient solution at 27 ± 30ºC for 15 days. All the plants used were healthy (intense lemon green color without root detachment).

2.2 Decolorization of Methylene blue and Congo Red assays

Methylene blue (triethylmethane dye, MB [$C_{16}H_{18}N_3SCl.3H_2O$; PM 372.90 gr/mol] and Congo Red (diazo dye [$C_{32}H_{22}N_6Na_2O_6S_2$; PM 696.7 gr/mol] were used as contaminants to be removed (Fig. 1). The initial pH of the colored solution was 6.9 to 7.2. The initial temperature ranged from 26.6ºC to 27.2ºC. During the treatment time intervals, absorbance and concentration of dyes were calculated using a UV/Vis spectrophotometer at a wavelength of 665 nm for methylene blue and 497 nm for Congo Red [17].

The decolorization test were carried out in 250 ml flasks containing 100 ml of colored solutions of MB and CR at concentration of 1 mg/L, 5 mg/L, 10 mg/L, and 15 mg/L. Four grams of *L. minor* was exposed to water contaminated with MB and CR at room temperature (24ºC ± 4ºC) under white lamp light at 10,000 to 25,000 lux meters [17]. For the trial, it was ensured that all plants roots reached the bottom of the beaker during the test to increase root sorption capacity and rhizosphere clearance mechanisms [22].

Samples of 2 ml of colored water were collected at 0, 30, 60, 90, 120, and 180 min; and 24, 48, 72 and 96 hours. Absorbance values were measured at each set point. All measurements were performed in triplicate. The removal of both dyes was evaluated by absorbance in terms of decolorization. The percentage of discoloration was calculated based on the following equation [23]:

$$Decolorization\ (\%) = \left(1 - \frac{At}{Ao}\right) \times 100$$

Where "A_0" describes the absorbance of the colored solution at time zero (before treatment), and "A_t" represents the absorbance measured after exposure to the colored solutions at time "t."

2.3. Statistical analysis

The results of the experiments were analyzed by one-way ANOVA with the Turkey-Kramer multiple comparison test using the GrapPad Prisms 8.0.2 program. The results were considered statistically significant with $p \leq 0.05$. Data are presented as the mean and standard deviation of three replicates.

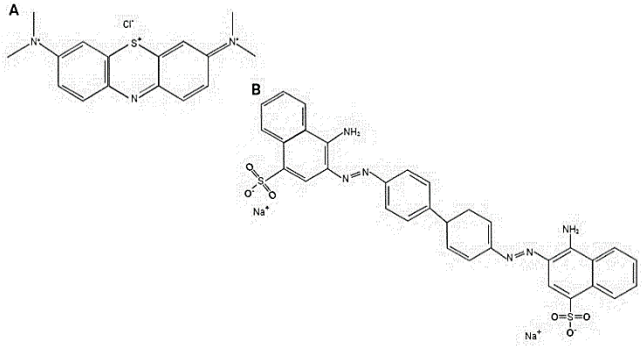

Figure 1. Chemical structure of A) Methylene blue (MB) and B) Congo Red (CR). The images were created using BioRender.com on-line tool (https://app.biorender.com/).
Source: adapted from the literature [20,21].

3 Results and discussion

The results of the effect of the exposure time and the initial concentration of MB and CR solutions on the absorbance of both dyes are presented in Fig. 2. For both colored solutions, a reduction of the absorbance was observed (Fig. 2A and 2B). The removal time was much shorter for the MB than the CR since the decrease of MB was observed after 30 minutes of the assay for all concentrations tested. Meanwhile, CR did not show any significant reduction in the first 180 minutes of the experiment for all concentrations, including the minor concentration of 1 mg/L (Fig. 2A).

Fig. 3 illustrates the maximum reduction of CR and MB over 96 hours with set points every 24 hours. Figure 3B indicates that the maximum reduction for CR was achieved at the 24-hour mark, and no further significant reduction was observed. On the other hand, Figure 3A shows that MB exhibited a more significant removal at 24 hours, which continued to be observable until 72 hours, including the highest concentration tested of 15 mg/L of dye. At 96 hours, a slight increase in the absorbance was seen in all concentrations except for the lowest concentration of 1 mg/L of MB.

Notably, when we compared the reduction between the 30-min mark vs. 180-min mark, a significant reduction of the MB dye was observed for all concentrations, with the percentage of decolorization of 63.74% vs. 80.73%, $p<0.01$ (1 mg/L, respectively), 28.67% vs. 73.05%, $p < 0.001$ (5 mg/L, respectively), 16.79% vs. 48.08%, $p < 0.001$(10 mg/L, respectively), and 5.32% vs. 34.77%, $p < 0.001$ (15 mg/L, respectively). In contrast, when we compared the reduction between the 30-min mark and 180-min mark on CR dye, we found the best percentage of remotion for the most concentrated solution of 15 mg/L of CR dye with a reduction of 9.97% at 30-min vs. 17.06% at 180-min with significant differences between both time marks ($p < 0.001$, Fig. 4B). Non-significant differences were found for all other concentrations.

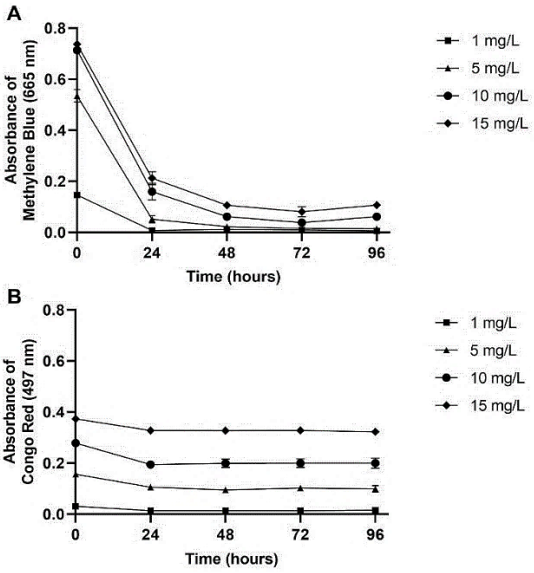

Figure 3. The absorbance of methylene blue (MB, A) and Congo red (CR, B) at concentrations of 1 mg/L, 5 mg/L, 10 mg/L, and 15 mg/L of dye, after treatment with L. minor for 24, 48, 72 and 96 hours. The values are presented as the average of the triplicate ± standard deviation. MB was measured at 665 nm and CR at 497 nm.
Source Self-made image.

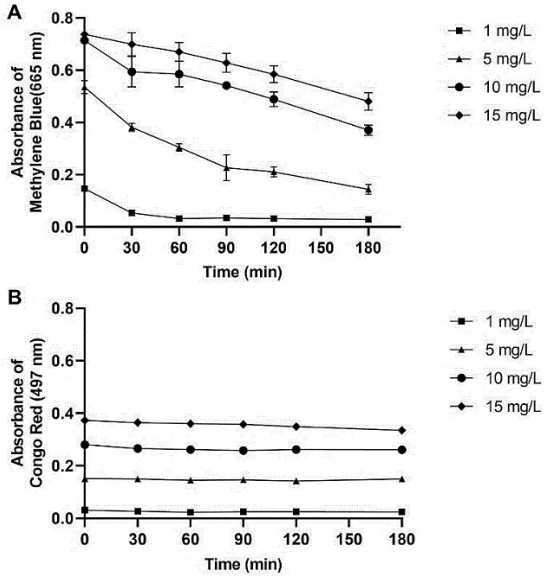

Figure 2. The absorbance of methylene blue (MB, A) and Congo red (CR) at concentrations of 1 mg/L, 5 mg/L, 10 mg/L, and 15 mg/L of dye, after treatment with *L. minor* for 30, 60, 90 and 180 min. The values are presented as the average of the triplicate ± standard deviation. MB was measured at 665 nm and CR at 497 nm.
Source: Self-mage image.

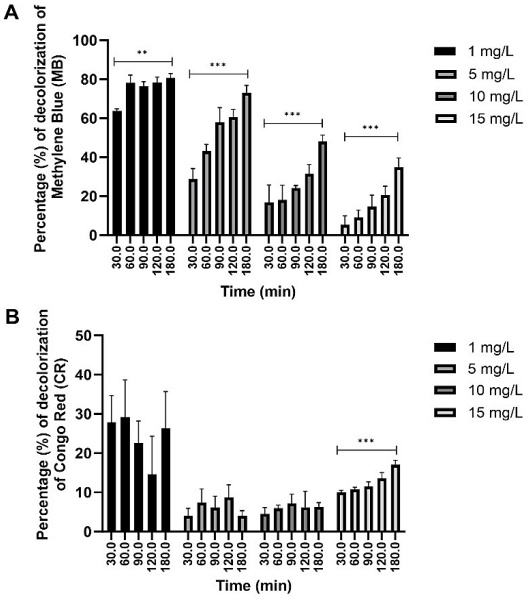

Figure 4. Percentage removal in terms of decolorization of methylene blue (MB, A) and Congo red (CR, B) dyes at concentrations of 1 mg/L, 5 mg/L, 10 mg/L, and 15 mg/L of dye after treatment with L. minor for 30, 60, 90, 120 and 180 minutes. Values are presented as the average of triplicate.
Source: Self-made image.

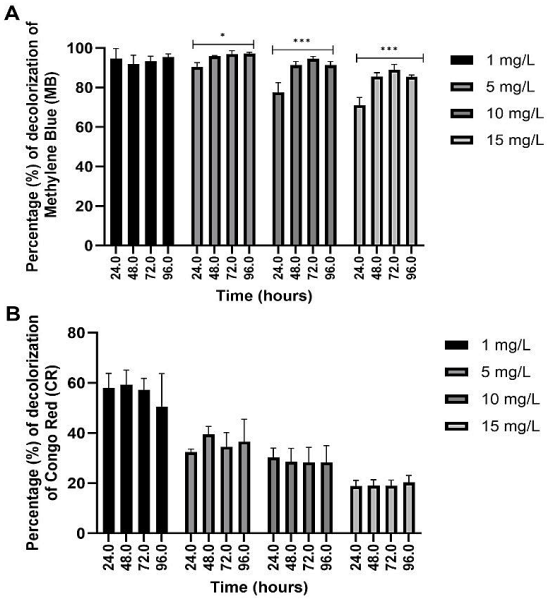

Figure 5. Percentage removal of methylene blue (A, C) and Congo red (B, D) dyes at concentrations of 1 mg/L, 5 mg/L, 10 mg/L, and 15 mg/L of dye after treatment with L. minor for 180 minutes (A, B) and 96 hours (C, D). Values are presented as the average of triplicate. Source: Self-made image.

The maximum removal efficiency of the MB dye at the lowest concentration of 1 mg/mL was reached in the full contact time of 96 hours, with percentages of decolorization of 95.49%. However, no significant differences were found in the 24-hour and 96-hour mark at this concentration (Tabl. 5A). Taking as reference the 96 hours, we found a significant difference among this set point and 24 hours, with the percentage of decolorization of MB of 97.26% vs. 90.35%, $p < 0.05$ on the concentration of 5 mg/L of dye (96-hour vs 24-hour, respectively), of 77.58% vs. 91.31%, $p < 0.001$ for 10 mg/L of dye (respectively), and of 71.13% vs. 85.43%, $p < 0.001$ for 15 mg/L of dye (respectively). However, non-significant differences were found between 48- and 72-hours vs. 96-hours of treatment (Fig. 5A).

Compared with previous studies where removal percentages of MB were 80.56% at 24 hours at a concentration of 50 mg/L [16] and 98% at a concentration of 15 mg/L [23], we observed a lower percentage of efficiency (71.13%, Fig. 5A) in the same exposure time (24 h). This could be explained by the amount of biomass used since in Can-Terzi et al. [24], 4.9 gr of the plant was used, while our study used only 4 gr. In addition, pH and temperature variations also modify the percentage of remotion of pollutants. This agrees with Torbati et al. [25], who evaluated the ability of duckweed to decolorize the acid dye Bordeaux B (ABB and aminoazo benzene dye) and determined that increasing the plant's temperature and weight, increases the efficiency of dye removal. In addition, the quantity of the biomass is of great importance since it has been found that the mechanisms of remotion are related to biosorption [24] and phytoadsorption through electrostatic interactions, hydrogen bonds, as well as degradation by desulfurization and denitrification processes [16,26]. Therefore, the greater the amount of plant present, the greater the sorption surface are disponible for the dye molecules [27]. However, in lower concentrations of 1 mg/L and 5 mg/L we achieve 94.59% and 90.4% of removal at 24 h (Fig. 5A).

Similar to the results obtained by Reema et al. [28] and Khataee et al. [27] that determined a removal percentage higher than 80% for MB after three days of contact, we observer a removal percentage of 88.93% of MB in 72 h at concentration of 15 mg/L (Fig. 5A). The MB-colored solution in lower concentrations of 1 and 5 mg/L reduced almost the maximum percentage of dye at 24 hours (94.59% and 90.41%, respectively); thus, no significant differences were observed between 24 and 96 hours of contact with ducked for the lower concentration tested (95.49%, Fig. 5A). On the contrary, the solutions with the highest concentration (10 and 15 mg/L) were significantly reduced at 96 hours compared to the contact time of 24 hours ($p < 0.001$, Fig. 5A). Therefore, the higher the concentration of MB, the longer contact time was required to increase the percentage of efficiency in the removal of the colored solution.

Regarding Congo Red (CR) dye, within the first 180 min of contact with duckweed, the removal percentage in terms of decolorization was 26.35% (1 mg/L), 4.04% (5 mg/L), 6.32% (10 mg/L) and 17.07% (15 mg/L), respectively (Fig. 4B). Likewise, the maximum decolorization of CR was performed at different set points for different concentrations. At 1 mg/L and 5mg/L of CR dye, maximal decolorization was found at 48 hours (59.32 %, and 39.43%, respectively); at 10 mg/L was found at 24 hours (30.27%), and at 15 mg/L was achieved at 96 hours (20.27%). Nevertheless, non-significant differences were found among all-time marks after 48 hours (Fig. 5B). This agrees with the survey by Kaur et al. [2], which determined that the contact time for maximum decolorization was 40 hours for the CR dye using *the Trachyspermum ammi* plant. In contrast with our study, Mahajan et al., [19] found a maximal removal percentage of 95% of CR dye solution at 24 hours using *Chara vulgaris*, however, these differences could be explained by the amount of algae used (5 g), the temperature (33ºC), the pH of 7, and different species used.

Notably, the decolorization rate of both MB and CR dyes decreases as the concentration of dye increases. This might be due to the high concentration of the dye molecule in the solution blocking the pores on the root surface and the rate of decolorization decrease [2]. Moreover, the toxic effects of the dye could be affecting the metabolic activity of the plant [29]. Indeed, previous studies have found that exposure to CR inhibits plant growth, decreasing biomass percentage and chlorophyll a biosynthesis [30]. On the other hand, the rapid adsorption in the initial phase of the experiment is caused by the large amount of dye particles in the solution and the large number of free sites available in the plant for the adsorption process [3].

In the CR dye assay, a significant reduction in absorbance was observed at 24 and 48 h, while after 72 h, an increase in absorbance was noticed (Fig. 3B and 5B). We observed that certain plants begin to die after 48 h (results not shown). Thus, previously adsorbed dye is released from the dead plants into the colored solution [31]. In addition, the difference between the removal percentage achieved for MB

vs CR and the removal time can be influenced by the chemical structure of both dyes. CR is an anionic dye and a more complex structure with higher molecular weight than MB, which is, in contrast, an ionic dye. Therefore, duckweed can potentially remove ionic dyes better than anionic ones. A study that compares the remotion process of MB vs. CR using *T. ammi* has also shown a better remotion for MB (99% or removal) compared to CR (91% of removal) [2]. Likewise, it seems that the sorption mechanisms of both dyes are different since we noticed that the root in the treatment with MB was colored after a few minutes of contact time and depleted about 72 hours; meanwhile, for the CR, the root remained attached to the frond, and it is the frond that is colored by the dye (data not show). Further studies will focus on the mechanisms of Congo Red removal by duckweed.

Other strategies for removing CR from wastewater include the use of adsorbents. This adsorbent comprises the use of *Azolla filiculoides* that removes 95% of CR dye in optimal conditions [32], *Vernonia amygalina* leaf powder with an adsorption capacity of 57.47 mg/g at pH 8 [33], nylon fiber waste with the removal of 95% for CR concentration of 400 mg/L at pH 6 [34], fly ash (waste material within SiO_2, Al_2O_3, Fe_2O_3, Na_2O, MgO and K_2O as constituents) within an adsorption capacity of 22.12 mg/g [35], hydrochard and MgAl layered double hydroxide (HC-MgAlLDH) nanocomposite with a removal capacity of 348.78 mg/g of CR [36], natural clay glauconite (a heterogeneous phyllosilicate mineral) activated with 2M NaOH with removal efficiency ~77% [37], metal-organic frameworks such as zeolitic imidazolate framework-8 with an adsorption capacity of 1250 mg/g [38], activated carbon, among others. However, even when adsorbents have taken a great interest due to the large quantities of available adsorbents and high adsorption capacity [39], some of these treatments, such as activated carbon, alumina, and silica gel, fly ash and zeolites require regeneration or disposal of adsorbents, thus limiting their application [24,33].

Furthermore, although phytoremediation is a promising method for dye removal, managing the plant biomass produced after the remediation process is crucial to avoid converting it into secondary pollution through post-mortem decomposition. Unfortunately, there is limited research on post-remediation management [40]. Simple harvesting will reduce the re-entry of pollutants by up to 75% [41]. Song et al. [40] demonstrated that it is possible to re-use remediation plant biomass (*Pharagmites australis* and *Typha angustifolia*) as a friendly fertilizer. It has also been suggested to use phytoremediation biomass for energy generation by incineration [42] or by production of bio-ethanol [43]. Nowadays, composting, leachate compaction, combustion, gasification, pyrolysis, torrefaction, and metal recovery are used for phytoremediation biomass management. The extraction and recovery of metalloids and metals from contaminated biomass is considered as a valuable bio-product [44].

Among dyes, Torbarti et al. [26] have shown that *L. minor* possessed reasonable reusability in the repetitive decolorization operation of triarylmethane dye with biodegradation in eight intermediate compounds, which could convert into the CO_2 and water (deep oxidation). Can-Terzi et al. [24] propose that the phytoremediation biomass of MB could be used as fertilizer since their desorption showed that MB that was removed by *L. minor* has remained within the plant structure and adhered into the functional groups of the plant. The authors also propose the use of *L. minor* biomass as biofuel production. Imron et al. [31] showed that rhizobacteria on *L. minor* could degrade MB into CO_2, water, and other intermediate products that will be adsorbed by *L. minor* onto leaves [31]. However, post-phytoremediation biomass treatment may be an essential issue to address.

4 Conclusion

The results demonstrate the ability of duckweed (*Lemna minor*) for the removal of Methylene Blue (MB) and Congo Red (CR) dyes in aqueous solutions. Duckweed has a higher percentage of efficiency in removing the Methylene Blue (95.49%, 1 mg/L; 97.24%, 5 mg/L; 91.30%, 10 mg/L; and 85.42%, 15 mg/L) compared to Congo Red dye (59.32%, 1 mg/L; 39.43%, 5 mg/L; 28.47%, 10 mg/L; and 20.27%, 15 mg/L) at al concentrations tested. Duckweed (*L. minor*) can reduce a percentage of ~90% of MB in less than 24 hours, while it reduces rates to ~ 20% of CR dye in the same contact period. Future studies will focus on exploring the mechanisms of adsorption and the toxicity of Congo Red in *L. minor*.

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A.L. Guerrero-Barrera, received a Dr. in Cellular Biology and a MSc. in Cellular Biology from CINVESTAV-IPN. PhD in Cellular Biology, Institute of Biotechnology of the UNAM. Publication of 73 scientific articles. Member of the National System of Researchers (SNI) Level II. Direction of 22 doctoral, 4 master's, and 33 undergraduate theses. UAA. Full-Time Research Professor since 2001. Visiting Professor at the University of Cergy Pontoise, France 2017. Sabbatical stay at Faculty of Veterinary Medicine, University of Montreal 2009. Participant in the "Researchers with Commercial Success" program, CONACYT-INFOTEC 2014, Leaders in Innovation Scholarship Program. Royal Academy of Engineering and Isis Group Oxford 2015 and "Researchers with commercial success," CONACYT-INFOTEC 2015.
ORCID: 0000-0002-0952-8544

F.J. Avelar-González, received a Dr. in Biotechnology from CINVESTAV-IPN. He is a BSc. Eng. in Biochemical Engineer who graduated from the Autonomous University of Aguascalientes, Mexico and has a MSc. in Biotechnology from CINVESTAV-IPN. He is a member of the National System of Researchers (SNI), Level I, and a full-time research professor at the Autonomous University of Aguascalientes.
ORCID: 0000-0002-0777-9243

F.Y. Ramírez-Castillo, is a BSc. Eng. in Biochemical Engineer and Dr. in Biological Sciences from the Autonomous University of Aguascalientes, Mexico. She has published 12 indexed articles on the detection of pathogens in water, antimicrobial resistance, and the detection of pathogens in respiratory diseases. She carried out a research stay at the Université de Montréal focused on the pathogenesis and antimicrobial resistance of *E. coli*, molecular diagnostic techniques of pathogens in aquatic environments, antimicrobial resistance, urinary tract infections, and respiratory infections, and studies of contamination in surface waters. She is a member of the National System of Researchers (SNI), Level I
ORCID: 0000-0003-1707-2441.

D.E. Guillén-Padilla, is a BSc. in Biological Pharmaceutical Chemist from the Autonomous University of Aguascalientes, Mexico. She is currently working on a project for the molecular characterization of clinical isolates of *P. aeruginosa* resistant to quinolones, where the presence or absence of genes that code for resistance to this antibiotic, mediated by plasmids or present in a determining region, is evaluated. Likewise, it participated in the phytoremediation mini-project, where the ability of the aquatic plant *Lemna minor* to remove dyes present in water contaminated with said dyes was evaluated.
ORCID: 0009-0000-3585-9681

C.I. Méndez Sandate, Bachelor´s degree in Biological Pharmaceutical Chemist at the Autonomous University of Aguascalientes, Mexico. The thesis project is "Evaluation of the phytotoxicity of the antibiotic enrofloxacin in the floating aquatic plant *Lemna minor* (duckweed). Likewise, he participated in the phytoremediation mini-project, where the ability of the aquatic plant *Lemna minor* to remove the methylene blue and Congo red dyes present in the aqueous solution was evaluated.
ORCID: 0009-0004-7637-4794