EDER CARLOS LOPES COIMBRA

EVALUATION OF PHYTOREMEDIATION IN WASTEWATER TREATMENT WITH A FOCUS ON MACROPHYTE SELECTION, POLLUTANT REMOVAL AND BIOMASS CHARACTERISTICS

Thesis submitted to the Agricultural Engineering Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Alisson Carraro Borges

Co-advisers: Ann Honor Mounteer
André Pereira Rosa

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Assent:

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To my family, for their unconditional love and support.

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"Life is not always about healing; you heal, feel better, and then do it all over again. Life is about knowing that you are wounded, hurt, and full of doubts, but you keep going anyway"

(Viola Davis, 1965)

ABSTRACT

COIMBRA, Eder Carlos Lopes, D.Sc., Universidade Federal de Viçosa, February, 2024. **Evaluation of phytoremediation in wastewater treatment with a focus on macrophyte selection, pollutant removal and biomass characteristics**. Adviser: Alisson Carraro Borges. Co-advisers: Ann Honor Mounteer and André Pereira Rosa.

The success of phytoremediation depends on carefully selecting plants to effectively remove water pollutants. The choice should consider the water type, specific pollutants, and treatment system conditions. This thesis comprises three technical-scientific papers. The first article conducted a review study on the relationship between environmental factors and the removal of iron (Fe), copper (Cu), and manganese (Mn), from wastewater, through phytoremediation. Emergent macrophytes showed the best results for Fe and Mn removal. Significant correlations were found between physical factors and the plants' ability to bioconcentrate Fe, Cu and Mn. Short exposures increased metal removal but hindered Fe and Cu absorption. Reduced light favored Cu and Mn removal, while lower pH favored Cu and Mn removal. High concentrations of phosphorus and dissolved oxygen increased Cu absorption in plants. In the second article, *Lemna minuta*, *Landoltia punctata*, *Azolla microphylla*, and *Salvinia minima* treated synthetic swine wastewater over a 10-day exposure period. *Azolla microphylla* experienced the least stress, and *L. minuta* showed the highest growth and lipid production. These two plants were more efficient in removing acute and chronic wastewater toxicity. All four plants exhibited removal rates exceeding 60% for COD and $P\text{-}PO_4^{3-}$, and over 40% for $N\text{-}NO_3^-$ and Cu. The Entropy-Fuzzy AHP TOPSIS method deemed *Azolla microphylla* the most suitable for treatment. This approach proved reliable in macrophyte selection, supported by individual evaluation and sensitivity analysis methods. The third article evaluated the impacts of LED light, considering its duration and intensity, as well as the phytohormone cytokinin, on *Azolla microphylla*'s performance in treating synthetic swine wastewater. Results confirmed that both LED light and varying cytokinin doses influenced reduced concentrations of organic matter, metals, and sulfamethoxazole (SMX) in swine wastewater. Under optimal treatment conditions, *Azolla* removed COD (89.2% to 90.8%), $N\text{-}NH_4^+$ (72.6% to 91.2%), $N\text{-}NO_3^-$ (84.4% to 88.6%), Cu (75.4% to 86.4%), SMX (77% to 79%), $P\text{-}PO_4^{3-}$ (54.1% to 59.9%), and dissolved organic carbon (67.4% to 71.3%). However, these conditions resulted in a moderate reduction for Zn. The influence of these factors varied for each pollutant, highlighting the importance of carefully considering treatment parameters. The research in this papers underscores the critical role of precise plant selection and treatment conditions in water remediation, emphasizing the need for multidisciplinary approaches and the potential for biomass reuse.

Keywords: Phytoremediation; Biotope; Doehlert matrix; Multicriteria decision-making; *Azolla*; Swine Wastewater.

RESUMO

COIMBRA, Eder Carlos Lopes, D.Sc., Universidade Federal de Viçosa, fevereiro, 2024. **Avaliação da fitorremediação no tratamento de águas residuárias com foco na seleção de macrófitas, remoção de poluentes e características da biomassa**. Orientador: Alisson Carraro Borges. Coorientadores: Ann Honor Mounteer e André Pereira Rosa.

O sucesso da fitorremediação depende da seleção cuidadosa de plantas para remover eficientemente poluentes da água. A escolha deve considerar o tipo de água, poluentes específicos e condições do sistema de tratamento. Esta tese é composta por três artigos técnico-científicos. No primeiro artigo, foi realizado um estudo de revisão sobre a relação entre fatores ambientais e a remoção de ferro (Fe), cobre (Cu) e manganês (Mn), de água residuária, por meio de fitorremediação. As macrófitas emergentes apresentaram os melhores resultados para remoção de Fe e Mn. Foram encontradas correlações significativas entre fatores físicos e a capacidade das plantas de bioconcentrar Fe, Cu e Mn. Exposições curtas aumentaram a remoção de metais, mas prejudicaram a absorção de Fe e Cu. Luz reduzida favoreceu a remoção de Cu e Mn, enquanto pH mais baixo favoreceu a remoção de Cu e Mn. Altas concentrações de fósforo e oxigênio dissolvido aumentaram a absorção de Cu nas plantas. No segundo artigo, *Lemna minuta*, *Landoltia punctata*, *Azolla microphylla* e *Salvinia minima* trataram águas residuárias sintéticas suinícolas ao longo de um período de 10 dias de exposição. *Azolla microphylla* experimentou o menor estresse, e *L. minuta* apresentou o maior crescimento e produção de lipídios. Essas duas plantas foram mais eficientes na remoção de toxicidade aguda e crônica das águas residuárias. Todas as quatro plantas apresentaram taxas de remoção superiores a 60% para DQO e $P\text{-}PO_4^{3-}$, e mais de 40% para $N\text{-}NO_3^-$ e Cu. O método Entropia-Fuzzy AHP TOPSIS considerou a *Azolla microphylla* a mais apropriada para tratamento. A abordagem proposta demonstrou ser confiável na seleção de macrófitas, sendo respaldada por métodos individuais de avaliação e análise de sensibilidade. O terceiro artigo avaliou os impactos da luz de LED, considerando sua duração e intensidade, bem como do fito-hormônio citocinina, no desempenho da *Azolla microphylla* em tratar de águas residuárias sintéticas suinícolas. Os resultados confirmaram que tanto a luz de LED quanto doses de citocinina influenciaram reduções das concentrações de matéria orgânica, metais e sulfametoxazol (SMX) nas águas residuárias suinícolas. Sob condições de tratamento ótimas, a *Azolla* removeu DQO (89,2% a 90,8%), $N\text{-}NH_4^+$ (72,6% a 91,2%), $N\text{-}NO_3^-$ (84,4% a 88,6%), Cu (75,4% a 86,4%), SMX (77% a 79%), $P\text{-}PO_4^{3-}$(54,1% a 59,9%), e carbono orgânico dissolvido (67,4% a 71,3%). No entanto, essas condições resultaram em uma redução mais moderada para Zn. A influência desses fatores variou para cada poluente, destacando a importância de considerar cuidadosamente os parâmetros de tratamento. A pesquisa desenvolvida nessa tese destaca a importância da seleção precisa de plantas e condições de tratamento para a fitorremediação de águas residuárias, enfatizando a necessidade de abordagens multidisciplinares e o potencial de reaproveitamento da biomassa vegetal.

Palavras-chave: Fitorremediação; Biótopo; Matriz Doehlert; Tomada de Decisão Multicritério; *Azolla*; Águas residuárias de suinocultura.

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

$[HM]_{plant}$: Heavy metal concentration in plant tissue
%R: Pollutant removal
$\tilde{A}^{-1}$: Inverse fuzzy value
$\mu_{\tilde{A}}$: Membership fuzzy function
A_c: Anthocyanin corrected absorbance
PSI: Phyto-Stress Index
A_i: Area between i^{th} and the $(i + 1)^{th}$ radius coordinates of the star plot.
AHP: Analytic Hierarchy Process
AIC: Akaike Information Criterion
Antho: Anthocyanin
BCF: Bioconcentration factor
BOD: Biochemical Oxygen Demand
BmK_i = Biomarker content measured at Day i
BmK_0 = Biomarker content measured at Day 0
BmK_{10} = Biomarker content measured at Day 10
Bp: Biomass productivity
Car: Carotenoids
Chl a: Chlorophyll a
Chl b: Chlorophyll b
$CI_{95\%}$: Confidence interval
COD: Chemical Oxygen Demand
CR: Consistency ratio (%)
Cu: Copper
$_{d}$COD: Dissolved Chemical Oxygen Demand
DOC: Dissolved Organic Carbon
DW: Dry Weight Basis
E-FAHP: Entropy-Fuzzy Analytic Hierarchy Process
FAHP: Fuzzy Analytic Hierarchy Process
FW: Fresh Weight basis
GLM: Generalized linear models
HI: High Influence
IBR: Integrated Biomarker Response
$IC_{50\%,8d}$: Concentration that caused 50% of inhibition, measured in 8 days
$LC_{50\%,3d}$: Concentration that caused 50% of lethality, measured in 3 days
LED: Light Emitting Diode
LI: Low Influence
LOD: Limit of detection
M: General fuzzy number
NH_3^+: Ammonia
NI: No Influence
$N\text{-}NO_3^-$: Nitrogen-nitrate
$N\text{-}NH_4^+$: Ammonium-nitrogen
PAR: Photosynthetic active radiation
$P\text{-}PO_4^{3-}$: Phosphorus-phosphate
PCA: Principal Component Analysis
R: Domain of a fuzzy function
R_i: Overall performance score
R_{COD}: Removal of COD (%)
RGR: Relative Growth Rate
$R_{N\text{-}NH4+}$: Removal of $N\text{-}NH_4^+$: (%)
$R_{N\text{-}NO3\text{-}}$: Removal of $N\text{-}NO_3^-$ (%)
$R_{P\text{-}PO43\text{-}}$: Removal of $P\text{-}PO_4^{3-}$ (%)
R_{Zn}: Removal of Zn (%)
R_{Cu}: Removal of Cu (%)
RSM: Response Surface Methodology
ROS: Reactive Oxygen Specie
S: Score
S_i and S_{i+1}: Consecutive clockwise individual biomarker scores
SMX: Sulfamethoxazole
SSW: Synthetic Swine Wastewater
tCOD: Total Chemical Oxygen Demand
TKN: Total Kjeldahl Nitrogen
TN: Total Nitrogen
Total Chl: Total Chlorophyll
TOPSIS: Technique for Order of Preference by Similarity to Ideal Solutions
TU: Toxicity Units
TUa: Toxicity Unit-Acute
TUc: Toxicity Unit-Chronic
VHI: Very High Influence
VLI: Very Low Influence
V_i^+: Ideal reference value point
V_i^-: Anti-ideal reference value point
V_{ij}: weighted normalized decision matrix
w^c: Compromised weight
w^s: Subjective (FAHP) weight
w^o: Objective (Entropy) weight
Zn: Zinc

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CHAPTER 1: INTRODUCTION AND OBJECTIVES

1.1 General Introduction

Human activities, including urban and industrial expansion, have increased the exploitation of water resources with impacts on aquatic ecosystems. Pollution, resulting from uncontrolled discharge of pollutants, compromises the quality of drinking water and harms freshwater organisms due to the release of wastewater (Häder et al., 2020).

While conventional methods can indeed enhance pollutant removal, the associated capital, operational, and maintenance costs are considerable. In light of this, there is a crucial need to explore environmentally friendly and cost-effective treatment technologies (Shahid et al., 2018). Bioremediation stands out as a sustainable and efficient alternative for reducing pollutant concentrations, whether through the involvement of plants or microorganisms (Mustafa and Hayder, 2021; Saeed et al., 2022).

Phytoremediation is a natural technology that harnesses plants to extract, sequester, or detoxify pollutants (Mustafa and Hayder, 2021). Its application in wastewater treatment is well-documented for removing potentially toxic elements (Rezania et al., 2016), nitrogen and phosphorus (Li et al., 2021), antibiotics (McCorquodale-Bauer et al., 2023), endocrine disruptors (Rajhi and Bardi, 2023) and even microplastics (Rozman et al., 2023). The phytoremediation advantages encompass low capital requirements, minimal energy consumption, potential biomass reuse, and a reduced carbon footprint (Mustafa and Hayder, 2021), although disadvantages may encompass phytotoxicity linked to the physical and chemical treatment conditions (Wang and Aghajani Delavar, 2023).

In phytoremediation in hydroponics, the root system or even the fronds of aquatic plants, associated with microorganisms, act as natural filters when coming into direct contact with contaminated water (Zhang et al., 2024). In this system, macrophytes employed in phytoremediation are categorized based on their structural habitat (biotope) or aquatic niche, which encompasses floating, submerged, or emergent habitats (Ekperusi et al., 2019).

Nevertheless, these plants may demonstrate notable sensitivity to specific wastewater types, leading to a reduction in their productivity (Akansha et al., 2020). Such wastewater, containing highly toxic compounds, can impede plant development and, consequently, affect treatment performance (Alkimin et al., 2020). Furthermore, reports indicate substantial variation among plant species in their capacity to remove pollutants (Wei et al., 2021), underscoring the potential for markedly improved pollutant removal through the careful selection of plant species aligned with favorable treatment conditions (Kumar Yadav et al., 2018).

However, this limitation can be mitigated through the careful selection of suitable plants and the implementation of strategies to modify and enhance the physical and aqueous environment. These measures aim to optimize plant performance and increase efficiency in pollutant removal. Strategies include modifications to the treatment system concerning operational factors that impact the growth/development of the macrophyte or enable plants to combat toxic compounds. This includes practices such as bioaugmentation by plant growth-promoting bacteria (Fahid et al., 2020), phytohormone (Yu et al., 2024), light supplementation (Walsh et al., 2021), artificial aeration (Xin et al., 2019), or phytoremediation assisted by microbial fuel cells (Colares et al., 2021), to name a few.

Therefore, it is crucial to understand the ecological niche (floating, emergent, submerged) that excels in pollutant removal from wastewater and the relationship between physical and aqueous environmental factors in the performance of macrophytes. Furthermore, among these niches, it is necessary to select a plant species that is more suitable for the effluent and treatment conditions. To achieve this, the selection of the species in wastewaters using multicriteria analysis methodologies is crucial. Moreover, factors such as photoperiod, LED light supplementation, and phytohormones, when combined, can enhance the removal of these pollutants and simultaneously increase plant biomass.

The thesis is structured in five chapters. In this first chapter, a general introduction and the objectives of the work are presented. In the second chapter, a review was conducted to assess the effectiveness of metal removal by different groups of macrophytes (submerged, emergent, or floating). In the third chapter, a multicriteria analysis tool was used to select a macrophyte for swine wastewater treatment. In the fourth chapter, a Doehlert-RSM matrix optimization was applied to the chosen macrophyte focusing on the removal of copper, zinc, organic matter, N, P, and the antibiotic sulfamethoxazole. In addition, plant biomass productivity was assessed through protein and lipid content after the treatments. In the fifth chapter, general conclusions and suggestions for future research are presented. Overall, the work addressed the use of multivariate statistical analyses in the selection, evaluation, and optimization of wastewater treatment through macrophytes using hydroponic phytoremediation. The second and third chapters have already been published in specialized scientific journals.[1] The choice and

[1] The second chapter was published in the journal "Toxics", volume 11(2), 158, 2023. The third chapter was published in the "Journal of Water Process Engineering", volume 53, July 2023, 103793, 2023.

motivation of research during the development of the thesis are described in section 1.2 (Thesis Overview), as follows.

1.2 Thesis' Overview

Our research group (Laboratório de Qualidade Ambiental/LQA, DEA/UFV) is dedicated to studying and implementing green technologies for wastewater treatment, particularly, but not limited to, those stemming from agro-industrial activities. Our goal is not only to treat these waters by removing organic matter, potentially toxic elements (heavy metals), nitrogen, phosphorus, and micropollutants but also to explore ways to recover resources from the sanitation process through bioenergy reuse and biofertilizers (fertigation), for example. Our studies involve the use of hydroponic phytoremediation systems, constructed wetlands, natural coagulants, and anaerobic digesters for treating wastewater and producing biogas. We are dedicated to creating efficient and environmentally responsible wastewater treatment solutions.

This doctoral research project focused on hydroponic phytoremediation. The study investigated how the selection of macrophytes and operational factors can affect both the efficiency of aquatic pollutant removal and the resulting biomass productivity. The phytoremediation system was seen as an integral part of a circular economy. There is a lack of studies in the current literature that address the systematic selection of the most suitable plant (macrophytes) for phytoremediation, taking into account operational factors, treatment conditions, types of wastewater, and specific pollutants present. This study aims to address this gap by emphasizing the need for a personalized approach to maximize the efficiency of hydroponic phytoremediation.

By being based on the principles of circular economy for wastewater treatment and sanitation resource recovery, the phytoremediation study developed in this thesis demonstrates a commitment to the Sustainable Development Goals, especially numbers 2, 6, 7, 12, and 13. The thesis is divided into five chapters. Chapter 1 consists of the general introduction, where the research problems addressed in the doctoral development were presented, including the details discussed in this section. The fifth chapter is the general conclusion and suggestions for future work, which serve as a reflection on the results obtained. In this chapter, the general conclusions derived from the study are presented, highlighting the most important findings and their implications. Additionally, suggestions for future research are offered, pointing out possible directions to extend or deepen the work done, to further contribute to the advancement of knowledge in the field.

The technical articles covered in Chapters 2, 3, and 4 are discussed and outlined below. The specific details including their design, deeper discussions, and findings are addressed in their relevant sections. In this section, I will present an overview of each of these chapters, highlighting their general objectives, the advances made, and the limitations encountered during the development of the thesis.

Chapter two, titled "Removing Mn, Cu and Fe from Real Wastewaters with Macrophytes: Reviewing the Relationship between Environmental Factors and Plants' Uptake Capacity," was developed during the author's doctoral qualification phase. The chapter investigates the capacity of macrophytes to remove and bioaccumulate iron (Fe), copper (Cu), and manganese (Mn) from real wastewater. This was achieved through a systematic literature review that looked into the influence of environmental factors from both the physical and aqueous environments on the plants' uptake capacity.

Various aquatic environmental factors were analyzed, including the initial concentrations of metals and levels of dissolved oxygen, nitrogen, and phosphorus. Some of the questions that were explored include: how do the initial concentrations of these metals impact the removal efficiency of Fe, Cu, and Mn? What is the influence of dissolved oxygen, nitrogen, and phosphorus on the removal and accumulation of these metals by plants? From the physical environmental factors, the study explored how treatment time (or exposure) and photoperiod affect metal removal and accumulation in plant tissues. Understanding these factors is critical to optimize phytoremediation under real field conditions and to develop effective treatment strategies.

Additionally, the three types of macrophytes used in wastewater phytotreatment were addressed, whose habitats can be floating, emergent, or submerged, to understand if metal accumulation and removal vary among these biotopes. he choice of metals was based on their environmental importance, as excessive amounts can be harmful and toxic despite being essential for plant metabolism. The disasters caused by the mining waste dams rupturing in Mariana (2015) and Brumadinho (2019) resulted in the release of large amounts of Iron (Fe) and Manganese (Mn) into waterways, highlighting the urgent need to find effective solutions to mitigate the environmental impacts of such catastrophic events.

The limitations of the study include the scarcity of available research, possibly due to restrictions and limitations of the studies themselves. Furthermore, the disparity in sample size between floating macrophytes, which are more commonly used in wastewater treatment by hydroponic phytoremediation, compared to emergent and submerged macrophytes, may have influenced the supposed advantage of emergent macrophytes. However, this study represents an important advancement as one of the first to systematically address the dynamics of metal removal, considering the capacity of the three macrophyte biotopes and the influence of environmental factors on the removal of iron, copper, and manganese from wastewater.

In Chapter 3, titled "Using wastewater treatment performance, biomass, and physiological plant characteristics for the selection of a floating macrophyte for phytoremediation of swine wastewater through the integrative Entropy-Fuzzy AHP-TOPSIS method", four species of floating macrophytes - *Lemna minuta*, *Landoltia punctata*, *Salvinia minima*, and *Azolla microphylla* - were employed to treat synthetic swine wastewater. The performance of these macrophytes in removing organic matter, nitrogen, phosphorus, copper, zinc, and ecotoxicity from wastewater was assessed. Additionally, biomass productivity was investigated by analyzing lipid, protein, and volatile solids content, as well as changes in photosynthetic pigments such as chlorophyll, carotenoids, anthocyanins, and specific relative growth caused by wastewater and experimental conditions.

The primary objective of this chapter was to select the most suitable species for treatment, considering aspects of water quality improvement, biomass production, and observed phytotoxicity during treatment. This was achieved through a systematic approach to macrophyte selection for wastewater treatment. For this purpose, a multicriteria analysis model called Entropy-Fuzzy AHP-TOPSIS was employed. The inputs considered were: treated water quality (focusing on pollutant removal and toxicity), amount of biomass produced (measured by lipids, volatile solids, and proteins), and phytotoxicity (changes in pigments during treatment). The goal was to select a plant that ideally exhibited the highest pollutant and toxicity removal, greater biomass production, and the least impact (lower phytotoxicity) caused by treatment.

In this multicriteria model, the selection of plants was based on the application of two weights. The first weight was calculated by entropy, which assessed variations in responses among the treatments by plants. It considered that the greater the variation, the greater the influence (weight) of the variable on the objective. The second weight was determined by expert opinion in an AHP (Analytic Hierarchy Process) analysis. In this analysis, experts were asked about the essential treatment aspects for choosing a plant for phytoremediation.

These aspects included water quality (evaluated by the removal of chemical oxygen demand, nitrogen, phosphorus, copper, zinc, and ecotoxicity), biomass produced (in terms of lipid, volatile solid, and protein content), and phytotoxicity aspects (changes in pigments). The assigned weights were transformed into Fuzzy numbers to mitigate any subjective bias in the selection. Subsequently, these weights were integrated and inserted into the TOPSIS

(Technique for Order of Preference by Similarity to Ideal Solution) model, which ranked the most appropriate species under the studied conditions. *Azolla microphylla* was chosen as the most suitable species for treatment as it obtained the highest ranking after the TOPSIS analysis

This study not only represents one of the first efforts for a thorough and systematic evaluation of plant selection for phytoremediation but also pioneers the application of objective (entropy) and subjective (Fuzzy AHP) approaches with the TOPSIS model. In addition, it is the first to quantify lipid and protein contents, aspects related to the quantity and quality of biomass produced in swine wastewater phytoremediation. Furthermore, this study marks the debut of *Azolla microphylla*'s use in swine wastewater treatment.

It is worth noting that although emergent macrophytes showed better responses in the previous chapter (Chapter 2), floating ones were chosen due to their local availability, easier management, and the desire to explore the potential of these widely studied macrophytes by our group. Finally, it was observed that phytotoxicity aspects play a crucial role in plant selection, representing a significant advancement since it is essential to investigate this aspect before using the plant on a real scale. However, it is important to consider other phytotoxicity aspects, such as enzymatic and non-enzymatic antioxidants, which were not addressed in this study.

Chapter 4, titled "Effects of LED Lights and Cytokinin on the Phytotreatment of Swine Wastewater by *Azolla* spp.: Pollutant Removal and Biomass Valorization," aimed to evaluate the effect of LED light (measured by light intensity and photoperiod) and the phytohormone cytokinin on the treatment capacity of *Azolla microphylla* in synthetic swine wastewater effluents. An innovation of this study is the combined use of LED light and cytokinin for the first time in evaluating phytoremediation of swine wastewater. The macrophyte species used was *Azolla microphylla*, chosen based on the results of the previous stage (Chapter 3). Biomass productivity and the levels of lipids, proteins, and carbohydrates were evaluated once again.

In this chapter, the use of factors such as light variation and phytohormones, which promote plant growth, is crucial to enhance phytoremediation for several reasons. Firstly, healthy plant growth can increase the rate of contaminant absorption from water, leading to more efficient pollutant removal. In this regard, the study investigated the impact of LED light exposure, measured by intensity and duration (photoperiod), along with the application of the exogenous phytohormone cytokinin, on the treatment of swine effluents by *Azolla microphylla* and its biomass production, within a resource recovery context. Utilizing the Doehlert matrix and Response Surface Methodology, the optimization of these factors led to low residual concentrations under optimal treatment conditions. It was observed that LED light and phytohormones affect pollutant removal differently. For instance, the removal of SMX antibiotic is highly dependent on the duration of light exposure, and the application of cytokinin only impacts SMX removal. On the other hand, nutrients such as nitrogen and phosphorus are more efficiently removed with extended light exposures.

This differential aspect of our study highlights the importance of adjusting treatment system operation to optimize specific pollutant removal, representing an advancement in understanding phytoremediation mechanisms. This work contributes to a deeper understanding of how light and phytohormones influence the removal of different types of pollutants in wastewater, going beyond merely identifying optimal treatment conditions and quantifying removal typically found in literature studies on the subject.

1.3 Objectives

General Objective

The general objective was to evaluate how operational factors and the use of different species of macrophytes influence the treatment of wastewater by phytoremediation in hydroponics with a focus on the removal of metals, antibiotics, nitrogen, phosphorus, and biomass produced in terms of lipids, carbohydrates and protein.

Specific objectives:

• To investigate the relationship between environmental factors (photoperiod, exposure time, dissolved oxygen, pH, nitrogen, phosphorus, and initial metal concentration) and the capacity of different macrophyte biotopes (emergent, floating, and submerged) in the removal and accumulation of iron (Fe), copper (Cu), and manganese (Mn) from wastewater (Chapter 2).
• To evaluate the treatment of synthetic swine wastewater using the macrophytes *Lemna minuta*, *Landoltia punctata*, *Salvinia minima*, and *Azolla microphylla*, and select, through the multi-criteria analysis, the most suitable macrophyte for treatment based on the presented quality of treated wastewater, biomass productivity, and phytotoxicity (Chapter 3).
• To optimize the removal of organic matter, copper, zinc, nitrogen, phosphorus and sulfamethoxazole from synthetic swine wastewaters by *Azolla microphylla* under the application of LED lights (in terms of photoperiod and intensity) and phytohormone cytokinin. Under optimized conditions, assess biomass production and starch, protein, and lipids (Chapter 4).

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CHAPTER 2: REMOVING Mn, Cu AND Fe FROM REAL WASTEATERS WITH MACROPHYTES: REVIEWING THE RELATIONSHIP BETWEEN ENVIRONMENTAL FACTORS AND PLANTS' UPTAKE CAPACITY

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Abstract: Heavy metal pollution creates environmental health concerns. Among these, iron (Fe), copper (Cu) and manganese (Mn) are commonly found in aquatic environments due to the release of wastewater. Phytoremediation in hydroponics uses macrophytes to treat contaminated environments, and this is influenced by environmental factors. However, the relationship between these factors and the removal of Fe, Cu and Mn by macrophytes is not known. Therefore, a meta-analysis serves to determine the correlations between environmental factors and the removal of these metals in real wastewater by macrophytes, as well as to identify the role of different aquatic forms of macrophytes in phytoremediation. Emergent macrophytes had higher concentrations of manganese in their tissues, and higher bioconcentrations factor of iron and manganese than floating plants. Regardless of the biotope, higher concentrations of Fe and Cu decreased the ability of plants to bioconcentrate them. The correlations among exposure time, pH, dissolved oxygen, nitrogen, phosphorus, photoperiod and metal phytoremediation by plants were also found. It can be concluded that the emergent macrophytes showed better performance in terms of the removal of Fe, Cu and Mn, and that the significant correlations between environmental factors and removal vary with the type of metal and the environmental factor analyzed.

Keywords: toxicity; phytoextraction; biotope; eco-friendly technology; full scale

1. INTRODUCTION

Urbanization, industrialization and increased extension of agricultural practices have been proportional to the increase in surface and/or groundwater pollution through the release of wastewater (Strokal et al., 2021). Heavy metals (HMs) are of great concern to human and natural health, as they are non-biodegradable, bioaccumulative and persistent pollutants in nature (Ali et al., 2013, 2020; Barbosa et al., 2010; Rezania et al., 2016). HMs are released into the environment through natural factors (e.g., rock weathering), but mainly through domestic and industrial activities such as mining, electroplating, metallurgy, textiles, battery manufacturing, tanneries, oil refining, paint manufacturing, pesticides, pulp and paper, printing and photographic industries (Zamora-Ledezma et al., 2021).

Iron (Fe), copper (Cu) and manganese (Mn) are HMs commonly found in aquatic environments. In proper concentrations, these metals play an essential role in plant metabolism (Broadley et al., 2011; Queiroz et al., 2021; Rehman et al., 2019; Rout and Sahoo, 2015). However, in inappropriate concentrations in the environment, these metals cause imbalances in metabolism and toxicity not only in plants but also in animals. For instance, studies that evaluated the metal average concentrations between 1972 and 2019 showed that concentrations of these three metals in rivers and lakes across the planet have more than doubled over the decades and are at levels well above the limits established by the World Health Organization (WHO) and by the United States Environmental Protection Agency (USEPA) (Kumar et al., 2019; Zhou et al., 2020). As there is no consensus in the literature on the definition of the term "heavy metals" (Duffus, 2002), here, we follow the recommendation of Appenroth (2010) (Appenroth, 2010), who stated that for plant sciences, the term "heavy metal" refers to those belonging to the transition metals, as is the case for Fe, Cu and Mn.

Some heavy metals, as they are not part of metabolism, are non-essential in living beings (e.g., Cr, Pb, As and Hg) (Tchounwou et al., 2012). Thus, in trace concentrations and under suitable conditions, they cause toxicity to aquatic organisms or humans. Therefore, they are cause for concern (Renu et al., 2017) and should be studied regarding their presence in the environment and their biological effects. However, due anthropogenic activities (industrial, domestic or agro-industrial), other metals are released in large quantities into the environment through wastewater. These metals, such as Fe, Cu, Mn, etc., are usually essential for living beings (Bhattacharya et al., 2016), although needed in microquantity. Nevertheless, in high concentrations, they are toxic to the natural environment (e.g., to aquatic biota) and, due to their bioaccumulative nature, they can biomagnify through the food web and be toxic to humans.

For instance, environmental and human toxicities have already been described for Fe, Cu and Mn. Iron accumulation in humans can cause hemochromatosis (an autosomal recessive disease) (Griffiths, 2015). In the aquatic environment, acute toxicity values (capable of causing death) of 6.7 mg/L have been reported in *Daphnia magna* (Okamoto et al., 2015). Manganese can produce neurological problems in humans (USEPA, 2004) and it has been shown that when inhaled by rats, it can cause damage to astrocytes in their central nervous system (Henriksson, 2000). Copper has been reported in fish species in an acute toxicity value of 14.61 µg/L (*Ptychocheilus oregonensis*) and chronic value (when it affects the organism's ability to reproduce) of 5.92 µg/L (*Oncorhynchus tshawytscha*) (USEPA, 2007). Thus, given the environmental importance of metals that, although essential to living beings, can be toxic in large quantities, we review the presence in the wastewater and discuss the ability to remove three of these metals (Fe, Cu and Mn) from wastewater.

Conventional technologies usually used in the removal of HMs include chemical precipitation, ion exchange, adsorption, membrane separation, coagulation–flocculation, photocatalytic degradation, flotation and electrochemical processes, which, though efficient, have disadvantages because of their high cost of implementation/operation and because they generate toxic by-products (Fu and Wang, 2011). It is, therefore, necessary to use technologies that are cleaner, less expensive and efficient in removing these pollutants.

Given this context, phytoremediation technology has become a viable and sustainable alternative, with recognized efficiency in the removal of aquatic pollutants, and with low implementation, operation and maintenance costs (Ali et al., 2020; Ansari et al., 2020). In addition, there is the possibility of recovering resources through the use of plant biomass in the production of energy or fertilizer (Kurniawan et al., 2021). It is a technology in which plants are used to remove, detoxify or immobilize contaminants from different environmental matrices, such as in contaminated water, soil or sediments (Wei et al., 2021).

In hydroponic phytoremediation, the root system or even the fronds of aquatic plants (macrophytes), in association with microorganisms, function as natural filters when they have direct contact with contaminated water (Chen et al., 2016; Colares et al., 2020; Olguín and Sánchez-Galván, 2012). In these systems, the macrophytes used in phytoremediation are classified by their aquatic structural habitat (biotope) or the aquatic form of the macrophyte, which can be floating, emergent or submerged (Ekperusi et al., 2019).

However, because of the concentration of contaminants in real wastewater, the occurrence of phytotoxicity can impede the phytoremediation of wastewater (Mustafa and Hayder, 2021). Despite this, the combination of the type of plant and environmental factors such as pH, photoperiod, nitrogen (N), phosphorus (P), dissolved oxygen (DO) and exposure time, which are recognized as promoting the growth and development of plants (Carr et al., 1997; Ramakrishna and Ravishankar, 2011), make such species able to better cope with these adverse situations, performing better in the removal of HMs. However, there are no reports of a systematic understanding of the existing relationship between the environmental factors and the removal of Fe, Cu and Mn by macrophytes.

A meta-analysis is an essential tool that makes it possible to aggregate information and discussions through a statistical analysis of the results from independent primary studies, providing support for conclusions about a studied phenomenon, with the capacity to contrast and identify patterns or sources of disagreement (Mikolajewicz and Komarova, 2019). There are few reports of this type of analysis in the area of phytoremediation of aquatic environments contaminated by metals (Li et al., 2015), as contaminated soils are studied more often (Audet and Charest, 2007; Huang et al., 2020; Tőzsér et al., 2017; Yu et al., 2018; Zhao et al., 2020). Furthermore, to best of our knowledge, there has been no study on the relationship of the environmental factors mentioned above and the removal of Fe, Cu and Mn from real wastewaters through phytoremediation by macrophytes.

Thus, the main objective of this study was to evaluate, through a meta-analysis, the relationship between the environmental factors and the capacity of macrophytes to remove and absorb Fe, Cu and Mn. The hypothesis was that the conditions of the physical and/or wastewater would influence the removal of these HMs from the wastewater and the absorption capacity of these plants, but in different ways, depending on the type of metal and the plants' aquatic habitat. For this, the specific objectives were to verify (i) the existing correlations between the conditions of the physical environment (photoperiod, exposure time) and wastewater (metal concentration in the wastewater ($[HM]_{wastewater}$) (pH, OD, N and P) on the capacity of different macrophyte types to absorb Fe, Cu and Mn, and (ii) whether the absorption of these three metals was different between floating macrophytes and those in emergent habitats.

2. MATERIAL AND METGODS

2.1. Literature Search and Data Selection

English-language publications were collected by searching the Scopus "http://www.scopus.com (accessed on 11 july 2022)" and Web of Science "http://apps.webofknowledge.com/ (accessed on 11 july 2022)" databases from January 1990 to 2022 (retrieved on 11 July 2022) to identify studies that had evaluated the use of macrophytes in the hydroponic phytoremediation of waters contaminated by the metals Fe, Cu and Mn. For this purpose, the following search terms were used: “macrophytes”, “phytoremediation”, “phytoextraction”, “heavy metals”, “metals”, “iron”, “Fe”, “copper”, “Cu”, “manganese”, “Mn”, “effluent”, “wastewater” and “wastewater treatment”.

The search string was (“macrophytes” AND (“phytoremediation” OR “phytoextraction”) AND (“heavy metals”) OR (“metals”) OR (“Fe”) OR (“iron”) OR (“Cu”) OR (“copper”) OR (“Mn”) OR (“manganese”) AND (“effluent”) OR (“wastewater”) OR (“wastewater treatment”)) from the search field TOPIC (Web of Science) or Title-ABS-Key (Scopus). Reviews, editorials, conference proceedings, books and book chapters were disregarded for further analysis.

The authors conducted a systematic literature review of the phytoremediation of real wastewater that involved removing Fe, Cu and Mn. The PRISMA methodology was used to identify the articles. It should be noted that prior screening of the articles (title/abstract screening) was performed using the StArt tool (Fabbri et al., 2016). The screening of articles by title/abstract was carried out in order to exclude articles that did not deal with phytoremediation in hydroponic systems, as well as overlapping (duplicates) or irrelevant articles. After that, all articles were read to select those that achieved the established criteria.

The selection of publications for the meta-analysis included the following criteria: (i) studies that evaluated the removal of Fe, Cu and/or Mn through phytoremediation of real wastewaters by macrophytes (submerged, emergent or floating) and (ii) studies that reported the concentrations of these metals in the wastewater (mg L^{-1}) and the tissues of aquatic plants ($[HM]_{plant}$, mg kg^{-1}).

Furthermore, we excluded studies that involved the biomonitoring of streams or lakes; those that did not provide or in which it was not possible to calculate the bioconcentration factor (BCF); those that studied, in association, two or more macrophytes in the same hydroponic tank; and those that used nonhydroponic constructed wetlands (CWs). The information extracted from the studies were BCF, $[HM]_{plant}$, percentage of metals removed from the water (%R), pH, $[HM]_{wastewater}$, N, P, photoperiod, DO and exposure time.

2.2. Meta-Analysis

For the analysis of data integration, it was assumed that the studies conducted in different locations with different plant growth habits were independent (Li et al., 2015). Studies that tested more than one species or type of wastewater but were not dependent were included in the analyses. Macrophytes were categorized into biotopes (emergent, submerged and floating) and into different families (USDA, 2020) to determine differences among them in the removal of Fe, Cu and Mn from the real wastewater. For the studies in which no BCF values were provided, these were determined according to (Rezania et al., 2016) as shown in Eq. (1).

$$BCF=\frac{\text{Metal concentration in the plant tissues (mg L}^{-1}\text{)}}{\text{Metal concentration in the wastewater (mg L}^{-1}\text{)}} \quad (1)$$

Moreover, for studies that provided values of BCF for the roots (BCF_{root}) and the aerial parts (BCF_{aerial}), the sum of BCF_{root} and BCF_{aerial} was considered to be the plant's BCF (Parwin and Karar Paul, 2019). When this was the case, stem and leaf parts were considered to be aerial parts of the plant. Nitrogen (N) and phosphorus (P) concentrations reported in different formats (nitrate, ammonium, ammoniacal nitrogen and phosphate) were converted to their respective molar concentrations of N (mmol N L^{-1}) or P (mmol P L^{-1}). The removals of metals from the wastewater, when not provided, were calculated according to Eq. (2):

$$\%\,R = \frac{C_i - C_f}{C_i} \times 100 \quad (2)$$

where C_i is the initial metal concentration in the wastewater, in mg L^{-1}; C_f is the final metal concentration in the wastewater, in mg L^{-1}; and %R represents the total removal of metals from the wastewater.

2.3. Statistical Data Analysis

To eliminate the differences caused by the experimental conditions, the species, the initial metal concentration and other sources of variation, the BCF and $[HM]_{plant}$ data were transformed to a Neperian logarithm (ln). This transformation allowed the observed variability to be compressed but did not change the relationship between the observed points (Li et al., 2015). The data of ln (BCF), $\ln[HM]_{plant}$ and the percentage of metal removed (%R) were correlated with the environmental factors ($[HM]_{wastewater}$, pH, exposure time/experimental duration, N, P, DO and photoperiod) according to Spearman's monotonic correlations (r_s) at the 5% significance level (Croux and Dehon, 2010; Lovie, 1995).

The normality of the distribution (Shapiro–Wilk, $\alpha = 0.05$) was checked beforehand. Correlations were examined when there were at least seven pairs of interactions and were always for the entire plant regardless of the type of structural habitat (May and Looney, 2020). Fisher's transformation Z test ($\alpha = 0.05$) was used to compare the r_s coefficients when they were significant (Diedenhofen and Musch, 2015).

The species were categorized into emergent and floating biotopes, and differences in their Fe, Cu, and Mn uptake were verified. For this purpose, comparisons were made between the two biotopes in terms of the uptake, ln (BCF) and $\ln [HM]_{plant}$ for the same metal according to the nonparametric Mann–Whitney test ($\alpha = 0.05$). Data in graphs and figures were extracted using Image J software "https://imagej.nih.gov/ij/ (accessed on 11 July 2022)", and statistical analyses were performed with Graph Prism v.6 (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. Literature Search

In total, 225 publications were selected from Scopus (72 publications) and Web of Science (153 publications). Of this total, only 13 studies met the selection criteria (Section 2.1). These thirteen studies were chosen because they were the only ones that met the selection criteria discussed in section 2.1. All studies were concentrated in Asia, with most conducted in India (76.9%), followed by Pakistan, Malaysia and Japan, with one study each (Table 1). The wastewaters studied were domestic (municipal and graywater) and industrial (pulp and paper, paper, palm oil, mining and metallurgy). In addition, 13 families (Table 1) and 16 species divided into the emergent (8), floating (7) and submerged (1) biotopes were reported in the selected studies.

The species *Lemna minor* (Lemnaceae) and *Pistia stratiotes* (Araceae) were the most frequently studied, with four studies each. In addition, the concentrations of the three metals in the wastewaters ranged across 0.104 - 22.91, 0.032 - 4.64 and 0.007 - 230 mg L^{-1} for iron, copper and manganese, respectively. Some studies performed phytoremediation in more than one type of wastewater but independently (Bokhari et al., 2016; Parwin and Karar Paul, 2019) (Table 1).

3.2. Accumulation of Fe, Cu and Mn in Plant Tissues

Overall, the bioconcentration factors, expressed as ln (BCF), and the concentrations of metals in plant tissues, expressed as ln $[HM]_{plant}$, presented great variability among the macrophytes and for each type of metal analyzed. Except for the emergent macrophytes that took up manganese, for which the coefficient of variation (CV) was 5% and 33% for ln $[HM]_{plant}$ and ln (BCF), respectively, all the others showed high CVs, some higher than 300%, as in the case of ln $[HM]_{plant}$ for the floating macrophytes used for phytoremediation of copper-contaminated waters. Despite this, all statistical analyses were performed with all data, without excluding the outliers.

The most and least frequently reported metals were copper and manganese, respectively (Table 1). The ln (BCF) data of the selected macrophyte species varied according to the type of metal and the type of family used in the study (Figure 1a, b). In general, the emergent macrophyte families showed higher ln (BCF) values for all three metals, although some species were reported only once.

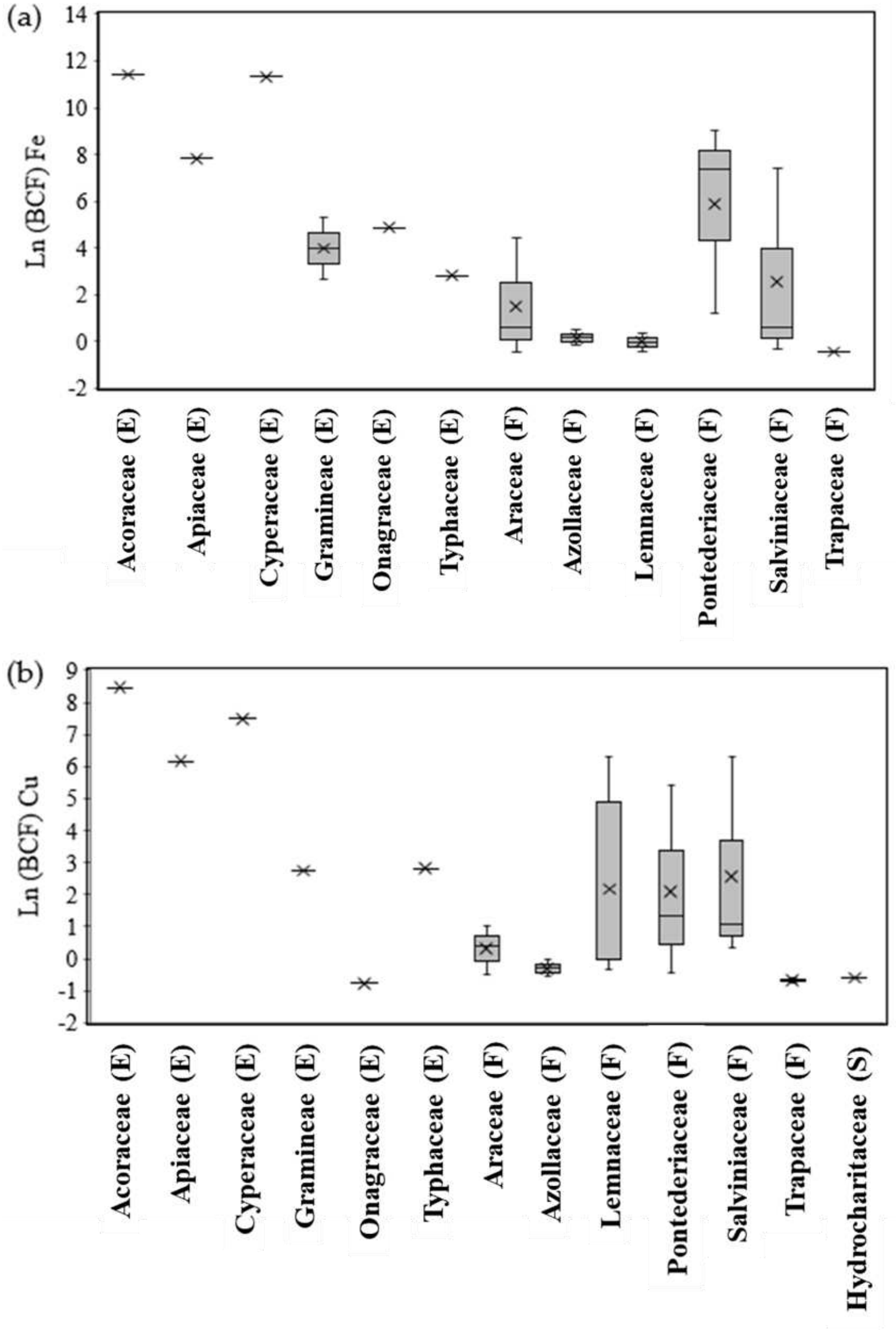
Table 1. Summary of some information extracted from the selected studies to evaluate Fe, Cu and Mn uptake capacities

Study	Country	Family	Initial metal concentration in the wastewater (mg L^{-1})			Removal (%)	Time of exposure (days)	Ref.
			Fe	Cu	Mn			
1	India	*Pontederiaceae*	0.15–0.98	0.062	/	-55.5-68.3 (Fe); 100 (Cu)	28	(Parwin and Karar Paul, 2019)
2	Pakistan	*Lemnaceae*	/	0.03–0.06	/	92.2-94.5 (Cu)	31	(Bokhari et al., 2016)
3	India	*Azollaceae, Lemnaceae*	22.91	2.04	9.61	93.1-95.4 (Fe); 93-98.8 (Cu); 98-99.5 (Mn)	7.0	(Bharti and Banerjee, 2012)
4	India	*Araceae, Pontederiaceae, Salviniaceae*	9.01	0.56	14.2		10	(Lakra et al., 2019)
5	India	*Apiaceae*	4.15	1.32	2.56	43.4 (Fe); 52.2 (Cu); 5.8 (Mn)	28	(Mazumdar and Das, 2021)
6	India	*Araceae, Lemnaceae Pontederiaceae, Rapaceae, Onagraceae, Hydrocharitaceae*	/	0.23	/	21.7,30.4, 60.8, 63.6, 71.4 (Cu)	20	(Mishra et al., 2013)
7	Japan	*Acoraceae, Cyperaceae*	0.10	0.14	0.01	7.7-13.5 (Fe); 1.5-5.2 (Cu); 1.5-7.1 (Mn)	85	(Soda et al., 2012)
8	India	*Azollaceae, Lemnaceae*	0.76	1.43	4.96	70-74 (Fe); 86-97 (Cu); 94-96 (Mn)	7.0	(Vaseem and Banerjee, 2012)
9	India	*Salviniaceae*	3.9	0.68	230	33.8 (Fe); 17.7 (Cu); 2.6 (Mn)	28	(Das and Mazumdar, 2016)
10	Malaysia	*Araceae, Gramineae, Onagraceae*	5	/	/	/	14	(Hamzah et al., 2016)
11	India	*Trapaceae*	6.75	4.64	2.16	41 (Fe); 37.7 (Cu); 60 (Mn)	60	(Kumar and Chopra, 2018)
12	India	*Gramineae, Typhaceae*	0.15	0.11	/	/ (Fe); 66.5-68.6 (Cu)	14	(Kumari and Tripathi, 2015)
13	India	*Araceae, Salviniaceae*	18.21	0.93	8.47	94.1-94.3 (Fe); 74.2-96.7(Cu); 96.2-98.5 (Mn)	10	(Lakra et al., 2017)

There were no significant differences in ln (BCF) and ln $[HM]_{plant}$ values among roots, aerial parts (leaves/stems) and whole plants for each metal, according to the Kruskal–Wallis nonparametric test at 5% significance (data not shown). However, other studies demonstrate differences in metal accumulation between the aerial parts and the roots (Shahid et al., 2017; Shojaei et al., 2021), which contradicts our findings. This discrepancy, besides being species-specific, is inherently associated with the type of treated wastewater and the metal species under study (Khalilzadeh et al., 2021). For instance, it has been reported that non-essential metals for plant metabolism are typically adsorbed onto the roots to prevent toxicity (Bonanno, 2011).

The ln (BCF) data for iron, copper and manganese showed, regardless of the family type or biotope, means (μ) of 3.462 (n = 21, median = 2.756, minimum = - 0.555, maximum = 9.998), 2.008 (n = 25, median = 0.380, minimum = - 0.777, maximum = 8.455) and 2.122 (n = 14, median = 0.113, minimum = - 0.146, maximum = 8.421), respectively.

For the differences in the three metals among families, ln (BCF) values higher than 6.9 (BCF > 1000) were observed for the families Acoraceae (*Acorus gramineus*), Cyperaceae (*Cyperus alternifolius L.*) and Apiaceae (*Centella asiatica*), which were reported only once, in works that studied the removal of these metals in wastewaters from the pulp and paper industry (Mazumdar and Das, 2021) or metallurgy (Soda et al., 2012) (Figure 1a–c). On the other hand, among the floating species and for the metal iron, the Pontederiaceae family, represented by *Eichhornia crassipes*, although they presented ln(BCF) values below 6.9 on average (Figure 1a), there was a case in which this species had ln(BCF) > 6.9 when used for the phytoremediation of graywater (Parwin and Karar Paul, 2019).


(a)
Ln (BCF) Fe
14
12
10
8
6
4
2
0
-2
Acoraceae (E)
Apiaceae (E)
Cyperaceae (E)
Gramineae (E)
Onagraceae (E)
Typhaceae (E)
Araceae (F)
Azollaceae (F)
Lemnaceae (F)
Pontederiaceae (F)
Salviniaceae (F)
Trapaceae (F)
(b)
Ln (BCF) Cu
9
8
7
6
5
4
3
2
1
0
-1
-2
Acoraceae (E)
Apiaceae (E)
Cyperaceae (E)
Gramineae (E)
Onagraceae (E)
Typhaceae (E)
Araceae (F)
Azollaceae (F)
Lemnaceae (F)
Pontederiaceae (F)
Salviniaceae (F)
Trapaceae (F)
Hydrocharitaceae (S)

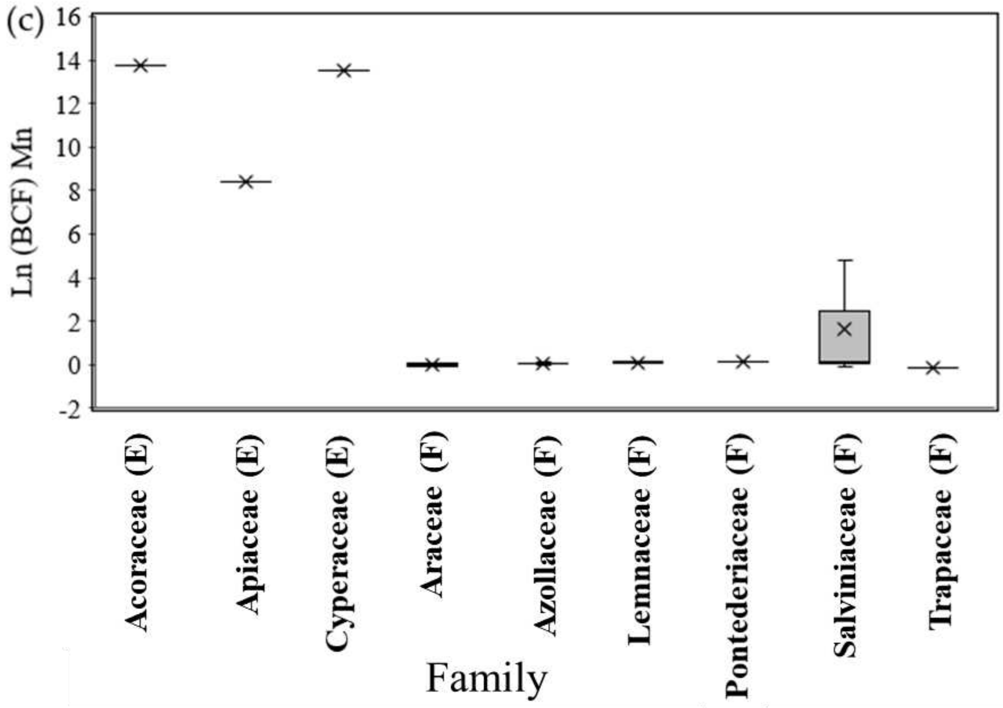
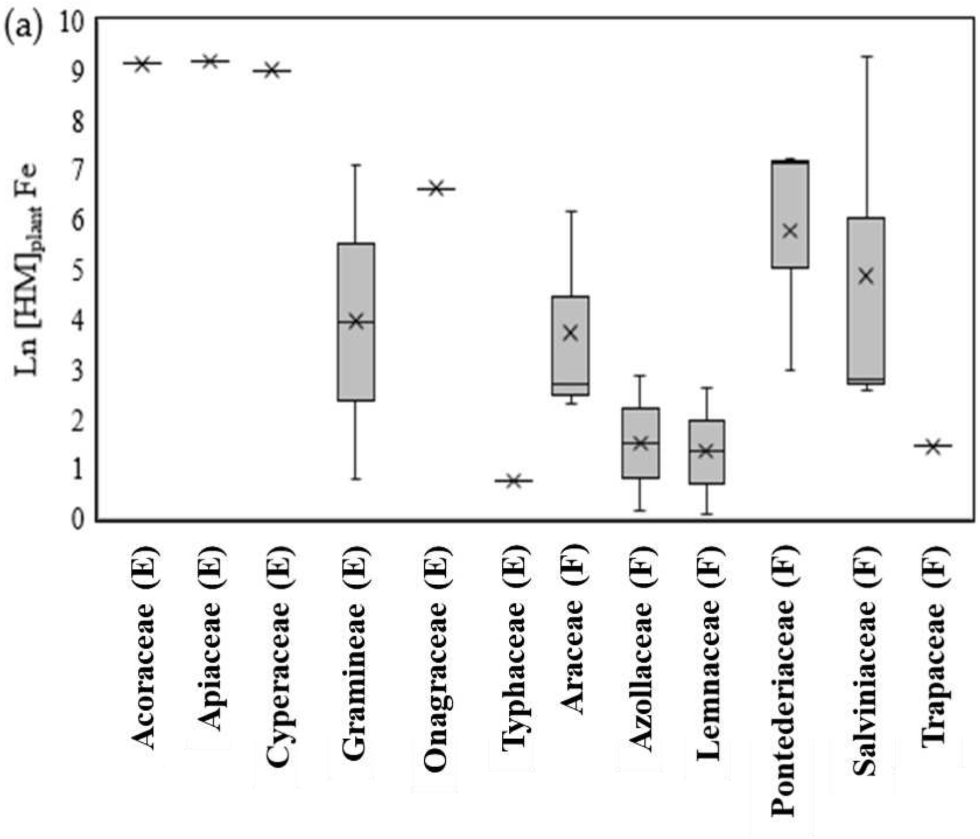


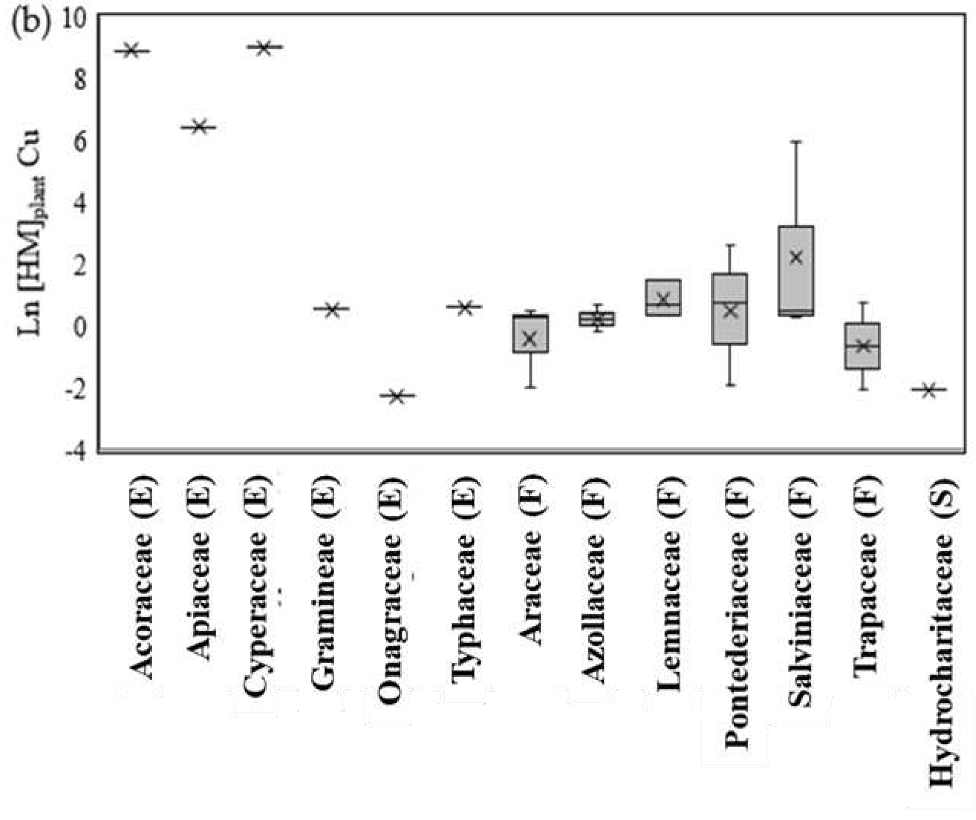
Fig. 1. Boxplot of bioconcentration factors (BCF), expressed as ln, for iron (Fe) (**a**), copper (Cu) (**b**) and manganese (Mn) (**c**). The symbol "X" represents the mean. (E) Emergent families of macrophyte; (F) floating families of macrophyte; (S) submerged families of macrophyte

The concentrations of metals in plant tissues, expressed as ln $[HM]_{plant}$, also varied with the type of metal and the type of family used in the study (Fig. 2a, b). Similar to ln (BCF), emergent macrophytes showed higher ln $[HM]_{plant}$ values for all three metals, although some were reported only once.

The concentrations of metals in plants, ln $[HM]_{plant}$, for iron, copper and manganese showed, regardless of the family type or biotope, means (μ) of 4.44 (n = 21, median = 2. 938, minimum = 0.12, maximum = 9.253), 1.268 (n = 25, median = 0.546, minimum = - 2.246, maximum = 8.997) and 4.134 (n = 14, median = 2.453, minimum = 0.624, maximum = 10.240), respectively.

For the differences among families, there were concentrations below 1.0 only for copper (ln $[HM]_{plant} < 0$) and mainly among the floating macrophytes (Figure 2b). Again, the highest ln$[HM]_{plant}$ values occurred for the families Acoraceae (*Acorus gramineus*), Cyperacea*e* (*Cyperus alternifolius L.*) and Apiaceae (*Centella asiatica*) in studies on the removal of these metals from the pulp and paper industry (Mazumdar and Das, 2021) or metallurgy (Soda et al., 2012) effluents (Fig. 2a–c).


(a)
Ln [HM]plant Fe
10
9
8
7
6
5
4
3
2
1
0
Acoraceae (E)
Apiaceae (E)
Cyperaceae (E)
Gramineae (E)
Onagraceae (E)
Typhaceae (E)
Araceae (F)
Azollaceae (F)
Lemnaceae (F)
Pontederiaceae (F)
Salviniaceae (F)
Trapaceae (F)


(b)
Ln [HM]plant Cu
10
8
6
4
2
0
-2
-4
Acoraceae (E)
Apiaceae (E)
Cyperaceae (E)
Gramineae (E)
Onagraceae (E)
Typhaceae (E)
Araceae (F)
Azollaceae (F)
Lemnaceae (F)
Pontederiaceae (F)
Salviniaceae (F)
Trapaceae (F)
Hydrocharitaceae (S)

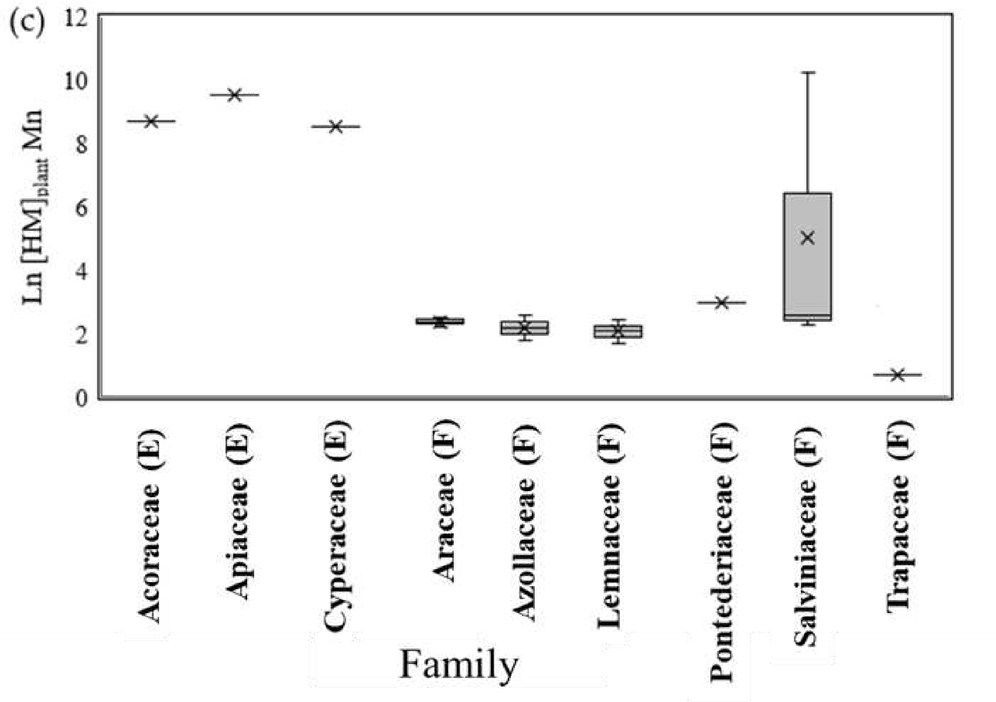


Fig. 2. Boxplot of the concentrations of HMs in plant tissues, expressed in ln, for iron (**a**), copper (**b**) and manganese (**c**). The symbol "X" represents the average. (E) Emergent families of macrophyte. (F) Floating families of macrophyte. (S) Submerged families of macrophyte.

The species were subgrouped into floating and emergent biotopes and showed differences between the two biotopes in the ranked mean comparison values (mean rank) of ln (BCF) and ln $[HM]_{plant}$ for metal uptake according to the Mann–Whitney test at 5% significance (Table 2). The submerged macrophyte *Hydrilla verticillata* (Hydrocharitaceae) was not considered because it was reported in only one study (ln (BCF): - 0.598; ln $[HM]_{plant}$: - 2.068) (Mishra et al., 2013).

Table 2. Comparison between emergent and floating biotopes in the absorption of Fe, Cu and Mn

Comparison	ln (BCF)			ln $[HM]_{plant}$		
	Mean Rank	U	*p*-Value	Mean Rank	U	*p*-Value
Floating–Fe vs. Emergent–Fe	8.64 15.71	16.0	0.012 *	9.86 13.29	33.0	0.247 ^{ns}
Floating–Cu vs. Emergent–Cu	11.06 16.83	28.0	0.089 ^{ns}	11.39 15.83	34,0	0.199 ^{ns}
Floating–Mn vs. Emergent–Mn	6.00 13.00	0.0	0.005 *	6.27 12.00	3.0	0.038 *

^{ns} Not significant. * Significant according to the Mann–Whitney test (p < 0.05). $N_{floating}$: 14 (Fe), 18 (Cu) and 11 (Mn). $N_{emergent}$: 7 (Fe), 6 (Cu) and 3 (Mn).

To sum up, emergent macrophytes had the highest ranked mean ln (BCF) values relative to floating macrophytes, with significant differences ($p < 0.05$) between the two biotopes for iron and manganese (Table 2). Furthermore, the U = 0 statistic between floating and emergent species in the ln (BCF) value for manganese indicates that all ln (BCF) values for the emergent macrophytes were greater than those for the floating ones. For ln $[HM]_{plant}$, the emergent species also showed the highest mean ranks compared with the floating species, but the emergent species tended to have significantly higher ln $[HM]_{plant}$ values ($p < 0.05$) only for manganese when compared with the floating species.

3.3. Correlations between environmental factors and the removal and concentration of metals in Plant Tissues

The conditions of the physical and wastewater environment (environmental factors), reported by the parameters $[HM]_{wastewater}$, pH, exposure time (t), N, P and DO concentrations, and the photoperiod was correlated with the responses ln (BCF), ln $[HM]_{plant}$ and %R (Table 3). The exposure time parameter obtained more significant Spearman correlation coefficients (r_s) ($p \leq 0.05$), with positive values of 0.727 (ln (BCF)) and 0.649 (ln $[HM]_{plant}$) for iron and 0.462 (ln $[HM]_{plant}$) for copper. On the other hand, for the photoperiod, there was a strong negative correlation with at least one of the three measured responses for all three metals, with r_s values between - 0.437 and - 0.760 for Fe, Cu and Mn (Table 3). Spearman coefficients, positive or negative, are considered to be strongly correlated or directional when they are between ± 0.5 and ± 1.0 (Adhianto et al., 2010).

Among the metals, there were more significant r_s coefficients ($p \leq 0.05$) for copper, which correlated positively or negatively with all factors except for nitrogen in terms of ln (BCF), ln $[HM]_{plant}$ or %R. Furthermore, for iron, only the exposure time and the concentration of the metal in the wastewater influenced the values of %R, ln (BCF) and ln $[HM]_{plants}$. For manganese, only pH, exposure time and photoperiod correlated significantly with at least one of the measured responses ln (BCF), ln $[HM]_{plant}$ or %R. In this case, the smaller number of environmental factors with significant correlations for Fe and especially for Mn could be attributed to the smaller sample sizes (Audet and Charest, 2007; Royall, 1986).

Table 3. Spearman's correlation coefficients (r_s) for the factors of metal concentration in the wastewater ([HM]wastewater), pH, time, nitrogen, phosphorus, DO (dissolved oxygen) and photoperiod with the ln of the bioconcentration factor (BCF), the metal concentration in the plant ([HM]plant) and the percentage of metal removed (%R) from the wastewater.

Factors	Fe			Cu			Mn		
	ln (BCF	ln $[HM]_{plant}$	%R	ln (BCF)	ln $[HM]_{plant}$	%R	ln (BCF)	ln $[HM]_{plant}$	%R
$[HM]_{wast}$	**-0.703** ^{a}	-0.184 ^{ns}	**0.876** ^{a}	**-0.422** ^{a}	-0.117 ^{ns}	0.146 ^{ns}	-0.233 ^{ns}	0.179 ^{ns}	0.366 ^{ns}
pH	0.366 ^{ns}	0.368 ^{ns}	-0.374 ^{ns}	-0.212 ^{ns}	-0.226 ^{ns}	**-0.616** ^{a}	**0.539** ^{a}	0.303 ^{ns}	**-0.543** ^{a}
t	**0.727** ^{a}	**0.649**	**-0.759** ^{ab}	0.380 ^{ns}	**0.462** ^{a}	**-0.574** ^{a}	0.431 ^{ns}	0.526 ^{ns}	**-0.759** ^{a}
N	0.254 ^{ns}	0.485 ^{ns}	-0.441 ^{ns}	-0.138 ^{ns}	0.229 ^{ns}	-0.465 ^{ns}	/	/	/
P	0.036 ^{ns}	0.291 ^{ns}	0.103 ^{ns}	**0.601** ^{a}	**0.842** ^{ab}	0.126 ^{ns}	/	/	/
DO	0.127 ^{ns}	0.461 ^{ns}	-0.438 ^{ns}	**0.506** ^{a}	**0.526** ^{b}	0.038 ^{ns}	0.410 ^{ns}	0.148 ^{ns}	-0.48 ^{ns}
Phot	0.220 ^{ns}	-0.235 ^{ns}	**-0.437** ^{b}	**-0.596** ^{a}	**-0.627** ^{b}	0.450 ^{ns}	**-0.760** ^{a}	**-0.755**	-0.25 ^{ns}

Units: $[HM]_{wast}$ ($[HM]_{wastewater}$) and DO (dissolved oxygen), mg L^{-1}; t (time of exposure/experiment), days; Phosphorus (P) and Nitrogen (N), mmol L^{-1}; photoperiod, hours. The significant correlation coefficients between environmental factors (factors) with ln (BCF), ln $[HM]_{plant}$ or %R (*p*-values less than or equal to 0.05) are indicated with a positive or negative sign and emphasized in bold. At the same time, different letters within each column indicate significant differences among the correlation coefficients according to Fisher's transformation Z-comparison test with $p < 0.05$. ^{ns}, not significant. The symbol (/) indicates that the calculation of r_s was not possible (n < 7).

For the macronutrient's nitrogen and phosphorus, only the concentration of phosphorus and copper correlated ($p < 0.05$), with the metal concentration in plant tissues showing positive rs values of 0.601 for ln (BCF) and 0.842 for ln $[HM]_{plant}$. The same behavior was obtained for dissolved oxygen, with coefficients of 0.506 for ln (BCF) and 0.526 for the correlation with ln $[HM]_{plant}$. There were no correlations between the parameter of nitrogen concentration with ln (BCF), ln $[HM]_{plant}$ or %R for any of the metals.

A strong positive correlation ($p \leq 0.05$) was obtained between $[HM]_{wastewater}$ and %R for iron, with $r_s = 0.876$. On the other hand, the strongest negative correlation ($p < 0.05$) was obtained between photoperiod and ln (BCF) for manganese with $r_s = - 0.760$. Comparisons of the order of magnitude between the significant coefficients indicated differences between the coefficients of photoperiod (- 0.437) and $[HM]_{wastewater}$ (0.876) and %R for the metal Fe ($p < 0.05$). On the other hand, for the ln $[HM]_{plant}$ of the metal Cu, there were differences ($p < 0.05$) between the correlation coefficients of DO (0.526), photoperiod (- 0.627) and time (0.462) (Table 3).

4. DISCUSSION

4.1. Accumulation of Fe, Cu and Mn in Plant Tissues

The concentration of metals in plants, expressed as $\ln[HM]_{plant}$, indicates how much of the metal was present in the plant tissue at harvest time (Audet and Charest, 2007). However, this concentration alone is not enough to determine the real capacity of plants to absorb metals into their tissues (Vymazal, 2016). A plant can have a high concentration of the metal in its tissues but not necessarily have a high capacity to remove it from the wastewater during the exposure time. Therefore, the bioconcentration factor, expressed here as ln(BCF), best represents the relative absorption of the plant for concentrating metal from the wastewater (Olguín and Sánchez-Galván, 2012).

Overall, among the families, emergent macrophytes showed the highest mean values of ln (BCF) and ln $[HM]_{plant}$ for all three metals, as shown in Figures 1a–c and 2a–c. This biotope, however, was comparatively less frequently reported than the floating biotope. In hydroponic phytoremediation systems, floating macrophytes are often used, given their greater ease of management and operation (Ali et al., 2020).

However, the individual performance of a macrophyte in the phytoremediation of contaminated water may vary depending on the type of wastewater and the environmental conditions of the experiments (Olguín and Sánchez-Galván, 2012). When we grouped the families into the emergent and floating biotopes to compare their performance (Table 2), the mean values of ln (BCF) for Fe and Mn and ln $[HM]_{plant}$ for Mn of the emergent species were higher than those of the floating ones, indicating that the former were more effective in the uptake of Fe and Mn compared with the latter group for the phytoremediation of real wastewater, except for copper.

Root architecture is an important factor that improves water quality (Nikolakopoulou et al., 2018) and plants with more roots can assist the development of mycorrhizal fungi, bacteria or algae. These organisms occur in symbiosis with macrophytes' roots and can improve the plants' uptake of pollutants (Akhtar et al., 2021). Thus, as the calculated BCF value did not distinguish between the accumulation in the roots and in the aerial parts (Section 2.2), it is possible that the roots of the selected emerging macrophytes contributed to the higher value of ln (BCF) than floating macrophytes. Macrophytes from emergent habitats are recognized for having voluminous roots (Nikolakopoulou et al., 2018).

Furthermore, among the metals, copper and manganese presented the lowest mobility from the wastewater to the plant tissues, with the lowest mean values of ln (BCF). Except for the macrophytes with emergent habits, all floating macrophytes presented ln (BCF) values lower than 6.9, indicating the low uptake capacity of these two metals by these macrophytes. An ln (BCF) value of less than 6.9, which corresponds to a BCF value of <1000, indicates the low capacity of plants to bioconcentrate an element in their tissues (Olguín and Sánchez-Galván, 2012).

The concentration of copper in plant tissues depends on the type of plant, its stage of development and environmental factors (Yruela, 2009). Furthermore, in wastewater, copper ionizes and forms complexes with the organic and inorganic matter present in the wastewater, which may affect its bioavailability to plants (Costa et al., 2018). Manganese is poorly mobile in plants and its oxidized form (Mn^{2+}), in which plants require it, is sensitively dependent on the optimal supply regulated by Mn transporters in the rhizosphere (Alejandro et al., 2020). In addition, although it has seldom been studied in aquatic systems, in multi-element environments, there may be competition for active binding sites on the macrophytes, increasing or decreasing the uptake of some metals relative to others (Broadley et al., 2011; Hua et al., 2012).

Finally, it should be noted that, although phytoremediation is a promising method for controlling pollution, the biomass produced by the remediation process must be managed; otherwise, it will eventually return to the environment and cause secondary pollution. In the literature, there are some routes for the disposal of biomass after phytoremediation. In all of them, volume reduction is essential for proper recovery of heavy metals.

For instance, in the incineration (e.g., combustion process), biomass can be used for energy generation and the remaining ash (which contains heavy metals) can be used as phyto(bio)ore (Kumar Yadav et al., 2018) or discarded under controlled conditions. Another solution is composting, through which, although it reduces the volume of biomass, high amounts of heavy metals or the presence of certain HMs make the agricultural use of the compost unfeasible (Song and Park, 2017). In this sense, stabilization/inertization techniques have been used, such as mixing with other materials or adding lime to reduce the leachability of metals (Kovacs and Szemmelveisz, 2017). Regarding leaching, this can be obtained by treating the compacted biomass with solvents in which the metallic components are extracted from the leachate and disposed of under suitable conditions (Vocciante et al., 2019). Therefore, the fate of these plants after their use in wastewater treatment should be part of the study for the implementation of the phytoremediation technique.

4.2. Correlations between Environmental Factors and the Removal and Concentration of Metals in Plant Tissues

The bioconcentration of Fe and Cu by plants indicates that the phytoextraction of these metals is less effective as their concentrations in the wastewater increase (Table 1 and Table 3). This may occur when, at high concentrations, the metabolic costs to plants of absorbing them are high (Audet and Charest, 2007) or as a result of the control that plants have in regulating the levels of metals in their plant cells in situations with high concentrations of heavy metals in the wastewater (Connorton et al., 2017; Printz et al., 2016). Furthermore, in the context of exposure to complex environments, such as in real wastewater, plants may expend more energy in counteracting possible oxidative stress than on the uptake of HMs (Antoniadis et al., 2017; Demidchik, 2015).

Interestingly, the exposure time showed an opposite relationship between the removal of metals from the wastewater and their absorption in plant tissues. For all three metals, the removal (%R) of metals was higher in tests with shorter durations (Table 1 and Table 3). On the other hand, for Fe and Cu, an increase in ln $[HM]_{wastewater}$ or ln (BCF) occurred with longer durations (Table 3). Plants, when exposed to a new adverse condition, may undergo an adaptation time before they begin remediation (Olguín and Sánchez-Galván, 2012). In high-toxicity environments, the oxidative stress defense system is triggered until the plants can adapt to the new environment and remediate it (Demidchik, 2015). In hydroponic phytoremediation systems, besides the uptake by plants, bacteria and algae, abiotic mechanisms such as binding to suspended solids and precipitation as insoluble compounds are among the main mechanisms of heavy metal removal (Wei et al., 2021). Thus, it might be that at the beginning of the tests, the metals are removed (%R) from the wastewater preponderantly through the action of other mechanisms such as rhizobacteria, but not by the plants.

The pH value is one of the most important factors influencing the bioavailability of metals in plants (Muthusaravanan et al., 2018), and small changes in this value can cause an increase or decrease in the concentrations by an order of magnitude compared with the surrounding environment (Król et al., 2020). Although the efficiency of metal uptake by plants varies in terms of the ideal pH range, in general, at a higher pH, metal ions form insoluble oxides, such as hydroxides, and plants are unlikely to take them up (Li et al., 2015). On the other hand, at a lower pH, there is an increase in the concentration in the medium and increased availability to the plants (Broadley et al., 2011; Król et al., 2020). In the selected studies, the vast majority of wastewater had neutral to alkaline pH ranges, which are likely to lead to negative correlations between the pH values and plant uptake values. However, there were negative correlations ($p \leq 0.05$) between pH and the removal (%R) of Cu and Mn, which indicates that greater removal of these metals occurs at lower pH values.

For manganese, however, the positive correlation ($p < 0.05$) between pH and ln (BCF) indicated that plants are also able to remove and bioconcentrate this metal in their plant tissues at an alkaline pH. For cationic species, such as Mn^{2+} (plant-available fraction), higher pH values result in lower mobility and, therefore, lower availability to plants. This is because as the pH increases, the hydrolysis of the metal increases, forming insoluble compounds. Moreover, at a higher pH, the electro-negative charge on the surfaces of the variable-charge colloids present in the wastewater also increases, which causes these metals to be retained (Antoniadis et al., 2017). However, increased metal availability may occur at higher pH values, which can be attributed to the precipitation of its soluble forms in carbonate fractions, which are mobilized in the acidic rhizosphere zone of the plants (Shaheen et al., 2017).

The bioconcentration factor does not distinguish between metal uptake and what can be adsorbed on the plants' root surface (Olguín and Sánchez-Galván, 2012), although it is customary to evaluate the absorption capacity of plants using the bioconcentration factor. In this sense, other indices should be considered for a better evaluation of absorption, such as the tolerance index (TI), the adsorption factor (AF) and the translocation factor (TF) (Olguín and Sánchez-Galván, 2012).

Phosphorus and dissolved oxygen correlated significantly ($p \leq 0.05$) with the ln (BCF) and ln $[HM]_{plant}$ of copper. On the other hand, in none of the metals were there any correlations ($p \leq 0.05$) of N with the variables of ln (BCF), ln $[HM]_{plant}$ and (%R), which indicates that nitrogen does not seem to affect the removal and/or uptake of Fe, Cu and Mn from real wastewater treated by macrophytes.

Dissolved oxygen, by increasing aeration in the rhizosphere region, plays an essential role in supporting the growth of aerobic bacteria (Mishra et al., 2013). These bacteria increase the capacity of plants to absorb heavy metals by changing their uptake properties, such as root growth, or by acting to reduce phytotoxicity (Antoniadis et al., 2017). Furthermore, it has been shown that aeration of the rhizosphere can induce the formation of more developed aerenchyma and increase the capacity for metal uptake and bioaccumulation (Xin et al., 2019).

Phosphorus is the second most important macronutrient for plant nutrition and growth (Gupta et al., 2014). In terrestrial environments, P compounds act as a source of heavy metals for plants either by direct metal adsorption, phosphate anion-induced metal adsorption and desorption or modification of the rhizosphere through acidification and mycorrhizal associations (Bolan et al., 2003). Copper is associated with its transporters from the surrounding medium to plant tissues, such as the COPT family of proteins, which also participate in the metabolism of phosphate (Carrió-Seguí et al., 2019; Printz et al., 2016; Puig, 2014).

Similar to P, N is also a macronutrient and is essential for plant growth (Ågren et al., 2012; Kraiser et al., 2011). In this case, it could have an indirect effect on the removal of metals since it is associated with an increase in plant biomass. Although species with a larger biomass generally have higher concentrations of elements, this does not necessarily imply their better performance in terms of the accumulation and translocation of elements, as a dilution effect of plant biomass can occur (Bonanno and Vymazal, 2017)Studies have suggested that several plant species living in environments that are susceptible to high pollutant loads, such as wetlands, tend to adopt a strategy of tolerance by relying on roots (and rhizomes) as the main accumulating organs, which can store most of the trace elements to protect themselves against the harmful effects of toxic concentrations on photosynthetic organs (Bonanno, 2011).

For instance, emergent macrophytes in a hydroponic system grow on a mat or raft floating on the surface of the water rather than being rooted in the sediment/substrate. The roots, normally more voluminous and developed, are below the floating mat and provide a large surface area for biofilms to attach. In these systems, the vegetation mat is larger and more consolidated than that of free-floating systems (here called floating macrophytes), which might make them more able to remove pollutants (Headley and Tanner, 2012).

The photoperiod is the amount of light in a 24-hour daily cycle. In plants, the photoperiod controls responses such as circadian rhythms and light signal detection in the leaves (Jackson, 2009). The uptake and concentration of Fe and Cu are higher in shorter photoperiods, with which strong negative correlations ($p \leq 0.05$) were found (Table 3). However, with longer periods of light exposure, there may be changes in the redox state of chloroplasts, which includes responses to environmental stress as one of its functions. Under conditions of high light exposure or high light intensity, there is a need for the acclimatization of the photosynthetic machinery to avoid damage to the antioxidant defense system (Suzuki et al., 2012). Therefore, with the combination of the stressful conditions of the wastewater environment and the longer light period, plants decrease the uptake of metals to avoid such damage.

It was observed in the meta-analysis that the environmental factors ($[HM]_{wastewater}$; pH; exposure time; the concentrations of N, P and DO; and photoperiod) affected the removal and uptake of Fe, Cu and Mn, but with significant correlations that varied with the type of metal and the parameter analyzed. However, it is important to verify the magnitude of the coefficient to the correlated variable. The magnitude does not depend on the correlation's sign but on the sample size (n) used for the correlation (Diedenhofen and Musch, 2015).

For this purpose, comparisons were made between the significant coefficients of the environmental factors with the same correlated variable. In almost all of them, there were no significant differences according to Fisher's Z-test of transformation ($p \leq 0.05$). For example, for the ln (BCF) of iron, there were no differences between the coefficients of time (0.727) and $[HM]_{wastewater}$ (- 0.703) regarding the ability of macrophytes to bioconcentrate this metal in their tissues (Table 3). On the other hand, for ln $[HM]_{plant}$ of copper, there were differences between the coefficients of exposure time (0.462) and OD (0.526) even though they were coefficients with strong correlations.

5. CONCLUSIONS REMARKS

In this study, we performed a meta-analysis of research from the past 30 years (1990–2022) on the relationship between factors in the physical and aqueous environments and the removal of Fe, Cu, and Mn from real wastewater through phytoremediation using hydroponically grown floating, emergent and submerged macrophytes. Emergent macrophytes, although less often reported, showed higher mean ln (BCF) values for iron and manganese, and higher ln $[HM]_{plant}$ values for manganese than their floating counterparts. There was only one report of a submerged macrophyte. Thus, research with submerged macrophytes treating wastewater and evaluating Fe, Cu, Mn should be encouraged in order to also understand the dynamics of removal of these HMs by this type of macrophyte.

In addition, Cu and Mn showed less mobility from the wastewater to plant tissues, regardless of the biotope. There were significant correlations between the factors of the physical medium (exposure time and photoperiod) and the wastewater (pH, initial metal concentration in the wastewater, N, P and DO), but in different ways: Regardless of the aquatic form of the macrophytes, higher initial concentrations of Fe and Cu in the effluent decreased the ability of plants to bioconcentrate them in their plant tissues.

-Shorter exposure times or test durations increased the removal of Fe, Cu and Mn from the wastewater, but hindered the uptake of Fe and Cu.

-Shorter daylengths increased the plants' ability to absorb the three metals and remove them from the wastewater.

-Lower pH increased the removal of Cu and Mn from the wastewater, but decreased the uptake of Mn.

-Higher concentrations of P and DO increase the uptake and concentration of Cu in plant tissues.

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CHAPTER 3: USING WASTEWATER TREATMENT PERFORMANCE, BIOMASS AND PHYSIOLOGICAL PLANT CHARACTERISTICS FOR SELECTION OF A FLOATING MACROPHYTE FOR PHYTOREMEDIATION OF SWINE WASTEWATER THROUGH THE INTEGRATIVE ENTROPY-FUZZY AHP-TOPSIS METHOD

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Abstract: Phytoremediation success depends on the selection of suitable plant species. To this end, the multiple-attribute decision-making process (MADM) is a useful tool for selecting plants to remediate wastewater. Here, macrophytes were used to treat synthetic swine wastewater (SSW) and the best plant alternative for phytoremediation of SSW was chosen based on the hybrid Entropy-Fuzzy AHP TOPSIS MADM method. The attributes used to feed the MADM method were treatment performance, physiological endpoint changes and biomass by-products. Macrophyte-treated wastewater achieved higher pollutant removal than controls. *Lemna*, *Landoltia*, *Salvinia*, *Azolla* and Control units had removals of 81 %, 79 %, 68 %, 81 %, 25 % for tCOD; 66 %, 71 %, 65 %, 68 %, 8 % for P-PO_4^{3-}; 51 %, 63 %, 40 %, 66 %, 14 % for N-NO_3^-; 36 %, 36 %; 26 %; 42 %; 7 % for N-NH_4^+; 51 %, 40 %, 52 %, 63 %, 3 % for Cu; and 25 %, 18 %, 26 %, 42 %, 14 % for Zn, respectively. Furthermore, Azolla and Lemna removed more SSW toxicity and presented the lowest phyto-stress index (0.09) and highest lipids production (14 % dw) during treatment. *Azolla* proved to be the best option for phytoremediation of swine wastewater since it had the highest rank as determined by the proposed method. Acute toxic units of the treated effluent (0.38) and phyto-stress index (0.49) had the greatest weight in choosing the best plant while the production of biomass volatile solids and removals of P-PO_4^{3-} and tCOD were the least influential. This study provided a reliable approach to selecting the best plant from available macrophytes capable of improving the physicochemical and ecotoxicological quality of wastewater and offering value-added plant biomass products.

Keywords: Crude protein; Duckweed; Integrated biomarker response; Multicriteria decision-making; Piggery wastewater treatment; Total lipids.

1 INTRODUCTION

Over the past decades, animal production has been transformed from subsistence farming to an intensive model where costs have decreased but the environmental impact has increased. Pig production is characterized by high loads of solid, liquid, and gaseous wastes, making it one of the agricultural activities with the greatest environmental impact (Andretta et al., 2021).

The composition of swine wastewater can vary according to age, number, nutritional needs, feed composition, housing methods (e.g., bedding material) and production phase (mating, gestating, farrowing, nursery and growing/finishing) of pigs (Varma et al., 2021). These wastewaters are composed of feces and urine mixed with washing water containing pathogenic microorganisms, unmetabolized antibiotics, nutrients and organic matter (Kunz et al., 2009).

Thus, the use of an efficient method of treating swine wastewater is essential. Conventional treatment methods, whether physical, chemical, or biological, eventually have limited capacity to remove pollutants and/or have high implementation and/or operational costs (Li et al., 2022). Commonly used in the treatment of pig farming wastewater, anaerobic digestion may reduce health risks and produce biogas and biofertilizers. However, digestion does not reduce the pollution load sufficiently to discharge this wastewater into waterbodies, requiring post-treatment, notably, by waste stabilization pond systems (Kunz et al., 2009). Even this combination may not eliminate N, P, and organic matter, which still need to be properly treated and recycled.

Bio-based remediation using plants, known as phytoremediation technology, is attractive, sustainable and being environmentally friendly alternative with low operation and maintenance costs (Ansari et al., 2020). Aquatic plants, namely macrophytes, are capable to removing metals, nutrients, or xenobiotic compounds from industrial, municipal and agricultural wastewaters (Ali et al., 2022; Saha et al., 2022).

However, different plants perform differently in improving wastewater quality (Geng et al., 2017) and although plants can be tolerant to toxic compounds, the success of using macrophytes lies in the choice of a suitable plant species. The appropriate selection of the phytoremediator species is considered the most important factor for biomass growth and nutrient removal efficiency (Li et al., 2020). For instance, Kadir et al. (2020) evaluated the capacity of the duck weeds *Lemna minor* and *Azolla pinnata* for phytoremediation of four dilutions (2.5, 5, 10 and 15%) of palm oil manufacturing effluent (POME) over ten days. The authors reported that the 2.5% POME dilution resulted in the highest growth of *L. minor* and *A. pinnata* (increase of 8.7g e 9.8g wet weight, respectively), and all the plants survived until the end of the 10-day exposure period, with *A. pinnata* presenting greater reductions in the parameters analyzed. The highest ammonia reductions were found in the 5% POME dilution (98% with *A. pinnata* and 95.5% with *L. minor*), while the highest phosphate removals were found in the 10% POME dilution (93.3% with *A. pinnata* and 87.7% with *L. minor*) and the highest COD removals were found in the 15% POME dilution (78% with *L. minor* and 66% with *A. pinnata*). The authors highlighted the importance of the plants in improving treated effluent quality while at the same time serving as a potential source of animal feed.

Swine at the wastewater offers a readily and cost-effective growth medium for macrophytes due to the high concentration of nutrients (Xu and Shen, 2011), but selecting the best plant from a collection of local strains is a prerequisite for establishing an effective culture system. Nevertheless, what if the selection of these plants involved the direct participation of stakeholders involved in the plant selection process? If plant performance varies by wastewater type and plant strain/species, what parameters should one take into account to evaluate which plant is best for a given wastewater or treatment condition?

For this purpose, the multiple-attribute decision-making process (MADM) is a useful tool for selecting the most suitable plant to remediate contaminated areas (Cao et al., 2022; Wang et al., 2019). MADM methodologies are based on various approaches and representations of the logic of the human decision-making process (Hwang and Yoon, 1981) and have been employed in choosing wastewater treatment alternatives (Kalbar et al., 2012).

Wang et al. (2019) used AHP, TOPSIS and triangular fuzzy numbers to choose the most suitable plants for phytoremediation of the two petroleum-contaminated soil sites in China, the Changing shale gas field and Liaohe oil field. The criteria used in the study included physiological characteristics, natural environmental conditions and polluted soil properties. The highest weight of the evaluation system for the Changning shale gas field was assigned to moisture content, followed by light intensity. For the Liaohe oil field, temperature had the greatest weight, followed by moisture content. The results showed that in the Changning shale gas field, *Festuca arundinacea* was the most suitable plant according to the AHP-TOPSIS fuzzy calculations. For the Liaohe oil field, *Festuca elata* Keng ex E. Alexeev had the highest score using the fuzzy AHP-TOPSIS method.

Cao et al. (2022) evaluated fifteen clones of *Salix* spp to phytoremediate soils contaminated by heavy metals (Cd, Zn and Pb) under flooded and non-flooded (control) conditions. The best clones for phytoremediation were selected based on the AHP-Entropy model fed with the attributes/criteria growth performance, photosynthetic parameters, accumulation and mobility of toxic metals. Flooding affected growth and behavior of photosynthetic parameters in different ways, with different clones showing an increase or decrease in height, stem diameter and leaf area as well as photosynthetic rate, transpiration rate or stomatal conductance in relation to the control condition. The flooded condition reduced Cd contents (11.7-90.1%) in all organs but increased Zn and Pb contents in the roots compared to the non-flooded condition. In general, the AHP-Entropy model indicated three clones with the greatest potential for phytoremediation of soils contaminated by metals, under the conditions studied. Thus, the use of the MADM methodology for selecting plants for phytoremediation of contaminated areas has proven to be a valuable approach. However, to date there have been no reports on phytoremediation of swine wastewater, reinforcing the innovative aspect of this study.

In view of the aforementioned, the present study compared the performances of four macrophyte species (*Lemna minuta*, *Landoltia punctata*, *Salvinia minima* and *Azolla microphylla*) in treatment of synthetic swine wastewater, biomass by-products production and physiological plant trait endpoints. We used multicriteria decision-making based on Entropy-fuzzy AHP for weighting followed by TOPSIS to rank alternatives to select the most suitable macrophyte phytoremediator of swine wastewater. Our results provide a comprehensive dataset on the potentials of the four macrophytes for the phytoremediation of swine wastewater as well as by-product production. Here we propose the use of the MADM methodology as a way to identify the most satisfactory macrophyte alternative, the one that guarantees not only wastewater reclamation but also that provides biomass in quality and quantity biomass for further upcycled.

2. MATERIAL AND METHODS

2.1. Plant stock cultivation

The plants used in this experiment were full-fledged floating aquatics plants *Azolla microphylla* Kaulf. (water fern), *Salvinia minima* Baker (water velvet) (Miranda and Schwartsburd, 2016) and the duckweeds *Lemna minuta* Kunth and *Landoltia punctata* Crawford (Pereira et al., 2016). The strains were collected at the Botanical Garden of the Federal University of Viçosa (UFV), Viçosa, Minas Gerais, Brazil (20°45'25.0" S 42°52'25.5" W) and sterilized by washing in 1% (v/v) commercial sodium hypochlorite for 1 min followed by rinsing repeatedly with deionized water, for 5 min. The plants were routinely cultivated in Hoagland solution (Hoagland and Arnon, 1950) with weekly replacement, at room temperature (23 ± 2 °C) under 40 µmol m^{-2} s^{-1} supplied by fluorescent lamps with a 16 h light: 8 h dark cycle.

2.2. Synthetic swine wastewater

Synthetic swine wastewater (SSW) was prepared to simulate a secondary wastewater treatment step that has passed a primary treatment stage (Table A.1, Appendix A). The inorganic composition was based on swine wastewater reported previously (Table A.1, Appendix A). The organic composition was designed to provide a total chemical oxygen demand (tCOD) of approximately 600 mg L^{-1} (Ramos et al., 2016). For this purpose, the organic fraction was composed of carbohydrates (glucose: 153 mg L^{-1}; starch: 50 mg L^{-1}), proteins (meat extract: 218 mg L^{-1}) and lipids (soy oil: 184 mg L^{-1} emulsified with neutral commercial detergent). The mass ratio of carbohydrates: proteins: lipids was based on that used by Pérez-Pérez et al. (2021). The final wastewater pH was adjusted to 6.5 and sodium bicarbonate (500 mg L^{-1}) was added for buffering capacity. This composition was chosen to meet both the organic content (protein, lipids, carbohydrates) and inorganic content (metals, nitrogen, phosphorus) found in swine wastewater.

2.3. Batch experimental condition

The experiment was conducted at bench-scale at room temperature (23 ± 2°C), 40 µmol m^{-2} s^{-1} light intensity (40W, fluorescent lamps, Ouro Lux®, São Paulo, SP, Brazil) and a 16 h light: 8 h dark cycle. Treatments were carried out in 2 L working volume plastic pots (10 cm height x 24 cm diameter) with a surface area of 0.045 m^2. Treatment units contained synthetic swine wastewater with floating plants and control units contained only SWW, without plants.

Each treatment consisted of 3 replicates, for a total of 15 experimental units (Fig. A.1, Appendix A). Each treatment consisted of a single plant, except for the control group, which had no plant. To reduce the bias of biomass production from different frond sizes, which occurs when the complete surface is covered when different species are used (Bergmann et al., 2000), the initial biomass (on a fresh weight basis) was adjusted to cover ½ of the total surface of the plastic pots. Thus, initial plant biomasses were 242.8 g_{FW} m^{-2} for *L. minuta*, 134.6 g_{FW} m^{-2} for *L. punctata*, 524.7 g_{FW} m^{-2} for *S. minima* and 368.7 g_{FW} m^{-2} for *A. microphylla*. Therefore, each treatment's three replicates contained the mentioned mass of each plant. The experimental period was 10 days, chosen to avoid nutrient suppression in the plastic pots that might affect plant growth, as discussed previously (Paolacci et al., 2018). Water lost due to (evapo)transpiration was replaced with deionized water twice a week.

Plant biomass weight was recorded for each plastic pot at the start and end of the experiment and during the exposition changes in fronds in each replicate were recorded. Chlorophylls (a, b and total), carotenoids and anthocyanin contents were quantified on a fresh weight (FW) basis, while crude proteins, total lipids and biomass total volatile solids were quantified on a dry weight (DW) basis. Treated wastewater quality was assessed by phosphate-phosphorus (P-PO_4^{3-}), nitrate-nitrogen (N-NO_3^-), ammonium-nitrogen (N-NH_4^+), tCOD, dissolved zinc (Zn), dissolved copper (Cu) and chronic/acute toxicity units (TU) for survival and reproduction of *Ceriodaphnia dubia*. The physicochemical and ecotoxicological characterization and biomass analysis were conducted at the beginning and end of the 10-day treatment period. Except toxicity assays, which were performed using composite samples, all other analyses were carried out in triplicate.

2.4. Analytical methods

Physicochemical characteristics of synthetic swine wastewater were determined at the beginning (raw) and end (treated) of the 10-day treatment. COD (5220 D), dissolved organic carbon (DOC) (5310B, (Shimadzu, TOC-L CSH analyzer), biochemical oxygen demand ($BOD_{5,20}$) (5210B), total phosphorus (TP) (4500-P B.4 and E), phosphorus-phosphate ($P\text{-}PO_4^{3-}$) (4500-P E), total Kjeldahl nitrogen (TKN) (4500-N_{org}C), ammonium-nitrogen ($N\text{-}NH_4^+$) (4500-NH_3 B and C), pH (4500- H^+, IntelliCAL™ PHC 101), zinc (3500-Zn), copper (3500-Cu) were quantified according to the Standard Methods (APHA, 2022). Total nitrogen (TN) was quantified by oxidative combustion-chemiluminescence (Shimadzu, TOC-L CSH coupled to chemiluminescence detector TNM-TN unit). Nitrate-nitrogen ($N\text{-}NO_3^-$) was analyzed according to Yang et al. (1998). A HACH DR 6000 UV-visible beam spectrophotometer with 1 cm matched cells was used for absorbance measurements. Dissolved COD ($_d$COD), DOC and heavy metals samples were filtered using nylon membrane filters with a pore size of 0.45 µm (Merck, Ireland). Sample pH was adjusted with 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl solutions.

For dissolved metals determination, the samples were digested with nitric-perchloric acid (3:1, v/v) and preserved at 4°C until analysis. The metals were determined by atomic absorption (PinAAcle 500®, PerkinElmer, MA, U.S.A) with limits of detection of 0.0002 mgL^{-1} for Zn and 0.0003 mgL^{-1} for Cu. The raw synthetic swine wastewater was characterized by quantification of dCOD, tCOD, DOC, $BOD_{5,20}$, Zn, Cu, TP, $P\text{-}PO_4^{3-}$, TKN, TN, $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ (Table 1).

Table 1: Raw synthetic swine wastewater physicochemical and ecotoxicological characteristics

Parameter	Value
pH	6.5
dCOD (mg L^{-1})	345
tCOD (mg L^{-1})	594
$BOD_{5,20}$ (mg L^{-1})	338
$BOD_{5,20}$/tCOD	0.57
DOC (mg L^{-1})	78.2
Zn (mg L^{-1})	4.20
Cu (mg L^{-1})	1.18
[1]Toxicity unit-chronic (TUc)	121.48
[2]Toxicity unit-acute (TUa)	116.63
TP (mg L^{-1})	109.5
$P\text{-}PO_4^{3-}$ (mg L^{-1})	97.3
TN (mg L^{-1})	268.0
TKN (mg L^{-1})	156.1
$N\text{-}NH_4^+$ (mg L^{-1})	85.0
$N\text{-}NO_3^-$ (mg L^{-1})	86.3

[1,2] Corresponding to $LC_{50\%,3d}$ ($CI_{95\%}$) = 0.857% (0.6917-1.063) and $IC_{50\%,8d}$ ($CI_{95\%}$) = 0.823% (0.6136-1.061)
dCOD: Dissolved Chemical Oxygen Demand, tCOD: Total Chemical Oxygen Demand, $BOD_{5,20}$: Biochemical Oxygen Demand, TP: Total phosphorus, TN: Total nitrogen, TKN: Total Kjeldahl nitrogen.

2.5. Toxicity assays

Toxicity of raw and treated wastewater was quantified using the *Ceriodaphnia dubia* Richard, 1894 (*Crustacea, Cladera*) survival and reproduction assay performed at 25 ± 2 °C and 16 h light: 8 h dark cycle (700 lux) (ABNT, 2017). The semi-static assays consisted of *C. dubia* neonates (≤ 24h age) placed in either 15 mL of control or different wastewater dilutions (0, 6.25, 12.5, 25, 50 and 100% for treated wastewater and 0, 0.1, 0.2, 0.4, 0.8, 1.6% for raw wastewater) with a renewal of test solutions every 72 h during 8 days. Each dilution included 10 replicates with one neonate per replicate. The total number of neonates produced per female adult, in each replicate per dilution, was counted after an 8-day test period and compared to the control group.

The effects of chronic exposure on *C. dubia* basic life-history parameters survival and total number of newborns were assessed. The lethal ($LC_{50\%,3d}$) and altered reproduction ($IC_{50\%,\ 8d}$) concentrations were determined by the *Trimmed Spearman-Karber method* and linear interpolation respectively, using Comprehensive *Environmental Toxicity Information System*™ software (Tidepool Scientific Software, McKinleyville, CA, U.S.A). The analyses were performed in a composite sample from the three replicates of each treatment. Results were expressed in chronic ($TUc = 100/IC_{50\%}$) or acute ($TUa = 100/LC_{50\%}$) toxicity units. The health of *C. dubia* test organisms were periodically monitored by evaluating their sensibility to sodium chloride (NaCl) as reference material.

2.6. Plant biomasses analyses

The analyses of biomass productivity, relative growth, and photosynthetic pigments were conducted to assess the treatment's effect on phytotoxicity and biomass production, considering its protein, lipid, and volatile solids content. These parameters were utilized to inform the multicriteria analysis model (Section 2.7).

2.6.1 Relative growth rate and biomass productivity

At the beginning and end of the experiment, plant biomass fresh weight (FW, g) was determined by blotting the plant on paper towel to remove excess water followed by weighing on an analytical balance. For plant dry weight (DW, g), a known FW mass of the sample was placed in an airflow oven at 65°C for 72 h until a constant weight was obtained. The relative growth rate (RGR, d^{-1}) of the plants was calculated based on dry biomass using Eq. 1.

$$RGR = \frac{\ln (DW_e / DW_b)}{t} \tag{1}$$

where: RGR is the average relative growth rate (d^{-1}); DW_b (g) and DW_e (g) represent the dry biomass at the beginning and end of the experiment, respectively; and t represents the duration of the experiment (10 days).

Biomass productivity (Bp) was calculated on dry biomass according to Eq. 2.

$$B_p = \frac{DWe - DWb}{t*A} \tag{2}$$

where, Bp is the biomass productivity (g m^{-2} d^{-1}); DW_e is the dry biomass weight at the end of the experiment (g); DW_b is the dry biomass weight at the beginning of the study (g); t is the duration of the experiment (10 days); and A is the surface area of plastic pots used (0.045 m^2). The DW_b was determined by measuring a known mass of the Hoagland solution at the exact day and time when the test was initiated.

2.6.2 Chlorophyll, carotenoids and anthocyanin contents

Chlorophyll a (Chl a), b (Chl b), total (Total Chl) and carotenoids (Car) extractions were performed according to Su et al. (2010). Absorbance readings at 470, 649 and 664 nm were used to calculate amounts of Chl a, Chl b, Total Chl and Car (Lichtenthaler, 1987) (Eqs 3-6). The results were expressed in mg pigment per g fresh weight of plant biomass (mg g^{-1} FW).

$$\text{Chlorophyll a (mg g}^{-1}) = (13.36A_{664} - 5.19A_{649})\frac{v}{Wx\,1000} \tag{3}$$

$$\text{Chlorophyll b (mg g}^{-1}) = (27.43A_{649} - 8.12A_{664})\frac{v}{Wx\,1000} \tag{4}$$

$$\text{Total Chl (mg g}^{-1}) = (5.24A_{664} + 22.24A_{649})\frac{v}{Wx\,1000} \tag{5}$$

$$\text{Carotenoids (mg g}^{-1}) = \left(\frac{1000A_{470} - 2.13Chl_a - 97.64Chl_b}{209}\right)\frac{v}{Wx1000} \tag{6}$$

where v (mL) is the volume of extracted liquid and W (g) is the fresh weight of the extracted plants.

The anthocyanin (Antho) content was extracted according to Close et al. (2004). For the anthocyanin content, prior absorbance readings at 529 and 650 nm were made to correct chlorophyll absorbance overlaps - A_c (Sims and Gamon, 2002) (Eq 7). Total anthocyanin content was calculated using corrected absorbance (A_c) and molar absorbance coefficient, *ε,* for anthocyanin at 529 nm of 30, 000 L mol^{-1} cm^{-1} (Murray and Hackett, 1991). Anthocyanin was expressed in mol per gram of fresh weight of plant biomass (mol g^{-1} FW).

$$A_C = A_{529}-(0.288A_{650}) \tag{7}$$

To compare pigment changes during synthetic swine wastewater treatment, the contents of Chl a, Chl b, Total Chl, Car and Antho were recorded before the start (plants harvested from Hoagland solution, Day 0) and at the end (plants harvested from synthetic swine wastewater, Day 10) of the experiment.

2.6.3 Phyto-stress index (PSI)

A general stress index, Integrated Biomarker Responses (IBR), from here on referred to as the Phyto-Stress Index (PSI), was calculated using the biomarkers Chl a, Chl b, Total Chl, Car and Antho contents following the methods proposed by Beliaeff and Burgeot (2002), Guerlet et al. (2010) and Devin et al. (2014).

Briefly, the mean value (x) for each macrophyte biomarker was standardized using the mean value (*m*) and standard deviation (*sd*) for all macrophyte biomarkers to produce the Y value as follows (Eq. 8).

$$Y = \frac{x - m}{sd} \tag{8}$$

The Z value was then defined as Z = Y or Z = -Y corresponding to activation or inhibition of a biomarker, respectively. The score value (S) for each biomarker content was obtained according to Eq. 9.

$$S = Z + |min| \tag{9}$$

where, min is the minimal Z value for all treatment groups for each biomarker.

The changes in biomarker content (BmK_i) at the beginning (day 0) and end (day 10) of the experiment, $BmK_i = BmK_{10} – BmK_0$, were measured to further estimate score values. The plant with the greatest reductions (inhibition, $BmK_i < 0$) in chlorophyll a, b and total chlorophyll (Ziegler et al., 2019) and greatest increases (induction, $BmK_i > 0$) in carotenoids and anthocyanin (Naing and Kim, 2021; Uarrota et al., 2018) contents in its tissues was considered to have the highest stress index level.

In this method, a higher IBR value meant a greater response (more stress) expressed by the exposed organisms. Moreover, a radar diagram plotted according to the score (S) value indicated the strength of biomarkers in response to pollutants. The highest score corresponded to the greatest biological effects. Thus, to calculate the scores of the pigments Chl a, Chl b and Total Chl, transformed BmK_i values were used as follows, $BmK_t = 1 - BmK_i$. For carotenoids and anthocyanin contents, the score (S) values were calculated using the direct BmK_i values.

The PSI index was calculated according to Eqs 10-11 (Beliaeff and Burgeot, 2002; Devin et al., 2014; Guerlet et al., 2010).

$$A_i = \frac{1}{2}\sin(2\pi/n)S_iS_{i+1} \qquad (10)$$

in which individual areas A_i connect the i^{th} and the $(i + 1)^{th}$ radius coordinates of the star plot; S_i and S_{i+1} represent consecutive clockwise individual biomarker scores and their successive star plot radius coordinates; n represents the number of radii corresponding to the biomarkers used in the study and the term (2π/n) is calculated in radians. The PSI corresponded to the total area displayed by the radar diagram (Eq 11) (Beliaeff and Burgeot, 2002).

$$PSI = \sum A_i \qquad (11)$$

However, since the PSI value depended on the arrangement of biomarkers, the median of all possible (n -1)! PSI matrices were used as the final PSI index value (Devin et al., 2014).

2.6.4. Biomass total lipids, crude protein and total volatile solids contents

Total lipid content in macrophyte biomass was estimated by Bligh and Dyer's method (Bligh and Dyer, 1959) and was expressed as % of DW (g of lipids per 100 g of dry plant weight). The crude protein content in each macrophyte tissue was estimated by N * 6.25 (Casal et al., 2000) whereas nitrogen (N) was measured by the Kjeldahl nitrogen method and expressed as % of DW (g of proteins per 100 g of dry plant weight). Volatile solids were determined by ignition of residue in a muffle furnace at 550 °C for 30 min (USEPA, 2001). The results were expressed as mg of volatile solids per g of dry plant biomass (mg g^{-1}DW).

2.7. Multi-criteria decision-making methods

The approach to choosing the best plant for the phytoremediation of swine wastewater, among the four alternative macrophytes (*A. microphylla*, *L. punctata*, *L. minuta* and *S. minima*) was based on the quality of the treated wastewater (Criteria I) and the physiological characteristics of the plants and their biomass by-products (Criteria II) (Fig A.2, Appendix A). Each of these criteria is composed of indicators and their respective attributes.

The supposition was that the most suitable plant was the one that ideally presented the maximum removal of wastewater physicochemical quality attributes (physicochemical quality indicators); minimized acute and chronic wastewater ecotoxicity, expressed as toxicity units (toxicity indicators); presented the highest production of lipids, proteins and volatile solids content (resource recovery indicators); and, finally, had greatest growth and lowest phyto-stress index (phytotoxicity indicators). A total of thirteen attributes were considered in the evaluation process (Fig A.2, Appendix A). The attributes used in decision-making were chosen through a literature survey of common pollutants studied in the phytoremediation process as well as biomass by-products and their phytotoxicity endpoints (RGR and PSI). The wastewater physicochemical quality indicators and resource recovery indicators were classified as benefit attributes while toxicity indicators and phytotoxicity indicators were classified as costs in the TOPSIS model (see section 2.8).

The multicriteria decision-making methods for the comparison of the four floating macrophytes were performed using combined Entropy and Fuzzy Analytic Hierarchy Process (E-FAHP) methods for weighting criteria and TOPSIS for ranking the alternatives. The purpose of the use of E-FAHP in this study was to combine the advantages of subjective (Fuzzy Analytic Hierarchy Process-FAHP) and objective (Entropy) evaluation methods to produce weights and reduce the limitations of each of the two methods.

A compromise weight (w^c) of the subjective FAHP ($W^S = w^{S1}, w^{S2},\dots, w^{Sn}$) and objective Entropy ($w^O = w^{O1}, w^{O2},\dots, w^{On}$) weighting methods were determined by a combination of weighting methods as reported previously (Chen, 2020) (Eq 12).

$$w^c = \frac{w_j^S * w_j^O}{\sum_{j=1}^{n} w_j^S * w_j^O} \tag{12}$$

The calculation of weights by two methods (E-FAHP) as well as TOPSIS for ranking alternatives is described in Appendix A (Text A.1).

2.8. Data analysis

The experiment was laid out in a completely randomized design. To determine the effect of treatments to improve swine wastewater quality, biomass by-products production and plant growth endpoints changes we used a statistical generalized linear model (GLM) with Gamma, Log normal or Normal continuous distributions in R software. The significant distribution that best fits the data was chosen based on the lowest value of the Akaike Information Criterion (AIC) among the three indicated distributions (Table A.9, Appendix A). We also performed a Tukey post-hoc test to assess pairwise differences between treatments. A significance level of 5 % was set for all statistical analyses. A sensitivity analysis was carried to assess the robustness of TOPSIS ranking preference of the four macrophytes alternatives. A detailed description can be found in the Appendix A (Fig A.4) and the section 2.8.

3. RESULTS AND DISCUSSION

3.1. Treated synthetic swine wastewater quality

3.1.1 Wastewater physicochemical quality indicators

All macrophyte treatments reduced significantly concentrations of N-NH_4^+ and N-NO_3^- compared to the control ($p \leq 0.05$, Fig 1). The removal of N-NH_4^+ ranged from 7% (control), 26.3% (*S. minima*), 36.4% (*L. minuta*), 36.4% (*L. punctata*) to 42.4% (*A. microphylla*). Similarly, for nitrate-nitrogen the removals represented 13.6% (control), 50.9% (*L. minuta*), 63.2% (*L. punctata*), 40.1% (*S. minima*) and 65.7% (*A. microphylla*).

Nitrogen removal in macrophyte treatment units can occur by plant uptake, nitrification/denitrification process as well as sedimentation of particulate nitrogen and volatilization of ammonia (Zimmo et al., 2004). Indeed, at the end of the treatments a bottom sludge was formed (including in the control treatment) (Fig A.3, Appendix A), but with no decaying plant biomass observed.

Wastewater pH increased from 6.5 (raw wastewater) to pH 7.5 (control), pH 7.6 (*L. minuta*), pH 7.6 (*L. punctata*), pH 7.2 (*S. minima*), and pH 7.8 (*A. microphylla*). Even though at pH levels above 7.0, where the equilibrium between NH_3 and NH_4^+ tends to shift towards the formation of ammonia, which can volatilize (Metcalf and Eddy, 2016), the pH values close to neutral do not strongly indicate proeminent ammonia volatilization, given that the pKa of ammonia is 9.3. However, it's important to note that the extent of ammonia volatilization can be influenced by factors such as temperature, airflow, and surface agitation, in addition to pH. Thus, sedimentation and volatilization seemingly represented small fractions of nitrogen removal, as corroborated by the low removals of N-NH_4^+ (7%) and N-NO_3^- (13%) in the control treatments.

Other routes (direct plant uptake and nitrification/denitrification) likely explain the removal of N-NH_4^+ and N-NO_3^-. Denitrifying bacteria develop in anaerobic/anoxic environments. Although the aerenchyma of aquatic plants release oxygen to the rhizosphere through photosynthesis (Reddy et al., 1989), overpopulation shades the units and decreases the oxygen content in the rhizosphere zone (Weisner et al., 1994) favoring denitrification conditions. In this study, it was not possible to measure the dissolved oxygen or oxidation-reduction potential in the treated wastewater.

Direct plant uptake contributed to the removal of nitrogen. Plants preferentially uptake NH_4^+ over NO_3^- since the energy required to assimilate NH_4^+ is lower than for NO_3^- (Hachiya and

Sakakibara, 2016), although this may depend on the species of plant under study. Nevertheless, in all treatments the nitrate-nitrogen removal was greater than ammonium-nitrogen removal. However, it has already been observed that the increase in nitrate/ ammonium ratio in growth medium led to a decrease in uptake of both N forms by roots and an increase the uptake of NO_3^- by fronds (Cedergreen and Madsen, 2002). Thus, we believe that the greater nitrate removals could be due mainly to denitrification and direct plant uptake. In contrast, ammonia removal of may have been mainly due to the direct plant uptakes.

Toyama et al. (2018) cultured four duckweeds species (*Spirodela polyrhiza*, *Lemna minor*, *Lemna gibba* and *Landoltia punctata*) over 4 days of exposition and evaluated their potentials to remove nitrogen from municipal, swine wastewater and anaerobic digestion wastewater. Unlike our results, they found an increase in $N\text{-}NO_3^-$ for all plants in swine-treated wastewater. They argued that microbial nitrification prevailed over denitrification due to the inhibition caused by the aerobic conditions formed in the rhizosphere by duckweed photosynthesis. In our case, this did not appear to have occurred.

For the initial concentration of 97.3 mg L^{-1} $P\text{-}PO_4^{3-}$, the treatments removed 7.9% (control), 66.3% (*L. minuta*), 71.5% (*L. punctata*), 64.7% (*S. minima*) and 68.3% (*A. microphylla*). Wetlands may provide a combination of physical, chemical and biological processes to remove phosphorus from wastewater. These may include precipitation of metal phosphates, adsorption onto clay or substrate particles, biological reduction or plant uptake (Shahid et al., 2018). Nevertheless, the main route of phosphorus removal in floating macrophyte tanks is direct uptake by the plant and plant-mediated microbial processes (Mohedano et al., 2012). Hence, the low phosphate removal in the control unit (7.9%) corroborates that the phosphate removal pathway required the presence of macrophytes.

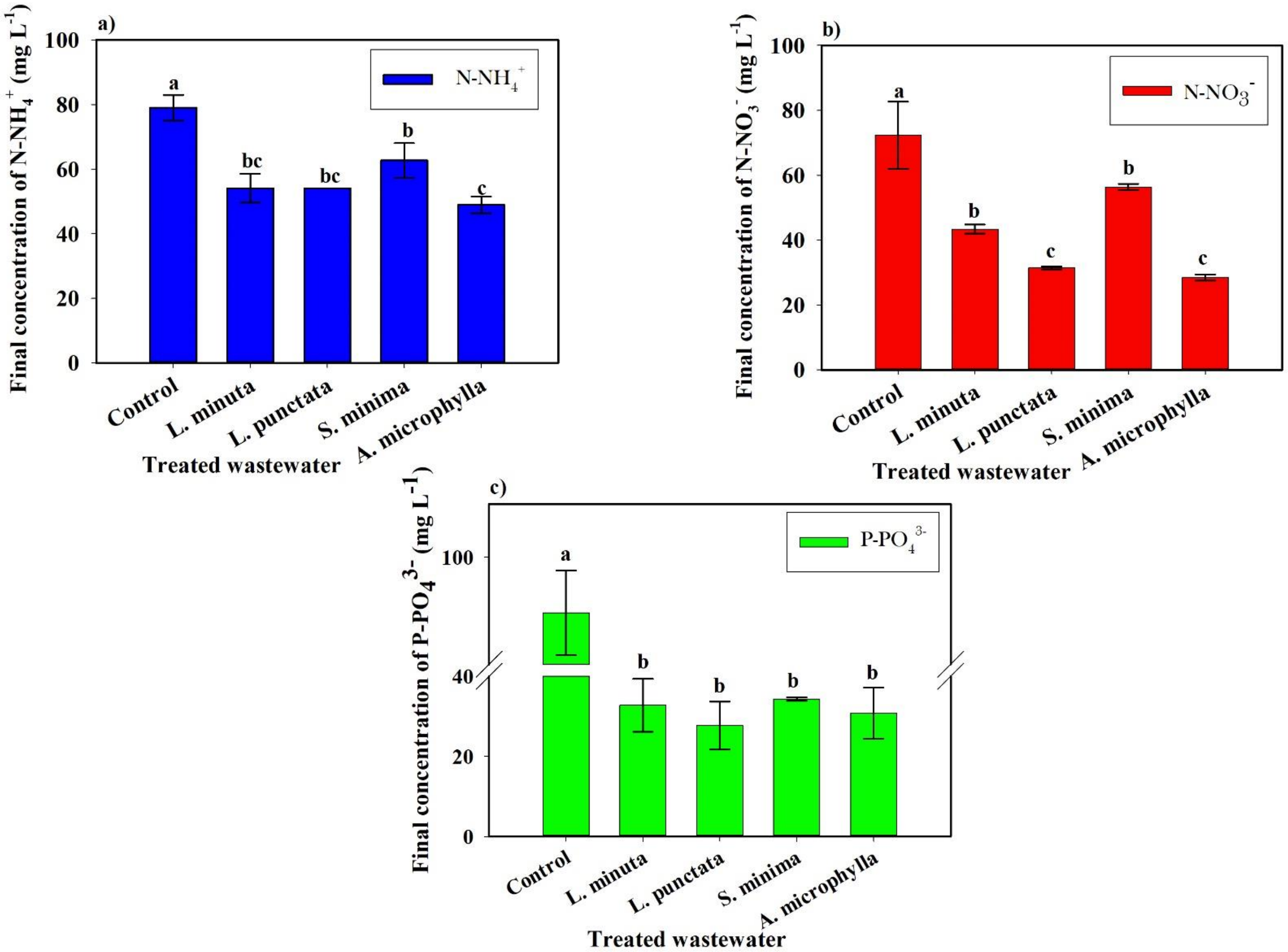

Fig 1: Concentration of a) N-NH_4^+, b) N-NO_3^- and c) P-PO_4^{3-} in the treated wastewater. Different letters show a significant difference ($p \leq 0.05$) between the treatments according to the Tukey test ($p \leq 0.05$), (n = 3. mean ± standard deviation).

Copper and zinc are essential pig dietary supplements and the unabsorbed Cu^{2+} and Zn^{2+} can enter swine wastewater via faeces and urine (Zeng et al., 2021). For Copper, all plant-mediated treatments showed significantly lower concentrations ($p \leq 0.05$) than the control unit (Fig 2a). On the other hand, no significant differences in Zn concentrations were observed in the wastewaters treated by the four plants and control (Fig 2b).

Macrophytes are recognized for their effectiveness in taking up heavy metals from wastewater (Rezania et al., 2016). Both metals are essential elements for these plants, with Zn serving as a co-factor for several enzyme systems and in protein synthesis (Zhou et al., 2018), while Cu acts in the synthesis of photosynthetic electron transport (Kumar et al., 2021).

Of all the physicochemical parameters, the lowest final concentrations were observed for organic matter, measured by tCOD (Fig 2c), representing removals that ranged from 25% (control), 68% (*S. minima*), 79.3% (*L. punctata*), 80.7% (*A. microphylla*) to 80.6% (*L. minuta*). All macrophyte treatments produced final tCOD significantly lower ($p \leq 0.05$) than the control treatment (Fig 2c).

Although synthetic wastewater was used, treatment did not take place under sterile conditions and therefore subject to the development of microorganisms, which may have colonized the plant roots and metabolized the organic matter. The synthetic wastewater presented a $BOD_{5,20}$/COD ratio of 0.57 (Table 1) was characterized by readily biodegradable (Metcalf and Eddy, 2016). Thus, it is likely that the microorganisms present in the wastewater contributed to the high tCOD removal.

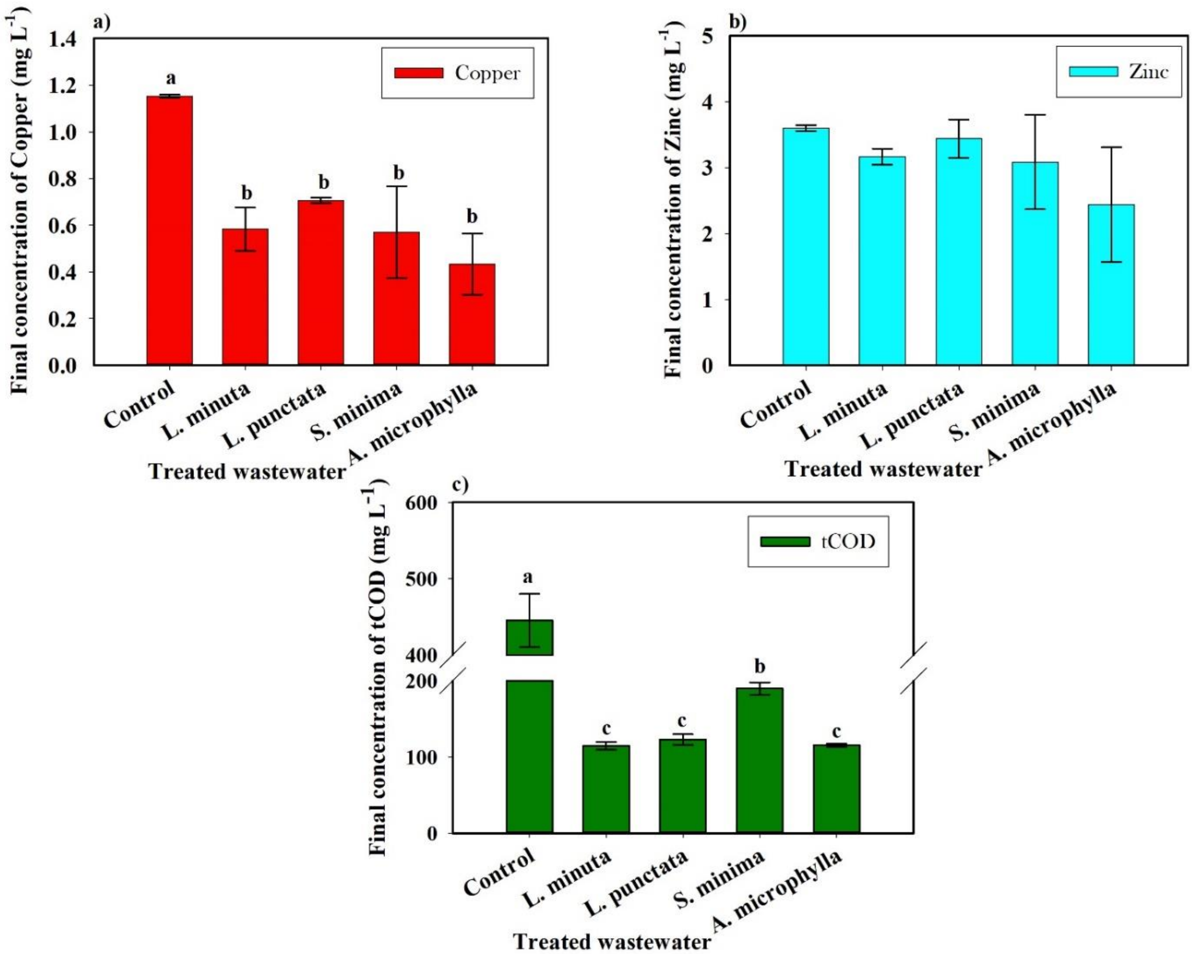

Fig 2: Final concentration of a) copper, b) zinc and c) tCOD in the treated wastewater. Different letters show a significant difference ($p \leq 0.05$) between the treatments according to the Tukey test ($p \leq 0.05$). ($n = 3$. mean ± standard deviation).

3.1.2 Ecotoxicological Wastewater Evaluation

The macrophyte-treated and control units significantly reduced acute and chronic toxicities to *C. dubia*. From initial value of 116.6, the acute toxic units (TUa) ranged from zero (no toxicity) in the *Lemna minuta* and *A. microphylla* wastewater to 2.16 units in the control (Table 2). On the other hand, the chronic toxic units ranged between 1.41 in *Lemna*-treated wastewater and 2.77 units in the control (Table 2) from initial value, 121.4.

C. dubia is extremely sensitive to metals and other compounds present in swine wastewater. Toxicity of some of these pollutants reported in the literature include $LC_{50\%,2d}$ =1.15 mg L^{-1} for $N\text{-}NH_4^+$ (Martins et al., 2007) to *D. magna* and 18 µg L^{-1} (Cu) or 173.5 µg L^{-1} (Zn) to *C. dubia* (Cooper et al., 2009). Likewise, in a multi-element medium synergistic/interactions occur and other compounds potentiate toxicity of the wastewater (Cooper et al., 2009; Villamar et al., 2012). Thus, it was expected that the high concentrations of the pollutants in the synthetic swine wastewater (Table 1, Table A.1) would cause high acute and chronic toxicities to *C. dubia*.

However, when comparing the residual concentrations of Cu, Zn, and $N\text{-}NH_4^+$ in the wastewater treated by *Azolla* and *Lemna* (as detailed in section 3.1.1) with the $CL_{50\%}$ concentrations for acute toxicity (1.15 mg L^{-1} for $N\text{-}NH_4^+$, 18 µg L^{-1} for Cu, and 173.5 µg L^{-1} for Zn), it was expected that the treated wastewater would exhibit at least acute toxicity attributed to these compounds since their concentrations were higher than the $CL_{50\%}$. However, this was not observed (as shown in Table 2). This apparent contradiction may be attributed to a possible antagonistic effect that occurred in the effluents treated by these plants.

Only one study was found in the literature that evaluated the ecotoxicological effects of swine wastewater using *C. dubia* as a model organism (Belin et al., 2000), in which acute toxicity ($LC_{50\%,\ 2d}$) of swine wastewater after secondary treatment was found in the range of 27.9% - 31.5% (TUa = 3.5 – 3.2). That wastewater, with lower acute toxicities, had concentrations of metals and nutrients about 10 to 100-fold less than in the wastewater used in our study.

A recent study showed that, despite being different species, *C. dubia* and *D. magna* have similar sensitivities in acute and chronic toxicity tests; therefore, both toxicity results may be comparable (Connors et al., 2022). Villamar et al. (2012) characterized toxicity in primary swine wastewater and estimated an $LC_{50\%,2d}$ = 3.5% (TUa = 28.5) to *D. magna*. Those authors observed that the increased toxicity of the wastewater to the test organism was related, for example, to the presence of $N\text{-}NH_4^+$.

Table 2: Toxicity of synthetic treated swine wastewater to *C. dubia* after three-day (acute toxicity) and eight-day (chronic toxicity) exposures

Treated wastewater	Acute toxicity		Chronic toxicity	
	$LC_{50\%, 3d}$ ($CI_{95\%}$)	TUa	$IC_{50\%, 8d}$ ($CI_{95\%}$)	TUc
Control	46.3 (25.93-86.64)	2.16	36.0 (29.53-43.19)	2.78
L. minuta	No toxicity	0.00	70.7 (66.84-71.43)	1.41
L. punctata	70.7 (56.79-88.04)	1.41	41.1 (35.29-45.96)	2.43
S. minima	65.3 (57.84-75.25)	1.53	35.8 (35.41-36.29)	2.80
A. microphylla	No toxicity	0.00	58.3 (47.97-64.95)	1.72

$CI_{95\%}$: Confidence interval; $LC_{50\%,3d}$: concentration that caused 50% lethality, measured after 3 days; $IC_{50\%,8d}$: concentration that caused 50% inhibition, measured after 8 days; TUa: acute toxicity units; TUc: chronic toxicity units

Wastewater treated with *A. microphylla* and *L. minuta* presented significantly lower acute and chronic toxicities than the control, *L. punctata* and *S. minima* wastewater-treated (Table 2). After three days, neither *L. minuta* nor *A. microphylla* wastewater presented acute toxicity to *C. dubia*. Furthermore, only the wastewater treated by these two plants showed significantly lower chronic toxicity than the control unit (Table 2).

3.2. Biomass by-products and physiological plant characteristics

3.2.1 Resource recovery indicators

Protein, lipids and total volatile solids contents were quantified in the *Landoltia punctata*, *L. minuta*, *S. minima* and *A. microphylla* harvested at the end of the experiment. Final protein contents spanned from 22.8 % DW to 33.7 % DW; lipids from 1.1 % DW to 14.1 % DW and volatile solids from 660.1 mg g^{-1} to 687.8 mg g^{-1} DW. There was no significant difference between treatments for volatile solids (Fig 3a).

Biomass productivity is an important parameter for assessing plant growth and its by-product potential yield (Hunt, 2017). All four macrophytes, grew vigorously in the synthetic swine wastewater over the 10-day experiment. *L. minuta* (15.94 g m^{-2} d^{-1}) and *A. microphylla* (15.44 g m^{-2} d^{-1}) presented the highest biomass productivity ($p \leq 0.05$) whereas *S. minima* (12.65 g m^{-2} d^{-1}) had the lowest production (Fig 3b).

L. punctata (33 ± 0.9 % DW) and *L. minuta* (22.7 ± 0.9 % DW) contained the highest and lowest ($p \leq 0.05$) protein content, respectively, while *S. minima* (28.5 ± 6.2 % DW) and *A.*

microphylla (28.4 ± 1.0 % DW) had similar intermediate protein contents (Fig 3c). The great potential of protein production by *L. punctata* treating swine farming wastewater has already been reported in the literature. For instance, Bergmann et al. (2000) compared 41 duckweed species in swine wastewater treatment and *L. punctata* was the species with the highest protein production. Mohedano et al. (2012) evaluated the efficiency of *L. punctata* ponds in the removal of nutrients from piggery farm wastewater as well as biomass productivity and protein content. The ponds produced 68-ton ha^{-1} y^{-1} of DW and 35% DW of protein revealing the great potential of *L. punctata* to polish swine wastewater and simultaneously produce added value.

L. minuta had the highest ($p \leq 0.05$) lipids content (14.1 ± 2.1 % DW), while. *S. minima* was the macrophyte with the lowest final lipids content (1.1 ± 0.2 % DW) and *A. microphylla* (7.8 ± 2.4 % DW) and *L. punctata* (9.8 ± 0.2 % DW) had statistically similar lipids contents ($p \leq 0.05$) (Fig 3d). To the best of our knowledge, no studies that evaluated lipid content in macrophyte biomass that treated swine farming wastewater were found in the literature.

However, for other types of wastewater, lipid concentrations vary between 4% and 14% DW (Brouwer et al., 2016; Miranda et al., 2018). The protein and lipids contents together with high biomass production make the postharvest macrophyte biomasses potentially value-added by-products such as biofuels and animal feeds (Appenroth et al., 2017), which may support circular economy efforts.

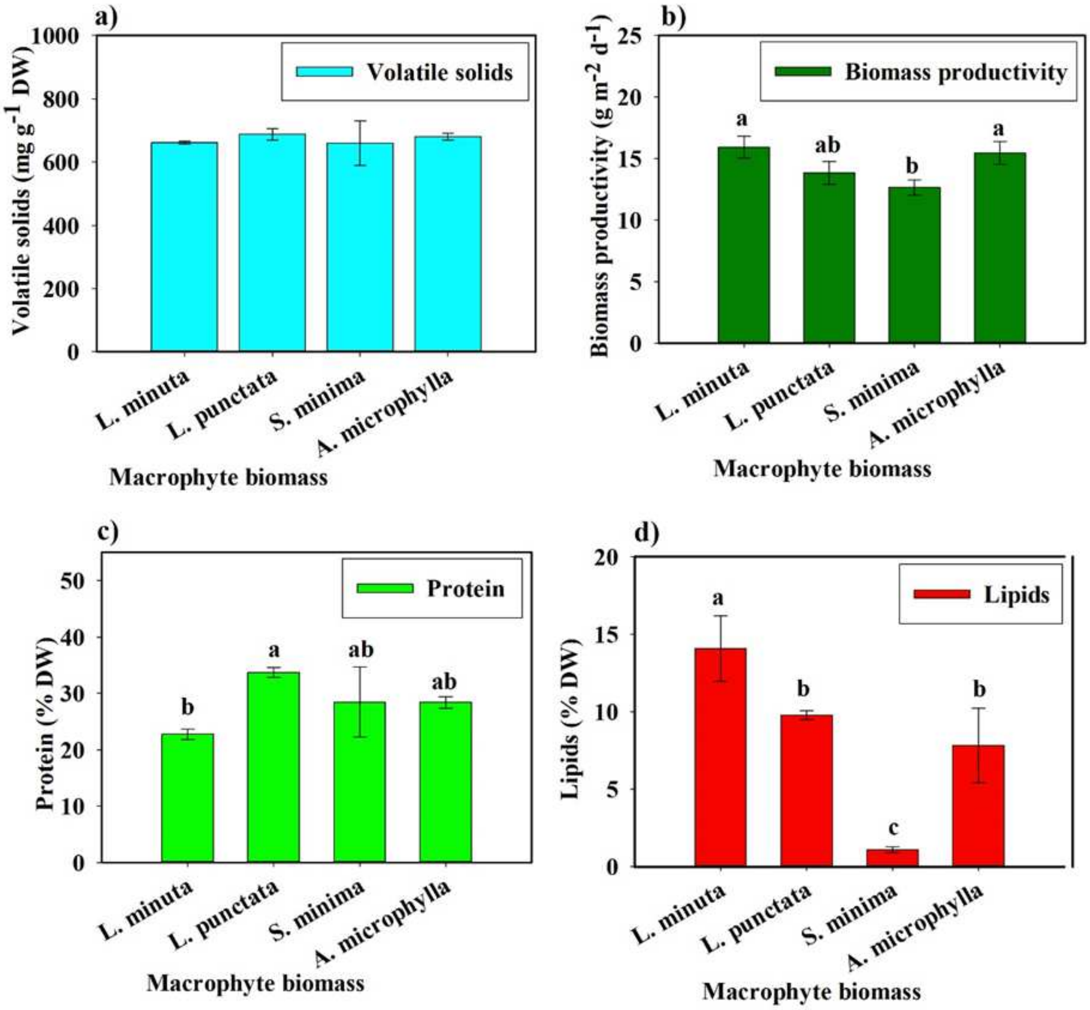

Fig 3: Total of a) Volatile solids, b) biomass productivity, c) Proteins and d) Lipids contents in the macrophyte biomasses after the experiment. Different letters show a significant difference ($p \leq 0.05$) between the treatments according to the Tukey test. (mean ± standard deviation, n = 3).

3.2.2 Phytotoxicity indicators

Overall, there was a decrease in chlorophylls (a, b and total) and an increase in carotenoids and anthocyanin in the fronds of the macrophytes at the end of the experiment (Table 3). The largest reductions in Chl a occurred in *L. minuta* (45.3%) followed by *L. punctata* (26.6%), *A. microphylla* (12.9%) and *S. minima* (9.8%). The behavior was slightly different for Chl b and total Chl. To Chl b, *S. minima* (44.9%) had the largest reductions followed by *L. minuta* (28.2%), *A. microphylla* (26.5%), *L. punctata* (14.5%). For the total Chl, this pattern was done by *L. minuta* (40.9%), *S. minima* (27.9%), *L. punctata* (23.6%), *A. microphylla* (15.9%). *L. punctata* had the highest contents ($p \leq 0.05$) of these pigments before and after treatment whereas *S. minima* or *A. microphylla* had the lowest ($p \leq 0.05$) (Table 3).

Photosynthetic pigment changes can indicate the potential productivity in plants and their reduction can be used as a test for wastewater-borne toxicity, although it does not indicate a specific substance that causes this toxicity (Ziegler et al., 2019). Although essential for plant metabolism, high concentrations of nutrients such as nitrogen and phosphorus might inhibit the growth of aquatic plants (Huang et al., 2013; Rastetter et al., 2017). Moreover, metals might be more easily absorbed by aquatic plants and more likely to be harmful when internalized (Ziegler et al., 2019). For instance, Henke et al. (2011) observed aberrations in the chloroplasts of *L. punctata* exposed to metals.

On the other hand, carotenoids and anthocyanin increased during all treatments with the maximum change occurring in *A. microphylla* and *S. minima* followed by *L. punctata* and *L. minuta* (Table 3). Living organisms have a redox system that aims to balance the production/increase of reactive oxygen species (ROS) and prevent damage. Under environmentally stressful conditions, the toxic effects of ROS in plants are counteracted by enzymatic and non-enzymatic mechanisms (Sharma et al., 2012). Representing the second line of defense against the ROS, the non-enzymatic antioxidant system includes carotenoids and phenolic compounds (i.e. anthocyanin) that act as scavengers for these species that impair plant growth and development (Naing and Kim, 2021; Uarrota et al., 2018).

L. minuta (0.3163 ± 0.006) presented the highest relative growth rate and *S. minima* (0.2309 ± 0.004) was the lowest. *L. punctata* (0.2857 ± 0.012) and *A. microphylla* (0.2665 ± 0.006) presented statistically similar rates. RGR is a useful indicator of plant growth under environmental stress and disturbance regimes. The higher the plant RGR during treatment, the better adapted the plant was to the wastewater (Sudiarto et al., 2019).

To sum up, the photosynthetic pigments (Chl a, b and total) decreased and carotenoids and anthocyanin increased in the four plants after phytoremediation of SSW. This indicates that the treatment conditions increased phytotoxicity in all the macrophytes. However, it is not possible to pinpoint which plant was most or least tolerant to the wastewater, since different responses were found in different plants.

To uncover this pattern, we used the phyto-stress index, which can be defined as any external factor that negatively affects plant growth, reproductive capacity and survival (Uarrota et al., 2018). The toxicity biomarkers related to photosynthesis including Chl a, Chl b, Total Chl, Car, and Antho were integrated and quantified in Phyto-Stress Index (PSI) values. PSI values were obtained from the median of all (n-1)! A_i matrices (Eqs. 10,11, see Methods section 2.6.3) are expressed in Table 3.

Overall, *A. microphyll* had the lowest stress index (0.09) during the treatment of synthetic swine wastewater (Table 3), while the highest PSI was found for *L. minuta* (3.52). Likely, the expressive PSI for *L. minuta* was attributed to the high reductions in chlorophyll a and total chlorophyll during wastewater treatment with this plant, which presented the highest scores for these two biomarkers among all biomarkers of all plants (Table A.5, Appendix A).

Only *Azolla* showed no visible signs of necrotic or chlorosis spots in their fronds (Fig A.3, Appendix A). To the best of our knowledge, no studies have used the IBR value from photosynthetic pigments for aquatic plants and our study contains the first account of this useful tool for measuring the phyto-stress of macrophytes using photosynthetic pigments.

Table 3: Pigment concentrations measured at the initial and final of the experiment and relative growth rate

Macrophytes	Biomarkers that made up the Phyto-Stress Index (PSI)										PSI	RGR (d^{-1})
	Chl a (mg g^{-1}FW)		Chl b (mg g^{-1}FW)		Total Chl (mg g^{-1}FW)		Car (mg g^{-1}FW)		Antho (µmol g^{-1}FW)			
	BmK_0	BmK_{10}	BmK_0	BmK_{10}	BmK_0	BmK_{10}	BmK_0	BmK_{10}	BmK_0	BmK_{10}		
L. minuta	0.7404^{b} ± 0.06	0.4049^{b} ± 0.04	0.2556^{ab} ± 0.01	0.1835^{ab} ± 0.01	0.996^{b} ± 0.07	0.588^{b} ± 0.05	0.2214^{ab} ± 0.02	0.5032 ± 0.01	0.0379 ± 0.03	0.3865^{a} ± 0.07	3.52	0.3163^{a} ± 0.006
L. punctata	0.8693^{a} ± 0.02	0.6381^{a} ± 0.01	0.3101^{a} ± 0.04	0.2617^{a} ± 0.01	1.179^{a} ± 0.06	0.900^{a} ± 0.02	0.2542^{a} ± 0.01	0.5282 ± 0.08	0.055 ± 0.04	0.2347^{b} ± 0.02	0.54	0.2857^{b} ± 0.012
S. minima	0.2656^{d} ± 0.03	0.2398^{c} ± 0.06	0.2927^{a} ± 0.01	0.1627^{b} ± 0.07	0.558^{c} ± 0.04	0.402^{c} ± 0.09	0.1595^{ab} ± 0.01	0.4427 ± 0.03	N. D	0.2519^{ab} ± 0.08	0.35	0.2309^{c} ± 0.004
A. microphylla	0.5016^{c} ± 0.07	0.4369^{b} ± 0.03	0.1705^{b} ± 0.07	0.1181^{b} ± 0.02	0.672^{c} ± 0.06	0.565^{b} ± 0.02	0.1218^{b} ± 0.07	0.4563 ± 0.04	N. D	0.3272^{ab} ± 0.03	0.09	0.2665^{b} ± 0.006

* Bmk_0: Concentration of target biomarker measured in plants collected in Hoagland solution, before treatment (Day-0). BmK_{10}: Concentration of target biomarkers measured in plants collected in synthetic swine wastewater after treatment (Day-10). Chl a: Chlorophyll a. Chl b: Chlorophyll b. Total Chl: Total Chlorophyll. Car: Carotenoids. Antho: Anthocyanin. RGR: Relative Growth Rate. FW: Fresh Weight. N.D: Not Detected. Means in the same column followed by the same letter (a, b, c and d) are not significantly different ($p \leq 0.05$) according to the Tukey test (n = 3. mean ± standard deviation).

3.3. Multi-criteria analysis

The weighting criteria were obtained from subjective (FAHP) and objective (Entropy) methods. For subjective weighting, out of all the fifteen experts' evaluations, only ten were considered for the calculation of the weights, since they obtained CR < 0.1 (Table A.2, Appendix A). For FAHP method (W^S), RGR, TUa, TUc and $R_{N\text{-}NH4+}$ were the four most important attributes while R_{COD} was the least (Table 4).

Table 4: Individual and compromise weights of the multicriteria methods

Attribute	FAHP weights (W^S)	Entropy weights (W^O)	[a]Compromise weights (W^C)
R_{COD}	0.0219	0.0014	0.00031
$R_{N\text{-}NH4+}$	0.1028	0.0082	0.00846
$R_{N\text{-}NO3-}$	0.0733	0.0106	0.00779
$R_{P\text{-}PO43-}$	0.0590	0.0004	0.00024
R_{Cu}	0.0785	0.0279	0.02199
R_{Zn}	0.0794	0.0074	0.00587
TUa	0.1209	0.0205	0.02495
TUc	0.1209	0.4064	0.49358
Lipids	0.0393	0.1211	0.04781
Volatile solids	0.0478	0.0001	0.00005
Protein	0.0368	0.0055	0.00205
RGR	0.1210	0.0038	0.00457
PSI	0.0984	0.3867	0.38233

[a]: Compromise weights refer to W^C (set of W^S and W^O weights-Eq. 12). RGR: Relative Growth Rate. PSI: Phyto-Stress Index. TUa: acute toxicity units; TUc: chronic toxicity units

The weights were also determined by the Entropy method (W^O), in which the variation of attribute responses between alternatives is considered and the greatest weight is attributed to the greatest variation. In this method, the attributes of greatest importance (highest weight) were TUc, PSI and lipids and the lowest importance were volatile solids and $R_{P\text{-}PO43}$. The RGR attribute had the highest weight in the FAHP method and one of the lowest in the Entropy method. This difference shows the importance of combining the methods since using only one would lead to different rankings of the alternatives.

Thus, the compromise weights (W^C) were calculated (Eq.12). Cost attributes were the most important criteria in compromise weights. In descending order, TUc, PSI and lipids were the attributes with the highest weights while R_{COD}, $R_{P\text{-}PO43-}$ and volatile solids were the least important (Table 4). The attribute response matrix (Table A.6, Appendix A) was normalized.

The normalized decision matrix was multiplied by the compromised weights given the weighted normalized decision matrix, Vij (Table A.7, Appendix A).

Among the alternatives, the largest response differences were found for TUa, lipids and PSI which caused the entropy value of these attributes to increase and therefore their weights. For instance, the higher PSI value obtained by *L. minuta* increased the entropy value of PSI and affected its combined weight. A combination of high weight and high PSI (cost type) value caused *Lemna*'s score to decrease. Indeed, for TUc, lipids and PSI there was an increase in Entropy weights compared to FAHP weights (Table 4).

Since the final weight (W^C) was calculated as a weighted average (Eq 12), it is possible that the Entropy method could have more importance than the FAHP on the final choice of plant. However, a comparison of TOPSIS rankings by the two methods showed greater similarity between the ranking of the combined weights (W^C) and the subjective weights (FAHP - obtained from the experts' evaluation) than between W^C and Entropy (Table 5). Likely, greater differences in TUc, lipids and PSI among the alternatives did not lead to advantages of the Entropy method over the fuzzy AHP weighting. In any case, in both rankings, *A. microphylla* was the highest ranked. The W^C scores are considered as the final TOPSIS rankings, and the best alternative found was *A. microphylla* followed by *L. minuta*, *L. punctata* and *S. minima* (Table 5).

Table 5: Selection of best alternatives for all macrophytes based on TOPSIS analysis for each weighting's methods

[a]Weights	Alternatives	V_i^+	V_i^-	Performance score	Ranking
W^C	*L. minuta*	0.3659	0.3642	0.4988	2
	L. punctata	0.3385	0.3204	0.4862	3
	S. minima	0.3653	0.3387	0.4811	4
	A. microphylla	0.0159	0.5156	0.9701	1
W^S	*L. minuta*	0.0987	0.1042	0.5137	2
	L. punctata	0.0961	0.0885	0.4796	3
	S. minima	0.1089	0.0884	0.4481	4
	A. microphylla	0.0193	0.1418	0.8801	1
W^O	*L. minuta*	0.3701	0.3101	0.4559	4
	L. punctata	0.2815	0.3276	0.5379	2
	S. minima	0.3114	0.3426	0.5238	3
	A. microphylla	0.0401	0.4776	0.9226	1

[a] W^C: Compromise weights (E-FAHP method). W^S: Subjective weights (FAHP method). W^O: Objective weights (Entropy method).

In the sensitivity analysis, the weights of the attributes were changed and ranking results were assessed. The sensitivity analysis also proved the preference of *Azolla*, regardless of the weights assigned (Fig A.4, Appendix A). Hence, the W^C and TOPSIS proved reliable in choosing a suitable macrophyte for phytoremediation of swine wastewater.

3.4. Advantages and recommendations of the present research approach

The hybrid Entropy-Fuzzy AHP weighting with ranking TOPSIS methods was considered to select the best-floating macrophyte for the phytoremediation of SSW. To the best of our knowledge, this study is the first to assess simultaneously the quality aspects of the treated wastewater, biomasses by-products and phytotoxicity endpoints as attributes in the multicriteria decision-making method.

By integrating the Entropy-FAHP and TOPSIS methods it was possible to find a plant that not only meets the expectations of the decision maker but also the intrinsic values obtained by its performance during wastewater treatment. From the attributes, quality criteria of the treated wastewater were considered concerning plant characteristics during (phytotoxicity) and at the end (biomass by-products) of the wastewater treatment.

This approach allowed us to evaluate not only if phytoremediation improves the quality of the SSW wastewater, but also which plants were less affected by the wastewater, as measured by the PSI. Moreover, thinking about a circular economy, the proposal innovates by incorporating the potential of value-added products from plants, prioritizing the choice of a plant with higher production of lipids and proteins, but also evaluating the volatile solids content of the biomass as a prospect for potential production of bioenergy.

Some works used the integration of multicriteria decision-making methods in the choice of plants for phytoremediation, including considering the aspects of physical-chemical quality of the remediated medium and plant physiology (Cao et al., 2022; Wang et al., 2019) but not for wastewater considering the quality attributes of the treated wastewater, physiological plant characteristics and biomass by-products. Furthermore, this is the first time *A. microphylla* has been reported for treating swine wastewater. Our study showed the as-yet unexplored potential of the species in treating swine wastewater.

The selection of wastewater treatment technologies using decision-making processes depends heavily on factors such as capital and operating and maintenance costs (Kalbar et al., 2012). Since this was a laboratory experiment, social and environmental aspects were not considered. We chose to work under conditions with no external variations, including using real wastewater that may vary from one collection to another. The objective was that the only variable was the different plants used.

It is recommended, however, that this methodology also be applied at a pilot scale under external variations, and that the economic costs of capital (CAPEX) and operation (OPEX), which are still relatively unexplored and discussed in constructed wetlands treatment systems, be incorporated into multicriteria decision-making models. Even so, the present work provided good results and met the objective, which was to choose which plant was best suited for the treatment of swine wastewater based on the quality of the treated wastewater, the health of the plants, and what biomass could provide as value-added by-products.

4. CONCLUSIONS

The macrophytes *L. minuta, L. punctata*, *A. microphylla* and *S. minima* were used for the treatment of synthetic swine wastewater, during 10 days of exposure. At the end of the experiment, the quality of the treated wastewater, biomass resources and growth parameters were accessed.

Overall, there were significant differences in the removal of the pollutants among the plants and almost always with the control. *A. microphylla* stood out as the plant with the lowest PSI caused by SSW. In turn, *L. minuta* presented treatment with the highest RGR and lipid production. Both plants performed very well in removing acute and chronic toxicity of the treated wastewater to *C. dubia*. However, *L. minuta* was strongly affected by wastewater as indicated by the highest PSI value.

These better performances of *A. microphylla* and *L. minuta* were shown in the ranking obtained by the hybrid MADM method proposed here. In this method, *A. microphylla* was chosen as the most suitable macrophyte for swine wastewater treatment. Ranking considering only the individual weight methods (FAHP and Entropy) and sensitivity analysis also indicated the best performance of this species.

Our study provides a reliable approach to decision-makers to choosing a suitable macrophyte that performs in dual advantage: optimal wastewater treatment performance and biomass with the greatest potential for recycling in a circular economy context.

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CHAPTER 4: EFFECTS OF LED LIGHTS AND CYTOKININ ON THE PHYTOTREATMENT OF SWINE WASTEWATER BY AZOLLA SPP.: POLLUTANT REMOVAL AND BIOMASS VALORIZATION

Abstract: Phytoremediation is an eco-friendly and affordable option for tackling wastewater pollutants. We investigated how LED light exposure, measured by intensity and duration (photoperiod), along with cytokinin, impacts *Azolla microphylla*'s swine wastewater treatment performance and biomass production over of ten days. Under optimal treatment conditions we observed high removals of COD (89.2% to 90.8%), $N\text{-}NH_4^+$ (72.6% to 91.2%), $N\text{-}NO_3^-$ (84.4% to 88.6%), Cu (75.4% to 86.4%), sulfamethoxazole (77% to 79%), $P\text{-}PO_4^{3-}$ (54.1% to 59.9%) and DOC (67.4% to 71.3%) while Zn presented a more moderate reduction (2% to 9.7%). Biomass productivity reached up to 34.8 t ha^{-1} yr^{-1}. Protein production accounted for 23% to 27% of dry weight, while lipids ranged from 20% to 34% of dry biomass. Starch content varied from 8% to 28% of fresh weight. Higher light intensities, with both high or low values of photoperiods, and low concentrations of cytokinin were identified as optimal conditions for removal of almost all pollutants. However, pollutant removal was impacted differently by LED light and cytokinin concentration. In treatment conditions with the shortest photoperiods (8 h), we observed the lowest residual concentrations of Cu and Zn, whereas with longer photoperiods (24 h), the lowest residual concentrations of $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ were recorded. On the other hand, SMX was the only parameter in which cytokinin had a clear influence on its removal, with the lowest residual concentration observed under 8-hour photoperiods combined with the lowest tested cytokinin concentrations (0.3 mg L^{-1}). For COD and $N\text{-}NO_3^-$, no discernible pattern was evident for any of the analyzed factors. Therefore, our study demonstrates the potential for treating swine wastewater using *Azolla microphylla*, aligned with its ability to produce biomass rich in high-value compounds.

Keywords: Phytoremediation; Optimization; Doehlert matrix; Sulfamethoxazole; Phytohormone; Piggery Wastewater.

1. INTRODUCTION

Worldwide expansion of the economy and population has spurred the growth of livestock farming, with pork playing an important part in the global meat industry. For instance, in 2022, Brazil produced approximately 4.9 million tons of pork meat of which 77% was intended for the domestic market. As a result, Brazil is currently the fourth-largest pork producer in the world, trailing only behind China, the European Union, and the United States (ABPA, 2023).

Swine wastewater is a source of pollution that requires appropriate management and treatment before release into the environment. These liquid residues arise from swine manure and the water used for barn cleaning, resulting in wastewater laden with pollutants, including organic matter, nutrients, metals, and antibiotics (Deng et al., 2023).

Common technologies used to treat swine wastewater are land application, stabilization ponds and anaerobic digesters (Deng et al., 2023; Kunz et al., 2009). Conversely, phytoremediation is a cost-effective and affordable option for tackling wastewater pollution. It is eco-friendly, solar-powered, and offers a sustainable economical alternative to treat wastewater. Moreover, macrophyte biomass can serve as alternative feedstock for producing biofuels, biofertilizers and animal feed (Chen et al., 2022; Kurniawan et al., 2021).

Although different types of macrophytes for treating swine wastewater have already been reported in the literature (Wang et al., 2021; Xu et al., 2020), some species, such as *Azolla microphylla*, have not been extensively studied, despite showing great potential for swine wastewater treatment (Coimbra et al., 2023). In addition, addressing certain challenges, such as phytotoxicity or plant growth impairment, is essential to ensure the efficient removal of pollutants by aquatic plants. To enhance plant growth, strengthen environmental stress responses, and enhance the effectiveness of phytoremediation, it is important to consider appropriate operational factors (Zhang et al., 2024).

Light intensity, photoperiod, and phytohormones are factors reported to affect plant development or improve the phytoremediation process (Kilian et al., 2022; van Dyck et al., 2021; Yu et al., 2024). The quantity of light, in terms of both duration (light exposure time or photoperiod) and intensity (e.g., measured in photon flux density), impacts plant growth and dictates how plants respond to adverse environmental conditions (Paik and Huq, 2019; Paradiso and Proietti, 2021). Furthermore, light sources of specific wavelengths and intensities enhance plant productivity by regulating metabolic processes and physiological responses (Paik and Huq, 2019). Within this context, light-emitting diodes (LEDs) offer advantages over conventional lighting as they are low-cost, have a longer lifespan, and have low heat emissions

(Paradiso and Proietti, 2021). Nevertheless, the use of light photoperiod and intensity in phytoremediation studies remains underexplored, with most research focusing solely on biomass increase (Liu et al., 2019, 2018; Yin et al., 2015), without considering the impact on the capacity to remove aquatic pollutants.

On the other hand, researchers have studied phytohormones for their potential role in macrophytes in the phytoremediation of wastewater and soils, particularly in the removal of potentially toxic elements (PTEs) such as chromium (Barbosa et al., 2023), fluoride (Vaz et al., 2023), and cadmium (Peng et al., 2023). However, many phytohormones have not yet been explored. Among these, cytokinin has produced remarkable results in biomass accumulation in duckweeds, surpassing the performance of other phytohormones (Liu et al., 2019). Nonetheless, it is still unclear how this phytohormone affects macrophytes in the phytoremediation of wastewater. Hence, it is necessary to investigate how light intensity, photoperiod, and cytokinin affect the phytoremediation of swine wastewater.

Most studies that examine the influence of operational factors on wastewater treatment apply only one variable at a time (Walsh et al., 2021; Yin et al., 2015). This approach has limitations, as it requires many experiments and fails to uncover the iterative behavior of process factors. Adopting a statistical experimental design, such as response surface methodology (RSM), allows for the collective optimization of factors, effectively circumventing these constraints. Furthermore, the Doehlert matrix design offers valuable insights into the response surface with fewer experiments required compared to designs like the central composite, providing experimental flexibility (Ferreira et al., 2004).

The main objective of this study was to evaluate the influence of LED, considering both its intensity and photoperiod and cytokinin on the performance of *Azolla microphylla* in the treatment of swine wastewater. This treatment targets the removal of organic matter, nutrients, metals, and antibiotic, alongside the production of high-value-added biomass. We hypothesized that these factors increase the removal of pollutants from swine wastewater and biomass production, albeit in different manners.

2. MATERIAL AND METHODS

2.1 Experimental setup

Bench-scale structures with dimensions of 120 cm × 50 cm × 70 cm (L × W × H) containing eight units (29 cm × 29 cm) were constructed of medium-density fiberboard (MDF) (Fig. 1A-C). The apparatus was equipped with white light emitting diode (LED) lamps (10W, LED lights, Avant Lux®, São Paulo, SP, Brazil) (Fig. 1C), controlled by dimmers (Qualitronix® Tecnologia Ltda, Santa Rita do Sapucaí, MG, Brazil), connected to timers (TM-22 Timer, Timer Elcon Indústria e Comércio Ltda, Belo Horizonte, MG, Brazil) (Fig. 1B). In each unit, 16.6 g (± 0.01) of healthy fresh plants were added to 2 L working volume plastic pots (0.045 m²) containing simulated swine wastewater (SSW), totaling 19 pots used, one for each experimental condition as per Table 1.

The plant species employed in this study was *Azolla microphylla* (water fern), selected from the previous stage (Chapter 3) as detailed previously (Coimbra et al., 2023). The strains were collected at the Botanical Garden of the Federal University of Viçosa (UFV), Viçosa, Minas Gerais, Brazil (20°45’25.0” S 42°52’25.5” W), sterilized and acclimatized in a half-strength Hoagland nutrient medium at room temperature (23 ± 2 °C) under 40 µmol m^{-2} s^{-1} LED lamps with a 16 h light: 8 h dark cycle. The experimental period was 10 days, during which deionized water was added every two days to compensate for evapotranspiration. The use of deionized water is to ensure that only water is introduced, without the presence of pollutants in concentrations that may alter the characteristics of the wastewater.

The swine wastewater was prepared following a previous study (Coimbra et al., 2023) to simulate a secondary wastewater treatment step. Initial concentrations of chemical oxygen demand (COD) (306 ± 9 mg L^{-1}), copper (Cu) (1.18 ± 0.1 mg L^{-1}), zinc (Zn) (4.2 ± 0.05 mg L^{-1}), dissolved oxygen demand (DOC) (42 mg L^{-1}), phosphate-phosphorus (P-PO_4^{3-}) (91.63 ± 8.25 mg L^{-1}), ammonium-nitrogen (N-NH_4^+) (85 ± 13.11 mg L^{-1}) and nitrate-nitrogen (N-NO_3^-) (83.2 ± 2.8 mg L^{-1}) were utilized. The sulfamethoxazole (SMX) concentration was 100 µg L^{-1} based on concentrations reported in swine wastewaters (Cheng et al., 2020). The analyses of wastewater and biomass were conducted at the beginning and end of the 10-day treatment period.

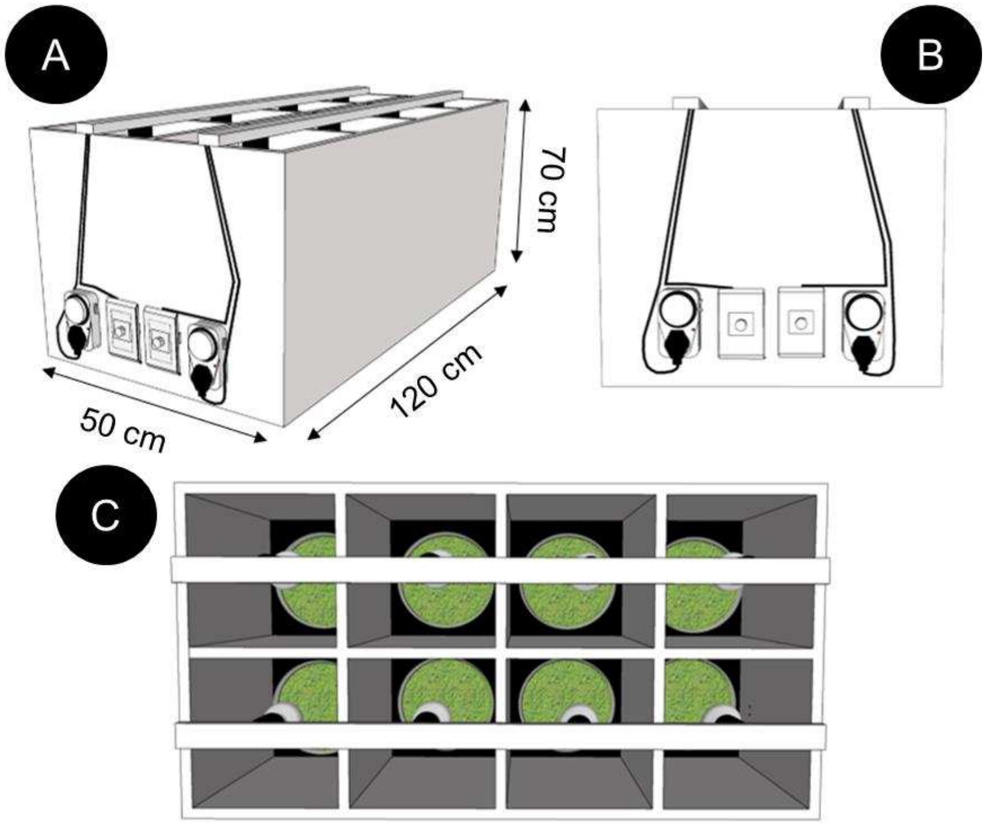

Fig 1: Experimental setup for a phytoremediation of simulated swine wastewater. Views (not to scale) isometric (A), front (B), and top (C) of the experimental apparatus. In C, the representation of the eight units of each MDF structure.

2.2 Experimental design

RSM with a Doehlert experimental design was used to evaluate the treatment of simulated swine wastewater. Residual concentrations of Zn, Cu, COD, P-PO_4^{3-}, N-NH_4^+, N-NO_3, and SMX and the removed concentration of DOC, after phytoremediation, were the response variables.

The experiment evaluated three independent variables (operational factors): photoperiod (hours), cytokinin (6-benzylaminopurine, B3408; Sigma Aldrich) concentration (mg L^{-1}) and light intensity (µmol $m^{-2}s^{-1}$), measured as photosynthetically active radiation (PAR) (HOBO® Photosynthetic Light Smart Sensor, Bourne, MA, USA). The lower and upper levels of photoperiod (6-24 hours) (Van Dyck et al., 2021; Yin et al., 2015), cytokinin (0.25-2.25 mg L^{-1}) (Liu et al., 2019) and light intensity (6-100 µmol m^{-2} s^{-1}) (Petersen et al., 2022; Yin et al., 2015) were selected based on previous research that employed these factors to evaluate plant growth or phytoremediation in various aqueous media. The experiment consisted of 19 experimental runs (Table 1) including the central point conditions (15-hour photoperiod, 1.25

mg L^{-1} cytokinin, and 53 µmol m^{-2} s^{-1} light intensity) conducted in septuplicate for Doehlert matrix analysis (Teófilo and Ferreira, 2006), except for SMX, for a total of 16 runs and four central points. The experiment was laid out completely randomized.

Table 1: Factor levels in each experimental run of the experimental design employed

Run	[a]Coded levels			Actual levels		
	A	B	C	Photoperiod (h)	Cytokinin (mg L^{-1})	Light Intensity (µmol m^{-2} s^{-1})
1	1	0	0	24	1.25	53
2	0.5	0.866	0	19.5	2.25	53
3	0.5	0.289	0.817	19.5	1.58	100
4	-1	0	0	6	1.25	53
5	-0.5	-0.866	0	10.5	0.25	53
6	-0.5	-0.289	-0.817	10.5	0.92	6
7	0.5	-0.866	0	19.5	0.25	53
8	0.5	-0.289	-0.817	19.5	0.92	6
9	-0.5	0.866	0	10.5	2.25	53
10	0	0.577	-0.817	15	1.92	6
11	-0.5	0.289	0.817	10.5	1.58	100
12	0	-0.577	0.817	15	0.58	100
13	0	0	0	15	1.25	53
14	0	0	0	15	1.25	53
15	0	0	0	15	1.25	53
16	0	0	0	15	1.25	53
17	0	0	0	15	1.25	53
18	0	0	0	15	1.25	53
19	0	0	0	15	1.25	53

[a]A: Photoperiod. B: Cytokinin. C: Light Intensity

To minimize external lighting interference (from both artificial and natural sunlight), during the experiment, we closed the windows of the laboratory room to reduce the entry of sunlight. The lamps in this room (ceiling height: 3.1 meters) were also usually turned off. Throughout the experimental period, the room temperature remained at 23 ± 2 °C.

2.3 Wastewater Analytical Methods

The simulated swine wastewater was characterized for its physical and chemical nature. Standard methods (APHA, 2022) were employed to quantify COD (5220D), DOC (310B, Shimadzu TOC-L CSH analyzer), P-PO_4^{3-} (4500-P), N-NO_3^- (4599-NO3E), N-NH_4^+ (4500-NH3 B and C), Zn (3500-Zn), and Cu (3500-Cu).

The SMX analysis was conducted based on a variation of the Bratton-Marshall method using Solid Phase Extraction and UV–Visible spectrophotometry (Errayess et al., 2017), with the

SMX concentration determined using a constructed calibration curve measured at 536 nm (R^2 > 0.995). Solid-phase extraction was performed using the Strata® - X cartridge (500 mg/6 mL, Phenomenex, USA) and sulfamethoxazole (≥ 98.0%) was purchased from ACS Científica® (Sumaré, SP, Brazil). All chemicals and reagents were used without further purification. For the N-NO_3^-, Zn, Cu and SMX analyses, samples were filtered using nylon membranes with a pore size of 0.45 μm (Merck, Ireland). A Hach DR 6000 UV–visible beam spectrophotometer with 1 cm matched cells was used for absorbance measurements. Cu and Zn were determined by atomic absorption (PinAAcle 500®, PerkinElmer, MA, USA).

2.4 Biomass analysis

After 10 days of phytoremediation, the plants were collected to evaluate the content of energy-rich molecules in *Azolla*. Prior to analysis, the fresh biomass (FW) was dried in an airflow oven at 65 °C for 72 h until a constant weight was obtained. Biomass productivity (Bp) was calculated as the difference between the dry biomass content (DW) after treatment and that on day zero (collected from Hoagland solution) per unit of square meter of plastic pot area over the ten days of exposure. The results were expressed in g_{DW} m^{-2} d^{-1}.

The Bligh and Dyer method, with minor modifications, was employed to ascertain the total lipid content. Initially, the dry macrophyte biomass (0.1 ± 0.004 g) was added to 20 milliliters of chloroform and methanol solution (1:1 v/v) and vortexed for one minute. The mixture was subjected to ultrasonic waves at 60 watts and 40 kHz for 30 minutes. Afterwards, 10 mL of 1% NaCl solution (m/v) was added and the solution was vortexed for one minute followed by careful removal of the aqueous phase. This process was repeated three times. The lipid-rich phase was transferred to a pre-weighed tube and dried in an airflow oven at 45 °C for 96 hours. Finally, the tube was weighed and the lipid content was expressed as % DW (g of lipid per 100 g of dry plant weight).

The starch content in *Azolla* biomass was determined using the spectrophotometric method (Magel, 1991), with minor modifications. Fresh plant materials (300 ± 0.005 mg) were first homogenized in 14 mL of an 18% (m/v) hydrochloric acid (HCl) solution. This homogenate was then refrigerated at 4 °C for 1 hour and subsequently shaken for 30 minutes at room temperature (23°C). Following this, the mixture was centrifuged at 5000 rpm for 5 minutes. After centrifugation, the supernatant (4 mL) was carefully collected and combined with an equal volume of Lugol's solution. Finally, the absorbance of the solution was measured at 605 nm and 530 nm. The starch content was determined as described previously (Magel, 1991) and was expressed as % FW (g of starch per 100 g of fresh plant weight).

The crude protein content in macrophyte tissue was determined by multiplying the total nitrogen content (TN) by a conversion factor of 6.25 (Casal et al., 2000). TN was quantified using the Kjeldahl method for solid samples (Bremner and Mulvaney, 1983). The protein content was expressed as % DW (g of protein per 100 g of dry plant weight).

2.5 Data Analysis

Quadratic or third-order hierarchical models were applied to the experimental data from the RSM-Doehlert experimental results. Optimization was performed to find the conditions resulting in the lowest residual concentration of the physicochemical parameters. Design-Expert® Software v13 (Stat-Ease Inc., Minneapolis, MN, USA) was used for data optimization. Analysis of variance (ANOVA) was conducted to assess the significance of effects and their interactions ($\alpha = 0.05$). Non-significant terms, excluding those needed to support the hierarchy, were removed. The predictive capacity of the models was evaluated by considering the significance of the regression models ($p \leq 0.05$), the non-significance of lack of fit ($p \geq 0.05$), as well as the coefficients of determination (R^2), adjusted coefficients of determination (R^2_{adj}) and predicted coefficients of determination (R^2_{pred}).

Multivariate analyses including principal component analysis (PCA), two-way hierarchical heatmap and Pearson correlation matrix were applied to track the relationships between optimal treatment condition and physicochemical parameters(Tang et al., 2023). Biomass products and wastewater parameters were analyzed using a statistical generalized linear model (GLM) with gamma, log normal, or normal continuous distributions (with Tukey post-test). The significant distribution was chosen based on the lowest Akaike Information Criterion (AIC) value (Table S9, supplementary material). All statistical analyses were conducted at a significance level of 5 % and outlier values (identified via the Grubbs test) were excluded from the original data. R program environment (version 4.1.2; R Core Team, 2018) was used to perform the statistical analysis.

3. RESULTS

Table 2 lists the residual concentrations of the physicochemical parameters of swine wastewater obtained in the experimental runs. DOC and $P\text{-}PO_4^{3-}$ concentrations were determined as the concentration removed and the residual concentration per biomass produced ($DOC_{removed}$/Bp and $P\text{-}PO_4^{3-}$/Bp), respectively. This approach was adopted to identify significant models ($p \leq 0.05$) while ensuring no significant lack of fit ($p \geq 0.05$) (Tables S1-8 in the supplementary material) for these parameters. Thus, model optimization aimed to generate wastewater with the lowest residual concentrations of Cu, Zn, COD, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, SMX and $P\text{-}PO_4^{3-}$/Bp and maximize $DOC_{removed}$/Bp responses.

COD ranged from 33 mg L^{-1} to 67 mg L^{-1}. Broader variations were observed for $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$, with ranges of 18 mg L^{-1} to 66.9 mg L^{-1} and 4.5 mg L^{-1} to 49.5 mg L^{-1}, respectively. Minimum residual concentrations of Cu and Zn were 3.34 mg L^{-1} and 0.38 mg L^{-1}, while maximum concentrations reached 4.16 mg L^{-1} and 1.22 mg L^{-1}, respectively. SMX concentrations varied from 0.019 mg L^{-1} to 0.092 mg L^{-1}. Variations in removed and residual concentrations of DOC and $P\text{-}PO_4^{3-}$ per unit of biomass produced were also observed (Table 2).

In all experimental runs, concentrations generally decreased, except for increased copper levels at a photoperiod of 10.5 hours, light intensity of 53 µmol m^{-2} s^{-1} and 0.25 mg L^{-1} of cytokinin. The copper concentration in this run reached 1.22 mg L^{-1}, which was higher than the initial concentration in the raw wastewater (1.18 mg L^{-1}). In phytoremediation experiments, such as those involving aquatic plants, there may be an increase in wastewater parameters due to potential plant declines, resulting from phytotoxicity caused by the wastewater during treatment (Hu et al., 2020).

Table 2: The 3-factor Doehlert matrix with the experimental response values for the treatment of swine wastewater by *Azolla microphylla*

Run	Factors			[a]Response							
	Photoperiod (h)	Cytokinin (mg L^{-1})	Light Int. (µmol m^{-2} s^{-1})	COD	DOC_{remov}/Bp	P-PO_4^{3-}/Bp	N-NH_4^+	N-NO_3^-	Zn	Cu	SMX
1	24	1.25	53	44	8.9	16.9	23.2	34.2	3.7	0.38	0.019
2	19.5	2.25	53	59	9.2	38	27.6	4.5	3.64	0.5	0.040
3	19.5	1.58	100	63	8.9	13	18	37.8	3.74	0.5	0.022
4	6	1.25	53	40	28.8	42.7	54.1	9.9	4.16	0.64	0.025
5	10.5	0.25	53	37	35.9	48.5	48.9	8.1	3.84	1.22	0.092
6	10.5	0.92	6	42	73.9	185.6	66.9	9.9	3.82	0.88	0.025
7	19.5	0.25	53	53	12	14.8	23.2	34.2	3.78	0.98	0.032
8	19.5	0.92	6	50	36	75	61.8	11.7	3.4	0.74	0.043
9	10.5	2.25	53	33	29.6	40.5	48.9	23.4	4.0	0.64	0.021
10	15	1.92	6	36	25.5	34.3	56.7	18.9	4.02	0.5	0.025
11	10.5	1.58	100	62	17.7	26.2	38.6	49.5	3.44	0.46	0.019
12	15	0.58	100	43	12.3	16.4	28.3	42.3	3.8	0.58	0.028
13	15	1.25	53	51	15.4	21.3	38.6	38.7	3.62	0.72	0.024
14	15	1.25	53	48	12.3	27.8	28.3	38.7	3.84	0.7	0.027
15	15	1.25	53	62	13.6	20.7	23.2	7.2	4.12	0.66	0.022
16	15	1.25	53	46	19.8	27.9	38.6	44.1	3.34	0.58	0.031
17	15	1.25	53	49	14	20.1	23.9	13.5	3.88	0.5	/
18	15	1.25	53	67	40.2	71.5	28.3	42.3	3.66	0.54	/
19	15	1.25	53	43	21.4	42	33.5	38.7	3.92	0.54	/

Light Int: Light Intensity. DOCremv/Bp: Concentration of DOC (Dissolved Organic Carbon) removed per produced biomass (Bp). SMX: Sulfamethoxazole.
[a] The units for COD, N-NH_4^+, N-NO_3^-, Zn, Cu, and SMX are in mg L^{-1}. For DOCremov/Bp and P-PO_4^{3-} we referred as mg L^{-1} Bp^{-1}. The symbol / represents unmeasured SMX data.

3.1 Regression models analysis and optimization

To demonstrate the influence of the selected factors (photoperiod, cytokinin, and light intensity) on pollutant removal, a significant and well-adjusted mathematical model was individually developed for each pollutant evaluated (Zn, Cu, COD, $DOC_{removed}$/Bp, $P\text{-}PO_4^{3-}$/Bp, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and SMX). The statistical analyses of the regression models (ANOVA) as well as diagnostic residual plots for all pollutants are presented in Tables S1-S8 and Figures S2-S9 of Appendix B, respectively.

Equations 1-8 represent, in non-coded units, the relationship between the residual concentrations of Zn, Cu, COD, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, SMX, and $P\text{-}PO_4^{3-}$/Bp as well as $DOC_{removed}$/Bp as a function of photoperiod, cytokinin, and light intensity. The data for COD, $DOC_{removed}$/Bp, $P\text{-}PO_4^{3-}$/Bp, Zn, and SMX are presented in transformed data. In addition, while quadratic models were those of best-fix and significant for the other pollutants, a third-order model was the most suitable for COD (Eq. 1). For all responses, both the photoperiod and light intensity showed significance ($p \leq 0.05$), either linearly or interactively (quadratic or 2-interaction) interaction. On the other hand, cytokinin did not exert influence on the responses of $DOC_{removed}$/Bp, $N\text{-}NH_4^+$, and $P\text{-}PO_4^{3-}$/Bp (see Tables S1-S8, Appendix B).

$$1/\text{COD} = 0.0423 - 0.001545\ \text{Photoperiod} - 0.035978\ \text{Cytokinin} + 0.000097\ \text{Light Intensity} + 0.002481\ \text{Photoperiod} * \text{Cytokinin} - 1.24\text{E-}04\ \text{Cytokinin} * \text{Light Intensity} + 0.018259\ \text{Cytokinin}^2 - 0.001073\ \text{Photoperiod} * \text{Cytokinin}^2 \quad (1)$$

$$\ln(DOC_{removed}/\text{Bp}) = 5.117 - 0.083384\ \text{Photoperiod} - 0.027111\ \text{Light Intensity} + 0.000137\ \text{Light Intensity}^2 \quad (2)$$

$$\ln(P\text{-}PO_4^{3-}/\text{Bp}) = 5.243 - 0.065313\ \text{Photoperiod} - 0.015772\ \text{Light Intensity} \quad (3)$$

$$N\text{-}NH_4+ = 95.179 - 1.869\ \text{Photoperiod} - 0.9157\ \text{Light Intensity} + 0.00527\ \text{Light Intensity}^2 \quad (4)$$

$$N\text{-}NO_3^- = -97.730 + 9.11028\ \text{Photoperiod} + 70.35175\ \text{Cytokinin} + 0.31585\ \text{Light Intensity} - 2.47768\ \text{Photoperiod} * \text{Cytokinin} - 0.179189\ \text{Photoperiod}^2 - 13.88\ \text{Cytokinin}^2 \quad (5)$$

$$\text{Cu} = 1.892 - 0.014\ \text{Photoperiod} - 1.14766\ \text{Cytokinin} - 0.007315\ \text{Light Intensity} + 0.004192\ \text{Cytokinin} * \text{Light Intensity} + 0.270192\ \text{Cytokinin}^2 \quad (6)$$

$$1/\text{Zn} = 0.232 + 0.004843\ \text{Photoperiod} - 0.027123\ \text{Cytokinin} - 0.000061\ \text{Light Intensity} - 0.000066\ \text{Photoperiod}*\text{Light Intensity} + 0.00048\ \text{Cytokinin}*\text{Light Intensity} + 4.79\text{E-}06\ \text{Light Intensity}^2 \quad (7)$$

$$\ln(\text{SMX}) = -1.489 - 0.06191\ \text{Photoperiod} - 2.99339\ \text{Cytokinin} + 0.01399\ \text{Light Intensity} + 0.0945\ \text{Photoperiod} * \text{Cytokinin} - 0.001131\ \text{Photoperiod} * \text{Light Intensity} + 0.5072\ \text{Cytokinin}^2 \quad (8)$$

The selection of models (linear, quadratic, or cubic) was based on fitting parameters, including adequate precision, R^2, R^2_{adj}, and R^2_{pred}. For well-adjusted, significant models, the principle of simplicity led to the selection of the lowest-order model. All models (Eqs. 1-8) were statistically significant ($p \leq 0.05$) with non-significant lack of fit ($p \geq 0.05$) (see Tables S1-8, Appendix B). In addition, for all models, the adequate precision values were greater than 4, indicating sufficient model discriminations (Table 3).

The accuracy of the models (Eqs. 1-8) was characterized by regression coefficients for all pollutants (Table 3). The coefficients of determination (R^2) ranged from 0.66 (for $P\text{-}PO_4^{3-}$/Bp) to 0.91 (for Cu), while the adjusted coefficients of determination (R^2_{adj}) ranged from 0.55 (for Zn) to 0.88 (for Cu), and the predicted coefficients of determination (R^2_{pred}) from 0.21 (for Zn) to 0.81 (for Cu). Models with R^2 above 0.8 are considered well-fitted, and a difference of less than 20% between R^2_{adj} and R^2_{pred} indicates that the models are capable of predicting responses (Dias et al., 2021). In this regard the results obtained for copper, $N\text{-}NH_4^+$, and $DOC_{removed}$/Bp suggested that the regression models fit the data well. On the other hand, the models obtained for Zn, COD, SMX, and $N\text{-}NO_3^-$, despite having R^2 values of 0.71, 0.81, 0.89 and 0.75, respectively, should be used with discretion due to their low predicted R^2 values in relation to R^2_{adj} values. Figs. S10-17 (Appendix B) plots the predicted residuals (or removed) concentration of each pollutant versus the experimental concentration. Narrower confidence intervals ($CI_{95\%}$) and prediction intervals ($PI_{95\%}$) demonstrate more precise estimates and increased certainty in the predictions for the Cu, $N\text{-}NH_4^+$ and $DOC_{removed}$/Bp models.

Table 3: Data from the adjusted models of treatment of simulated swine wastewater by *Azolla microphylla*

[a] Parameter	*F value* (regression)	*p value* (Lack of fit)	R^2	R^2_{adj}	R^2_{pred}	Adeq. precision
COD	6.11	0.53	0.81	0.68	0.29	9.03
$DOC_{removed}$/Bp	24.51	0.34	0.84	0.81	0.72	16.91
$P\text{-}PO_4^{3-}$/Bp	14.40	0.15	0.66	0.61	0.46	12.58
$N\text{-}NH_4^+$	27.49	0.44	0.85	0.82	0.76	17.83
$N\text{-}NO_3^-$	5.56	0.84	0.75	0.62	0.34	6.91
Cu	24.90	0.62	0.91	0.88	0.81	17.32
Zn	4.41	0.90	0.71	0.55	0.21	7.85
SMX	10.43	0.22	0.89	0.80	0.36	12.63

[a] The units for COD, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, Zn, Cu, and SMX are in mg L^{-1}. For DOC_{remv}/Bp and $P\text{-}PO_4^{3-}$ we referred as mg L^{-1} Bp^{-1}

In the optimization stage, the target for all models was to achieve the lowest residual concentrations of Zn, Cu, COD, $P\text{-}PO_4^{3-}$/Bp, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and SMX or highest

$DOC_{removed}$/Bp ratio. The contour plots depict the regions of these concentrations resulting from the influence of photoperiod, cytokinin, and light intensity (Fig. S1, Appendix B). These plots were generated by maintaining one of the factors at its optimal value.

Overall, it was found that optimal regions occurred with higher light intensities combined with both high and low photoperiod values (Fig. S1, Appendix B). In addition, lower to intermediate concentrations of cytokinin resulted in more optimal regions for all pollutants. We observed that Cu and SMX were highly sensitive to light intensity and photoperiod, respectively. This is evident as significantly low concentrations, equivalent to more than 66% and 95% removal, were found across a large range of light intensities and photoperiods (Fig. S1-f, h, Appendix B)

For COD (Fig. S1-a) and N-NO_3^- (Fig. S1-e), two optimal regions were identified, which can be attributed to the complexity of the model, whether due to the transformations involved, its order, or the number of terms comprising it. Moreover, the model for N-NO_3^- showed regions with negative concentrations, indicating its ability to predict even lower concentrations than those observed. In cases where there were multiple optimal regions, the most economical factor levels were adopted, as long as the region had an individual desirability function close to or equal to 1 (Vera Candioti et al., 2014).

In this regard, the optimized treatment conditions for simulated swine wastewater by *Azolla* were achieved for: COD (7 hours, 0.3 mg L^{-1}, and 99 µmol m^{-2} s^{-1}); P-PO_4^{3-}/Bp (23 hours, 0.25 mg L^{-1}, and 96 µmol m^{-2} s^{-1}); N-NH_4^+ (24 hours, 0.25 mg L^{-1}, and 86 µmol m^{-2} s^{-1}); N-NO_3^- (7 hours, 0.3 mg L^{-1}, and 65 µmol m^{-2} s^{-1}); Cu (24 hours, 1.3 mg L^{-1}, and 99 µmol m^{-2} s^{-1}); Zn (23 hours, 0.76 mg L^{-1}, and 6.5 µmol m^{-2} s^{-1}); and SMX (19 hours, 0.76 mg L^{-1}, and 99.7 µmol m^{-2} s^{-1}) and the maximum $DOC_{removed}$/Bp ratio (6 hours, 0.25 mg L^{-1}, and 8 µmol m^{-2} s^{-1}) in terms of photoperiod, cytokinin, and light intensity, respectively. In all cases, the desirability function reached an individual unitary value.

These results indicated that the conditions of phytoremediation and its effectiveness depended on the target pollutant. Although there were similar optimal conditions, each pollutant achieved its maximum removal under specific treatment conditions. In this regard, we sought global optimal conditions that would represent the overall optimal conditions for each pollutant. These conditions are referred to here as optimal promising trial conditions. To achieve this, four conditions were selected: Trial A: 24 h, 0.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}. Trial B: 8 h, 0.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}. Trial C: 24 h, 1.3 mg L^{-1} and 100 µmol m^{-2} s^{-1} and Trial D: 8 h, 1.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}.

3.2 Assessment of Azolla treatment performance under optimal promising trial conditions

New experiments were carried out with the four promising optimal trial conditions (Trial A-D), in which pollutant concentrations were measured after treatment. For $DOC_{removed}$/Bp, P-PO_4^{3-}/Bp, N-NH_4^+, and Zn, significant differences were observed (Table 4) representing percentage removals ranging from 2% for Zn to 91% for N-NH_4^+. The results from these four conditions were then validated by comparing the observed results with those estimated by the models (Eqs. 1-8). This validation was performed by checking whether the mean value of the observed results fell within the predicted interval ($PI_{95\%}$) calculated from the models (Tables S10-S11, Appendix B).

Table 4: Residual or removed concentrations of pollutants under optimal promising trial conditions ([a] means ± standard deviation).

Parameter (unit)	[b]Trial			
	A	B	C	D
COD (mg L^{-1})	33 ± 5 a	28 ± 8 a	33 ± 9 a	32 ± 8 a
$DOC_{removed}$/Bp (mg $L^{-1}Bp^{-1}$)	3.15 ± 0.18 ab	4.04 ± 0.40 a	3.01 ± 0.31 b	4.16 ± 0.19 ab
P-PO_4^{3-}/Bp (mg $L^{-1}Bp^{-1}$)	4.22 ± 0.81 ab	6.02 ± 1.69 a	3.85 ± 0.23 b	5.79 ± 0.06 ab
N-NH_4^+ (mg L^{-1})	9.4 ± 1.6 b	23.3 ± 1.4 a	7.5 ± 1.6 b	22.6 ± 2.3 a
N-NO_3^- (mg L^{-1})	10 ± 3 a	9.5 ± 1.3 a	9.5 ± 2.6 a	13 ± 2.6 a
Cu (mg L^{-1})	0.16 ± 0.02 a	0.19 ± 0.04 a	0.29 ± 0.08 a	0.16 ± 0.07 a
Zn (mg L^{-1})	4.11 ± 0.05 ab	3.82 ± 0.17 ab	4.13 ± 0.11 a	3.79 ± 0.25 b
SMX (mg L^{-1})	0.022 ± 0.001 a	0.022 ± 0.005 a	0.023 ± 0.002 a	0.021 ± 0.004 a

[a] n = 4. [b]Trial A: 24 h, 0.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}. Trial B: 8 h, 0.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}. Trial C: 24 h, 1.3 mg L^{-1} and 100 µmol m^{-2} s^{-1} and Trial D: 8h, 1.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}. Different letters, within the rows, indicate a statistically significant difference ($p \leq 0.05$) between the four promising optimal trial conditions, as determined by the Tukey test.

Furthermore, our findings unveiled a strong, positive, and significant correlation ($p \leq 0.05$) in the pairs N-NH_4^+-$DOC_{removed}$/Bp (r = 0.88) and $DOC_{removed}$/Bp-P-PO_4^{3-}/Bp (r = 0.77), along with moderate positive correlations for N-NH_4^+-P-PO_4^{3-}/Bp (r = 0.67), and negative correlations between $DOC_{removed}$/Bp-Zn (r = -0.66), N-NH_4^+-Zn (r = -0.58) and $DOC_{removed}$/Bp-Cu (r = -0.52) (Ratner, 2009) (Fig. 2).

This suggests that the mechanisms involved in the removal of organic matter, measured as DOC, are positively associated with the removal of nutrients in the form of N-NH_4^+ and P-PO_4^{3-} (measured as P-PO_4^{3-}/Bp). Moreover, the removal of nitrogen in the form of N-NH_4^+ is associated with the removal of phosphorus, measured by P-PO_4^{3-}/Bp. There were no relationships in the concentration of COD, SMX, and N-NO_3^-.

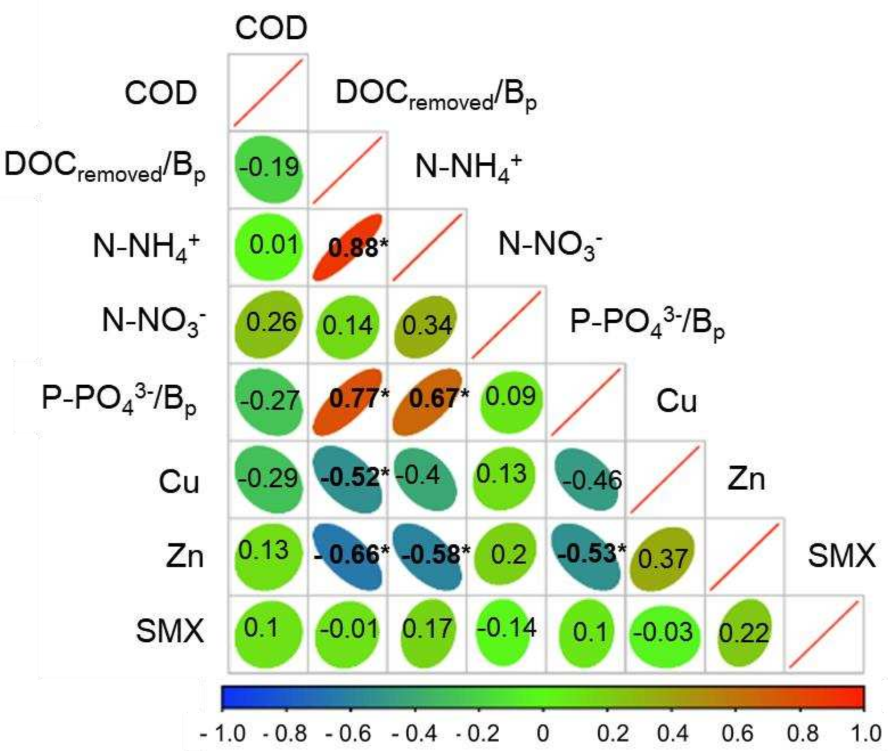

Fig. 2: Pearson correlation matrix among parameters of simulated swine wastewater treated under the four promising optimal trial conditions. Asterisks highlight those with significant Pearson correlation coefficients ($p \leq 0.05$).

Principal Component Analysis (PCA) was employed to assess the variation in pollutant concentrations across the four promising optimal trial conditions. This technique is useful in identifying associations between responses and their sources of variation (Jolliffe and Cadima, 2016). The analysis of the four conditions and the pollutants showed that the first and second principal components accounted for 42.61% and 17.76% of the variance, respectively, for a total of 60.37% overall (Fig. 3).

The first component of the study is majorly influenced by $DOC_{removed}$/Bp, P-PO_4^{3-}/Bp, and N-NH_4^+. On the other hand, COD, N-NO_3^-, and SMX primarily contribute to the second component. Moreover, the PCA distinctly separated the four promising optimal treatment conditions in the coordinate systems. Trials B and D (regions a and d) in the positive direction of the first axis showed high correlation with pollutants of the first component. In contrast, trials A and C (regions b and c) exhibited high correlation with factors in the negative direction of the first axis, primarily influenced by Cu, Zn, and COD.

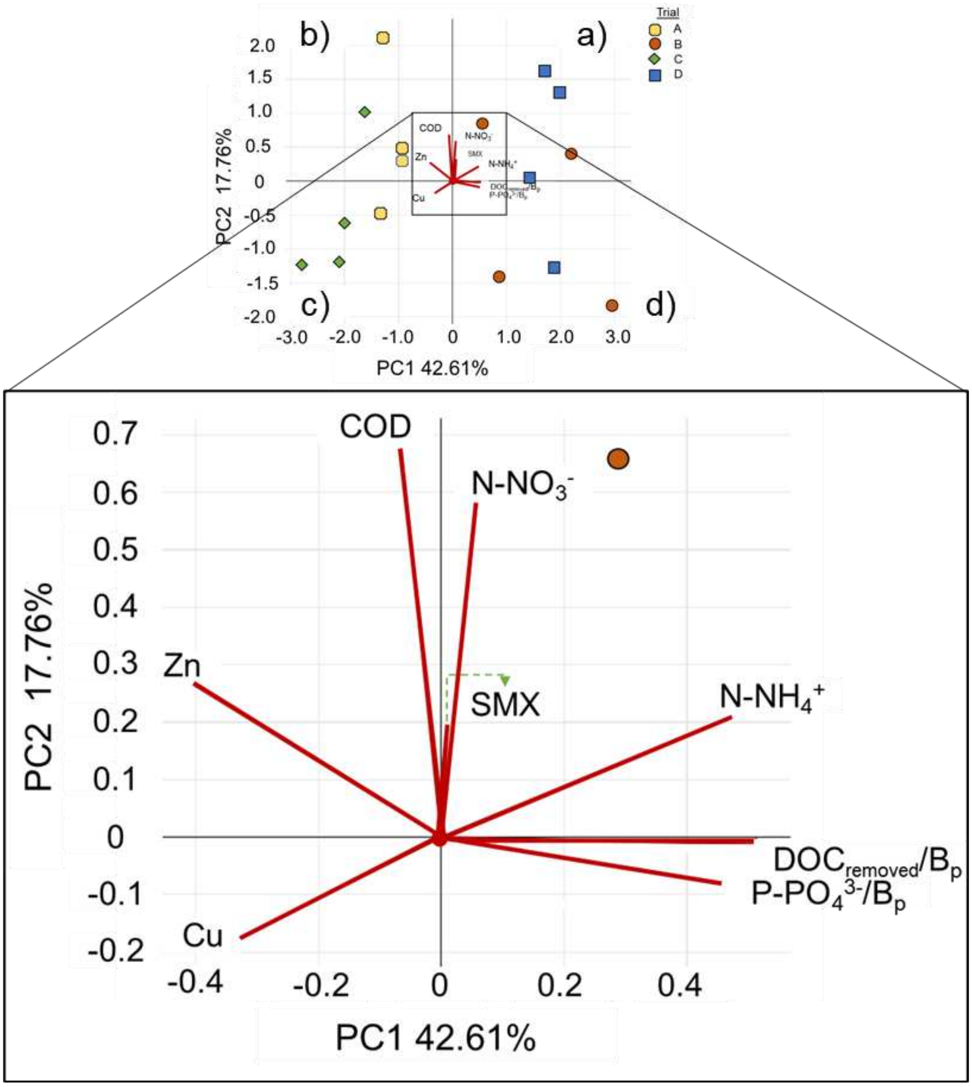

Fig. 3: PCA analysis of the four promising treatment conditions based on the pollutants analyzed. trials' condition: Trial A: 24 h, 0.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}. Trial B: 8 h, 0.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}. Trial C: 24 h, 1.3 mg L^{-1} and 100 µmol m^{-2} s^{-1} and Trial D: 8h, 1.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}.

The results of hierarchical clustering and the heatmap were used to determine the similarity among pollutants and the four promising optimal trial conditions (Fig. 4). The clusters in the dendrograms were obtained from the cluster analysis performed using the ward method with Euclidean distance as the similarity measure (Murtagh and Legendre, 2014).

For pollutants, three distinct clusters can be identified: $DOC_{removed}$/Bp, P-PO_4^{3-}/Bp, N-NH_4^+ and SMX (Cluster I); N-NO_3^- and COD (Cluster II); and Zn and Cu (Cluster III). On the other hand, the conditions were identified in two distinct groups: Trials B and D (Group 1) were separated from Trials A and C (Group 2). In addition, Cluster I showed a positive relationship (similarity) with Group I (trials B and D), while Cluster III exhibited a positive intensity with Group 2 (trials A and C). This indicates that higher concentrations of pollutants from Cluster I and Cluster III were expected in trials B and D (Group 1) and trials A and C (Group 2), respectively. The cluster analysis strongly confirmed the PCA results, indicating that conditions within the same cluster, in terms of the analyzed pollutants, demonstrated a high correlation in the PCA.

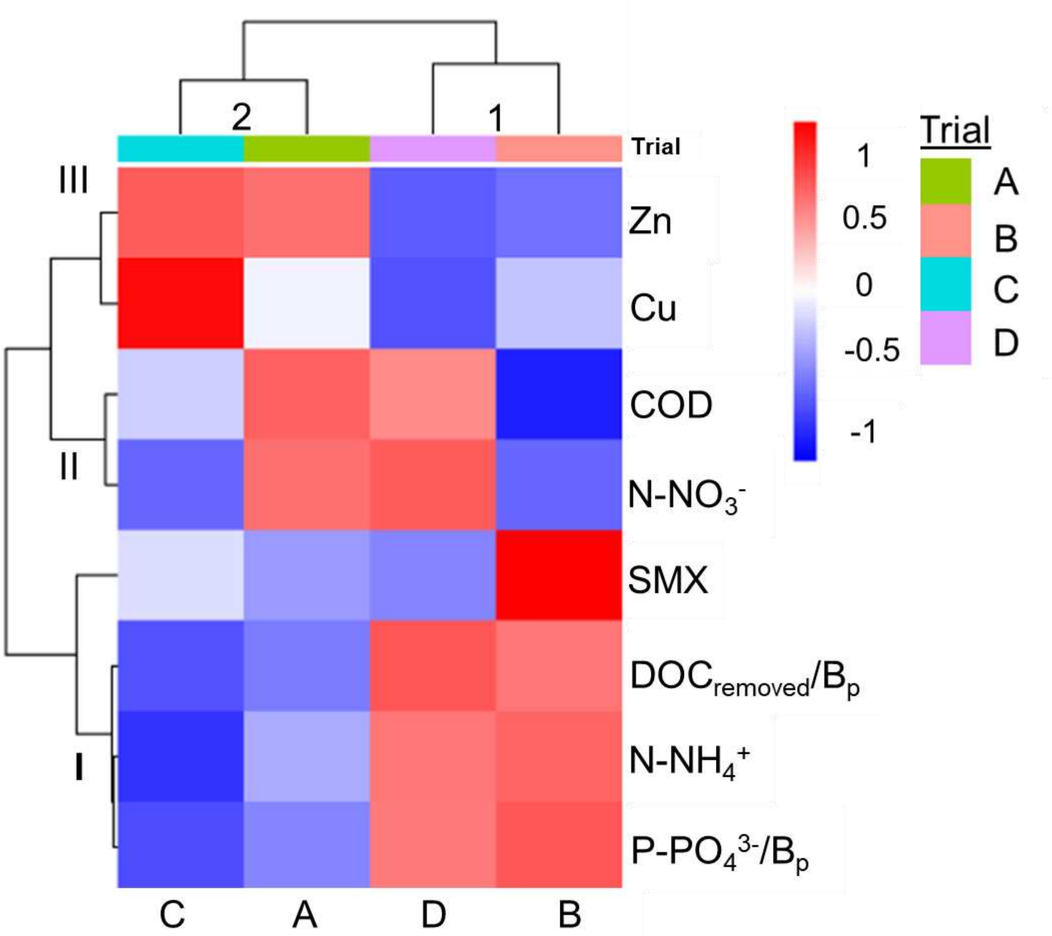

Fig. 4: Two-way heatmap cluster analysis of the four promising optimal trial conditions based on the pollutants. Trials' condition: Trial A: 24 h, 0.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}. Trial B: 8 h, 0.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}. Trial C: 24 h, 1.3 mg L^{-1} and 100 µmol m^{-2} s^{-1} and Trial D: 8h, 1.3 mg L^{-1} and 100 µmol m^{-2} s^{-1}.

3.3 Biomass contents

The biomass productivity (Bp) and the contents of protein, lipids, and starch were also measured in the four promising optimal trial conditions (Table 5). Among them, Trials A and C had the significantly highest biomass productions with 9.3 gDW m^{-2} d^{-1} (equivalent to 33.9 t ha^{-1} yr^{-1}) and 9.5 gDW m^{-2} d^{-1} (equivalent to 34.8 ha^{-1} yr^{-1}), respectively. Starch production levels were approximately 2 to 3 times higher ($p \leq 0.05$) in conditions A and C at the end of the treatment. Despite variations ranging from 20.32% DW to 34.05%DW for lipids and 23.05% DW to 26.61%DW, no significant differences were observed in the production of protein and lipids.

Table 5: Biomass by-product contents under optimal promising trial conditions

Parameter (unit)	[a]Trials			
	A	B	C	D
[b] Bp ($gDW\ m^{-2}\ d^{-1}$)	9.30 ± 0.1 a	7.01 ± 0.16 b	9.54 ± 0.40 a	7.20 ± 0.12 b
Proteins (%DW)	23.05 ± 5.28 a	25.83 ± 0.44 a	23.74 ± 0.19 a	26.61 ± 0.87 a
Lipids (%DW)	34.05 ± 22.80 a	22.8 ± 13.74 a	28.04 ± 12.31 a	20.32 ± 7.62 a
Starch (%FW)	28.01 ± 5.76 a	9.69 ± 2.64 c	17.92 ± 1.45 b	8.16 ± 1.35 c

[a] Trial A: 24 h, 0.3 mg L^{-1} and 100 µmol $m^{-2}\ s^{-1}$. Trial B: 8 h, 0.3 mg L^{-1} and 100 µmol $m^{-2}\ s^{-1}$. Trial C: 24 h, 1.3 mg L^{-1} and 100 µmol $m^{-2}\ s^{-1}$ and Trial D: 8h, 1.3 mg L^{-1} and 100 µmol $m^{-2}\ s^{-1}$. [b] Bp: Biomass productivity. Different letters, within the rows, indicate a statistically significant difference ($p \leq 0.05$) between the four promising optimal trial conditions, as determined by the Tukey test. (mean ± standard deviation, n = 4).

4. DISCUSSION

Our results indicate low residual pollutant concentrations in simulated swine wastewater under optimal trial conditions, influenced by LED intensity and duration, as well as cytokinin dosage. Most pollutants achieved removal rates surpassing 70%, notably COD, N-NH_4^+, N-NO_3^-, Cu, and SMX. P-PO_4^{3-} removal rates were at least 54%, while DOC removal rates were a minimum of 67% when considering the produced biomass (Table 5), whereas Zn removal rates reached a maximum of 9.7%.

High removal rates of organic matter and SMX were observed during phytoremediation. However, since plants do not directly remove these compounds or contribute in a very minimal way it is essential to consider alternative removal pathways such as photolysis and photodegradation, especially given the use of LED light. Photolysis involves the breakdown of contaminants by direct absorption of light energy, while photodegradation involves the degradation of pollutants through reactions initiated by light exposure (Olatunde et al., 2020). Although a detailed analysis of these pathways was not possible in this study, future research could explore mass balance analysis to determine the specific removal pathways of these compounds.

Furthermore, the three factors had distinct effects on pollutant removal under optimal trial conditions. The lowest residual concentration of N-NH_4^+ was achieved in treatments with the longest light exposure (24 hours). Higher $DOC_{removed}$/Bp ratios were observed under the shortest photoperiod (8 hours). Extended photoperiods or low cytokinin concentrations combined with brief LED light exposure may result in lower pollutant concentrations for SMX (Figs. 3, 4). Cytokinin and LED light did not demonstrate a pattern of impact on residual COD and $N\text{-}NO_3$

Higher removals of Cu and Zn occurred during treatments with the shortest light exposure periods (8 h). Under prolonged exposure or high light intensity, the photosynthetic machinery needs to acclimate to prevent damage to the antioxidant defense system (Suzuki et al., 2012). Therefore, due to the combination of stressful conditions in the wastewater environment and extended light periods, plants may decrease metal absorption as a strategy to avoid such damage.

Our results emphasize the importance of properly considering operational parameters when aiming to remove specific pollutants, for example, employing phytoremediation for the removal of micropollutants. Com base nos resultados e do ponto de vista da engenharia, como não houve uma condição que se destaque claramente como a melhor, se fosse necessário escolher uma condição de trabalho para o tratamento de águas residuais de suinocultura tratadas pela Azolla microphylla, recomendaria as condições A (8 horas de fotoperíodo, 1.3 mg L^{-1} de citocinina e 100 µmol m^{-2} s^{-1} de intensidade luminosa) e B (8 horas de fotoperíodo, 0.3 mg L^{-1} de citocinina e 100 µmol m^{-2} s^{-1} de intensidade luminosa). Isso se deve principalmente ao fato de que a citocinina não possui influência significativa na remoção de nenhum dos poluentes, com exceção do SMX, para o qual concentrações menores resultam em menores concentrações residuais. Do ponto de vista técnico-econômico, usar a menor concentração de citocinina seria mais vantajoso. Por outro lado, considerando que os principais problemas da suinocultura são a remoção de nutrientes (como nitrogênio amoniacal) e compostos micropoluentes, essas duas condições atenderiam a essas necessidades.

Based on the results and from an engineering perspective, since no condition clearly stands out as the best, if it were necessary to choose a working condition for treating swine wastewater by *Azolla microphylla*, we would recommend conditions A (24 hours of photoperiod, 0.3 mg L^{-1} cytokinin, and 100 µmol m^{-2} s^{-1} light intensity) and B (8 hours of photoperiod, 0.3 mg L^{-1} cytokinin, and 100 m^{-2} s^{-1} light intensity). This is primarily because cytokinin does not have a significant influence on the removal of any of the pollutants, except for SMX, for which lower concentrations result in lower residual concentrations. From a technical-economic standpoint, using the lower cytokinin concentration would be more advantageous. On the other hand, considering that the main issues in swine farming are the removal of nutrients (such as ammoniacal nitrogen) and micropollutants, these two conditions would meet those needs.

We aimed to optimize conditions for *Azolla* growth to enhance treatment efficiency. However, implementing artificial light on a large scale for wastewater treatment via phytoremediation may raise concerns due to increased costs and operational complexity. However, relying solely on natural light has limitations, including dependence on weather conditions, reduced efficiency during low light periods, and difficulties in maintaining long-term process control, especially in areas with periods of low solar luminosity.

However, the use of artificial light in wastewater treatment, particularly in phycoremediation with microalgae, is well-documented (González-Camejo et al., 2019) especially when considering its potential for biorefineries (Amaro et al., 2020). On the other hand, research has explored light types, photoperiods, and light intensity, primarily to assess macrophyte biomass growth (Xu et al., 2019). Most of these studies focus on how light influences submerged macrophytes' growth, crucial for their metabolism in submerged environments (Zhao et al., 2023).

In contrast, research regarding the utilization of LED lighting in phytoremediation remains in its early stages, with few studies reporting its use for floating macrophytes (Bawiec et al., 2020; Kilian et al., 2022). Nevertheless, despite the scarcity of studies on the effects of LED light on floating macrophytes, our study advances in elucidating the influence of LED lighting on the performance of *Azolla microphylla* in treating synthetic swine wastewater.

Our findings indicate that *Azolla* has great potential for biomass production after treatment of swine wastewater, with high protein, lipid, and carbohydrate (starch) contents. Particularly for starch, where the highest accumulation was observed under 24-hour photoperiod conditions, the previous report indicates that increased light exposure and intensity contribute to the elevated production and accumulation of starch (Yin et al., 2015). Plants tend to store carbon in the form of starch under high-stress conditions caused by prolonged exposure to light as a preferred means of storing energy (Van Dyck et al., 2021).

Ultimately, the present study revealed a high capacity of *Azolla* to enhance the quality of simulated swine wastewater. The analysis showed that the pollutants were removed at elevated rates, while the biomass productivity was notably high. However, it is important to note that this study, the first to assess *Azolla*'s application in swine wastewater treatment using LED lights and cytokinin has limitations that warrant further investigations.

Initially, the objective was to test how the macrophyte would react to high light levels when it comes to removing pollutants. The findings indicated that certain pollutants, including SMX, can indeed be removed effectively in a shorter time when exposed to light. To confirm the effectiveness of this behavior, it is necessary to examine other micropollutants of interest, both individually and together. High pollutant removals such as organic matter may suggest the involvement of other mechanisms, such as chemical oxidation or photodegradation, as previously reported in hydroponic phytoremediation systems (Polińska et al., 2021). we recommend conducting a mass balance analysis to identify the main pollutant removal pathways.

Moreover, the results suggest that *Azolla* is more sensitive to LED lights (in terms of duration and intensity) than to the presence of cytokinin, as demonstrated by $DOC_{removed}/Bp$ ratio and removals of $P\text{-}PO_4^{3-}/Bp$ and $N\text{-}NH_4^+$. Cytokinins regulate shoot growth and are linked to nutrient absorption and high biomass production (Arkhipova et al., 2007). Research indicates that cytokinins significantly enhance metal absorption compared to their absence (Yu et al., 2024). However, in our study, we did not observe a clear cytokinin influence on most parameters, possibly due to interactions with other factors. We recommend assessing cytokinin effects individually and in combination with other phytohormones to understand their potential in swine wastewater phytoremediation and analyze various pollutants.

5. CONCLUSION

In conclusion, our data confirm the initial hypothesis by revealing that LED light, in terms of intensity and duration as well as the variation of applied cytokinin doses, produces swine wastewater with low residual concentrations of organic matter, metals, and sulfamethoxazole, simultaneously generating biomass with high protein, lipid, and starch content. Under optimal treatment conditions, we observed removal rates exceeding 70% for COD, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, Cu, and SMX. Moreover, removal rates for DOC and $P\text{-}PO_4^{3-}$ were above 54%, while Zn exhibited lower removal rates ranging from 2% to 10%.

However, we found that each of these pollutants is uniquely influenced by the three factors. Under optimal treatment conditions, $DOC_{removed}$/Bp and lower residual concentrations of Cu and Zn are found in shorter photoperiods, while $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$/Bp, show lower residual concentrations in longer light exposure treatment conditions. On the other hand, for COD and $N\text{-}NO_3^-$, there is no pattern for any of the analyzed factors. Therefore, our study demonstrates the potential for treating swine wastewater using *Azolla microphylla*, aligned with its ability to produce biomass rich in high-value compounds when subjected to LED light and cytokinin.

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CHAPTER 5: CONCLUSION AND SUGGESTIONS

5.1 General Conclusion

In general, phytoremediation, despite being extensively studied, still lacks a comprehensive understanding of the parameters influencing its effectiveness, such as the relationship between pollutant removal, such as potentially toxic elements, and environmental variables. The second chapter of this thesis addressed this gap, emphasizing the effectiveness of emergent macrophytes in removing iron and manganese and identifying significant correlations between physical and water factors (e.g., initial pollutant concentration). In the third chapter, the MADM methodology was employed to select the most suitable macrophyte for simulated swine wastewater treatment, with *Azolla microphylla* being the preferred choice. The fourth chapter optimized the efficiency of *A. microphylla* in the removal of pollutants in simulated swine wastewater, revealing that under ideal treatment conditions, these plants were effective in reducing various pollutants.

These results indicate that careful selection of macrophytes and optimization of treatment conditions are crucial for the success of phytoremediation in hydroponics. Moreover, papers published in scientific journals such as "Toxics" and "Journal of Water Process Engineering" validate the relevance and contribution of these findings to the scientific community. Collectively, the findings support the use of phytoremediation as a sustainable approach for swine wastewater treatment, emphasizing the importance of considering not only pollutant removal but also biomass production with potential for recycling in a circular economy.

5.2 Suggestions for Future Research

The use of multicriteria tools proves to be intriguing as it allows for the multivariate assessment of various factors considered in the success of phytoremediation. This includes not only the ability to improve the wastewater quality but also the productivity of the generated biomass, taking into account the perspective of biomass reuse within the circular economy framework. Furthermore, this approach enables an analysis of how plants respond to the treatment and the pollutants present, which is crucial for a long-term evaluation, given that the survival of plants is inherently linked to this context. In this regard, future may studies take into account aspects of oxidative stress. For this purpose, analyses of enzymatic antioxidants (peroxidase (POD), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), alkaline phosphatase (AP)) and non-enzymatic antioxidants (malondialdehyde (MDA), Glutathione (GSH)) should be incorporated. This approach ensures a more in-depth discussion of the phyto-stress aspects addressed in chapter three.

Finally, considering the perspective of the circular economy, the biomass produced is a valuable byproduct of the phytoremediation process. Among its potential applications, due to its high protein content, one could explore the use of biomass as a source of animal feed, such as for fish, for instance. However, effectively managing the use of plants in this context poses challenges. For instance, what is the risk to human health when consuming fish fed with this residue due to the bioaccumulation of potentially toxic elements or micropollutants in plant biomass and their biomagnification in fish tissues? Therefore, to further delve into this underexplored area, a quantitative study of probabilistic, chemical, and microbiological risks to human health associated with the consumption of fish that might be fed with this biomass can be suggested. This should consider variations in the accumulation of these pollutants in plant biomass, the frequency and duration of fish exposure to the plants, and human consumption of the fish.

APPENDIX A – SUPPLEMENTARY MATERIAL CHAPTER 3

Table A.1. Inorganic and organic chemical composition of synthetic swine wastewater

Chemical	Concentration (mg L^{-1})	Reference
H_3BO_3	3.89	(Bergmann et al., 2000)
$MnCl_24H_2O$	3.17	
$ZnSO_47H_2O$	13.5	
$CuSO_45H_2O$	4.74	
$Na_2MoO_42H_2O$	0.05	
$CoCl_26H_2O$	0.10	
KH_2PO_4	428	
$CaCl_2$	666.5	
$MgCl_26H_2O$	334	
K_2SO_4	150.5	
$FeSO_47H_2O$	39	
NH_4NO_3	497.50	(Dan et al., 2020; Xiao et al., 2022)
$NaHCO_3$	495.71	(Pérez-Pérez et al., 2021)
Glucose	153	—
Starch	50	
Beef extract	218	
Lipids	184	

Table A.2: Weights calculated in Fuzzy Analytic Hierarchy Process matrices and their consistency values of the respective decision-makers (DM)

Attribute	DM1	DM2	DM3	DM4	DM5	DM6	DM7	DM8	DM9	DM10	DM11	DM12	DM13	DM14	DM15	N-GM
R_{DQO}	0.023	0.006	0.002	0.123	0.008	0.045	0.007	0.149	0.026	0.096	0.030	0.154	0.071	0.097	0.057	**0.022**
$R_{N\text{-}NH4+}$	0.075	0.156	0.142	0.123	0.081	0.082	0.084	0.114	0.026	0.128	0.093	0.026	0.117	0.097	0.140	**0.103**
$R_{N\text{-}NO3-}$	0.117	0.152	0.016	0.110	0.114	0.080	0.115	0.059	0.007	0.086	0.091	0.000	0.123	0.059	0.143	**0.073**
$R_{P\text{-}PO43-}$	0.074	0.159	0.102	0.063	0.003	0.080	0.045	0.059	0.026	0.086	0.061	0.065	0.161	0.059	0.143	**0.059**
R_{Zn}	0.119	0.118	0.067	0.008	0.079	0.082	0.082	0.017	0.077	0.014	0.063	0.026	0.117	0.055	0.103	**0.078**
R_{Cu}	0.119	0.118	0.067	0.008	0.030	0.082	0.084	0.017	0.121	0.014	0.066	0.026	0.156	0.097	0.103	**0.079**
TUa	0.154	0.073	0.142	0.160	0.121	0.112	0.084	0.149	0.121	0.128	0.093	0.117	0.061	0.055	0.062	**0.121**
TUc	0.154	0.073	0.142	0.160	0.121	0.112	0.084	0.149	0.121	0.128	0.093	0.117	0.061	0.055	0.062	**0.121**
Lipid	0.023	0.020	0.019	0.028	0.030	0.010	0.045	0.017	0.077	0.057	0.066	0.005	0.011	0.055	0.012	**0.039**
Volatile Solids	0.023	0.023	0.067	0.028	0.079	0.112	0.084	0.114	0.007	0.014	0.093	0.117	0.030	0.055	0.017	**0.048**
Protein	0.023	0.006	0.019	0.028	0.030	0.010	0.084	0.017	0.077	0.057	0.066	0.117	0.030	0.055	0.009	**0.037**
RGR	0.075	0.068	0.108	0.080	0.153	0.082	0.115	0.070	0.157	0.096	0.093	0.074	0.017	0.131	0.140	**0.121**
PSI	0.023	0.027	0.108	0.080	0.153	0.112	0.087	0.070	0.157	0.096	0.093	0.154	0.044	0.131	0.009	**0.098**
Consistency Ratio (%)	1.771	7.567	2.549	10.638	4.705	1.424	0.064	14.752	5.288	6.115	7.947	33.112	36.203	7.177	13.604	_

RGR: Relative Growth Rate. PSI: Phyto-Stress Index. N-GM: Normalized Geometric Mean. The weights used in the present research are the values obtained by the geometric mean of each attribute. **The DM4, DM8, DM12, DM13 and DM15 matrices were disregarded because they presented a consistency ratio above the allowed (CR > 10%)** (Saaty, 2008).

N-GM: , where n represents the attributes, x_i is the weight of the corresponding attribute and

Table A.3: Cronbach's Alpha results

<table>
<tr><th>Parameter</th><th>DM1</th><th>DM2</th><th>DM3</th><th>DM5</th><th>DM6</th><th>DM7</th><th>DM9</th><th>DM10</th><th>DM11</th><th>DM14</th></tr>
<tr><td>R_{DQO}</td><td colspan="10">0.900</td></tr>
<tr><td>$R_{N\text{-}NH4+}$</td><td colspan="10">0.902</td></tr>
<tr><td>$R_{N\text{-}NO3\text{-}}$</td><td colspan="10">0.910</td></tr>
<tr><td>$R_{P\text{-}PO43\text{-}}$</td><td colspan="10">0.929</td></tr>
<tr><td>R_{Zn}</td><td colspan="10">0.832</td></tr>
<tr><td>R_{Cu}</td><td colspan="10">0.956</td></tr>
<tr><td>TU_a</td><td colspan="10">0.856</td></tr>
<tr><td>TU_c</td><td colspan="10">0.879</td></tr>
<tr><td>Lipid</td><td colspan="10">0.657</td></tr>
<tr><td>Volatile solids</td><td colspan="10">0.908</td></tr>
<tr><td>Protein</td><td colspan="10">0.746</td></tr>
<tr><td>RGR</td><td colspan="10">0.894</td></tr>
<tr><td>PSI</td><td colspan="10">0.923</td></tr>
</table>

Note: The matrices with adequate consistency ratios (< 10%) were considered for calculation of Cronbach's Alpha. DM: Decision-maker. RGR: Relative Growth Rate. PSI: Phyto-Stress Index.

Table A.4: Categorization of the thirteen attributes studied and their visualization in the language of TOPSIS in ideal (V^+) and anti-ideal (V^-) values

Attribute (unit)	**Attribute type**	**TOPSIS algorithm**	
		V+	**V-**
R_{COD} (%)	Benefit	Maximum	Minimum
$R_{N\text{-}NH4+}$ (%)	Benefit	Maximum	Minimum
$R_{N\text{-}NO3\text{-}}$ (%)	Benefit	Maximum	Minimum
$R_{P\text{-}PO43\text{-}}$ (%)	Benefit	Maximum	Minimum
R_{Cu} (%)	Benefit	Maximum	Minimum
R_{Zn} (%)	Benefit	Maximum	Minimum
TUa ($LC_{50\%,\ 3d}$) (dimensionless)	Cost	Minimum	Maximum
TUc ($CI_{50\%,\ 8d}$) (dimensionless)	Cost	Minimum	Maximum
Lipids (% DW)	Benefit	Maximum	Minimum
Protein (% DW)	Benefit	Maximum	Minimum
Volatile solids (mg g^{-1}DW)	Benefit	Maximum	Minimum
RGR (d^{-1})	Benefit	Maximum	Minimum
Phyto-Stress Index (dimensionless)	Cost	Minimum	Maximum

TUa: Toxicity-Unit Acute. TUc: Toxicity-Unit Chronic. DW: Dry weight basis. RGR: Relative growth rate

Table A.5: The scores (S) of the Phyto-Stress Index (PSI), in four macrophytes treating synthetic swine wastewater.

Biomarker	*L. minuta*	*L. punctata*	*S. minima*	*A. microphylla*
Chl a	2.30	1.53	0.00	0.28

Chl b	0.46	0.00	1.60	0.08
Total Chl	2.28	1.31	0.37	0.00
Car	0.13	0.00	0.15	1.01
Antho	1.29	0.15	0.33	0.00

Chl a: Chlorophyll a. Chl b: Chlorophyll b. Total Chl: Total Chlorophyll. Car: Carotenoids. Antho: Anthocyanin

Table A.6: Responses of each attribute to use in TOPSIS method

Attribute	*L. minuta*	*L. punctata*	*S. minima*	*A. microphylla*
R_{COD}	80.69	79.30	68.03	80.56
$R_{N\text{-}NH4+}$	36.36	36.36	26.26	42.42
$R_{N\text{-}NO3-}$	50.85	63.17	40.06	65.74
$R_{P\text{-}PO43-}$	66.29	71.48	64.72	68.34
R_{Cu}	50.77	40.37	51.90	63.43
R_{Zn}	24.51	18.00	26.42	41.84
TUa	0.00	1.41	1.53	0.00
TUc	1.41	2.43	2.79	1.72
Lipids	14.06	9.79	1.09	7.83
Protein	22.77	33.73	28.47	28.40
Volatile solids	661.13	687.81	660.07	680.58
RGR	0.32	0.29	0.23	0.27
PSI	3.52	0.54	0.35	0.09

RGR: Relative Growth Rate. PSI: Phyto-Stress Index.

Table A.7: Weighted-normalized decision matrix, Vij.

Attribute	*Lemna minuta*	*Landoltia punctata*	*Salvinia minima*	*Azolla microphylla*
R_{COD}	0.000162	0.000159	0.000137	0.000162
$R_{N\text{-}NH4+}$	0.004293	0.004293	0.003101	0.005009
$R_{N\text{-}NO3-}$	0.003543	0.004402	0.002792	0.004581
$R_{P\text{-}PO43-}$	0.000118	0.000127	0.000115	0.000120
R_{Cu}	0.009282	0.006815	0.010005	0.015842
R_{Zn}	0.002851	0.002267	0.002915	0.003562
TUa	0.008166	0.014045	0.016141	0.009908
TUc	0.000000	0.334808	0.362657	0.000000
Lipids	0.035634	0.024807	0.002772	0.019833
Protein	0.000022	0.000023	0.000022	0.000023
Volatile solids	0.000814	0.001207	0.001019	0.001016
RGR	0.002612	0.002360	0.001906	0.002201
PSI	0.375988	0.057603	0.037284	0.010105

RGR: Relative Growth Rate. PSI: Phyto-Stress Index

Table A.8: Data mapping evaluation and corresponding triangular fuzzy number (Adapted from Sakhardande and Prabhu Gaonkar, 2022)

Differences in responses in linguist terms	Definition	Fuzzy assignment
VHI-VHI HI-HI LI-LI VLI-VLI NI-NI	Equally Important	(1,1,3)
VHI-HI HI-LI LI-VLI VLI-NI	Slightly Important	(1,3,5)
VHI-LI HI-VLI LI-NI	Essentially Important	(3,5,7)
VHI-VLI HI-NI	Strongly Important	(5,7,9)
VHI-NI	Extremely Important	(7,9,10)

FAHP: Fuzzy Analytic Hierarchy Process. VHI: Very High Influence. HI: High Influence. LI: Low Influence. VLI: Very Low Influence. NI: No Influence. Reciprocals: If the comparative was HI-VHI the M fuzzy number was (5,3,1).

Table A.9: Akaike Information Criterion (AIC) values of generalized linear family models associated to wastewater treatment performances, biomass by-products and plants growth endpoints

Parameter	GLM family regression models					
	Gamma		Normal		Log normal	
	AIC	Model (p-value)	AIC	Model (p-value)	AIC	Model (p-value)
tCOD	113.52	< 0.001	132.62	< 0.001	**111.41**	< 0.001
Zinc	33.29	0.223	29.12	0.146	35.78	0.272
Copper	-10.98	0.002	**-16.76**	< 0.001	-7.39	0.007
$P\text{-}PO_4^{3-}$	104.71	< 0.001	**102.48**	< 0.001	108.14	< 0.001
$N\text{-}NO_3^-$	91.31	< 0.001	98.70	< 0.001	**89.12**	< 0.001
$N\text{-}NH_4^+$	**87.79**	< 0.001	88.24	< 0.001	88.05	< 0.001
Protein	**67.14**	0.019	82.12	0.002	67.29	0.018
Lipids	**42.59**	< 0.001	50.58	< 0.001	48.98	< 0.001
Volatile Solids	126.35	0.746	125.75	0.737	126.68	0.751
Productivity	**34.91**	0.005	35.18	0.005	34.92	0.005
RGR	-79.03	< 0.001	-78.26	< 0.001	**-79.28**	< 0.001
Chl a – D0	-32.20	< 0.001	**-33.29**	< 0.001	-29.38	< 0.001
Chl a – D10	-28.62	< 0.00	**-38.52**	< 0.001	-22.47	< 0.001
Chl b – D0	-31.99	0.042	**-37.12**	0.014	-28.41	0.074
Chl b – D10	-39.03	0.015	**-40.87**	0.007	-37.59	0.021
Total Chl – D0	**-28.42**	< 0.001	-28.35	< 0.001	-27.19	< 0.001
Total Chl – D10	-24.08	< 0.001	**-30.90**	< 0.001	-20.16	0.001
Car – D0	-26.89	0.067	**-39.20**	0.011	-18.98	0.135
Car – D10	-35.56	0.135	-34.86	0.155	-35.81	0.128
Antho – D10	**-31.07**	0.039	-30.63	0.031	-30.66	0.047
Antho – D0*	---------------------------------- ND --------------------------------------					

D0: Concentration of target biomarker measured in plants collected in Hoagland solution, before treatment (Day-0). D10: Concentration of target biomarkers measured in plants collected in synthetic swine wastewater after treatment (Day-10). GLM: Generalized linear model. Chl a: Chlorophyll a. Chl b: Chlorophyll b. Total Chl: Total Chlorophyll. Car: Carotenoids. Antho: Anthocyanin. RGR: Relative Growth Rate. ND: Not Detected. * In D0, no anthocyanin was detected in plant biomass. The GLM distribution emphasized in bold means the significant ($p \leq 0.05$) distribution used to fitted to the data.

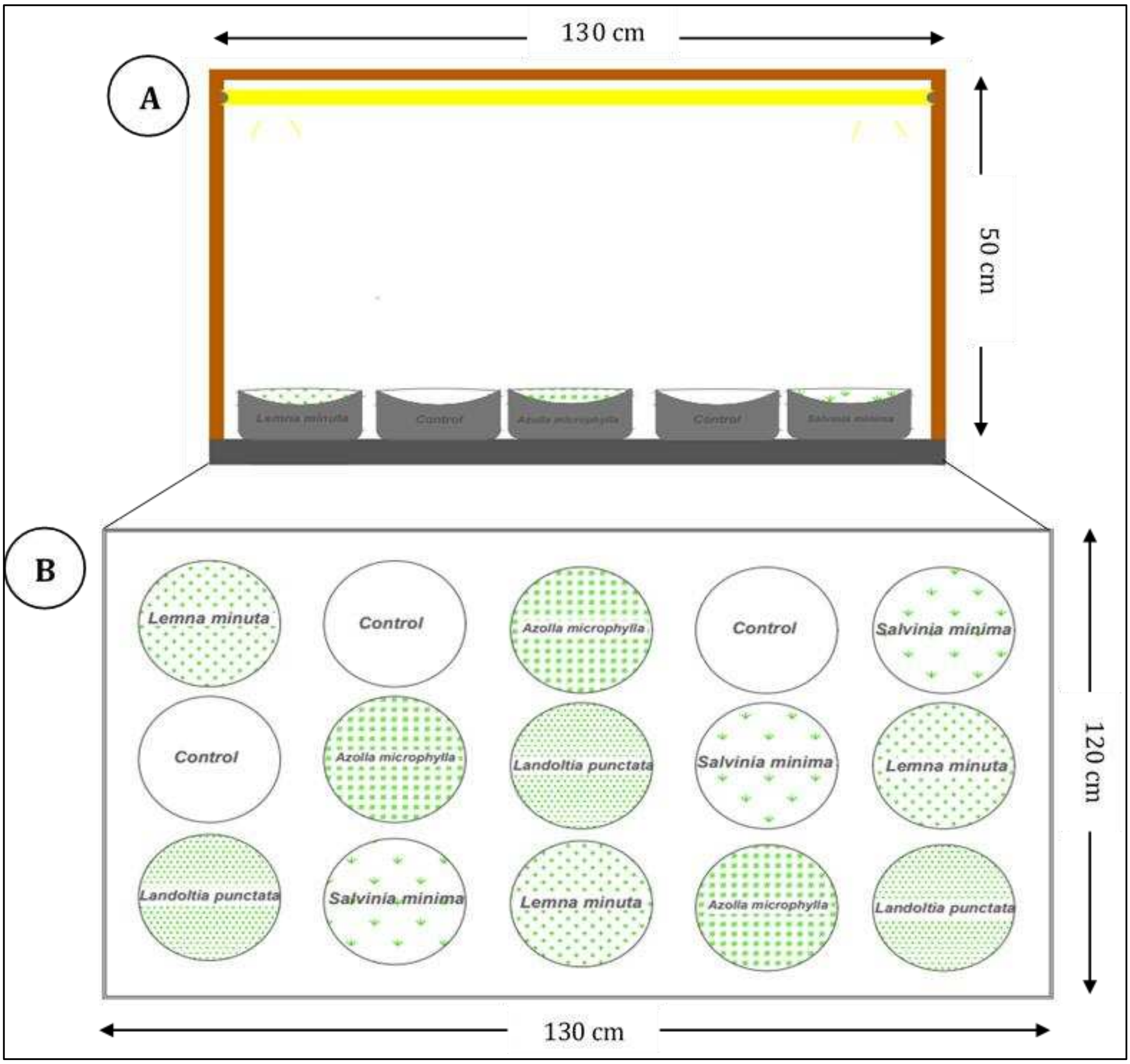


Figure A.1: Side (A) and upper (B) views of schematic set-up for synthetic swine wastewater phytoremediation process.

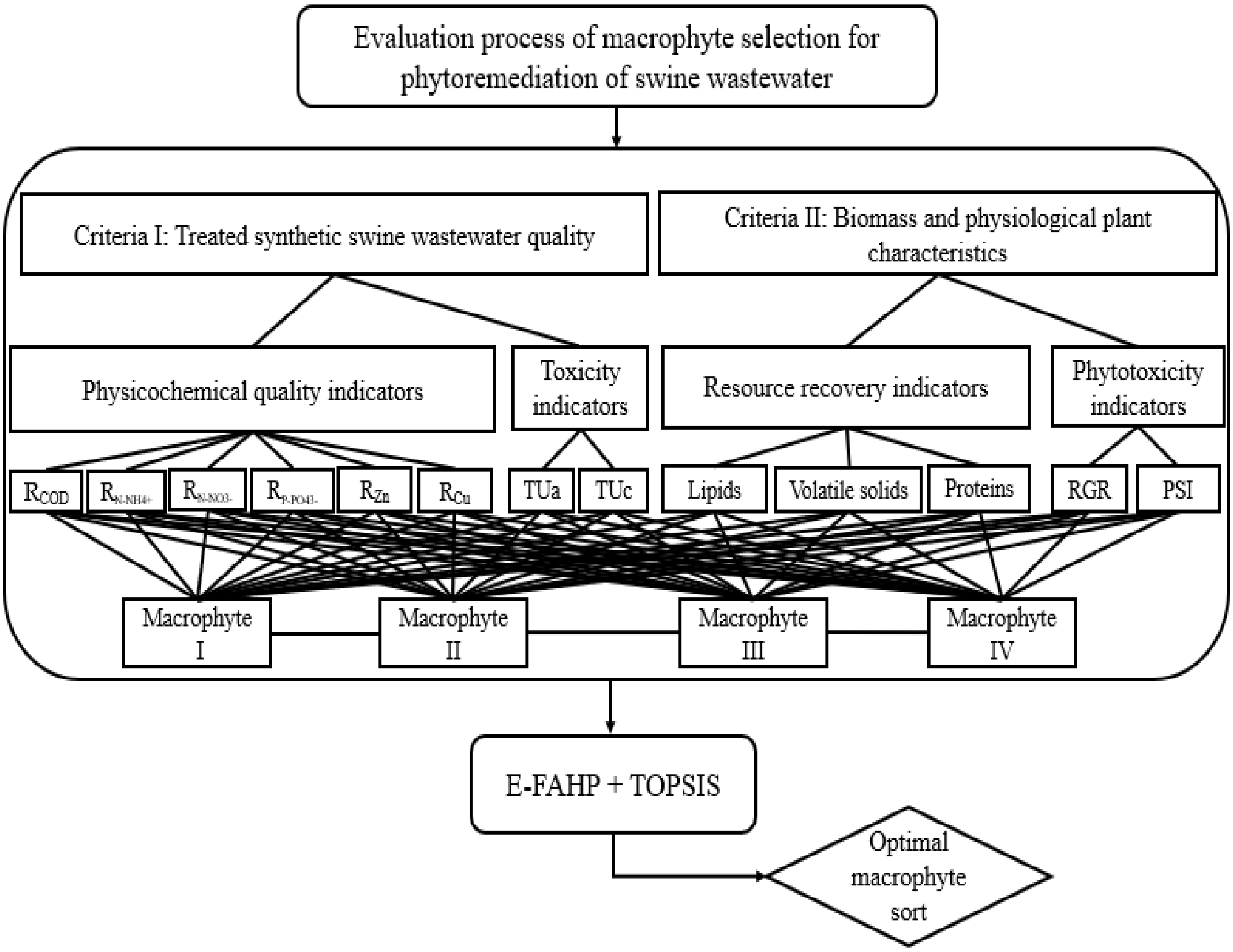


Figure A.2: Flow chart to stepwise microphyte selection for phytoremediation of swine wastewater. R means removal, in %, of target pollutants. TUa: Acute toxicity units. TUc: Chronic toxicity units. RGR: Relative Growth Rate. PSI: Phyto-Stress Index.

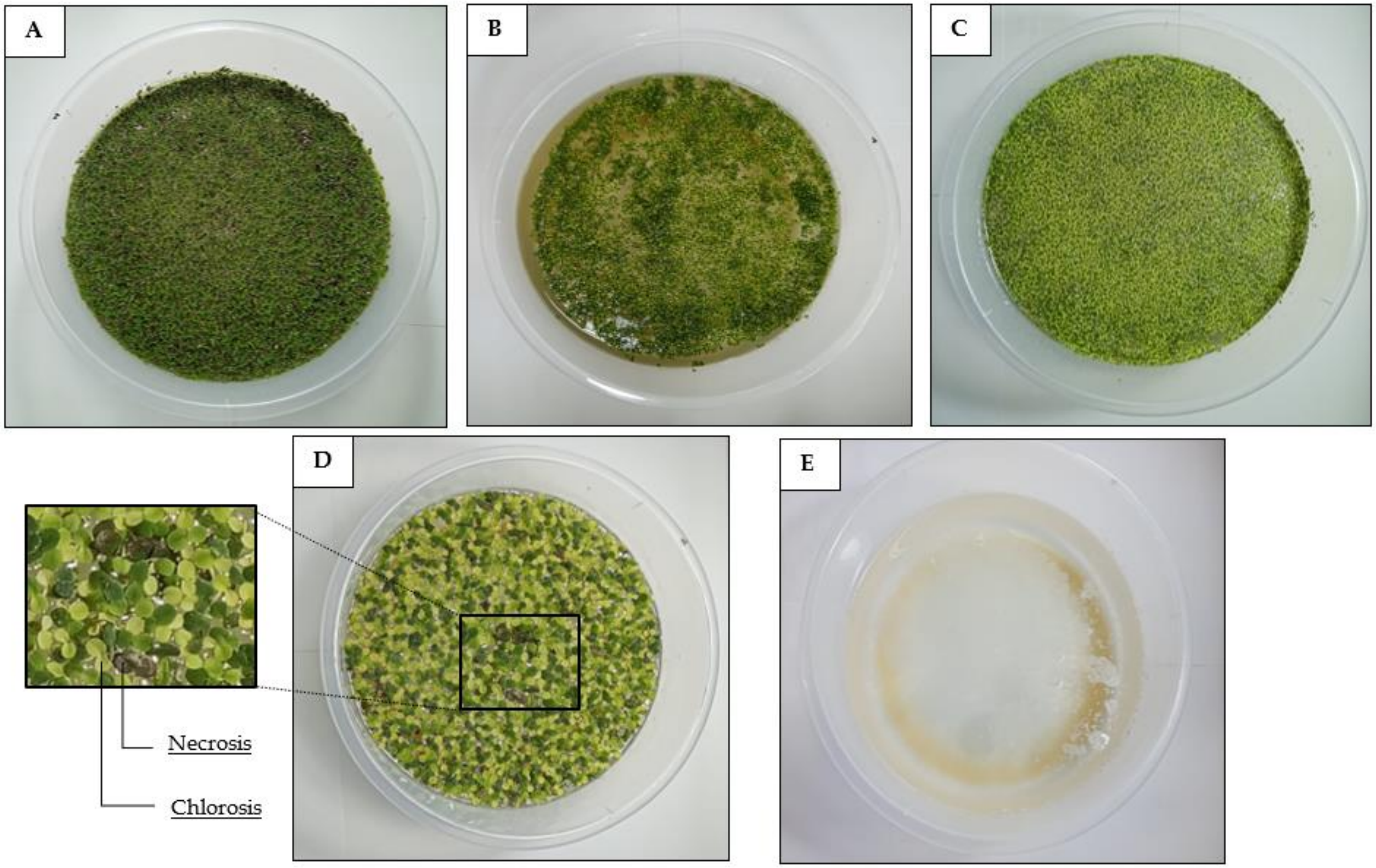


Figure A.3: Final appearance of floating plants in the synthetic swine wastewater. **A:** *Azolla microphylla.* **B:** *Landoltia punctata.* **C:** *Lemna minuta.* **D:** *Salvinia minima* (highlighting the signs of chlorosis and necrosis). **E:** Contro

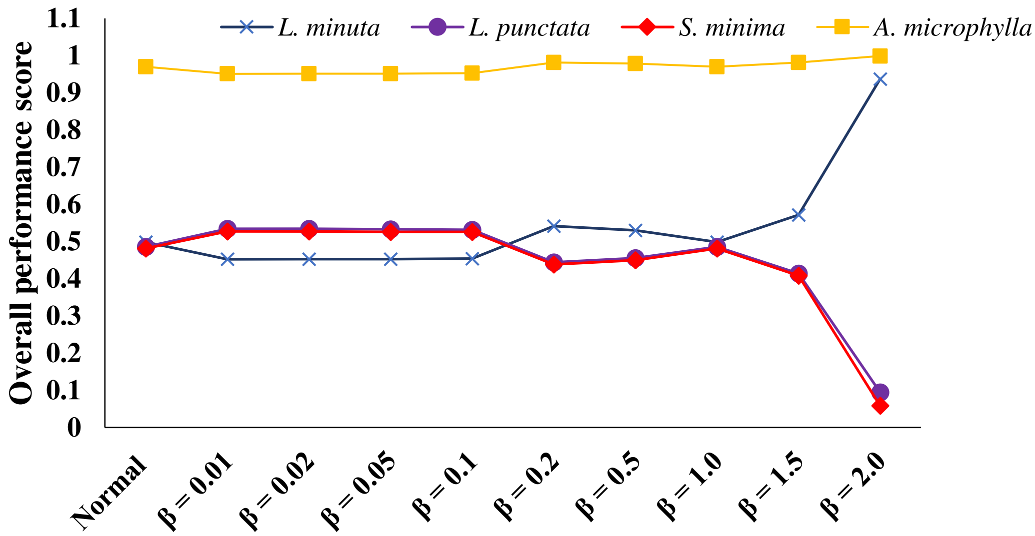


Fig A.4: Sensitivity analysis due to the alterations of unitary ratios β_k. The "normal" refers to the original performance score

Text A.1 Theoretical backgrounds and calculations

Fuzzy Analytical Hierarchy Method

A total of fifteen decision-makers were invited to evaluate, in degrees of influence, the importance that each of the attributes had as a criterion for choosing a plant. The degrees of influence were evaluated using the linguistic terms Very High Influence (VHI), High Influence (HI), Low Influence (LI), Very Low Influence (VLI), and No Influence (NI) (Sakhardande and Prabhu Gaonkar, 2022).

Evaluations were compared by measuring the differences between them and assigned to a Fuzzy Analytic Hierarchy Process (FAHP) (Table A.8) based on the methodology proposed by Sakhardande and Prabhu (2022). For instance, if the decision-maker gave the relative importance of High Influence (HI) to two attributes, the comparison matrix mapped the difference to Equally Important and assigned the corresponding triangular fuzzy number of (1,1,3) (Table A.8, Appendix A). In the present study we considered that when the comparison was reciprocal, the inverse value of the fuzzy number was assigned the fuzzy inverse value property (Eq A.1).

$$\tilde{A}^{-1} = (k, m, u)^{-1} = (1/u, 1/m, 1/k) \tag{A.1}$$

Where k, m, u natural crisp numbers, k, m, u $\in$ Ni (I = 1, 2, 3, …, 10).

Evaluation of fuzzy comparison judgments was based on triangular fuzzy numbers using the membership function $\mu_{\tilde{A}}$ ($R \rightarrow [0,1]$) where the R is the domain of the general fuzzy number M expressed as (k, m, u) (Eq.A.2).

$$\mu_{\tilde{A}}(x) = \begin{cases} \frac{x-k}{m-k}, & k \leq x \leq m \\ \frac{u-x}{u-m}, & m < x \leq u \\ 0, \text{otherwise} \end{cases} \tag{A.2}$$

The normalized weights of each attribute were calculated based on fuzzy extend methods proposed by Chang (Chang, 1996). However, in this method, there is the possibility to allocate a zero as an attribute weight value which can be unrealistic (Wang et al., 2008). To solve this, Tyagi et al. (2018) proposed an enhanced extended analysis (STEP 2). The following steps described the determination of the Fuzzy-AHP weights using enhanced extend the analysis (Eqs. A.3-A.11).

STEP 1: Determining the fuzzy extend (S_i) values of each i^{th} attribute

$$S_i = \sum_{j=1}^{m} M_{g\,i}^{j} \otimes \left[\sum_{i=1}^{n} \sum_{j=1}^{m} M_{g\,i}^{j}\right]^{-1} = \left(\frac{1}{\sum_{i=1}^{n} u_i}, \frac{1}{\sum_{i=1}^{n} m_i}, \frac{1}{\sum_{k=1}^{n} k_i}\right) \tag{A.3}$$

Where $M_{g\,i}^{j}$ are all triangular fuzzy numbers

STEP 2: Determining the degree of possibilities of triangular fuzzy numbers $V(M_2 \geq M_1)$ where M_2 (k_2, m_2, u_2) and M_1 (k_1, m_1, u_1) are two convex triangular fuzzy numbers. The degree of possibility of $M_2 \geq M_1$ is presented as

$$V(M_2 \geq M_1) = \sup_{y \geq x} \min [(\mu M_2 (y), \mu M_1 (x))] \tag{A.4}$$

where x and y represent the values corresponding to the membership function of each attribute

$$V(M_2 \geq M_1) = 1, \text{ if and only if } m_2 \geq m_1 \tag{A.5}$$

$$V (M_2 \geq M_1) = hgt (M_2 \cap M_1) = \mu M_2 (d) \tag{A.6}$$

where d is the ordinate of the highest point of intersection between μM_1 and μM_2.

The expression in Eqs (A.5, A.6) can be expressed in Eq. A.7 as follows (Tyagi et al., 2018).

$$V(M_2 \geq M_1) = \begin{cases} 1, & m_2 \geq m_1 \\ \dfrac{-(k_1 - u_2)}{(m_2 - u_2)-(m_1 - u_1)}, & k_1 \geq u_2 \\ \dfrac{(k_1 - u_2)}{(m_2 - u_2) - (m_1 - u_1)}, & k_1 < u_2 \end{cases} \tag{A.7}$$

A minimum degree of possibility of a convex number to be greater than j convex fuzzy numbers M_i (i = 1,2,3… j) as defined by

$$V (M_1, M_2 \ldots M_k) = \min V (M \geq M_i), i = 1, 2..., k \tag{A.8}$$

$$d' (C_i) = \min V (M_i \geq M_j), j = 1, 2\ldots, n; j \neq i \tag{A.9}$$

STEP 3: The weight vector (W_i) and their normalization (N-W_i) are given by

$$W_i = \left[d'(C_1), d'(C_2), d'(C_3),\ldots, d'(C_n)\right]^T \tag{A.10}$$

$$N\text{-}W_i = \frac{\left[d'(C_1), d'(C_2), d'(C_3),\ldots, d'(C_n)\right]^T}{\sum W_i} \tag{A.11}$$

The fifteen decision-makers produced respective weights (Eq. A.11) from their FAHP matrices. Overall weights of each attribute were selected by the normalized geometric mean of ten selected decision-maker weight matrices (consistency ratio < 0.1) (Table A.2, Appendix A). The consistency ratios (CR) of each fuzzy matrix were made using Saaty's crisp consistency method (Saaty, 2008, 1977), which is suitable for all types of fuzzy sets (Liu et al., 2020; Mahmoudzadeh and Bafandeh, 2013). Moreover, the data of each attribute was validated using Cronbach's alpha test for internal consistency (Taber, 2018) (Table A.3, Appendix A).

Entropy method

The objective elicitation method of Entropy was based on Shannon's entropy theory (Shannon, 1948), with weighting calculated based on the data itself (Chakraborty and Chakraborty, 2022). The steps are shown below (Eqs. A.12-A.16).

STEP 1: Construction of the matrix X_{ij} formed by original attribute responses (i.e. % of removal COD - R_{COD}, PSI index, etc) for all alternatives.

$$X = \begin{bmatrix} x_{11} & \cdots & x_{1m} \\ \vdots & \ddots & \vdots \\ x_{n1} & \cdots & x_{nm} \end{bmatrix} \tag{A.12}$$

where x_{ij} represents the value of m attribute for each n alternative

STEP 2: Data normalization

$$p_{ij} = \frac{x_{ij}}{\sum_{i=1}^{n} x_{ij}} \tag{A.13}$$

STEP 3: Determining entropy value - e_j

$$e_j = -\frac{1}{\ln(n)} \sum_{i=1}^{n} p_{ij} * \ln p_{ij} \tag{A.14}$$

If $p_{ij} = 0$, and then define $p_{ij} \ln p_{ij} = 0$

STEP 4: Determining the divergence coefficient dj for the attribute j

$$d_j = 1 - e_j \tag{A.15}$$

STEP 5: Determining entropy weight – w"

$$w'' = \frac{d_j}{\sum_{i=1}^{m} d_j} \tag{A.16}$$

TOPSIS (Technique for Order Preference by Similarity to Ideal Solution)

The R program TOPSIS package (Mosalman Yazdi, 2015) was used to rank the four macrophytes and select the best for use in the phytoremediation of swine wastewater using TOPSIS, as described below (Li et al., 2013) (Eqs.A.17-A.24).

STEP 1: Construction of the decision matrix (C) with m alternatives and n criteria

$$\mathbf{C} = \begin{bmatrix} c_{11} & \cdots & c_{1n} \\ \vdots & \ddots & \vdots \\ c_{m1} & \cdots & c_{nm} \end{bmatrix} \tag{A.17}$$

where c_{ij} is the observed value of attribute m for each alternative n

STEP 2: Data normalization of the initial matrix C by the vector normalization method

$$s_{ij} = \frac{x_{ij}}{\sqrt{\sum_{i=1}^{n} x_{ij}^{2}}}, \ i = 1, 2, ..., m; \ j = 1, 2, ..., n. \tag{A.18}$$

STEP 3: Weighted normalized matrix (Vij)

$$a_{ij} = w_{ij} \text{ x } s_{ij} \tag{A.19}$$

where a_{ij} is the weighted standardized value w_{ij} is the weight of each attribute.

STEP 4: Determining ideal (V^+) and anti-ideal (V^-) reference points.

$$V^+ = (v_1^+, v_2^+, ..., v_m^+) \tag{A.20}$$

$$V^- = (v_1^-, v_2^-, ..., v_m^-) \tag{A.21}$$

where,

v_m^+ = (max v_{ij}, if j is a benefit attribute or min v_{ij}, if j is a cost attribute)

v_m^- = (min v_{ij}, if j is a benefit attribute or max v_{ij}, if j is a cost attribute)

STEP 5: Determining Euclidean distances between the alternatives (V_i) and the ideal (V^+) and anti-ideal (V^-) reference points.

$$V_i^+ = \sqrt{\sum_{j=1}^{n} (v_m^+ - v_{ij})^2}, i = 1, \ldots, m. \tag{A.22}$$

$$V_i^- = \sqrt{\sum_{j=1}^{n} (v_m^- - v_{ij})^2}, i = 1, \ldots, m. \tag{A.23}$$

STEP 6: Determining overall performance score - R_i

$$R_i = \frac{V_i^-}{V_i^+ + V_i^-} \tag{A.24}$$

The optimal alternative was that with the highest R_i value.

As aforementioned, the indicators for each criterion were divided into thirteen attributes (Fig A.2,Appendix A). In the TOPSIS language, the attributes were categorized into benefit and/or cost types. For the benefit types, the best response was the highest, and for cost types, the best response was the lowest response, while the worst response was the opposite for the two attribute types (Table A.4, Appendix A). The best and worst values for each attribute were called the ideal (V^+) and anti-ideal (V^-) reference values points. Thus, each normalized attribute response (Eq. A.19, STEP 3) was compared among the four plant alternatives and the highest or lowest value was selected according to the classification of that attribute (Table A.4, Appendix).

The most suitable aquatic plant was the one that afforded highest removal of COD (R_{COD}), N-NH_4^+ (R_{N-NH4+}), N-NO_3^- (R_{N-NO3-}), P-PO_4^{3-} ($R_{P-PO43-}$), Cu (R_{Cu}), Zn (R_{Zn}) from the swine wastewater and contained the highest lipids, proteins, volatile solids and RGR contents. Therefore, these attributes were classified as benefit attributes (Table A.4, Appendix A).

On the other hand, the wastewater treated by the best plant should have the lowest acute and chronic toxicities (TUa and TUc). In addition, that plant should present the lowest phyto-stress index, measured by primary production biomarkers (pigments) and integrated into the overall stress index (PSI). These toxicity attributes were categorized as cost types (Table A.4, Appendix A).

Sensitivity analysis of the compromised weights for the TOPSIS method

Sensitivity analysis was performed according to the methodology proposed by Li et al. (2013). For this, variations in the compromised weights (w^c) were considered. w^c; where c = 1, 2, 3, ..., n ∀ n equals the number of attributes in which w^c is converted into w^{cc} (Eq. A.25)

$$w^{cc} = y_k * w^c \tag{A.25}$$

The relationship between w^i e w^{cc} is the unitary ratio β_k where w^i represents unitary weights for parameters 1, 2, k and n (Eq. 37).

$$\beta_k = \frac{w^i}{w^{cc}} \tag{A.26}$$

The new compromised weights (w^{cc}) of each attribute are calculated as follows (Eq. A.27)

$$w^{cc} = \frac{w^c}{1+ (y_k - 1)w^c} \quad \text{(A.27)}$$

Where y_k represents the initial variation ratio ($y_k > 0$) and it is expressed as (Eq. A.28)

$$y_k = \frac{\beta_k - \beta_k w^c}{1 - \beta_k w^c} \quad \text{(A.28)}$$

In this paper, nine values of β_k were used according to that proposed by Li et al. (2013), i.e., β_k = 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0. 1.5 and 2.0. The new w^{cc} is calculated and variations on TOPSIS ranks were analyzed (Fig A.4, Appendix A).

APPENDIX B – SUPPLEMENTARY MATERIAL CHAPTER 4

Table S1: The ANOVA table for the effects of Photoperiod, Cytokinin, and Light Intensity on the residual concentration of COD in synthetic swine wastewater

Factor	SS	df	MS	F	*p*-value
Model	0.0002	7	0.0000	6.11	0.0056
Photoperiod (A)	3.300E-06	1	3.300E-06	0.5866	0.4614
Cytokinin (B)	3.565E-08	1	3.565E-08	0.0063	0.9381
Light intensity (C)	0.0000	1	0.0000	7.90	0.0185
AB	3.647E-06	1	3.647E-06	0.6484	0.4394
BC	0.0000	1	0.0000	7.61	0.0202
B^2	0.0000	1	0.0000	2.27	0.1632
AB^2	0.0001	1	0.0001	11.68	0.0066
Residual	0.0001	10	5.625E-06		
Lack of Fit	0.0000	5	5.454E-06	0.9412	0.5257
Pure Error	0.0000	5	5.795E-06		
Cor Total	0.0003	17			

R^2 = 0.8105; Adjusted R^2 = 0.6779; Predicted R^2 = 0.2962; Adeq. Precision = 9.0288

Table S2: The ANOVA table for the effects of Photoperiod, Cytokinin, and Light Intensity of the DOC in synthetic swine wastewater (in terms of DOC removed per unit of biomass produced).

Factor	SS	df	MS	F	*p*-value
Model	4.73	3	1.58	24.51	< 0.0001
Photoperiod (A)	2.25	1	2.25	35.06	< 0.0001
Light intensity (C)	2.11	1	2.11	32.80	< 0.0001
C^2	0.3652	1	0.3652	5.68	0.0318
Residual	0.8996	14	0.0643		
Lack of Fit	0.6558	9	0.0729	1.49	0.3431
Pure Error	0.2438	5	0.0488		
Cor Total SS	5.63	17			

R^2 = 0.8401; Adjusted R^2 = 0.8058; Predicted R^2 = 0.7182; Adeq. Precision = 16.9090

Table S3: The ANOVA table for the effects of Photoperiod and Light Intensity of the P-PO_4^{3-}, per unit of biomass produced, in synthetic swine wastewater

Factor	**SS**	**df**	**MS**	**F**	***p*-value**
Model	4.67	2	2.34	14.40	0.0003
Photoperiod (A)	1.38	1	1.38	8.51	0.0106
Light intensity (C)	3.29	1	3.29	20.30	0.0004
Residual	2.43	15	0.1622		
Lack of Fit	2.04	10	0.2039	2.58	0.1532
Pure Error	0.3946	5	0.0789		
Cor Total SS	7.11	17			

R^2 = 0.6576; Adjusted R^2 = 0.6120; Predicted R^2 = 0.4608; Adeq. Precision = 12.5838

Table S4: The ANOVA table for the effects of Photoperiod and Light Intensity on the residual concentration of N-NH_4^+ in synthetic swine wastewater

Factor	**SS**	**df**	**MS**	**F**	***p*-value**
Model	3361.94	3	1120.65	27.49	< 0.0001
Photoperiod (A)	1132.32	1	1132.32	27.77	0.0001
Light intensity (C)	1686.73	1	1686.73	41.37	< 0.0001
C^2	542.89	1	542.89	13.32	0.0026
Residual	570.76	14	40.77		
Lack of Fit	390.29	9	43.37	1.20	0.4420
Pure Error	180.47	5	36.09		
Cor Total SS	3932.70	17			

R^2 = 0.8549; Adjusted R^2 = 0.8238; Predicted R^2 = 0.7647; Adeq. Precision = 17.8257

Table S5: The ANOVA table for the effects of Photoperiod and Light Intensity on the residual concentration of N-NO_3^- in synthetic swine wastewater

Factor	SS	df	MS	F	*p*-value
Model	2880.29	6	480.05	5.56	0.0071
Photoperiod (A)	131.68	1	131.68	1.53	0.2425
Cytokinin (B)	12.30	1	12.30	0.1426	0.7129
Light intensity (C)	1322.22	1	1322.22	15.32	0.0024
AB	551.40	1	551.40	6.39	0.0281
A^2	338.98	1	338.98	3.93	0.0730
B^2	552.42	1	552.42	6.40	0.0280
Residual	949.21	11	86.29		
Lack of Fit	315.79	6	52.63	0.4155	0.8425
Pure Error	633.42	5	126.68		
Cor Total SS	3829.50	17			

R^2 = 0.7521; Adjusted R^2 = 0.6169; Predicted R^2 = 0.3386; Adeq. Precision = 6.9092

Table S6: The ANOVA table for the effects of Photoperiod, Cytokinin and Light Intensity on the residual concentration of Zn in synthetic swine wastewater

Factor	SS	df	MS	F	*p*-value
Model	0.0025	6	0.0004	4.41	0.0163
Photoperiod (A)	0.0006	1	0.0006	6.19	0.0302
Cytokinin (B)	0.0000	1	0.0000	0.1617	0.6953
Light intensity (C)	0.0000	1	0.0000	0.4737	0.5055
AC	0.0008	1	0.0008	8.04	0.0162
BC	0.0007	1	0.0007	7.03	0.0225
C^2	0.0004	1	0.0004	4.64	0.0542
Residual	0.0011	11	0.0001		
Lack of Fit	0.0003	6	0.0000	0.3259	0.8976
Pure Error	0.0008	5	0.0002		
Cor Total	0.0036	17			

R^2 = 0.7064; Adjusted R^2 = 0.5463; Predicted R^2 = 0.2135; Adeq. Precision = 7.8489

Table S7: The ANOVA table for the effects of Photoperiod, Cytokinin, and Light Intensity on the residual concentration of Cu in synthetic swine wastewater

Factor	SS	df	MS	F	*p*-value
Model	0.6651	5	0.1330	24.90	< 0.0001
Photoperiod (A)	0.0625	1	0.0625	11.70	0.0051
Cytokinin (B)	0.3333	1	0.3333	62.41	< 0.0001
Light intensity (C)	0.0570	1	0.0570	10.68	0.0067
BC	0.0490	1	0.0490	9.17	0.0105
B^2	0.1984	1	0.1984	37.14	< 0.0001
Residual	0.0641	12	0.0053		
Lack of Fit	0.0340	7	0.0049	0.8050	0.6177
Pure Error	0.0301	5	0.0060		
Cor Total SS	0.7292	17			

R^2 = 0.9121; Adjusted R^2 = 0.8755; Predicted R^2 = 0.8099; Adeq. Precision = 17.3188

Table S8: The ANOVA table for the effects of Photoperiod, Cytokinin, and Light Intensity on the residual concentration of SMX in synthetic swine wastewater

Factor	SS	df	MS	F	*p*-value
Model	2.05	6	0.3410	10.43	0.0020
Photoperiod (A)	0.0046	1	0.0046	0.1410	0.7170
Cytokinin (B)	0.5071	1	0.5071	15.51	0.0043
Light intensity (C)	0.1169	1	0.1169	3.57	0.0953
AB	0.7228	1	0.7228	22.11	0.0015
AC	0.2064	1	0.2064	6.31	0.0362
B^2	0.6571	1	0.6571	20.10	0.0020
Residual	0.2616	8	0.0327		
Lack of Fit	0.2405	6	0.0401	3.79	0.2233
Pure Error	0.0211	2	0.0106		
Cor Total SS	2.31	14			

R^2 = 0.8866; Adjusted R^2 = 0.8016; Predicted R^2 = 0.3559; Adeq. Precision = 12.6258

Table S9: Akaike Information Criterion (AIC) values of generalized linear family models associated with wastewater treatment performances and biomass by-products to four promising optimal treatment conditions

Parameter (unit)	[a]GLM family regression models					
	Gamma		Normal		Log normal	
	AIC	Model (p-value)	AIC	Model (p-value)	AIC	Model (p-value)
[b]Bp ($gDWm^{-2}d^{-1}$)	1.432	< 0.001	3.78	< 0.001	**0.65**	< 0.001
Protein (%DW)	87.27	0.297	82.393	0.246	90.004	0.324
Lipids (%DW)	131.82	0.551	137.77	0.601	130.94	0.543
Starch (%FW)	**81.17**	< 0.001	88.97	< 0.001	83.61	< 0.001
$N\text{-}NO_3^-$ ($mg\ L^{-1}$)	88.29	0.311	92.322	0.373	87.05	0.289
$N\text{-}NH_4^+$ ($mg\ L^{-1}$)	98.51	< 0.001	**88.83**	< 0.001	108.99	< 0.001
Zn ($mg\ L^{-1}$)	-6.916	0.020	**-7.474**	0.016	-6.613	0.021
Cu ($mg\ L^{-1}$)	-34.34	0.167	-33.01	0.132	-33.15	0.2
COD ($mg\ L^{-1}$)	126.22	0.557	123.04	0.523	128.91	0.579
SMX ($mg\ L^{-1}$)	-112.0	0.255	-104.0	0.352	-115.33	0.216
DOC_{remv}/Bp ($mg\ L^{-1}Bp^{-1}$)	10.94	< 0.001	**10.651**	< 0.001	11.611	< 0.001
$P\text{-}PO_4^{3-}$/Bp ($mg\ L^{-1}Bp^{-1}$)	44.82	0.007	48.989	0.014	**43.468**	0.005

[a]GLM: Generalized linear model. The GLM distribution emphasized in bold means the significant ($p \leq 0.05$) distribution used to fit the data. [b]Bp: Biomass productivity

Table S10: Validation results for promising optimal treatment conditions A and B using *Azolla microphylla*

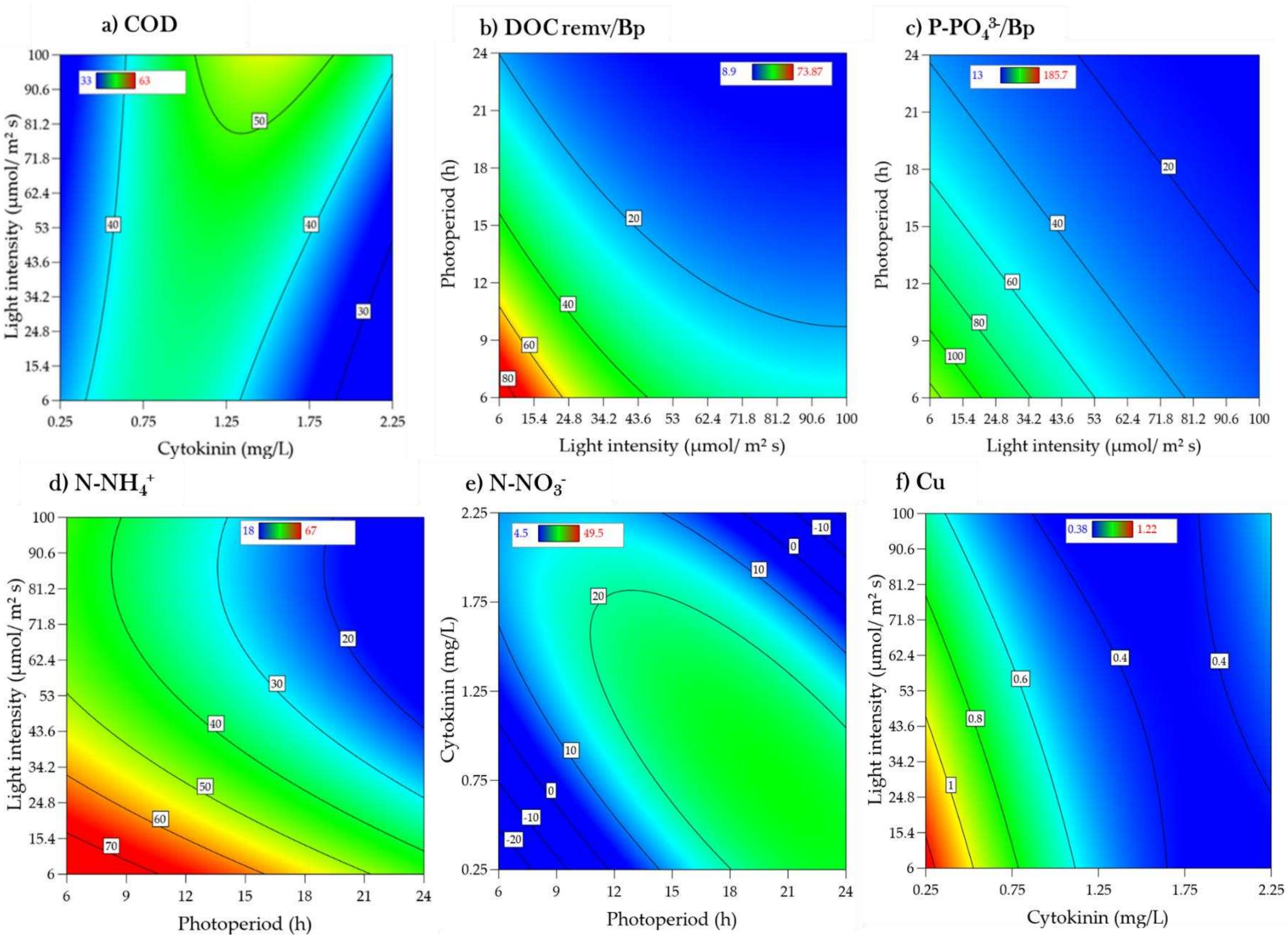
	Trial A		Trial B	
Photoperiod (h)	24		8	
Cytokinin (mg L^{-1})	0.3		0.3	
Light intensity (µmol m^{-2} s^{-1})	100		100	
Parameter (unit)	[a]Observed value (CV, median, G.M)	[b]Model-estimated value ($PI_{95\%}$)	[a]Observed value (CV, median, G.M)	[b]Model-estimated value ($PI_{95\%}$)
COD (mg L^{-1})	33.1 (14.29, 32.8, 32.8)	57.82 (35.98 a 134.71)	27.64 (29.28, 28.71, 26.79)	31.45 (24.25 to 44.04)
DOC_{remv}/Bp (mg L^{-1} Bp^{-1})	3.15 (5.79, 3.23, 3.15)	6.08 (2.97 to 11.67)	4.04 (9.80, 4.06, 4.03)	23.08 (11.52 to 43.45)
N-NH_4^+ (mg L^{-1})	9.4 (17.32, 8.46, 9.31)	11.48 (-5.76 to 28.71)	23.26 (6.06, 22.56, 23.46)	41.39 (24.70 to 58.07)
N-NO_3^- (mg L^{-1})	10 (34.64, 8, 9.64)	51.31 (16.05 to 86.56)	9.5 (13.59, 9.5, 943)	9.18 (-21.56 to 39.92)
P-PO_4^{3-}/Bp (mg L^{-1} Bp^{-1})	4.22 (19.31, 3.89, 4.17)	8.84 (3.71 to 23.09)	5.3 (20.31, 5.90, 5.84)	25.14 (8.48 to 63.37)
Cu (mg L^{-1})	0.16 (12.5, 0.16, 0.16)	0.63 (0.39 a 0.88)	0.17 (13.32, 0.16. 0.17)	0.86 (0.62 to 1.09)
Zn (mg L^{-1})	4.11 (1.29, 4.1, 4.11)	4.2 (3.59 a 5.04)	3.82 (4.48, 3.76, 3.81)	3.76 (3.29 to 4.36)
SMX (mg L^{-1})	0.022 (6, 0.022, 0.021)	0.012 (0.0054 to 0.025)	0.022 (22.09, 0.02, 0.022)	0.123 (0.0567 to 0.256)

[a] CV (Coefficient of variation, in %). GM. Geometric Mean [b]$PI_{95\%}$ - Prediction interval at 95%.

Table S11: Validation results for promising optimal treatment conditions C and D using *Azolla microphylla*

	Trial C		Trial D	
Photoperiod (h)	24		8	
Cytokinin (mg L^{-1})	1.3		1.3	
Light intensity (µmol m^{-2} s^{-1})	100		100	
	[a]Observed value (CV, median, G.M)	[b]Model-estimated value ($PI_{95\%}$)	[a]Observed value (CV, median, G.M)	[b]Model-estimated value ($PI_{95\%}$)
COD (mg L^{-1})	33.4 (27.37, 29.53, 24.82)	0.73 (42.54 to 99.15)	31.9 (25.45, 35.29, 31.0)	53.64 (39.46 to 79.83)
DOC_{remv}/Bp (mg L^{-1} Bp^{-1})	3.01 (10.23, 3.03, 3.00)	6.08 (2.97 to 11.67)	4.16 (4.64, 4.21, 4.15)	23.08 (11.52 to 43.45)
N-NH_4^+ (mg L^{-1})	7.52 (21.65, 5.64, 5.12)	11.48 (-5.76 to 28.71)	22.6 (10.21, 22.56, 22.47)	41.39 (24.70 to 58.07)
N-NO_3^- (mg L^{-1})	9.5 (27.85, 9, 9.24)	39.98 (11.99 to 67.97)	13.00 (19.86, 13, 12.80)	37.49 (12.52 to 62.48)
P-PO_4^{3-}/Bp (mg L^{-1} Bp^{-1})	3.85 (5.94, 3.79, 3.85)	8.84 (3.71 to 23.09)	5.79 (1.01, 5.77, 5.79)	25.14 (8.48 to 63.37)
Cu (mg L^{-1})	0.29 (28.29, 0.28, 0.28)	0.34 (0.14 to 0.53)	0.16 (45.16, 0.15, 0.14)	0.56 (0.37 to 0.75)
Zn (mg L^{-1})	4.13 (2.70, 4.11, 4.13)	3.86 (3.40 to 4.45)	3.79 (6.48, 3.71, 3.78)	3.48 (3.14 to 3.91)
SMX (mg L^{-1})	0.023 (7.94, 0.023, 0.023)	0.013 (0.0064 to 0.025)	0.021 (26.08, 0.021, 0.020)	0.029 (0.016 to 0.053)

[a] CV (Coefficient of variation, in %). GM. Geometric Mean [b] $PI_{95\%}$ - Prediction interval at 95%.



a) COD
Light intensity (µmol/ m² s)
Cytokinin (mg/L)
b) DOC remv/Bp
Photoperiod (h)
Light intensity (µmol/ m² s)
c) P-PO4 3-/Bp
Photoperiod (h)
Light intensity (µmol/ m² s)
d) N-NH4 +
Light intensity (µmol/ m² s)
Photoperiod (h)
e) N-NO3 -
Cytokinin (mg/L)
Photoperiod (h)
f) Cu
Light intensity (µmol/ m² s)
Cytokinin (mg/L)

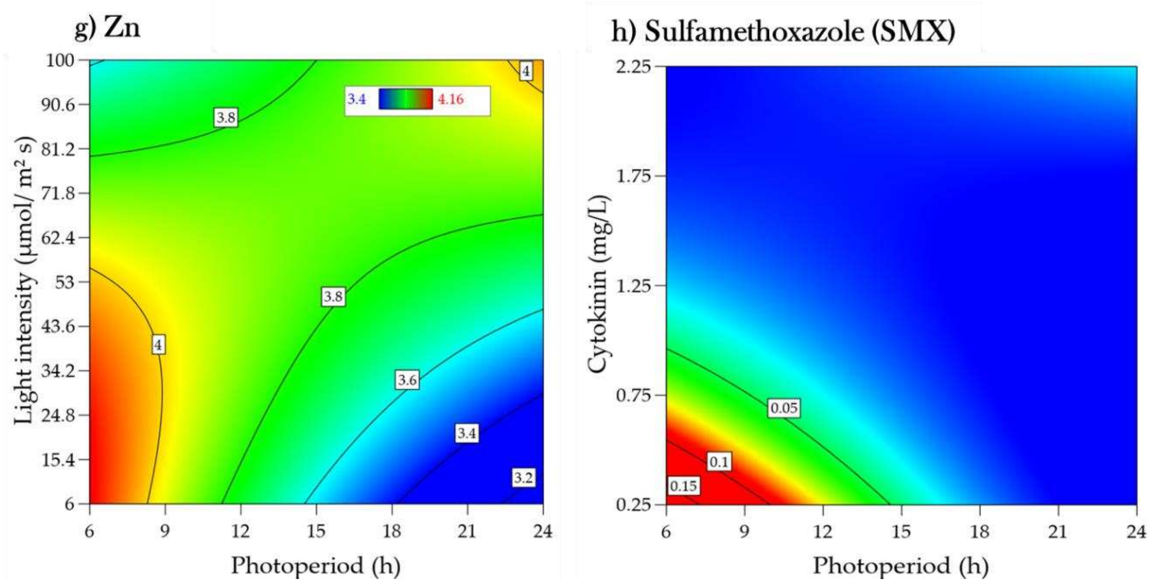


Fig. S1: Contour plot of the influence of photoperiod, cytokinin, and/or light intensity on the remaining parameter in synthetic swine wastewater. a) COD (mg L^{-1}) (photoperiod = 7 h); b) DOC rem/Bp (mg L^{-1} Bp^{-1}) (cytokinin = 0.25 mg L^{-1}); c) P-PO_4^{3-}/Bp (mg L^{-1} Bp^{-1}) (cytokinin = 0.25 mg L^{-1}); d) N-NH_4^+ (mg L^{-1}) (cytokinin = 0.25 mg L^{-1}); e) N-NO_3^- (mg L^{-1}) (light intensity = 65 µmol m^{-2} s^{-1}); f) Cu (mg L^{-1}) (photoperiod = 24 h); g) Zn (mg L^{-1}) (cytokinin = 0.76 mg L^{-1}); h) SMX (mg L^{-1}) (light intensity = 99.7 µmol m^{-2} s^{-1})

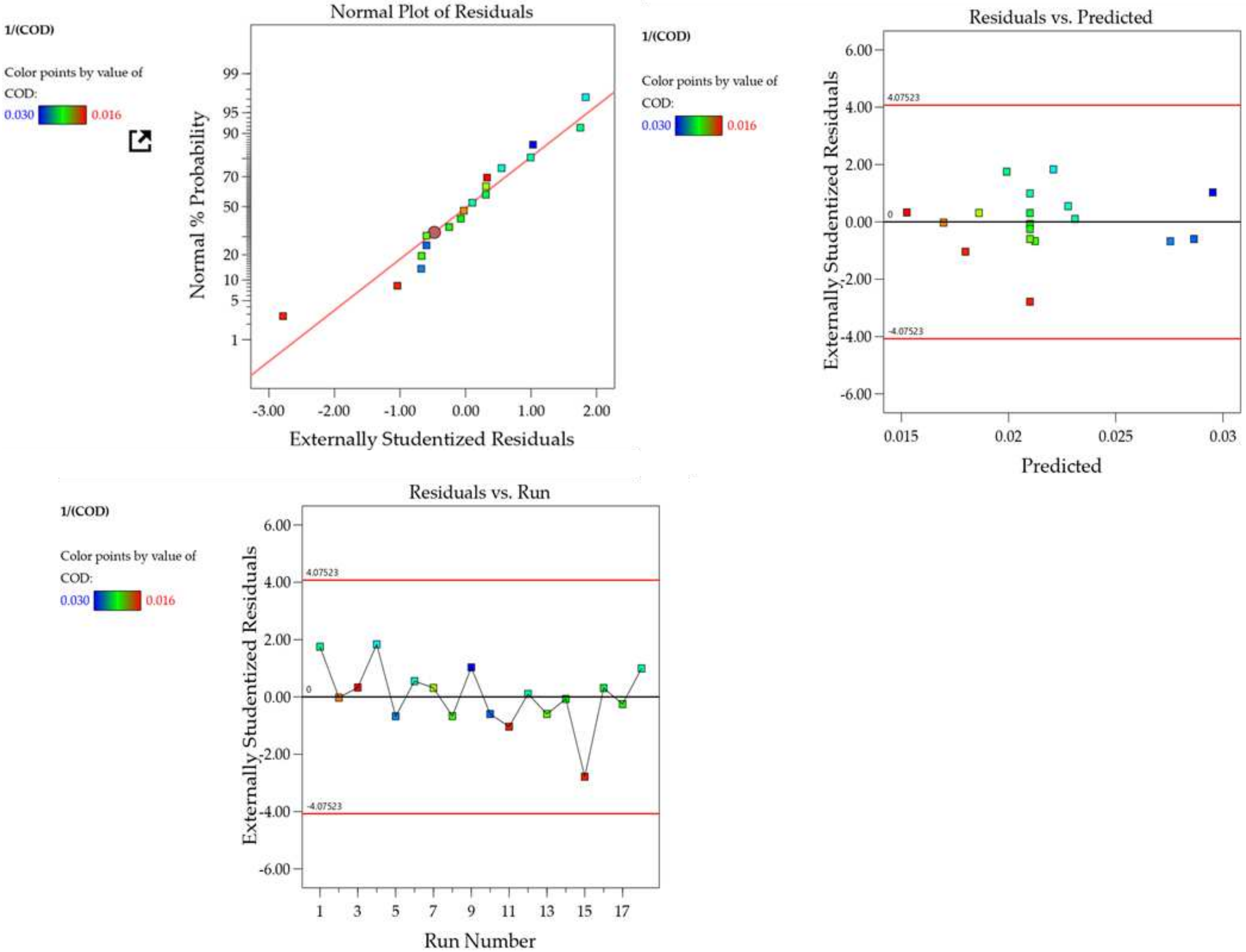


Fig. S2: Residual plots for COD analysis

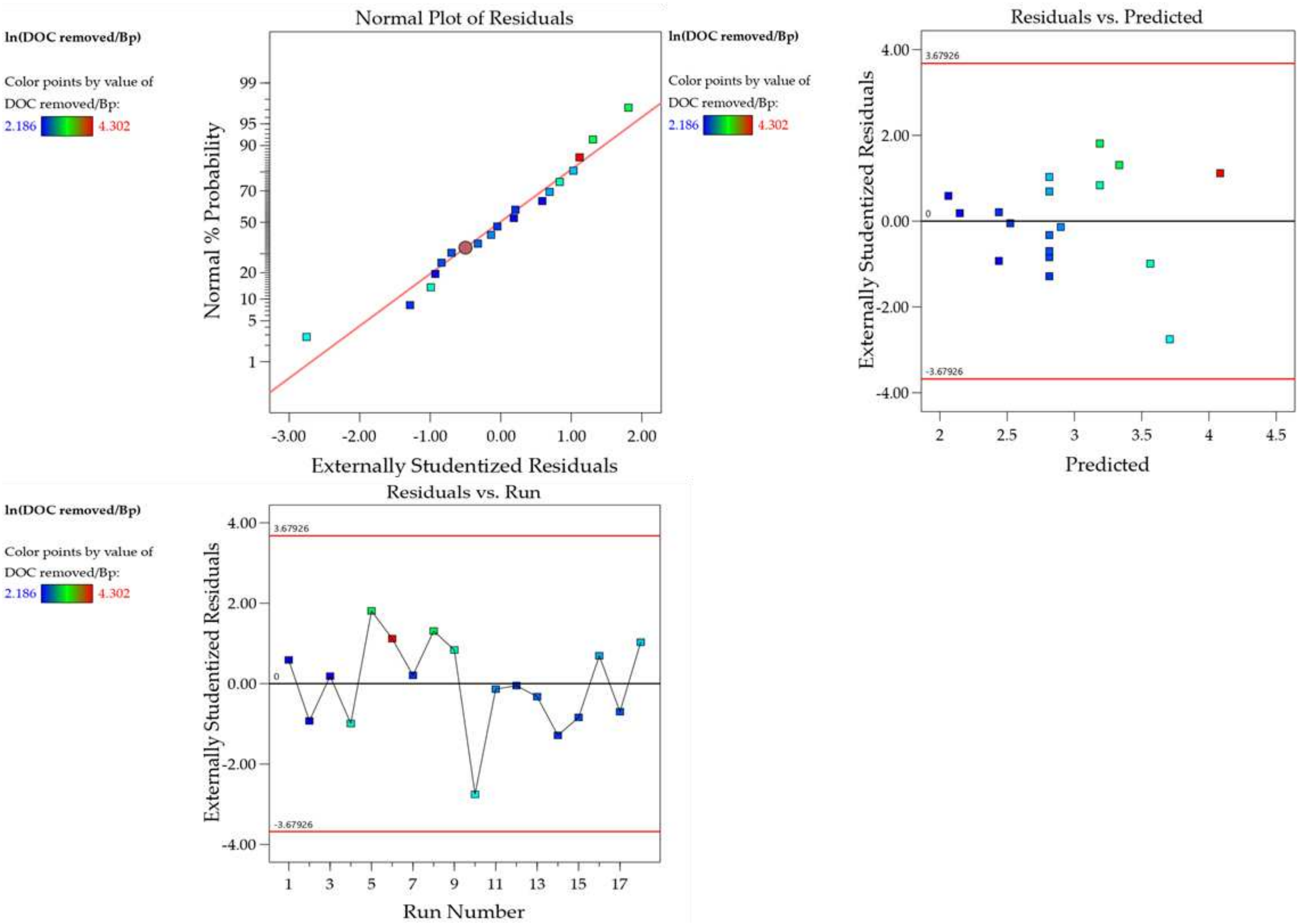


Fig. S3: Residual plots for $DOC_{removed}/Bp$ analysis

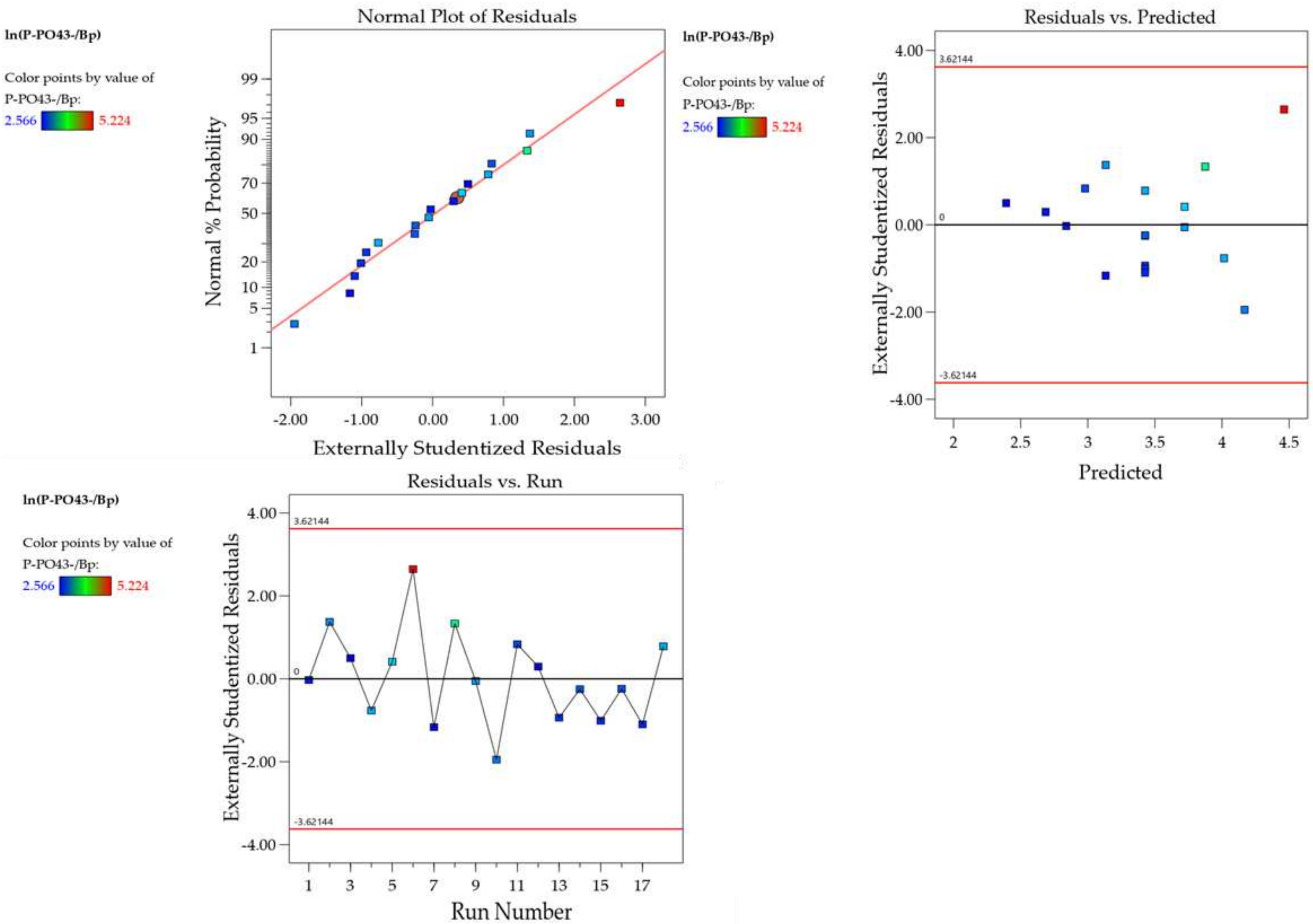


Fig. S4: Residual plots for P-PO_4^{3-}/Bp analysis

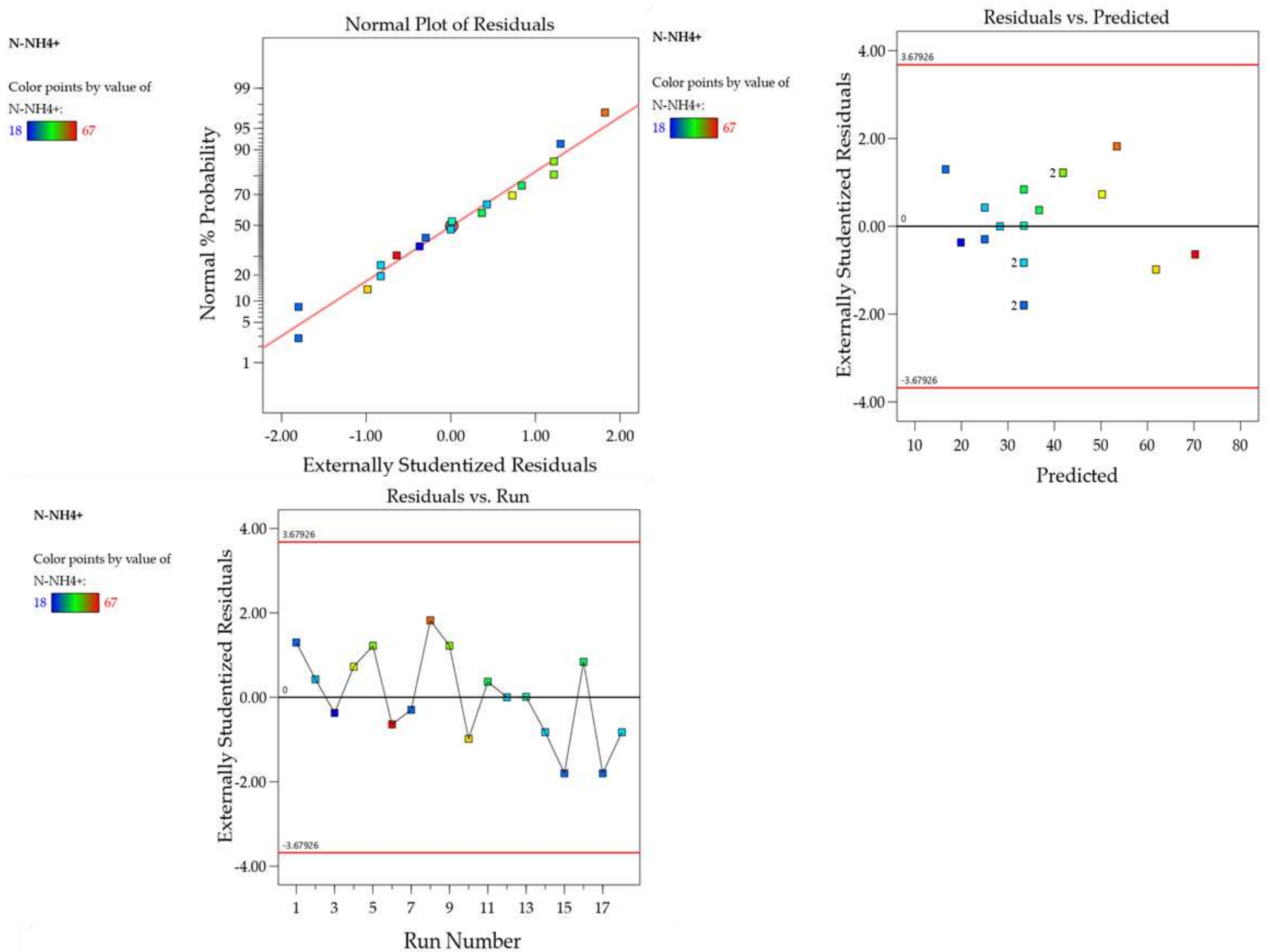


Fig. S5: Residual plots for $N\text{-}NH_4^+$ analysis

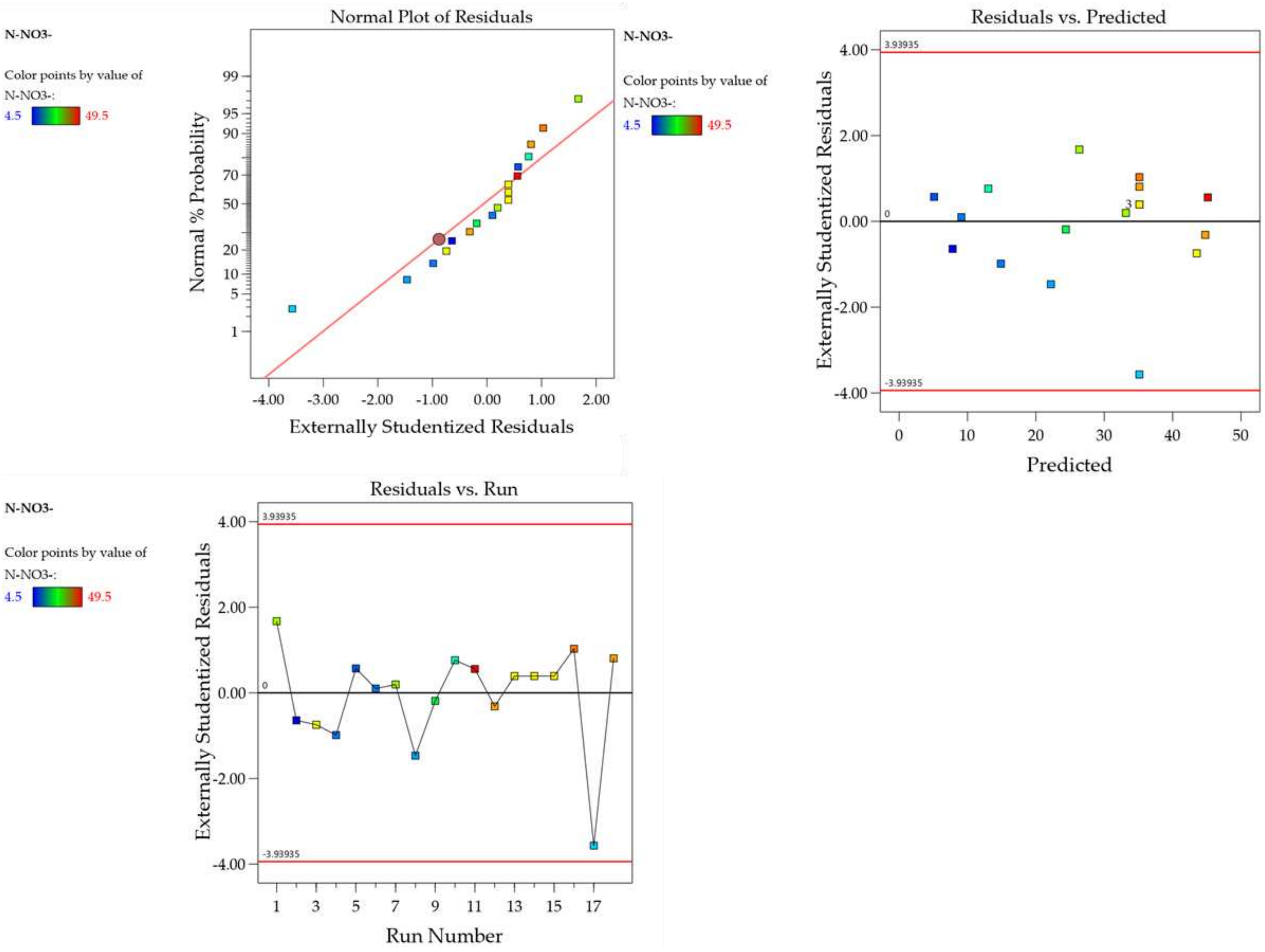


Fig. S6: Residual plots for N-NO_3^- analysis

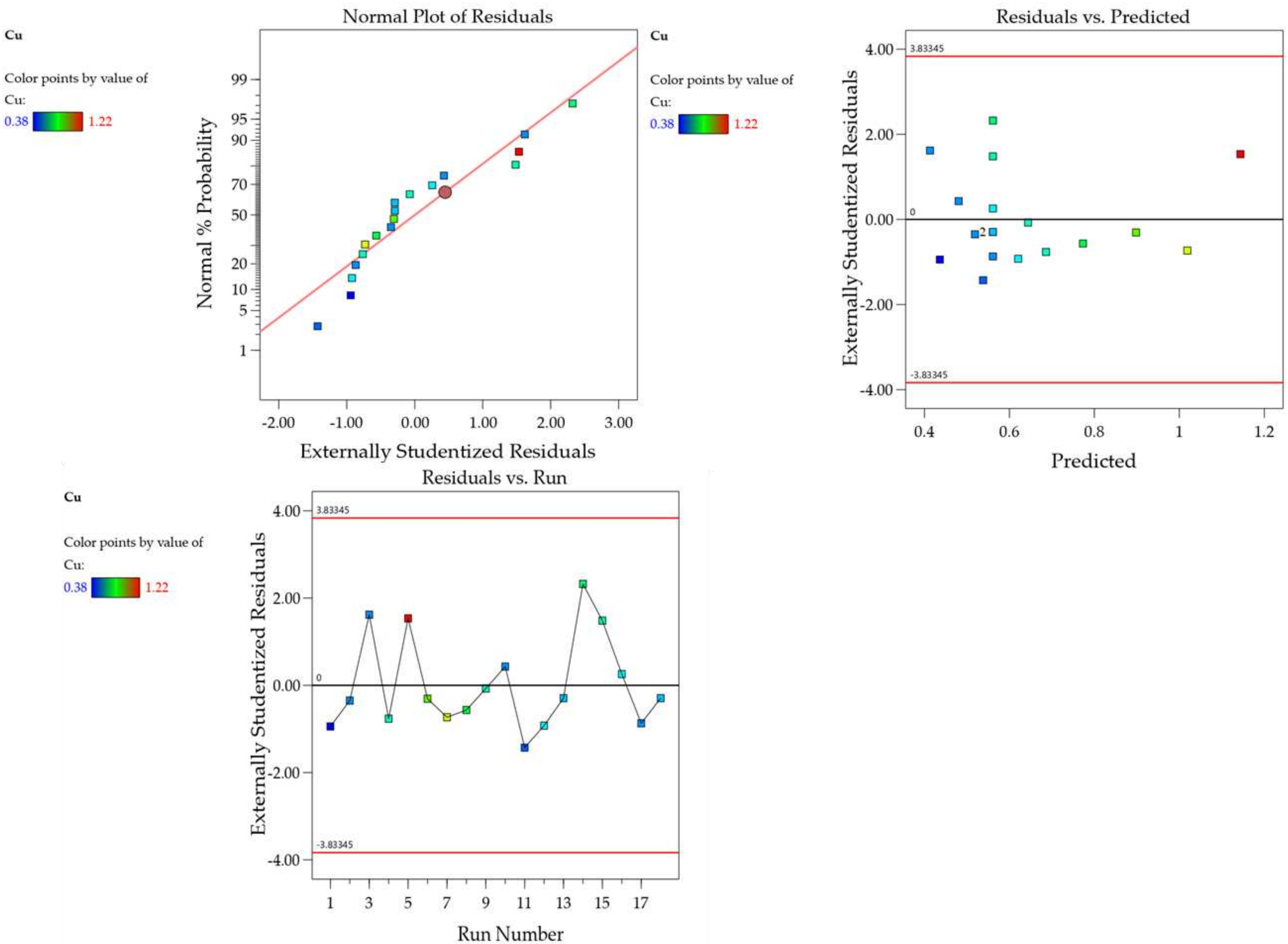


Fig. S7: Residual plots for Cu analysis

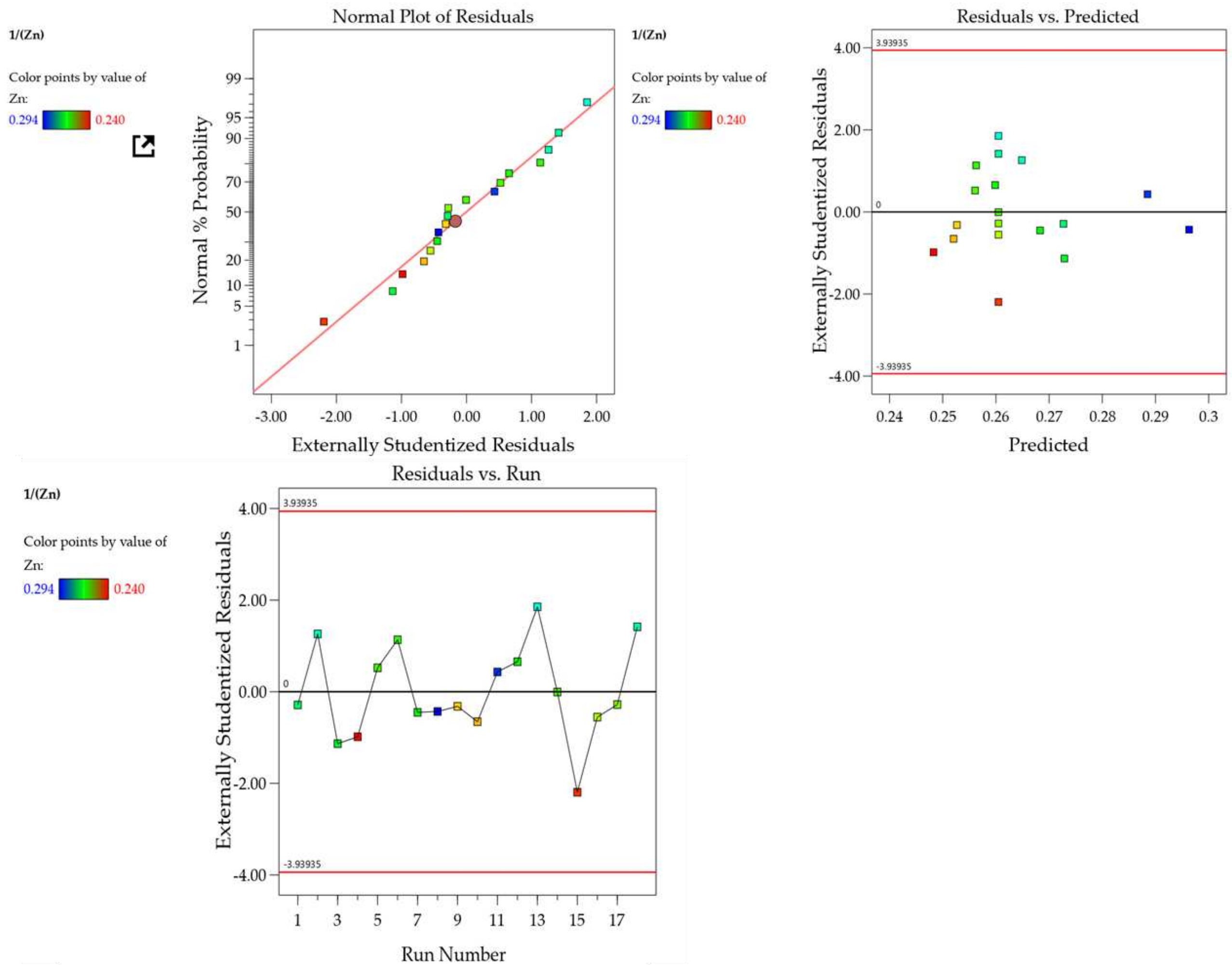


Fig. S8: Residual plots for Zn analysis

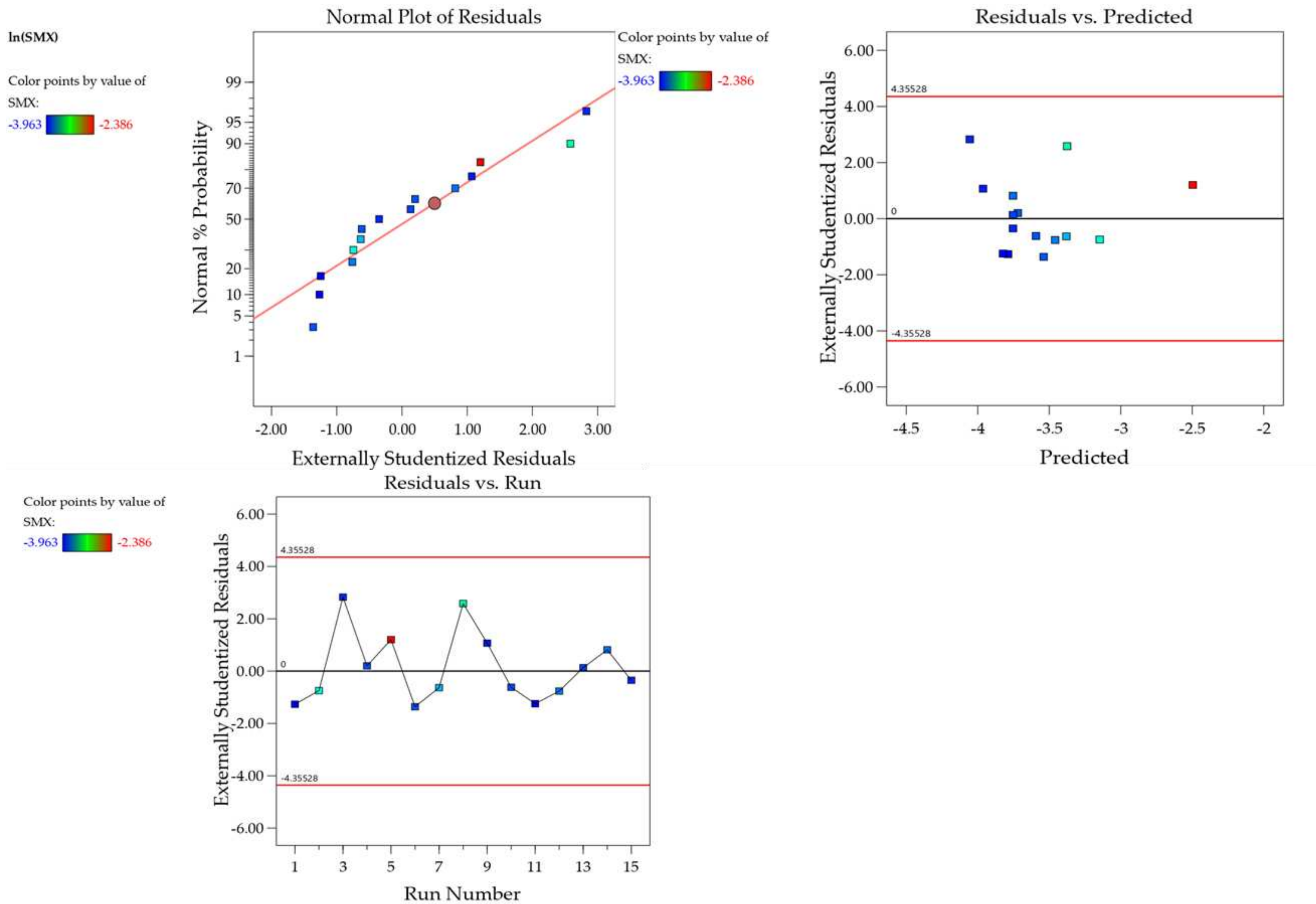


Fig. S9: Residual plots for SMX analysis

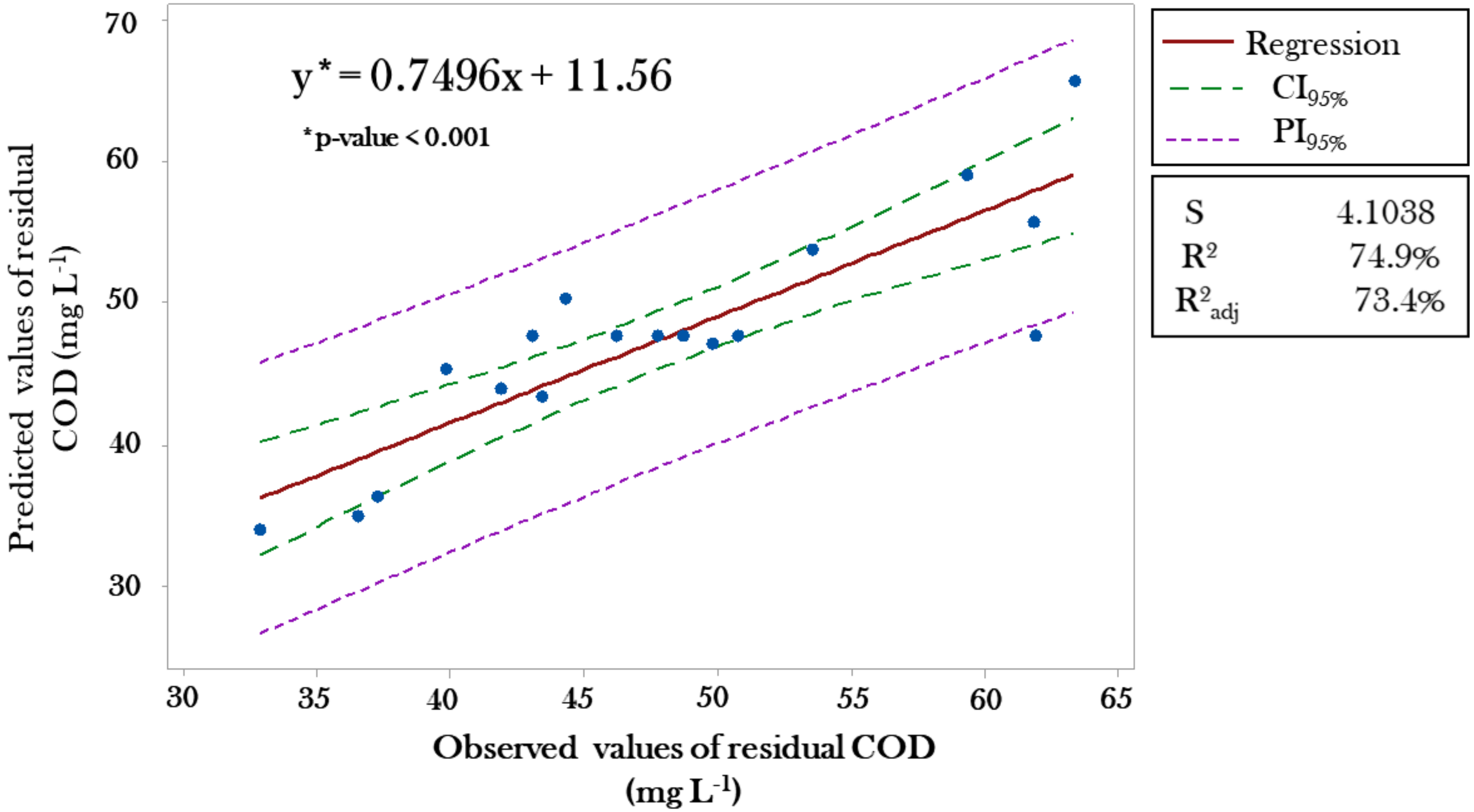


Fig. S10: Predicted values versus observed values for the residual concentration of COD

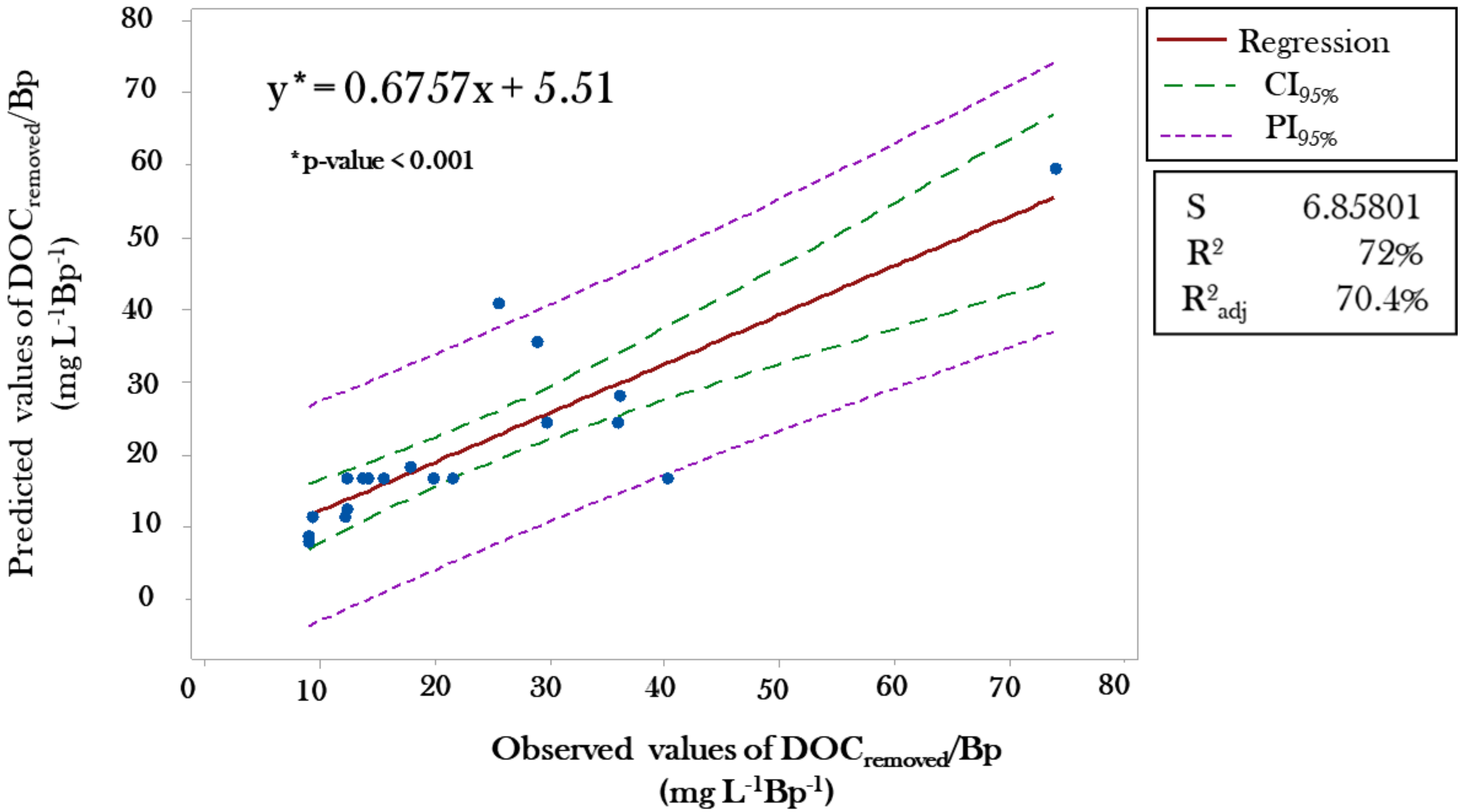


Fig. S11: Predicted values versus observed values of DOC removed per unit of biomass produced

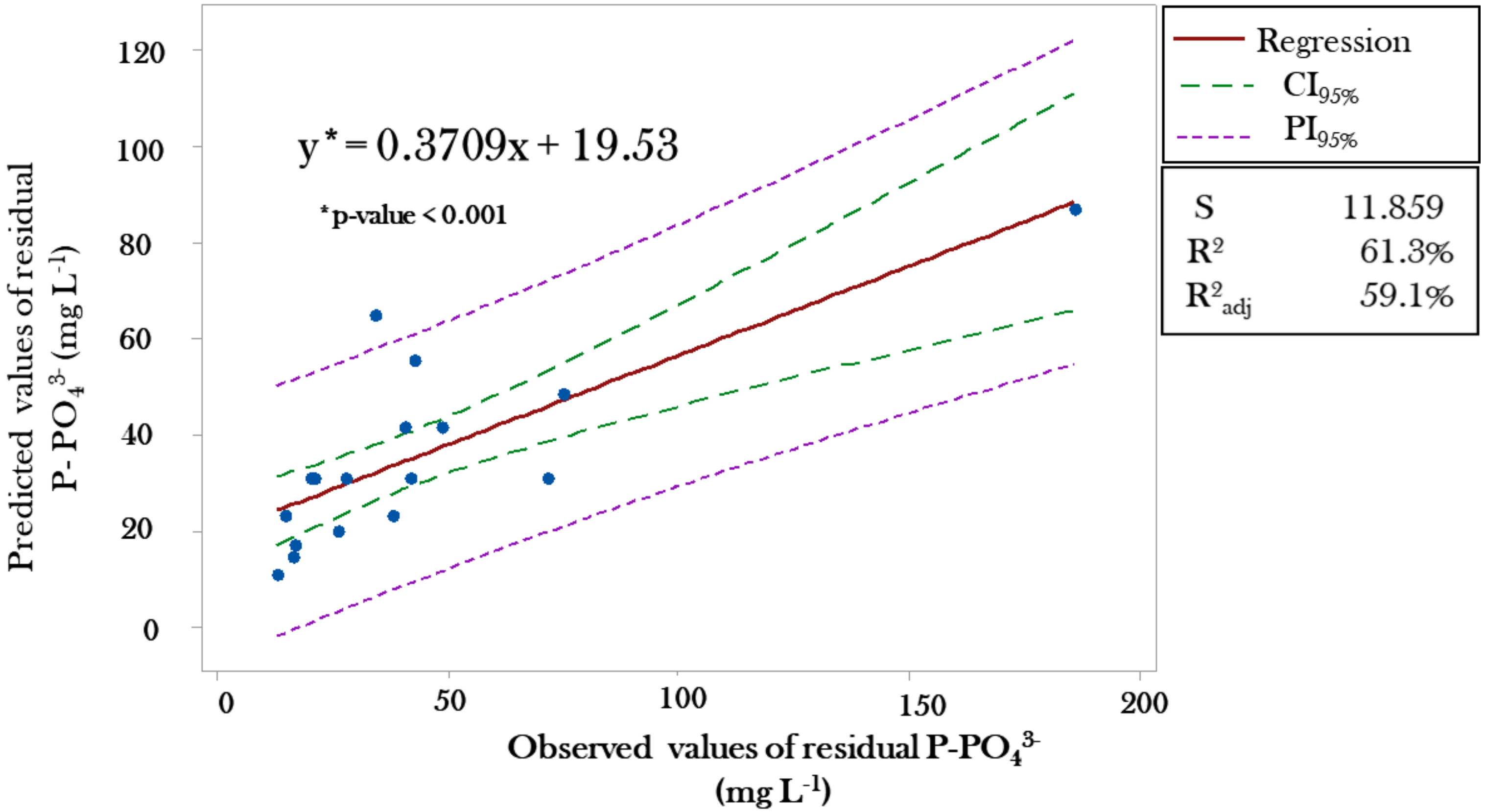


Fig. S12: Predicted values versus observed values of P-PO_4^{3-} per unit of biomass produced

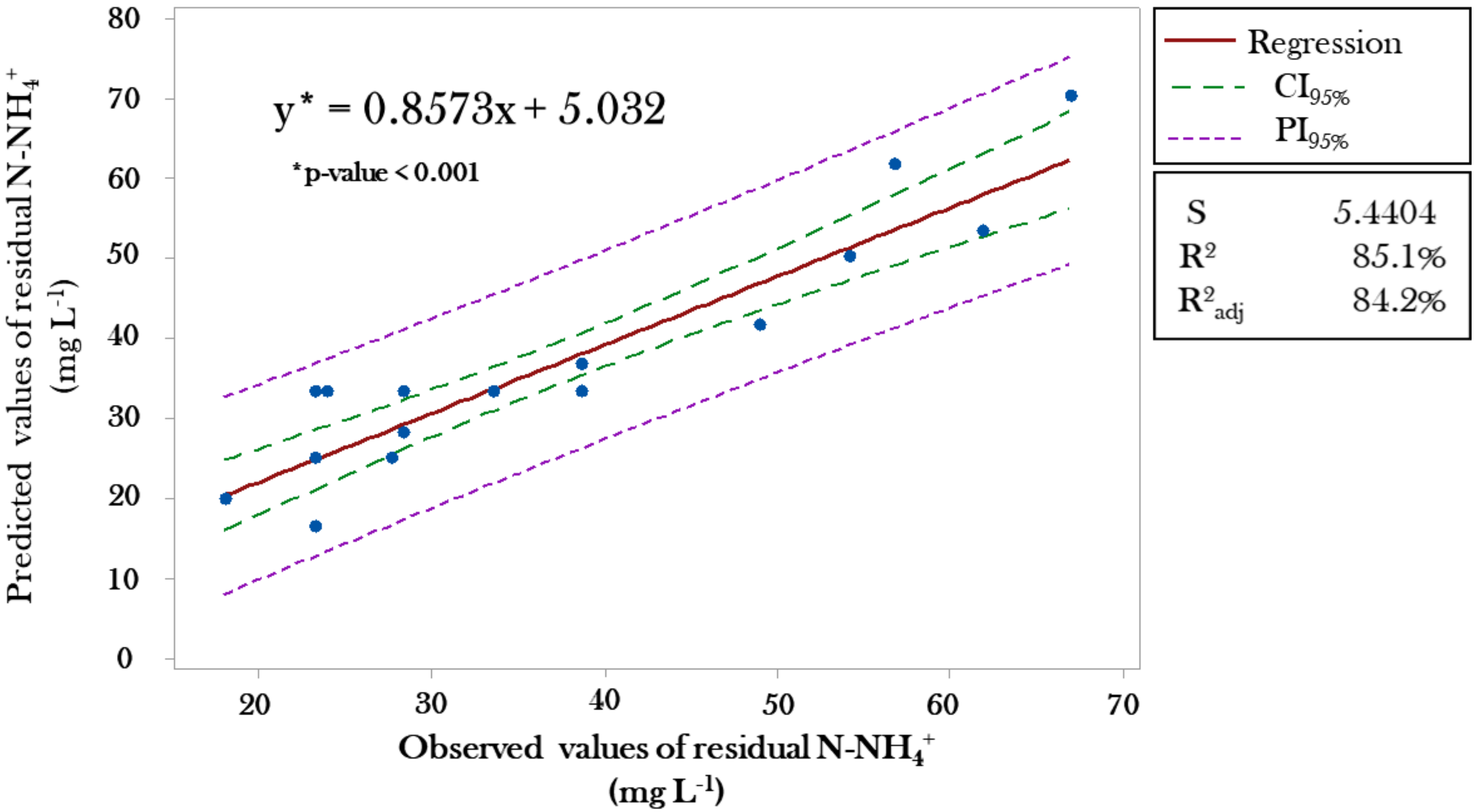


Fig. S13: Predicted values versus observed values for the residual concentration of $N\text{-}NH_4^+$

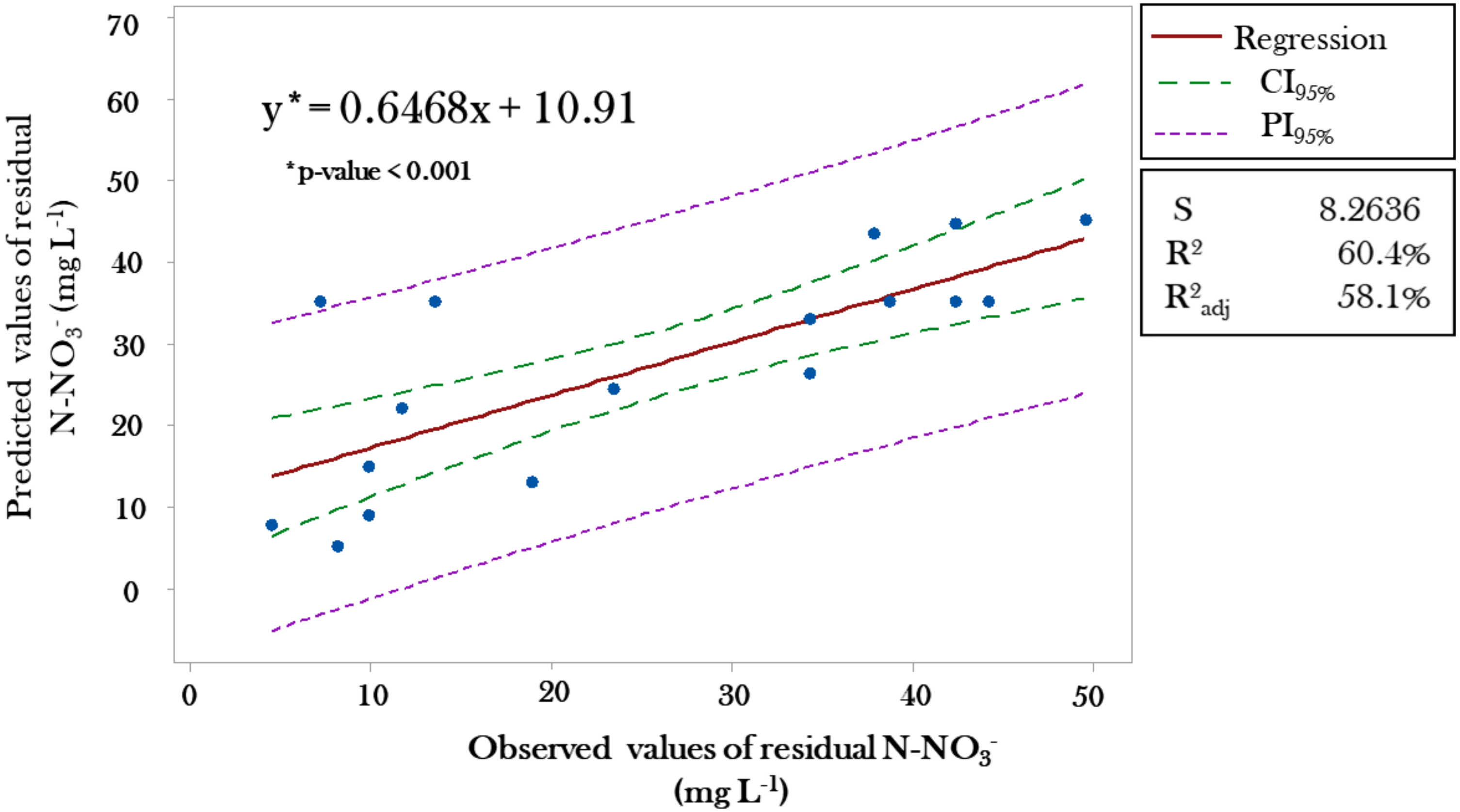


Fig. S14: Predicted values versus observed values for the residual concentration of $N\text{-}NO_3^-$

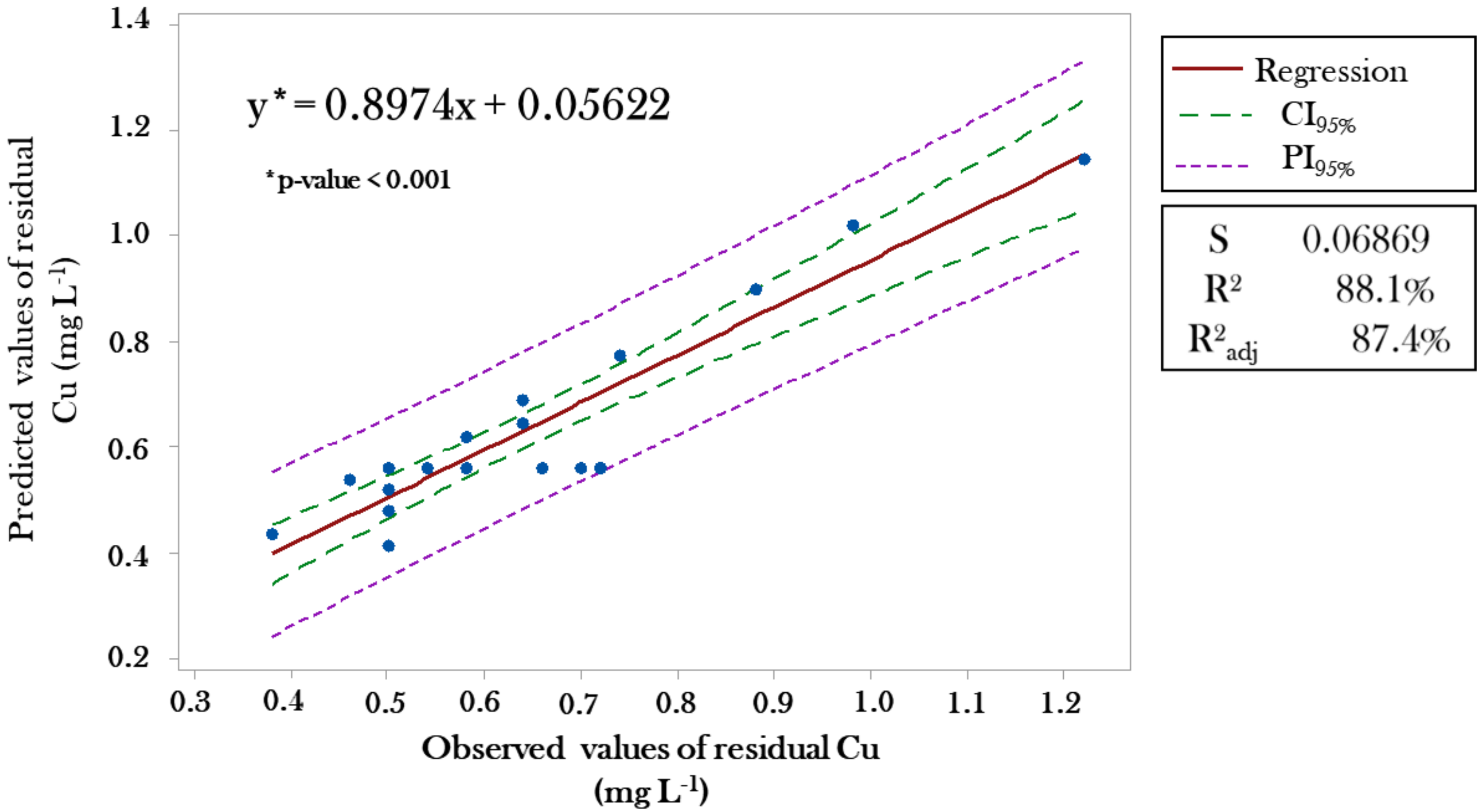


Fig. S15: Predicted values versus observed values for the residual concentration of Cu

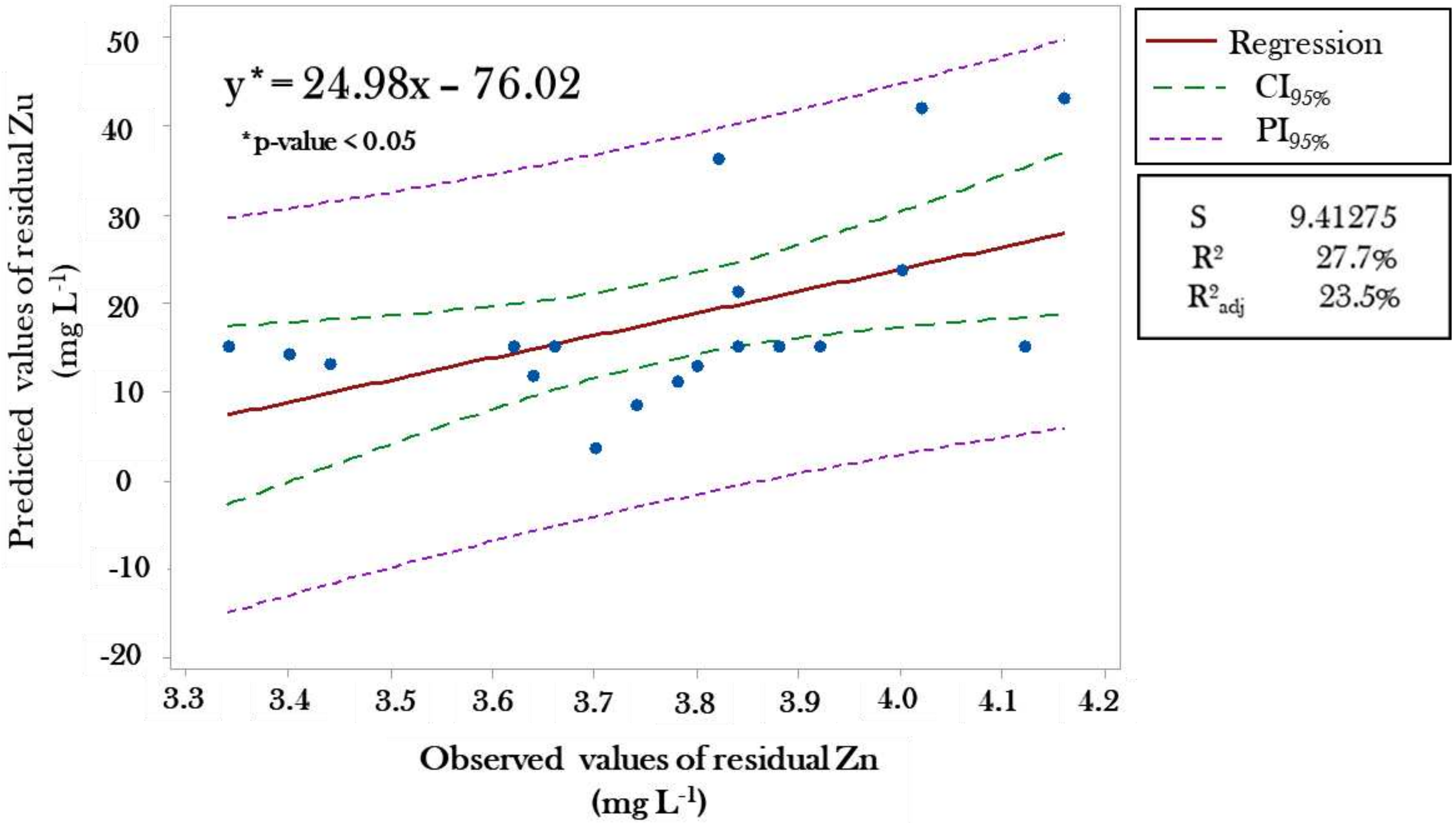


Fig. S16: Predicted values versus observed values for the residual concentration of Zn

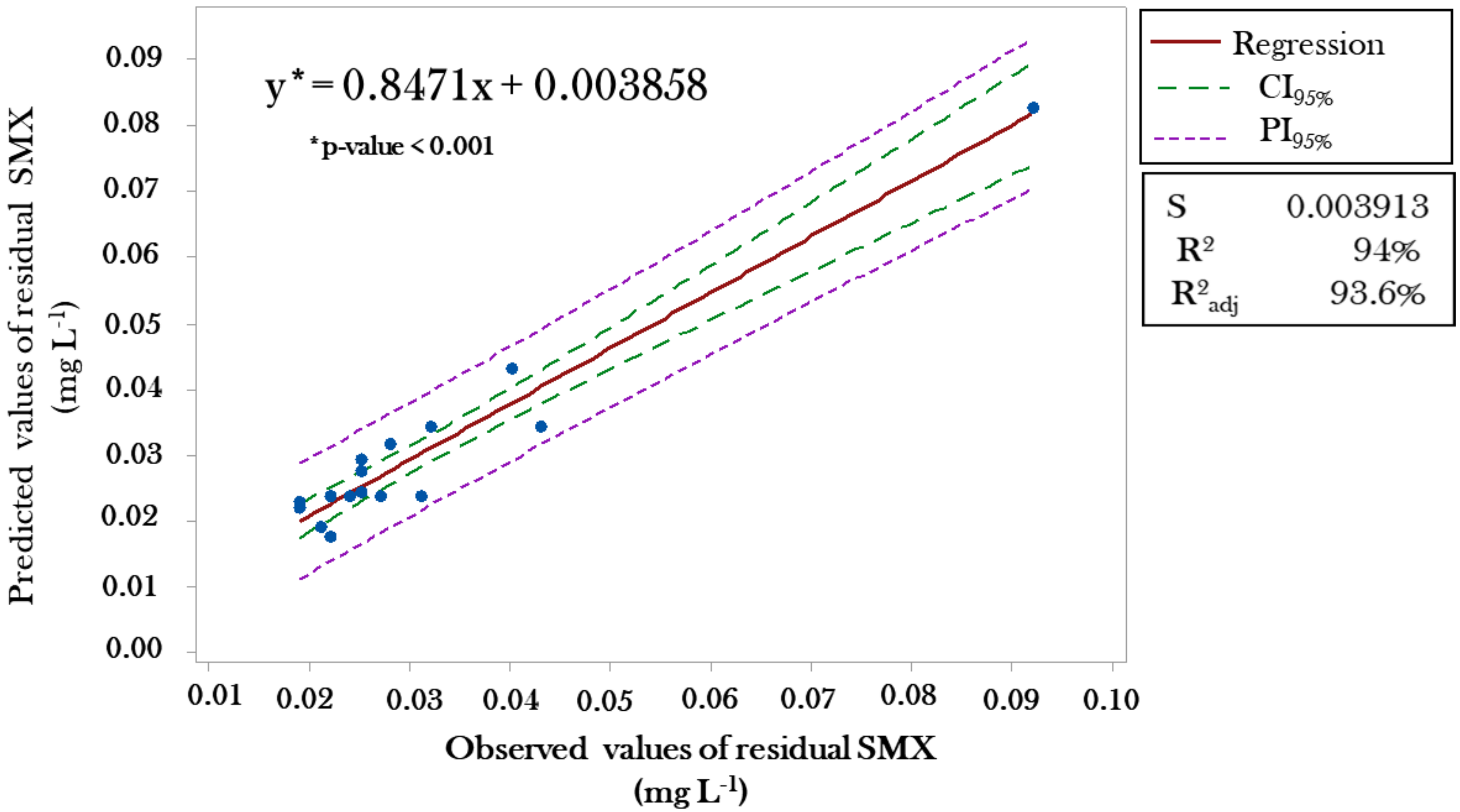


Fig. S17: Predicted values versus observed values for the residual concentration of SMX