University of KwaZulu-Natal

Harnessing the Power of Microalgae and Daphnia for Bioremediation of Nutrients, Pharmaceuticals, and Heavy Metals in Wastewater

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A thesis submitted to the School of Chemistry and Physics, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, for the degree of [Doctor of Philosophy in Chemistry].

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Format 2: As a set of papers which are published, in press, submitted, or intended for submission.

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Dr Precious N. Mahlambi

Abstract

Excess nutrients in aquatic ecosystems promote eutrophication, which significantly affects oxygen-dependent organisms. Furthermore, toxic microalgae, such as microcystin, cylindrospermopsis, etc., thrive during eutrophication, releasing poisonous compounds harmful to human health and other aquatic organisms. Pharmaceutical compounds and heavy metals in aquatic environments further exacerbate these global concerns. Thus, addressing such problems is paramount and aligns with Sustainable Development Goals (SDGs) 6 - Clean Water and Sanitation, 12 - Responsible Consumption and Production, and 14 - Life Below Water. Bioremediation of wastewater with biological microorganisms such as microalgae and daphnia provides an excellent solution due to their remarkable properties that enable them to efficiently eliminate many contaminants, including heavy metals, nutrients, and pharmaceuticals. So, this study explored the novel approach to nutrient removal by combining *Chlorella* spp. and *Daphnia magna* (*D. magna*). *Chlorella* spp. completely removed nitrate and nitrite from wastewater by converting them into compounds like amino acids, proteins, and lipids. When *D. magna* was employed alone, it faced significant challenges due to the absence of primary producers like bacteria and microalgae, which they mainly feed on. However, combining it with *Chlorella* spp. proved exceptionally effective, as 100% removal was obtained for nitrate and nitrite, possibly driven by *D. magna* grazing on *Chlorella* spp. that had assimilated nitrate and nitrite into their biomass. Challenges arose for ammonia and phosphate removal as they achieved up to 27% removals. This can be ascribed to nutrient release, *Chlorella*'s saturation capacity, and environmental changes such as pH.

For pharmaceuticals, the study successfully developed and validated the LC-PDA method for separating sulfamethoxazole, nevirapine, and efavirenz. The optimum conditions were a mobile phase (90:10% acetonitrile: water with 0.1% formic acid), run time (8 minutes), flow rate (0.4 mL/min), and wavelength (220 nm). The study also developed and validated the solid-phase extraction (SPE) method for extracting analytes of interest in water matrices. The optimum conditions were conditioning solvent (mixture of acetonitrile and methanol in a 70:30 (v/v) ratio), sample volume (50 mL), and pH 7. The study then assessed *Chlorella*'s capacity to remove sulfamethoxazole, nevirapine, and efavirenz. The obtained removal efficiencies for efavirenz, nevirapine, and sulfamethoxazole ranged from 0–60%, 5–51%, and 10–50%, respectively. Furthermore, the results exhibited lower removal efficiency (up to 15%) for higher concentrations (1 and 5 mg/L), whereas lower initial concentrations (0.5 and 0.25 mg/L)

showed higher removal rates (up to 60%). Low removals could be due to factors like toxic metabolite accumulation and pharmaceutical toxicity. This study also explored copper, lead, and zinc adsorption capacity on *Chlorella* spp. biomass. Batch cultures were assessed in triplicate at 150 rpm in an orbital shaker under different biomass dosages, pH levels, contact times, and metal concentrations. The optimum conditions were pH 7, 60 minutes contact time, biomass dosage of 12.5 mg, and 0.5 m/L concentration. The optimal conditions yielded complete removal of lead and zinc, with copper reaching up to 80% removal. The study also assessed the adsorption of target heavy metals employing Freundlich, Langmuir, and Temkin isotherms, with the Langmuir isotherm better fitting copper (R^2 = 0.9888) while the Freundlich isotherm best-fitted lead (R^2 = 0.976) and zinc (R^2 = 0.968). Lead and zinc favoured the pseudo-first-order kinetic model, whereas copper favoured the pseudo-second-order kinetic model. Thermodynamic studies exhibited an endothermic and spontaneous process for copper and zinc.

The results of this PhD underscored *Chlorella*'s potential as an environmentally safe and effective option for removing nutrients, pharmaceutical compounds, and heavy metals. Mechanisms for removal included surface adsorption, photodegradation, bioaccumulation, and enzymatic degradation. The Fourier transform infrared spectroscopy (FTIR) confirmed the existence of functional groups like alkene, amide, carbonyl, carboxyl, ethers, hydroxyl, and methyl, which participate in the adsorption of these contaminants through various interactions. Surface morphology analysis through scanning electron microscopy (SEM) shows changes in *Chlorella* spp. cells after exposure to target compounds (nutrients, pharmaceutical compounds, and heavy metals), suggesting the possibility of interaction that aids their removals. Thus, this study contributed valuable insights for improving wastewater treatment strategies and addressing water scarcity concerns. Additionally, it promotes a circular economy as *Chlorella* spp. and daphnia biomass can be harvested at the end of the treatment process for diverse uses, including biogas production, organic fertiliser, animal feed, etc. Going forward, future research should focus on optimising bioremediation by exploring different combinations of microalgae and other biological agents to enhance the removal efficiencies of heavy metals and pharmaceuticals. Moreover, genetic modification of *Chlorella* spp. to improve resilience and uptake capacity is crucial, and integrating advanced monitoring technologies like biosensors are promising directions. In-depth studies on removal mechanisms, such as adsorption, photodegradation, bioaccumulation, and enzymatic degradation, are essential. Also, scaling up to pilot and full-scale applications is crucial for evaluating feasibility and economic viability.

Lastly, collaboration with industrial partners and policymakers can help develop regulatory frameworks and incentives. These efforts can advance bioremediation and support global SDGs related to clean water, responsible production, and life below water.

Abbreviations

ACN	Acetonitrile
df	Degrees of Freedom
DO	Dissolved oxygen
F	F-statistic
HLB	Hydrophilic-lipophilic base
HPLC	High-pressure liquid chromatography
ICP-OES	Inductively coupled plasma – optical emission spectroscopy
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MS	Mass spectroscopy
MS	Mean Square
PDA	Photodiode array
SPE	Solid phase extraction
SS	Sum of Squares
TDS	Total dissolved solids
UKZN	University of KwaZulu – Natal
UV-Vis	Ultraviolet-visible
WHO	World Health Organization
WWTPs	Wastewater Treatment Plants
%RSD	Percentage relative standard deviation

Declarations

Declaration 1 – Plagiarism

I, **Ntombiphumile Perceverence Tenza** declare that

1. The research reported in this thesis is my original research, except where otherwise indicated.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
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    b. where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References section/s.

Signed ……

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Declaration 2-Publications

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Details of publications:

Paper 1: Pharmaceutical bioremediation in water resources: A review. Submitted to Journal of Environmental Sciences and Engineering

Paper 2: Microalgae and Daphnia as Potential Removers of Nutrients in Wastewater Samples from South Africa. Formatted for submission to the Journal of Environmental Management

Paper 3: An Innovative Approach for Bioremediation of Antibiotics and Antiretroviral Drugs Contaminants in Wastewater using *Chlorella* spp. Formatted for submission to Science of Total Environment

Paper 4: A Green Revolution in Heavy Metal Removal. Formatted for submission to the Journal of Hazardous Materials

In the aforementioned publications, I conducted all experimental work and collaborated with my supervisor (Dr PN Mahlambi) in the writing process.

Signed: ……

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No achiever stands alone; they ascend on the wings of collective support, soaring to new heights fueled by the strength of unity – Bilawar.

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Chapter 1

1.1 Introduction

This chapter covers an introduction, delineates the challenges that prompted this research topic's focus, and outlines the specific objectives pursued to achieve the research's overall aim.

1.2 Background / Importance of topic:

Emerging pollutants like pharmaceutical compounds from illegal disposal of medications, improperly treated wastewater, and industrial discharges have become a pressing challenge in many water environments (Horn et al. 2022). Their presence causes many detrimental effects on human health and aquatic life and a cascading impact on aquatic environments. The proliferation of nutrients like nitrogen and phosphorous stemming primarily from agricultural runoff and untreated sewage exacerbates aquatic environment effects as they cause the degradation of aquatic ecosystems through detrimental phenomena like eutrophication and harmful algal blooms (Devlin and Brodie 2023). In addition to pharmaceuticals and excess nutrients, heavy metals further worsen water problems. Heavy metals such as copper, lead, and zinc originate from natural sources that include geological formations. However, human activities such as incorrect electronic waste disposal, industrial processes, mining operations, and urbanization contribute significantly to their release into the water environment. The release of these compounds can result in their accumulation in aquatic environments and pose significant environmental threats due to toxicity. This shows an urgent need to develop innovative and sustainable strategies that efficiently remove heavy metals, excessive nutrients, and pharmaceuticals in water environments. Bioremediation using microorganisms like microalgae and daphnia has gained much attention from the vast array of remediation approaches as they present a promising and environmentally friendly method for tackling these concerns. Microalgae, single-celled photosynthetic organisms, possess remarkable metabolic capabilities that efficiently eliminate various organic pollutants and nutrients from wastewater (Shah and Shah 2020, Sepúlveda-Muñoz et al. 2023). Such contaminants are removed through bio-adsorption, bioaccumulation, and biotransformation, which ultimately controls pollution and promotes resource recovery (Maryjoseph and Katheesan 2020, Mustafa et al. 2021, Reddy et al. 2021). The ability of microalgae to thrive in altered environmental conditions and cohabit with other wastewater processes renders them great candidates for integration into already existing treatment systems (Varshney et al. 2015).

Similarly, Daphnia, a small filter-feeder freshwater zooplankton genus, has shown promise to contribute to the intertwined challenges of nutrients and pharmaceuticals in wastewater (Shiny et al. 2005, Mahaye and Musee 2022). These small microorganisms possess extraordinary biological features that enable them to be ideal organisms for sustainable bioremediation approaches. Such features include their feeding behaviour, rapid growth, and reproduction rate, which help prevent excess nutrient concentrations (Stevčić et al. 2020, Tanjung et al. 2020). Moreover, their ability to bioaccumulate and metabolize organic compounds like pharmaceuticals can significantly assist in breaking down and bio-transforming these compounds to a less toxic form, reducing their effects (Abdullahi et al. 2022).

Non-steroidal anti-inflammatory drugs (NSAIDs), antiretroviral drugs (ARVDs), and antibiotics are the most prevalent pharmaceuticals frequently detected in many wastewater treatment plants (Abafe et al. 2018, Mtolo et al. 2019, Hlengwa and Mahlambi 2020, Madikizela et al. 2020, Ngumba et al. 2020, Adeola and Forbes 2021, Horn et al. 2022). This can be ascribed to the inability of traditional treatment processes to efficiently remove such compounds, which leads to the contamination of receiving freshwater bodies (Horn et al. 2022). Thus, tapping into bioremediation through conducting a comprehensive study focusing on the utilization of microalgae and daphnia to eliminate heavy metals, nutrients, and pharmaceuticals is of utmost importance. This is more important in developing countries like South Africa, which faces immense water quality and quantity challenges. Such research endeavours could shed light on the optimal conditions and mechanisms these organisms use to sequester and degrade contaminants effectively.

Furthermore, obtaining more insights into the fate of pharmaceutical compounds within microalgae and daphnia cells and their subsequent transformations could enable the development of efficient, innovative, and sustainable wastewater treatment approaches. Such studies could also mitigate the pervasive issue of nutrient-associated risks in aquatic environments. It is imperative to note that such studies can revolutionize the design of future wastewater treatment processes, safeguard aquatic environments, and, most importantly, protect aquatic organisms and human health, and usher in a greener and more sustainable environment for the present and future generations.

1.3 Scope of Research

This research project was aimed to assess innovative, and sustainable wastewater treatment methodologies employing *Chlorella* spp. and *Daphnia magna* to effectively remove nutrients (nitrogen and phosphorus), heavy metals (copper, lead, and zinc) and pharmaceuticals (ARVs, NSAIDs, and antibiotics) from South African wastewater. The inadequacy of conventional treatment processes in addressing these persistent pollutants underscores the urgent need for novel solutions as they pose environmental and health risks. Implementing these novel methodologies not only addresses the shortcomings of conventional treatment processes but also offers a sustainable approach to mitigate environmental and health risks associated with persistent pollutants in wastewater.

1.4 Justification of the Study

Excess nutrients and heavy metals in water environments have emerged as a pressing and multifaceted environmental concern with far-reaching consequences for humans and aquatic organisms. Moreover, the production and consumption of pharmaceutical drugs are increasing alarmingly, worsening ecological problems worldwide. Several factors can be attributed to the overall increase and the consumption of pharmaceuticals, including improved access to healthcare services and over-the-counter medication, particularly in developing countries. Advancements in medical science also play a huge role in this upsurge as it results in drugs that treat numerous conditions that were untreatable. Improper disposal of expired pharmaceuticals by hospitals and industries, as well as extensive use in the agriculture sector (such as antibiotics in animal farming) and veterinary medicine, also contribute to the overall load of pharmaceutical compounds in aquatic environments. Wastewater treatment plants also play a huge role in pharmaceutical pollution in aquatic environments, as most conventional treatment processes cannot effectively eliminate pharmaceutical compounds from sewage. As a result, treated effluents released into water bodies may still contain residual pharmaceuticals.

In South Africa, antiretroviral drugs (ARDVs), non-steroidal anti-inflammatory drugs (NSAIDs), and antibiotics have been detected in many final wastewater effluents (Abafe et al. 2018, Mtolo et al. 2019, Hlengwa and Mahlambi 2020, Madikizela et al. 2020, Ncube et al. 2021, Horn et al. 2022). Most of the studies conducted thus far focus primarily on the prevalence of these compounds with little focus on their removal. Hence, it is pivotal to delve into strategies that could revolutionize wastewater treatment, offering a novel, sustainable, and

environmentally conscious approach to address the dual challenge. Thus far, no studies have evaluated the removal of the selected group of pharmaceuticals (ARVDs, NSAIDs, and antibiotics), nutrients (nitrogen and phosphorus), and heavy metals (copper, lead, and zinc) in South African wastewater using microalgae and daphnia species; hence, this project was proposed. The proposed treatment process could profoundly affect South Africa's potential to achieve Sustainable Development Goals (SDG), particularly SDGs 6 (clean water and sanitation), 12 (ensure sustainable consumption and production patterns), and 14 (life below water), by 2030. Furthermore, this research project advocates for the advancement of a circular bio-economy. This is because, after the separation of clean water and biological agents, microalgae biomass and daphnia carcasses can be harvested for various uses. These include methane gas production, liquid fuel, various high-value chemicals, fertilizers, alcohol production (ethanol), animal feed supplements, etc. (Encarnação et al. 2020, Giwa et al. 2022).

1.5 Aim

The study aimed to assess the bioremediation potential of *Chlorella* spp. and *Daphnia magna* in the context of removing commonly detected heavy metals, nutrients, and pharmaceuticals in South African wastewater.

1.6 Objectives

To reach the aim, the following objectives were followed:

1. Explored and synthesized existing literature, identified gaps, and justified further studies to enhance pollutant removal systems using *Chlorella* spp. and *D. magna* for nutrients, pharmaceuticals, and heavy metals in water.
2. Developed and validated the liquid chromatography-mass spectrometry (LC-MS) methods for separating ARVDs, and antibiotics in water samples.
3. Developed and validated solid phase extraction method for extracting antibiotics, ARVDs, and antibiotics in South African water resources.
4. Examined the influence of varied light conditions on the growth rates and biomass accumulation of *Chlorella* spp.
5. Assessed the impact of different *Chlorella* spp. culture concentrations on growth dynamics and nutrient utilization.
6. Evaluated the ability of *Chlorella* spp. microalgae to remove nitrates, nitrites, ammonia and phosphate from wastewater.

7. Evaluated the ability of *Daphnia magna* to completely remove nitrates, nitrites, ammonia, and phosphate from wastewater.
8. Investigated the synergy of *Chlorella* spp. microalgae and *Daphnia magna* remove to excess nutrients, particularly nitrogen and phosphorus, from wastewater.
9. Investigated the effectiveness of *Chlorella* spp. microalgae in removing antibiotics (sulfamethoxazole) and antiretroviral drugs (efavirenz and nevirapine) from water
10. Characterized dried *Chlorella* spp. biomass for its adsorption capacity towards heavy metals such as copper, lead, and zinc
11. Optimize the process parameters, including pH levels (3–11), biomass dosage (5–100 mg), contact times (15–110 minutes), and metal concentrations (0,25–16 mg/L) to enhance the adsorption efficiency of *Chlorella* spp. biomass towards heavy metals
12. Investigated copper, lead, and zinc adsorption kinetics onto *Chlorella* spp. biomass using the Pseudo-first-order model, Pseudo-second-order model, and Intraparticle diffusion model.
13. Evaluated the adsorption isotherms (Freundlich, Langmuir, and Temkin) to determine the adsorption mechanisms and capacities of copper, lead, and zinc on *Chlorella* spp. Biomass.
14. Assessed the thermodynamic parameters (ΔG°, ΔH°, and ΔS°) to understand the spontaneity, heat of adsorption, and entropy changes during the adsorption process of heavy metals by *Chlorella* spp. biomass.

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Chapter 2 Bioremediation of pharmaceutical, heavy metals, and nutrients in water resources: A review

Abstract

The contamination of water sources by pharmaceuticals, heavy metals, and excess nutrients is a pressing global environmental concern, presenting substantial risks to aquatic ecosystems and human health. Therefore, this review examined the potential of biological organisms (microalgae, fungi, bacteria, and aquatic plants) as effective solutions for remedying pharmaceuticals, heavy metals, and nutrients in contaminated water sources. Microalgae species such as *Chlorella*, demonstrated adaptability and diverse metabolic capabilities, positioning them as efficient alternatives for wastewater treatment, albeit with challenges with toxicity, land use and energy-intensive harvesting process. Fungi, such as *Phanerochaete chrysosporium* and *Trametes versicolor*, exhibited potent enzymatic abilities for mycoremediation but require further research to optimize operational complexities. Bacteria species like *Flavobacterium, Pseudomonas,* and *Bacillus* play pivotal roles in bioremediation, showing robust capabilities in degrading pollutants and necessitating exploration of biotransformation pathways for enhanced efficacy. Aquatic plants, including *Pistia stratiotes* and *Eichhornia crassipes*, demonstrated promising capabilities in removing contaminants, though challenges like invasive species management and biomass disposal optimization remain. Overall, leveraging these biological organisms offers versatile and effective strategies for addressing complex water contamination challenges, underscoring the need for continued research and technological advancements to ensure scalable, sustainable, and globally applicable remediation methods.

Keywords: Bioremediation, Heavy Metals, Nutrients, Pharmaceuticals

2.1 Introduction

Water contamination by pharmaceuticals, heavy metals, and excess nutrients has become a significant global environmental issue. These pollutants pose serious risks to both aquatic ecosystems and human health. Conventional treatment methods, such as chemical precipitation, ion exchange, membrane filtration, and many others, often fall short of achieving efficient removal and can be expensive, energy-intensive, and generate secondary pollutants. In contrast, bioremediation offers a promising alternative. This approach leverages

microorganisms, including aquatic plants, bacteria, fungi, and microalgae, to degrade or sequester contaminants, providing sustainable solutions for enhancing water quality. So, this review aimed to synthesize current research on bioremediation using aquatic plants, bacteria, fungi, and microalgae to address pharmaceuticals, heavy metals, and excess nutrients in water environments. To reach the aim, the following objectives were achieved: (1) assessed the sources and pathways of pharmaceuticals, heavy metals, and excess nutrients in water environments, (2) investigated the underlying mechanisms and pathways involved in bioremediation processes, (3) compared the performance of different bioremediation strategies, such as phycoremediation, mycoremediation, and phytoremediation, in removing pharmaceuticals, heavy metals, and nutrients, identify gaps in current research, and (4) provided actionable recommendations to researchers and stakeholders on improving bioremediation techniques, optimizing treatment processes, and addressing emerging contaminants in water environments.

2.1.1 Pharmaceuticals in water environments

Pharmaceuticals are among the emerging contaminants that have an adverse effect on water resources. They are mainly used to prevent, treat, and cure various diseases and thus improve health. Many pharmaceutical compounds have been detected in water resources. Their occurrence is attributed to wastewater effluents, poor disposal mechanisms at domestic sites and hospitals, aquaculture facilities, and animal farming activities (Figure 2.1) (Encarnação et al. 2020, Mamta et al. 2020). Also, poor sanitation services can be attributed to pharmaceutical contamination because faecal matter leaches to nearby water resources during rainy seasons (Ngqwala and Muchesa 2020). The pharmaceutical compounds are well documented for being present in various water resources such as groundwater, lentic as well as lotic water bodies, and their presence raises public health concerns (Ebele et al. 2017, Hu et al. 2017, Mamta et al. 2020, Montesdeoca-Esponda et al. 2021, Patel et al. 2019).

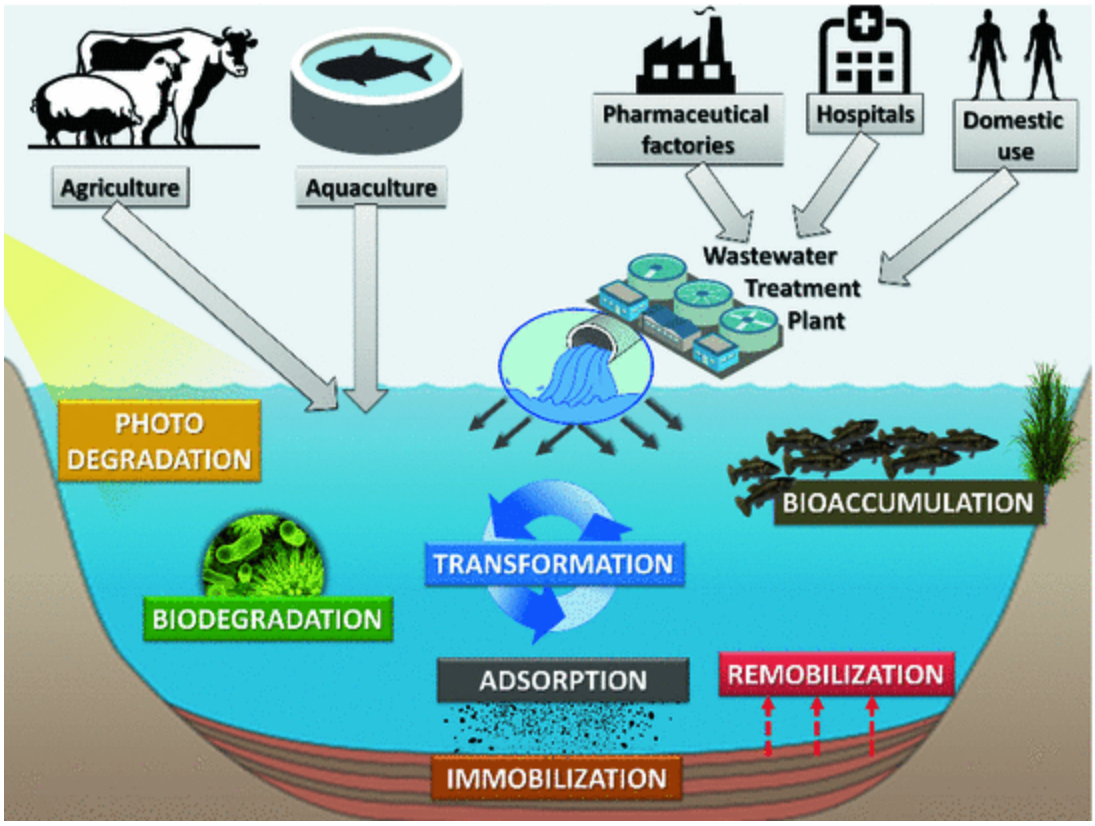

Figure 2.1 Sources of pharmaceutical contaminants in aquatic environments (Reis 2022)

Pharmaceutical compounds have high polarity and bioaccumulation tendency; furthermore, they are persistent to biodegradation which causes serious threats to many aquatic invertebrates and vertebrates (Fernandes et al. 2021, Maryjoseph and Katheesan 2020, Shah and Shah 2020). Rivera-Jaimes et al. (2018) documented that NSAIDS like naproxen, carbamazepine, bezafibrate, and acetaminophen cause a toxic effect on daphnia species while trimethoprim, indomethacin, and sulfamethoxazole negatively affect microalgal species. According to studies conducted by Fernandes et al. (2021), mianserin, which belongs to the tetracyclic antidepressant group, can change the physiological and biochemical parameters of *Danio rerio* (zebrafish larvae), which inhibit their growth. A study by Guiloski et al. (2017) projected that exposure to paracetamol caused genotoxicity, oxidative stress, and significant changes in the steroid hormones of *Rhamdia quelen* (catfish). Elsewhere, Maryjoseph and Katheesan (2020) documented those steroid pharmaceuticals can act as endocrine disruptors, resulting in feminisation and reproductive disruption in fishes. Furthermore, the frequent use of antibiotics causes antibiotic-resistant bacteria and antibiotic-resistance genes in water resources (Ngqwala and Muchesa 2020). This is problematic in clinical therapy as antimicrobial resistance reduces

the effectiveness of antibiotics, leading to therapeutic failure, higher mortality rates, and increased health costs (Ngqwala and Muchesa 2020).

Pharmaceutical global consumption and personal care products (PPCPs) amount to approximately 10,000 tons annually (Maryjoseph and Katheesan 2020). This high consumption rate is attributed to the fast-growing global population and increasing demand for healthier lifestyles, thereby enhancing average life expectancy. Between 70% to 90% of pharmaceutical compounds pass through the human system without full metabolization, eventually being excreted (unchanged or partially metabolized) via faeces and/or urine (Encarnação et al. 2020, Ngqwala and Muchesa 2020). Consequently, excreted pharmaceuticals enter wastewater treatment plants (WWTPs), which are not typically equipped to efficiently remove such compounds, thereby contaminating surface waters, groundwater, and treated drinking water. In this context, several studies have reported significant findings. For instance, Mtolo et al. (2019) identified efavirenz, an antiretroviral drug, in wastewater effluents in Durban, KwaZulu-Natal, with concentrations ranging from 2.79 to 93.1 µg/L. Similarly, Ncube et al. (2021) detected antibiotics in the Klip River in Gauteng Province, South Africa, with concentrations ranging from 0.0618 to 0.133 µg/L. Antibiotic concentrations have also been documented in other African countries such as Kenya (0.00008–0.049562µg/L), Ghana (0.00011–0.00964 µg/L), and Mozambique (0.0008–0.053828 µg/L), comparable to levels observed in South Africa, where Segura et al. (2015) reported concentrations of 0.00038–0.010568 µg/L in surface waters. NSAIDs like diclofenac, ibuprofen, and naproxen have been quantified in wastewater and river waters of KwaZulu-Natal Province, South Africa, showing concentration ranges of 0.6–221 µg/L (Madikizela and Chimuka, 2017a; Madikizela et al. 2017a). Additionally, Hlengwa and Mahlambi (2020) detected NSAIDs, including carbamazepine, diclofenac, fenoprofen, ibuprofen, and naproxen in KwaZulu-Natal wastewater (influent and effluent) and river waters, with concentrations ranging from 0.00899 to 0.0669 µg/L.

In this regard, Mtolo et al. (2019) reported efavirenz, an antiretroviral drug in 4 wastewater effluents in Durban, KwaZulu-Natal, with concentrations ranging from 2.79–93.1 µg/L. Ncube et al. (2021) reported antibiotics in the Klip River from Gauteng Province in South Africa with concentrations ranging from 0.0618–0.133 µg/L. The concentrations of antibiotics have also been reported in other African countries like Kenya (0.00008–49.562 µg/L), Ghana (0.00011–9.64 µg/L), and Mozambique (0.80–53828 ng/L). Such concentrations are comparable with antibiotics found in South Africa as Segura et al. (2015) reported 0.00038–10.568 µg/L in

surface waters. NSAIDs like diclofenac, ibuprofen, and naproxen have been quantified in wastewater and river waters of KwaZulu-Natal Province in South Africa, and the results exhibited a concentration range of 0.6–221 µg/L (Madikizela and Chimuka 2017a, Madikizela et al. 2017a). In another study, Hlengwa and Mahlambi (2020) detected NSAIDs such as carbamazepine, diclofenac, fenoprofen, ibuprofen, and naproxen in KwaZulu-Natal wastewater (influent and effluent) and river waters with concentrations ranging from 8.99–66.9 µg/L.

Despite the extensive coverage in existing literature on pharmaceutical contamination in water environments, several critical gaps persist. Previous reviews have predominantly focused on individual classes of pollutants, such as non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, and antiretroviral drugs, documenting their occurrence, sources, and potential impacts on aquatic ecosystems and human health. However, there is a significant gap in understanding the interactions between different classes of pharmaceuticals and their combined effects in aquatic environments. For example, while studies may separately investigate the effects of antibiotics on microbial communities or NSAIDs on aquatic organisms, there is limited research exploring how simultaneous exposure to multiple classes of pharmaceuticals may exacerbate or mitigate ecological impacts. Future research should prioritize integrated studies that examine the joint effects of numerous classes of pharmaceuticals to provide a more comprehensive understanding of their environmental consequences. Moreover, there is a notable lack of long-term studies evaluating how chronic pharmaceutical exposure affects biodiversity and ecosystem function in aquatic environments. Current research primarily focuses on short-term effects and overlooks potential cumulative impacts on species composition, ecosystem dynamics, nutrient cycling, and food webs. So, addressing this gap requires sustained, multi-seasonal studies to fully understand and mitigate the ecological risks associated with pharmaceutical contamination. Furthermore, a significant gap exists in understanding regional variability in pharmaceutical contamination levels. Current knowledge on this variability is limited, with studies often focusing on specific locations and short-term sampling periods. This lack of comprehensive data across different regions and seasons hampers efforts to accurately assess the extent and persistence of pharmaceutical pollution in various aquatic environments. So, addressing this gap requires systematic, long-term monitoring efforts that capture geographical and seasonal variations in pharmaceutical concentrations. Closing the aforementioned gaps is essential for advancing our understanding

of pharmaceutical pollution dynamics, improving environmental monitoring practices, and ensuring effective water quality and ecosystem health management.

It is also important to note that the effectiveness of remediation strategies aimed at removing pharmaceuticals from wastewater is an area that has not been thoroughly explored. There is a need for a more extensive evaluation of various treatment methods to determine their capability to eliminate pharmaceutical residues from wastewater comprehensively. Currently, research has primarily focused on conventional treatment processes, such as activated sludge, oxidation, and filtration techniques, which do not adequately address pharmaceutical compounds' diverse and complex chemical properties. Furthermore, several drawbacks are associated with the said treatments, including added energy requirements, large amounts of chemicals, and the development of unwanted and harmful by-products (Encarnação et al. 2020, Silva et al. 2019). Other technologies like chemical precipitation, electrocoagulation, and anaerobic bed reactors have also been explored; however, they have been found to be inefficient, especially at dilute concentrations. Also, the operation costs make it almost impossible for these technologies to reach the implementation stages (Ali et al. 2018). More efficient tertiary technologies like reverse osmosis, ultrafiltration, nanofiltration, ozonation, and photolysis are currently being investigated, but their application in developing countries like South Africa is relatively expensive. Hence, there is a critical need to explore emerging technologies like biological treatments to evaluate their effectiveness in removing pharmaceuticals under diverse environmental conditions and in different wastewater compositions. Understanding the capabilities and limitations of these methods is vital for developing robust, sustainable solutions to combat pharmaceutical pollution in water systems.

2.1.2 Heavy metals in water environments

Heavy metals are continually discharged into water sources from both anthropogenic activities such as mining industries, fossil fuel combustion, pesticide and fertilizer use, sewage sludge, fungicides, used batteries, detergents, and medical devices and natural sources like natural rocks, volcanic eruptions, wind-blown dust, particles, and aerosols (Figure 2.2) (Zamora-Ladezma et al. 2021, Aziz et al. 2023). Arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), and mercury (Hg) are common heavy metals of environmental concern due to their toxicity and widespread presence in water sources. In numerous locations around the world, their average concentrations in surface water bodies, along with manganese (Mn), iron (Fe), cobalt (Co), and others, exceed the maximum allowable values for drinking

water, posing significant risks to human health and the environment (Qasem et al. 2021). Even at very low concentrations, many heavy metal ions are toxic or carcinogenic, causing damage to multiple organs such as the lungs, kidneys, liver, brain, heart, prostate, oesophagus, stomach, and skin (Zamora-Ladezma et al. 2021, Aziz et al. 2023). They can also lead to neurodegenerative disorders like Alzheimer's and Parkinson's diseases (Zamora-Ladezma et al. 2021). In water environments, heavy metals can accumulate in organisms such as phytoplankton, zooplankton, and fish, causing oxidative damage, endocrine disruption, immune system depression, and negatively impacting survival and growth (Aziz et al. 2023).

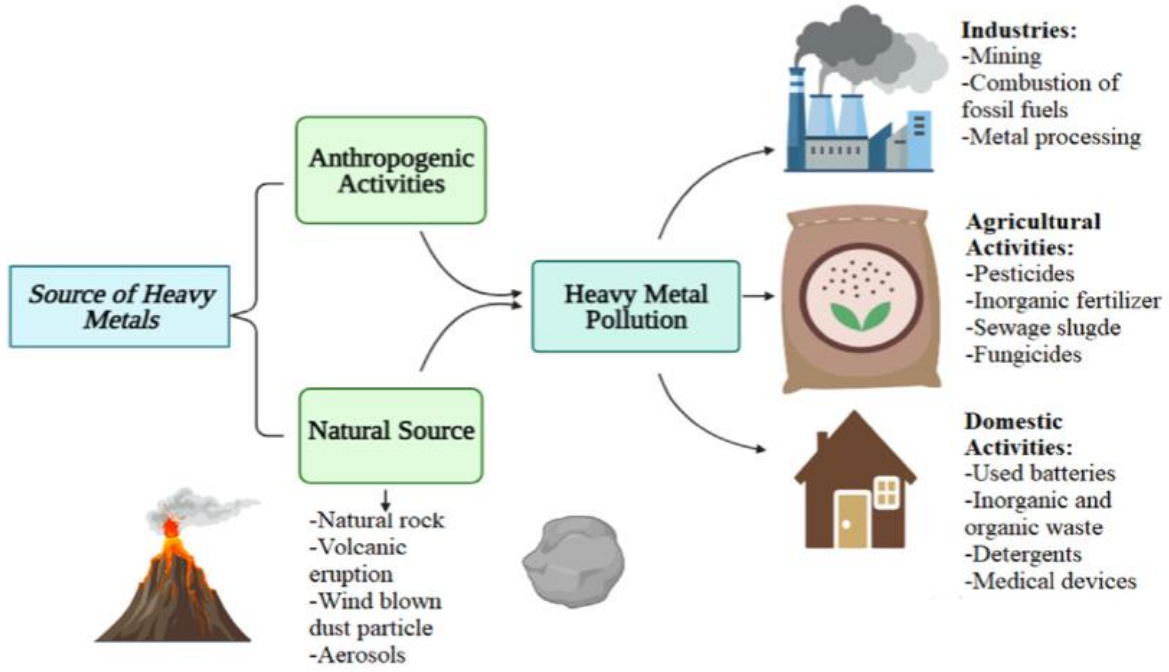

Figure 2.2 Sources of heavy metals (a) and their human health impacts (b) (Zamora-Ladezma et al. 2021)

Much research has been conducted across various African countries, consistently identifying significant concentrations of heavy metals in diverse water bodies. For example, Kamzati et al. (2020) documented elevated levels of cadmium (0.002 mg/L), zinc (2.308 mg/L), and iron (8.807 mg/L) in water sites across Malawi, surpassing WHO drinking water standards for iron and zinc (0.300 mg/L and 0.100 mg/L, respectively). In Nigeria, Chris et al. (2023) reported on lead (0.595 mg/L), chromium (0.085 mg/L), nickel (0.004 mg/L), and cadmium (0.001 mg/L) concentrations in water samples from artisanal oil mining areas, illustrating localized impacts of industrial activities. Along the Isipingo River in South Africa, Adeyinka et al. (2023) identified detectable iron levels ranging from 0.010–0.047 mg/L in surface water, highlighting regional variations in heavy metal pollution. Similarly, Addo-Bediako et al. (2018)

documented chromium (0.020 –0.060 mg/L), copper (0.010–0.012 mg/L), manganese (0.270–0.660 mg/L), nickel (0.010–0.020 mg/L), and zinc (0.020–0.070 mg/L) concentrations exceeding permissible limits in the Steelpoort River system in Limpopo Province, South Africa). In another research endeavour, Edokpayi et al. (2018) analyzed borehole water in Limpopo Province, noting varying concentrations of chromium (0.005–0.150 mg/L), iron (0.150–1.860 mg/L), manganese (0.010–1.220 mg/L), copper (0.010–0.410 mg/L), lead (0.002–0.026 mg/L), and zinc (below 5.0 mg/L), emphasizing the localized impacts of agricultural practices and industrial discharges. Atangana and Oberholster (2021) investigated wastewater treatment effluents and groundwater in the Olifants River catchments in Limpopo, revealing diverse concentrations of copper (0.030–0.150 mg/L), iron (0.120–0.310 mg/L), nickel (0.050–0.110 mg/L), zinc (0.010–0.020 mg/L), barium (0.030–0.170 mg/L), and manganese (1.410–8.220 mg/L), highlighting the complex interplay of anthropogenic activities and natural processes in influencing heavy metal distributions. In the Western Cape of South Africa, Olujimi et al. (2015) observed fluctuating levels of arsenic (0.001–0.024 mg/L), cadmium (0.00009–0.015 mg/L), lead (0.004–0.087 mg/L), and mercury (0.0001–0.008 mg/L) in river water affected by both industrial and domestic effluents. These reported concentrations often exceed permissible limits set by regulatory bodies like the WHO and/or the Department of Water Affairs and Forestry (DWAF), which establish standards for safe aquatic organism habitat and human consumption. Furthermore, these studies have predominantly focused on identifying the presence and concentrations of various pollutants across different regions. However, these studies often lack comprehensive assessments of effective mitigation strategies, such as evaluating the efficiency and applicability of adsorption processes for removing heavy metals from water sources.

Researchers worldwide have developed methods and techniques to address heavy metal contamination in wastewater, which include physical, chemical, and biological processes. Physical methods such as membrane technologies (including reverse osmosis and nanofiltration) are widely recognized for their effectiveness in capturing heavy metals (Qasem et al. 2021, Aziz et al. 2023). However, they often require high energy input and can be costly to operate and maintain. Additionally, these methods may suffer from membrane fouling, which reduces their efficiency over time and necessitates regular cleaning or replacement of the membranes. Chemical methods like precipitation and coagulation-flocculation are well-established and cost-effective but generate significant sludge that requires sedimentation (Qasem et al. 2021). While effective, electrochemical processes (like electrocoagulation and

electrooxidation) are often costly due to electrode passivation and high energy consumption (Aziz et al. 2023). Among these methods, biological treatments like adsorption stand out due to their simplicity, broad applicability, high removal rates, and potential for cost-effective reusability (Qasem et al., 2021). Therefore, a critical gap exists in the literature regarding the comprehensive evaluation and application of bioremediation processes such as adsorption tailored explicitly to African water systems. This gap necessitates further research to assess the feasibility, efficiency, and environmental implications of adsorption techniques for mitigating heavy metal pollution in the region effectively.

2.1.3 Excess nutrients in water environments

Nitrogen and phosphorus are vital nutrients for essential biological processes such as protein and DNA synthesis, cellular growth, and reproduction in numerous organisms, including plants, animals, and microbes (Shen et al. 2020). In aquatic environments, their excessive presence has been linked to significant global impacts, affecting human health, aquatic organisms, and ecosystem integrity. Several pathways introduce these contaminants into aquatic environments, including runoff from farmlands, aquaculture effluents, as well as industrial and municipal discharges, contributing to socio-economic and environmental challenges (Mudaly and van der Laan 2020, Akinnawo 2023). Seasonal rainfall and storm events exacerbate this by further transporting nutrients from land to water bodies (Lukhele and Msagati 2024).

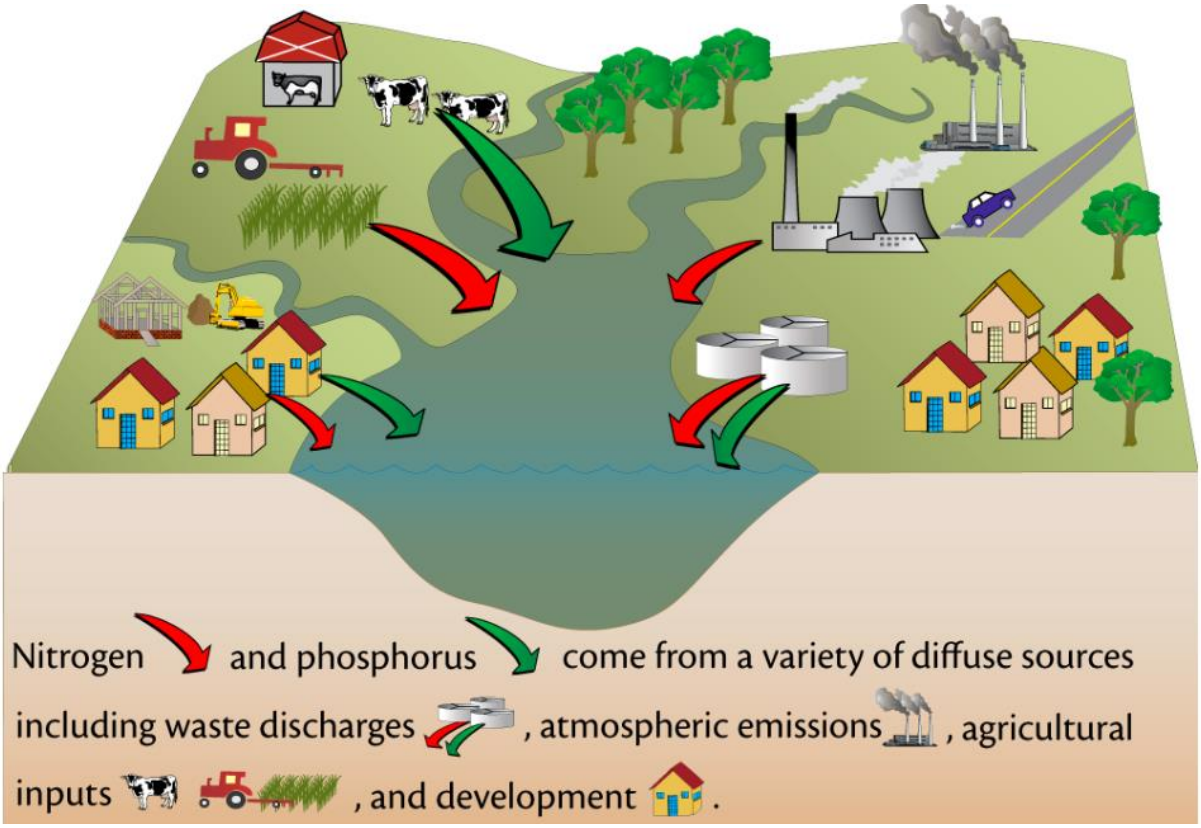

Figure 2.3 Sources of nitrogen and phosphorus in aquatic environments (Thomas 2007)

Excessive nitrogen and phosphorus in water bodies lead to eutrophication, which causes a series of harmful effects in aquatic ecosystems. It leads to increased growth of aquatic plants and algae, forming dense blooms that make water bodies unsuitable for drinking, recreation, and supporting aquatic life (Beusen et al. 2022, EPA 2024). This process also clouds the water and turns it green, reducing sunlight penetration and hindering photosynthesis in underwater plants (Akinnawo, 2023). As the excess algae die and decompose, they deplete dissolved oxygen, resulting in the death of fish and other aquatic organisms (Isiuku and Enyog 2020). This decrease in oxygen also affects mammals and birds that rely on fish as a food source (Beusen et al. 2022, Akinnawo, 2023). In humans, nitrates in drinking water can form carcinogenic compounds such as nitrosamines and contribute to methemoglobinemia, particularly harming infants (Johnson 2019, Beaudet et al. 2014). Methemoglobinemia, characterized by inadequate tissue oxygenation, can lead to fatalities (Beaudet et al. 2014). Additionally, eutrophication promotes the growth of cyanobacteria, which produce toxins causing liver damage, diarrhoea, skin irritation, and neurotoxicity in humans (Kubickova et al. 2019, Lukhele and Msagati, 2024). Furthermore, nutrient pollution and resulting algal blooms annually cost the tourism industry billions of dollars, impacting recreational activities like

fishing and boating (Alvarez et al. 2024, EPA, 2024). Algal blooms can degrade waterfront property values due to their unsightly appearance and unpleasant odour (Bechard 2021). Moreover, excessive algae can block water pipes and turbines, causing problems in transportation and power generation (Akinnawo, 2023). Lastly, algal blooms in drinking water sources can drastically increase treatment costs, which can also amount to billions of dollars for cleaning up (EPA, 2024, Igwaran et al. 2024).

Increased awareness of human activities affecting the nitrogen and phosphorus cycles has spurred extensive research aimed at improving nutrient management practices (Shen et al. 2020). For instance, a study conducted in Southeastern Nigeria evaluated nitrate and phosphate levels in water bodies that include Abadaba River, Agulu Lake, Njaba River, Oguta Lake, and Nike Lake during both dry and wet seasons (Isiuku and Enyog, 2020). In this study, the overall range of nitrate concentrations varied from 13.163±2.30 mg/L to 36.173±7.22 mg/L, whereas phosphate concentrations ranged from 2.144±0.513 mg/L to 40.204±6.024 mg/L, with lower levels observed during the dry season and higher levels during the wet season. In South African rivers, extensive studies have focused on nitrogen and phosphorus levels (Villiers et al. 2007, Oberholster et al. 2011, van Ginkel 2011, Dabrowski et al. 2014, Huchzermeyer et al. 2017, Griffin 2017, and Neil 2017. Analysis of long-term water quality monitoring data from the Department of Water Affairs and Forestry indicates that nutrient concentrations in 95% of the largest river catchments in South Africa surpass recommended guidelines for plant life (De Waal et al. 2023). Moreover, the National Eutrophication Monitoring Program (NEMP) reports that 18 out of 25 major river systems in South Africa are eutrophic, including significant rivers like the Olifants, Orange, Crocodile, Vaal, and others (Lukhele and Msagati, 2024). Even though the above-mentioned research exists on nitrogen and phosphorus in African waters, including South Africa, accessing comprehensive data remains extremely challenging due to several factors. Limited monitoring infrastructure and resources in many regions make conducting regular and comprehensive water quality assessments difficult. Research efforts on water quality and nutrient levels are often fragmented across different institutions and regions, leading to inconsistencies in data collection and reporting. Moreover, limited access to historical and current datasets further complicates the assessment of trends in nutrient concentrations over time accurately. Although the Department of Water Affairs and Forestry conducts long-term water quality monitoring in South Africa, coverage may be uneven across various rivers and catchments, and the existing data is not easily obtainable. To close the research gap on nutrient levels, researchers are required to undertake comprehensive studies to

assess nitrogen and phosphorus across various regions. Developing and validating methods to detect these nutrients in diverse water matrices is crucial. Furthermore, publishing findings in scientific journals and collaborating with stakeholders can facilitate knowledge dissemination and inform targeted strategies for managing nutrient pollution effectively.

Various techniques are utilized to mitigate nutrient pollution, including dredging, aeration, infiltration-percolation, membrane methods, chemical processes, and others. Dredging removes sediment enriched with nitrates and phosphates; however, it can deepen water bodies and disrupt sediment habitats, cause significant disturbance to the benthic environment, and potentially release previously settled contaminants back into the water column, and it requires careful environmental management (Akinnawo 2023). Aeration increases oxygen levels in water, reducing stratification and aiding in nutrient control. However, it requires high energy consumption and operational costs and can lead to the release of volatile organic compounds (VOCs) and other gaseous pollutants into the atmosphere, potentially causing air quality issues (Gu et al. 2023). Infiltration-percolation effectively treats secondary wastewater effluents but demands suitable geology and significant area for implementation (Bali and Gueddari 2019). Membrane separation methods like reverse osmosis produce high-quality water but are energy-intensive and costly to maintain (Akinnawo 2023). Chemical techniques such as coagulation/flocculation remove contaminants but can alter water pH, require technical expertise, and generate sludge, whereas aluminium-based coagulants pose health risks (Akinnawo 2023). Therefore, there is an urgent need for further research and development of effective strategies for nutrient removal in water bodies, particularly focusing on biological organisms to advance sustainable and efficient treatment methods.

2.2 Bioremediation mechanisms and possible pathways

Bioremediation offers an elegant solution for removing many environmental contaminants in water resources, and it is the most suitable, economically practical, lucrative, and environmentally friendly alternative for cleaning water. Bioremediation is defined as a biological treatment that uses organisms and their enzymes to convert recalcitrant and xenobiotic contaminants into a less toxic form (Silva et al. 2019, Shah and Shar 2020). In this process, biological organisms use pollutants as a source of energy and/or as nutrients (Divya et al. 2015). A wide range of organisms including aquatic plants, fungi, bacteria, and microalgae have been used in bioremediation. These organisms can adapt to extreme conditions

and they can bio-absorb many contaminants, even if they occur in extremely low concentrations where the conventional methods do not work (Coelho et al. 2015).

Different mechanisms can degrade excess nutrients, heavy metals, and pharmaceutical compounds in WWTPs, including biodegradation, sorption, and photodegradation. Biodegradation is the principal removal mechanism that can occur either through co-metabolisms or through sole substrate degradation. In co-metabolism, microorganisms degrade target compounds that cannot be utilised as the sole nutrient and energy source in the presence of a primary substrate; which supports the microorganism's growth and induce the secretion of enzymes and cofactors which enables the bio-transforming of the pollutants (Kannes-Veiga et al. 2022). In sole substrate degradation, microorganisms use target compounds as a source of carbon and energy for their growth (Tiwari et al. 2016, Kannes-Veiga et al. 2022). It is important to note that the solubility of target compounds plays a crucial role in biodegradation efficiency. Hydrophobic compounds have low solubility which enables them to be retained in sewage sludge; this promotes microbial degradation through catabolic microbial enzymes, or these compounds are used by microorganisms as carbon sources (Tiwari et al. 2016). On the other hand, hydrophilic compounds have high solubility which causes them to quickly escape WWTPs without being biodegraded (Tiwari et al. 2016).

Sorption is another removal mechanism that has been reported to degrade excess nutrients (nitrogen and phosphorus), heavy metals (copper, lead, cadmium, cobalt, mercury, zinc, etc), and pharmaceutical compounds like tetracycline, norfloxacin, clotrimazole, haloperidol, and ciprofloxacin in WWTPs (Kim et al. 2014, Wu et al. 2019, Horsing et al. 2022, Rajendran et al. 2022). Sorption occurs due to the hydrophobic interaction of the aliphatic and aromatic group, microorganism cell membrane, electrostatic interaction of positively charged compounds to negatively charged microorganisms or sludge, and due to sludge lipid molecules (Tiwari et al. 2016). The sorption of contaminants is highly dependent on the octanol-water coefficient, sludge adsorption coefficient, and acid dissociation constant (Tiwari et al. 2016). Through this mechanism, contaminants can be removed by either adsorption or absorption. In adsorption, contaminants accumulate on the biomass surface that has active and energy-rich sites (Karungamye 2020). The positively charged contaminants interact with the negatively charged surface of microorganisms through electrostatic interactions, causing ions and molecules to bind to the surface (Wang 2009). In absorption, contaminants enter the biomass (Lucas et al. 2018). This occurs when the aliphatic and aromatic groups of the target compound

interact with the lipophilic cell membrane of the microorganism and the lipid fraction of the sludge through hydrophobic interactions (Wang 2009). Unlike biodegradation, sorption is regarded as the minor removal mechanism for most compounds. The findings of Lucas et al. (2018) are in agreement with this statement, as their study revealed that sorption was responsible for 3 – 13% of the removal of pharmaceuticals, with the rest of the removal being achieved through biodegradation.

Photodegradation is a well-documented removal mechanism in WWTPs, and it entails direct and indirect pathways. Direct photodegradation occurs when target compounds absorb electromagnetic radiation to break the bond or rearrange the compound to form a stable product (Karungamye 2020). Indirect photodegradation occurs when dissolved organic matter absorbs irradiation and secretes some reactive species capable of removing pharmaceuticals and other micro-pollutants (Karungamye 2020). Indirect photodegradation can be coupled with hydroxyl radicals and triplet excited state effluent organic matter to maximise the photodegradation of target compounds (Karungamye 2020). For example, a study by Ryan et al. (2011) showed that sulfamethoxazole photodegradation was divided into 48%, 36%, and 16% by indirect photolysis, hydroxyl radicals, and triplet excited state effluent organic matter, respectively. In the same study, trimethoprim removal was divided into 18%, 62%, and 20% by indirect photolysis, hydroxyl radicals, and triplet excited state effluent organic matter.

2.3 The use of microalgae (Phycoremediation)

Microalgae are highly adaptive organisms that can grow autotrophically, heterotrophically, or mixotrophically (Silva et al. 2019). They can withstand extreme environmental conditions that include varying levels of nutrients, carbon dioxide, pH, temperature, salinity, light, etc. (Varshney et al. 2015). Their tolerant ability is governed by their spontaneous mutation and/or physiological adaptations (Bhandari et al. 2021). Microalgae bioremediation is an innovative solution that has the potential to perform better than conventional wastewater treatment processes. Phycoremediation can remove non-natural anthropogenic contaminants such as pharmaceuticals, pesticides, and industrial contaminants (Encarnação et al. 2020, Bhandari et al. 2021). Phycoremediation also has valuable economic advantages by allowing resource recycling of water, bioresource for biofuel, sewage sludge, and manure production, as well as high biomass production which can be harvested for alcohol production, and animal feed supplements (Encarnação et al. 2020).

The uptake of contaminants by different microalgae entails 3 processes. First is bio-adsorption, where contaminants such as heavy metals are adsorbed on microalgal cell walls or onto extracellular polymeric substances (Figure 2.2) (Maryjoseph and Katheesan 2020). For example, under basic conditions (higher pH), the negative charge at the active site of the algal cell wall increases, causing metallic cations to be attracted. Consequently, these metals become adsorbed on the cell wall (Bhandari et al. 2021). According to Mustafa et al. (2021), the presence of sulfated polysaccharides, carbohydrates, intercellular spaces, fibril matrix, and binding sites (OH^-, SO_4^{2-}, NH_2^- and COO^- functional groups) on the algal cell wall assists in achieving high removal efficiency. Such properties enable microalgal biomass to be a good biosorbent that treats wastewater effluents.

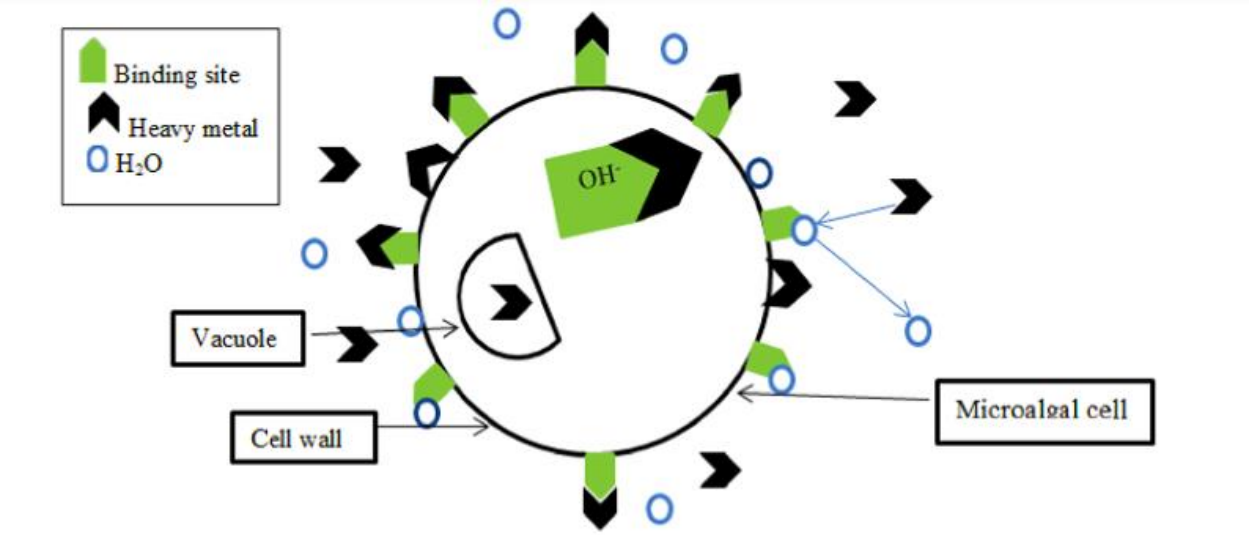

Figure 2.4 Bio-adsorption by microalgal cells (Mustafa et al. 2021)

Bioaccumulation is the second phycoremediation process where contaminants are transferred through the cell wall into the interior of living microalgal cells where it binds to intracellular proteins and other substances (Maryjoseph and Katheesan 2020). During this process, microalgae uptake contaminants; they then accumulate and/or metabolize them inside their cells (Bhandari et al. 2021). This process has been reported to remove pollutants like heavy metals, pesticides, nitrates, sulphates, and phosphates (Mustafa et al. 2021). The third is the biodegradation/biotransformation pathway which can be intracellular and/or extracellular (Reddy et al. 2021). Intracellular degradation occurs through enzymatic reactions, whereas extracellular degradation involves exopolymeric substances (Reddy et al. 2021). In this pathway, contaminants are not only biologically filtered, like adsorption and bioaccumulation, but are also transformed into simpler breakdown molecules through catalytic metabolic

degradation. This makes it the most promising pathway because it overcomes drawbacks related to the disposal of produced microalgal biomass (Sutherland and Ralph 2019).

The ability of microalgal species to remove pharmaceutical contaminants in water has been widely explored in recent years, with the majority focusing on the use of monocultures (Bai and Acharya 2017, Ali et al. 2018, Xiong et al. 2019). de Wilt et al. (2016) assessed micropollutant removal in an algal-treated system using *Chlorella sorokiniana.* They found removal efficiencies between 60 – 100% for diclofenac, ibuprofen, paracetamol, and metoprolol. Matamoros et al. (2016) evaluated the removal efficiencies of *Chlorella* and *Scenedesmus sp*. from urban wastewater. They reported up to 99% removal of 4-octylphenol, galaxolide, and tributyl phosphate which was achieved via volatilization; whereas 40% removal of ibuprofen was attributed to biodegradation. In the same study, the removal of nutrients such as phosphorus and nitrogen were close to 100%. In a study that used *Nannochloris sp* from Lake Mead Water, ciprofloxacin projected 100% removal, whereas triclosan showed 48 – 73% removal (Bai and Acharya 2017). These 2 compounds were rapidly removed to concentrations below the detection limit after 14 days (Bai and Acharya 2017). Elsewhere, Ding et al. (2020) evaluated the simultaneous removal of mixed pharmaceutical compounds by freshwater diatom (*Navicula* sp). In this study, the removal efficiencies of atenolol, carbamazepine, ibuprofen, and naproxen were found to be greater than 90% through bioaccumulation after 21 days. Peng et al. (2014) used *Scenedesmus obliquus* and *Chlorella pyrenoidosa* to assess the removal of progesterone and norgestrel. Their results showed that both organisms achieved greater than 95% removal of progesterone while norgestrel had 95% removal by *S. obliquus* and 60% removal by *C. pyrenoidosa.* Several other studies evident the use of microalgae to remove compounds like ciprofloxacin (Bai and Acharya 2017, Gentili and Fick 2017), levofloxacin (Xiong et al. 2017), salicylic acid (Escapa et al. 2017), sulfamethoxazole (Ding et al. 2020), tramadol (Ali et al. 2018), triclosan (Bai and Acharya 2016), and many others.

Microalgae species have also exhibited significant potential in removing heavy metals in water. For example, acid-tolerant *Chlorella* spp. and *Desmodesmus sp*. removed 2 mg/L of cadmium (II) with an efficiency greater than 58% (Abinandan et al. 2019). A study by Valdez et al. (2018) found that *Chlorella* spp. could achieve up to 60% cadmium (II) biosorption at a concentration of 20 mg/L. Elsewhere, *Chlorella sorokiniana* tolerated up to 100 mg/L of chromium (VI) for three days, reaching a removal efficiency of 99.7% after 24 hours of contact

time (Husien et al. 2019). Research by Cherifi et al. (2016) showed that *Navicula subminuscula* achieved nearly 98% removal in cultures with 20 mg/L Cr (VI). Furthermore, *Pseudanabeane mucicola* and *Pediastrum duplex* can tolerate chromium (VI) concentrations up to 1.936 g/L and 0.224 g/L, respectively, with the former showing a removal efficiency of 71% (Dao et al. 2018). Many other studies have also demonstrated the effectiveness of microalgae in heavy metal removal (Kwak et al. 2015, Molazedah et al. 2015, Das et al. 2016, Suganya et al. 2016, Dirbaz and Roosta 2018, Shen et al. 2019).

Experimental findings have also demonstrated the efficacy of microalgae in removing phosphate and nitrate from water matrices. Over 8 days, microalgae exhibited significant removal capabilities, with *Chlorella vulgaris* and *Spirulina platensis* achieving maximum nitrate removal rates of 89.80% and 81.49%, respectively (Sayadi et al. 2016). In the same study, *Chlorella vulgaris* showed high removal efficiency for phosphorus-orthophosphate, recording 100%. In another study, *Chlorella* spp. achieved ammonia-nitrogen removal efficiencies of 81% after 1.25 hours and 98.81% after 4 hours, while phosphorus-orthophosphate removal efficiencies were 77.4% after 1.25 hours and 100% after 4 hours (Zhang et al. 2012). Research by Rasoul-Amini et al. (2014) reported that *Chlamydomonas* spp. nearly eliminated phosphorus from urban sewage with an initial concentration of 19.11 mg/L. *Nannochloropsis* spp. achieved a 100% phosphorus removal rate from urban wastewater (Fallahi et al. 2020). In another research endeavour, Xin et al. (2010) found that *Scenedesmus* spp. effectively removed both nitrogen and phosphorus, achieving 90% nitrogen removal and nearly 100% phosphorus removal. Even though microalgae exhibit so many promising results, challenges still exist when it comes to large-scale applications. Such challenges include a vast amount of land required, predatory zooplankton, and high oxygen concentrations (Maryjoseph and Katheesan 2020, Singh et al. 2022). Also, the toxicity effect of wastewater on some microalgal species is documented in the literature (Silva et al. 2019). So, employing genetically modified organisms for experiments could offer a solution that can overcome some of these challenges (Silva et al. 2019). The genetic adaptations will enable microalgae to withstand severe conditions; for example, when they are introduced to extreme conditions, they will produce toxic degrading enzymes (Maryjoseph and Katheesan 2020). Another challenge is presented by the treatment's final step (harvesting), which requires high production costs and energy consumption. Environmentally friendly methods like bio-flocculation coupled with co-pelletization are recommended. In co-pelletization, other microorganisms such as bacteria and fungi could be added since they do not need additional chemicals or energy inputs for efficient

and uniform exposure to sunlight in order to achieve high efficiencies (Silva et al. 2019, Zhao et al. 2019).

2.4 The use of fungi (Mycoremediation)

Fungi are extensively used as biosorbents for the removal of toxic contaminants, and they show good capacities for metal uptake and recovery (Igiri et al. 2018). Fungi can use their non-stereoselective, non-specific intracellular and extracellular oxidative enzymes to transform a wide variety of compounds like pharmaceuticals (Silva et al. 2019). According to Assress et al. (2019), fungi are one of the most important groups of microbial communities in wastewater treatment plants, and they help break down organic chemicals from protein to complex compounds. The first studies that investigated the fungal potential to remove contaminants were carried out in sterile conditions and they used single spiked contaminants (Marco-Urrea et al. 2009, 2010). To date, very limited studies have been conducted under complex matrices that are non-sterile. Still, the few that exist have exhibited great success in transforming and/or removing contaminants such as heavy metals, nutrients, and pharmaceuticals. Fungi use 4 pathways to degrade compounds in wastewater. Such pathways can occur separately or synergistically, and they include (i) bio-absorption and immobilisation of the compounds, (ii) reactive oxygen species production like hydrogen peroxide (H_2O_2), superoxide anion radicals (O_2^-), and hydroxyl radical (OH^-), (iii) extracellular enzymatic processes that include peroxidase, and phenoloxidase enzymes as well as (iv) intracellular enzymatic processes that involves cytochrome P450 (Figure 2.2), (Akerman-Sanchez et al. 2021).

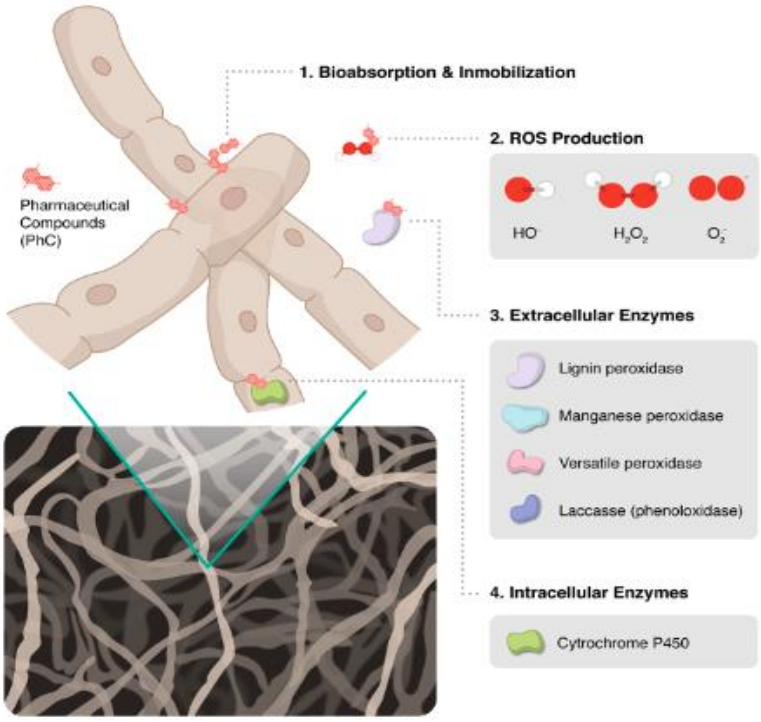

Figure 2.5 Fungi pathways for degrading pharmaceutical compounds in wastewater (Akerman-Sanchez et al. 2021)

Phanerochaete chrysoporium and *Trametes versicolor* are the most widely used species in wastewater bioremediation. Zhang and Geißen (2012) evaluated the removal of carbamazepine using *P. chrysoporium* under non-sterile conditions. Results from this study showed a high elimination of approximately 60 – 80%. In the same study, researchers found that adding sufficient nutrients to the treatment systems is crucial as it increases the removal efficiency. Elsewhere, Rodarte-Morales et al. (2012) conducted a study that assessed the transformation of diclofenac, ibuprofen, and naproxen by pellets of *P. chrysosporium* in fed-batch bioreactors. Results projected high elimination percentages (75–99%) for removing the 3 pharmaceutical compounds. In 2013, Cruz-Morato et al. studied the degradation of pharmaceutical compounds by *T. versicolor* in non-sterile urban wastewater. In this study, 7 compounds were removed entirely. *T. versicolor* was investigated in a different study, and 32/39 antibiotics were removed by greater than 50% after 24 hours (Becker et al. 2016). The removal efficiency of *T. versicolor* was also evaluated by Mir-Tutusaus et al. (2017) in non-sterile hospital wastewater for 2 months. *T. versicolor* efficiently removed the majority of the detected pharmaceutical contaminants, including the most intractable ones. In this study, the treated wastewater effluent was found to be non-toxic, meaning that the treatment was able to remove potentially toxic metabolites (Mir-Tutusaus et al. 2017). Furthermore, *T. versicolor* was documented to remove

diverse recalcitrant analgesics, anti-inflammatories, antibiotics, and psychotropic drugs (Mir-Tutusaus et al. 2017). Moreover, the capability of *Trichoderma harzianum* and *Pleurotus ostreatus* to degrade carbamazepine and clarithromycin in aqueous aerobic conditions was investigated by Buchicchio et al. 2016. After 7 days, *P.ostreatus* removed 68% of carbamazepine and 55% of clarithromycin, whereas T. *harzianum* removed 72% of carbamazepine and 57% of clarithromycin, respectively. Elsewhere, *P. ostreatus* achieved 100, 70, and 60% removal for diclofenac, ketoprofen, and atenolol, respectively (Palli et al. 2017).

Several studies have explored the ability of fungi to remove heavy metals (Xu et al. 2013, Amirnia and Ray 2015, Bazrafshan et al. 2016). Srivastava and Thakur (2006) investigated the effectiveness of *Aspergillus* spp. in removing chromium from tannery wastewater. The study reported that *Aspergillus* spp. removed 85% of chromium from a synthetic medium at pH 6, compared to 65% removal from tannery effluent, likely due to the presence of organic pollutants that inhibit microbial growth. In another investigation, *Coprinopsis atramentaria* demonstrated significant bioaccumulation abilities, removing 76% of cadmium (II) and 94.7% of lead (II), highlighting its potential as an effective accumulator of heavy metal ions for mycoremediation (Lakkireddy & Kües, 2017). Elsewhere, Luna et al. (2016) studied *Candida sphaerica* and found that it produces biosurfactants with removal efficiencies of 95% for iron, 90% for zinc, and 79% for lead. The bio-removal of chromium (VI) by the native fungal isolate *Aspergillus fumigatus* from mine drainage revealed a maximum uptake of 48.2 mg/g under optimized conditions, in line with the Freundlich isotherm (Dhal et al. 2018). Other studies have demonstrated the potential of various fungal strains to remove nutrients like phosphorus and nitrogen. Zeng et al. (2021) investigated *Aureobasidium* sp., which was found to remove over 53.5% of phosphorus from actual wastewater aerobically. In separate studies, Zeng et al. (2020) and Fang et al. (2021) identified that the *Sporidiobolus pararoseus* and *Barnettozyma californica* are capable of nitrogen removal via heterotrophic nitrification-aerobic denitrification in wastewater. Elsewhere, Dalecka et al. (2020) explored the phosphorus, nitrogen, and total organic carbon removal capabilities of two fungal species (*T. versicolor* and *A. luchuensis*). This study found that *T. versicolor* achieved total phosphorus removal (99.9%) after a 6-hour incubation period, while *A. luchuensis* reached the same removal efficiency after 24 hours. In another study, Isnadina et al. (2019) analyzed the effectiveness of *Aspergillus niger*, and *Fusarium solani* in treating domestic wastewater containing liquid cleaning materials. The study revealed that the average phosphate removal percentages were 70.8% with

fungi addition and 69.6% without, highlighting the notable impact of fungal addition in wastewater treatment processes.

Despite the promising results demonstrated in various studies on using fungi for bioremediation, several critical research challenges warrant further investigation. Such challenges include factors that affect the oxidative metabolism of fungi, the prevalence of nutrients, pH, immobilization, and agitation/static growth conditions (Silva et al. 2019). Contamination with bacteria is another key factor hindering mycoremediation's full success. The bacteria and fungi compete for the substrate, damaging fungi mycelium, inhibiting biomass growth, and destabilizing fungal activity (Silva et al. 2019). As a way to overcome these challenges, Mir-Tutusaus et al. (2016) documented that starting with the coagulation-flocculation step before the fungal treatment tends to decrease the microbial load of the influent, enabling good maintenance of the fungal activity for 28 days. A partial biomass renovation was recommended as a potential way of improving the treatment because it will slow down the biomass ageing process (Mir-Tutusaus et al. 2016). Operating in an acidic condition could also help address these challenges because fungi have lower optimum pH for growth than bacteria's optimum pH (Silva et al. 2019). Furthermore, using disinfectants that permit the selection and/or inactivation of bacteria could play a huge role in improving the efficiency of fungal technologies (Silva et al. 2019). Singh et al. (2022) documented that insufficient removal of target compounds by fungal communities could also be attributed to mass transfer restriction, removal of excess biomass, death zones formation, high operational and maintenance costs, unfeasibility to large volume, inefficient mixing, as well as extreme agitation that shears pallets which may interrupt enzymes secretion. Thus, more attention should be directed to finding in-depth knowledge about these possible attributes to maximise the removal efficiencies. Genetic modifications in fungal species could also contribute to the breakthrough of this technology by reducing the limitations faced. Through genetic modification, fungal species will acquire new genetic traits that will enhance their metabolic and adaptive processes and thus achieve efficient bioremediation (Silva et al. 2019). Therefore, addressing these will enhance our understanding of fungal bioremediation and support the development of more effective and sustainable approaches for water treatment.

2.5 The use of bacteria for bioremediation

Bacteria are proficient microorganisms for bioremediation and can thrive in environments with toxic elements, even at concentrations that are lethal (Igiri et al. 2018). Species such as *Flavobacterium*, *Pseudomonas*, *Enterobacter*, *Bacillus*, and *Micrococcus* are documented to be efficient removers of environmental contaminants due to their high surface-to-volume ratios as well as the presence of teichoic acid on their cell wall (Igiri et al. 2018). Technologies that involve sludge (activated, granular, aerobic, etc.) have been used extensively in wastewater treatment due to the involvement of bacterial communities, and they show varying degrees of success. There seems to be a growing body of literature on the potential of bacteria to remove pharmaceutical compounds in wastewater over recent years. Tiehm et al. (2011) conducted a biodegradation study in water samples of the Jordan Valley, and the results revealed possible biodegradation for pharmaceutical residues such as naproxen, ibuprofen, diclofenac, and bezafibrate. In a pilot study that treated hospital wastewater with a membrane bioreactor that contained bacterial batch cultures, ibuprofen was completely degraded. At the same time, diclofenac achieved 60% removal which required post treatment for complete removal (Langenhoff et al. 2013). In another study that assessed the bacterial consortium's removal efficiency of a mixture of pharmaceutical compounds, researchers found that bacteria species achieved 100% removal for ibuprofen and 56% for diclofenac, (Aissaoul et al. 2017). Elsewhere, De Gusseme et al. (2011) used *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* to efficiently remove greater than 90% of paracetamol in a bioreactor. In a study conducted by Lin et al. (2015) on activated sludge, *Pseudomonas* spp. (CE22) achieved 88.1–100% removal efficiency for sulfamethoxazole, 66.3–74.1% for caffeine, 64.5–96.9% for salicylic acid, and 77.8–87.1% for chloramphenicol. For nutrients, *Pseudomonas* and *Paracoccus* spp. strains have been found to be highly effective for nitrate removal from wastewater (Foglar et al. 2004). The mixed bacterial strains in this study reduced nitrate concentrations from 200 mg/L to 0.11 mg/L. In another study, *Pseudomonas aeruginosa*, demonstrated a high capacity for mercury ion adsorption, with a maximum uptake reaching approximately 180 mg/g (Yin et al. 2016). Elsewhere, Zhang et al. (2012) identified *Pseudomonas putida* as an effective agent for mercury bioremediation in marine environments. This strain can adsorb nearly all total mercury, showcasing its potential to address this contaminant. In a separate study, Hlihor et al. (2017) investigated the adsorption capabilities of both live and dead *Arthrobacter viscosus* biomass for chromium (VI). They found that these biomasses not only exhibit high adsorption capacity and efficient recovery but also possess the ability to regenerate. Remarkably, they can

also reduce chromium (VI) to chromium (III) in aqueous solutions, achieving complete removal of chromium (VI) at initial concentrations below 100 mg/L. Research by Dave et al. (2010) explored the use of *Eichhornia* spp. biomass harvested from Chandola Lake for copper removal. Their findings indicated that this biomass removed 85.0% of copper from a solution containing 100 mg/L of copper (II). A study by Magnin et al. (2014) examined the bacterium *Rhodobacter capsulatus*, which absorbed zinc (II) with a maximum uptake capacity of 164 mg/g.

These studies highlight the diverse capabilities of bacteria-based solutions in effectively different contaminants from wastewater. However, more research is still needed in this field, especially on different pathways like biotransformation, which has yet to be thoroughly investigated. Widening the knowledge of the role that bacterial communities play in bioremediation is very important for future applications in technologies designed to remove emerging contaminants efficiently. Furthermore, long-term monitoring studies are essential to assess the stability and reliability of bacterial treatments under varying environmental conditions. Optimization of bioremediation processes, including substrate availability and growth conditions, could further enhance treatment efficiencies. Additionally, exploring novel bacterial strains tailored for specific contaminants and integrating bacterial bioremediation with advanced technologies will be critical in tackling challenges such as incomplete removal and the presence of recalcitrant pollutants.

2.6 Aquatic plants (Phytoremediation)

The term phytoremediation was coined in 1983. However, its implementation started in 1991 when rooted plants and trees were used to assimilate, metabolize, degrade, or detoxify metal contaminants and organic chemical contaminants (Henry 2000, Ojha et al. 2021). This technology is relatively easy to actualise, easy to maintain, environmentally friendly, relevant for long-term application, and cost-effective. Phytoremediation technologies can treat contaminants like nutrients, chlorinated solvents, heavy metals, pesticides, pharmaceuticals, explosives, crude oil, phenols, and many other organic and inorganic contaminants (Ansari et al. 2020, Ojha et al. 2021). Several aquatic plants are widely used to eliminate contaminants in water resources. They include *Pistia stratiotes* (water lettuce), *Eichhornia crassipes* (water hyacinth), *Chrysopogon zizanioides* (vetiver grass), *Phragmites australis* (common reed), *Azolla* (duckweed fern), *Spirodela* (duckweed), *Potamogeton* (pondweed), and many more (Ansari et al. 2020, Özengin and Emaci 2016). Aquatic plants have 4 pathways to reduce and/or

eliminate contaminants in water. First is phytoextraction, which is also referred to as phytoaccumulation; through this technique, contaminants are accumulated on plant roots, shoots, or leaves (Ojha et al. 2021). The plants that are involved in this treatment must beremoved from the wastewater to ensure that contaminants are not being recycled back into the surrounding medium (Suman et al. 2018). The second is phytotransformation or phytodegradation, in this pathway, contaminants are absorbed and transformed into a more stable, less mobile, and less toxic form. The third is phytovolatilization, which is the process whereby plant roots absorb contaminants, convert them into a gaseous state, and then release them into the atmosphere (Ojha et al. 2021). Fourth is rhizofiltration, which is the technique that is responsible for removing contaminants from flowing water (Ojha et al. 2021). During this process, filtration and sorption by plant roots and microorganisms found within the rhizosphere of wastewater contaminants play a crucial role in reducing contaminants.

The use of plants for the removal of pharmaceutical contaminants is said to be an economically practical solution due to the immense availability of a wide range of plant species that have the bioremediation capability for pharmaceuticals (Madikizela et al. 2020). For example, *Eichhornia crassipes* is a naturally occurring aquatic plant found in South African rivers, it has the capability to reduce NSAIDs like naproxen, ibuprofen, and diclofenac (Sibeko et al. 2019, Madikizela et al. 2020). *Typha angustifolia* is a perennial herbaceous plant that has been used for bioremediation purposes. Li et al. (2020) assessed the bioremediation capability of this plant in a constructed wetland, and the removal efficiency for ibuprofen was 76% (Table 5). Findings from this study also revealed that ibuprofen removal efficiency increased with plant development, with maximum efficiency at the bolting or mature stage. In a study by Matamoros et al. (2012), *Salvinia molesta, Lemna minor, Ceratophyllum demersum,* and *Elodea canadensis* were planted in microcosm wetland systems to investigate their phytoremediation potential. Greater than 90% removal of triclosan, diclofenac, and caffeine was achieved in 38 days. A paper by Ilyas et al. (2020) reported high to moderate plant removal efficiencies in constructed wetlands. Such efficiencies were for compounds like monensin (93%), Ofloxacin (89%), Oxytetracycline (87%), sulfapyridine (83%), caffeine (80%), salicylic acid (79%), naproxen (62%), ibuprofen (57%) and sulfapyridine (51%). Elsewhere, Özengin and Elmaci (2016) evaluated *Phragmite australis* (common reed) potential to treat pharmaceutical products in an aqueous solution. In this study, removal efficiencies for carbamazepine, ibuprofen, and sulfadiazine were 89.23, 95.94, and 89.50%, respectively. Numerous studies have explored

phytoremediation, especially on a laboratory scale and they have shown varying degrees of success (de Oliveira et al. 2019, Nguyen et al. 2019, Hwang et al. 2020, Ilyas et al. 2020).

Numerous investigations have explored the proficiency of *Lemna* spp. in detoxifying heavy metals from wastewater. In their study, Sekomo et al. (2012) documented substantial removal rates: 94% for chromium, 36% for lead, 33% for cadmium, 29% for copper, and between 51% and 82% for zinc from a mixed metal solution. In a different study, Basile et al. (2012) achieved notable removal efficiencies of 95% for cadmium, 93% for lead, 81.2% for zinc, and 86.5% for copper using *Lemna* spp., highlighting diverse metal accumulation patterns within plant tissues. Abdallah (2012) illustrated that *Lemna gibba* achieved removal rates of 86.2–94.8% for chromium and 91.0–96.4% for lead in laboratory settings. Additionally, Goswami et al. (2014) showcased that *Lemna minor* effectively removed over 70% of arsenic over a 22-day period, emphasizing its potential as a hyperaccumulator of arsenic at low initial concentrations and its application in arsenic remediation efforts. Research has underscored water lettuce (*Pistia stratiotes*) as a highly efficient bio-accumulator of heavy metals across various environmental settings. Vesely et al. (2011) illustrated significant lead removal rates exceeding 90% within a week in greenhouse experiments, while cadmium removal efficiency remained constant regardless of concentration increases. In stormwater detention ponds, as observed by Lu et al. (2011), water lettuce roots absorbed more than 50% of calcium, cadmium, copper, iron, magnesium, manganese, and zinc. Aurangzeb et al. (2014) documented water lettuce's capability to remove 70.7% of lead and 66.5% of copper from steel effluent. Recent studies have also explored water hyacinth (*Eichhornia crassipes*) as a robust bio-accumulator of heavy metals across diverse environmental settings. In research by Sekomo et al. (2012), conducted using up-flow anaerobic packed bed reactors, impressive removal rates were observed: 98% for cadmium, 99% for copper, 84% for zinc, and 98% for lead.

Several studies have highlighted the effectiveness of various aquatic plants for nutrient removal. For example, Rezania et al. (2021) reported that species such as *Azolla* and *Eichhornia crassipes* achieved the highest uptake abilities, removing up to 90% of phosphorus. Sricoth et al. (2018) found that *Eichhornia crassipes* achieved maximum total phosphorus (TP) removal of 42.9% in 7 days. Water lettuce (*Pistia stratiotes*) demonstrated a high TP removal efficiency of 93.6%, attributed to its long roots (approximately 49 cm) and high rhizofiltration capacity (Qin et al. 2016, Lu et al. 2018a). Kumar and Deswal (2020) used four species of free-floating plants (duckweed, water lettuce, Salvinia, and water hyacinth) and reported an average

TP reduction of 80%. Keizer-Vlek et al. (2014) utilized *Iris pseudacorus* and *Typha angustifolia* in floating treatment wetlands (FTWs) for phosphorus removal from urban surface waters, *Iris pseudacorus* had a significantly higher TP removal efficiency of 92% compared to 23% for *Typha angustifolia*. In another study, switchgrass (*Panicum virgatum*) was identified as the top-performing species, removing up to 64.7% of phosphorus in floating wetlands (Spangler et al. 2019). *Spartina maritima* showed positive and consistent phosphate removal with an efficiency of 89.0 ± 7.2% (Jesus et al. 2017). Du et al. (2017) found that vertical-flow constructed wetlands planted with *Canna generalis* achieved 77% TP removal with a three-day hydraulic retention time in autumn. Numerous other studies have also discussed nutrient uptake by macrophytes and aquatic plants, emphasising their potential in wastewater treatment (Daneshgar et al. 2018, Prabakaran et al. 2019, Yadav et al. 2018, Ali et al. 2020, Mustafa and Hayder 2021). The application of macrophytes for removing pharmaceuticals, heavy metals, and nutrient compounds from contaminated water has shown promising results. However, this technology has been predominantly explored outside African countries, leaving a significant research gap in the African context, where it holds great potential as a viable strategy. Furthermore, current research has typically focused on a limited number of analytes and studied them in isolation, even though pharmaceuticals, heavy metals, and nutrients often co-exist in water resources. Therefore, future studies should investigate using a wide range of plant species to remove these multiple compounds from water simultaneously. Despite phytoremediation being environmentally friendly and efficient in treating contaminated waters, it has been reported that sometimes the aquatic plants selected pose a huge threat to the aquatic ecosystems as they become invasive (Ansari et al. 2020). Therefore, well-defined management strategies that include chemical, biological, physical, and mechanical techniques are required to remove and dispose of produced biomass to prevent the propagation of potentially invasive species (Ansari et al. 2020). It is also important to note that the disposal mechanism of the generated biomass should be well-planned so that the contaminants are not returned to the environment where they can cause future problems. Climatic conditions are part of the limiting factors for this treatment process because plant growth and performance rely heavily on them. Hence, phytoremediation cannot be viable in all areas.

There are still several factors that need to be investigated to understand the potential of this treatment approach fully. Such factors include soil type used, the interaction of contaminants with soil, the retention time for phytoremediation, the accumulation capacity of plant species, the bioavailability of contaminants, the effect of invasive plant species, and pest and disease

attacks on plants. Genetic engineering needs to be considered as it uses recombinant technologies that give potential plants traits that enable them to withstand harsh conditions and generate a high yield. Researchers should also consider water-soluble protein from plant seeds as they present a promising solution for removing pharmaceutical compounds in water (Madikizela et al. 2020). As an example, Kebede et al. (2019) showed 72.4 – 91.5% removal of antibiotic drugs like sulphonamides, fluoroquinolones, macrolides, and tetracycline in wastewater by using water-soluble proteins extracted from *Moringa stenopetala* seeds. The use of aquatic plants for bioremediation has been well documented. However, more research is still needed as limited studies focus on pharmaceutical removal, especially in African countries.

2.7 Areas that should be further addressed, and recommendations to researchers

Factors like temperature, pH, and seasonal variation are the main limiting factors to large-scale applications, yet ways to overcome these factors are not documented in the available literature. So, an in-depth understanding of these factors is of paramount importance. Another predicament faced in bioremediation is the deficiency of important nutrients that microorganisms need. Such nutrients include carbon availability, nitrogen, phosphorus, and micro-nutrients like iron, manganese, zinc, sulfur, copper, potassium, and magnesium (Maryjoseph and Katheesan 2020). Insufficient nutrients reduce microorganisms' growth and reproduction, consequently affecting the biodegradation rate and effectiveness. This can be overcome through bio-stimulation, which allows the addition of required nutrients by increasing the metabolic activity of employed microorganisms and thus improving the biodegradation rate (Abatenh et al. 2017). Bioremediation can also produce toxic end products, threatening aquatic environments and neighbouring ecosystems. Singh et al. (2022) stated that the development of nanoparticles, bio-composite, and immobilized cells improve the removal efficiency, however, these are associated with additional chemicals and types of equipment which hike the overall cost of the treatment process. Therefore, more efforts should be directed toward developing low-cost compound substrates. Furthermore, bioremediation should be carried out together with other physical and chemical techniques.

Bio-catalysis and bio-surfactants should also be incorporated into these treatment systems to increase their efficiency (Shah and Shah 2020). Genetic engineering is another promising technology that uses recombinant DNA to alter microorganisms' genetic material so that they have specialized characteristics that enable them to perform specific functions during the treatment process. Genetic engineering shows promising results in bench-scale, petri dish, and

bioreactor studies. However, there is sparse knowledge regarding their application in real in situ bioremediation. Therefore, more investigations should be directed to real-world applications. It is important to note that employing fast-growing communities in bioremediation presents an issue of an inevitable build-up of unwelcomed biomass, so researchers need to explore alternative optimum clean-up communities that reach maximum catalytic ability with minimum cell mass (Joutey et al. 2013).

2.8 Conclusion

For pharmaceuticals, heavy metals, and nutrients, a substantial amount of work is still needed in African countries, as the majority of efforts focused primarily on analysis and occurrence, with limited research dedicated to bioremediation studies. This review highlighted the efficacy of biological organisms such as microalgae, fungi, bacteria, and aquatic plants in remediating pharmaceuticals, heavy metals, and nutrients from contaminated water sources. *Chlorella* microalgae has been prominently featured in numerous studies due to its adaptability and effectiveness in removing a wide range of pollutants. However, challenges like toxicity, and energy-intensive harvesting necessitated ongoing research into genetic modifications and innovative harvesting techniques for scalable and sustainable applications. Fungi, such as *Phanerochaete chrysosporium* and *Trametes versicolor*, demonstrated potent enzymatic abilities for mycoremediation, effectively targeting pharmaceuticals and heavy metals. However, continued research is crucial to overcoming operational complexities and advancing fungi-based treatments for sustainable water purification. Bacteria were pivotal in bioremediation due to their resilience in toxic environments. Species like *Flavobacterium, Pseudomonas,* and *Bacillus* showed robust capabilities in degrading pharmaceuticals, adsorbing heavy metals, and removing nutrients; highlighting their versatility in addressing diverse contaminants in wastewater treatment. However, further exploration of biotransformation pathways and the development of tailored bacterial strains are essential for enhancing the effectiveness and reliability of bioremediation technologies over the long term. Plants such as *Pistia stratiotes, Eichhornia crassipes*, and *Typha angustifolia* exhibited promising capabilities in eliminating pharmaceuticals, heavy metals, and nutrients from water bodies. Despite their environmental benefits, challenges such as invasive species control, climatic variability, and biomass disposal optimization require focused research efforts.

In conclusion, leveraging the unique strengths of microalgae, fungi, bacteria, and aquatic plants offers versatile and effective strategies for addressing complex water contamination

challenges. However, continued research and technological advancements are essential to optimize these biological remediation methods, ensuring their scalability, and sustainability.

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Chapter 3 Microalgae and Daphnia as Potential Removers of Nutrients in Wastewater Samples

Abstract

Excess nutrients, particularly nitrogen and phosphorus, in aquatic environments leads to eutrophication, which has several detrimental effects. It harms aquatic life by depleting oxygen, threatens human health by promoting toxic algal blooms, and significantly impacts the economy, particularly industries that rely on clean water, like ecotourism. High concentrations of these nutrients have been detected, yet there remains limited knowledge on how to completely remove them, and current methods are not sufficiently effective in eliminating them entirely. So, developing innovative and efficient wastewater treatment processes is imperative to address this challenge. Bioremediation, using microorganisms such as microalgae and daphnia, is a promising tool due to their remarkable characteristics. Therefore, this study delved into the synergy of *Chlorella* spp. and *Daphnia magna* (*D. magna*) as a bioremediation dynamic duo, working in concert to efficiently remove nutrients in water. In bioremediation, *Chlorella* spp. completely removed nitrate and nitrites from wastewater by using them as nitrogen sources or converting them into crucial compounds like amino acids, proteins, and lipids. However, removing ammonia and phosphate presented challenges, with incomplete removals ranging from 0–45%. The research also highlighted that *D. magna* faced substantial hurdles in nutrient removal when operating alone. However, when these microorganisms were combined, they remarkably decreased nutrient concentrations, mainly nitrate and nitrite. This study identified various possible mechanisms for removing contaminants: assimilation, adsorption, photodegradation, and ingesting nutrient-rich *Chlorella* by daphnia. Still, challenges emerged regarding ammonia and phosphate removals by combining both microorganisms. This can be ascribed to factors like *Chlorella*'s oversaturation, nutrient release through excretion or decay, environmental pH changes, etc. The *D. magna* also played a vital role in controlling excessive algal growth by consuming *Chlorella* spp., which helped maintain a balanced algal population. This synergistic approach enhanced nutrient reduction and improved water clarity and quality. Furthermore, changes were observed in the material structure and SEM images before and after the bioremediation experiments, implying that the contaminants present in the wastewater were transformed or removed by the bioremediation process. Based on the overall results, *Chlorella* spp. and *D. magna* for nutrient removal hold promise for future wastewater treatment. Moving forward, future studies should focus on

improving the nutrient removal capabilities of *Chlorella* spp. and *D. magna*, especially for ammonia and phosphate. Exploring optimized environmental conditions and co-cultivation strategies to enhance *D. magna*'s nutrient assimilation, along with studying their interactions under different environmental scenarios, will advance bioremediation strategies. These efforts aim to enhance wastewater treatment and support sustainable water management that is aligned with global environmental goals.

Keywords: Ammonia, *Chlorella* spp., *Daphnia magna*, Nitrate, Nitrate, Wastewater

3.1 Introduction

Wastewater is a by-product of human activity that contains various pollutants, including nutrients such as nitrogen and phosphorus, which have caused significant public concerns worldwide. These nutrients are often in high concentrations due to wastewater discharge, industries, and agricultural runoff. If not properly treated, these nutrients can cause severe environmental problems. In water environments, high nutrients can lead to a phenomenon called eutrophication, where there is an overgrowth of microalgae and other aquatic plants. Eutrophication can lead to increased water acidity, increased cyanotoxins, preventing sunlight penetration, and reduced oxygen levels in the water, resulting in a condition known as hypoxia (Tan et al. 2023). Hypoxia can result in the death of many fishes (catfish, trout, bass, etc.) and other aquatic organisms that depend on oxygen to survive (Zhao et al. 2020, Bulbul Ali and Mishra 2022, Lucchesi et al. 2022). There have been several documented cases of hypoxia in various water bodies worldwide. For example, the Gulf of Mexico, the Black Sea, and Lake Victoria are other water bodies that have recently experienced hypoxia (Ogendi et al. 2021, Robinet et al. 2022). Hypoxia has also been reported in South Africa. For example, research conducted at the Vaal Dam demonstrated that the dam underwent yearly oxygen depletion, with hypoxia occurring in the dam's deepest areas during the summer season (Ncube et al. 2020)

Eutrophication not only leads to significant disruptions in aquatic organisms' ecosystems but also affects human public health. Some species that thrive during algal blooms produce elevated toxins and bacterial growth, resulting in numerous health risks in humans, from skin irritation, liver damage, respiratory problems, and even death if ingested (Zhidkova et al. 2020, de Vries 2021). The toxins can be consumed by eating contaminated seafood, drinking contaminated water, or inhaling as aerosols when waves or wind stir the water. Eutrophication

can also have significant economic impacts. The loss of fish and other aquatic organisms can have repercussions for both commercial and recreational fishing sectors, as well as a detrimental impact on water quality. This, in turn, can harm the tourism industry, leading to economic losses and heightened expenses for water treatment (Sampat et al. 2021). Thus, proper management and treatment of wastewater are essential to prevent the negative impacts of excess nutrients.

In most regions, wastewater treatment processes consist of 2 systems; the first is the primary treatment, where large solids are segregated from water. This is followed by the secondary treatment, where degradable organic matter is eliminated biologically (Tan et al. 2023). Nonetheless, treated wastewater still consists of a significant amount of nitrogen and phosphorus, which generally results in eutrophication. To overcome this, much innovation has emerged over the recent years about conventional treatment processes. These included advancements such as chemical and physical methods like Sequencing Batch Reactors (SBR), Anaerobic-aerobic method（A/O), Anaerobic-anoxic-aerobic method (A^2/O), oxidation ditch, ion exchange, and coagulation precipitation (Li et al. 2021). However, several drawbacks are associated with such methods, including the large number of chemicals required and complex and high operational costs, and they tend to produce secondary pollution and greenhouse gasses (Li et al. 2021, Tan et al. 2023). Despite these advancements, there remains a clear research gap in developing wastewater treatments that are both efficient and cost-effective, especially as global populations continue to grow rapidly.

In response to these challenges, this study focuses on wastewater bioremediation using microalgae, a promising alternative that harnesses living microorganisms to remove nitrogen and phosphorus pollutants efficiently. Bioremediation has several advantages over traditional wastewater treatment methods. First, they reduce greenhouse gas production, are relatively easy to construct, have cheaper capital costs, and require less power consumption (Tan et al. 2023). Microalgae emerged as an ideal choice for bioremediation because they have a high capacity to absorb nutrients due to their rapid growth rate and high surface area to volume ratio (Li et al. 2021, Yaakob et al. 2021) (Figure 5.1). They have strong adaptability in wastewater due to their diverse physiological and biochemical traits (Nguyen et al. 2022). Moreover, microalgae can produce large biomass containing numerous metabolites like lipids, protein, pigments, and carbohydrates that can be used for high-valuable products, biofuels, and aquaculture feed supplements (Li et al. 2021, Ebhodaghe et al. 2022, Khan et al. 2023, Tan et

al. 2023). According to Li et al. (2021) and Sadvakasova et al. (2023), microalgae can reduce carbon dioxide emissions by photosynthesizing and storing carbon. Other advantages of using microalgae include low operational cost and the possibility of recycling assimilated nitrogen and phosphorus within the biomass as a fertilizer. Furthermore, it avoids the sludge handling problem and direct discharge of oxygenated effluent water into the water bodies (Makkena 2022, Guo et al. 2023). Freshwater crustaceans (*Daphnia* spp.) also show promising potential in wastewater treatment and nutrient removal (Figure 3.1). Over recent years, these microorganisms have attracted much attention in environmental research, especially in ecotoxicology and water quality, due to their remarkable filtering capabilities (Ebert 2022, Rodrigues et al. 2022). Having specialized thoracic limbs designed for efficient particle capture, daphnia sp. efficiently filters suspended particles, including microalgae and bacteria, from the water column (Gophen 2022). This natural propensity for filter feeding serves as a mechanism for nutrient acquisition. It facilitates the removal of nutrient-rich particulate matter, thus actively contributing to nutrient reduction in aquatic environments.

Figure 3.1 Microalgae and daphnia species used in wastewater bioremediation (Kinsman 2013, Marks 2013, Klepnev 2017)

In pursuing sustainable and cost-effective wastewater bioremediation, the synergistic usage of *D. magna* and *Chlorella* microalgae has emerged as a promising approach for nutrient removal. *Chlorella* microalgae can effectively convert nitrogen and phosphorus compounds into biomass during photosynthesis. *D. magna* is renowned for its proficiency in filtering and feeding on suspended particles such as microalgae. It can actively remove microalgae that have assimilated nitrogen and phosphorus into their biomass, thus contributing indirectly to the reduction of nutrients (Stevčić 2020, Gorzelnik et al. 2023). Therefore, combining these 2 microorganisms in wastewater bioremediation presents a sustainable means of mitigating nutrient pollution and holds promise in a more ecologically balanced aquatic environment. Therefore, this research aimed to investigate the potential of *Chlorella* spp. and *D. magna*-

based bioremediation to address nutrient pollution in South African wastewater. This could provide a sustainable and cost-effective solution to improve water quality and significantly impact achieving the Sustainable Development Goals (SDGs) of clean water and sanitation, life below water, affordable and clean energy, and responsible consumption and production.

3.2 Methodology

3.2.1 Study site

The Darvill wastewater treatment plant was used as a study site based on the available historical data, which suggests a high prevalence of nutrient concentration (0.1 – 31.60 mg/L) in the final wastewater effluents of this si te (Jwara et al. 2020, Sosibo 2022). The samples were taken from the influent and effluent points using a brown glass bottle to prevent light exposure. Light exposure can ruin the sample's integrity by changing the chemical and biological properties, potentially affecting the experiment's results. Before analysis, samples were kept in the refrigerator at about 1–8 °C to eliminate microbial growth or chemical reactions that may change with the sample integrity. Psycho-chemical parameters like pH, electrical conductivity (EC), temperature, and dissolved oxygen (DO) were quantified using a YSI multiparameter (Appendix 1).

3.2.2 Analytical reagents

Nutrient kits were bought from Lasec (South Africa), and they contained all required reagents (Ammonia VARIO HR TT, Nitrate VARIO TT, Nitrite T, and Phosphate HR T) and the MD600 instruments. The *D. magna* kit was purchased from ToxSolutions (South Africa), which consisted of growth media reagents, tubes with ephippia (*D. magna* eggs), concentrated hatching, and toxicant dilution medium. Nutrients were obtained from Sigma Aldrich (South Africa), and this study used ammonia, nitrate, nitrite, and phosphate.

3.2.3 Instrumentation

The MD600 multiparameter photometer from Lasec (Durban, South Africa) was used for nutrients, and the analysis was performed according to the manufacturer's protocol.

3.2.4 Preparation of stock solution

To prepare the standard stock solutions for ammonia, nitrate, nitrite, and phosphate, 10 mg of each analyte was accurately weighed and individually transferred into separate 100 mL

volumetric flasks. Distilled water was added to each flask up to the 100 mL mark, resulting in a concentration of 100 mg/L for each standard solution. To calibrate the MD600 instrument, standard working solutions were subsequently prepared by diluting the respective standard stock solutions. The concentration range (0.01 – 80 mg/L) was tailored to accommodate the different levels and detection limits associated with each analytical method. However, care was taken to ensure that the concentrations fell within a suitable calibration range to ensure the accuracy of the analysis. All prepared solutions, both the stock and working solutions, were carefully stored in a refrigerator at about 1 – 8°C to maintain their stability and integrity until they were ready for experiment use.

3.2.5 Method validation

The performance of the nutrient methods was investigated through regression equations. To determine the specificity of the MD600 instrument, a recovery test was performed by spiking samples and measuring the recovery rate. The recovery rate should be close to 100% to indicate that the method is specific and accurate for measuring the nutrients of interest. Precision was investigated through repeatability and reproducibility, and the results were given as the percentage relative standard deviation (%RSD). Equation 5.1 was utilized to calculate the percentage recovery.

$$\%\ Recovery = \frac{measured\ nutrient\ concentration}{spiked\ nutrient\ concentration} x\ 100 \qquad (3.1)$$

3.2.6 Algal strain

In this study, *Chlorella* spp. strain FSB41.2 obtained from the uMgeni-uThukela Water (Pietermaritzburg, South Africa) was utilized. Tris-acetate phosphate (TAP) medium was prepared according to Burgess (2016). Upon the initial analysis, cultures revealed the presence of both bacteria and *Chlorella* spp. cells, prompting the exploration of two methods for isolation and cultivation. Method 1 involved dilution streaks to enable pure *Chlorella* spp. growth, while method 2 focused on removing acetic acid from the TAP medium, which normally attracts unwanted organisms or contaminants. Then, cultures were maintained in a controlled environment (temperature at approximately 23°C and 20 hours: 04 hours light-dark cycle) to support growth. Monitoring was conducted by measuring absorbance at 680 nm using a UV/Visible Spectrophotometer to assess culture health and productivity.

3.2.7 Lab experimental set-up (microalgae)

The experiments were conducted on 500 mL conical flasks. All experiments were performed in triplicate. The wastewater effluent samples were filtered through a 55 mm Whatman filter paper to remove large particles and aerated continuously in the dark at room temperature before experiments to ensure sufficient oxygen content (> 5 mg/L). Wastewater pH was adjusted to ~ 7.5 before use. This pH condition is the most favourable to culture freshwater algae (Bai and Acharya 2017). The wastewater effluent samples were then sterilized by autoclaving for 30 min to eliminate bacteria and protozoa. At the beginning of each series of experiments, 250 mL of autoclaved wastewater was inoculated in the flasks with pre-cultured *Chlorella* spp. culture to reach an initial optical density of 1. The flasks were incubated under 20 hours of light: a 4-hour dark period, constant temperature of about 23°C with continuous mixing at 150 rpm. Routinely, 25 mL samples were taken aseptically for analytical measurements. Before analysis, drawn samples were centrifuged for 20 minutes at 4000 RPM to separate the microalgae from the liquid. Then, the supernatant was analyzed by the MD 600 multiparameter photometer.

3.2.8 Lab experimental set-up (daphnia)

The clean and sterile 500 mL glass beakers were filled with 250 mL of sterile and spiked wastewater. A known number of *D. magna* individuals (10 – 50 individuals per 100 mL) was transferred to each beaker using a pipette. The nitrogen and phosphorus levels in the beakers were measured every 24 hours. All experiments were performed in triplicate as well.

3.2.9 Lab experimental set-up (microalgae and daphnia)

The same procedure, as documented in section 3.2.7, was followed. However, an additional step was required where a known number of *D. magna* individuals (50 individuals per 100 mL) were added to each beaker utilizing a plastic pasteur pipette.

Equation 5.2 was used to calculate the nutrient uptake rate (%)

$$\%\ Recovery = \frac{R0}{Rt} x\ 100 \qquad (3.2)$$

% Recovery represents the nutrient removal efficiency, and Rt and R0 are the nutrient concentrations at day t and day 0, respectively.

3.3 Results & Discussion

3.3.1 Method validation

The MD600 instrument was highly accurate and reliable for measuring the concentrations of phosphate, nitrate, nitrite, and ammonia in samples, with an R^2 value ranging from 0.993 to 1, indicating a strong, positive linear correlation between the measured variables (Table 3.1). For the specificity of the MD600 instrument, recovery ranged from 94–105%, indicating that the method is specific and accurate for estimating the nutrients of interest (Table 3.1).

Table 3.1 Method validation parameters tested for the MD600 instrument (n=3)

Compounds	R^2	Measuring Range	Recoveries ± % RSD		
			Wastewater effluent	Repeatability	Reproducibility
Ammonia	0.993	1.0–50 mg/L N	95±1.0	100±0.6	99±0.9
Nitrate	0.999	1–30 mg/L N	96±1.5	102±0.9	96±0.9
Nitrite	1.000	0.01–0.5 mg/L N	94±1.9	98±0.4	97±0.5
Phosphate	0.998	1–80 mg/L P	96±1.2	105±0.9	102±0.7

3.3.2 The effect of culture concentration

The optical density (OD) measures the concentration of microalgae in the culture medium. An OD of 1 typically corresponds to a cell density of approximately 107 cells/mL. This study revealed that the 8% culture had higher growth than the 4% culture, which can be attributed to cell-cell interactions at lower cell densities (Figure 3.2). *Chlorella* spp. may not be able to produce sufficient growth factors, hormones, or signalling molecules to promote growth (Brennan et al. 2019, Ganuza et al. 2020), which could lead to slower growth in the medium with 4% inoculum.

High optical density was obtained for the 8% culture relative to the 4% culture (Figure 3.2a). That could be ascribed to higher biomass, resulting in a heightened nutrient uptake rate due to the more significant number of cells available. The media's pH and dissolved oxygen levels can also affect *Chlorella*'s growth and nutrient uptake. The higher biomass density of the 8% culture could have caused changes in pH and dissolved oxygen levels, resulting in more favourable nutrient uptake conditions. Cell-to-cell communication can also impact microalgal

nutrient uptake and growth. In particular, some microalgae species engage in quorum sensing, where cell-to-cell communication coordinates population-level behaviours (Ganuza et al. 2020).

Figure 3.2b also revealed high removal of nitrate and nitrites in both cultures; this can be ascribed to the ability of microalgae to use nitrates and nitrites as a nitrogen source for growth and metabolism (Wang et al. 2021, Pozzobon et al. 2021). The high biomass concentration in the 4% and 8% cultures could have facilitated efficient nitrogen utilization. This resulted in the complete conversion of nitrates and nitrites into other compounds, such as amino acids, proteins, and lipids, thereby contributing to their effective removal (Wang et al. 2020, Pozzobon et al. 2021, Yaakob et al. 2021). For subsequent experiments, the 8% Culture was used.

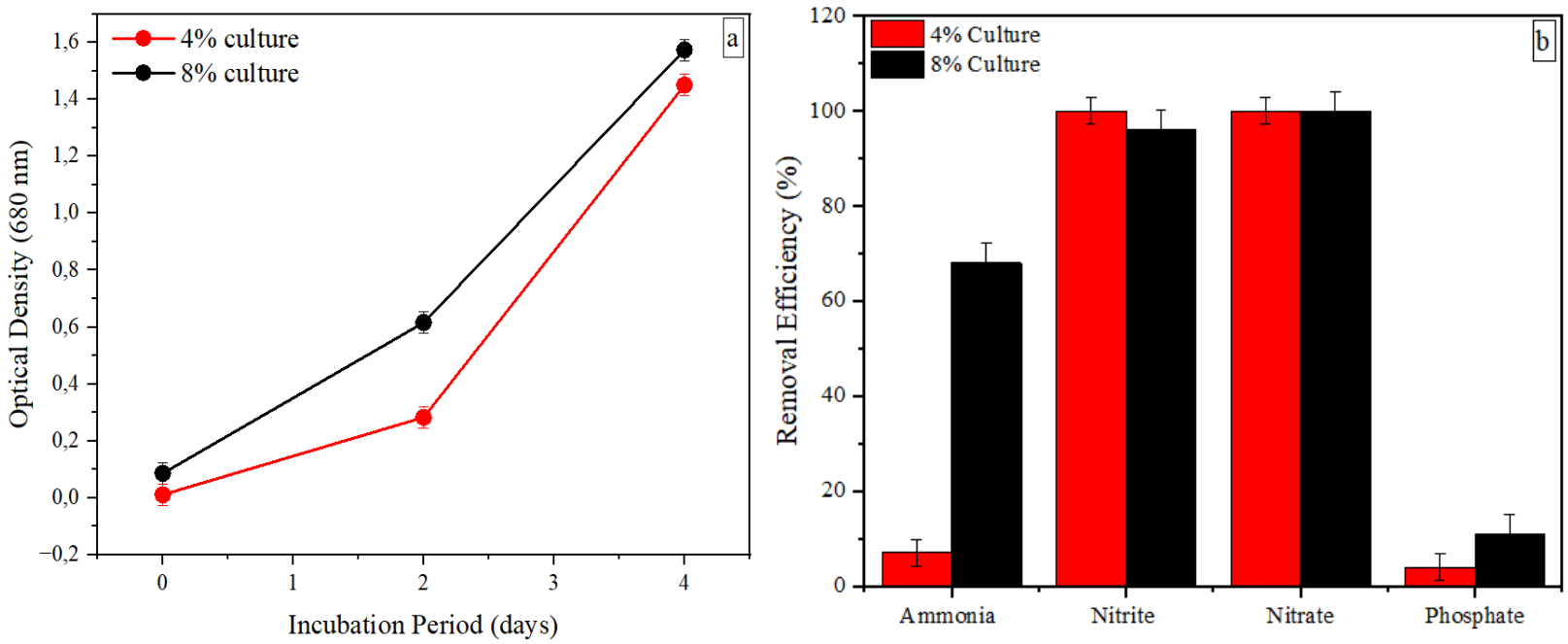

Figure 3.2 The graph depicts the relationship between Chlorella spp. culture concentration and Chlorella spp. optical density (a), as well as the corresponding nutrient removal efficiencies (b), with n=3 replicates. The graph depicts the relationship between *Chlorella* spp. culture concentration and *Chlorella* spp. optical density (a), as well as the corresponding nutrient removal efficiencies (b), with n=3 replicates.

3.3.3 Effect of Chlorella spp. on removing nutrients in wastewater effluent

For the NH_3, NO_3, NO_2, and PO_4 groups, the optimal density values increase with incubation time (Figure 3.3). This suggests that the microalgal population was actively utilizing the nutrients and that the bioremediation process was progressing.

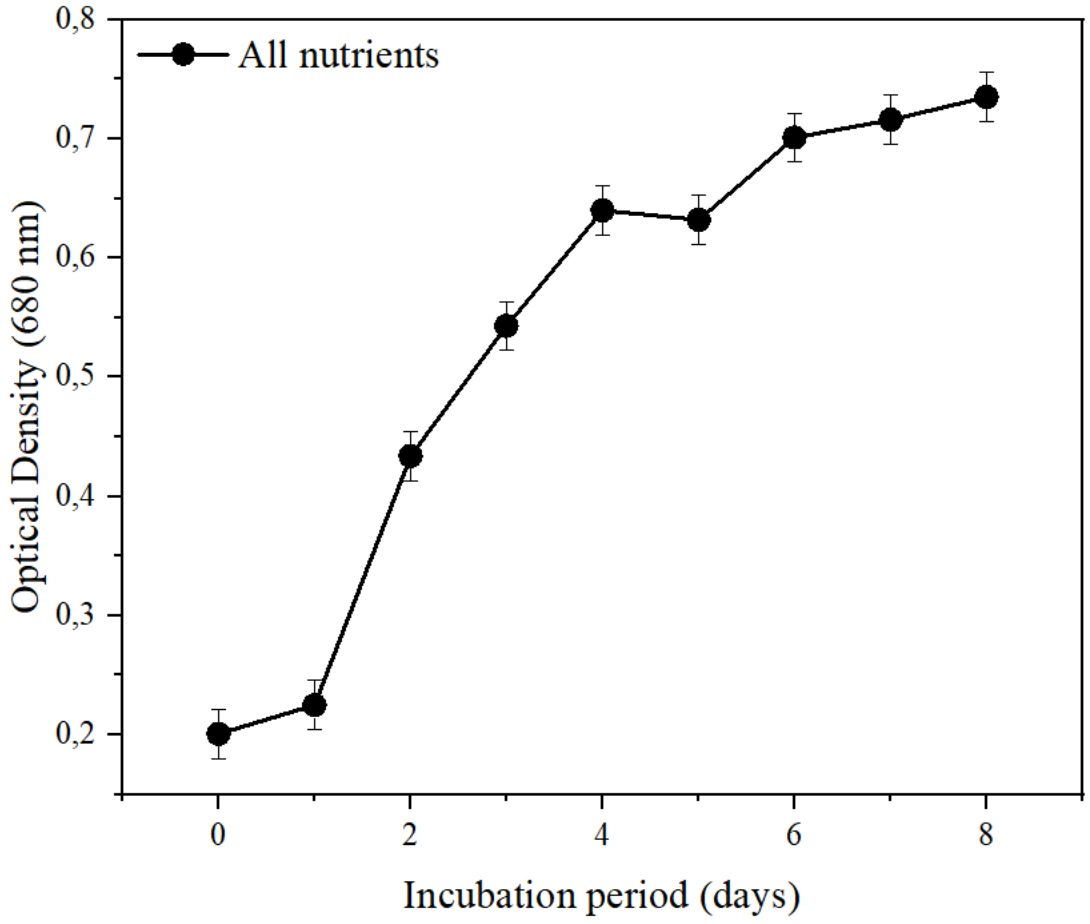

Figure 3.3 The effect of all nutrients (NH_3, NO_2, NO_3, and PO_4) on microalgal growth with n=3 replicates

After the second day of the experiment, nitrate was found to be below the detection limit due to several factors (Figure 3.4). Firstly, *Chlorella* spp. is known to have a higher affinity for nitrate than other pollutants such as ammonium and phosphate (Zhang et al. 2019). This means that *Chlorella* spp. may preferentially take up nitrate over other environmental nutrients, leading to more rapid removal of this pollutant. Secondly, the enzymes involved in assimilatory nitrate reduction in *Chlorella* spp., such as nitrate reductase and nitrite reductase, may be more efficient at converting nitrate into ammonium ions than the enzymes involved in other nutrient removal pathways (Sivakumar et al. 2020). This would result in faster nitrate removal than

other pollutants. The results of this study also exhibited an efficient reduction of nitrite (Figure 3.4).

Similarly, to nitrate, nitrite may be preferentially taken up by *Chlorella* spp. due to its structure and function. It is also important to note that due to water-solubility, nitrate and nitrite are more easily degraded than other pollutants, and *Chlorella*'s ability to proliferate and tolerate a wide range of environmental conditions makes them effective in bioremediation (Ma et al. 2019). According to Nguyen et al. (2022), microalgae possess the capability to remove nitrate and nitrite from wastewater through nitrogen assimilation. This is where inorganic nitrogen is converted to an organic form, a building block of numerous peptides, proteins, enzymes, chlorophylls, and energy transfer molecules like adenosine diphosphate (ADP) and adenosine triphosphate (ATP). Through the nitrogen assimilation process, both nitrate and nitrite are converted to ammonium by nitrate and nitrite reductase enzymes found within *Chlorella* spp. cells. Kumar and Bera (2020) highlighted that nitrogen compounds can be converted into ammonium within algal cells through enzymatic pathways involving nitrate reductase and nitrite reductase. In another research endeavour, Kaviraj and Kumar (2024) reported the conversion of complex nitrogen complex such as nitrate to ammonium in both prokaryotes and eukaryotes through enzymic reactions involving nitrate reductase, nitrite reductase and ammonium assimilating enzymes. Besson et al. (2022) further emphasized the sequential reduction of nitrate to nitrite and then ammonium for anabolic purposes. Additionally, Lui et al. (2022) elucidated the pathway in plants where nitrate was initially reduced to nitrite by nitrate reductase in the cytoplasm, followed by conversion to ammonium by plastidic nitrite reductase within plastids. These studies collectively supported the understanding that in *Chlorella* spp., as in other organisms, nitrate and nitrite were enzymatically converted to ammonium as part of the nitrogen assimilation process, crucial for cellular growth and metabolic functions. Ammonium is utilized in the intracellular amino acid glutamine, which possesses significant and unique metabolic functions (Sivakumar et al. 2020, Nguyen et al. 2022).

Regarding ammonia, it exhibited a high removal rate in the first 2 days, possibly due to *Chlorella*'s high affinity for nitrogen compounds (Huang et al. 2021). Furthermore, the activity of enzymes involved in ammonia assimilation may be higher in *Chlorella* spp. during the early stages of the experiment, leading to a faster removal rate of ammonia (Zhang et al. 2021). After day 2, the removal rate decreased; this can be ascribed to the reduction of ammonia, which is

often linked to the availability of essential nutrients like nitrates and nitrites (Figure 3.4). If these nutrients deplete over time, *Chlorella* spp. may struggle to maintain ammonia reduction rates. Furthermore, other nutrients, such as phosphate, may limit the growth and activity of *Chlorella* spp., resulting in a slower ammonia removal rate (Hong et al. 2021). Additionally, ammonia can form complexes with various anions, such as phosphate and organic acids, reducing the availability and uptake of ammonia by *Chlorella* spp.

A critical step in *Chlorella* spp.'s nitrogen assimilation processes involves the enzymatic conversion of ammonia, nitrate, and nitrite into ammonium (NH_4^+). However, it is essential to acknowledge the dynamic nature of ammonium speciation, particularly in response to changes in pH within the experimental environment. This research showed an increase in pH levels, which could have led to the conversion of ammonium into ammonia (NH_3) in alkaline conditions. This shift from ammonium to ammonia notably impacted our experimental findings. On the 6th and 7th days of the experiment, the results showed a sudden increase in ammonia concentrations, contrary to our initial expectations of continuous decreases. Also, ammonia can form complexes with various anions, such as sulfate, phosphate, and organic acids, reducing the availability and uptake of ammonia by *Chlorella* spp. (Kumar et al. 2015). If the concentrations of these anions in the medium become too high, they can precipitate with ammonia, forming insoluble salts that are unavailable for uptake. Furthermore, the formation of ammonia complexes or precipitates can affect the pH of the medium, which can further impact the uptake and utilization of ammonium by *Chlorella* spp. For example, forming ammonium sulfate complexes can increase the medium's acidity, inhibiting *Chlorella*'s growth and metabolic activity (Liang et al. 2018). Also, it is essential to note that *Chlorella* spp. has a limited capacity to take up and utilize ammonia from the medium. This capacity may become saturated if the initial concentration of ammonia in the medium is high (Kumar et al. 2015). Once the uptake capacity of *Chlorella* spp. is reached, any excess ammonia in the medium will remain unutilized and can accumulate over time, leading to an increase in ammonia levels.

Microalgae play a vital role in removing phosphorus from wastewater through 2 main processes: extracellular adsorption and intracellular uptake. Extracellular adsorption relies on extracellular polymeric substances (EPS), primarily composed of proteins and carbohydrates (Wu et al. 2021). These EPS contain amine groups that can bind to phosphate ions, leading to their adsorption onto EPS. Intracellular phosphorus uptake involves various mechanisms, including phosphate metabolism and polyphosphate formation (Wu et al. 2021). According to Nguyen et al. (2022), inorganic phosphorus can undergo phosphorylation to become part of

intracellular organic compounds like proteins, lipids, and nucleic acids. During this process, many phosphate transporters found within microalgae plasma membranes are utilized. This study revealed an alternative pattern, with inorganic phosphorus displaying limited removal (Figure 3.4). This phenomenon can be attributed to the uptake capacity of *Chlorella* spp., which may become saturated when phosphate concentrations in the medium are too high, resulting in a reduced removal rate (Bhatti et al. 2019). Secondly, phosphate may precipitate in insoluble minerals such as calcium phosphate or iron phosphate, making it unavailable for uptake by *Chlorella* spp. (Bhatti et al. 2019). While the presence of nitrate, nitrite, and ammonium can enhance *Chlorella's* growth and metabolism, an imbalance in the ratio of nutrients can affect the availability of phosphorus and limit its removal (Chen et al. 2019, Li et al. 2021). Moreover, phosphorus biochemicals like ribosomal RNA need nitrogen for synthesis. Thus, drawbacks in nitrogen from the growth medium may hinder phosphorus usage (Tan et al. 2023). The medium's pH is another factor that might affect phosphate removal. At low pH levels, the solubility of phosphate decreases, making it less available for uptake by *Chlorella* spp. On the other hand, at high pH levels, phosphate may precipitate as insoluble minerals, reducing its availability for uptake (Wang et al. 2019). The optimal pH range for *Chlorella* spp. growth and metabolism is typically between 7.0 and 8.5. Outside this range, the activity of enzymes involved in phosphate uptake and utilization may be affected, leading to reduced phosphate removal (Chen et al. 2019). This study showed that nitrogen assimilation is faster than phosphorus for microalgae growth. This complements the empirical formula for microalgae ($C_{106}H_{181}O_{45}N_{16}$ P), which suggests that much nitrogen is required to produce cells (Nguyen et al. 2022).

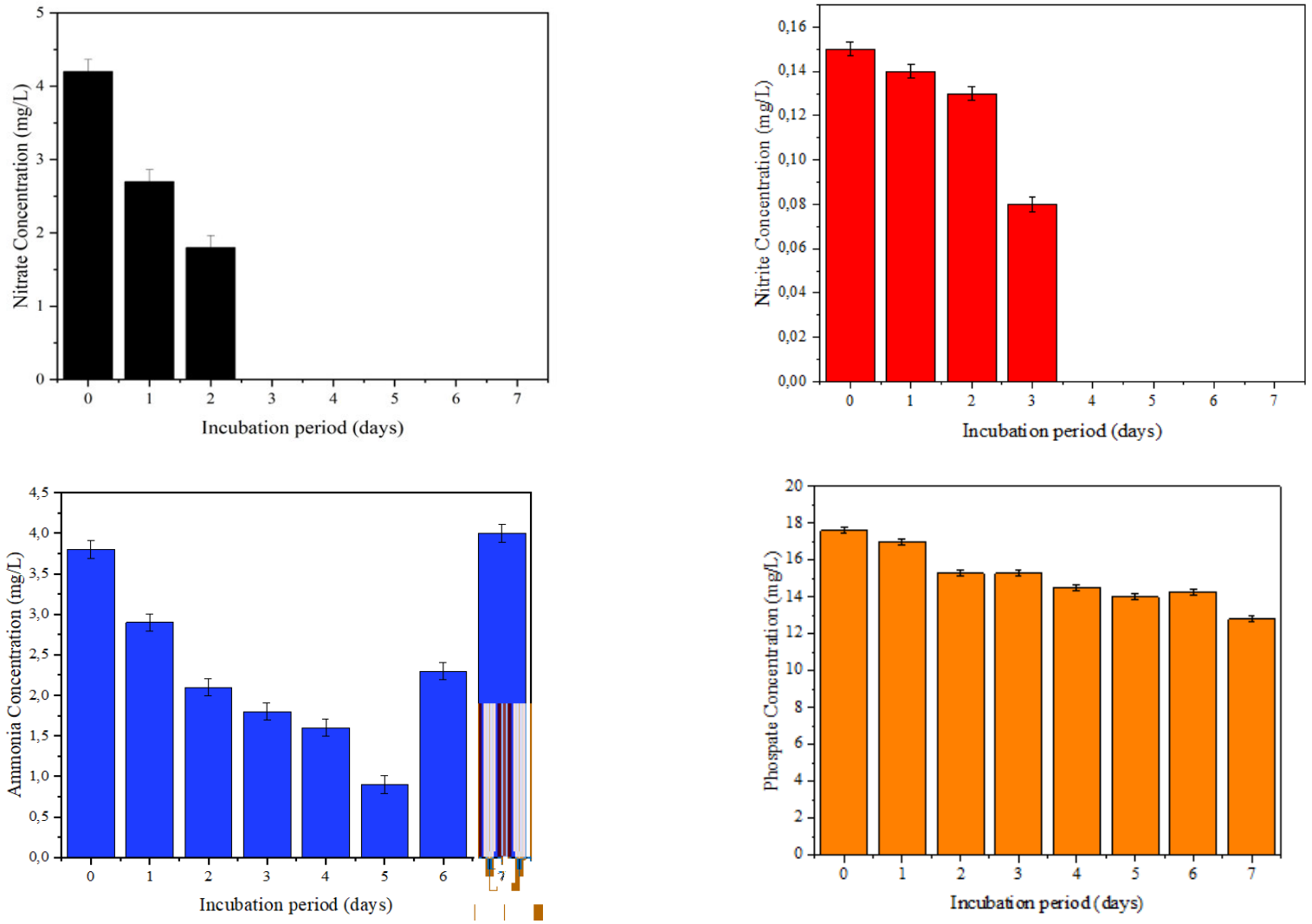

Figure 3.4 The effect of *Chlorella* spp. on removing nutrients ((NH_3-N, NO_2-N, NO_3-N and PO_4-P) from wastewater effluent with n=3 replicates.

Photodegradation can significantly affect the concentration and speciation of nitrogen and phosphorus compounds in water systems (Babaei et al. 2019). Photodegradation is a process by which nutrients can be broken down and transformed through light exposure. The study's findings indicated that photodegradation also played a role in nutrient removal, although on a smaller scale than *Chlorella* spp. (Figure 3.5). Ammonia, for example, can be converted to nitrate and nitrite through photochemical reactions, such as photolysis and photo-oxidation, particularly under high light intensity, low pH, and low oxygen levels (Ding et al. 2017). Similarly, nitrate and nitrite can also undergo photodegradation, forming other nitrogen compounds or releasing nitrogen gas (Qiu et al. 2016). Phosphate can also experience photodegradation through photolysis, where the phosphate molecule absorbs light energy and breaks down into more minor compounds, such as orthophosphate and polyphosphate (Guo et al. 2018).

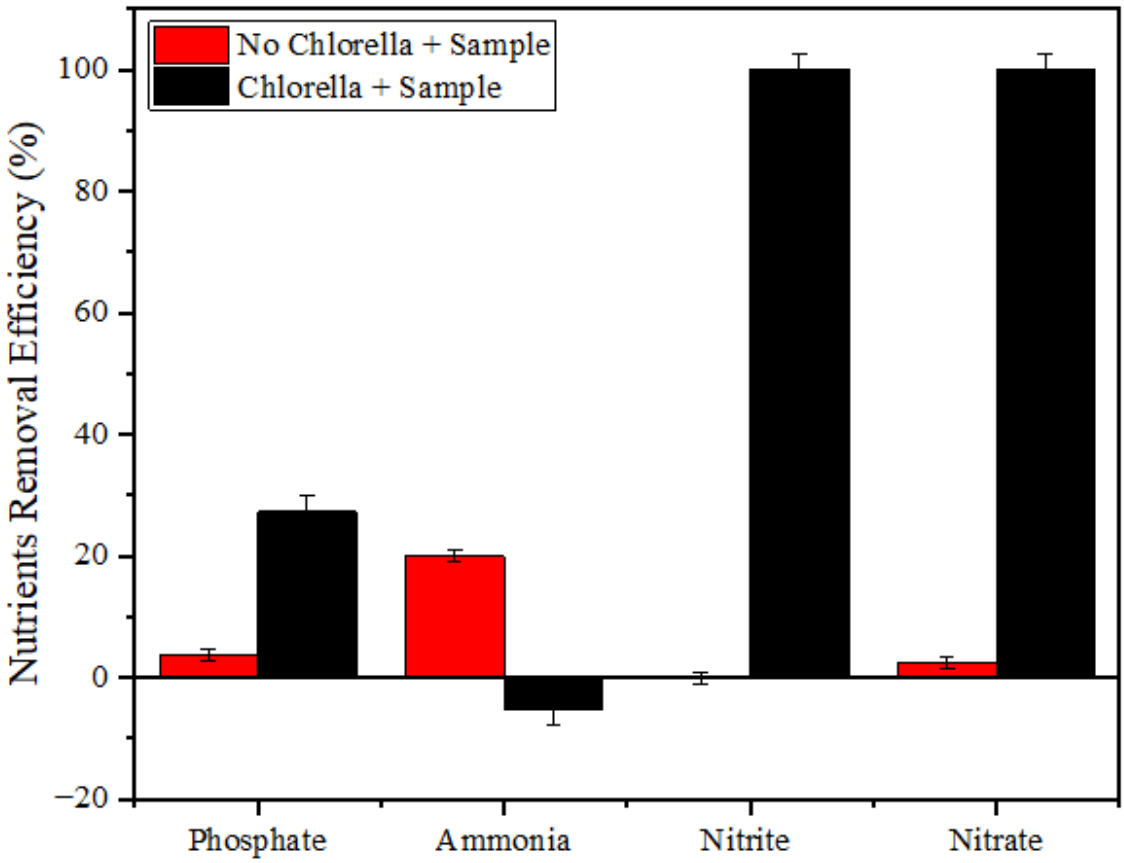

Figure 3.5 The effect of photodegradation and *Chlorella* spp. on removing nutrients from wastewater effluent with n=3 replicates.

3.3.4 The effect of Chlorella spp. on removing nutrients in wastewater influent

Similar trends were observed when assessing wastewater influent, with only slightly higher initial concentrations when compared to effluent samples. The removal efficiency trend was consistent for nitrate and nitrite, which were removed to below detection limits by the end of bioremediation (Figure 3.6). However, ammonia decreased, followed by a steady stage with little removal. On the other hand, phosphate showed a slow reduction, similar to that observed in wastewater effluent.

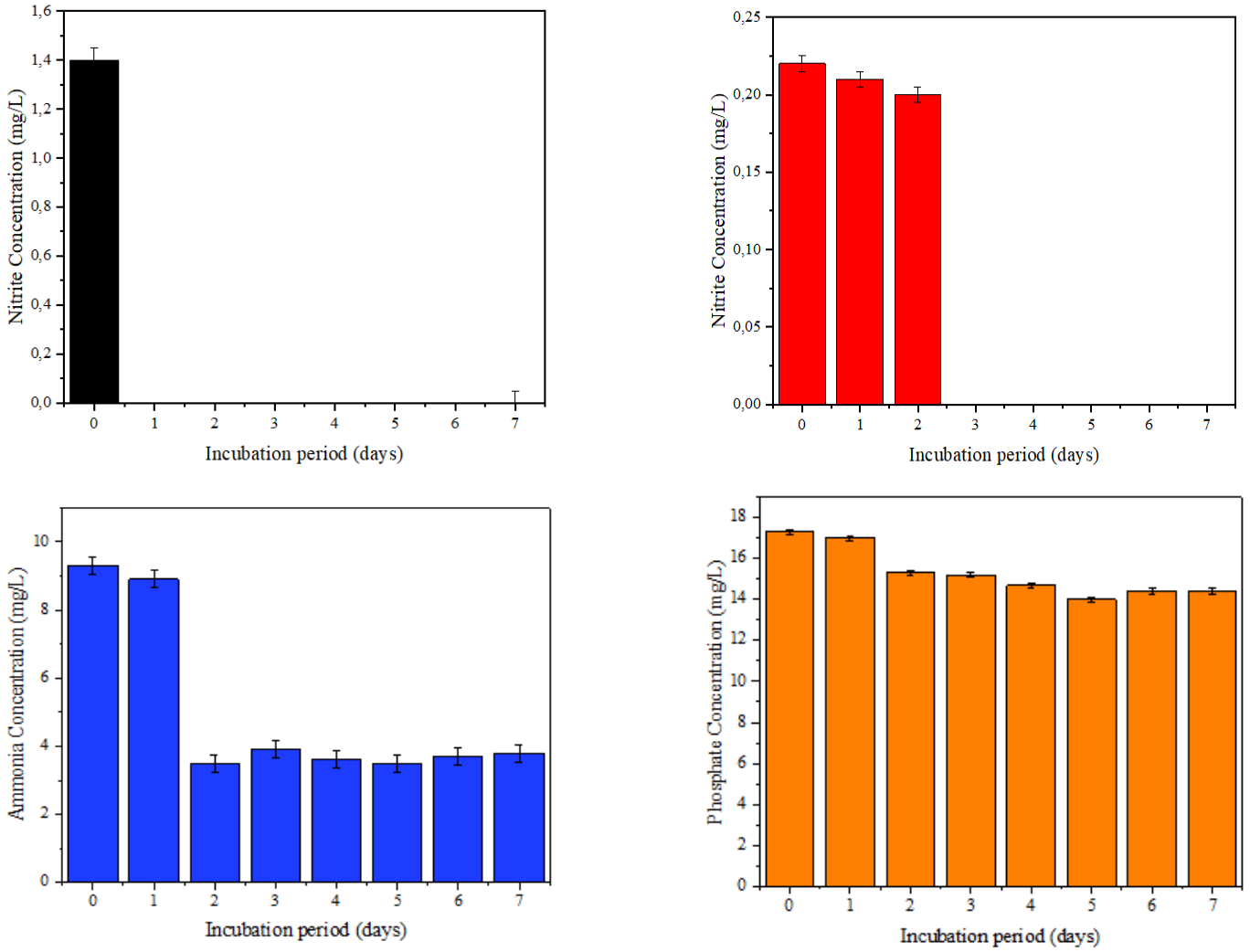

Figure 3.6 The effect of *Chlorella* spp. on removing nutrients (NH_3-N, NO_2-N, NO_3-N and PO_4-P) from wastewater influent with n=3 replicates

3.3.5 The efficiency of D. magna to remove nutrients in wastewater

To determine the population size that will yield better removal of the study nutrients, different daphnia population sizes were investigated and ranged from control with no individuals to 10, 20, and 50 individuals per 100 mL (Figure 3.7). The experiment showed no consistent trend of decreasing nutrient concentrations with larger *D. magna* populations, except phosphate, which elucidated relatively minor changes. One key factor contributing to these observations could be the absence of primary producers like microalgae in the experimental setup. *D. magna* primarily feeds on microalgae, bacteria, and detritus in the water (Stevčić et al. 2020). Therefore, *D. magna* did not directly consume the tested nutrients, resulting in no observable removal by these organisms.

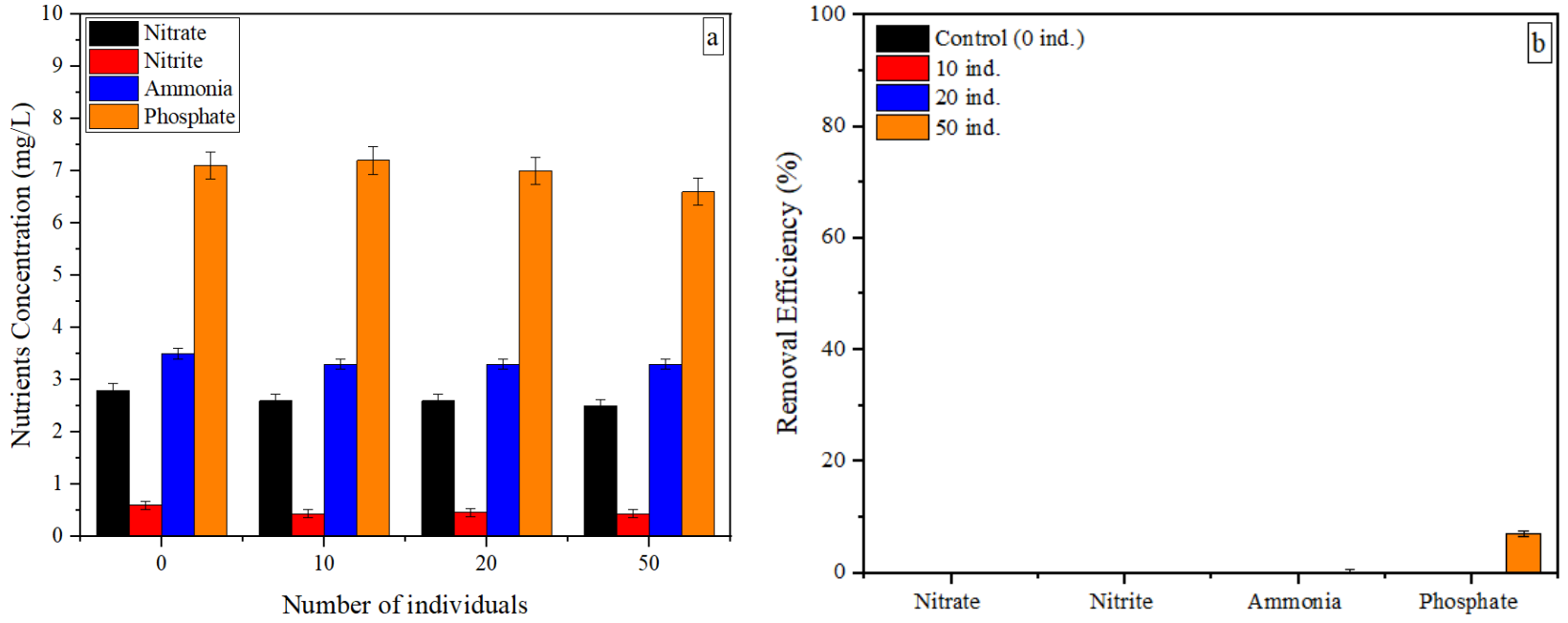

Figure 3.7 The effect of *D. magna* population size on nutrient removal with n=3 replicates. (a) illustrates the nutrient levels measured after the bioremediation experiment, while (b) shows the corresponding removal efficiencies achieved.

To see whether daphnia performance can be enhanced, the microorganisms were exposed to varying nutrient concentrations, including high, medium, and low levels (Figure 3.8). *D. magna* exhibited the capacity to utilize some inorganic phosphate. This aligns with the findings of a study by Parker and Olsen (1966), which elucidated that daphnia can absorb "2P-phosphate" from the water and subsequently store it within their ovaries and muscle tissues within a mere 30 minutes. This reservoir of accumulated phosphate sustains their metabolic processes and supports their reproductive activities. In another research endeavor, Urabe et al. (1997) provided evidence of daphnia's ability to assimilate ample inorganic phosphorus from the surrounding water directly. Ammonia, nitrate, and nitrite concentration showed no significant changes. In aquatic ecosystems, the conversion of ammonia (NH_3) and nitrite (NO_2^-) to nitrate (NO_3^-) usually occurs through a process called nitrification, which is carried out by specific bacteria (Baskaran et al. 2020). Nitrifying bacteria are crucial in oxidizing ammonia and nitrite to less toxic nitrate. However, it's worth noting that the presence of these bacteria was eliminated in the specific study under consideration. This absence of bacteria could explain why ammonia and nitrite were not removed. Regarding nitrates, *D. magna* typically does not directly absorb nitrate ions (NO_3^-) from the water through its structures. They rely heavily on primary producers like microalgae, as nitrate uptake in aquatic organisms is more complex.

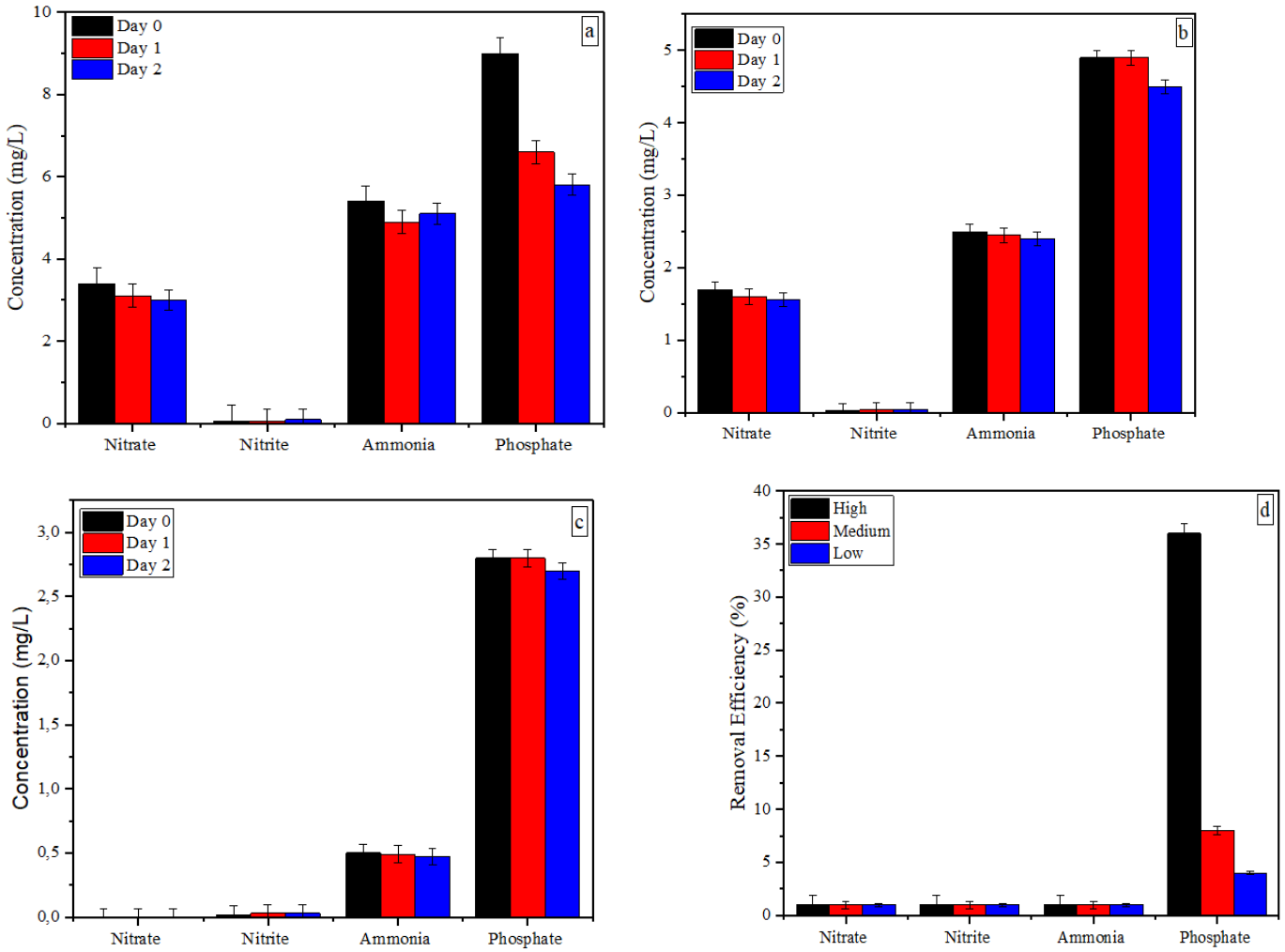

Figure 3.8 Nutrient concentrations measured over a 48 hours period with n=3 replicates: (a) high concentration, (b) medium concentration, (c) low concentration. (d) The overall removal efficiencies after 48 hours of incubation.

3.3.6 Nutrient removal by Chlorella spp. and D. magna

The next phase of this study aimed to evaluate the potential enhancement of *D. magna*'s performance by co-culturing it with *Chlorell*a spp. for nutrient removal. The initial objective was to assess 3 experimental conditions: (1) simultaneous introduction of microalgae and daphnia on the same day, (2) introduction of microalgae first followed by the later introduction of daphnia, and (3) introduction of daphnia first, followed by the later introduction of *Chlorella* spp. This was vital because *Chlorella* spp. can directly uptake nutrients from the water, while daphnia graze on these nutrient-rich microalgae, indirectly aiding the overall nutrient removal process.

Several notable trends emerged during the 7-day experiments. In treatments characterized by high concentrations, where both *Chlorella* spp. and *D. magna* were introduced concurrently on day zero 'High (A+D)', phosphate exhibited a small reduction by 2.2 mg/L on day 4, which equates to a maximum of 13% removal, indicating the slight potential of *D. magna* and *Chlorella* spp. to adsorb and assimilate this nutrient (Figure 3.9a). More significantly, nitrate and nitrite concentrations were below the detection limit by day 2, reflecting effective removal likely driven by microalgae's assimilation of these nitrogen compounds (Figure 3.9a). In treatments characterized by medium concentrations, where *Chlorella* spp. and *D. magna* were introduced concurrently on day zero 'Medium (A+D)', nitrate and nitrite levels consistently dropped below the detection limit by day 2 (Figure 3.9b). Notably, ammonia concentrations increased during High (A+D) and Medium (A+D) experiments, possibly attributed to factors including ammonia release during biological processes or complex ecosystem interactions. Stevčić et al. (2020) documented that nutrients such as ammonia can be re-released to water via the breakdown of microalgal cells and the excretion by daphnia. Daphnia can primarily excrete nitrogen primarily as ammonium (Stevčić et al. 2020).

In the experiments with high and medium concentrations, where algae were introduced first, followed by daphnia, 'High (A 1st+D 2nd)' and 'Medium (A 1st+D 2nd)' (Figure 3.9d-e), removal patterns resembled those observed in the 'High (A+D)' and 'Medium (A+D)' treatments. However, the removal rates were slower due to the delayed introduction of these organisms. Consequently, it took longer to remove compounds like nitrate completely. In the high concentration treatment where daphnia was introduced first, followed by the introduction of microalgae 'High (D 1st + A 2nd)', phosphate concentrations decreased by 3 mg/L, which

equates to a maximum removal of 18% on day 4 (Figure 3.9c). This could potentially be linked to phosphate removal by both *Chlorella* spp. and *D. magna*. In this treatment, nitrate was completely removed while achieving greater than 80% removal, driven by *D. magna* grazing on microalgae that had assimilated these nitrogen compounds into their biomass. Similar to the other experiments, ammonia concentrations increased, possibly due to ammonia release during biological processes or shifts in environmental conditions.

Notably, by day 7, phosphate concentrations revealed an increasing trend in the different treatments, possibly indicating nutrient release in the experiments through excretion and egestion by daphnia. Daphnia primarily excrete phosphorus as dissolved phosphate (Stevčić et al. 2020). Like daphnia, when microalgae cells die or undergo senescence, phosphate can be released back into the water as organic matter decomposes (Gao et al. 2013). The study findings align with Dang et al. (2022), which showed that phosphate was not fully treated due to the potential release through microalgae biomass decay.

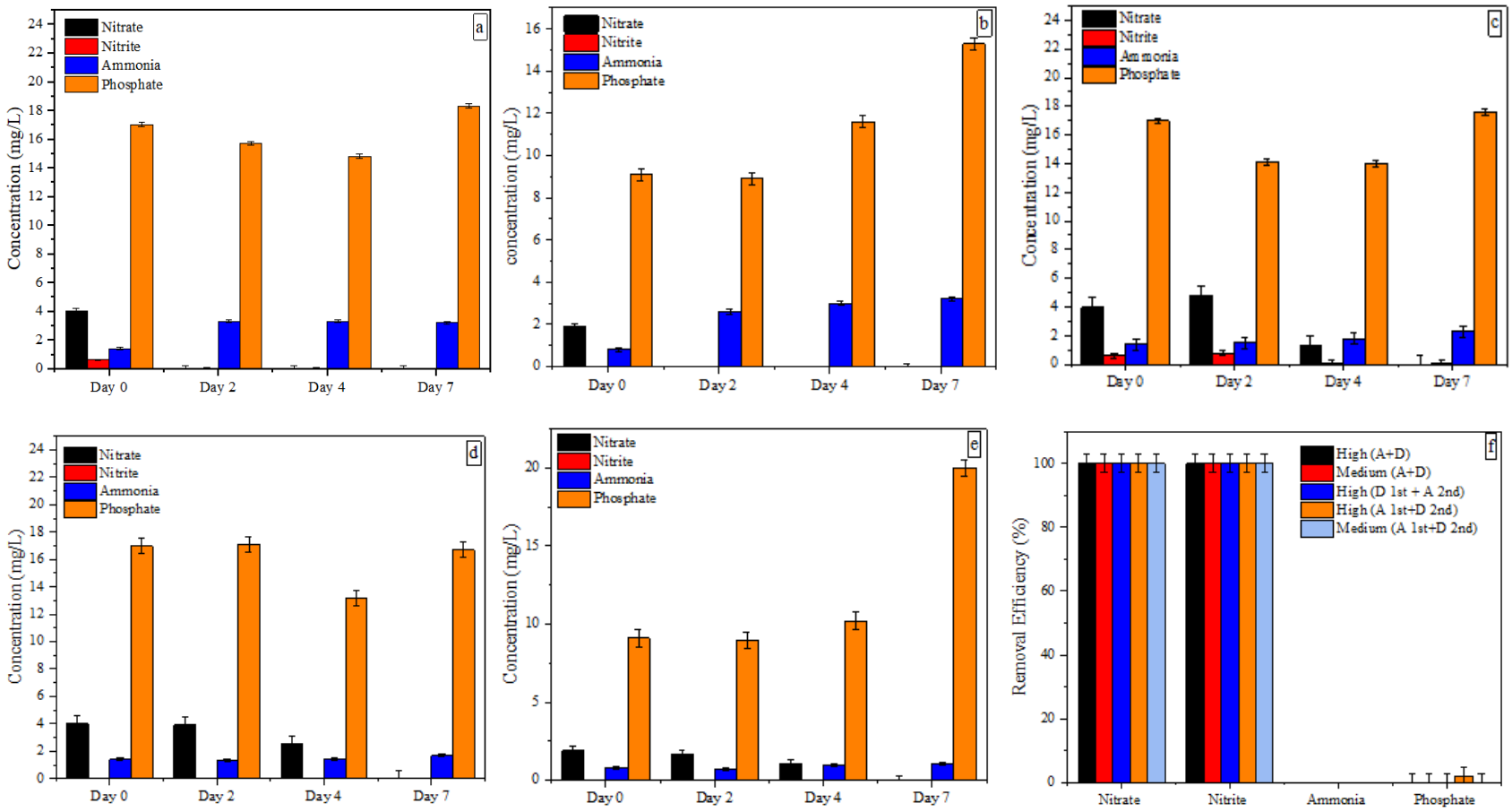

Figure 3.9 Synergistic effect of *Chlorella* spp.and *D. magna* on nutrient removal under different experimental conditions with n=3 replicates. (a) High (A+D), (b) Medium (A+D), (c) High (D 1st + A 2nd), (d) High (A 1st + D 2nd), (e) Medium (A 1st + D 2nd), and (f) overall removal efficiency.

3.3.7 The FTIR results before (a) and after (b) bioremediation experiments using Chlorella **spp.**

The FTIR results indicate the presence of various biomolecules in the *Chlorella* spp. biomass, including lipids, proteins, carbohydrates, and phosphates. These biomolecules play a crucial role in the bioremediation of pollutants by *Chlorella* spp., and their identification through FTIR analysis can provide valuable insights into the mechanisms of bioremediation.

Before bioremediation, the peak at 3279.19 cm^{-1} correlated with the hydroxyl (-OH) functional group (Morais et al. 2020). This peak shifted to 3283.98 cm^{-1} after bioremediation, possibly indicating hydrogen bonding (Figure 3.10). Hydrogen bonds affect nutrients' solubility, transport, and availability to *Chlorella* spp. In addition to the shift, the O-H stretching peak showed broadness and intensity before treatment, indicating a strong presence of hydrogen-bonded hydroxyl groups. However, it became narrower and less intense after treatment, suggesting a decrease in hydrogen-bonded hydroxyl groups, likely due to their involvement in adsorption or chemical reactions with the contaminants. The peak at 2924.76 cm^{-1} corresponds to the symmetric stretching vibrations of CH groups in lipids, indicating the presence of lipids in the biomass (Sánchez-López et al. 2021, Silva et al. 2022). This peak also shifted slightly to 2921.50 cm^{-1} after bioremediation, suggesting a decrease in lipids (Wang et al. 2020) (Figure 3.10). The reduction in the amount of lipids observed in the bioremediation experiment could be due to the metabolism of the microalgae, which may have prioritized other cellular processes over lipid synthesis in response to the environmental conditions. The peak at 1635.51 cm^{-1} corresponds to the C=C bending vibrations of alkene groups (Kee et al. 2019). This peak shifted to 1627.99 cm^{-1} after bioremediation, indicating the possible participation of these functional groups in the metabolism or biosorption process (Figure 3.10). The peak at 1402.43 cm^{-1} corresponds to the C-H bending vibrations of CH_3 groups in proteins and lipids (Nasir et al. 2023), indicating the presence of these biomolecules in the biomass. This peak shifted slightly to 1403.78 cm^{-1} after bioremediation, suggesting a possible increase in the amount of these biomolecules (Figure 3.10). The change observed in the FTIR spectrum from 1234.91 cm^{-1} (before) to 1233.13 cm^{-1} (after) is attributed to the stretching vibration of a C-N bond (Zhou et al. 2016). The C-N (carbon-nitrogen) bonds in *Chlorella* spp. primarily facilitate the adsorption of nitrogen-containing nutrients such as nitrate, nitrite, ammonium, and other forms of nitrogen compounds. Moreover, this peak's appearance or increase in intensity after treatment also highlighted the interactions with nitrogen-containing compounds. The peak at 1031.90 cm^{-1}

corresponds to the P=O stretching vibrations in phosphates (Silva et al. 2022), indicating the presence of phosphates in the biomass. This peak shifted slightly to 1030.24 cm^{-1} after bioremediation, suggesting a possible decrease in phosphate. After bioremediation, the P=O peak became broader and more intense, indicating an increase in phosphate group interactions and suggesting that phosphate groups were involved in complex interactions with the contaminants. Furthermore, the COO peak increased intensity and broadened after treatment, indicating carboxylate groups actively binding with the contaminants, with the broadening suggesting heterogeneous interactions involving multiple binding sites (Šimonovičová et al. 2021).

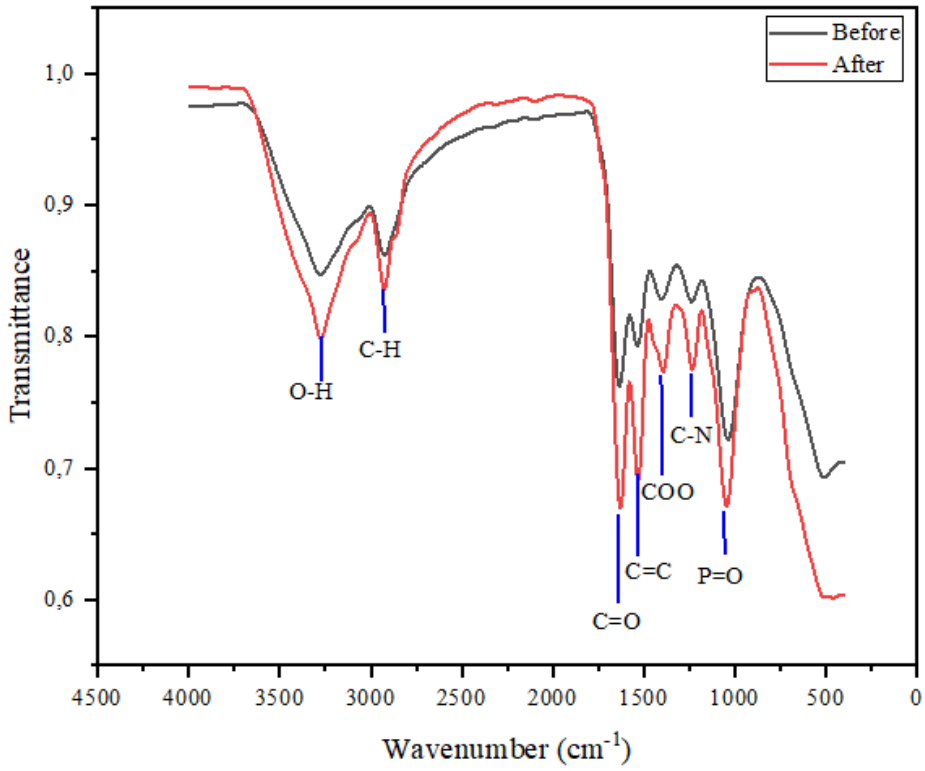

Figure 3.10 The FTIR spectra before and after bioremediation experiments using *Chlorella* spp.

3.3.8 Morphological changes assessment using Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) assessed *Chlorella* spp. cells' surface morphology before and after nutrient uptake (Figure 3.11). The SEM micrographs exhibited enlarged cells, possibly suggesting an interaction between the nutrients and the functional groups on the cell surface and internal structures through nutrient assimilation.

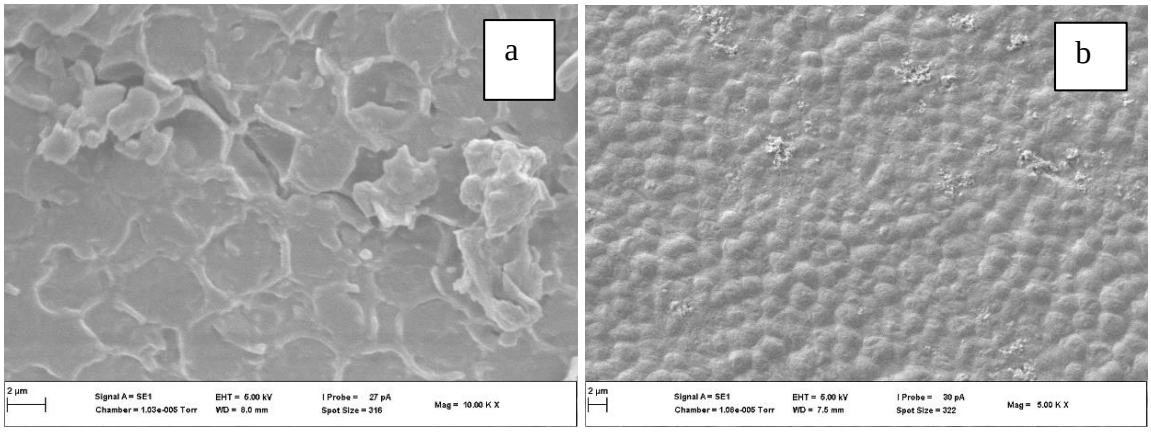

Figure 3.11 The SEM micrograph of *Chlorella* spp. (a) before (b) after pharmaceuticals bioremediation

3.4 Conclusion

The MD600 instrument proved reliable and precise for measuring nutrient concentrations within wastewater effluent samples. The regression equations showed a robust and positive linear relationship between the measured variables, while the recovery rates were close to 100%. It was evident that a higher initial *Chlorella* spp. culture (8%) fostered accelerated growth rates, directly correlating with heightened removal efficiencies. This phenomenon can be attributed to various factors, including but not limited to faster growth rates, cell-cell interactions, pH levels, dissolved oxygen concentrations, and other influencing factors. The findings also shed light on the exceptional capability of *Chlorella* spp. to effectively remove nitrate and nitrite from the medium, either by using them as a nitrogen source or converting them into alternative compounds. However, the challenging nature of ammonia and phosphate removal is worth noting, underscoring the need for comprehensive investigations into the underlying mechanisms governing nutrient dynamics. Contrary to *Chlorella* spp., *D. magna* demonstrated limited ability to directly eliminate nutrients from wastewater, except phosphate, which exhibited marginal removal. However, when these 2 microorganisms are employed together, they show remarkable synergy in eliminating nitrate and nitrite, albeit with limited phosphate removal. Nevertheless, it should be noted that ammonia and phosphate tend to reaccumulate during the treatment process, primarily due to nutrient release via daphnia excretion or egestion, as well as the decay of microalgae biomass.

The combined efforts of microalgae and daphnia are pivotal in efficiently removing nutrients, as microalgae harness these pollutants for their growth. In contrast, daphnia plays a crucial role by consuming the growing microalgae. This collaborative approach ultimately generates a

sustainable source of biomass with substantial economic potential. Moreover, the grazing ability of daphnia assists in managing excessive algal proliferation, thereby enhancing water clarity. Furthermore, the FTIR spectrum reveals shifts in functional groups such as -OH, CH_2, -C≡N, C-C, and P=O, signifying interactions between *Chlorella* spp. and the nutrients. SEM images further support these observations, which depict alterations in *Chlorella* spp. morphology. Thus, the study outcomes lay the foundation for a sustainable and environmentally friendly approach to wastewater treatment and bioremediation. Still, there is a great need for future research and innovation to build upon these findings. An in-depth exploration of the specific mechanisms governing nutrient removal and the intricate interplay between *Chlorella* spp. and *D. magna* could provide fertile ground for technological advancements to cater to diverse environments, from urban wastewater treatment plants to natural wetlands. Moving forward, future studies should focus on enhancing the effectiveness of *Chlorella* spp. and *D. magna* in nutrient removal processes, particularly in addressing the challenges observed with ammonia and phosphate in this study. Exploring novel methodologies, such as optimizing environmental conditions or employing several co-cultivation strategies to augment D. magna's nutrient assimilation capabilities, could prove pivotal. Additionally, further investigations into the interplay between *D. magna* and *Chlorella* spp. under diverse environmental scenarios will deepen our understanding and improve the overall efficacy of bioremediation strategies. These advancements will not only contribute to advancing wastewater treatment technologies but also support sustainable water management practices in alignment with global environmental goals.

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Chapter 4 An Innovative Approach for Bioremediation of Antibiotics and Antiretroviral Drugs Contaminants in Wastewater using *Chlorella* spp.

Abstract

South Africa has implemented the world's most considerable antiretroviral treatment program, serving over 5 million individuals. Within this program, antibiotics are frequently prescribed alongside antiretroviral drugs to mitigate the risk of opportunistic infections in HIV patients. However, 30–90% of these compounds are excreted in urine and faecal waste, overwhelming the conventional wastewater treatment plants, which are not designed to treat such a high spectrum of compounds efficiently. Consequently, inadequately treated wastewater is discharged into freshwater bodies, threatening water quality. High amounts of pharmaceuticals have been detected in water environments, including wastewater final effluents and rivers. Hence, there is a clear gap that necessitates the development of innovative and efficient wastewater treatment processes to mitigate this issue effectively. Bioremediation, employing microalgae, emerged as a promising strategy. Microalgae can incorporate pollutants into their metabolic processes and accumulate, absorb, or adsorb these compounds. Therefore, this research focused on utilizing *Chlorella* spp. to remove antibiotics (sulfamethoxazole) and antiretroviral drugs (efavirenz and nevirapine) from water. In this study, the prolonged exposure to light (20 hours of light: 4 hours of darkness) resulted in the highest growth rates for *Chlorella* spp. compared to other tested conditions (04:20, 16:8, and 12:12). This indicated that an extended light period positively influenced both photosynthetic activity and biomass accumulation. When assessing the impact of different *Chlorella* spp. culture concentrations (8%, 16%, and 32%), higher initial algal concentrations (16% culture) promoted accelerated growth. However, excessively high algal densities (32% culture) tend to limit growth due to overcrowding and the rapid depletion of primary nutrients. In contrast, lower algal densities showed a lower growth rate due to the sparser cell distribution, significantly reducing cell-to-cell interactions. Regarding the removal efficiencies, efavirenz, nevirapine, and sulfamethoxazole ranged from 10–60%, 5–51%, and 10–50%, respectively. The removal percentages obtained in this study can be ascribed to the compound's ability to interact with the polar functional groups on the *Chlorella* spp. cell surface, enhancing their adsorption. To support this, the FTIR spectrum displayed shifts in functional groups, including O-H, C-H

bonds, COO-, C-N, and C=O, indicating interactions that occurred. To investigate the surface morphology of *Chlorella* spp. cells, SEM images revealed alterations, such as irregular texture of the cell surface and swelling compared to its regular and flat cell appearance before exposure to pharmaceuticals. After bioremediation, *Chlorella* spp. cells exhibited noticeable signs of damage or stress to the cell wall, suggesting potential interactions between the pharmaceutical compounds and the *Chlorella* spp. cells. Future research should investigate the mechanisms of pharmaceutical removal by *Chlorella* spp., including bioaccumulation and surface interactions. Further studies should also assess the scalability and sustainability of *Chlorella*-based bioremediation systems in real-world applications, supported by advanced analytical techniques. Additionally, exploring co-cultivation strategies with other microorganisms such as *Daphnia magna*, bacteria, and other microalgae species could enhance the overall effectiveness of bioremediation systems. These efforts aim to advance wastewater treatment technologies, ensuring cleaner freshwater ecosystems and supporting sustainable water management practices.

Keywords: Bioremediation, *Chlorella* spp., Antiretroviral Drugs, Antibiotics, Removal Efficiency

4.1 Introduction

In 2022, approximately 39 million people were living with HIV/AIDs globally; out of that, 25.7 million people were from African countries (UNAIDS 2023). Among these, South Africa exhibited the highest number, with an estimated 7.9 million people; making it the most prominent and high-profile HIV epidemic globally. To address and control this virus, South Africa has the most extensive antiretroviral treatment (ART) program, involving over 5 million individuals (Horn et al. 2022). The prevalence of high ART programs is correlated with the persistence of many ARVDs in water environments, as most pharmaceutical compounds (including ARVDs) do not undergo full biotransformation in human systems. This results in up to 90% of these compounds being excreted unchanged through urine and faeces, consequently reaching wastewater treatment plants (Swanepoel et al. 2015). The conventional wastewater treatment plants (WWTPs) cannot efficiently remove such compounds as they were not designed to remove such a high spectrum of compounds, so these drugs are discharged to receiving freshwater bodies (Hlengwa and Mahlambi 2020). Aside from WWTPs, numerous other channels transport ARVDs into freshwater environments, and they include improper and

unlawful disposal of excess and expired drugs by the manufacturers, leachate from landfills, underground sewer pipes, etc (Ebele et al. 2017, Horn et al. 2022).

Over recent years, a growing body of literature has focused on antiretrovirals in South African water environments and reporting concentrations from 0.35–140 µg/L (Abafe et al. 2018, Adeola et al. 2021, Horn et al. 2022). Antiretrovirals have also been reported in water environments of other African countries such as Kenya (1.1–228 µg/L) and Zambia (0.21–119 µg/L), (Wooding et al. 2017, Ngumba et al. 2020). Notably, the ARVDs observed in Europe are relatively low compared to the concentrations seen in African countries. For example, Prasse et al. (2010) reported concentrations ranging from 0.02–0.032 µg/L in German wastewater (influence and effluence). In Belgium, Vergeynst et al. (2015) also reported lower ARVD concentrations of less than 0.2 µg/L. These low concentrations could be ascribed to European advanced wastewater treatment processes, which have far better performance capabilities relative to the conventional treatment processes utilized in African countries. Additionally, the European region generally has lower HIV/AIDS prevalence rates and higher treatment adherence, contributing to reduced amounts of ARVDs entering the wastewater system. Thus, the combination of advanced wastewater treatment and lower overall usage of ARVDs due to these public health factors likely contributes to the observed low concentrations of ARVs in European wastewater. This highlights a massive need for immediate interventions in African countries to remove ARVDs and other pharmaceuticals in aquatic ecosystems.

HIV-positive individuals are at elevated risk of being infected by opportunistic diseases like pneumonia, tuberculosis, diarrhoea, and others. Thus, antibiotics are typically given to such individuals to fight that (Jendrzejewska and Karwowska 2018, Faiela and Sevene 2022, Omuferen and Maseko 2022). Since the advent of penicillin in 1929 by Fleming, many other antibiotics have been manufactured to either stop bacterial growth (bacteriostatic) or kill bacteria (bactericidal) (Faleye et al. 2018, Omuferen and Maseko 2022). Antibiotics are synthesised for treating numerous bacterial infections of the skin, intestines, urinary tract, respiratory tract, genitals, lymph nodes, and other body systems (Behzadi and Yazdanbakhsh, 2022). This all-purpose application has resulted in a global surge in antibiotic consumption. Omuferen and Maseko (2022) reported that approximately 100 000 tons – 200 000 tons of antibiotics are consumed worldwide. Moreover, about 76% of this global upsurge can be attributed to low- to medium-income countries like Brazil, China, India, Pakistan, Vietnam, Russia, Turkey, South Africa, and Egypt (Ngqwala and Munchesa 2020, Omuferen and

Maseko 2022). The global upsurge can also be ascribed to increase per capita growth (GDP), permitting many individuals to afford these drugs. Furthermore, many farmers in the agriculture sector have adopted antibiotics to meet high demands for animal products (Faleye et al. 2018). Even though antibiotics promote better advancement, the residual effects they cause on aquatic ecosystems cannot be ignored as the human system partially transforms these compounds. The partially transformed compounds promote antimicrobial resistance in humans and immensely impact aquatic organisms (Jendrzejewska and Karwowska 2018, Hassan et al. 2021). The antimicrobial resistance phenomenon leads to a substantial challenge that compromises the overall efficiency of antibiotics, leading to heightened mortality rates, increased treatment expenses, prolonged illness durations, and a growing demand for the continuous development of improved drugs to combat infectious diseases (Nqwesa and Munchesa 2020, Omuferen and Maseko 2022). Research has revealed bacterial resistance's effect in numerous nations such as Europe, the United States of America, and Africa, particularly South Africa (Faleye et al. 2018, Murray et al. 2022, Omuferen and Maseko 2022).

Even though a substantial body of literature centers around identifying and quantifying antibiotics and ARVDs in water environments, there is a deficiency in literature focusing primarily on risk assessment. Vankova (2010) showed that zidovudine causes haematological toxicity and carcinogenic risks in rodents. In a study by Ngumba et al. (2016), zidovudine and nevirapine were associated with ecotoxicological risks on microalgae, daphnia, and fish species. In a separate research endeavour, Minguez et al. (2016) and Guo et al. (2015) verified the ecotoxicological exposure risk of ARVDs toward aquatic microalgae. Elsewhere, efavirenz and nevirapine were shown to affect the embryonic development of *Bulinus tropicus* (Vogt et al. 2020). In the same study, Vogt and co-authors documented that ARVDs significantly impacted the embryonic length, hatching, and mortality rate of *B. tropicus*. Swanepoel et al. (2015) revealed stavudine, didanosine, nevirapine, efavirenz, and abacavir presence in *Clarias gariepinus* (African catfish) plasma with concentrations of 110 ng/L, 55 ng/L, 90 ng/L, 135 ng/L, and 36 ng/L respectively. In another study, tenofovir disoproxil hindered *Microcystis novacekil* growth, immobilized *Artemia salina,* and caused a 50% loss of bioluminescence for *Aliivibrio fischeri* (Silva et al. 2019). Robison et al. (2017) assessed the effect of efavirenz on *Oreochromis massambicus* (Mozambique tilapia). This study showed that the prevalence of efavirenz in water caused liver impairment, such as hepatic steatosis, in *Oreochromis massambicus*.

Research has also demonstrated the synergistic impacts of ARVDs and antibiotics on organisms. For example, nevirapine with mixed antibiotics such as sulfamethoxazole and trimethoprim induced histopathology in the ovaries of female *Oreochromis massambicus* (Nibamureke and Wagenaar 2021). This causes infertility and results in reproduction problems. In a study by Faleye et al. (2018), tetracycline levels of about 0.1 – 50 μg/L in water environments were documented to suppress fish immune systems. The same study also noted that antibiotics like norfloxacin and sulfamethoxazole at concentration levels of about 200 μg/L cause detrimental effects on zebrafish (*Danio rerio*) growth and reproduction rate. Even though antibiotics and ARVDs are saving millions of lives, the existing literature makes it clear that these compounds pose severe threats to non-target organisms. Therefore, their risks can no longer be ignored.

Despite efforts to quantify and monitor these contaminants, a critical gap exists in developing effective strategies to mitigate their presence in aquatic ecosystems. Wastewater bioremediation brings forth an excellent remedy for treating and purifying wastewater efficiently. Bioremediation is the process of using biological microorganisms to detoxify environmental contaminants. Such microorganisms include microalgae, daphnia, fungi, bacteria, macrophytes, etc. However, this study focused primarily on microalgae (Figure 4.1). Nutritional flexibility and the ability to thrive in extreme environmental conditions make microalgae an ideal candidate for executing this task. Moreover, they possess numerous intracellular and extracellular enzymes capable of converting complex contaminants into carbon and energy sources. For example, microalgae can use nitrogen and phosphorus in water for growth (Delrue et al. 2016). Microalgae can also bio-transform perilous compounds into less toxic metabolites, bio-degrade them into non-toxic end products, or adsorb these compounds in their cell surfaces. A substantial body of literature advocates for the effective utilization of microalgae in bioremediation in many regions of the world (Bai and Acharya 2017, Escapa et al. 2017, Gentili and Fick 2017, Xiong et al. 2017, Ali et al. 2018, Xiong et al. 2019, Escudero et al. 2020, Ding et al. 2020). However, limited research exists in African countries like South Africa, creating ample opportunity for further exploration.

Figure 4.1 Microalgae in natural environments, controlled laboratory settings, and diverse algal cells (Marks 2013, Klepnev 2017, Wuest 2019).

Given the pressing requirement for economical and proficient technologies for treating wastewater in developing countries like South Africa, this study evaluated using *Chlorella* spp. to remove selected antibiotics and ARVDs in wastewater. *Chlorella* spp. was chosen over other microalgae due to its exceptional versatility and efficiency. *Chlorella* spp. can thrive in various cultivation conditions—autotrophic, heterotrophic, and mixotrophic—and in both open and closed systems (Ungureanu et al. 2021). This adaptability makes it suitable for diverse wastewater types, including municipal, agricultural, zootechnic, and industrial effluents. Its proven ability to remove nutrients, pharmaceuticals, and a wide array of pollutants, through mechanisms like bio-adsorption, bioaccumulation, and biodegradation, underscores its effectiveness (Ungureanu et al. 2021, Amaral et al. 2023). The study aimed to develop and validate effective methods for extracting and simultaneously determining selected antibiotics and ARVDs in wastewater effluent samples. This was followed by optimizing microalgae conditions to achieve high removal efficiency. Finally, the evaluation of the removal capacities of *Chlorella* spp. was conducted. This research endeavour presented a viable treatment solution with low-cost technology that is relatively easy to develop and operate, meaning municipalities and water utilities with low budgets could adopt this treatment. Furthermore, this research promotes a circular bio-economy because, after bioremediation, clean water can be separated from microalgae. Separated microalgae biomass can be used for a variety of uses, including methane gas production, liquid fuel, various high-value chemicals, fertilizers, alcohol production (ethanol), animal feed supplements, etc.

4.2 Materials and Methods

4.2.1 Study site

The exact sampling points coordinates are 29,60106º S; 30,42794º E for influent and 29,59720º S; 30,43882º E for effluent at the Darvill wastewater treatment plant (Figure 4.2.). The use of

an amber sample bottle was crucial to protect the analytes from light, as it can alter their chemical and biological properties. The samples were transported to the laboratory in a cooler box where they were stored in the refrigerator at approximately 1-8°C. This was done to prevent microbial growth or chemical reactions that may affect sample integrity. Physical parameters such as pH, electrical conductivity (EC), temperature, and dissolved oxygen (DO) were measured using a YSI multiparameter meter (Appendix 1).

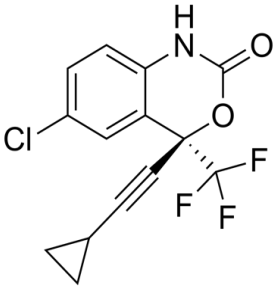
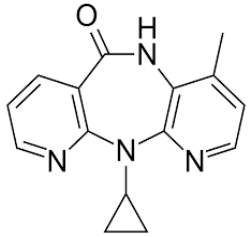
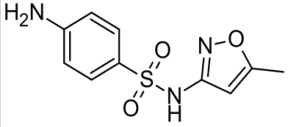
Figure 4.2 Darvill Wastewater Treatment Plant. a = influent and b = effluent

4.2.2 Analytical reagents

High-performance liquid chromatography (HPLC) grade solvents, including 99.9% acetonitrile and 99.9% methanol, were procured from Sigma Aldrich (Durban, South Africa) to ensure their high purity and suitability for analytical applications. Antibiotic (sulfamethoxazole ≥98%) and ARVD (nevirapine ≥98%, and efavirenz ≥98%) standards were also obtained from Sigma Aldrich to establish accurate calibration curves and validate the analytical method.

Table 4.1. presents the chemical and physical properties of the analytes of interest. Efavirenz poses a high molecular weight that correlates with a larger size and surface area, increasing its chance of adsorption (Sun and Bai 2023). It is also characterised by minimal water solubility and higher log Kow values representing pronounced lipophilicity. Lipophilic compounds can dissolve in fats and oils rather than water (Lindsley 2014), easily bypassing water-based treatment systems. Nevirapine is characterised by less lipophilicity and higher water solubility, making it more available in water than Efavirenz. Sulfamethoxazole is practically insoluble in water, indicating constrained availability in the aqueous phase but more in organic phases or sediments.

Table 4.1 Chemical structure and physicochemical properties (Eugene-Osoikhia and Emesiani 2019, Adeola et al. 2021)

Compounds	Chemical Structure	MW (g/mol)	Water solubility (mg/L)	Log K_{ow}	pKa
Efavirenz		315.68	0.093	4.70	10.20/12.52
Nevirapine		266.30	0.705	3.89	2.80
Sulfamethoxazole		253.28	practically insoluble	0.89	5.7

MW: molar weight; Log K_{ow}: octanol–water partition coefficient; pKa:-log of acid-dissociation constant

4.2.3 Instrumentation

The SPE vacuum manifold used to ensure reliable and efficient sample preparation was bought from Sigma Aldrich (Steinheim, Germany). The accompanying vacuum pump was purchased from Edwards (Munich, Germany), providing the necessary suction for the SPE process. Chromabond hydrophilic-lipophilic balance (HLB) cartridges with a capacity of 60 mg and 3 mL were procured from Separation Scientific (Gauteng, South Africa), functioning as the medium for analyte enrichment and purification through solid-phase extraction.

A liquid chromatography system (LC-2020) from Shimadzu (Tokyo, Japan) was employed to analyze analytes of interest. The LC-2020 system had essential components, including a degasser, quaternary pump, auto-sampler, and an LC-2030/2040 photodiode array detector (PDA) sourced from Germany and Europe. A Shim-Pack GIST analytical C18-HP column (4.6 mm x 150 mm ID, 3.5μm) obtained from Shimadzu (Tokyo, Japan) was utilized to separate and detect the target compounds, and the column temperature was maintained at 30°C. The LC-2020 system was operated with specific parameters to achieve optimal separation and detection of the selected antibiotic and ARVDs. The photodiode array detector was set to wavelengths of 220 nm, providing suitable sensitivity for the targeted analytes. A sample injection volume of 10 μL and a 0.4 mL/min flow rate were employed to ensure accurate and precise analysis. The LC isocratic program comprised 90% acetonitrile and 10% water (in 0.1% formic acid). This program was designed to achieve efficient elution and separation of the target analytes during the chromatographic run.

4.2.4 Preparation of stock solution

To make the standard stock solution, 10 mg of each analyte, namely efavirenz, naproxen, and sulfamethoxazole, was carefully measured and transferred into one 100 mL volumetric flask. Acetonitrile was added to the mark to achieve a 100 mg/L concentration. Standard working solutions were then prepared by diluting the standard stock solution to calibrate the LC-PDA instrument. The concentrations of the working solutions ranged from 0.2 to 1 mg/L, ensuring a suitable calibration range for accurate analysis. All prepared solutions, including the stock and working solutions, were stored in a refrigerator at a temperature of approximately 4°C to maintain the stability and integrity of the solutions until further use in the LC-PDA analysis.

4.2.5 LC-PDA method optimization

The LC-PDA method described by Kunene and Mahlambi (2023) was selected as the basis for separating the target analytes in this study with further optimization. The optimization process aimed to enhance the efficiency and reliability of the analytical method, thereby facilitating accurate and precise analysis of the analytes of interest. Specific attention was given to key analytical conditions, such as the mobile phase composition and the selection of the detector wavelength (220, 225, and 254 nm). These parameters were fine-tuned to ensure excellent peak separation of all the target analytes within a reasonable timeframe.

4.2.6 Solid-phase extraction (SPE) procedure optimization

Initially, the SPE procedure described by Mtolo et al. (2019) served as a foundation for the extraction method, which was subsequently fine-tuned to maximise recovery for all analytes of interest. Several parameters were optimized during the process, including the conditioning solvent (acetonitrile, methanol, and a mixture of both at various ratios (v/v)), sample loading volume (25, 50, and 100 mL), and sample pH (2.5, 7, 9, 11). Methanol, acetonitrile, were selected due to their ability to dissolve polar compounds effectively, as the analytes of interest are also polar (Hlengwa and Mahlambi, 2020). For the SPE optimization process, wastewater effluent samples were spiked with the standard stock solution to make a final 1 mg/L concentration. The final SPE procedure included conditioning the sorbent to activate the functional groups for effective interaction with the analytes of interest. This was done using 2 mL of acetonitrile and methanol in a 70:30 (v/v) ratio followed by 2 mL of distilled water. Then, 50 mL of the wastewater effluent sample (pH adjusted to 7) was passed through the conditioned sorbent to trap the analytes of interest. A 2 mL of distilled water was utilised to wash off impurities then 2 mL of acetonitrile was passed on to the sorbent to elute adsorbed analytes. The eluted analytes were then concentrated to 1 mL using a gentle stream of nitrogen and analysed with LC-PDA.

4.2.7 Method validation

The optimised analytical methods were subjected to further evaluation and validation through various parameters, including linearity, precision, the limit of quantification (LOQ), the limit of detection (LOD), and percentage recoveries. Linearity was determined by analysing the coefficient of determination (R^2) obtained from the analytical curves constructed using concentrations ranging from 0.2 to 1 mg/L. Precision was assessed by investigating repeatability and reproducibility, and the results were expressed as the percentage relative

standard deviation (%RSD). The method's sensitivity was determined by establishing the LOD and LOQ, representing the lowest concentrations of the analyte that can be accurately and precisely detected and quantified, respectively. Percentage recoveries were evaluated by spiking wastewater with known concentrations of the target analytes. These spiked samples were analyzed in triplicate to assess the accuracy and reliability of the method in real environmental matrices.

4.2.8 Bioremediation experiments

4.2.8.1 Growth medium: Tris-acetate phosphate (broth and agar)

Broth tris-acetate phosphate (TAP) medium was prepared following instructions published by Harris (1989). The agar medium was prepared by adding 15 g of bacteriological agar to 1 liter of the broth medium. The bacteriological agar was chosen for its high gel strength and compatibility with numerous culture media. The medium was then autoclaved at 121°C and 0.11 KPa pressure for 30 minutes to ensure sterility. Subsequently, the medium was cooled to a temperature of 45–50 °C in a water bath before carefully pouring onto sterile plates. These plates were then allowed to cool undisturbed for approximately 24 – 48 hours, enabling complete solidification of the medium. Following the cooling period, the plates were ready for streaking or inoculation with the desired samples, facilitating further experimentation and analysis. For broth culture medium, 50 mL TAP medium was poured into 100 mL Erlenmeyer flasks, which were then covered with foil and autoclaved.

4.2.8.2 Ensuring culture purity and exploring cleaning methods for Chlorella spp. cultures

The *Chlorella* spp. (strain FSB41.2) used in this study was provided by uMgeni-uThukela Water (Pietermaritzburg, South Africa). A microscopic culture analysis was conducted to assess the presence of contaminants, revealing the coexistence of bacteria and *Chlorella* spp. cells. Various cleaning methods have been suggested in the literature to address this issue, and 2 methods were explored. Method 1 involved performing a series of dilution streaks. This approach aimed to isolate and eliminate contaminants, allowing the growth of pure *Chlorella* spp. cultures. A study by Zhang et al. (2019) emphasized the importance of serial dilution streaking in minimizing microbial contamination, providing insights into its successful application for culture purification. Method 2 focused on removing the carbon source, specifically acetic acid, from the Tris-Acetate-Phosphate (TAP) medium. This modification aimed to reduce the attraction of undesired microorganisms, enhancing the selectivity for

Chlorella spp. growth. Given this information, the TAP media was prepared without acetic acid, enabling optimal conditions for *Chlorella* spp. growth and minimizing the risk of contamination from unwanted microorganisms. Figure 4.3 shows the schematic representation of steps to take for axenic verification.

Figure 4.3 Schematic representation of steps to take for axenic verification

4.2.8.3 Ensuring sterility and accuracy

Creating a controlled and sterile environment was essential to initiate the culture streaking procedure. This was achieved by securely closing the laboratory door and switching off the air conditioning system to minimize air disturbances. Next, it was imperative to ensure gas availability by turning on the gas gauge. Then, the Bunsen burner was safely ignited to allow a reliable heat source throughout the streaking process. The working environment was maintained sterile by cleaning the bench surface with 70% ethanol before working, minimizing contaminants. Then, a wire loop for streaking was prepared through the heat until it reached a vibrant red-hot state. The loop was allowed to cool briefly for a few seconds, ensuring it was still warm but not excessively hot. Then, a warm sterile loop was submerged into the culture of interest, ensuring that the lid of the Petri dish remained turned upside down to minimize potential contamination. The streak was initiated by gently drawing 3 to 4 lines on one side of the agar surface using the loop. Following this, the wire loop was reheated to ensure sterilization, and streaking was continued by repeating the process on the remaining 3 sides of the agar plate. This comprehensive approach facilitated optimal distribution and isolation of the culture.

For broth medium contained within Erlenmeyer flasks, it was first essential to sterilize the flask's mouth using the Bunsen burner flame. This precautionary step minimized the possibility of introducing external contaminants. Once the mouth of the flask was suitably sterilized, the wire loop was placed into the agar medium and then straight into the liquid medium, ensuring the loop did not come into contact with the mouth of the flask during the process. Aseptic conditions were maintained throughout the culture streaking procedure by diligently following these steps, facilitating accurate and reliable microbiological analysis.

4.2.8.4 Conditions for microalgae cultivation and growth

The cultures were placed in a temperature-controlled room at approximately 23°C, promoting an optimal growth rate. Additionally, it was crucial to supply a light-dark cycle (20 hours:04 hours), preferably white fluorescence, to support photosynthesis and metabolic activities. By maintaining a consistent temperature and providing light, the microalgae cultures could undergo proper photosynthetic processes, leading to visible growth within 3 to 4 days. These carefully controlled conditions facilitate the cultivation of healthy and thriving microalgae, enabling further research and applications in various scientific fields.

4.2.8.5 Storage of media

Proper storage of media was essential to maintain their viability and integrity. Broth media was stored in a refrigerator at 1 to 8 °C. This cold environment assists with preserving the nutrients and inhibits any microbial growth, ensuring the longevity of the media. As for agar media, after pouring them into petri dishes and allowing them to solidify, they were sealed with parafilm to create an airtight environment. It was advised to store the plates in a dark area, away from direct light exposure. This darkness helped to prevent any potential light-induced changes. By following these storage practices, the quality and viability of media were maintained, ensuring their suitability for future experiments.

4.2.8.6 Microalgae growth analysis

Regularly monitoring microalgae growth is crucial. This involves measuring optical density, which reflects its absorbance and provides insights into the culture's health and productivity. This study accomplished this by aseptically collecting 3 mL samples from the growing culture and quantifying absorbance at 680 nm using a UV/Visible Spectrophotometer (JENWAY 7310) (Christwardana and Hadiyonto, 2017, Rawati, 2021).

4.2.8.7 Optimization of conditions before application

Prior studies and existing literature have demonstrated valuable insights on optimal pH, temperature, and orbital shaker speed for microalgae growth. Thus, in the current study, those conditions were adopted and maintained constant throughout the study as justified by the existing literature. Optimising 2 other parameters was of paramount importance to enhance microalgae removal efficiencies. First was the light/dark intervals to pinpoint the most favourable interval for maximizing microalgae removal potential. In this case, 4 distinct intervals were investigated: 16 hours of light followed by 8 hours of darkness, 12 hours of light followed by 12 hours of darkness, 20 hours of light followed by 4 hours of darkness, and 4 hours of light followed by 20 hours of darkness. This was followed by carefully examining the initial stock inoculum concentration to identify the optimal concentration that yielded the highest removal rate. Three concentrations of the culture were tested: 8%, 16%, and 32%. To achieve these specific concentrations, the corresponding volumes of the culture were added to each experimental flask, ensuring that they accurately represented the *Chlorella* spp. content within the medium at these defined levels. Needed volumes were calculated using the following:

$$V\ culture = \frac{Percentage\ x\ V\ medium}{100} \quad (4.1)$$

Where V culture is the volume of culture to be added, Percentage is the desired percentage (e.g., 8%, 16%, or 32%), and V medium is the total volume of the medium.

For pharmaceutical removal, equation 2 was used (Xiong et al. 2017):

$$Pt = \frac{= C0 - Ct}{t0} x\ 100 \quad (4.2)$$

Where, C0 represents the initial concentration of pharmaceutical compound at time zero, and Ct represents the concentration at time t.

4.2.8.8 Bioremediation using optimized conditions

For this study, the synthetic approach of spiking the growth medium with pharmaceuticals provided precise control over initial target compound concentrations, facilitating investigations into removal rates and the effects of varying concentrations. This approach also ensured

reproducibility through consistent conditions and simplified the experimental setup by eliminating potential interferences from real wastewater constituents. The pH was adjusted to approximately 7.5, and the sample was sterilised by autoclaving for 30 minutes to eliminate bacteria and protozoa that might be present. Furthermore, the pharmaceutical stock solution containing the target compounds and the pre-cultured *Chlorella* spp. culture was added to the media to reach an initial optical density of 1. This study utilized spike concentrations of 5 mg/L, 1 mg/L, 0.5 mg/L, and 0.25 mg/L. The flasks were then placed in the same environmental conditions as their typical culture environment (section 4.2.8.4.) for 10 days. Samples (60 mL) were taken routinely for analytical measurements. Before analysis, 55 mL samples were centrifuged for 20 minutes at 4000 rpm to separate the microalgae from the liquid. The supernatant was then subjected to solid-phase extraction before LC-PDA analysis. Additionally, 3 mL of the remaining subsample was used to assess cell density. The cell density allows for quantifying algal growth dynamics throughout the experiment.

4.3 Results and Discussion

4.3.1 Optimization of LC-PDA instrument

The method was adopted from Kunene and Mahlambi (2023) and further refined. Initially, a gradient elution approach was utilized with a mobile phase composition containing a mixture of 50% acetonitrile and 50% water (in 0.1% formic acid) from 0 to 5 minutes, 70% acetonitrile, 30% water (in 0.1% formic acid) from 6 to 20 minutes. The flow rate was 0.4 mL/min, and the detector wavelengths were 225 nm and 254 nm. Under these conditions, sulfamethoxazole and nevirapine were co-elutioned. The mobile phase was then adjusted to 70% acetonitrile and 30% water (in 0.1% formic acid) from 0 to 10 minutes, but sulfamethoxazole and nevirapine still did not entirely separate. Subsequently, the mobile phase was modified to consist of 80% acetonitrile and 20% water (in 0.1% formic acid) while keeping the other parameters constant. With these changes, all the compounds successfully separated. However, there was a significant elution gap of approximately 3 minutes between nevirapine (4.787 minutes) and efavirenz (7.568 minutes). Therefore, a further mobile phase adjustment was made, which comprised 90% acetonitrile and 10% water (in 0.1% formic acid), while the run time, flow rate, and detector wavelength remained unchanged. These conditions led to shorter retention times for all the compounds, with sulfamethoxazole eluting at 4.207 minutes, nevirapine at 4.880 minutes, and efavirenz at 5.882 minutes. Moreover, the analysis was conducted at 220, 225,

and 254 nm detector wavelengths, where 220 nm improved peak absorbance and thus was taken as the optimum for all analytes (Figure 4.4).

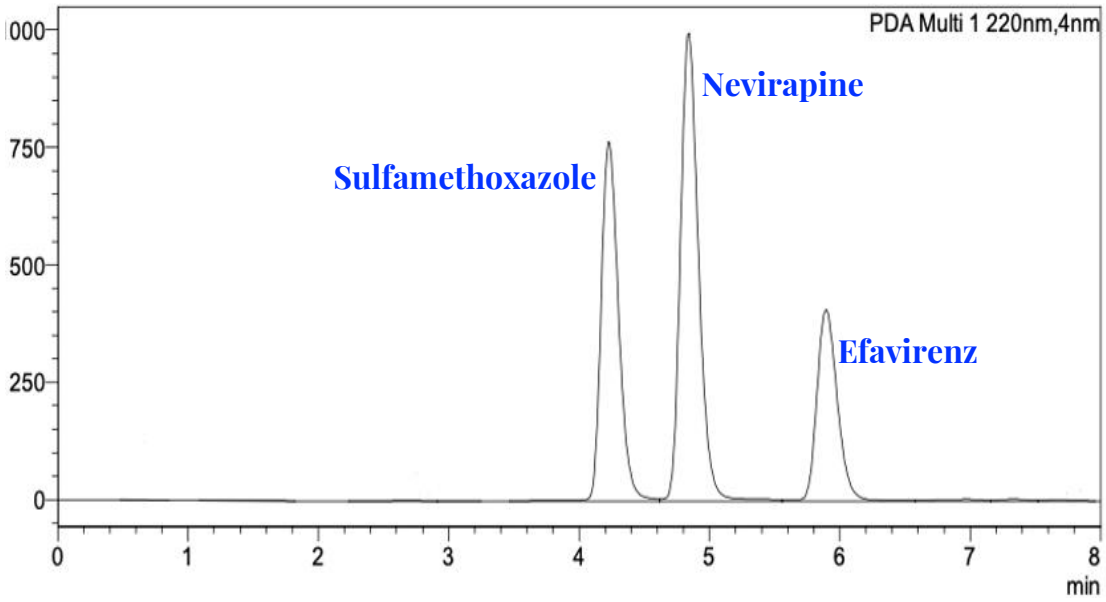


Figure 4.4 The LC-PDA chromatogram showing 1 mg/L antibiotic and ARVDs separation (Sulfamethoxazole = 4.229, Nevirapine = 4.839, and Efavirenz = 5.895).

4.3.2 Calibration of LC-PDA method

The correlation coefficient (R^2) obtained for the analysis ranged from 0.997 to 1, which falls within the acceptable range specified by official published guidelines. These results indicate robust and positive linear relationships achieved through the newly developed method (refer to Figure 4.5 and Table 4.2).

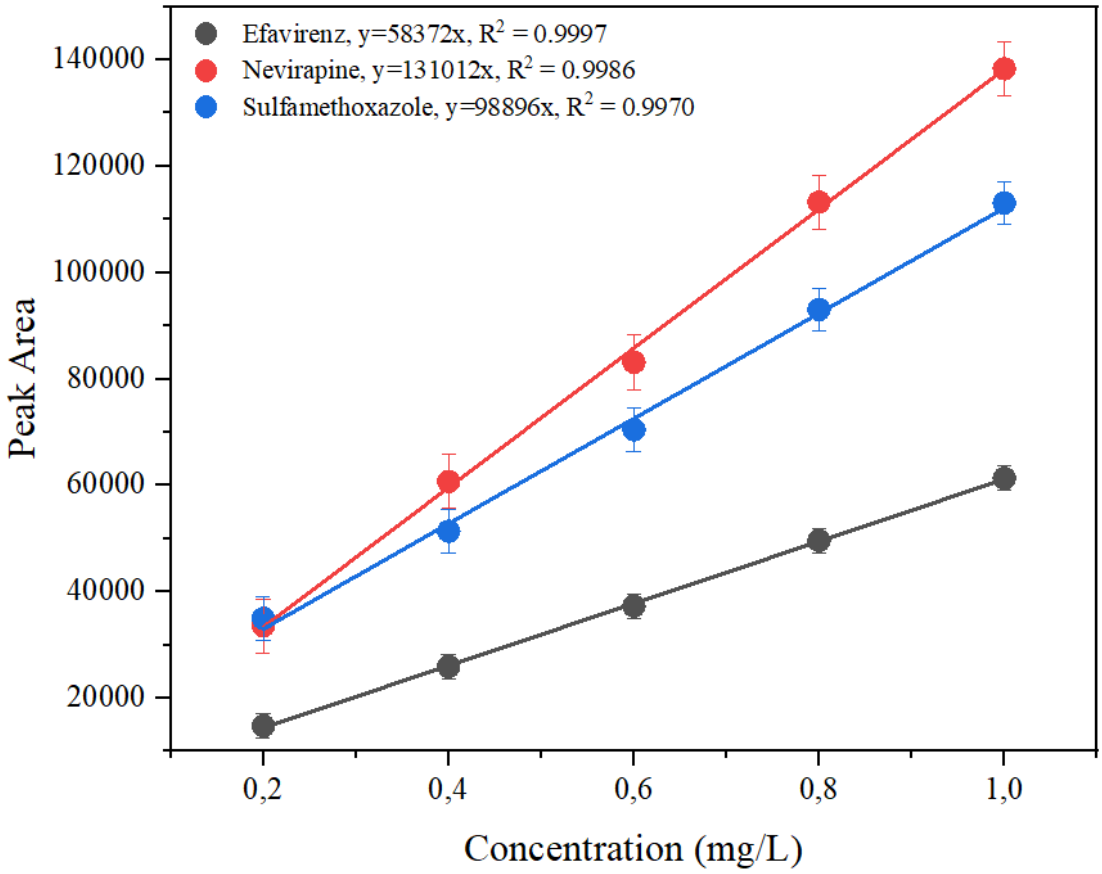


Figure 4.5 The LC-PDA calibration from standard working solution ranging from 0.2– 1 mg/L with n=3 replicates.

4.3.2.1 The effect of conditioning solvent on the recoveries of the analytes

Acceptable recoveries ranging from 91% to 110% were achieved regardless of the conditioning solvent used (Figure 4.6). The results of the ANOVA tests also indicated no statistically significant differences in mean percentage recoveries between the different conditioning solvents for any of the compounds ($p = 0.814$), which is higher than 0.05. Notably, the highest recovery of 110% was obtained when a mixture of acetonitrile and methanol in a ratio of 70:30 (v/v) was employed as the conditioning solvent. This indicated that the combined solvent mixture efficiently activated the functional groups of the sorbent better than individual solvents, leading to an increased surface area and facilitating strong interactions between the target analytes and the sorbent. The effectiveness of the solvents can be attributed to their miscibility with water and their medium to high polarity properties (0.460 – 0.762) (Dailey et al. 2015). Furthermore, this solvent mixture could remove any residues from the packing material that might interfere with the analysis. Consequently, the optimum conditioning solvent

selected for subsequent experiments was a mixture of acetonitrile and methanol in a ratio of 70:30 (v/v).

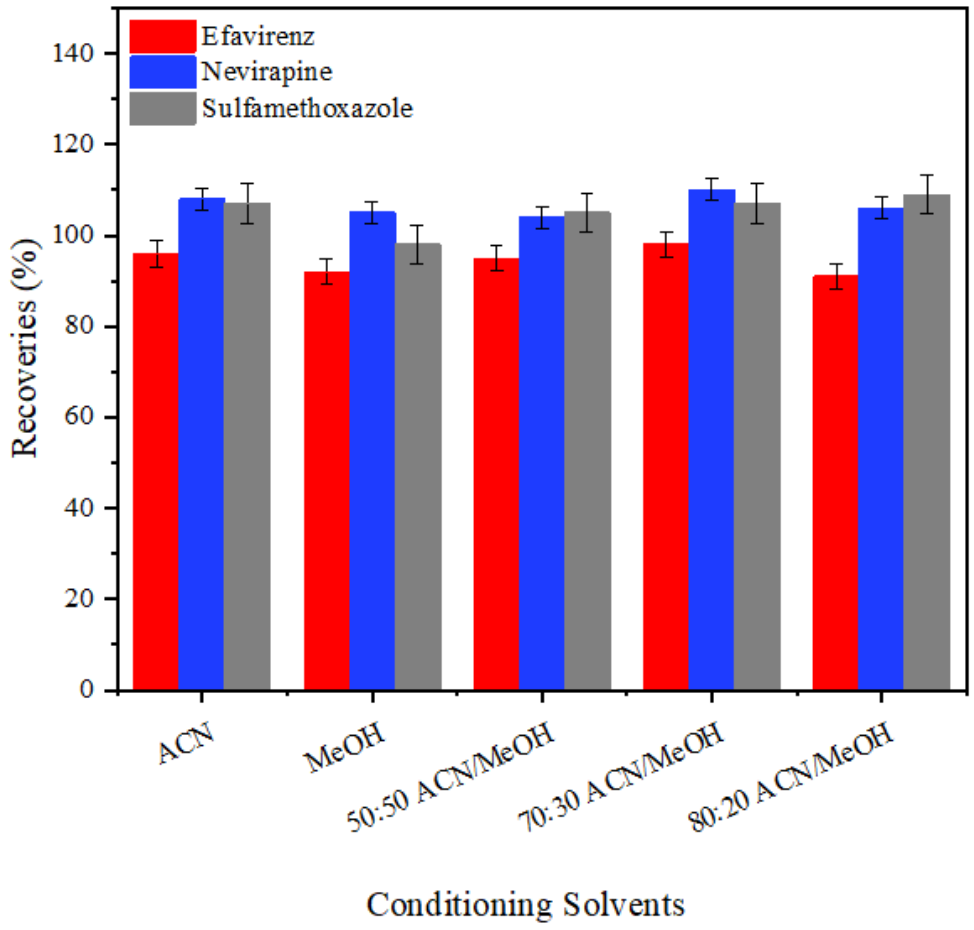


Figure 4.6 The effect of conditioning solvent on the recoveries of SPE with n=3 replicates. Extraction conditions were a sample volume: 50 mL, sample pH: 7, eluting solvent: acetonitrile.

4.3.2.2 The effect of sample loading volume on the analyte's recoveries

Optimizing the sample volume in solid-phase extraction (SPE) is crucial, as it directly impacts various factors such as extraction time, potential matrix effects, and pre-concentration factors. When a sample volume of 25 mL was employed, all analytes displayed recoveries below 80% (Figure 4.7). The lower recoveries observed (65% to 77%) at this volume could be attributed to insufficient target analytes available to interact with the sorbent. In contrast, all analytes exhibited greater recoveries at a sample volume of 50 mL, ranging from 98% to 107%. This indicates that increasing the sample load volume enhances the analyte retention within the sorbent's activated sites. These findings align with the results reported by Moranata et al.

(2021), who observed higher recoveries with increased sample volumes. However, when a sample volume of 100 mL was utilized in this work, a reduction in recoveries was observed, ranging from 74% to 85%. This phenomenon can be attributed to the breakthrough volume effect, wherein the sorbent becomes oversaturated, causing a shift in the adsorption/desorption equilibrium and resulting in the loss of the adsorbate (Ngubo, 2018). A similar trend was observed in the study by Mtolo et al. (2019). Low recoveries observed at 100 mL sample load may also be attributed to the displacement of the sorbent material as additional samples flow through, potentially modifying the interaction between the active sites and the analytes. Based on these observations, a sample volume of 50 mL was deemed sufficient, providing satisfactory recoveries for all analytes. According to the ANOVA analysis, a p-value of 0.001 was obtained for all tested volumes, indicating a significant difference between the volumes investigated. However, when conducting pairwise analysis, the following p-values were obtained: 25- and 50-mL = 0.002; 25 and 100 = 0.127; 50- and 100-mL = 0.006. Since higher recoveries were obtained at 50 mL, all subsequent experiments were carried out using that volume.

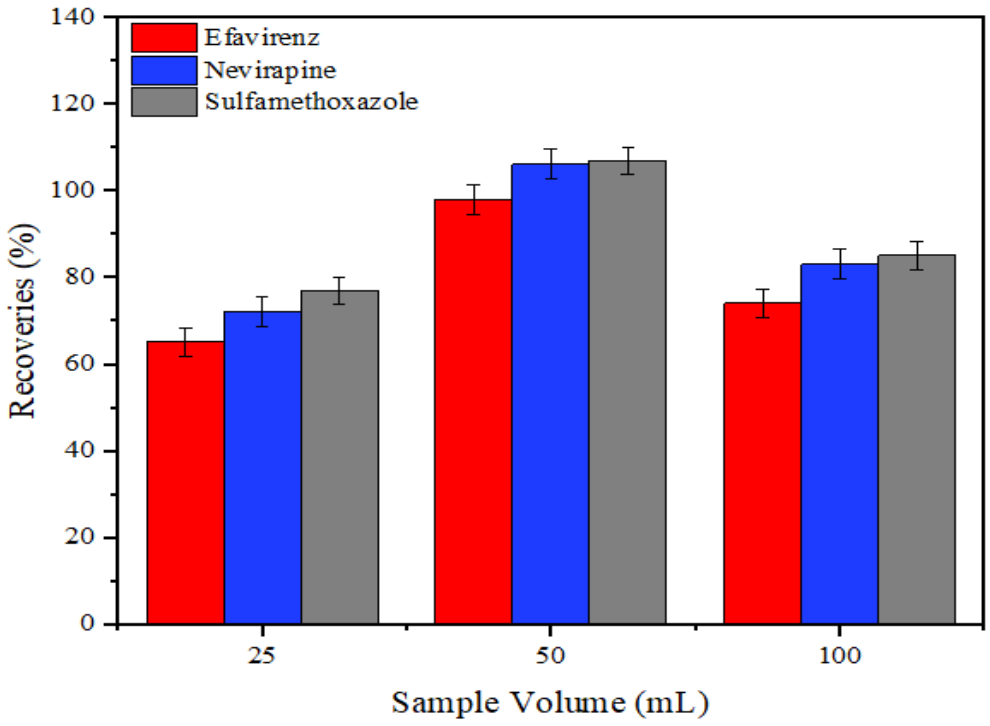


Figure 4.7 The Effect of sample volume on the recoveries of SPE with n=3 replicates. Extraction conditions were a conditioning solvent: 70:30 ACN/MeOH, sample pH: 7, eluting solvent: acetonitrile.

4.3.2.3 The effect of different pH on the analyte's recoveries

The pH can influence adsorbents' surface properties and compounds' speciation in a solution. Neutral pH (7) resulted in 114% to 120% recovery, attributed to lipophilic divinyl benzene and hydrophilic N-vinyl pyrrolidone on the Chromabond HLB cartridges (Figure 4.8). These properties promote sorption at neutral pH, thereby improving the recovery of analytes (Madikizela et al. 2017, Hlengwa and Mahlambi 2020). Similar findings were reported by Lindholm-Lehto et al. (2018), where higher recoveries were obtained at neutral pH when using HLB cartridges. On the other hand, acidic and basic pH conditions resulted in lower recoveries, with sulfamethoxazole exhibiting the lowest recoveries, particularly at basic pH. The decrease in recoveries at acidic pH can be attributed to the protonation of the NH group in pharmaceutical compounds. Protonation can alter the compound's chemical properties, including its solubility in aqueous solution, reactivity, and stability in aqueous solutions. The low recoveries obtained at basic pH can be ascribed to hydrolysis, where analytes exist in their anionic form (Hlengwa and Mahlambi, 2020). The findings of this study are in accordance with John et al. (2018), who stated that the adsorption of anions decreases with increasing pH due to the higher concentration of competitive anions such as OH^-. The ANOVA analysis exhibited a p-value of 0.039, indicating a significant difference between the pH investigated. Thus, neutral pH was determined to be the optimum pH for the extraction process.

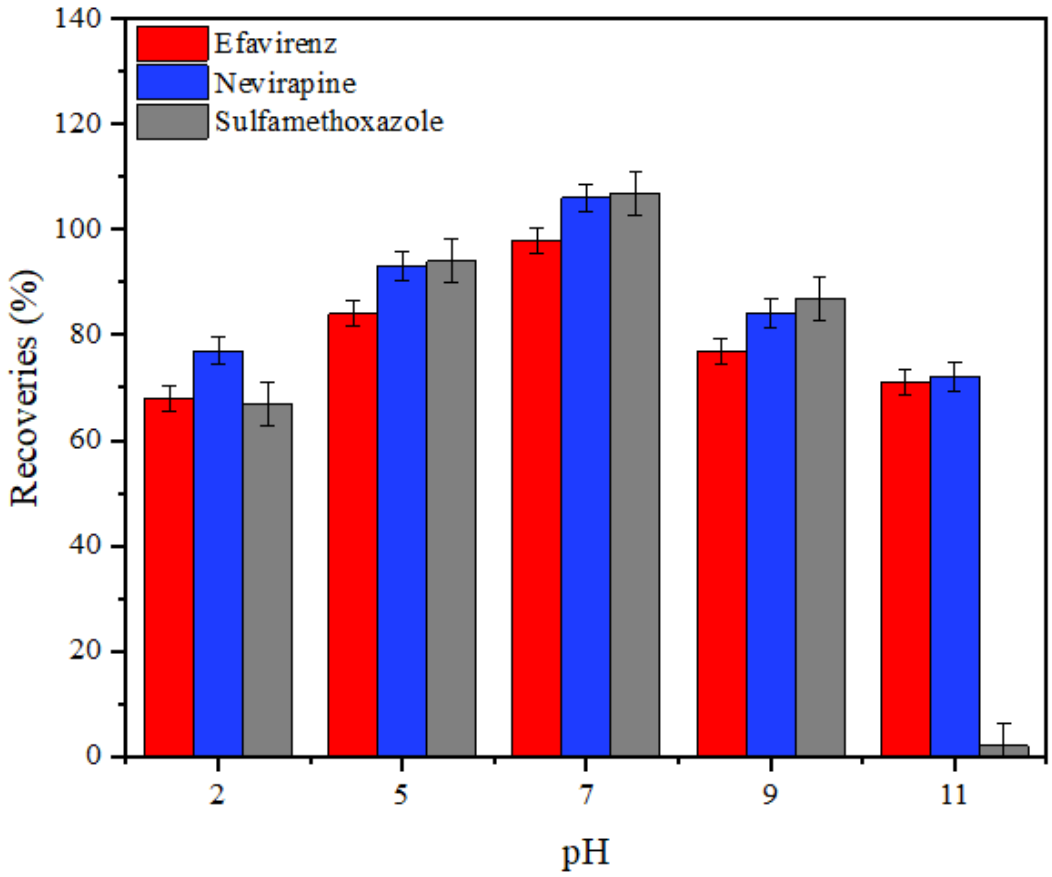


Figure 4.8 The effect of pH on the recoveries of SPE with n=3 replicates. Extraction conditions were a conditioning solvent: 70/30 ACN/MeOH, sample volume: 50 mL, eluting solvent: acetonitrile.

The analysis of variance (ANOVA) was employed to assess the percentage contribution of each SPE parameter to the overall optimization. The results revealed that conditioning solvent exerted the most substantial influence, contributing 66.7% to the optimization process. This underscores the critical role of selecting and preparing an appropriate conditioning solvent in achieving optimal extraction efficiency. On the other hand, pH and sample volume, while contributing 16.7% each, demonstrated significant though relatively smaller individual impacts on the overall optimization. Despite their small percentage contributions, pH and sample volume play crucial roles in SPE. Small adjustments in these parameters can significantly impact the success of the method, underscoring the importance of careful consideration in their optimization. In conclusion, this optimized method holds great promise for advancing pharmaceutical compound analysis in water matrices, offering a valuable contribution to environmental monitoring and public health.

4.3.3 Validation of the analytical method

The proposed methods were comprehensively evaluated for accuracy, sensitivity, and precision. The recoveries ranged from 99% to 120% in wastewater, indicating the method's good accuracy (Table 4.2). Notably, efavirenz demonstrated a slightly lower percentage of recoveries than the other 2 compounds. This observation could be attributed to its higher octanol-water partition coefficient value (4.70) relative to nevirapine (3.89) and sulfamethoxazole (0.89) (Table 4.1). According to Gao et al. (2019), solid-phase extraction efficiency tends to diminish when the octanol-water partition coefficient falls within the range of 4.0 – 6.0. This decline can be attributed to the pronounced hydrophilicity and weaker hydrophobic interaction exhibited by the compounds in this range. The method showed good sensitivity, as evidenced by the limit of detection ranging from 0.056 µg/L to 0.176 µg/L and the limit of quantification ranging from 0.187 µg/L to 0.585 µg/L. This indicates the capability of the method to detect trace levels of the analytes. Precision was evaluated through repeatability and reproducibility, expressed as %RSD. Repeatability and reproducibility yielded %RSD values of less than 1% for all analytes, demonstrating the method's high precision and reliable performance.

Table 4.2 R^2, LODs, LOQs, % Recoveries (n=3) of the analytical method

Compounds	**R^2**	**LOD (µg/L)**	**LOQ (µg/L)**	**% Recoveries ± % RSD**		
				Wastewater effluent	**Repeatability**	**Reproducibility**
Efavirenz	1.000	0.176	0.585	99±0.03	94±0.01	96±0.06
Nevirapine	0.999	0.069	0.230	120±0.01	102±0.00	104±0.07
Sulfamethoxazole	0.997	0.056	0.187	102±0.00	106±0.00	106±0.06

4.3.4 Chlorella spp. culture purity

A series of aseptic dilution streaks were performed to ensure the cultures' purity. Following a 7-day incubation period, bacteria were detected alongside microalgae in the cultures. Consequently, the carbon source (acetic acid) was eliminated from the TAP medium due to its propensity to attract undesirable microorganisms. Weekly dilution streaks were implemented. After 4 months, the culture was confirmed to be free from contaminants and pure, as Figure 4.9 shows.

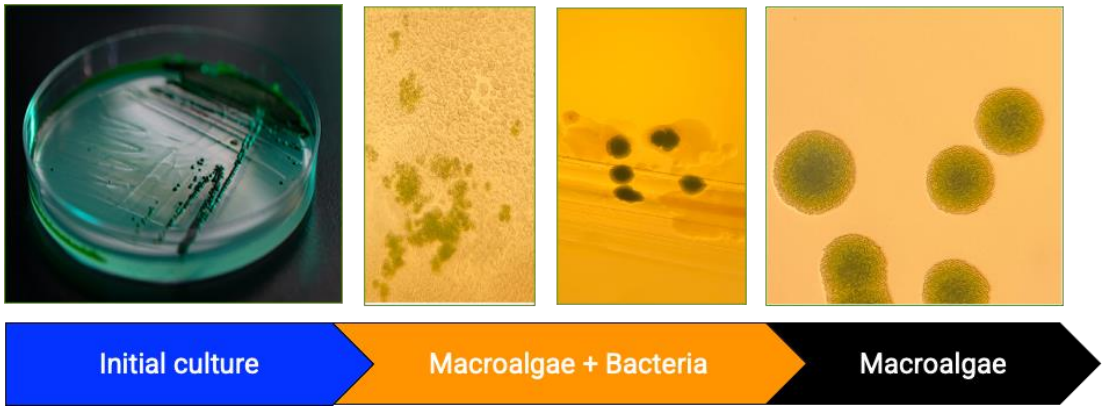


Figure 4.9 Schematic illustrating the different stages of culture until purity was achieved.

4.3.5 Bioremediation experiments

Extensive research efforts have focused on determining the most favourable light: dark cycle for microalgae growth. Understanding and controlling the light: dark cycle allows researchers to optimize microalgae growth, photosynthetic activity, metabolic processes, nutrient uptake, and energy efficiency, thereby maximizing the effectiveness of the bioremediation process. The optical density measurements provided insights into the growth patterns of the algal culture under different light regimes. The study's results revealed that longer light illumination (20 hours of light: 4 hours of darkness) promoted the highest growth rates, as indicated by the consistently increasing optical densities over time (Figure 4.10a). This suggests that the microalgal culture benefited from the availability of extended periods of light, enabling enhanced photosynthetic activity and biomass accumulation. Consistent with these research findings, a study by Zhang et al. (2015) investigated the effects of different light conditions, mainly varying light/dark cycles, on the growth of *Scenedesmus dimorphus*, a type of microalgae. Their study revealed a positive relationship between microalgae production and extended illumination periods. The highest microalgae concentration of 700 mg/L was observed during a 24:0 light/dark cycle. These results support the idea that longer light illumination can significantly boost microalgae growth, increasing biomass accumulation. Also, a study by Rosmahadi et al. (2022) further supports the findings regarding the benefits of extended periods of light on microalgae growth and biomass accumulation. In their research, Rosmahadi and colleagues implemented a 20:4 photoperiod regime consisting of 4 hours of darkness followed by 20 hours of illumination. They observed that this specific light/dark cycle produced the highest microalgal density. Several other studies collectively indicate that more prolonged light exposure is beneficial for microalgae growth, including species like *Chlorella* (Khoury 2020, Pozzobon 2022, Urbina-Suarez et al. 2022). Longer light periods enhance

growth rates, biomass productivity, lipid content, and photosynthetic efficiency, making it favourable for optimizing microalgae cultivation and related applications. The 16-hour light condition and 12-hour light condition also supported algal growth but to a lesser extent than 20 hours of light condition (Figure 4.10a). The increasing optical densities in these conditions indicate that the algal culture could still carry out photosynthesis and accumulate biomass, although at a slower rate relative to continuous light exposure. Contrary to the results above, prolonged darkness (20 hrs darkness) resulted in a decline in algal growth, indicated by decreasing optical densities. This highlights the importance of light as an energy source for photosynthesis and biomass production in microalgae (Figure 4.10a). Therefore, subsequent experiments were conducted under continuous 20-hour light: 4-hour darkness exposure conditions.

The removal efficiency of the investigated compounds showed a correlation with the optical density results, indicating that 20 hours of illumination resulted in better removal of the compounds relative to the other regime (Figure 4.10b). A more extended illumination period allowed the microalgae to continuously engage in the light-dependent reactions of photosynthesis, which are vital for energy production and biomass accumulation (Urbina-Suarez et al. 2022). Moreover, the increased light period can reduce self-shading in dense algal cultures (Khan et al. 2018, Giraldo et al. 2021). Self-shading refers to the shadowing effect caused by densely packed algal cells, which can limit light penetration to deeper layers of the culture. Furthermore, a more extended light period has a huge potential to foster a larger overall algal population, thereby increasing the chances of contact between the target pharmaceutical compounds and the microalgae. Consequently, leading to enhanced removal rates. Moreover, more prolonged light exposure stimulates the metabolic activity of the microalgae, resulting in a more efficient enzymatic breakdown and transformation of the targeted pharmaceutical compounds. When comparing the 3 compounds, sulfamethoxazole exhibited superior removal as it is more prone to photodegradation through photolysis (Zhou et al. 2015).

Efavirenz also has the propensity to undergo photodegradation; hence, it showed better removal than nevirapine (Mthiyane (2022). Furthermore, efavirenz's lipophilic nature and relatively high kPa values indicate the possibility of enhanced interactions with *Chlorella* spp. during prolonged light exposure. According to Rothman et al. (2015), higher kPa correlated with the likelihood of volatilization.

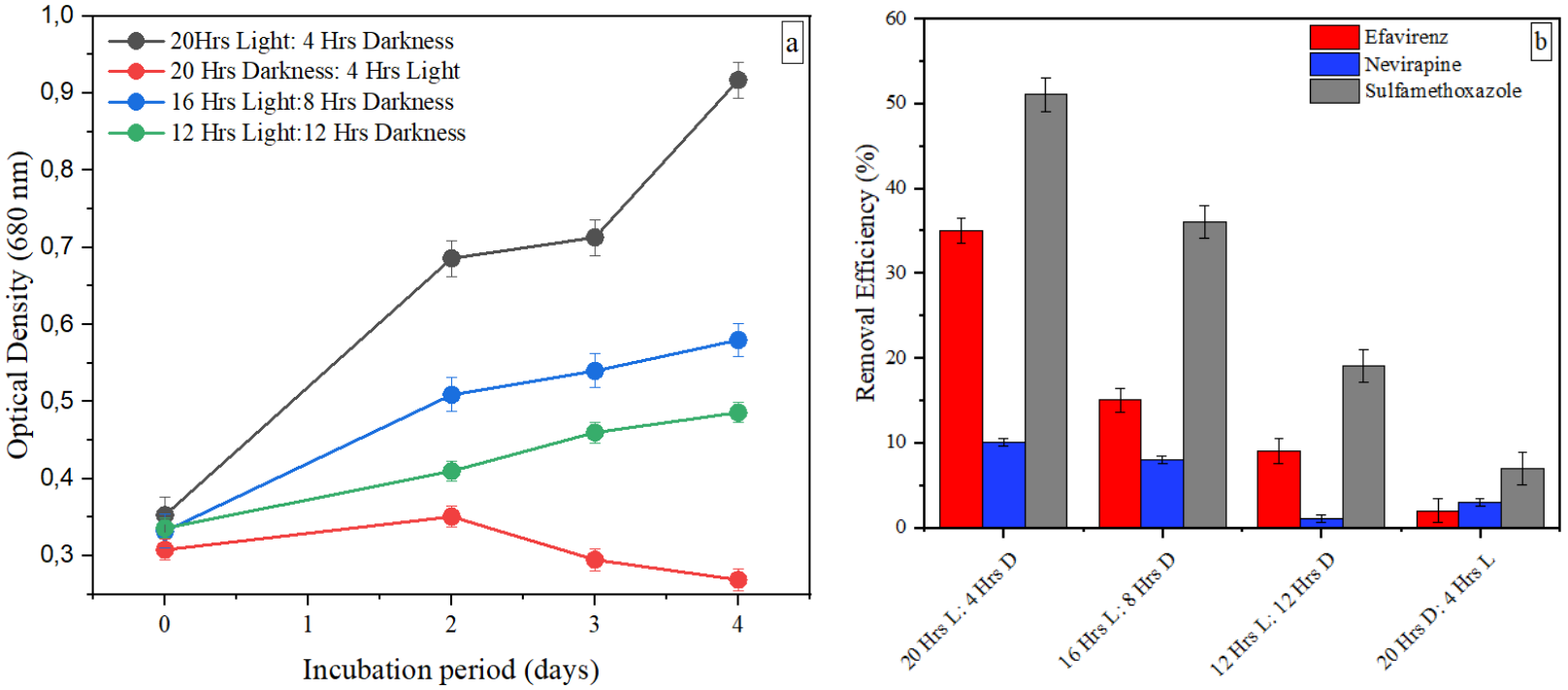


Figure 4.10 The influence of varied light: dark cycles on pharmaceutical removal. (a) depicts the *Chlorella* spp. optical density over four days of incubation whereas (b) illustrates corresponding removal efficiencies with n=3 replicates.

After determining the most suitable light: dark period that resulted in better removal efficiency of efavirenz, nevirapine, and sulfamethoxazole; the investigation was expanded to assess the impact of different *Chlorella* spp. culture concentrations (Figure 4.11a). The study results suggest that the initial algal concentration significantly impacts the subsequent growth of microalgae. Higher initial algal concentrations (16% culture) promoted accelerated growth, primarily due to increased nutrient availability and the increased enzymic and metabolite levels involved in carbon fixation and cell growth. According to Peng et al. (2014), the increase in biomass concentrations corresponded to a greater extent of contaminant adsorption. However, excessively high algal densities (32% culture) can limit growth. This can occur due to the rapid depletion of primary nutrients such as phosphorus and nitrogen, the primary limiting nutrients (Yaakob et al. 2021). Additionally, overcrowding within dense populations can lead to inhibitory effects that further hinder growth. These effects arise from the early depletion of nutrients and the resulting adverse conditions caused by overcrowding. Therefore, balancing the algal concentration to provide sufficient resources and space while avoiding overcrowding is essential for maximizing growth and productivity.

The study results exhibited a lower growth rate when employing 8% culture; this can be attributed to the sparser cell distribution, significantly reducing cell-to-cell interactions (Figure 4.11a). These interactions are crucial as they provide benefits such as enhanced nutrient sharing and cooperative growth that promote higher growth rates (Brennan et al. 2019). Furthermore, cell-to-cell interactions in microalgae can encourage the exchange of signaling molecules and genetic information, facilitating coordinated responses to environmental changes and optimizing the population's overall fitness (Wong et al. 2017). In the 8% culture, the lower cell density may result in reduced allelopathic interactions, which further contributes to the slower growth rates compared to denser cultures, as microalgae release allelochemicals that can have inhibitory or stimulatory effects on other individuals (Amaro et al. 2023). Thus, based on the trends observed in the given data, the 16% algal concentration was a promising choice for further experiments as it resulted in higher optical densities relative to the 8% and 32% concentrations, indicating a more pronounced growth response.

Regarding the removal efficiency of pharmaceutical compounds, the study demonstrates that varying the initial microalgal concentration affects the removal efficiency (Figure 4.11b). Increasing the initial microalgal concentration from 8% to 16% generally enhanced removal efficiency, but increasing it to 32% decreased removal efficiency. This suggests that an

intermediate microalgal concentration (16% *Chlorella*) is more effective in removing these compounds. Additionally, the optimal algal concentration for nevirapine removal is 16%, exhibiting the highest removal efficiency. The study's findings highlight the importance of considering the initial algal concentration in both microalgae growth and the removal efficiency of pharmaceutical compounds, with optimal concentrations leading to enhanced growth and improved contaminant removal.

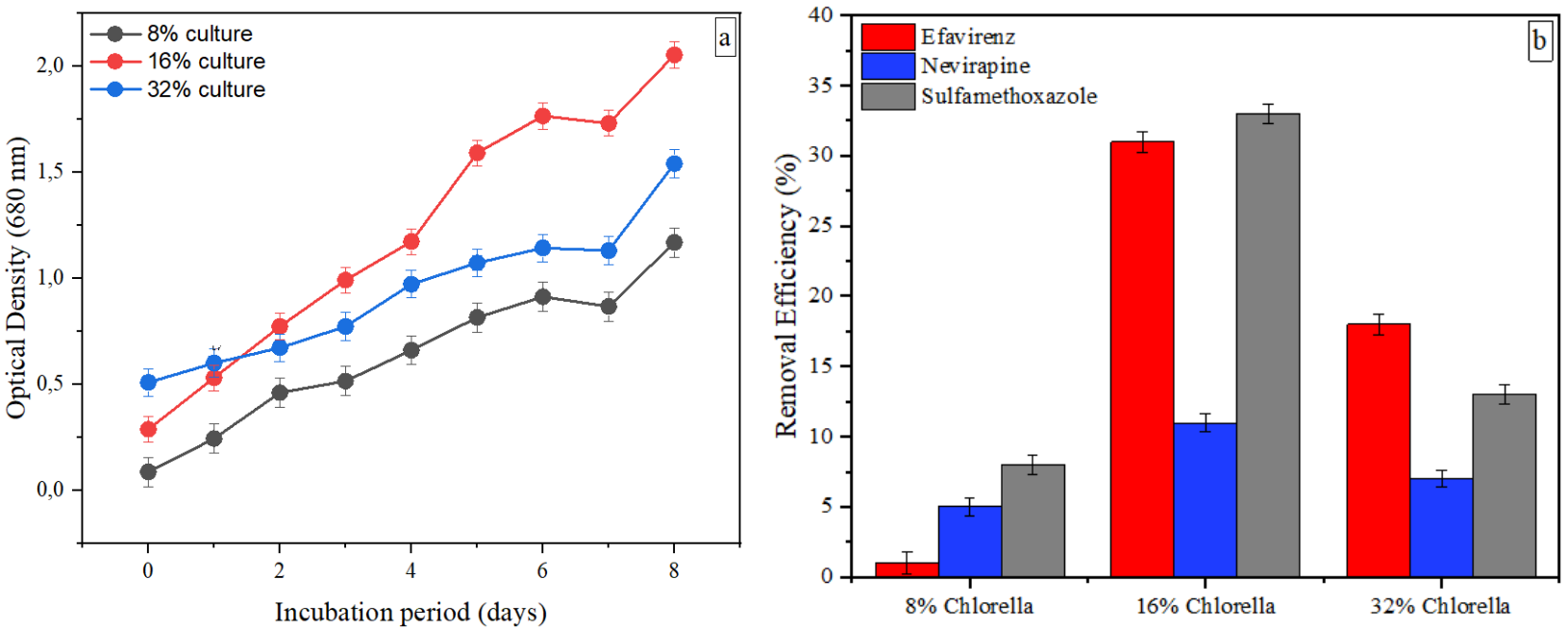


Figure 4.11 The influence of varied culture *Chlorella* spp. concentration with n=3 replicates. (a) exhibits *Chlorella* spp. optical density over 8 days of incubation, while (b) depicts the corresponding removal efficiencies.

4.3.6 Effect of concentration spike on pharmaceutical removal

The performance of *Chlorella* spp. in this experiment was found to vary significantly depending on the concentration of the spiked media. The samples spiked with 0.25 and 0.5 mg/L generally performed slightly better than those spiked with 1 and 5 mg/L (Figure 4.12). The *Chlorella* spp. cells potentially developed specific mechanisms to efficiently metabolize, adsorb, or detoxify the pharmaceuticals at lower concentrations, thereby promoting their growth and proliferation (Abdelfattah et al. 2023). The observed results could also be attributed to the accumulation of toxic metabolites emerging as a contributing factor. Studies by Hao et al. 2017 and Cheng et al. 2020 documented that intermediate metabolites or breakdown product accumulation generally increases as the *Chlorella* spp. cells metabolize and degrade the pharmaceutical compounds. Thus, at higher concentrations of 1 and 5 mg/L, the accumulation of these toxic metabolites might have reached higher levels, further exacerbating their toxic effects on the cells. In contrast, the lower concentrations of 0.25 and 0.5 mg/L reduced toxic metabolite accumulation, thereby enabling better cell survival and growth.

Nutrient imbalance is another crucial aspect that might have contributed significantly to the observed results. The higher concentrations of pharmaceuticals might have disrupted the nutrient balance in the growth media to a greater extent. This disturbance interfered with nutrient uptake and caused imbalances in essential elements necessary for *Chlorella* spp. growth. In contrast, the lower concentrations caused a lesser disturbance in nutrient availability, allowing the *Chlorella* spp. cells to maintain more favourable growth conditions. Lastly, the observed results can be ascribed to the combination of efavirenz, nevirapine, and sulfamethoxazole at higher concentrations potentially yielded synergistic or additive effects, resulting in heightened toxicity to the *Chlorella* spp. cells compared to individual compounds alone. Elevated concentrations of toxicants can disrupt the microalgae cell wall's integrity, induce cell aggregation, or result in cell division dysfunction (Zhang et al. 2019). Thus, the increased overall toxicity led to the observed decline and death of the *Chlorella* spp., particularly at 5 mg/L (Figure 4.12). At lower concentrations, each compound reduced the synergistic or additive effect, thus promoting improved cell viability and growth.

The current study's findings align with previous research by Ding et al. (2021), who observed that low concentrations of ibuprofen promoted the development of the freshwater diatom *Navicula* sp, which belongs to the microalgae group. Likewise, in the study by Dai et al. (2021),

Chlorella vulgaris displayed increased growth in the presence of low concentrations of triclosan. However, higher levels of triclosan led to significant inhibition of the microalgae's growth. In the study conducted by Geiger et al. (2016), the toxicity of individual and binary mixtures of pharmaceuticals and chlorophenols on freshwater microalgae *Chlorella vulgaris* was investigated. The findings revealed that low concentrations of the tested mixtures resulted in higher toxic effects than individual compounds. Additionally, the study identified predominantly additive effects in the mixture toxicity, highlighting the importance of understanding the combined effects of multiple contaminants in aquatic environments. These findings contribute valuable insights into the impact of varying pollutant concentrations on aquatic organisms and underscore the significance of considering pollutant interactions when assessing environmental risks and management strategies.

In terms of the removal efficiencies, results showed that at the higher concentrations (1 and 5 mg/L), the removal percentages were generally lower than the 0.25 and 0.5 mg/L concentrations (Figure 4.12). These findings are consistent with a study by Reddy et al. (2023), which assessed the removal of nevirapine using *Coelastrella tenuitheca* and *Tetradesmus obliquus*. Reddy et al. observed a slight decrease in the removal rate as the nevirapine concentration increased in the medium, indicating the importance of considering the initial concentration levels when designing bioremediation strategies using microalgae. This shared finding suggests that higher concentrations of pharmaceuticals can have a more pronounced toxic effect on microalgae, hindering their growth and reproduction and ultimately leading to reduced optical density measurements. Moreover, a study by Zheng et al. in 2022 focused on enhancing the tolerance and biodegradation capacity of *Chlorella vulgaris* through sulfamethoxazole acclimatization. Interestingly, the study found that the removal rate of sulfamethoxazole exhibited a decreasing trend as the concentration of sulfamethoxazole increased. These findings emphasize the significance of considering initial concentration levels and adjusting media concentration when designing effective bioremediation strategies involving *Chlorella* spp. to remove specific compounds.

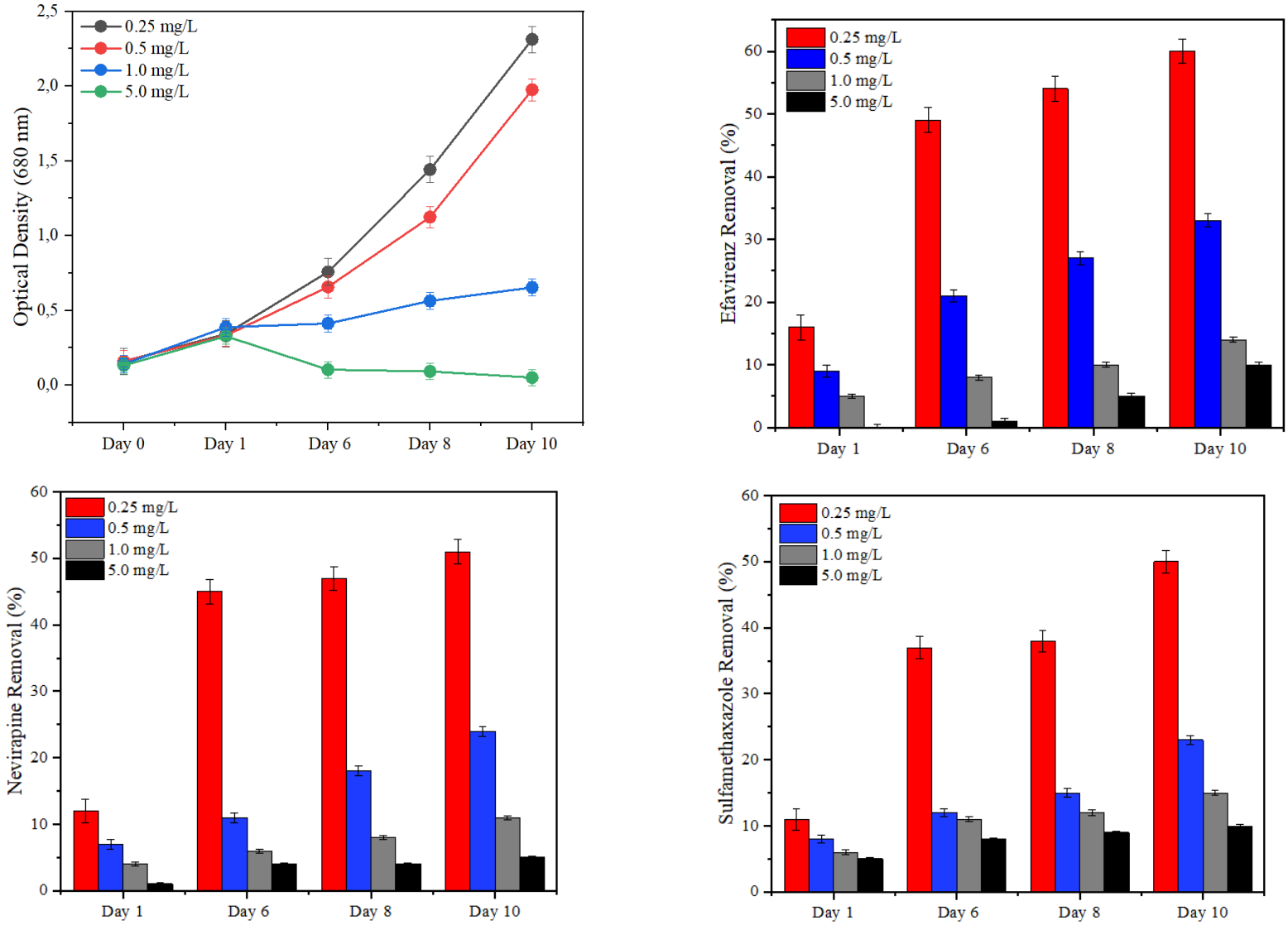


Figure 4. 12 The influence of different concentrations on pharmaceutical removal efficiency (n=3 replicates)

4.3.7 FTIR and SEM

The observed removal percentages of these 3 compounds in this study can also be attributed to their ability to interact with the polar functional groups on the *Chlorella* spp. cell surface, enhancing the adsorption of these compounds. For example, sulfamethoxazole has sulfonamide and amino groups ($-NH_2$) that can form hydrogen bonds with hydroxyl (-OH) and carboxyl (-COOH) groups on *Chlorella* spp. (Khan et al. 2021, Lu et al. 2021, Chu et al. 2022). Efavirenz contains hydroxyl (-OH) and amino ($-NH_2$) groups, which also share the hydrogen-bonding capability, potentially facilitating its adsorption on the cell surface. Nevirapine, with its hydroxyl (-OH), positively charged amino ($-NH_2$) groups, and aromatic rings, not only forms hydrogen bonds but also electrostatic interactions with *Chlorella* spp. but also contributes to hydrophobic interactions due to its aromatic rings (Adeola et al. 2021, Barik et al. 2021). Also, it is noteworthy to highlight that photodegradation is another mechanism that can be attributed to the observed pharmaceutical removals, as reduction was also observed in controls where microalgae was not added. Through photodegradation, pharmaceutical compounds absorb photons, which excites them and undergo chemical reactions, usually resulting in transformed compounds that are less harmful or more readily biodegradable.

The bioremediation experiment on day 10 showed a noticeable shift in the FTIR spectrum, with a peak shifting from 3278.97 cm^{-1} (before) to 3276.74 cm^{-1} (after) (Figure 4.13). This peak corresponds to the stretching vibration of the hydroxyl (-OH) functional group, which is abundant in carbohydrates, proteins, nucleic acids, and other organic molecules within *Chlorella* spp. (Morais et al. 2020). The observed slight decrease in wavenumber suggests a potential reduction in the concentration of hydroxyl groups, possibly due to their involvement in the bioremediation process. These hydroxyl groups are known to bond hydrogen and interact with polar compounds like efavirenz, nevirapine, and sulfamethoxazole (Klecker and Nair 2017, Hutchins 2018). Consequently, these interactions facilitate the adsorption and binding of pharmaceuticals onto the surface or within their cellular structures, ultimately enhancing their removal from the environment (Bilal et al. 2018, Silva et al. 2019, Abdelfattah et al. 2023). It is important to note that after treatment, the narrower and less intense O-H peak suggested a reduction in hydrogen-bonded hydroxyl groups, implying their involvement in adsorption or chemical interactions with the pharmaceuticals.

Another shift in the FTIR spectrum was observed, with a peak changing from 2923.73 cm^{-1} (before) to 2925.38 cm^{-1} (after) (Figure 4.13). This peak corresponds to the stretching vibration of C-H bonds in aliphatic hydrocarbons (C-H alkane groups), as reported by Goher et al.

(2016), El-Naggar et al. (2020) and Morais et al. (2020). According to Vidyadharani and Dhandapani (2013), this peak indicates the abundance of lipid substances. Usually, the cell membranes contain lipids with C-H bonds in their hydrocarbon tails, which contribute to the membrane's integrity, fluidity, and selective permeability (El-fayoumy et al. 2023). This structural feature is crucial for the selective uptake of pharmaceutical compounds and other molecules during bioremediation processes. Furthermore, microalgae possess enzymes capable of enzymatically breaking down complex organic compounds like pharmaceuticals through oxidation or reduction reactions (Xiong et al. 2021), where C-H bonds often play a role. Moreover, some pharmaceutical compounds, like sulfamethoxazole, have hydrophobic regions in their molecular structure (Niu et al. 2023). The C-H bonds in *Chlorella* spp. can interact with and adsorb these hydrophobic compounds, sequestering them within the cell or onto its surface. This adsorption process contributes to the efficient removal of pharmaceutical pollutants from the surrounding environment.

The FTIR spectrum analysis revealed significant shifts in the peaks related to the C=C bonds in the bioremediation experiment (Figure 4.13). The peak at 1638.82 cm^{-1} (before) and 1634.86 cm^{-1} (after) corresponds to the alkene group with a variable C=C bond between atoms and medium intensity. Its shift after adsorption suggested a possible change in functional groups, indicating the metabolism of the target compounds by *Chlorella* spp. (Kee et al. 2019, Morais et al. 2020). Additionally, the peak at 1534.57 cm^{-1} (before) and 1534.39 cm^{-1} (after), associated with the stretching vibration of C=C bonds in aromatic compounds, exhibited a slight decrease in wavenumber, indicating potential modification or degradation of aromatic components in the target compounds. It's worth noting that after bioremediation, the C=C peaks showed an increase in intensity, indicating interactions with unsaturated compounds (such as aromatic rings) present in pharmaceutical molecules.

The shift in the FTIR spectrum from 1394.37 cm-1 (before) to 1395.77 cm-1 (after) was observed, and it corresponds to the symmetric stretching vibration of a carboxylate group (COO-) (Šimonovičová et al. 2021) (Figure 4.13). After treatment, the COO- peak increased intensity and broadened, suggesting active involvement of carboxylate groups in binding or interacting with the pharmaceuticals. These carboxylate groups in *Chlorella* spp. play a crucial role in the bioremediation of pharmaceutical compounds like efavirenz and nevirapine through electrostatic interactions, leading to the formation of complexes or chelation complexes that sequester and immobilize the compounds (Danouche et al. 2021, Ankit et al. 2022, Osman et al. 2023). Additionally, in the case of sulfamethoxazole, ion-exchange reactions take place

(Anness and Conoby 2013, Shu et al. 2023), facilitating its uptake and subsequent removal by *Chlorella* spp. from contaminated water sources.

The shift in the FTIR spectrum from 1237.79 cm^{-1} (before) to 1232.20 cm^{-1} (after) is associated with the stretching vibration of a C-N bond (Zhou et al. 2016) (Figure 4.13). This decrease in wavenumber suggests a possible modification of compounds containing amine functional groups. After bioremediation, the increased intensity of C-N suggested interactions with nitrogen-containing pharmaceutical compounds. The C-N bonds play a crucial role in substrate binding and catalysis, transforming the pollutants into less toxic forms. Pharmaceutical compounds often have nitrogen-containing functional groups, such as amines or amides, which can interact with C-N bonds in *Chlorella* spp. cells, leading to the adsorption and binding of pharmaceuticals that facilitate their removal from the environment. C-N bonds within *Chlorella* spp. allow for surface modifications that enhance the cell's effectiveness in adsorbing and interacting with pharmaceutical compounds during bioremediation. Another shift in the FTIR spectrum from 1042.52 cm^{-1} (before) to 1044.58 cm^{-1} (after) was observed, which corresponds to the stretching vibration of a C-O bond (Zhou et al. 2016, Kee 2019, El-Naggar et al. 2020) (Figure 4.13). The slight increase in wavenumber suggests possible changes in the concentration of these functional groups. The C-O bonds are documented in carbohydrates, essential to *Chlorella*'s structural components (Andreeva et al. 2021, Aghazadeh et al. 2023). Carbohydrates provide energy and support for the bioremediation process and maintain the viability of microalgae during the bioremediation process (Andreeva et al. 2021).

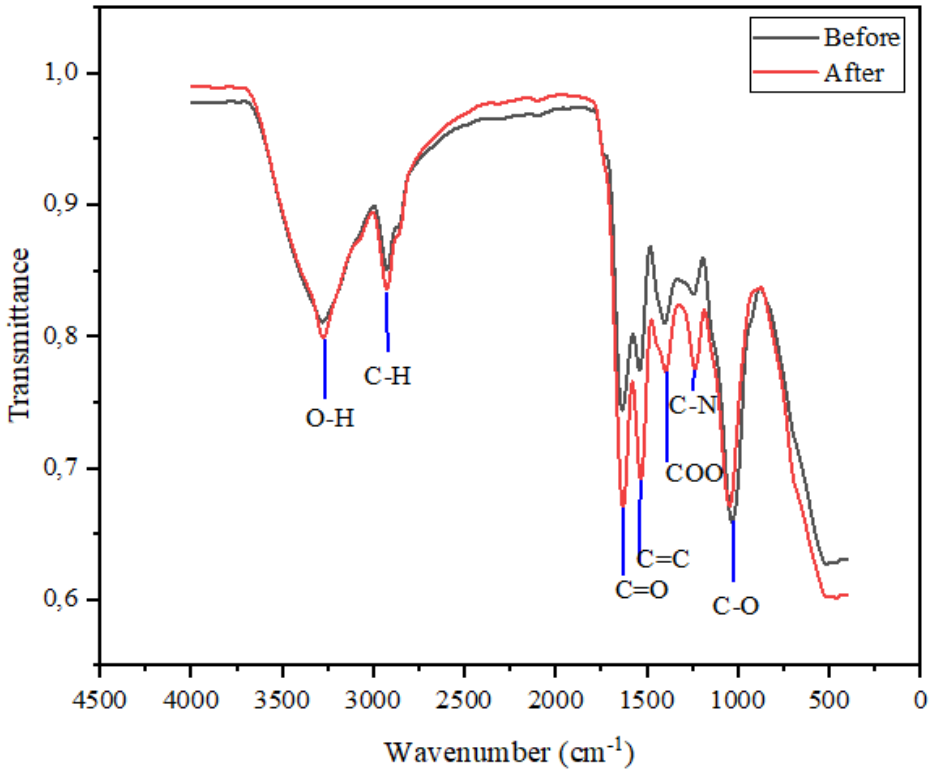


Figure 4.13 The FTIR spectrum analysis for the bioremediation experiment.

The scanning electron microscopy (SEM) was used to access the morphology of *Chlorella* spp. before and after the bioremediation experiments (Figure 4.14 a–b). The cells had specific dimensions and slightly flat cell features before exposure. After bioremediation, the SEM images revealed alterations, including a more irregular texture of the cell surface, and cells displayed slight swelling. The cells initially exhibited a healthy state with intact cell walls; post-experiment, there were noticeable signs of damage or stress to the cell wall, indicating potential interactions between the pharmaceutical compounds and the functional groups on the *Chlorella* spp. cell surface.

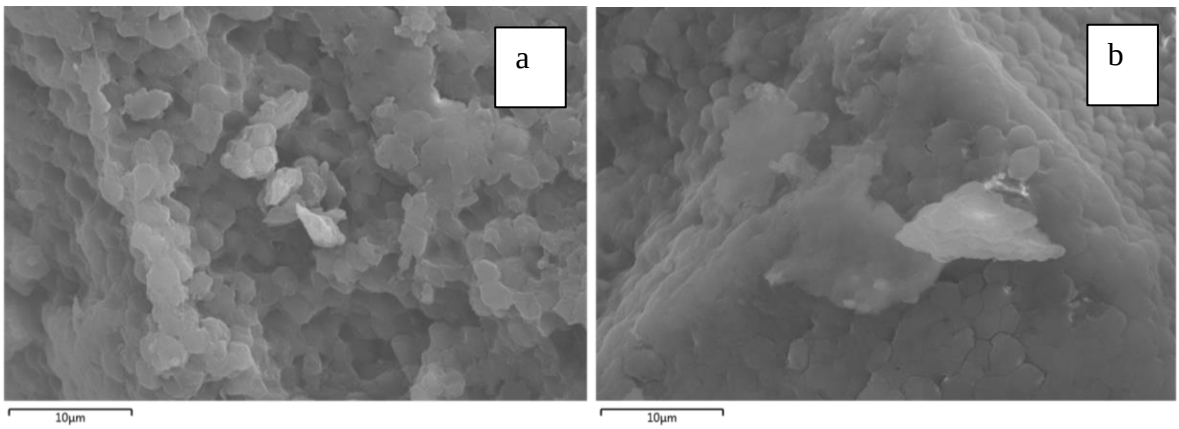


Figure 4.14 SEM micrograph of *Chlorella* spp.(a) before (b) after pharmaceuticals adsorption.

4.4 Conclusion

The optimized LC-PDA method conditions, including a mobile phase (90:10% acetonitrile: water with 0.1% formic acid), 8-minute run time, flow rate of 0.4 mL/min, and a wavelength of 220 nm provided a reliable framework for multi-residue analytical method, allowing for the simultaneous detection and quantification of multiple contaminants (antibiotic and antiretrovirals) in a single sample. In the SPE method, using a mixture of acetonitrile and methanol in a 70:30 (v/v) ratio as a conditioning solvent, 50 mL sample volume, and neutral pH demonstrated efficient recoveries for all target analytes. This analytical approach is essential for continuously monitoring such compounds in water matrices, helping identify and address environmental and public health concerns associated with pharmaceutical residues. Removal of antiretroviral drugs (ARVDs) and an antibiotic using *Chlorella* spp. exhibited concentration-dependent patterns. Higher initial concentrations (5 mg/L and 1 mg/L) resulted in lower removal efficiency, whereas lower initial concentrations (0.5 mg/L and 0.25 mg/L) demonstrated higher removal rates. Several factors contributed to this, including the potential accumulation of toxic metabolites, nutrient imbalance in the growth medium, increased pharmaceutical toxicity, and more. The study also uncovered a possible positive relationship between microalgae growth and pharmaceutical removal efficiency, influenced by factors such as cell-to-cell interactions, nutrient availability, pH, and others. The FTIR spectrum displayed shifts in functional groups, including -OH, C-H bonds, COO-, C-N, and C=O, indicating that they played a role in adsorbing these compounds through various interactions like hydrogen bonding, electrostatic forces, and hydrophilic interactions. Supporting this observation, SEM images depicted alterations on the *Chlorella* spp. cell surface, suggesting possible interactions between the targeted compounds and *Chlorella* spp. cells. It is crucial to underscore that the findings of this study lay a solid groundwork for the application of microalgae-based bioremediation. Nevertheless, further research is imperative to delve into the intricate mechanisms, such as biochemical and molecular mechanisms, that drive pharmaceutical removal. A comprehensive understanding of these intricacies will empower us to develop more efficient and sustainable solutions, ultimately contributing to cleaner water bodies and a healthier environment. Future studies should focus on scaling up the process for real-world applications, exploring the genetic and metabolic pathways involved in microalgae's removal capabilities, and assessing the long-term stability and efficacy of the bioremediation process under various environmental conditions. Additionally, investigating co-cultivation with other organisms such as *D. magna*, bacteria, and other microorganisms could enhance removal efficiency and potentially achieve 100% contaminant removal.

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Chapter 5 Unlocking the Potential of *Chlorella* spp. Biomass: Effective Adsorption of Copper, Lead, and Zinc from Wastewater

Abstract

Heavy metals in aquatic environments pose a significant environmental and health concern globally. These metals are often detected in wastewaters and rivers, where conventional treatment methods frequently fall short in completely removing them. Existing approaches are often inefficient and unsustainable, relying on costly and chemically intensive processes that do not effectively address the broad spectrum of heavy metal contaminants. This research focuses on addressing this critical gap by investigating the potential of *Chlorella* spp. biomass for adsorbing and removing heavy metals such as copper, lead, and zinc from wastewater. By optimizing conditions and exploring adsorption mechanisms, the study aims to contribute to the development of more efficient and sustainable wastewater treatment strategies, supporting environmental sustainability goals and ensuring cleaner water ecosystems for future generations. Fourier transform infrared spectroscopy results exhibited functional groups such as hydroxyl, methyl, carbonyl, alkene, carboxyl, amide, and ether responsible for binding heavy metal ions on the *Chlorella* spp. surface. Batch cultures, conducted in triplicate at 150 rpm in an orbital shaker, evaluated the removal of heavy metals through adsorption under varied biomass dosage (5–100 mg), pH levels (3–11), contact times (15–115 minutes), and metal concentrations (0.25–16 mg/L) at a constant temperature of 25 °C. The optimum conditions for all three metals were pH 7, 60 minutes contact time, biomass dosage of 12.5 mg, and a metal concentration of 0.5 mg/L. 100% removal efficiencies were obtained for lead and zinc, while copper reached the highest removal rate of 80%. Adsorption of copper, lead, and zinc were also investigated using Freundlich, Langmuir, and Temkin isotherms, with the Langmuir isotherm best fitting copper (R^2 = 0.9888). In contrast, the Freundlich isotherm best-fitted experimental data for lead (R^2 = 0.976) and zinc (R^2 = 0.968). In terms of the kinetics model, copper, lead, and zinc followed the pseudo-first-order. Thermodynamic studies revealed an endothermic and spontaneous process for copper and zinc, while a complete removal was obtained for lead at all tested temperatures, so calculations were impossible. Given the high removal percentages observed for the assessed heavy metals, *Chlorella* spp. biomass proved to be a promising and sustainable option for effectively removing heavy metals from polluted wastewater. Based on these findings, future research should explore co-cultivation with other microorganisms, and scale up to pilot applications. Collaboration among

researchers, industry, and policymakers is essential for integrating these technologies into environmental management, supporting sustainable water quality and global goals.

Keywords: Adsorption, *Chlorella* spp., metal pollution, Copper, Lead, Zinc

5.1 Introduction

Recent global assessments indicate that heavy metal contamination affects drinking water sources for approximately 40% of the lakes and rivers worldwide, highlighting the pervasive nature of this environmental and public health issue (Zamora-Ledezma et al. 2021, Pandey and Kumari 2023, Piwowarska et al. 2024). Heavy metals such as copper (Cu), cadmium (Cd), chromium (Cr), mercury (Hg), and lead (Pb) are persistent pollutants in water bodies, originating mainly from industrial discharges, agricultural runoff, mining activities, and improper waste disposal (Ali et al. 2019, Briffa et al. 2020, Balali-Mood et al. 2021, Spain et al. 2021). The toxicity of heavy metals poses significant health risks to human populations. Acute exposures can lead to severe neurological disorders, gastrointestinal disturbances, and cardiovascular diseases (Balali-Mood et al. 2021, Spain et al. 2021). Chronic exposure, even at low levels, is linked to various cancers, kidney diseases, and reproductive disorders (Cherono et al. 2021, Prabakaran and Rajan 2021). Vulnerable groups such as children, pregnant women, and the elderly are particularly susceptible due to their higher sensitivity to toxic substances and prolonged exposure durations. In addition to direct health impacts, heavy metals bioaccumulate in aquatic organisms, entering the food chain and posing ecological threats. Fish and shellfish, vital protein sources for millions, can accumulate high levels of these contaminants, leading to food safety concerns and economic impacts on fisheries (Tattibayeva et al. 2022). Furthermore, heavy metal contamination jeopardizes ecosystem health, disrupting aquatic biodiversity and ecosystem services crucial for human well-being, such as water purification and nutrient cycling.

Moreover, there are varying degrees of the effectiveness of conventional wastewater treatment processes in eliminating heavy metals through different methods, potentially leading to the discharge of heavy metals into receiving rivers despite treatment efforts. In addition to conventional treatment methods such as activated sludge and chemical precipitation, cutting-edge techniques such as reverse osmosis, membrane filtration, and ion exchange can achieve significant heavy metal removal (Ni et al. 2019, Pfeifer and Skerget 2020, Tattibayeva et al. 2022). However, these advanced methods have numerous drawbacks, like expensive

operational costs, employing chemicals that generate secondary pollution, less efficiency at low concentration levels, disposal mechanisms of residual sludge, and complex processes requiring highly skilled individuals for operation and maintenance (Cherono et al. 2021, Tattibayeva et al. 2022). This highlights a critical gap in the need for efficient, innovative, sustainable, environmentally friendly, and cost-effective approaches to remediate heavy metal pollution.

Biological adsorbents like microalgal biomass are a promising and environmentally sustainable alternative for removing heavy metals from metal-polluted water. Microalgae, such as members of the genus *Chlorella*, are considered ideal candidates due to their fast growth, high surface area exhibiting metal-binding functional groups (e.g., amide, carboxyl, hydroxyl) aiding in metal removal via bonding at the microalgal cell wall water interface (Yadav et al. 2021, Kula-Maximenko et al. 2022, Ma and Jian 2023). The processes that govern the sequestration of heavy metals by microalgae like *Chlorella* spp. include ion exchange, complexation, surface complexation, precipitation, and reduction (Giarikos et al. 2021). Therefore, this study examined the relationship between the adsorption of different heavy metals (copper - Cu^{2+}, lead Pb^{2+}, and zinc - Zn^{2+}) and *Chlorella* spp. biomass at varying pH, biomass dose, contact time, and metal concentration. These heavy metals were chosen based on their frequent detection in water environments, which can be ascribed to their non-biodegradable nature (Ni et al. 2019). The Langmuir, Freundlich, and Temkin isotherm models described copper, lead, and zinc adsorption on the *Chlorella* spp. biomass. Pseudo-first- and pseudo-second-order kinetics were explored with an intraparticle diffusion model to understand the reaction rates and mechanism between the studied metals and *Chlorella* biomass. Thermodynamic calculations were also employed to assess heavy metals' adsorption behavior interacting with *Chlorella* spp. biomass.

5.2 Materials and Methods

5.1.1 Study site, sample collection, storage, and preparation

The Darvill WWTP in Pietermaritzburg, KwaZulu-Natal, South Africa, was selected as the study site. The water samples were collected from the effluent point using high-quality plastic containers (polyethylene, Lasec) to prevent sample contamination and adsorption on the walls of the container. The containers were then sealed airtight to prevent the heavy metals from oxidizing and forming insoluble compounds in the presence of air (Abd-El-Nabey et al. 2022).

The samples were temporarily stored in a cooler box with ice during transportation and kept in a fridge at about 4 °C at the laboratory. Parameters such as pH, conductivity, temperature, total dissolved solids (TDS), salinity, and dissolved oxygen (DO) were measured using a YSI multiparameter meter. Before experiments, the wastewater effluent samples underwent filtration using a 55 mm chm shift filtration paper (F1001 grade) to eliminate large particles.

5.1.2 Reagents and certified reference material

Purelab ultrapure water with a conductivity of 18.2 MΩ·cm was employed to prepare calibration standards, and all glassware was cleaned using a diluted nitric acid solution. The 1000 mg/L ICP heavy metals CRMs (Copper – Cu, Lead – Pb, and Zinc – Zn) procured from Sigma Aldrich (Johannesburg, South Africa) were used in the analysis.

5.1.3 ICP-OES Instrument Calibration

The metal determination in water was conducted using the Varian 720-ES ICP-OES instrument (Varian, Johannesburg, South Africa). The instrument operated at a frequency of 40 MHz, with an RF power of 1.00 kW. A pneumatic concentric nebulizer was utilized, delivering a flow rate of 0.75 L/min, and an inert carrier gas (Argon) was introduced at a rate of 15 rpm (Naicker et al. 2023). The wavelengths acquired were 327.396 nm for copper, 220.353 nm for lead, and 202.548 nm for zinc

5.1.4 Validation of the analytical method for the determination of heavy metals

The evaluation of the performance of the analytical methods involved assessments of linearity, limits of detection (LOD), limits of quantification (LOQ), and percentage recovery tests. The ICP-OES calibration and the method linearity assessment were performed using a mixture of heavy metals with concentrations ranging from 0.1 to 20 mg/L. The intensities of ten blank samples (n = 10) were measured. To determine the LODs and LOQs for the specific analytes, these values were calculated as 3 and ten times the standard deviation (σ) of the average of the ten individually prepared blank solutions. The method's accuracy was evaluated by spiking wastewater effluent samples with one mg/L standard containing a mixture of heavy metals of interest. Subsequently, the percentage recoveries were computed using equation 1.1.

$$\%\ Recovery = \frac{measured\ heavy\ metal\ concentration}{spiked\ heavy\ metal\ concentration}\ x\ 100 \qquad (5.1)$$

5.1.5 Preparation of Chlorella spp. biomass

5.1.5.1 Chlorella spp. culture and maintenance

The uMgeni-uThukela Water (Pietermaritzburg, South Africa) provided the *Chlorella* spp. strain used in this study, with cultivation routinely done using tris-acetate phosphate (TAP) medium, as outlined by Harris (1989). An axenic culture was established by sequential subculturing and purification of single colonies of *Chlorella* spp. strain FSB41.2 using acetate free medium under autotrophic growth conditions with regular culture-based (Nutrient agar) and microscopic (phase contrast) monitoring as reported previously (Adedoyin and Schmidt, 2023). Subsequently, biomass production occurred in a controlled growth environment (approximately 23°C with a 20-hour light-dark cycle) using 1000 mL Erlenmeyer flasks, each filled with 600 mL of the TP culture medium following autoclaving. The growth of cultures was monitored over time by absorbance measurements at 680 nm using a UV/Visible Spectrophotometer (JENWAY 7310). The cells were harvested from the TP culture medium after reaching the late exponential growth phase by centrifugation (5000 rpm, 15 min) and washed 3 times with distilled water to remove the remaining medium. Following centrifugation, the resulting *Chlorella* spp. cell pellets were subjected to overnight oven drying at 80 ^{o}C, since very low or exceedingly high temperatures negatively affect the cell structure (Tattibayeva et al. 2022). Subsequently, an electric grinder (MRC SM-450) was used to grind the dried pellet, followed by sieving (KINGTEST sieve shaker VB200/300) and storing the dried biomass in a sealed container to prevent rehydration.

5.1.6 Characterisation of Chlorella spp. biomass

The dried *Chlorella* spp. biomass was characterized by analyzing its elemental composition, surface properties, and functional groups using Fourier-Transform Infrared Spectroscopy (PerkinElmer Inc.), Scanning Electron Microscopy (SEM Zeiss EVO LS15), and X-ray diffraction (Rigaku MiniFlex 600) techniques. After that, the optimization for the adsorption studies was performed by adjusting parameters such as biomass dosage, pH, contact time, and initial heavy metal ion concentration.

5.1.7 Adsorption studies

Dried *Chlorella* spp. biomass (12.5 ± 0.1 mg) obtained from axenic cultures was added to conical flasks with 30 mL of the metal ion solution at a known concentration ranging from

0.25–16 mg/L. Experiments were performed to determine the adsorption using Freundlich, Langmuir, and Temkin isotherms. Intraparticle diffusion models, pseudo-first- and pseudo-second-order, were assessed for adsorption kinetics. The samples were incubated in a rotary shaker at 150 rpm and 25 °C for 60 minutes in these experiments. The samples were centrifuged at 5000 rpm for 15 minutes to separate the *Chlorella* spp. cells from the liquid. The liquid samples were then filtered with a 0.22 μm pore size filter (Merck Millex) and a 30 mm sterile syringe to remove residual algal cells before analysis with ICP OES. The impact of temperature on adsorption equilibrium was explored by altering the temperature within the 298 to 333 K range (Tang et al. 2017, Soto-Ramírez et al. 2023).

The adsorption process was quantified using the provided formula (5.2):

$$qe = \frac{(C0-Ce)}{m}\ x\ V \qquad (5.2)$$

Where qe is the adsorption capacity (mg/g), C0 is the initial concentration of the adsorbate (mg/L), Ce is the equilibrium concentration of the adsorbate (mg/L), V is the volume of the solution (L), m is the mass of the adsorbent (g).

The degree of removal (R) of heavy metal ions was calculated by the following formula (5.3):

$$\%\ Recovery = \frac{(C0-Ce)}{C0} x\ 100 \qquad (5.3)$$

Where: RE is the removal efficiency in percentage, C0 is the initial concentration before adsorption, and Ce is the concentration at equilibrium.

The kinetic models of pseudo-first order, pseudo-second order, and intraparticle diffusion were calculated using the following formulas (5.4, 5.5, and 5.6) (Spain et al. 2021, Shi et al. 2023):

$$qt = qe(1 - e)^{-K^1.t} \qquad (5.4)$$

Where: *qt*: Adsorption capacity at time *t* (mg/g), *qe*: Adsorption capacity at equilibrium (mg/g), *t*: Time (min), k^1: Rate constant of the pseudo-first-order kinetics (1/min).

$$qt = \frac{qe^2 k_2 t}{1+qtk_2 t} \qquad (5.5)$$

Where: *qt*: Adsorption capacity at time *t* (mg/g), *qe*: Adsorption capacity at equilibrium (mg/g), *t*: Time (min), *k*2: Rate constant of the pseudo-second-order kinetics (1/min).

$$qt = k_{diff}\, x\, t^{1/2} + C \quad (5.6)$$

Where: *qt*: Adsorption capacity at time *t* (mg/g), *k:* intraparticle diffusion rate constant, *t:* Time (min), C: is the intercept that represents the boundary layer effect

The following equations were used to calculate Freundlich, Langmuir, and Temkin isotherms (5.7, 5.8) (Cherono et al. 2021):

$$log(qe) = log\ log\left(k_f\right) + \frac{1}{n}\, x\, log\ (Ce) \quad (5.7)$$

Where log(*qe)* is the logarithm of the amount of solute adsorbed per unit mass of adsorbent at equilibrium, log (K_f)*:* is the logarithm of the Freundlich constant, 1/*n* is the reciprocal of the Freundlich exponent or non-linearity factor, log (*Ce):* is the logarithm of the equilibrium concentration of the solute in the liquid phase.

$$qt = \frac{1}{k_2} x\, qe^2 + \frac{1}{qe} \quad (5.8)$$

Where: *t* is the time (min), *qt* is the amount of adsorbate adsorbed at time *t* (mg/g), *qe* is the amount of adsorbate adsorbed at equilibrium (mg/g), *k*2 is the rate constant of the pseudo-second-order kinetics (g/mg·min).

For thermodynamics, the parameters and their associated formulas are (5.9 - 5.12):

$$K_2 = \frac{qe}{Ce} \quad (5.9)$$

$$\Delta G = -RT\ x\ Ln\ x\ KL \quad (5.10)$$

$$\Delta H = -Slope\ x\ R \quad (5.11)$$

$$\Delta S = Intercept\ x\ R \quad (5.12)$$

Where: Δ*G* is the Gibbs free energy change (kJ/mol), Δ*H* is the enthalpy change (kJ/mol), Δ*S* is the entropy change (kJ/mol), *T* is the absolute temperature (K), R = Gas constant.

5.2 Results and Discussion

5.2.1 Method validation for the ICP-EOA instrument

The performance of the ICP-OES was assessed through regression equations from concentrations ranging from 0.1 - 20 mg/L. The correlation coefficients (R^2) ranged from 0.9996 to 0.9998, signifying a strong linear relationship between the concentrations assessed and the ICP-OES, indicating exceptional accuracy and precision (Table 1).

Table 5.1 additionally represents the optimum wavelengths, limit of detection, limit of quantification, repeatability, and reproducibility for the selected heavy metals. When using ICP-OES, selecting optimum wavelengths is paramount for producing accurate and sensitive results as they maximize sensitivity, decrease spectral interferences, and increase measurement precision (Naicker et al. 2023). The LOD and LOQ obtained in this study ranged from 0.06 - 0.255 mg/L and 0.196 - 0.851 mg/L respectively. This implied a high sensitivity level, affirming the methods' capability to detect and quantify trace levels effectively. This study assessed precision by repeatability and reproducibility, expressed as %RSD. The results produced %RSD values of less than 1% for all analytes, indicating high precision and reliable performance.

Table 5.1 Method validation parameters established for the ICP-OES instrument (n=3)

Heavy Metals	Wavelength (nm)	R^2	LOD (mg/L)	LOQ (mg/L)	Repeatability	Reproducibility
					% Recoveries ±% RSD	
Copper	327.396	0.9998	0.128	0.426	93.6±0.8	90±0.9
Lead	220.353	0.9995	0.06	0.196	91.1±0.1	89±0.5
Zinc	202.548	0.9998	0.255	0.851	94.5±0.3	92±0.7

5.2.2 Characterisation of Chlorella biomass as the adsorbent

Scanning electron microscopy (SEM) is a powerful tool for visualizing the surface features and morphology of microorganisms such as *Chlorella* spp. (Nasir et al. 2023). As expected, the

Chlorella spp. even in the dried state, cells showed an almost intact surface area, enabling the binding of metal ions via interaction with exposed functional groups (Spain et al. 2021, Tattibayeva et al. 2022). An image taken after adsorption indicated morphological changes resembling cell shrinkage and increased surface irregularities, suggesting an interaction of the cell surface with metal ions (Figure 1b).

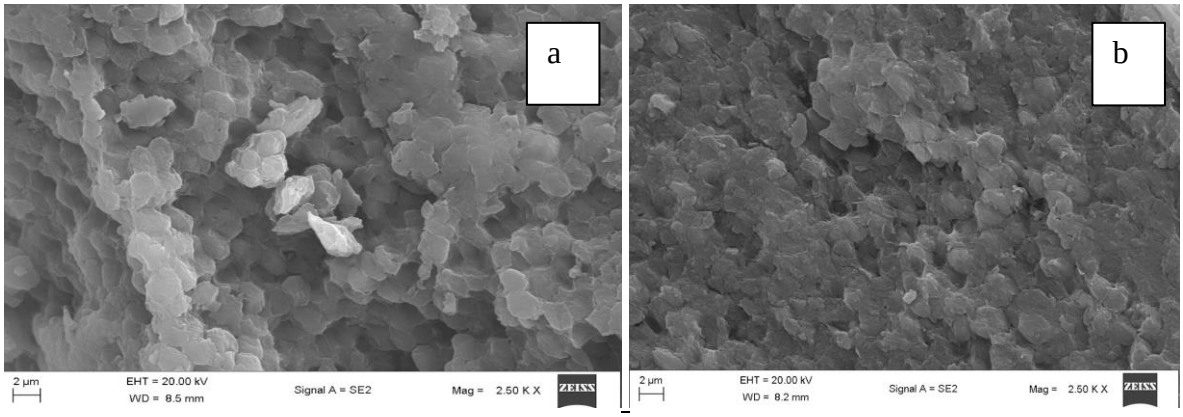

Figure 5.1 SEM micrograph of dried *Chlorella* spp. cells before (a) and after (b) metal binding.

The results of the analysis of the elemental composition of *Chlorella* spp. biomass, which comprises carbon (C), nitrogen (N), oxygen (O), phosphorus (P), and sulfur (S) are shown in Figure 5.2. The obtained elemental composition in this study closely aligns with the literature values reported for *Chlorella* spp. by Mandalam et al. (1998), with the exception of slightly higher levels of nitrogen (N) and sulfur (S) in this study. The obtained elements are associated with microbial biomass, such as *Chlorella* spp., and are key elements present in microalgal proteins, nucleic acids, lipids, carbohydrates, and organic acids (Ma et al. 2020, Yaakob et al. 2021). Organic functional groups, such as carboxyl (-COO) and hydroxyl (-OH), are abundant on the cell surface of *Chlorella* spp. and are crucial in the adsorption of heavy metals by providing binding sites (Yang et al. 2019). Similarly, nitrogen-containing functional groups such as amide (C-N) associated with cell surface proteins form various interactions with metal ions (Giarikos et al. 2021), which applies as well to phosphate and sulfur-containing groups.

Analytical methods such as Fourier-transform infrared spectroscopy (FTIR) are vital in identifying functional groups present in microbial biomass. For *Chlorella* spp., the functional groups present in biomass samples analysed included hydroxyl (-OH), methyl (-CH3), carbonyl (C=O), alkene (C=C), carboxyl (-COO), amide (C-N), and ether (C-O) (Figure 2b). These functional groups increase the overall binding capacity and reactivity of *Chlorella* spp. biomass. This study results agree with the findings of Ahmad et al. (2018), El-Nagger (2020),

Sultana et al. (2020), and Shi et al. (2023), who reported the occurrence of similar functional groups that play a significant role in heavy metal adsorption. Notably, a shift from 3270 to 3281 cm-1 after adsorption was observed in the peak associated with O-H stretching vibrations. As hydroxyl groups enable hydrogen bonding interactions (Wohlert et al. 2022, Wang and Guo 2023), the observed changes in peak intensity and broadness confirmed the interactions of hydroxyl groups with metal ions. The observed shift in C-H stretching vibrations from 2925 to 2968 cm^{-1} provided evidence of their involvement in hydrophobic interactions (Barchi and Strain 2023). The increased intensity, as well as shifts in C=O, C=C, and C-N stretching vibrations from 1618 to 1638 cm^{-1}, 1538 to 1528 cm^{-1}, and 1234 to 1250 cm^{-1}, respectively, suggests the interaction of carbonyl, alkene, and amide groups with metal ions during the adsorption process through metal coordination (Spain et al. 2021, Li et al. 2022, Barchi and Strain 2023). Other noticeable shifts in the carboxyl group (1407 to 1398 cm^{-1}) and ether group (1036 to 1028 cm^{-1}) were observed. The carboxyl groups promote electrostatic interactions through negatively charged sites, and ether groups play a crucial role in the overall surface polarity (Barczak 2019, Zhang et al. 2019). This exhibited a diversity of functional groups, which enabled the complexity of interactions and ultimately impacted the adsorption capacity of *Chlorella* spp. Biomass for target heavy metals.

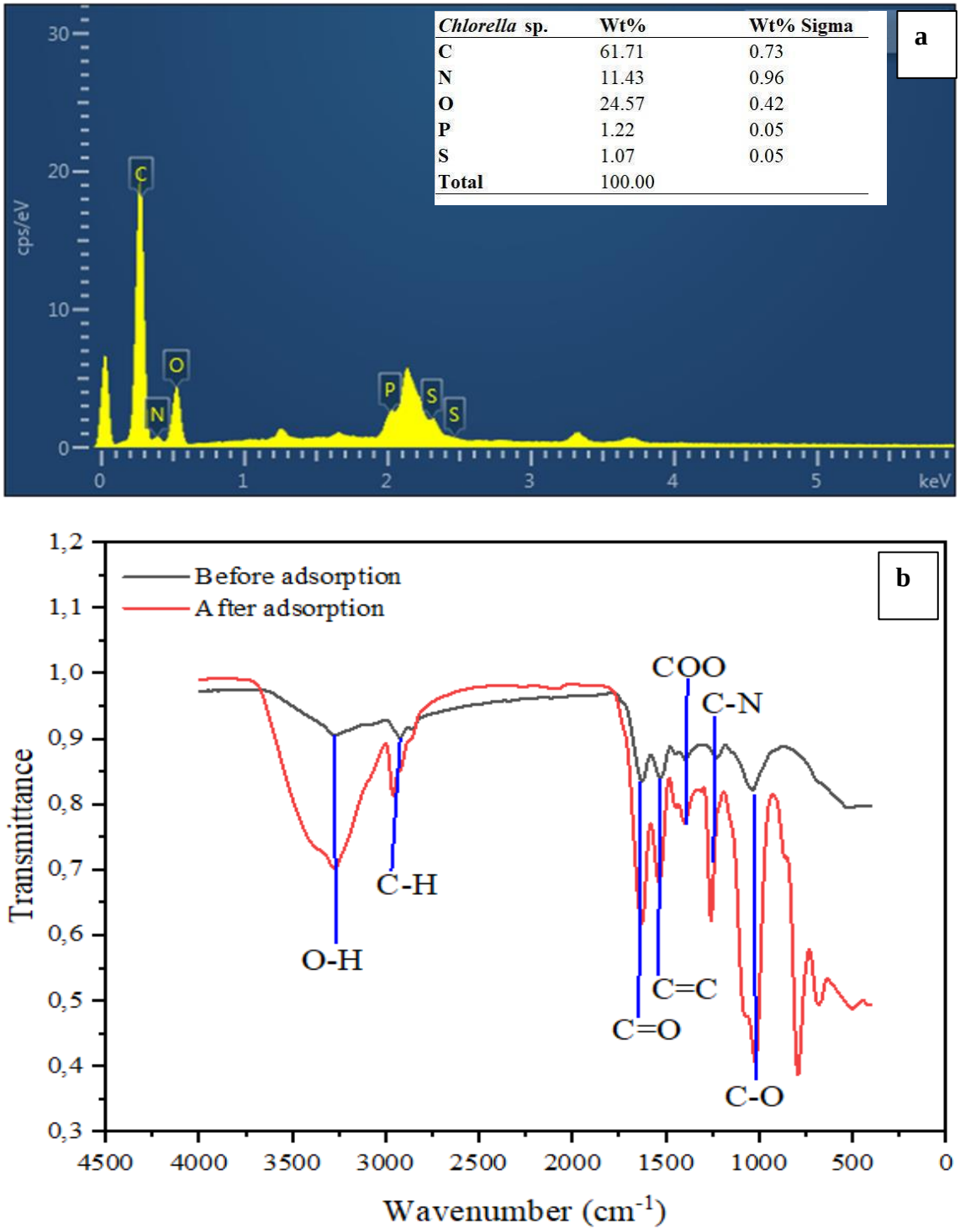

Figure 5.2 Energy Dispersive X-ray Spectroscopy (EDX) before adsorption (a) and Fourier transform infrared spectroscopy (b) of *Chlorella* spp. biomass.

5.2.3 Optimisation of the adsorption conditions of metals to Chlorella spp.

5.2.3.1 Effects of the biomass dosage on the degree of removal efficiency

Finding optimum conditions for critical parameters such as biomass dosage is crucial in *Chlorella*-biomass-based adsorption studies as it ensures cost-effectiveness and enhances the efficiency and environmental sustainability of the process. The results depicting the effect of biomass dosage are illustrated in Figure 5.3a. Hence, copper removal efficiency decreased as the biomass dosage increased, with the highest removal being 56% at 5 mg. This could be ascribed to competitive adsorption as the heavy metals were present in a mixture, with the three metals potentially competing for the same available binding sites. These findings align with the observations made by Ni et al. (2019), who documented competitive adsorption behaviour between cadmium and lead, where cadmium showed a decreased adsorption in the presence of lead. This was ascribed to lead's higher electronegativity and binding affinity, larger hydrolysis constant, atomic weight, ionic radius, as well as a larger Misono softness value which increases its binding favourability (Ni et al. 2019, Al Hamouz and Ali 2013, Musumba et al. 2020). In this study, the *Chlorella* spp. biomass consistently removed lead and zinc with high removal efficiencies, with maximum values of 86% and 95% recorded at 25 mg. This trend was observed across various biomass dosages, except for the 100 mg dosage, where removal efficiency was substantially reduced for zinc. Aggregation of biomass in the experimental flasks tends to increase at high mass dosages (Pfeifer and Skerget 2020), which could reduce the surface area exposed to the solution, severely impacting the adsorption capacity. A biomass dosage of 12.5 mg was selected for the later experiments. This selection was justified by the nearly identical removal efficiencies observed at 12.5 mg and 25 mg for lead and zinc, as well as notably better removal for copper.

5.2.3.2 Effects of pH on the degree of removal efficiency by Chlorella spp. biomass

Figure 5.3b illustrates the effect of a varying pH at constant biomass dosage, contact time, temperature, and metal concentration upon the metal removal efficacy. The low removal efficiencies observed at acidic conditions (pH 3-5), particularly for copper and zinc, could be attributed to the protonation of metal binding functional groups (e.g., $-NH_2$, -OH, -SH) in *Chlorella* spp. biomass, causing them to behave like positively charged species that repel metal cations (Cherono et al. 2021, Tattibayeva et al. 2022). Similarly, several studies (Kiruba et al. 2014, Gonte and Balasubramanian 2016, Shi et al. 2023, Waqar et al. (2023) reported that low pH promoted competition between metal ions and protons for binding sites, resulting in reduced adsorption of their analytes. Additionally, Almomani and Bhosale (2021) documented

that the solubility of heavy metals in water increases in acidic conditions, resulting in decreased adsorption rates. The removal efficiencies for copper, lead, and zinc remained relatively stable between pH 7 and 9, with values of 56.35% and 55.75% for copper, 89.68% and 89.86% for lead, and 88.98% and 89.40% for zinc, respectively. This suggests an equilibrium point between pH 7-9 regarding metal binding to *Chlorella* spp. biomass. When the pH becomes too basic (pH 11), a decrease in removal efficiencies was observed, particularly for copper and to a lesser degree for lead and copper, which can be attributed to the formation of insoluble metal hydroxides, hindering metal adsorption onto binding sites (Al-Saadi et al. 2013, Cherono et al. 2021). These findings match studies by Zhang (2011) and Depci et al. (2012) that assessed the removal of heavy metals employing dairy manure compost and activated carbon derived from apple pulp. Thus, pH 7 was chosen as the optimum condition as it helps to prevent the disruption of pH-sensitive biological processes and the accumulation of toxic substances in receiving water bodies, contributing to the overall health and sustainability of the environment.

5.2.3.3 Effects of time on the degree of removal efficiency by Chlorella spp. biomass

The effect of contact time on removing selected heavy metals was investigated (Figure 5.3c). A slight increase in lead and zinc removal efficiencies from 15 to 30 minutes was observed, attributable to lead and zinc having a larger ionic radius than copper, resulting in stronger interactions with binding sites on the *Chlorella* spp. biomass surface, leading to higher and faster adsorption than small copper ions (Ni et al. 2019). As the contact time increased (45 - 115 minutes), the removal of these 2 compounds did not increase substantially, indicating saturated binding sites and the establishment of an equilibrium between metal ions and the binding sites available on the *Chlorella* spp. biomass surface. Contrary to lead and zinc, the copper removal efficiency increased from 30 to 60 minutes, stipulating different copper binding kinetics for the *Chlorella* spp. Biomass. However, at >= 75 -115 minutes, slightly lower removal efficacy indicated partial saturation or changes in binding site accessibility. The decrease observed could also indicate desorption, where previously adsorbed copper ions are released from the binding sites back into the solution (Chatterjee and Abraham 2019). It is important to note that at 15 minutes, copper removal was not apparent, possibly due to the *Chlorella* spp. biomass not having enough time to adsorb copper ions effectively. Thus, the optimum time was established as 60 minutes, with maximum removals of 70% observed for copper, 95% for lead, and 100% for zinc.

5.2.3.4 Effects of metal concentration on the degree of removal efficiency by Chlorella spp. biomass

This study explored the effect of metal concentration on the adsorption by *Chlorella* spp. biomass to understand the dynamic relationship between initial metal ion concentrations and their adsorption behaviour. For this analysis, the other four variables (e.g., biomass dosage, pH, temperature, and contact time were kept constant (Figure 5.3d). A notable increase in copper removal efficiency was observed, from 37% at 0.25 mg/L to 79% at 1.0 mg/L. This could be due to the increased availability of copper ions at higher concentrations (0.5 and 1.0 mg/L), providing more opportunities for adsorption onto the *Chlorella* spp. biomass surface. Al-Homaidan et al. (2015) reported a similar trend when assessing the removal of cadmium ions by *Spirulina platensis* dry biomass. However, the lead and zinc removal efficiencies from 0.25 mg/L to 1.0 mg/L did not change notably. This can also be ascribed to the abundant availability of the binding sites on the biomass surface at low metal concentrations. Above metal concentrations of 1 mg/L, a decrease in copper and zinc removal efficiencies was observed, but to a lesser degree for lead. The increased concentration of heavy metal ions in the solution may overwhelm the active sites, resulting in heightened competition among available ions for limited binding sites (Musumba et al. 2020). Consequently, the lower adsorption of copper contributed to it being less efficiently removed than the other two metals. Kiruba et al. (2014) also reported reduced removal as cadmium concentrations increased due to the saturation of the active sites of the surface-modified Eucalyptus seeds system.

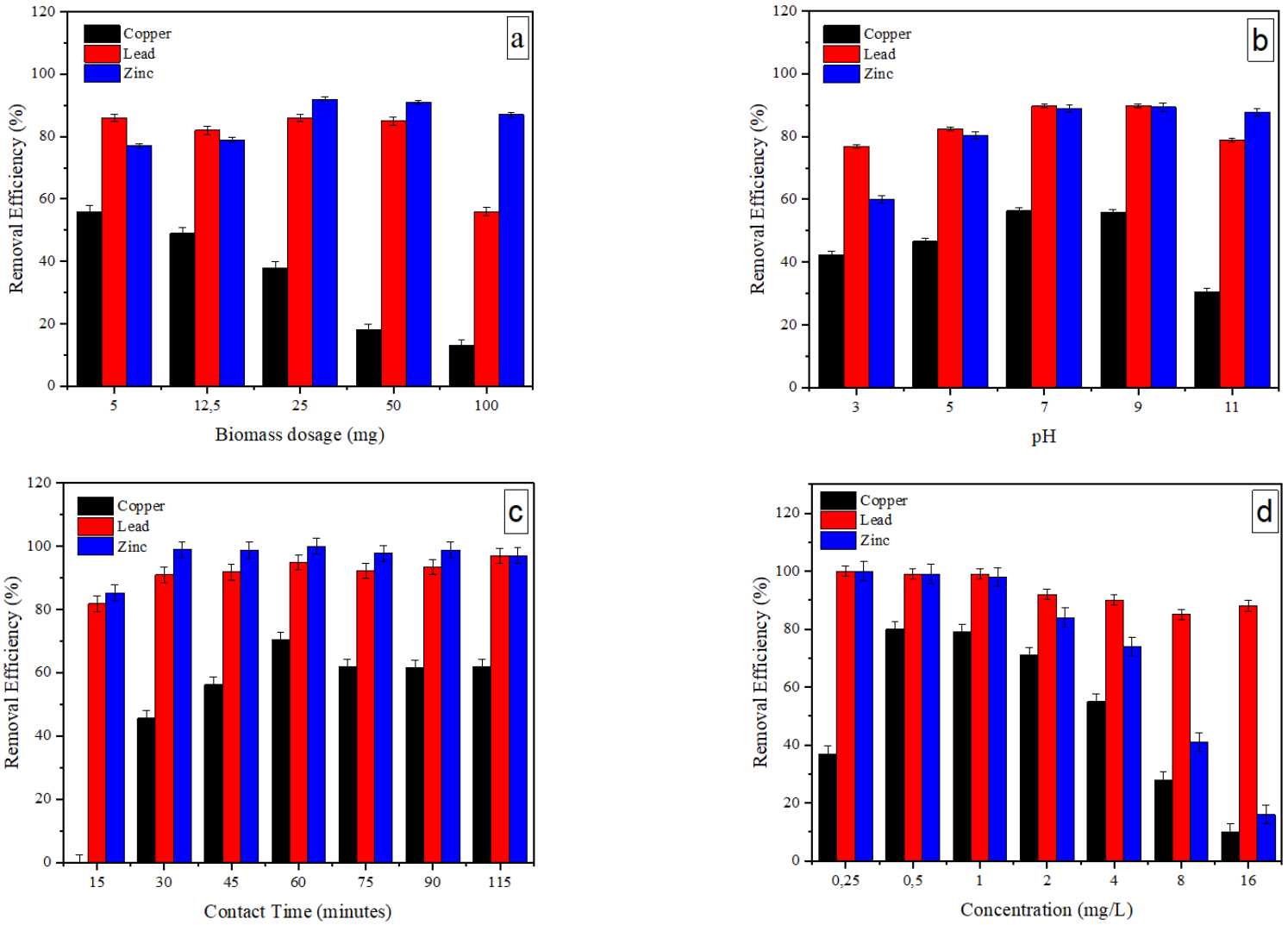

Figure 5.3 The effect of biomass dosage (a), pH (b), contact time (c), and metal concentration (d) on the metal removal efficiency of *Chlorella* spp. dried biomass with n=3 replicates. a) Stable pH (5), 60 minutes contact time, metal concentration of1mg/L, and temperature at25°C. b)

Chlorella spp. biomass dosage of 12.5 mg, 60 minutes contact time, temperature of 25°C, and metal concentration of 1 mg/L. c) *Chlorella* spp. biomass dosage maintained at 12.5 mg, pH held constant at 7, temperature at 25°C, and metal concentration of 1 mg/L. d) *Chlorella* spp. biomass dosage set at 12.5 mg, pH maintained at 7, temperature at 25°C, and 60 minutes contact time.

5.2.4 Kinetic studies

The results of adsorption kinetics model coefficients using *Chlorella* spp. as a biosorbent are presented in Table 5.2. The pseudo-first-order kinetic model produced a calculated equilibrium adsorption capacity close to the experimentally determined values, suggesting the accuracy and reliability of the model's predictive capabilities. The rate constant (k_1) for copper illustrated a slow adsorption process (0.045 min^{-1}), while lead and zinc exhibited fast adsorption rates (0.117 and 0.131 min^{-1}, respectively). This can be ascribed to lead and zinc possessing larger electronegativity, hydrolysis constant, atomic weight, ionic radius, and Misono softness values than copper (Ni et al. 2019, Al Hamouz and Ali 2013, Musumba et al. 2020). The coefficient of determination (R^2) ranged from 0.9979 to 0.9974, indicating a perfect fit of the first-order model to the experimental data. The pseudo-second-order kinetic model yielded a slightly less accurate fit for copper, lead, and zinc. The calculated equilibrium adsorption capacities were closer to the experimental values for all 3 metals. The obtained rate constants (k_2) indicated slow copper adsorption and a faster lead and zinc adsorption process. The coefficients of determination values were ≥ 0.987 for all three metals. For the intraparticle diffusion model, copper showed slower intraparticle diffusion - K_{diff}, while lead and zinc exhibited faster rates. The lower R^2 values (0.611 - 0.884) relative to the first and second-order models confirm that intraparticle diffusion alone cannot be the only controlling factor. Thus, the pseudo-first-order kinetic model was the best-fitted model.

Table 5.2 The results of adsorption kinetics model coefficients for three metals and Chlorella spp. as a biosorbent.

Kinetics Type		Copper	Lead	Zinc
First order	qe ex(mg/g)	0.424	1.250	2.423
	qe cal(mg/g)	0.412	1.247	2.402
	k_1	0.045	0.117	0.131
	R^2	0.998	0.990	0.994
Second order	qe ex(mg/g)	0.401	1.259	2.393
	qe cal(mg/g)	0.474	1.308	2.490
	k_2	0.137	0.223	0.156
	R^2	0.992	0.987	0.992
Intraparticle	K_{diff}	0.040	0.105	0.199
	C	0.046	0.366	0.744
	R^2	0.884	0.641	0.611

5.2.5 Isotherm studies of adsorption

The isotherm results in Table 5.3 shed light on the distribution of heavy metal ions between the *Chlorella* spp. biomass surface and the aqueous phase. The Freundlich isotherm results exhibited favourable adsorption behaviours for the 3 heavy metals. The Freundlich exponent (1/n) values were below one for all heavy metals, suggesting that a heterogeneous surface in view of available adsorption sites characterized the adsorption of the heavy metal ions (Abin-Bazaine et al. 2022). The Freundlich constants obtained for the heavy metals affirmed the adsorption capacities. The high R^2 values obtained collectively confirmed the suitability of the Freundlich model, which suggests heterogeneous and multi-layered adsorption onto *Chlorella* spp. biomass (Saleh 2022). However, copper exhibited highly favoured adsorption by Langmuir isotherms compared to Freundlich, as evidenced by a substantially higher R^2 value of 0.988, indicating monolayer adsorption on a homogeneous surface (Kalam et al. 2021). The dimensionless separation factor (R_L) values were less than 1 but greater than zero, indicating favourable adsorption. Selahle et al. (2022) documented that when the R_L values are less than 1, the adsorption is favourable, meaning that the adsorbent material efficiently removes these target analytes from the solution. The Temkin isotherm parameters provided information about the interaction strengths between the heavy metal ions and the *Chlorella* spp. Biomass surface. Different Temkin constants (K_T) indicated varied levels of interaction strength, with lead and zinc showing higher strength. The R^2 values obtained (0.8 – 0.927) exhibited a reasonable fit of the Temkin model.

Table 5.3 The results of Freundlich, Langmuir, and Temkin models biosorption isotherms for three metals and Chlorella spp. biomass as biosorbent.

Isotherm	Parameters	Copper	Lead	Zinc
Freundlich	1/n	0.372	0.247	0.320
	k_f	2.094	5.041	8.973
	R^2	0.939	0.976	0.968
Langmuir	q max (mg/g)	2.777	2.743	7.513
	K_L (L mg)	4.962	256.7	42.39
	R_L	0.004	0.0001	0.0004
	R^2	0.988	0.908	0.907
Temkin	K_T (L mg^{-1})	52.26	2681	169.8
	B_T ($Jmol^{-1}$)	0.563	0.524	1.854
	R^2	0.923	0.927	0.800

5.2.6 Thermodynamic studies of adsorption

Table 5.4 presents the effect of temperature on copper, and zinc adsorption. For copper and zinc, the adsorption thermodynamic parameters calculated illustrate endothermic processes, indicated by the positive enthalpy change (ΔH) of 48.80 kJ/mol for copper, 9.48 kJ/mol for zinc, matching data reported by Musumba et al. (2020). Increased enthalpy during adsorption was denoted by the positive entropy change (ΔS) of 163.74 kJ/mol for copper and 43.63 kJ/mol for zinc, matchingSaleh (2022). For the same compounds, negative Gibbs free energy change (ΔG) values (-0.20 kJ/mol to -5.81 kJ/mol) were obtained across tested temperatures, along with via high R^2 values (0.989, 0.933), signifying the spontaneous nature of copper and zinc adsorption onto the *Chlorella* spp. biomass surface, and confirm the reliability of the model employed and the data resulting from it.

Table 5.4 Adsorption thermodynamics of copper and zinc onto Chlorella spp. biomass.

Heavy metals	**ΔH (kJ mol^{-1})**	**ΔS (kJ mol^{-1})**	**ΔG (kJ mol^{-1})**				**R^2**
			298	**308**	**318**	**333**	
Copper	48.804	163.743	-0.198	-1.300	-3.303	-5.810	0.989
Lead	*	*	*	*	*	*	*
Zinc	9.482	43.627	-3.406	-4.089	-4.456	-4.961	0.933

* Not enough data. Lead ions were completely removed across all temperatures at the assessed concentration levels, hence the absence of thermodynamic parameters. However, such complete removals indicated the high affinity of *Chlorella* spp. biomass for lead ions within the assessed concentration levels.

5.2.7 Real Wastewater Effluent Samples Analysis

The collected wastewater effluent samples' physicochemical properties were measured to assess the water quality (Table 5.5). The samples showed at the time of sampling a low dissolved oxygen (DO) content, which was below the recommended limits for USEPA (6–9 mg/L) and DWAF (7.5 mg/L) (USEPA 2009, DWAF 2010). Such low levels can be ascribed to oxygen consumption by reducing agents (e.g., hydrogen sulfide) as well as biological processes taking place in the presence of organic or inorganic electron donors driving microbial respiration. In addition, aquatic organisms can be negatively affected as copper, lead, and zinc

toxicity increase at low DO (Kang et al. 2019, Olabode et al. 2020). At the time of sampling, the temperature of the Darvill effluent was found to be 24.7^{o}C, which would impact the rate and extent of metal adsorption based on thermodynamic grounds (e.g., increased molecular movement and collision frequency) as well as biochemical reactions taking place in water. The pH value is another important parameter affecting the availability of heavy metals. For example, acidic pH levels enhance the solubility of heavy metals, increasing their availability in water. In contrast, alkaline pH levels reduce heavy metals solubility via the production of less soluble hydroxide compounds (Saalidong et al. 2022). However, the pH value of the water sample was 7.39, which is within the acceptable range (6-9) for discharging domestic and industrial wastewater into water bodies (DWAF 1999). The measured conductivity was 538 µS. This value suggests the presence of dissolved ions, including the possibility of heavy metals (CWT 2004, Sosibo 2022). The salinity was 0.27 psu, which agrees with the results of Kunene and Mahlambi (2019), which reported 0.3 psu in the same site. Total dissolved solids (TDS) denote the overall concentration of dissolved substances, including inorganic salts and other small quantities of organic matter. High TDS can suggest a possibility for the presence of high levels of heavy metals since TDS serves as a carrier for dissolved substances, including heavy metals. In this study, 272 mg/L was obtained.

Good performance was demonstrated in the effluent samples, achieving greater than 98% removal for all heavy metals. The exhibited success in all heavy metals tested signifies that *Chlorella* spp. It is indeed a promising, sustainable, and reliable adsorbent for heavy metal removal in real-world applications.

Table 5.5 Darvill WWE physicochemical parameters and removal efficiencies for copper, zinc, and lead by dried Chlorella spp. biomass with n=3 replicates

Sampling point	DO (mg/L)	Temp. °C	pH	Conductivity (µS)	Salinity (psu)	TDS (mg/L)	Removal Efficiency (%)		
							copper	lead	zinc
Darvill Effluence	1.56	24.7	7.39	538	0.27	272	100	98	100

5.3 Conclusion

This study highlighted the promising efficacy of *Chlorella* spp. dried biomass as an adsorbent for removing 3 heavy metal ions from water environments. Characterizing the *Chlorella* spp. dried biomass exhibited crucial functional groups, such as carboxyl, hydroxyl, methyl, carbonyl, and amide, that are essential in removing heavy metals. The adsorption of heavy metals onto *Chlorella* spp. biomass was influenced by parameters such as biomass dosage, pH, contact time, and initial heavy metal ion concentration. A biomass dosage of 12.5 mg was selected as optimum because it demonstrated removal efficiencies exceeding 80% for zinc and lead. For all three metals, maximum heavy metal ion removal occurred at pH 7, with a contact time of 60 minutes and an initial concentration of 0.5 mg/L. The pseudo-first-order kinetic model exhibited a good fit for copper, lead, and zinc experimental data. Isotherm studies indicated that Freundlich parameters (1/n and K_f) were favourable in describing adsorption for lead and zinc while Langmuir isotherms best described copper. Varied Temkin constants suggested different interaction strengths, with lead and zinc exhibiting higher adsorption strength.

The thermodynamic analysis highlighted endothermic processes (positive ΔH), increased randomness during adsorption (positive ΔS), and spontaneous adsorption, supported by the obtained negative ΔG. In real wastewater effluent samples, complete removal was observed for copper and zinc, while lead exhibited 98% removal. These findings underscore the promising potential of *Chlorella* spp. biomass in real-world applications for efficient heavy metal removal from complex environmental matrices.

Future research directions should prioritize the development and optimization of *Chlorella* spp. biomass-based technologies for heavy metal removal in wastewater treatment. This includes the co-cultivation with other microorganisms or engineered strains, to enhance biomass productivity and metal adsorption capacities. Additionally, advancing understanding of the underlying mechanisms of heavy metal adsorption by *Chlorella* spp. through advanced analytical techniques and modelling approaches will be crucial. Scaling up laboratory findings to pilot and full-scale applications will also be essential to validate these bioremediation strategies' feasibility and economic viability. Collaboration between researchers, industry partners, and policymakers will facilitate the integration of these innovative technologies into practical environmental management practices, thereby contributing to sustainable water quality management and supporting global environmental goals

5.4 Acknowledgements

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Chapter 6 Synthesis, and Recommendations

6.1 Synthesis Section

This study focussed on using biological microorganisms like *Chlorella* spp. and *D. magna* to remove excess nutrients, pharmaceuticals, and heavy metals in water environments, which posed significant environmental challenges globally. Excess nutrients such as nitrogen and phosphorus harm human health, aquatic environments, and ecotourism by contributing to eutrophication, harmful algal blooms, and oxygen depletion in aquatic ecosystems. The rising production and consumption of pharmaceutical drugs introduced diverse pharmaceutical compounds into water bodies, exacerbating impacts on aquatic life and human health. According to the literature, the human body does not efficiently utilize pharmaceuticals, so large amounts are excreted into the environment, together with improper disposal of unused or expired drugs. Concurrently, heavy metals like copper, lead, and zinc, originating from industrial discharges and urban runoff, persisted in water sources, posing toxicological risks to ecosystems and human populations. Given these conditions, exploring effective biological methods for removing these contaminants is essential. The research aimed to develop sustainable and environmentally friendly strategies to remove excess nutrients, pharmaceuticals, and heavy metals in wastewater environments. By filling this research gap, the study contributed to advancements in water quality management and environmental sustainability. The thesis results effectively demonstrated that combining *Chlorella* spp. and *D. magna* significantly removed nitrate and nitrite from wastewater, highlighting a synergistic approach to nutrient management. For pharmaceuticals, *Chlorella* spp. demonstrated high removal efficiency for the studied pharmaceuticals, particularly at lower concentrations, suggesting promising avenues for future research in this area. The study results also highlighted *Chlorella* spp. biomass was highly effective in adsorbing heavy metals, facilitating their complete removal from water matrices.

Insights from the study revealed varying effectiveness among the tested materials for nutrient, pharmaceutical, and heavy metal removal. When used alone, *D. magna* faced challenges in nutrient removal due to its dependency on primary producers like bacteria and microalgae. However, combining *D. magna* with *Chlorella* spp. resulted in effectively removing nitrate and nitrite. Challenges persisted in removing ammonia and phosphate, even when using both organisms, suggesting further opportunities for future studies to explore the underlying intricacies of the removal mechanisms of these contaminants. Regarding pharmaceuticals,

Chlorella spp. showed significant removal efficiencies for sulfamethoxazole, nevirapine, and efavirenz, but only at lower concentration levels. This warrants further research to understand factors that could enhance *Chlorella*'s performance, especially at higher concentration levels. Overall, *Chlorella* spp. demonstrated promising results across three key areas explored in this study.

6.2 General Conclusions

The nutrients were analyzed according to the manufacturer's protocol. The suggested methods were successfully validated, exhibiting reliability and good precision for determining ammonia, nitrate, nitrite, and phosphorus concentrations in water samples. The R^2 values above 0.993 were obtained, indicating a strong positive linear relationship. The specificity of the methods was verified through recovery rates, and results exhibited recoveries ranging from 94 – 105%. When optimizing conditions for *Chlorella* spp. culture, 8% initial culture was deemed optimum as it yielded heightened growth and high removal rates. The results of this chapter showed complete nitrate and nitrite removals by *Chlorella* spp. This can be attributed to the microorganism's ability to utilize these compounds as their nitrogen source and convert them to other valuable compounds like lipids, protein, etc. Ammonia and phosphate removal rates were constrained, reaching up to 22%. This can be ascribed to factors like the saturation of *Chlorella*'s nutrient uptake capacity, depletion of essential nutrients, pH fluctuations, and the production of complexes or precipitates, which reduce the availability and uptake. *D. magna* demonstrated challenges in nutrient removal; however, when combined with *Chlorella* spp., their collective effectiveness was remarkable, resulting in a 100% removal of nitrate and nitrite.

The proposed LC-PDA and SPE methods were successfully developed and found appropriate for separating and extracting target pharmaceutical compounds in water samples. The LC-PDA optimum conditions were 90:10% (acetonitrile: water with 0.1% formic acid) as a mobile phase, 8-minute run time, 0.4 mL/min flow rate, and 220 nm wavelength. The SPE optimum conditions included 70 acetonitrile:30 methanol (v/v) for conditioning solvent, 50 mL sample volume, and neutral pH (7). The LOD and LOQ for the developed SPE method ranged from 0.056–0.176 µg/L and 0.187–0.585 µg/L, respectively. The recoveries ranged from 99–120% in wastewater, with repeatability and reproducibility reported as %RSD values were less than 1% for all analytes, indicating high precision. The R^2 between 0.997–1 at a concentration range of 0.1–1.0 mg/L. Twenty hours light: 4 hours darkness condition and 16% initial culture

concentration were optimal for *Chlorella* spp. culture. Additionally, antibiotics and antiretroviral removals showed concentration-dependent trends, with higher initial concentrations yielding lower removals, while lower initial concentrations achieved higher removals. *Chlorella* spp. also demonstrated a good capacity for removing heavy metals like copper, lead, and zinc through adsorption. For optimizations, the following conditions were deemed optimum: 12.5 mg biomass dosage, pH 7, 60 minutes contact time, and 0.5 mg/L concentration. The pseudo-first-order kinetic model and Freundlich parameters best-fitted zinc and lead, while copper experimental data showed a good fit on the pseudo-second-order and Langmuir isotherm. The thermodynamics revealed endothermic processes, increased randomness during adsorption, and spontaneous adsorption. Regarding removal efficiencies, complete removals were observed for copper and zinc, while lead exhibited 98% removal in wastewater effluent samples.

In these sub-studies, the mechanisms responsible for removals included surface adsorption, photodegradation, bioaccumulation, and enzymatic degradation. The FTIR showed the presence of functional groups like alkene, amide, carbonyl, carboxyl, ethers, hydroxyl, and methyl, which participated in the sequestration of these contaminants through various interactions. SEM images also exhibited changes in *Chlorella* spp. cells after exposure to nutrients, pharmaceutical compounds, and heavy metals, suggesting the possibility of interaction that aids their removals. In conclusion, the findings from this study provided a solid foundation for this body of work, and it showed a promise to improve the effectiveness of future wastewater treatment processes. However, there is a massive need for additional research to understand these microorganism-based bioremediation processes fully. Overall, combining both *Chlorella* spp. and *D. magna* appeared to yield more desirable and efficient nutrient removal relative to using them alone, especially for nitrogen compounds like nitrates and nitrites. This synergistic approach not only addressed nutrient removals but also contributed significantly to controlling excessive algal growth, and it increased the overall water clarity. It is worth noting that in this study, the impact of *D. magna* on pharmaceuticals and heavy metals was not explored, due to its performance alone being inefficient in the nutrient removal section. Therefore, innovative study designs, including *D. magna*, require thorough assessments and more time and resources.

6.3 Recommendations and Future Directions

- Future experiments should focus on extending contact time between pharmaceuticals and *Chlorella* spp. cells to reach equilibrium and assess its effect on removal efficiency. Additionally, in-depth investigations into specific mechanisms such as surface adsorption, photodegradation, bioaccumulation, and enzymatic degradation are crucial to enhance process efficiency.
- To enhance the efficiency of pharmaceutical and heavy metal removal by *Chlorella* spp., future studies should focus on developing methods to monitor and adjust essential nutrients throughout the bioremediation process. Continuous monitoring and adjustment of nutrient levels could enhance *Chlorella*'s growth, metabolism, and biosorption capacity, thereby improving overall pollutant removal efficiency.
- Due to incomplete removal of ammonium and phosphates by *Chlorella* spp. and *D. magna* observed in this study, comprehensive investigation into molecular and biomolecular mechanisms is crucial to enhance their removal efficiency. Research should focus on unravelling the roles of specific biomolecules, such as enzymes, transport proteins, and regulatory factors, alongside molecular pathways involved in nutrient uptake and metabolism within these organisms.
- Given that the synergistic effects between *D. magna* and *Chlorella* spp. have primarily been investigated for nutrient removal only in this study, future research should expand to include their co-cultivation for the removal of pharmaceuticals and heavy metals.
- Future studies should also explore diverse combinations of microalgae, bacteria, and other microorganisms, in order to harness synergistic effects to enhance pollutant removal efficiencies. Employing different microalgal strains would shed more light on the success of the microalgal-based bioremediation treatment systems. Different strains may yield variations in resistance and affinity for target analytes, enabling researchers to choose the most effective strains.
- It is necessary to test the applicability of *Chlorella* spp. and *D. magna* bioremediation treatments on a larger scale, like bioreactors, in order to evaluate the practical feasibility and economic viability of this approach
- Genetic engineering must be conducted as it offers another avenue to improve *Chlorella's* biodegradation capabilities. By manipulating the genetic makeup of *Chlorella* spp, researchers can introduce specific traits or modifications that enhance its efficiency (Hassanien et al. 2023). This genetic manipulation boosts the cell's ability

to degrade pollutants, improving removal efficiency (Li et al. 2022). For instance, genetic engineering can confer resistance to inhibitory compounds that may adversely affect *Chlorella* spp. growth or metabolism. By introducing genes encoding efflux pumps or detoxification enzymes, microalgae cells can better tolerate and degrade the pollutants (Ranjbar et al. 2022, Sreenikethanam et al. 2022). Additionally, genetic engineering allows for the enhancement of stress response mechanisms in *Chlorella* spp., making it more resilient and capable of maintaining high biodegradation efficiency under adverse conditions such as fluctuating nutrient availability, temperature variations, or oxidative stress. By enhancing photosynthetic efficiency, nutrient utilization, or growth rates, genetically modified *Chlorella* spp. strains can produce more biomass, leading to higher pollutant uptake and degradation capacities (Kumar et al. 2020, Hassanien et al. 2023, Hu et al. 2023).

- Conducting research that taps into the circular economy is paramount because it allows researchers to delve into applications for harvested biomass, such as the production of bioenergy, sustainable agriculture use, or as a source of valuable bioactive compounds. Future research should also integrate advanced monitoring technologies such as biosensors for real-time assessment of contaminant levels, which can further streamline the bioremediation process. Also, collaboration with industrial partners and policymakers can help develop regulatory frameworks and incentives for adopting sustainable wastewater treatment technologies.

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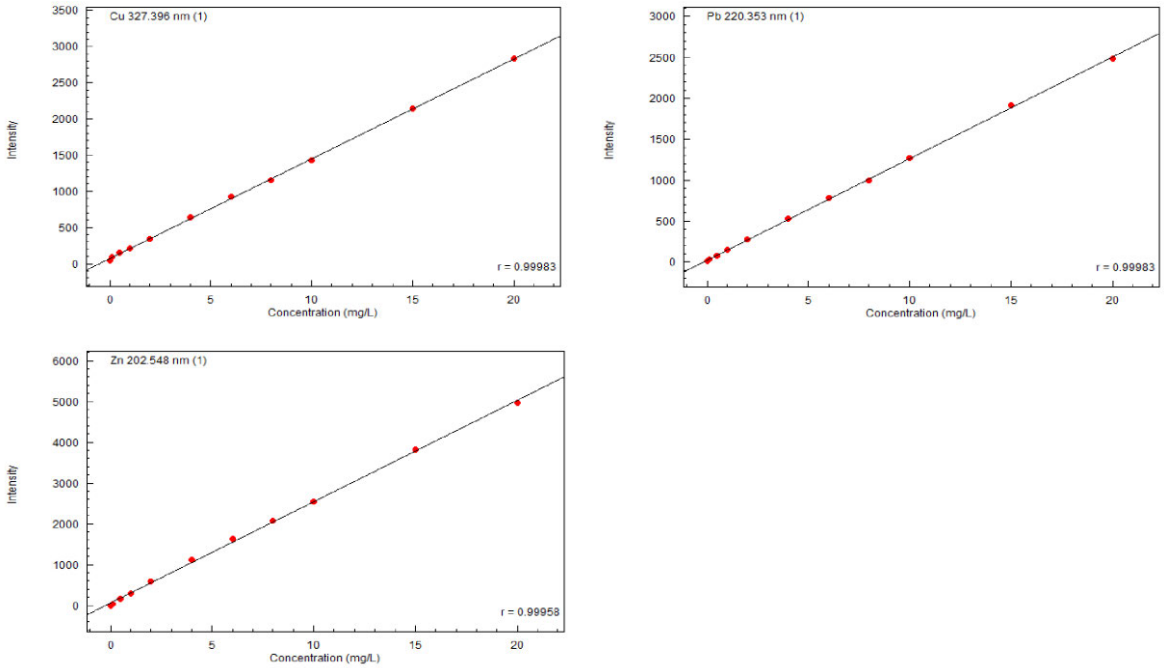
Appendixes

Appendix 1 Physicochemical parameters at Darvill sampling sites.

Sampling points	DO (mg/L)	Temp. oC	pH	Conductivity (µS)	Salinity (psu)	TDS (mg/L)
Effluence	1.56	24.7	7.39	538	0.27	272
Influence	0.32	22.5	7.53	833	0.49	407



Appendix 2 Calibration curves for copper (Cu), lead (Pb) and zinc (Zn)

Appendix 3 ANOVA results for the effect of conditioning solvent on SPE

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
ACN	3	311	103,6667	44,33333
MeOH	3	295	98,33333	42,33333
50:50 ACN/MeOH	3	304	101,3333	30,33333
70:30 ACN/MeOH	3	315	105	39
80:20 ACN/MeOH	3	306	102	93

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	76,93333	4	19,2333	0,386212	0,813827	3,47805

Within Groups	498	10	49,8	
Total	574,933	14		

Appendix 4 ANOVA results for the effect of sample volume on SPE

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
25 mL	3	214	71,3333	36,3333
100 mL	3	242	80,6666	34,3333
50 mL	3	311	103,666	24,3333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1661,556	2	830,7778	26,23509	0,001081	5,143253
Within Groups	190	6	31,66667			
Total	1851,556	8				

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
25 mL	3	214	71,3333	36,3333
100 mL	3	242	80,6666	34,3333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	130,6667	1	130,6667	3,698113	0,126826	7,708647
Within Groups	141,3333	4	35,33333			
Total	272	5				

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
100 mL	3	242	80,6666	34,3333
50 mL	3	311	103,666	24,3333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	793,5	1	793,5	27,05114	0,006511	7,708647
Within Groups	117,3333	4	29,33333			
Total	910,8333	5				

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
25 mL	3	214	71,33333	36,33333
50 mL	3	311	103,6667	24,33333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1568,167	1	1568,167	51,6978	0,001982	7,708647
Within Groups	121,3333	4	30,33333			
Total	1689,5	5				

Appendix 5 ANOVA results for the effect of pH on SPE

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
pH 2	3	212	70,66667	30,33333
pH 5	3	271	90,33333	30,33333
pH 7	3	311	103,6667	24,33333
pH 9	3	248	82,66667	26,33333
pH 11	3	145	48,33333	1610,333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*

				3,83378	0,03857	
Between Groups	5280,4	4	1320,1	5	5	3,47805
	3443,33		344,333			
Within Groups	3	10	3			
	8723,73					
Total	3	14				

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
			90,3333	30,3333
pH 5	3	271	3	3
			103,666	24,3333
pH 7	3	311	7	3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	266,666		266,666	9,75609	0,03540	7,70864
Between Groups	7	1	7	8	8	7
	109,333		27,3333			
Within Groups	3	4	3			
Total	376	5				

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
			103,666	24,3333
pH 7	3	311	7	3
			82,6666	26,3333
pH 9	3	248	7	3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
				26,1118	0,00693	7,70864
Between Groups	661,5	1	661,5	4	4	7
	101,333		25,3333			
Within Groups	3	4	3			
	762,833					
Total	3	5				

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
			70,6666	30,3333
pH 2	3	212	7	3
			103,666	24,3333
pH 7	3	311	7	3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1633,5	1	1633,5	59,7622	0,001508	7,708647
Within Groups	109,3333	4	27,33333			
Total	1742,833	5				

Appendix 6 ANOVA results for the percentage contribution of SPE parameters

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
pH	3	311	103,6667	24,33333
Conditioning solvents	3	315	105	39
Sample volume	3	311	103,6667	24,33333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3,555556	2	1,777778	0,060837	0,941549958	5,143253
Within Groups	175,3333	6	29,22222			
Total	178,8889	8				

Appendix 7 Sum of squares (SS) for SPE parameters

SPE parameters	SS
pH	0,592592593
Conditioning solvent	2,37037037
Sample volume	0,592592593

Appendix 8 Percentage contribution of each parameter on the overall extraction process

Parameters	% Contribution
pH	16,67
Conditioning solvent	66,67
Sample volume	16,67