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Efficacy and mechanisms of antiretroviral drugs removal by algae from wastewater treatment plants

Submitted in fulfilment of the requirements of the degree of

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Karen Reddy

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Prof. Brenda Moodley

DECLARATION

Efficacy and mechanisms of antiretroviral drugs removal by algae from wastewater treatment plants

Karen Reddy

I hereby declare that the dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

4 April 2024

Karen Reddy **Date**

APPROVAL

I hereby approve the final submission of the following dissertation.

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This 4th day of April 2024, at the Durban University of Technology.

ABSTRACT

The presence, risks, and fate of pharmaceutical pollutants in the environment have raised concerns worldwide. South Africa, with the largest population consuming antiretroviral (ARV) drugs in Africa, faces challenges in efficiently removing these compounds from water bodies. This study's primary focus was to investigate the efficiency and mechanisms of nevirapine (NVP) removal by algae isolated from wastewater treatment processes. It included the isolation and screening of algal strains from wastewater treatment plants for their potential to remove ARV drugs, optimizing culture conditions to enhance removal efficiency, determining the potential mechanisms employed by selected algal strains for NVP remediation, and assessing the associated metabolic responses of algal cells to NVP using gene expression and metabolomics analyses. Eleven green indigenous fresh water microalgal isolates were screened from wastewater treatment plants (WWTPs) in KwaZulu-Natal, resulting in the selection of two strains, *Coelastrella tenuitheca* and *Tetradesmus obliquus*, based on their growth rates, biomass productivity and toxicity tolerance. In the ecotoxicity study, the calculated IC_{50} values of NVP (0–100 mg L^{-1}) on selected algal strains after 96 h of exposure were 23.45 mg L^{-1} (*C. tenuitheca*) and 18.20 mg L^{-1} (*T. obliquus*), which far exceeds the concentration of NVP found in wastewater. Hence, *T. obliquus* and *C. tenuitheca* was selected for further NVP remediation studies using different cultivation conditions. A concentration range of 0-4000 ng L^{-1} of NVP was tested to assess the potential for NVP removal by both microalgae (autotrophic cultivation). Lower concentrations of NVP (up to 200 ng L^{-1}) have shown to have a positive impact on microalgae growth. Specifically, in *T. obliquus*, the highest dry cell weight of 941.27 mg L^{-1} was obtained when exposed to a NVP concentration of 50 ng L^{-1}. Both microalgae showed varying removal efficiencies (19.53–74.56%) when exposed to different NVP concentrations. During the late log phase on day 8, *T. obliquus* achieved the highest NVP removal efficiency, removing 74.56% of the NVP, while *C. tenuitheca* achieved a removal rate of 48% at an NVP concentration of 50 ng L^{-1}. The photosynthetic efficiency (Fv/Fm and rETR) of both microalgal species was found to be unaffected by environmental concentrations of NVP (up to 4000 ng L^{-1}) during the mid-log phase of growth. Furthermore, the scanning electron microscopy (SEM) analysis demonstrated that both algal species produced distinct ridges on their cell surfaces after NVP uptake. Additional evaluations were conducted on the microalga, *T. obliquus*, for the removal of NVP at 4000 ng L^{-1}, as well as their cellular response (expression of antioxidant enzymes and metabolomics) and biomass production under different cultivation modes (autotrophic, heterotrophic, and mixotrophic). The highest NVP removal

efficiency was observed under mixotrophic (80.13%) growth on day 8, whilst heterotrophic and autotrophic cultivation modes removed 70.30% and 64.40%, respectively. Mass balance calculations showed that the primary removal mechanism was identified as biodegradation, with a relatively low contribution from bioadsorption (2.39-3.36%) and bioaccumulation (0.55-0.87%). Fourier-transform infrared (FTIR) spectroscopy results of harvested microalgal cells displayed bands in the region of 950-1000 cm^{-1}, indicating the presence of aromatic C-H rings found in NVP. Additionally, 6 possible biotransformation products of NVP were identified by untargeted liquid chromatography-time of flight mass spectrometry. Additionally, under autotrophic conditions, the gene expression analysis revealed heightened activities of superoxide dismutase (*sod1*), glutathione peroxidase (*gpx1*) and catalase (*cat2*) in *T. obliquus*. The upregulation of antioxidant genes enhances the organism's ability to defend against oxidative stress induced by NVP. The expression levels of antioxidant genes were significantly reduced during heterotrophic and mixotrophic growth, suggesting microalgae can overcome oxidative stress with glucose supplementation. To further investigate the cellular level response of microalgal cells to NVP, metabolomic analysis was carried to out to identify and quantify key algal metabolites during mixotrophic cultivation. The increase in activity of the fatty acid biosynthesis pathway and carbohydrate synthesis was observed by *T. obliquus* in the presence of NVP under mixotrophic growth conditions. The findings from this study emphasize the significant potential of microalgae in the field of ARV drug remediation.

PREFACE

Research outputs:

Journal articles

Reddy, **K**., Renuka, N., Kumari, S., Bux, F. (2021). Algae-mediated processes for the treatment of antiretroviral drugs in wastewater: Prospects and challenges. *Chemosphere*, 280, 130674. https://doi.org/10.1016/j.chemosphere.2021.130674. (Impact Factor 8.94)

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Book chapter

Reddy, **K**., Jose, S., Faiz, T., Renuka, N., Ratha, S.K., Kumari, S., Bux, F. (2022). Microbe-assisted bioremediation of pesticides from contaminated habitats: Current status and prospects. Bioremediation for Sustainable Environmental Cleanup. CRC Press, Taylor & Francis Group. ISBN 9781032234915.

Conference presentations

Reddy, K., Renuka, N., Kumari, S., Ratha, S.K., Moodley, B., Pillay, K., Bux, F. (2022). Assessing the potential for nevirapine removal and its ecotoxicological effects on microalgae in an aqueous environment. Indo-African International Conference on Emerging Materials Science and Technologies (IAFICEMST'2022), 3-5 August 2022 (Online -Invited lecture).

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DEDICATION

To my parents

The late **Mr & Mrs Reddy**

and

Dr & Mrs Moses

All of you have played a pivotal role in making me the person I am today.

Throughout my life, you have taught me to be determined, to believe in myself, and to keep working no matter what.

I am truly thankful and honoured to have you as my parents.

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ABBREVIATIONS

Abbreviation	Definition
ABC	Abacavir
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AOP	Advanced oxidation processes
APX	Ascorbate peroxidase
ART	Antiretroviral therapy
ARV	Antiretroviral
BBM	Bold's basal medium
BG-11	Blue-green medium
BNR	Biological nutrient removal
CAT	Catalase
CBZ	Carbamazepine
Chl	Chlorophyll
COVID-19	Coronavirus
DCW	Dry cell weight
DMRT	Duncan's multiple range test
D.O	Dissolved oxygen
DRV	Darunavir
ddA-TP	2,3-dideoxyadenosine-5-triphosphate

EFF	Effluent
EFV	Efavirenz
EC_{10}	Effective concentration (10% growth inhibition)
EC_{50}	Effective concentration (50% growth inhibition)
EMP	Embden-Meyerhof pathway
EMPs	Emerging micropollutants
EPS	Exopolymeric substances
ETV	Entecavir
FDA	Food and drug administration
FAO	Fosamprenavir
FTIR	Fourier-transform infrared spectroscopy
Fv/Fm	Maximum quantum efficiency of photosystem II
GC-LC/MS	Gas chromatography- liquid chromatography/ mass spectrometry
GC-TOF/MS	Gas chromatography time-of-flight mass spectrometry
GPX	Glutathione peroxidase
HRAPs	High-rate algal ponds
HPLC-UPLC-MS/MS	High-pressure liquid chromatography/ Ultra performance liquid chromatography-tandem mass spectrometry
HIV	Human immunodeficiency virus
HRT	Hydraulic retention time
IBP	Ibuprofen

Inf	Influent
3TC	Lamivudine
LI	Light intensity
LOQ	Limit of quantification
LC-HRMS	Liquid chromatography-high resolution mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
K_{ow}	n-octanol/water partition coefficient
MBR	Membrane reactor
Min	Minutes
N/A	Not available
No.	Number
NVP	Nevirapine
PAM	Pulse Amplitude Modulation Flourometry
P. e	Population equivalent
PCs	Pharmaceutical compounds/contaminants
PCR	Polymerase chain reaction
POX	Peroxidase
PP	Photoperiod
PSII	Photosystem II
rETR	Photosynthetic electron transport rate
ROS	Reactive oxygen species

RT-qPCR	Real time quantitative polymerase chain reaction
RTV	Ritonavir
SEM	Scanning electron microscopy
SD	Standard deviation
SOD	Superoxide dismutase
SPE	Solid-phase extraction
SPSS	Statistical package for social sciences
SRT	Solid retention time
T	Temperature
TDF	Tenofovir
TON	Total oxidised nitrogen
TPs	Transformation products
USA	United states of America
UV/H_2O_2	Ultraviolet
WWTP	Wastewater treatment plants
YSI	Yellow Springs Instrument
ZDV	Zidovudine

SYMBOLS

Symbol	Definition	Symbol	Definition
<	Less than	ng	Nanogram
>	Greater than	ng L^{-1}	Nanogram per litre
≥	Greater than or equal to	µg	Microgram
%	Percentage	µg L^{-1}	Microgram per litre
±	Plus-minus	mg L^{-1}	Milligram per litre
X	Multiplication	ng ml^{-1}	Nanogram per millilitres
~	Approximate	mg ml^{-1}	Milligram per millilitres
d	Days	g mol^{-1}	Gram per mole
h	Hours	cm^{-1}	Centimetres
^{o} C	Degrees Celsius	Nm	Nanometres
L	Litre	O_2	Oxygen
µ	Micro	CO_2	Carbon dioxide
µl	Microlitre	H_2O	Water
ml	Millilitre	$	Dollar
µm	Micrometer	€	Euro

CHAPTER ONE: INTRODUCTION

The preservation of freshwater resources has gained significant global attention owed to the increased discharge of pollutants, particularly emerging micropollutants (EMPs), into the aquatic environment (de-Oliveira *et al.,* 2020, Liu *et al.,* 2023). Among the various EMPs, the occurrence of pharmaceutical compounds/contaminants (PCs) has emerged as a significant environmental threat (Majumder *et al.,* 2019, Rebello *et al.,* 2018, Samal *et al.,* 2022). The presence of hormones, antibiotics, non-steroidal anti-inflammatory drugs, analgesics, and ARV drugs in aquatic environments has raised concern due to their toxic effects and potential to induce microbial drug resistance (Majumder *et al*., 2019, Adhikari, 2019; Kudu *et al.*, 2022). South Africa has the largest antiretroviral therapy (ART) program worldwide, with millions of individuals accessing these drugs daily as long-term therapy for HIV (Akullian *et al.*, 2021). More than 35 different ARV drugs, including tenofovir (TDF), abacavir (ABC), lamivudine (3TC), nevirapine (NVP), efavirenz (EFV), and zidovudine (ZDV), are utilized for HIV treatment (Mlunguza *et al.,* 2020). Some of these drugs are administered in combination (Schinazi *et al.,* 2022, Perazzolo *et al.*, 2022). The rising HIV infection rates each year have led to a significant increase in the production and consumption of ARV drugs globally (Nannou *et al.*, 2020). Studies have shown that within the human body, these drugs may undergo metabolic reactions such as hydroxylation, cleavage or glucuronidation (Marin *et al.*, 2021).

Pharmacokinetics studies have shown that most of these drugs are usually not fully metabolized within the human body and are excreted *via* faeces and/or urine in its original form, or as transformation and degradation products (Schoeman *et al.*, 2017). In urban areas, excreted drug products, along with urine and faeces, enter sewage networks and eventually reach WWTPs, where they may undergo further degradation or transformation processes (Horn *et al.*, 2022). However, conventional treatment processes, such as activated sludge and sedimentation, are not specifically designed to target these compounds. As a result, a considerable portion of PCs can pass through the treatment process. Failing to adequately remove these compounds during the treatment process can lead to the contamination of receiving freshwater resources, such as rivers and lakes, and pose significant ecological risks (Horn *et al.*, 2022). The removal of PCs from wastewater can be challenging due to their complexity and hazardous nature (Pal, 2018). Conventional biological treatment processes, such as activated sludge and trickling filters, are not specifically designed for PC removal (Ismail and Mokhtar, 2020). Consequently, the likelihood of these drugs passing through different stages of wastewater treatment processes is

generally high, as evidenced by the detection of numerous PCs, including ARV drugs, in the effluents of conventional WWTPs worldwide (Schoeman *et al.*, 2017). Advanced wastewater treatment techniques like reverse osmosis and ozonation have demonstrated effectiveness in treating various compounds. However, their extensive implementation is limited due to the significant operational expenses involved, as highlighted in studies by Gienau *et al.*, 2020, and Silva *et al.*, 2017. Algae-based systems have emerged as an alternative and sustainable method for removing residual nutrients (tertiary treatment) from wastewater in WWTPs (Abdelfattah *et al.*, 2023, Ansari *et al.*, 2019). This approach offers a dual benefit of nutrient removal and the production of microalgal biomass, which can be utilized for various applications such as biofuels, biofertilizers, pigments, and feed based on the quality of the biomass produced (Andreotti *et al.*, 2020, Ansari *et al.*, 2019, Guo *et al.*, 2016, Renuka *et al.*, 2018). Literature has revealed the ability of algae to effectively eliminate a wide range of PCs, including anti-inflammatories (de Wilt *et al.*, 2016, Ding *et al.*, 2020), antibiotics (Leng *et al.*, 2020), steroids (Sami and Fatma, 2019) and other organic pollutants (Nguyen *et al.*, 2021) from the aqueous environment (Bai and Acharya, 2017, Ding *et al.*, 2020, Xiong *et al.*, 2019). Given the limited information available on the removal of ARV drugs by algae, it becomes crucial to address this research gap. Their persistence in the environment can have potential ecological and public health implications, making it imperative to gain a comprehensive understanding of how algae-based systems interact with and remove these contaminants. The unique metabolic activities and surface interactions of algae suggest their potential effectiveness in adsorbing, transforming, or metabolizing these pharmaceutical compounds. Comprehensive research in this direction will not only enhance our understanding of algae's capabilities but also contribute to the development of innovative and eco-friendly approaches for wastewater treatment.

SCOPE OF THE STUDY

Research on the efficiency of microalgal strains in the removal of PCs from aquatic environments is an active and evolving field. Numerous studies have investigated the potential of various microalgae species to uptake and metabolize PCs. However, there are still important gaps in our understanding of ARV drug removal. One significant research gap lies in assessing the efficiency of different algal strains for ARV drug removal. Not all microalgal species have the same capacity to uptake and degrade PCs, and the factors influencing their efficiency need further exploration. Thus, understanding the specific characteristics and traits of different algal strains that contribute to their effectiveness in ARV drug removal is crucial for optimizing treatment processes. Another area that requires attention is the mechanisms involved in the removal of ARV drugs by microalgae. While it is known that microalgae can take up PCs through passive diffusion or active transport, the detailed processes and interactions involved are not fully elucidated. Investigating the specific pathways and transporters involved in ARV drug uptake by microalgae can provide valuable insights into improving removal efficiency. Furthermore, the transformation products resulting from the interaction between ARV drugs and algae are still not well-characterized. Algae have the potential to biotransform PCs into metabolites that may have different properties and environmental impacts. Understanding the transformation pathways and identifying the resulting metabolites is crucial for assessing the fate and behaviour of pharmaceuticals in aquatic ecosystems. In addition, the extent of tolerance and toxicity of ARV drugs on microalgae remains an important research gap. Microalgae can be exposed to ARV drugs at various concentrations, and their response to these compounds can vary greatly. Evaluating the tolerance levels and potential toxic effects of persistent ARV drugs, such as nevirapine, on microalgae is essential for understanding the potential ecological risks associated with their presence in aquatic environments. Closing these research gaps will contribute to the development of effective and sustainable strategies for the removal and management of ARV drugs in aquatic ecosystems.

AIM AND OBJECTIVES

Aim

To investigate the efficiency and mechanisms of removal of selected antiretroviral drugs by algae isolated from various wastewater treatment processes

Objectives

- Isolation and screening of algal strains from wastewater treatment plants for antiretroviral drugs removal potential
- Optimization of culture conditions for higher antiretroviral drugs removal by selected algal strains
- Determination of potential mechanisms of antiretroviral drugs remediation by selected algal strains
- Assessment of the associated metabolic responses of algal cells to antiretroviral drugs through gene expression and metabolomics analyses.

REDDY, **K**., RENUKA, N., KUMARI, S., BUX, F. (2021). Algae-mediated processes for the treatment of antiretroviral drugs in wastewater: Prospects and challenges. *Chemosphere*, 280, 130674.

2.1 Pharmacokinetics of antiretroviral drugs in the human body

The Food and Drug Administration (FDA) has approved multiple ARV drugs for the treatment of HIV, as stated by the U.S. Department of Health and Human Services in 2020 (Figure 2.1). These drugs are categorized into nucleoside and non-nucleoside reverse transcriptase inhibitors, protease inhibitors, fusion inhibitors, entry inhibitors, and integrase strand transfer inhibitors based on their mechanism of action. Understanding pharmacokinetics, which encompasses the absorption, metabolism, and elimination of orally administered drugs, is crucial (Kenakin, 2019). The bioavailability of a drug refers to the fraction that reaches the systemic circulation. Different ARV drugs exhibit bioavailability values ranging from 30% to 80% (Table 2.1), indicating that none of these drugs is fully absorbed by the body, resulting in a significant portion being excreted directly into the environment through faeces and urine (Ncube *et al.*, 2018). For instance, the ARV drug TDF has limited bioavailability, with a median terminal elimination half-life of approximately 17 hours (hrs) (Vaidya *et al.*, 2016). While Quercia *et al.* (2018) reported that the bioavailability percentages of 3TC and TDF were 86% and 70-80%, respectively. Metabolism of ARV drugs in the human body can form various metabolites (Gopalan *et al.*, 2017, Sinxadi *et al.*, 2015). Some of these metabolites have been identified in urine (Rakhmanina and van den Anker, 2010, Ray *et al.*, 2016). These ARV drugs and their metabolites may undergo further degradation and transformation in the environment, potentially forming compounds with persistent biological activity (Tak *et al.*, 2020, Wood *et al.*, 2016).

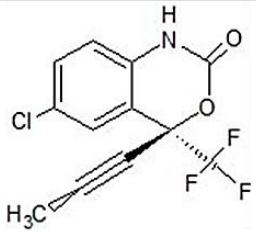
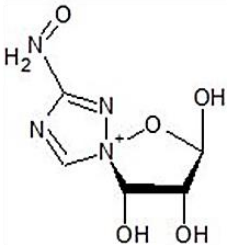
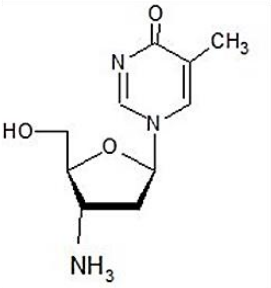
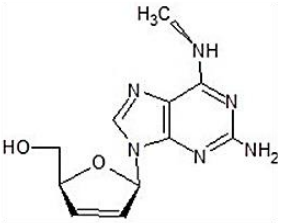
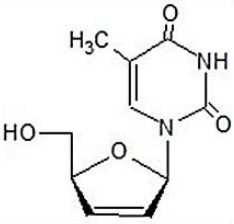
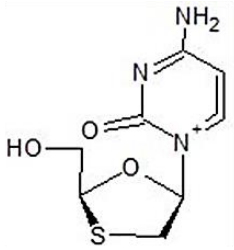
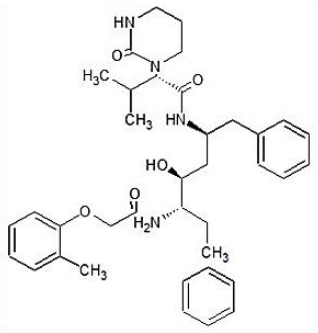
 **Efavirenz** $C_{14}H_9ClF_3NO_2$	 **Nevirapine** $C_{15}H_{14}N_4O$
 **Ribavirin** $C_8H_{12}N_4O_5$	 **Zidovudine** $C_{10}H_{13}N_5O_4$
 **Abacavir** $C_{14}H_{18}N_6O$	 **Stavudine** $C_{10}H_{12}N_2O_4$
 **Lamivudine** $C_8H_{11}N_3O_3S$	 **Lopinavir** $C_{37}H_{48}N_4O_5$

Figure 2.1 Structures of selected ARV drugs (Adapted from https://www.ncbi.nlm.nih.gov (accessed June 2023).

Table 2.1 Classes, bioavailability, and metabolites of different antiretroviral drugs.

Class	Mechanism of action	ARV drug	Bioavailability (%)	Half-life (h)	Metabolites	Reference
Nucleoside reverse transcriptase inhibitors	Blocks reverse transcriptase	Zidovudine	65	0.5-3	3′-azido-3′-deoxy-5′- O-beta-D-glucopyranuronosylthymidine, isomeric cycloaddition products	(Kupiec *et al.*, 2014, Kurmi *et al.*, 2020)
		Lamivudine	82-86	5–7	Lamivudine sulfoxide, Lamivudine 5' triphosphate	(Kumar and Patel, 2010, Quercia *et al.*, 2018)
		Didanosine	30-54	1.5-12	2,3-dideoxyadenosine-5-triphosphate (ddA-TP), hypoxanthine, xanthine and uric acid	(Adams *et al.*, 1998, Wada *et al.*, 2000, Severino *et al.*, 2012)
		Stavudine	>80	3.5	Stavudine-5- triphosphate	(Lea and Faulds, 1996, Panhard *et al.*, 2007)

Table 2.1 continued.

Class	Mechanism of action	ARV drug	Bioavailability (%)	Half-life (h)	Metabolites	Reference
Non-nucleoside reverse transcriptase inhibitors	Causes allosteric inhibition of the transcriptase	Abacavir	83	1.5	5′-carboxylic acid, 5′-glucuronide, carbovir triphosphate	(Adetokunboh *et al.*, 2015, De Clercq, 2001)
		Tenofovir	39	17	Tenofovir disoproxil fumarate, Tenofovir alafenamide	(Taylor and Triggle, 2007, Ray *et al.*, 2016)
		Zalcitabine	>80	1.1-1.8	Zalcitabine triphosphate, 2',3'-dideoxycytidine 5'-triphosphate	(Adkins *et al.*, 1997)
		Efavirenz	40–45	45	7-hydroxy-efavirenz 8-hydroxy-efavirenz 8,14- dihydroxy-efavirenz	(Rakhmanina and van den Anker, 2010, Grilo *et al.*, 2016)
		Nevirapine	>90	45	2 -hydroxynevirapine glucuronide 3- hydroxynevirapine glucuronide 8-hydroxynevirapine glucuronide 12-hydroxynevirapine glucuronide 4-carboxynevirapine	(Cammett *et al.*, 2009)

Table 2.1 continued.

Class	Mechanism of action	ARV drug	Bioavailability (%)	Half-life (h)	Metabolites	Reference
Protease inhibitors	Directly bind to HIV protease and prevent subsequent cleavage of polypeptides	Ritonavir	60-80	3-5	isopropylthiazolyl oxidation metabolite	(Kubin and Hammer, 2010)
		Indinavir	65	8	2-dealkylated/monooxygenated	(Gatti *et al.*, 2000)
		Lopinavir	NA	6	Oxidative metabolites	(Kubin and Hammer, 2010)
Fusion inhibitors	Blocks the HIV envelope from merging with the host CD4 cell membrane	Enfuvirtide	84.3	1.5-4	Amino acids	(Aquaro *et al.*, 2006, Cheng *et al.*, 2016)
Integrase strand transfer inhibitors	Blocks integrase (an HIV enzyme)	Raltegravir	32	3.1-9	-	(Evering and Markowitz, 2008, Ananworanich *et al.*, 2012)
		Elvitegravir	NA	5.2	naphthyridine carboxamide, dihydroquinoline carboxylic acid	(Shimura *et al.*, 2008, Schafer and Squires, 2010);

2.2 Occurrence of ARV in environmental matrices

Research findings have indicated that significant concentrations of ARV drugs have been detected in various environmental matrices, such as wastewater, surface water, groundwater, and drinking water (Wood *et al.*, 2015, Abafe *et al.*, 2018, Boulard *et al.*, 2018, Funke *et al.*, 2016, Rimayi *et al.*, 2018, Adeola and Forbes, 2022). The geographical distribution of ARV drugs globally is depicted in Figure 2.2. A notable observation is that most studies investigating ARV drugs have focused on wastewater treatment plants (WWTPs) and surface waters (Boulard *et al*., 2018; Fisher *et al*., 2016; Rimayi *et al.*, 2018). However, limited data is available regarding the occurrence of ARV drugs in groundwater and drinking water, and such studies have been restricted to specific geographic regions (Appendix one).

Among the other countries, the African continent exhibits the highest concentrations of ARV drugs (Fig. 2.2, Appendix one). This observation can be attributed to the fact that Africa possesses the largest standard ART program worldwide. Consequently, the per capita consumption of ARV drugs is notably higher in Africa than in other regions globally, as the World Health Organization reported in 2013. This finding serves to justify the elevated concentrations of ARV drugs observed in the African continent. It is important to note that the majority of research on ARV drug occurrence on the African continent has been conducted in South Africa and Kenya, indicating a lack of investigation in other African countries (Fig. 2.3). Additionally, studies examining the detection and prevalence of ARV drugs in WWTPs, and surface water have consistently reported higher levels in developing countries, particularly in the African continent, in comparison to developed countries (Ngumba *et al.*, 2016a, Prasse *et al.*, 2010, Vergeynst *et al.*, 2015, Wood *et al.*, 2015). Considering the potentially harmful implications of ARV drugs to aquatic organisms (Kudu *et al.*, 2022), it becomes crucial to gather data from unexplored regions globally where ARV drugs are extensively used. The implications of these drugs in the aquatic environment necessitate a comprehensive understanding of their occurrence and potential ecological risks beyond the currently studied areas.

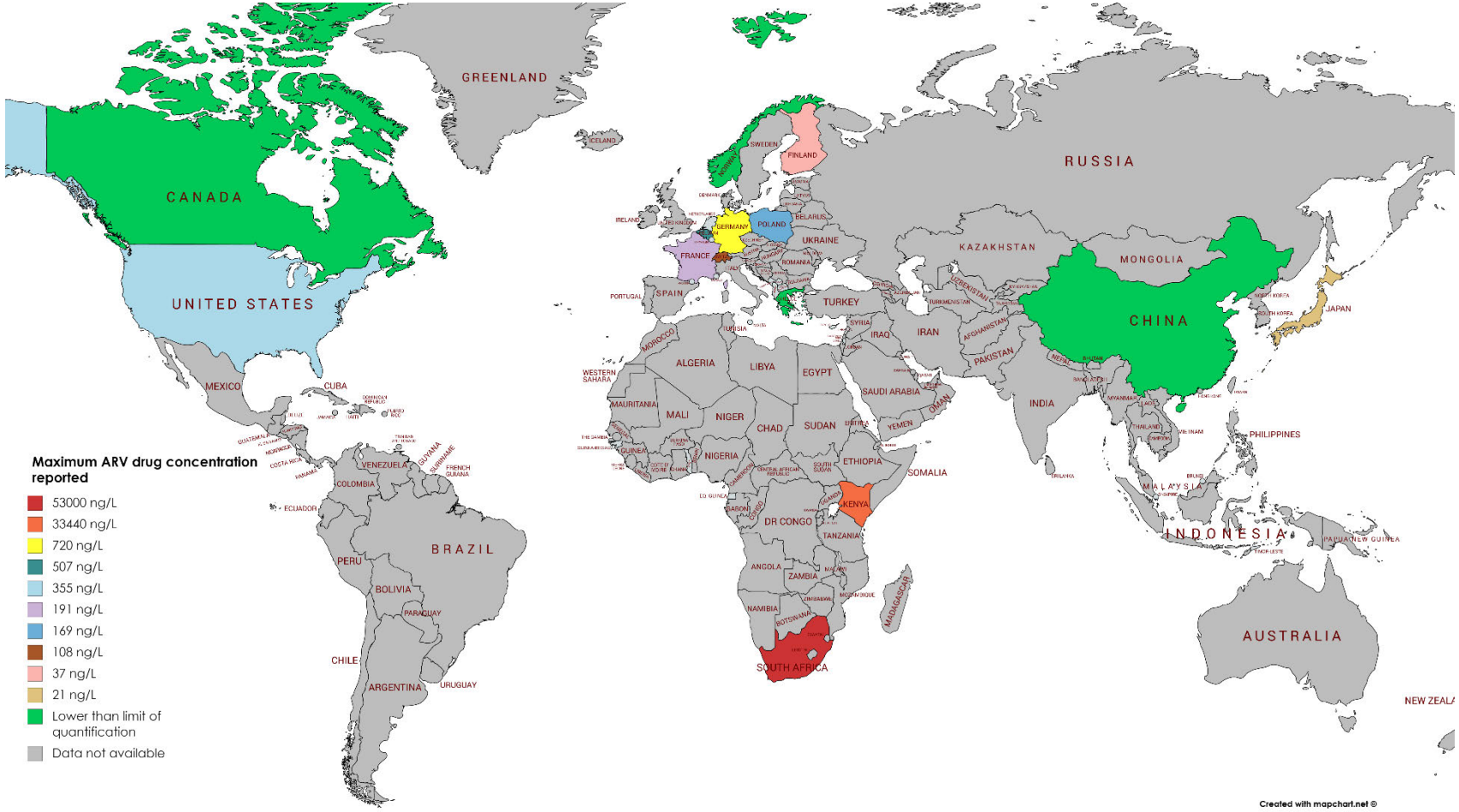


Figure 2.2 Global occurrence of antiretroviral drugs based on available reports (Appendix one).

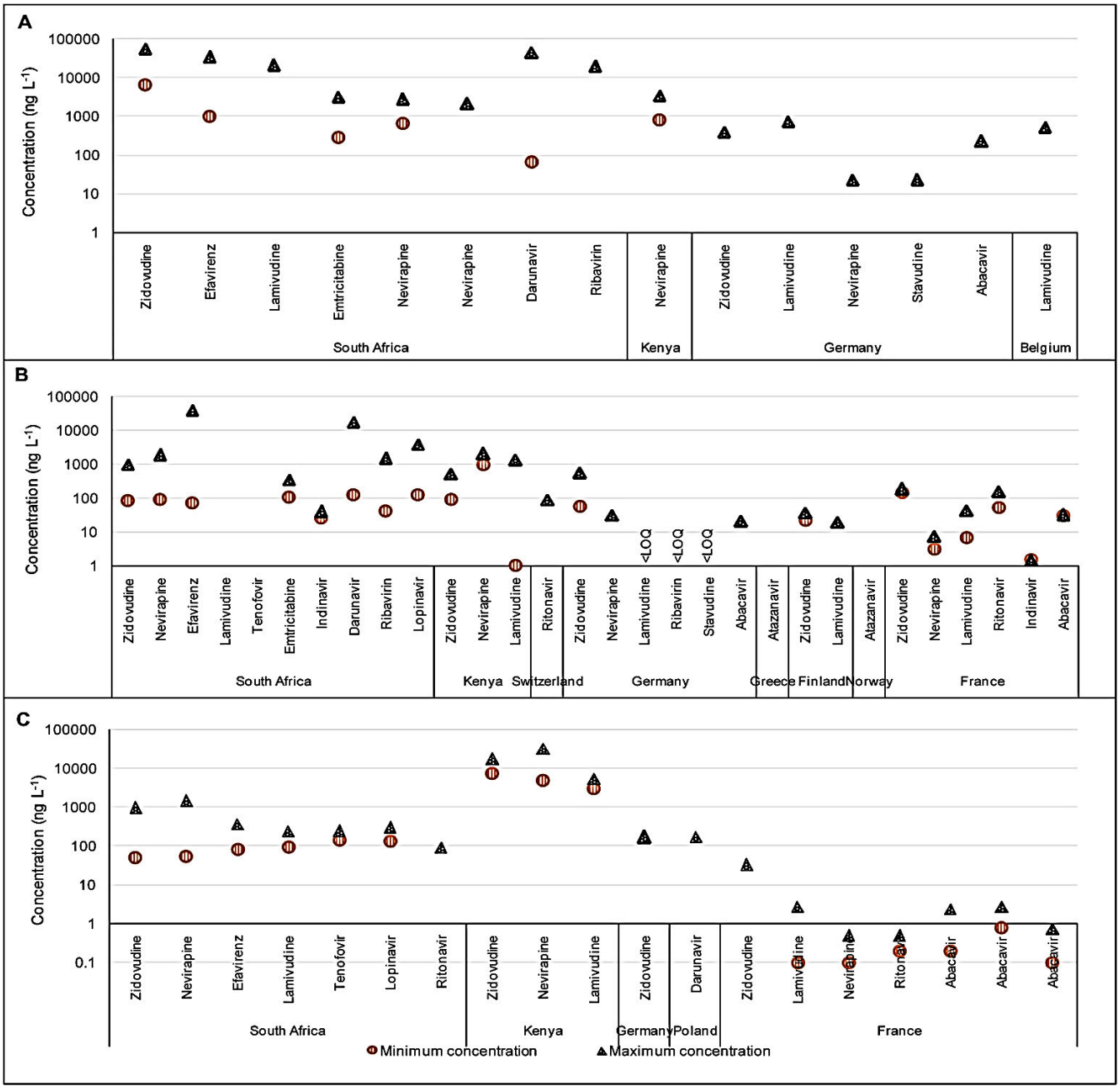


Figure 2.3 Concentration of antiretroviral drugs in different environmental matrices reported from various countries (**A**) wastewater influent (**B**) wastewater effluent (**C**) surface water (Appendix one). LOQ- Limit of quantification.

The lack of standard detection protocols and inaccessibility to high-end equipment to detect low levels of ARV drugs from the environment are major challenges (Bouwman *et al.*, 2020). Advanced methods such as high-pressure liquid chromatography/ Ultra performance liquid chromatography-tandem mass spectrometry (HPLC-UPLC-MS/MS) (Wood *et al.*, 2017, Zheng *et al.*, 2020, Mhuka *et al.*, 2020), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Mosekiemang *et al.*, 2019) and gas chromatography time-of-flight mass spectrometry (GC-TOF/MS) (Wooding *et al.*, 2017, Arvaniti *et al.*, 2023) are proven efficient in the detection of a wide range of ARV drugs from different environmental matrices at lower concentrations (Table 2.2). Funke *et al.* (2016), studied the fate of five ARV drugs (ABC, 3TC, ZDV, emtricitabine and ganciclovir) and their transformation products (TPs) in wastewater treatment plants, surface water and drinking water using liquid chromatography-high resolution mass spectrometry (LC-HRMS). Their findings indicated that all ARV drugs followed a comparable pathway of biodegradation, resulting in the oxidation of the terminal hydroxyl group and the generation of hydroxy TPs. Notably, when analysing wastewater treated with ozonation on a pilot scale, the removal of carboxylic groups was observed. However, it remained uncertain whether this process resulted in the formation of stable oxidation products (Funke *et al.*, 2016). Majority of the studies on ARV drugs in the aquatic environment have focused on the detection of the original parent compound rather than their behaviour or TPs (Abafe *et al.*, 2018, Azuma *et al.*, 2019, Mosekiemang *et al.*, 2019). Gaining a comprehensive understanding of the fate and behaviour of PCs under environmental conditions can be extremely challenging due to their inherent complexity. Therefore, conducting more extensive studies on detecting ARV drugs and their TPs is imperative. Such investigations are essential for unravelling the occurrence, fate, and potential accumulation of these compounds in various environmental matrices.

Table 2.2 Summary of techniques used for the detection of antiretroviral drugs in various environmental matrices.

Antiretroviral drug	**Sample**	**Detection technique**	**Limits of detection ($ng\ L^{-1}$)**	**Limits of quantification ($ng\ L^{-1}$)**	**Reference**
Efavirenz	Wastewater	Gas Chromatography-Time of Flight Mass Spectrometry	7.8	25.9	(Schoeman *et al.*, 2015)
Efavirenz	Wastewater	Liquid Chromatography-Tandem Mass Spectrometry	9	31	(Abafe *et al.*, 2018)
Efavirenz	Wastewater	Ultra-high-performance- Liquid chromatography	510	1690	(Horn *et al*., 2022)
Nevirapine	Wastewater	Gas Chromatography-Time of Flight Mass Spectrometry	1.8	6	(Schoeman *et al.*, 2015)
Nevirapine	Surface water	Liquid Chromatography-electrospray ionization-Tandem Mass Spectrometry	4	12	(Ngumba *et al.*, 2016a)
Nevirapine	Surface water	Liquid Chromatography-Tandem Mass Spectrometry	2	5	(Ngumba *et al.*, 2016b)

Table 2.2 continued

Antiretroviral drug	**Sample**	**Detection technique**	**Limits of detection (ng L^{-1})**	**Limits of quantification (ng L^{-1})**	**Reference**
Nevirapine	Wastewater	Liquid Chromatography-Tandem Mass Spectrometry	6	20	(Abafe *et al.*, 2018)
Nevirapine	Wastewater	Ultra-high-performance- Liquid chromatography	10	50	(Horn *et al*., 2022)
Lamivudine	Surface water	Liquid Chromatography-electrospray ionization-Tandem Mass Spectrometry	3	10	(Ngumba *et al.*, 2016a)
Lamivudine	Surface water	Liquid Chromatography-Tandem Mass Spectrometry	2	6	(Ngumba *et al.*, 2016b)
Lamivudine	Wastewater	Liquid Chromatography-Tandem Mass Spectrometry	20	65	(Abafe *et al.*, 2018)
Zidovudine	Surface water	Liquid Chromatography-electrospray ionization-Tandem Mass Spectrometry	37	122	(Ngumba *et al.*, 2016a)

Table 2.2 continued

Antiretroviral drug	Sample	Detection technique	Limits of detection (ng L^{-1})	Limits of quantification (ng L^{-1})	Reference
Zidovudine	Surface water	Liquid Chromatography-Tandem Mass Spectrometry	13	44	(Ngumba *et al.*, 2016b)
Zidovudine	Wastewater	Liquid Chromatography-Tandem Mass Spectrometry	4	15	(Abafe *et al.*, 2018)
Zidovudine	Wastewater	Ultra-high-performance- Liquid chromatography	500	1670	(Horn *et al*., 2022)
Lopinavir	Wastewater	Liquid Chromatography-Tandem Mass Spectrometry	5	16	(Abafe *et al.*, 2018)
Lopinavir	Wastewater	Ultra-high-performance- Liquid chromatography	580	1940	(Horn *et al*., 2022)
Atazanavir	Wastewater	Liquid Chromatography-Tandem Mass Spectrometry	2	12	(Abafe *et al.*, 2018)

Table 2.2 continued

Antiretroviral drug	**Sample**	**Detection technique**	**Limits of detection (ng L^{-1})**	**Limits of quantification (ng L^{-1})**	**Reference**
Ritonavir	Wastewater	Ultra-high-performance- Liquid chromatography	240	800	(Horn *et al*., 2022)
Didanosine	Wastewater	Ultra-high-performance- Liquid chromatography	10	50	(Horn *et al*., 2022)
Abacavir	Wastewater	Liquid Chromatography-Tandem Mass Spectrometry	4	15	(Abafe *et al*., 2018)

2.3 Fate of antiretroviral drugs in wastewater treatment plants

Wastewater treatment plants have emerged as crucial components in addressing the presence and fate of ARV drugs in the environment. These plants hold significant potential in facilitating the transformation and degradation of ARV drugs, as highlighted in Figure 2.4 and Table 2.3. Research studies have provided valuable insights into the removal efficiency of various ARV drugs within WWTPs (Mosekiemang *et al.*, 2019, Ngumba *et al.*, 2016a, Schoeman *et al.*, 2017). It has been observed that the effectiveness of drug removal can be influenced by several factors, including the specific treatment processes implemented and the inherent complexity of the compound (Fernandes *et al.*, 2021). The complexity of ARV drugs includes a range of characteristics, such as their physical properties, chemical composition, and hydrophobic nature. Additionally, the reactivity of these drugs towards the different treatment processes also plays a crucial role in their removal (Eryildiz *et al.*, 2022). It is essential to consider these factors when evaluating the performance of WWTPs in mitigating ARV drugs and their potential impact on the environment.

Observations have indicated that in conventional WWTPS, both primary and secondary treatment processes play a significant role in the removal of ARV drugs (Moreira *et al.*, 2016). The hydrophobic characteristics of ARV drugs enable their sorption onto solid particles, facilitating their subsequent removal along with the solid particles or sludge during the primary treatment stage (Wood *et al.*, 2015, Patel *et al.*, 2019). During the secondary/biological treatment stage, ARV drugs can undergo a series of intricate processes, including dilution, dispersion, partitioning, biodegradation, and the sorption of both the parent compound and its TPs onto the sludge (Schoeman et al., 2017). While a minor amount of these compounds could also be removed through volatilization into the environment (Verlicchi et al., 2012).

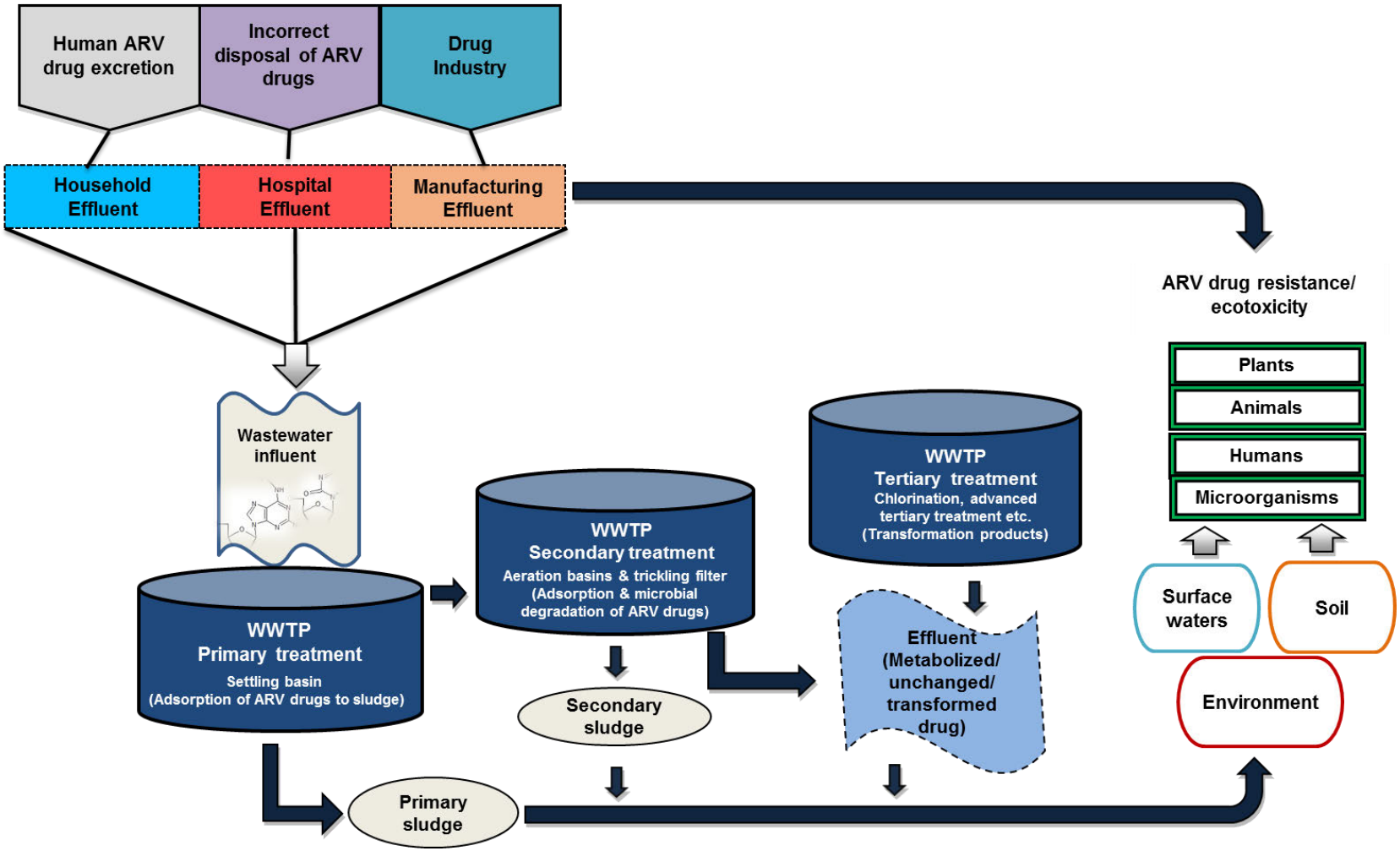


Figure 2.4 Sources of antiretroviral drugs and their fate at conventional wastewater treatment plants.

Compounds containing esters, nitriles and aromatic alcohols have functional groups that are readily biodegradable, whereas aromatic amines, iodide, nitro and azo groups remain persistent (Sipma *et al.*, 2010, Cai *et al.*, 2023, Zhou *et al.*, 2020). Therefore, aromatic or unsaturated cyclic rings in ARV drugs make them more toxic and resistant to biological degradation processes (Jain *et al.*, 2013). Similarly, these compounds could be sorbed onto the lipid molecules found on the cell membrane of microorganisms and sludge through the hydrophobic interaction of aliphatic and aromatic groups (Suárez *et al.*, 2008). The duration of solid retention time (SRT) or sludge age has been identified as a significant factor influencing the efficiency of ARV drug removal during the secondary treatment process (Jelic *et al.*, 2011). Generally, a longer SRT promotes the growth of a diverse biological community, enhancing the effective removal of ARV compounds during both the primary and secondary treatment processes (Cydzik-Kwiatkowska and Zielińska, 2016). Furthermore, the electrostatic interactions between positively charged compounds and negatively charged microbes and sludge are influenced by the octanol-water coefficient, acid dissociation constant, and sludge adsorption coefficient (Tiwari *et al.*, 2017). The removal efficiency of ARV drugs primarily depends on their solubility in wastewater. A compound with low solubility (hydrophobic) is more likely to be retained in the sewage sludge (Luo *et al*., 2014; Shraim *et al*., 2017). Studies on the fate of ARV drugs in WWTPs have also indicated their presence in the final effluent and the formation of diverse TPs during the tertiary treatment process (chlorination) due to oxidation reaction (Nannou *et al.*, 2020, Schoeman *et al.*, 2015, Wood *et al.*, 2016). For example, Wood *et al.* (2016) revealed the presence of the ARV drug NVP and its TPs in a tertiary treated (chlorination) wastewater. Their study also showed that the TPs were non-toxic; however, they retained the antiviral activity. In another study, Schoeman *et al.* (2017) observed higher concentrations of NVP in the final effluent than the influent and suggested that deconjugation of hydroxylated TPs of NVP at the WWTP (anaerobic pond) and the absence of binding of NVP to the primary settling tank sludge could be possible explanations for the increased concentrations. However, due to the physical removal of ARV drugs by solids, transformation into lower molecular compounds and conjugates that can later be hydrolyzed and released as the parent compound, the fate of ARV drugs in WWTPs remains uncertain. Nevertheless, it is evident from the literature that conventional biological WWTPs are not effective in removing ARV drugs and their by-products from wastewater, necessitating the exploration of alternative and sustainable technologies for their removal.

T**able 2.3.** Antiretroviral drugs detected in wastewater treatment plants.

Compound	Location	Source (WWTP)	Treatment processes	LOQ (ng L^{-1})	Concentration (ng L^{-1})	Reference
Efavirenz	South Africa	Influent	Anoxic, aeration, chlorination	12.9[a]	5500-14000	(Schoeman *et al.*, 2017)
	South Africa	Effluent	Fine bubble diffused aeration	-	74.1	(Wood *et al.*, 2015)
	South Africa	Influent	Activated sludge, MBR, chlorination	430-920	15400	(Mosekiemang *et al.*, 2019)
	South Africa	Effluent		430-920	1930	(Mosekiemang *et al.*, 2019)
	South Africa	Influent	Anoxic, aeration, chlorination	25.9	17400	(Schoeman *et al.*, 2015)
	South Africa	Effluent		25.9	7100	(Schoeman *et al.*, 2015)
	South Africa	Effluent	BNR	179	580	(Mhuka *et al.*, 2020)
	Germany	Influent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	21.8	(Prasse *et al.*, 2010)

Table 2.3 continued.

Compound	Location	Source (WWTP)	Treatment processes	LOQ (ng L^{-1})	Concentration (ng L^{-1})	Reference
Nevirapine	Kenya	Effluent	Stabilization ponds (anaerobic, facultative and maturation ponds)	8 - 122	1357	(Ngumba *et al*., 2016a)
	Germany	Effluent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	32.1	(Prasse *et al*., 2010)
	Kenya	Influent	Screening bars, anaerobic, facultative and maturation ponds, trickling filters	-	850-3300	(K'Oreje *et al.*, 2016)
	Kenya	Effluent		-	1030-2080	(K'Oreje *et al*., 2016)
	South Africa	Influent	Anoxic, aeration, chlorination	6.0	2100	(Schoeman *et al*., 2015)
	South Africa	Effluent		6.0	350	(Schoeman *et al*., 2015)
	South Africa	Effluent		11.4[a]	92-473	(Schoeman *et al*., 2017)

Table 2.3 continued.

Compound	Location	Source (WWTP)	Treatment processes	LOQ (ng L^{-1})	Concentration (ng L^{-1})	Reference
Zidovudine	South Africa	Effluent	Fine bubble diffused aeration	-	450-970	(Wood *et al*., 2015)
	Germany	Influent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	380	(Prasse *et al*., 2010)
	Germany	Effluent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	564	(Prasse *et al*., 2010)
	Kenya	Effluent	Screening bars, anaerobic, facultative and maturation ponds, trickling filters	-	90-110	(K'Oreje *et al*., 2016)
	Kenya	Effluent	Stabilization ponds (anaerobic, facultative and maturation ponds)	8 - 122	513	(Ngumba *et al*., 2016a)
	Germany	Effluent	BNR, ozonation	5 - 100	57-180	(Funke *et al.*, 2016)
	Finland	Effluent	BNR	5 - 63	22-37	(Ngumba *et al*., 2016b)
	France	Effluent	BNR	-	154-191	(Aminot *et al.*, 2015)

Table 2.3 continued.

Compound	Location	Source (WWTP)	Treatment processes	LOQ (ng L^{-1})	Concentration (ng L^{-1})	Reference
Stavudine	Germany	Influent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	22.8	(Prasse *et al*., 2010)
	Germany	Effluent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	<LOQ	(Prasse *et al*., 2010)
	Germany	Influent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	720	(Prasse *et al*., 2010)
	Kenya	Effluent	Stabilization ponds (anaerobic, facultative and maturation ponds)		3985	(Ngumba *et al*., 2016a)
Lopinavir		Effluent	Fine bubble diffused aeration	-	130	(Wood *et al*., 2015)

Table 2.3 continued.

Compound	Location	Source (WWTP)	Treatment processes	LOQ (ng L^{-1})	Concentration (ng L^{-1})	Reference
Lamivudine	Germany	Effluent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	<LOQ	(Prasse *et al*., 2010)
	Germany	Effluent	BNR, ozonation	20000	20	(Boulard *et al*., 2018)
	South Africa	Influent	BNR	430 – 920	20900	(Mosekiemang *et al*., 2019)
	South Africa	Effluent	BNR	430 - 920	<LOQ	(Mosekiemang *et al*., 2019)
Acyclovir	Greece	Effluent	BNR	-	308000	(Arvaniti *et al.*, 2023)
Tenofovir	South Africa	Influent	BNR	100	250	(Mlunguza *et al.*, 2020)

Table 2.3 continued.

Compound	Location	Source (WWTP)	Treatment processes	LOQ (ng L^{-1})	Concentration (ng L^{-1})	Reference
Abacavir	Germany	Influent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	225	(Prasse *et al*., 2010)
	Germany	Effluent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	<LOQ	(Prasse *et al*., 2010)
	Germany	Effluent	BNR, ozonation	20000	10	(Boulard *et al.*, 2018)
	Germany	Effluent		5 - 100	21	(Funke *et al*., 2016)
	Germany	Influent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	22.8	(Prasse *et al*., 2010)
	South Africa	Influent	Anoxic, aeration, chlorination	360000 - 6810000	19600	(Osunmakinde *et al.*, 2013)

Table 2.3 continued.

Compound	Location	Source (WWTP)	Treatment processes	LOQ (ng L^{-1})	Concentration (ng L^{-1})	Reference
Ribavirin	South Africa	Influent	Anoxic, aeration, chlorination	360000 - 6810000	19600	(Osunmakinde *et al.*, 2013)
	South Africa	Effluent		360000 - 6810000	42	(Osunmakinde *et al.*, 2013)
	Germany	Influent	Aerated grit-removal tank, primary clarifier, anaerobic compartment	0.2 - 10	<LOQ	(Prasse *et al*., 2010)
	Germany	Effluent		0.2 - 10	<LOQ	(Prasse *et al*., 2010)

*LOQ-Limit of quantification; WWTP-Wastewater treatment plant; BNR- Biological nutrient removal; MBR- Membrane reactor; [a] mg kg^{-1}

2.4 Ecotoxicological effect of ARV drugs

The toxicity of antiretroviral drugs and their metabolites classifies them as highly hazardous therapeutic agents, posing risks to living organisms (Castiglioni *et al.*, 2006, Adeola and Forbes, 2022, Cid *et al.*, 2021). The presence of certain ARV drugs in the environment can result in their persistence, leading to the emergence of drug resistance in microorganisms and posing direct risks to aquatic organisms (Álvarez-Muñoz *et al.*, 2015, Zhou *et al.*, 2015, Kudu *et al.*, 2022).

Assessing the ecotoxicity of ARV drugs on microbial cells within a specific exposure period can serve as a valuable indicator of the compounds' toxicity levels and tolerance in microorganisms. Limited studies have investigated the acute and chronic toxicity of ARV drugs on various aquatic organisms, including *Pseudokirchneriella subcapitata, Desmodesmus subspicatus, Vibrio fischeri, Artemia salina, Skeletonema marinoi,* and *Daphnia magna* (Table 2.4). Microalgal species, such as *P. subcapitata* and *D. subspicatus*, have demonstrated a tolerance for high concentrations of ARV drugs, even at levels of 100 mg L^{-1} (Guo *et al.*, 2015, Minguez *et al.*, 2016). Minguez *et al.* (2016), investigated the ecotoxicity effect of ARV drug abacavir (ABC) on green algae, diatoms and crustaceans using freshwater and marine assays. Their findings indicated that the effective concentration (EC_{50}) value of ARV drug ABC for *D. magna* and *A. salina* was significantly higher (>100 mg L^{-1}) as compared to *P. subcapitata* (57 mg L^{-1}), which indicated the potential of these organisms to tolerate high concentrations of ARV drugs.

A comprehensive review by Guo *et al.* (2015) compiled toxicity data on several ARV drugs, including 3TC, NVP, ABC, EFV, Entecavir (ETV), darunavir (DRV), and telzir/fosamprenavir (FAO), specifically focusing on their effects on algal species. The majority of ecotoxicological studies in this review involved green algae or blue-green algae and were conducted over an exposure period ranging from 72 hours to 12 days. Among these studies, the microalga *P. subcapitata* emerged as a commonly used bioindicator species for assessing the presence of toxic substances in freshwater environments. The EC_{50} values for most ARV drugs (3TC, NVP, ABC, DRV and ETV) ranged from >0.012 mg L^{-1} to >110 mg L^{-1} for *P. subcapitata* after a 72-hr exposure period (Chen *et al.*, 2015).

Based on the data presented in Table 2.4, it can be postulated that microalga *P. subcapitata* exhibits a high tolerance to most ARV drugs, although the lowest EC_{50} value (0.012 mg L^{-1})

was observed for EFV. In a study by Russo *et al.* (2018), the toxic effects of ZDV (4.5 mg L^{-1}) and STV (4.35 mg L^{-1}) on bacteria (*Aliivibrio fischeri*, *Salmonella typhimurium*), crustacean (*Daphnia magna*) and algae (*Raphidocelis subcapitata*) were evaluated using UV254 and UV254/H_2O_2 processes. The results indicated that the tested ARV drugs did not exhibit any adverse effects on bacterial and crustacean species. However, ZDV showed slightly higher toxicity (~25% growth inhibition) to the alga *R. subcapitata* compared to STV (<20% growth inhibition). The toxicity of ZDV increased by 36% and 44% respectively when subjected to UV254 and UV254/H2O2 processes in synthetic wastewater. Russo *et al.* (2018), suggested that the ARV drugs might undergo different molecular changes such as transformation and conjugation, which result in the production and accumulation of a wide array of transformation compounds in the environment. Therefore, potential studies on the ecotoxicological effect of these TPs are critical to unravel their possible negative impact on living organisms.

The literature shows that different algal species such as *P. subcapitata*, *A. salina*, and *R. subcapitata* could tolerate high concentrations of ARV drugs (Table 2.4). However, it can also be deduced that the toxicity effect and tolerance of algal species to ARV drugs could vary among different genera/species. Additionally, their toxicity effect could vary depending on the type and combination of ARV drugs. These ARV drug-tolerant microorganisms could play a possible role in the remediation of ARV drugs.

Table 2.4 Ecotoxicity of antiretroviral drugs in aquatic microorganisms.

Antiretroviral drug/ Product	**Organism**	**Test duration**	**EC_{50} (mg L^{-1})**	**Reference**
Abacavir	*Pseudokirchneriella subcapitata*	72 h	>49.06	(Guo *et al.*, 2015)
	Pseudokirchneriella subcapitata	72 h	57.32	(Minguez *et al.*, 2016)
	Daphnia magna	48 h	>100	(Minguez *et al.*, 2016)
	Artemia salina	48 h	>100	(Minguez *et al.*, 2016)
	Skeletonema marinoi	72 h	N/A	(Minguez *et al.*, 2016)
Efavirenz	*Pseudokirchneriella subcapitata*[a]	72 h	>0.012	(Guo *et al.*, 2015)
	Microcystis aeruginosa	12 d	>0.076 (EC_{10})	(Guo *et al.*, 2015)
	Echinometra lucunter	60 min	11.46	(Cid *et al.*, 2021)
Telzir	*Desmodesmus subspicatus*	72 h	>100	(Guo *et al.*, 2015)
Darunavir	*Pseudokirchneriella subcapitata*	72 h	>43	(Guo *et al.*, 2015)
Nevirapine	*Pseudokirchneriella subcapitata*	72 h	>43	(Guo *et al.*, 2015)

Table 2.4 continued.

Antiretroviral drug/ Product	**Organism**	**Test duration**	**EC_{50} (mg L^{-1})**	**Reference**
	Echinometra lucunter	60 min	84.61	(Cid *et al*., 2021)
Lamivudine	*Pseudokirchneriella subcapitata*	72 h	>96.9	(Guo *et al*., 2015)
	Pseudokirchneriella subcapitata	96 h	463	(Guo *et al*., 2015)
Entecavir	*Pseudokirchneriella subcapitata*	72 h	>110	(Guo *et al*., 2015)
Atazanavir	*Pseudokirchneriella subcapitata*	72 h	>4.1	(Guo *et al*., 2015)
	Echinometra lucunter	60 min	73.04	(Cid *et al*., 2021)

d-Days; h-Hours; min-Minutes; EC_{50}-Effective concentration (50% growth inhibition); EC_{10}-Effective concentration (10% growth inhibition); N/A-Not available; [a]*Raphidocelis subcapitata* formerly known as *Pseudokirchneriella subcapitata*.

2.5 Available technologies for the treatment of ARV drugs

Advanced oxidation processes (AOPs) such as ozonation (O_3/H_2O_2), photocatalysis (UV/TiO_2), ultraviolet (UV/H_2O_2), and electrolysis have been recently recognized for the removal of complex inorganic and organic contaminants, including ARV drugs in water and wastewater (Anjali and Shanthakumar, 2019). Removal percentage, however, varied and ranged from 20-100% (An *et al.*, 2011, Anjali and Shanthakumar, 2019, Li *et al.*, 2012, Lucena *et al.*, 2020, Michael-Kordatou *et al.*, 2018, Ngumba *et al.*, 2020, Wang *et al.*, 2010). Although these techniques displayed efficient ARV drug removal, the possible formation of persistent

compounds and high operational costs limits their widespread application. Table 2.5 summarizes the advantages and disadvantages of current treatment technologies used for the treatment of ARV drugs from the aqueous phase.

Kebede *et al*. (2020) conducted research on the utilization of activated carbon and nanofibers for the elimination of RTV and EFV. The study demonstrated that activated carbon exhibited a high removal efficiency, ranging from 75.07% to 92.85%, without generating toxic byproducts. However, it is important to note that the contaminants were removed through phase-by-phase separation rather than complete mineralization. Although this technology is simple and adaptable to many treatment formats, it requires relatively high added cost for incineration to eliminate the adsorbent (Crini and Lichtfouse, 2019). Few others have also investigated the microbial degradation of ARV drugs, a simple process that can be carried out by a number of species as a mixed or pure culture (Jain *et al.*, 2013). A study by K'Oreje *et al.*, (2016) explored the removal of different ARV drugs using activated sludge from conventional WWTPs as inoculum for the biodegradation studies. Their results showed poor to moderate removal of ARV drugs, with no removal observed for 3TC, NVP and ZDV (Vaňková, 2010).

Table 2.5 Comparison of different technologies for the treatment of ARV drugs from the aqueous phase.

Treatment	Advantages	Limitations	Method/Technique	ARV Drug (% Removal)	Reference
Advanced oxidation processes	▪ Rapid reaction rate for most of compounds ▪ No generation of solid residuals ▪ Degradation of pollutant, rather than concentrating it ▪ Able to obtain complete mineralization of most PCs	▪ Formation of persistent and toxic transformation products ▪ Subsequent process needed to quench residual oxidant ▪ High operational and maintenance costs ▪ Background water quality can interfere with treatment efficiency	UV_{254} UV_{254}/H_2O_2 UV/H_2O_2 UV/Cl_2	Stavudine (90) Zidovudine (90) Stavudine (100) Zidovudine (100) Lamivudine (72.2; 97.33) Nevirapine (52.9) Zidovudine (93.90) Lamivudine (77.4) Nevirapine (20.8)	(An *et al.*, 2011, Anjali and Shanthakumar, 2019, Li *et al.*, 2012) (Linden and Mohseni, 2014, Lucena *et al.*, 2020), (Michael-Kordatou *et al.*, 2018, Ngumba *et al.*, 2020, Wang *et al.*, 2010)

Table 2.5 continued

Treatment	Advantages	Limitations	Method/Technique	ARV Drug (% Removal)	Reference
Adsorption	▪ Technologically simple and adaptable to many treatment-formats ▪ High removal of PCs without the generation of toxic active products ▪ High surface area ▪ Wide range of commercial products available	▪ Contaminants removed from aqueous phase *via*. phase-by-phase separation instead of mineralization ▪ Relatively high investment ▪ Performance depends on the type of material ▪ Elimination of the adsorbent requires incineration, regeneration or replacement of the material	Iron–carbon micro-electrolysis process Fabricated nanofibers, white root extract	Didanosine, sulfamethoxazole, lidocaine, amitriptyline hydrochloride, prednisolone, isoniazid, dexamethasone, ritonavir, efavirenz, and fluconazole (75.07–92.85)	(Crini and Lichtfouse, 2019, Garba *et al.*, 2019, Kebede *et al.*, 2020, Ni and Li, 2009, Zietzschmann *et al.*, 2016)

Table 2.5 continued

Treatment	Advantages	Limitations	Method/Technique	ARV Drug (% Removal)	Reference
Biological Algal Technology	▪ The application of microorganisms for the biodegradation of organic contaminants is a simple and economical ▪ Several species can be used in mixed cultures (consortia) or pure cultures	▪ Slow process may lead to problems of kinetics ▪ Low biodegradability of certain molecules ▪ Requires management and maintenance of the microorganisms ▪ Sludge disposal	Conventional activated sludge Conventional activated sludge Conventional activated sludge	Lamivudine, nevirapine, and zidovudine (0) Efavirenz (27-71) Nevirapine (11-49)	(Crini and Lichtfouse, 2019, Vaňková, 2010, K'Oreje *et al.*, 2016, Schoeman *et al.*, 2017)
	▪ Algae adapt to wide range of conditions ▪ Promotes pollutant and pathogen removal ▪ Formation of valuable biomass	▪ Large land requirements ▪ Environmental and operational conditions at large scale may hinder contaminant removal ▪ Harvesting and dewatering of algae requires high energy inputs	-	-	(Kohlheb *et al.*, 2020, Molinuevo-Salces *et al.*, 2019)

2.6 Algae-mediated wastewater treatment

The concept of using algae for wastewater bioremediation dates back to 1957 when Oswald and his co-workers reported the potential of photosynthetic nutrient removal in out-door waste stabilization ponds (Oswald *et al.*, 1957). Presently, algae-based wastewater treatment processes have been successfully demonstrated in the laboratory (Mennaa *et al.*, 2019), pilot (Arbib *et al.*, 2017) and demonstration scales (Delrue *et al.*, 2016). High-rate algal ponds (HRAPs) have received considerable attention in recent years due to various advantages such as low input energy, high-quality effluent (treated wastewater), production and recovery of algal biomass for diverse applications such as biofuels, nutraceuticals, protein-rich feed and as fertilizer supplement (Craggs *et al.*, 2012, Chinnasamy *et al.*, 2014, Ansari *et al.*, 2019, Rueda *et al.*, 2020). Other significant advantages of using algae for wastewater treatment include their capability of CO_2 sequestration and their ability to survive in extreme environmental conditions by switching the metabolism between autotrophism, heterotrophism and mixotrophism (Terrado *et al.*, 2017). Algae can also remove the excess inorganic and organic compounds present in wastewater (López-Serna *et al.*, 2019). These compounds also include but are not limited to various ECs such as pesticides, hormones, nanoparticles, flame retardants and PCs (Tolboom *et al.*, 2019).

2.6.1 Pharmaceutical removal using algae

The ability of algal species for PCs removal from the aqueous environment has been explored, with most of the research focusing on the use of monocultures (Bai and Acharya, 2017, Yu *et al.*, 2017). de Wilt *et al.* (2016), investigated the removal of PCs from urine using *Chlorella sorokiniana*. The key removal mechanisms reported in their study included biodegradation and photolysis that led to 60–100% removal of diclofenac, IBP, paracetamol and metoprolol. Matamoros *et al.* (2016) demonstrated the efficiency of *Chlorella* sp. and *Scenedesmus* sp. to remove a wide range of contaminants *viz*. caffeine, ibuprofen (IBP), galaxolide, tributyl phosphate, 4-octylphenol, tris (2-chloroethyl) phosphate and carbamazepine (CBZ) from urban wastewater using aerated batch reactor. They reported up to 99% removal of 4-octylphenol, galaxolide, and tributyl phosphate through volatilization; whilst 40% removal of IBP was achieved through biodegradation.

Pharmaceuticals such as CBZ and sulfamethoxazole are considered recalcitrant during conventional and advanced wastewater treatment, however, research has revealed the potential of algae to remove these PCs from the aqueous environment (Bai and Acharya, 2017, Ding *et al.*, 2020, Xiong *et al.*, 2016). Xiong *et al.* (2016) reported 35% and 28% degradation of CBZ by *Chlamydomonas mexicana* and *Scenedesmus obliquus*, respectively, under laboratory conditions. However, a high CBZ concentration (200 mg L^{-1}) inhibited the growth of *S. obliquus* and *C. mexicana* by 97% and 30%, respectively. They also observed the formation of biodegradation products of CBZ (10, 11-dihydro-10, 11-expoxycarbamazepine and n-hydroxy-CBZ) by *C. mexicana*. In another study, Bai and Acharya (2017) evaluated the removal of sulfamethoxazole, CBZ, trimethoprim, ciprofloxacin, and triclosan by green alga *Nannochloris* sp. from lake water. It was observed that algae-mediated sorption contributed to the removal of 11% trimethoprim and sulfamethoxazole, 13% CBZ, and 27% triclosan. A study by Ding *et al*. (2020) explored the removal of individual and mixed PCs (CBZ, atenolol, IBP and naproxen) by *Navicula* sp. under controlled growth conditions. They found a higher removal rate for CBZ under individual treatment conditions as compared to the mixed treatment. However, the removal efficiency of atenolol (100%), IBP (96.74%) and naproxen (94.8%) were better in mixed conditions. The major PCs removal mechanisms by *Navicula* sp. were found to be through bioaccumulation with higher efficiency for CBZ (Ding *et al.*, 2020).

Steroid hormones have also been identified as biologically active in the aqueous environment and have been classified as Group 1 carcinogens (Sami and Fatma, 2019). Numerous laboratory-scale studies were conducted to evaluate the potential of various algal species for the removal of steroid hormones (Hom-Diaz *et al.*, 2015, Maes *et al.*, 2014, Peng *et al.*, 2014, Sami and Fatma, 2019). The removal mechanism of progesterone and norgestrel was studied by Peng *et al.* (2014) using *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in an aqueous solution during 5 days of cultivation. Both the species were able to degrade >95% of progesterone, while higher removal of norgestrel was observed in *S. obliquus* (95%) as compared to *C. pyrenoidosa* (60%). They also observed the removal of progesterone and norgestrel (<20%) through adsorption to dead algal biomass. A study by Sami and Fatma (2019) evaluated the potential of 16 different aquatic cyanobacterial species for the removal of estrone using standard growth media. The highest removal of estrone (53.7-94.5%) at 20 mg L^{-1} concentration was achieved using *Spirulina* CPCC-695. Their findings form a basis for the potential exploration of filamentous cyanobacterial species for PCs removal, which are known for their ease of harvesting.

Researchers have also evaluated the removal of antibiotics such as sulfamethoxazole (Ding *et al.*, 2020, Xiong *et al.*, 2019), clarithromycin, erythromycin (Escudero *et al.*, 2020), levofloxacin (Xiong *et al.*, 2017b), ciprofloxacin (Bai and Acharya, 2017, Gentili and Fick, 2017), cefradine (Chen *et al.*, 2015, Li *et al.*, 2015) and amoxicillin (Li *et al.*, 2015) by several algal species. Chen *et al.* (2015) compared the efficiency of *C. pyrenoidosa* for cefradine removal at different concentrations (10, 30 and 60 mg L^{-1}) in a sequence batch reactor. The inhibition rate of *C. pyrenoidosa* was determined at 24-hour intervals. Their results showed a slight increase in the population of *C. pyrenoidosa* after 24 hours of exposure to cefradine. However, at 48-96 hours of exposure and increasing the concentration of antibiotic significantly inhibited algal growth. It was observed that the antibiotic removal rate highly depends on the type of algae, its growth rate, the antibiotic concentration and type, environmental factors etc. (Leng *et al.*, 2020). This warrants future studies in understanding the possible mechanisms and interaction of different chemical, biological and environmental factors on algae-mediated PC removal.

The influence of biotic and abiotic factors on PCs removal and the formation of TPs in algae-mediated PCs removal has also been reported in different studies (Hom-Diaz *et al.*, 2015, Maes *et al.*, 2014). A study by Ismail *et al.* (2017) investigated the effect of continuous illumination on PCs (ketoprofen, paracetamol and aspirin) removal using a microalgal-bacterial consortium in a stirred-tank photobioreactor fed with standard growth medium. They found that applying continuous illumination achieved the complete removal of aspirin along with 20% and 80% removal of ketoprofen and paracetamol, respectively, during the short HRT of 3-4 days, even at high concentrations (0.5 mM) of each drug. Maes *et al.* (2014) investigated the effect of nutrients on the uptake, removal and biotransformation of 17α-ethinylestradiol (EE2) by *Desmodesmus subspicatus.* Their findings showed 68% of EE2 removal by *D. subspicatus* within 72 h of cultivation in M4 medium. They further noted that the presence of NaBr in the medium resulted in the formation of brominated analogues as a transformation product. However, no transformation product of EE2 was observed in the absence of NaBr. Based on these observations, they have concluded that the transformation of EE2 was mainly induced by an abiotic factor such as nutrients (in this case, it was NaBr). A later finding by Hom-Diaz *et al.* (2015) reported 88-100% and 71-100% removal of 17β-estradiol and 7α-ethinylestradiol, respectively using *Selenastrum capricornutum* and *Chlamydomonas reinhardtii* from anaerobic digester centrate. They also revealed the formation of several degradation products of 17β-estradiol and 17α-ethinylestradiol in the samples. However, only one transformation product

was found to be directly related to the degradation by *S. capricornutum*. Their observations indicated the possible influence of the prevailing abiotic factors and biotic factors on the formation of toxic TPs and their removal in the natural environment (Hom-Diaz *et al.*, 2015).

Nevertheless, recent studies have been conducted at pre-pilot and pilot-scale to evaluate the efficacy of PCs removal from wastewater using photobioreactors and HRAPs, which showed the potential influence of environmental factors on PCs removal (de Godos *et al.*, 2012, Hom-Diaz *et al.*, 2017). A study conducted by de Godos *et al.* (2012) demonstrated the efficient removal of tetracycline (>80%) from wastewater using a pilot-scale HRAP using a mixture of alga *C. vulgaris* and heterotrophic microorganisms. In this study, the main mechanisms responsible for tetracycline removal were presumed to be photodegradation and biosorption. In another study, Matamoros *et al.* (2015), demonstrated the removal of twenty-six pollutants, including PCs, by *Chlorella vulgaris* at pilot scale HRAPs using urban wastewater and achieved nearly 90% removal for caffeine, acetaminophen and IBP. They also observed that longer hydraulic retention time (HRT) assisted in better removal of PCs from wastewater during the winter season, whilst in the warmer season, a shorter HRT was found sufficient to achieve the same efficiency.

A study by Gentili and Fick (2017) investigated the removal efficiency of 79 types of PCs by a naturally grown consortium of wild strains of freshwater algae grown in urban wastewater in an open photobioreactor under outdoor conditions. The findings of their study showed varied removal rates for different PCs ranging from 10% - 90% by the indigenous algal consortium dominated by *Dictyosphaerium* sp. Similarly, Hom-Diaz *et al.* (2017) demonstrated the efficiency of an outdoor pilot-scale photobioreactor for the removal of anti-inflammatory drugs, IBP, acetaminophen, salicylic acid, and codeine from toilet wastewater (after primary settling) and achieved 98% treatment efficiency by alga *Chlamydomonas reinhardtii*. They have also demonstrated the role of environmental factors on PCs removal by microalgae. A better removal performance was reported in the warmer period compared to the winter season due to greater microbial activity at higher temperatures.

Literature suggests that different algal strains are capable of removing various PC compounds efficiently from an aqueous environment (Xiong *et al.*, 2016). However, despite the increased abundance of ARV drugs in the environment, there is limited information available on the exploitation of algae for ARV drugs removal from the environment (Table 2.6). Therefore, based on available studies on PCs removal by algae, chemical properties of ARV drugs and

ecotoxicological studies; the potential role and pathways of ARV drugs removal by algae are postulated and discussed in the following section.

Table 2.6 Remediation of pharmaceutical compounds (PCs) in aqueous medium by different algal species.

Algal species	Pharmaceutical compound	Compound type	Medium	Culture conditions			Removal (%)	Reference
				PP	LI	T		
Navicula sp.	Carbamazepine	Anticonvulsant	D1 medium	12:12	54	23	93.60	(Ding *et al.*, 2020)
	Sulfamethoxazole	Antibiotic	D1 medium	12:12	54	23	85.65	
	Ibuprofen	Anti-inflammatory	D1 medium	12:12	54	23	96.74	
	Naproxen	Anti-inflammatory	D1 medium	12:12	54	23	94.81	
Chlamydomonas acidophila	Clarithromycin	Antibiotic	Artificial wastewater	24	43	22	80	(Escudero *et al.*, 2020)
	Carbamazepine	Anticonvulsant	Artificial wastewater	24	43	22	N/A	
	Erythromycin	Antibiotic	Artificial wastewater	24	43	22	60	
	Cefradine	Antibiotic	BG-11	12:12	56	25	23	

Table 2.6 continued

Algal species	Pharmaceutical compound	Compound type	Medium	Culture conditions			Removal (%)	Reference
				PP	LI	T		
Scenedesmus obliquus	Sulfamethazine	Antibiotic	BBM	16:8	45-50	27	17.3	(Xiong *et al.*, 2019)
	Sulfamethoxazole	Antibiotic	Wastewater influent	16:8	45-50	27	29.3	
Scenedesmus obliquus	Levofloxacin	Antibiotic	BBM	16:8	50	27	10-92	(Xiong *et al.*, 2017b)
Scenedesmus obliquus	Carbamazepine	Anticonvulsant	BBM	16:8	50	27	28	(Xiong *et al.*, 2016)
Scenedesmus obliquus (strain CPCC 5)	Ibuprofen	Anti-inflammatory	BBM	16:8	90-160	22	60	(Larsen *et al.*, 2019)
	Ciprofloxacin	Antibiotic	F/2	24	2.02	20	100	(Bai and Acharya, 2017)

Table 2.6 continued

Algal species	**Pharmaceutical compound**	**Compound type**	**Medium**	**Culture conditions**			**Removal (%)**	**Reference**
				PP	LI	T		
Chlorella pyrenoidosa	Amoxicillin	Antibiotic	BG-11	12:12	56	25	77	(Chen *et al.*, 2015)
	Cefradine	Antibiotic	BG-11	12:12	56	25	23	(Li *et al.*, 2015)
Chlorella pyrenoidosa	Progesterone	Steroid hormone	BG-11	12:12	42	25	95	(Peng *et al.*, 2014)
Chlorella sorokiniana	Norgestrel	Steroid hormone	BG-11	12:12	42	25	40	(de Wilt *et al.*, 2016)
	Diclofenac	Anti-inflammatory	M8a	N/A	68	35	40-60	
	Ibuprofen	Anti-inflammatory	M8a	N/A	68	35	100	
	Paracetamol	Painkiller	M8a	N/A	68	35	100	

Table 2.6 continued

Algal species	**Pharmaceutical compound**	**Compound type**	**Medium**	**Culture conditions**			**Removal (%)**	**Reference**
				PP	LI	T		
Carbamazepine	Anticonvulsant	M8a	N/A	68	35		30-37	(de Wilt *et al.*, 2016)
17α-ethynylestradiol	Hormone	P49	N/A	172	25		60-95	
Chlorella sp.	7-amino cephalosporanic acid	Antibiotic	BG-11	N/A	200	26	100	(Guo *et al.*, 2016)
Chlorella vulgaris (strain CPCC 90)	Ibuprofen	Anti-inflammatory	BBM	16:8	90-160	22	60	(Larsen *et al*., 2019)
Desmodesmus subspicatus	Estrogen	Steroid hormone	M4	N/A	70	20	N/A	(Maes *et al.*, 2014)
Chlamydomonas reinhardtii	Estradiol	Steroid hormone	P49	N/A	172	25	88-100	(Hom-Diaz *et al.*, 2015)

Table 2.6 continued

Algal species	**Pharmaceutical compound**	**Compound type**	**Medium**	**Culture conditions**			**Removal (%)**	**Reference**
				PP	LI	T		
Microalgae	Sulfamethoxazole	Antibiotic	Domestic wastewater		Sunlight	20.7	85.3	(García-Galán *et al.*, 2020)
Microalgae	Metronidazole	Antibiotic	Domestic wastewater		Sunlight	20.7	>89	(García-Galán *et al.*, 2020)
Mixture dominated by *Chlorella* sp., diatoms and *Stigeoclonium* sp.	Carbamazepine and Triclosan	Anticonvulsant, Antibacterial and antifungal	Urban wastewater	N/A	1288.7 (Summer); 338.2 (Winter)	N/A	59-84	(Matamoros *et al.*, 2015)
Arthrospira and *Spirulina* species	Estrone	Steroid hormone	Zarrouk's and BG-11	12:12	25	27	53.7-94.5	(Sami and Fatma, 2019)
Selenastrum capricornutum	17β-estradiol	Steroid hormone	BG-11	12:12	24.8	25	91	(Wu *et al.*, 2021)

Table 2.6 continued

Algal species	**Pharmaceutical compound**	**Compound type**	**Medium**	**Culture conditions**			**Removal (%)**	**Reference**
				PP	LI	T		
Chlamydomonas mexicana	Carbamazepine	Anticonvulsant	BBM	16:8	50	27	35	(Xiong *et al.*, 2016)
Dictyosphaerium	Carbamazepine, Ciprofloxacin, Trimethoprim, Metoprolol and Tramadol	Anticonvulsant, Antibiotic, Cardioselective beta-blocker, Analgesic	Wastewater influent	N/A	293.5-500.4	10 – 32	>90	(Gentili and Fick, 2017)
Synechocystis sp.	Sulfathiazole	Antibiotic	BG-11	-	Sunlight	24	100	(Vassalle *et al.*, 2020)
Synechocystis sp.	Trimethoprim	Antibiotic	BG-11	-	Sunlight	24	78	(Vassalle *et al.*, 2020)

* PP-Photoperiod, light-dark cycle (Hours); LI-Light intensity (µmol photon m^{-2} s^{-1}); T-Temperature (˚C); BBM-Bold's Basal Medium; F/2- nutrient medium; N/A-Not available

2.6.2 Mechanisms of ARV drug removal by algae

The capability of algae to remove PCs from wastewater is well described in the literature. The literature shows that algae can tolerate elevated concentrations of ARV drugs in the aquatic environment (Table 2.6), which are greater than the reported concentrations in the aquatic environment (Table 2.3). Even though tolerance to ARV drugs cannot directly indicate their potential for the biotransformation of these PCs, it can still be used as a basis for understanding their ability to deal with stress and survive in an environment having ARV drugs. Additionally, algae have revealed potential for eliminating a wide array of PCs in the aquatic environment (Table 2.6). The active ingredients found in ARV drugs are comparable to the structure of normally studied PCs (Fortunak *et al.*, 2014). Hence, it can be suggested that algae could be capable of removing ARV drugs from the aqueous environment, dependent on their chemical constituents. Antiretroviral drug removal mechanisms by algae can be described based on the known mechanisms of PC removal, which include bioadsorption, biodegradation and bioaccumulation (Figure 2.5).

A. Adsorption

Algae possess the capability to serve as a biosorbent for a variety of environmental contaminants. This is attributed to the composition of the cell wall, which includes cellulose, alginate, chitin, and glycan, thereby offering crucial sorption sites (de Wilt *et al.*, 2016, Flemming and Wingender, 2010, Gadd, 2009, Wang *et al.*, 2017). The algal cell wall carries a negative charge primarily as a result of prevalent functional groups like carboxyl, phosphoryl, and amine. This enables electrostatic interactions between the microalgae cells and pharmaceutical contaminants that carry positive charges, as noted by Hena *et al.*, 2021. Additionally, these organisms possess assemblages of polymers resembling cellulose, hemicellulose, and lignin, as observed in the study by Peng et al. (2014). Because adsorption occurs externally, the mechanism can exhibit variability dependent on factors like drug hydrophobicity, structural attributes, the presence of functional groups, and the specific algal species involved (Ghernaout, 2014). The process of bioadsorption is pH-dependent, where the initial pH of the solution influences the surface charge of microalgae, thereby affecting the efficiency of pharmaceutical contaminant removal (Daneshvar *et al.* 2018). It has been reported that bioadsorption is more effective with PCs containing cationic groups that are

actively attracted towards the algal surface through electrostatic interactions (Ayangbenro and Babalola, 2017). Therefore, it can be presumed that cationic ARV drugs would have a high affinity for negatively charged algal cell wall. Moreover, microalgae cells release various extracellular polymeric substances into their surrounding environment, consisting of polysaccharides, proteins, nucleic acids, enzymes and lipids (Xiong *et al*., 2018). These extracellular polymeric substances, both bonded to microalgae cells and present in suspension, contribute available binding sites for PCs through mechanisms such as adsorption, chelation, ion exchange, micro-precipitation, and complexation (Hena *et al.*, 2021, Xiong *et al.*, 2021). Xie *et al.* (2020) showed that extracellular polymeric substances (EPS) from microalgae played a crucial role in bioadsorption, with the highest absorption of ciprofloxacin by EPS equivalent to the overall bioadsorption capacity. The impact of bioadsorption to the removal of PCs is influenced by the intrinsic properties of both the contaminants and microalgae species involved. The effectiveness of bioadsorption varies based on the functional groups, structure and hydrophobicity of different PCs (Xiong *et al.*, 2018). For instance, Xie *et al.* (2020) noticed that extracellular polymeric substances from *Chlamydomonas* sp. Tai-03 exhibited a 10-fold higher biosorption capacity for ciprofloxacin compared to sulfadiazine. Furthermore, *S. quadricauda* displayed a higher adsorption capacity (295.3 mg g^{-1}) for tetracycline compared to *T. suecica*, where the greatest adsorption ability of tetracycline after lipid extraction treatment was only 56.3 mg g^{-1} (Daneshvar *et al.*, 2018). Additionally, the efficiency of bioadsorption is influenced by factors such as contact time, concentration of PCs, and microalgae concentration. The bioadsorption capacity of *S. quadricauda* showed a linear increase with the initial concentration of tetracycline, ranging from 2.5 to 300 mg L^{-1}. The bioadsorption rate escalated during the first 60 minutes and then reached equilibrium within the subsequent 120 minutes (Daneshvar *et al.*, 2018). However, the bioadsorption of estrogen by *S. dimorphus*, even after 8 days, contributed only minimally (less than 10%) to the deduction of estrogen (up to 90%) (Zhang *et al.*, 2014). Therefore, for certain PCs, bioadsorption may not be the primary process responsible for their removal, while other mechanisms play a more significant role.

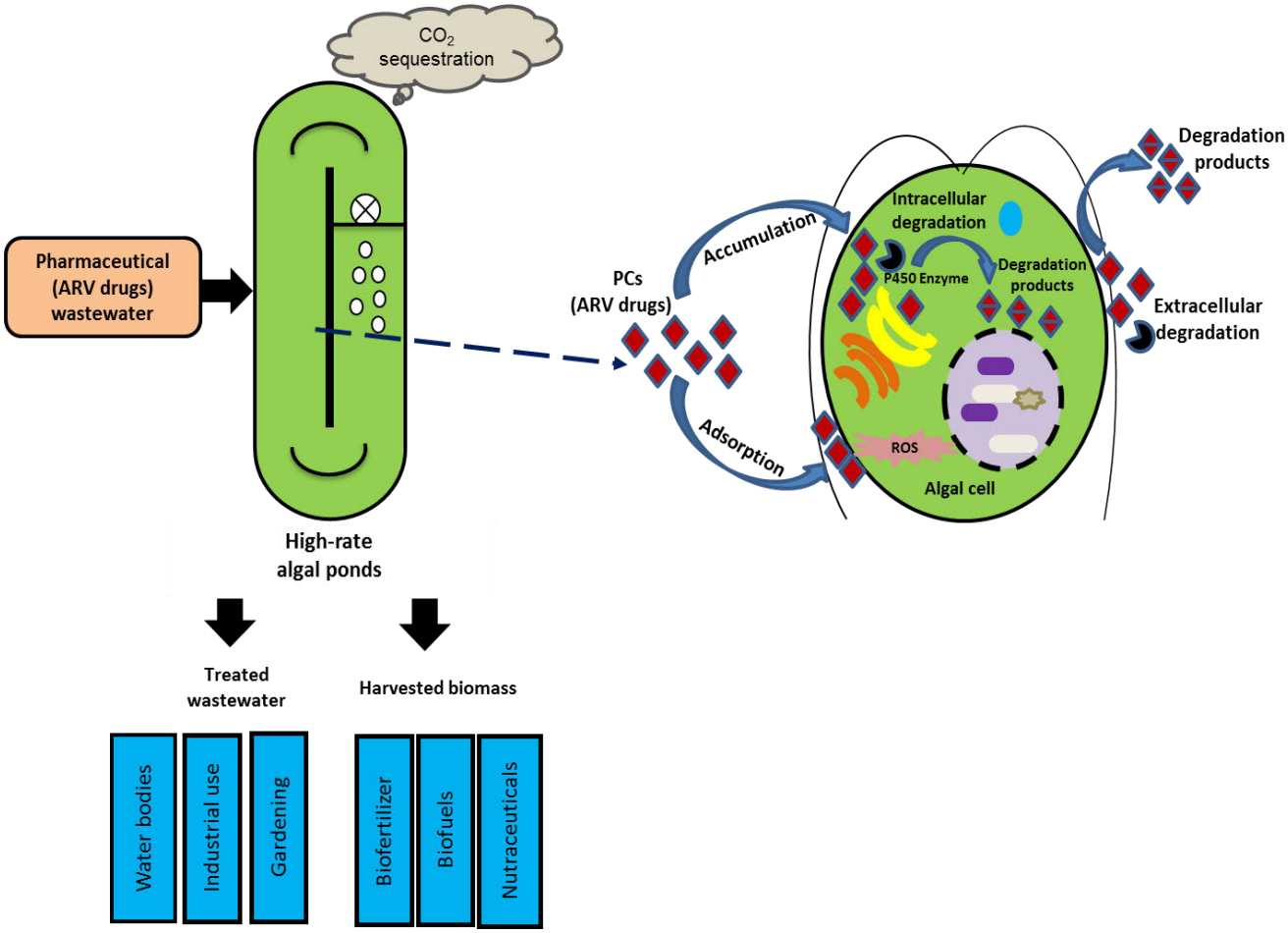


Figure 2.5 Proposed mechanisms and benefits of algae-mediated ARV drugs removal from wastewater.

B. Bioaccumulation

Bioaccumulation is an effective process that requires energy for algae to uptake PCs from aquatic conditions through adsorption within microalgal cells, as stated by Leng *et al.* (2020). Some PCs have the capability to pass within microalgal cell layers, enabling their assimilation by the cells (Leng *et al.*, 2020). There are three main routes for the transportation of PCs into the interior of microalgal cells: (1) passive diffusion, where pollutants with low molecular weight, particularly non-polar and lipid-soluble, move from external regions of high concentration to lower internal concentration; (2) passive-facilitated diffusion, which involves transporter proteins; and (3) active uptake, where pollutants move against a concentration gradient using energy (Xiong *et al.*, 2021). Previous studies have detected the presence of

various PCs, such as florfenicol, triclosan, carbamazepine, and levofloxacin, in microalgal cell extracts (Song *et al.*, 2019, Xiong *et al.*, 2017b, Xiong *et al.*, 2018). Xiong *et al.* (2017b) reported that levofloxacin bioaccumulated in *C. vulgaris* cells and achieved 101 μg g^{-1} biomass by 11 days of cultivation with the supplementation of 1% NaCl. The bioaccumulation of certain PCs in microalgae cells may be enhanced when they co-exist with other PCs. *Navicula* sp. cells were exposed to various pharmaceuticals and the intracellular components of bezafibrate and atenolol at 216 hours were 35.7 μg g^{-1} and 114.2 μg g^{-1}, respectively, which were higher than the levels observed in individual treatments (1.8 μg g^{-1} for bezafibrate and 0 μg g^{-1} for atenolol) (Ding *et al.*, 2020).

Additionally, the n-octanol/water partition coefficient (K_{ow}) value of any chemical (drug) is an imperative parameter to determine their bioaccumulation potential and toxicity, which assists in determining their fate in the environment and/or inside living organisms (Harris and Logan, 2014, Hermens *et al.*, 2013). K_{ow} value is also a key parameter to determine the hydrophilicity or lipophilicity of the compounds and is defined as an equilibrium ratio of the concentration of the chemical compound in a two-phased system of n-octanol and water (Cumming and Rücker, 2017).

$$\boldsymbol{K_{ow} = \frac{C_O{}^{equilibrium}}{C_w{}^{equilibrium}}}$$

Equation 2.1 The n-octanol/water partition coefficient (Kow) value.

In equation 2.1, $C_O{}^{equilibrium}$ is the concentration of the chemical compound in n-octanol under equilibrium conditions, while $C_w{}^{equilibrium}$ is the concentration of the chemical compound in water in the equilibrium.

Generally, the K_{ow} values of organic compounds are found to be of several orders of magnitude, therefore, the decadic logarithm of $K_{ow,}$ i.e. log K_{ow} values are considered to represent the characteristics of these compounds (Cumming and Rücker, 2017). Log K_{ow} values of organic compounds typically range between -3 (water-soluble) and +10 (water-insoluble) and are also used to determine the accumulative potential of the compounds (Dimitrov *et al.*, 2019). According to Dimitrov *et al.* (2019), compounds with log K_{ow} values lower than 4.5 are not bioaccumulative. However, Ding *et al.* (2020) reported that in algae, the bioaccumulation potential was dependent on the functional group of PCs. They further revealed that the bioaccumulation potential of PCs (atenolol, CBZ, IBP, sulfamethoxazole, bezafibrate and naproxen individually and in combination) in freshwater diatom *Navicula* sp. increased with the increase in log K_{ow} values in PCs with amino groups, while an opposite trend was observed for the carboxylic group-containing PCs (Ding *et al.*, 2020).

Most of the ARV drugs are found to possess an amino group in their cyclic ring structure (Fig 2.1) and have different log K_{ow} values (Table 2.7). Therefore, the trend of ARV drug removal by algae can also be expected to increase with the increase in log K_{ow} values for ARV drugs. Bioaccumulation of PCs in algal cells can induce the generation of reactive oxygen species (ROS) (Xiong *et al.*, 2018). Reactive oxygen species generation at normal levels acts as a signalling molecule to regulate cellular metabolism under normal and stressed conditions. Algal species can become more resistant to PCs at low environmental concentrations through the generation of ROS. However, at higher levels, ROS could cause damage to cellular components (lipids, proteins, and DNA) and affect PC removal efficiency (Kumar *et al.*, 2016, Xiong *et al.*, 2018). Monitoring of ROS generation through the expression of related genes could therefore assist in understanding the algal response to increased PCs level (Xiong *et al.*, 2018).

Table 2.7 Water Partition Coefficient (K_{ow}) values and solubility of different ARV drugs.

Antiretroviral drug	Log K_{ow}	Solubility in water
Zidovudine	0.05	10-50
Lamivudine	-9.54	70
Didanosine	-1.24	10-50
Stavudine	-0.72	50-100
Abacavir	1.2	77
Tenofovir	-	13.4
Zalcitabine	-1.30	50-100
Efavirenz	4.7	0.00855
Nevirapine	3.89	0.7046
Ritonavir	6.27	Insoluble
Indinavir	2.92	Very soluble
Lopinavir	5.94	7.7E-06
Enfuvirtide	-	Negligible solubility
Raltegravir	0.40	53.6
Elvitegravir	-	0.0003

*All information obtained from https://www.ncbi.nlm.nih.gov (accessed September 2023).

While bioaccumulation is recognized as a significant pathway for the elimination of PCs, the accumulated contaminants can promote the production of reactive oxygen species, which can inhibit the growth of microalgae. Therefore, the bioaccumulation of PCs in microalgae can serve as an guide in evaluating ecological hazards (Abdelfattah *et al.*, 2023).

C. Biodegradation

An alternative mechanism of PC removal by algae involves intracellular and extracellular biodegradation (Xiong *et al.*, 2019). Biodegradation involves the breakdown and transformation of PCs into simpler intermediates or final products (CO_2 and H_2O) through enzymatic reactions and the involvement of extracellular polymeric substances (Mojiri *et al.*, 2022, Xie *et al.*, 2020). Biodegradation of PCs and the formation of different TPs have been reported in algae-based treatment processes. Simpler molecules are formed through catalysis of the complex parent compound by algae (Peng *et al.*, 2014). The detailed mechanisms of PC removal through intracellular and extracellular degradation by algal systems have been reviewed and illustrated by Xiong *et al.* (2018). Intracellular degradation is generally achieved through enzymatic reactions (chlorination, chlorine substitution, dehydration, hydroxylation, side-chain breakdown, and ring cleavage), whilst exopolymeric substances (EPS) play an important role in the extracellular degradation of PCs (Zhou *et al.*, 2021, Xie *et al.*, 2020). Algae are reported to possess phase I and phase II enzyme families, which play an essential role in biodegradation and biotransformation of chemical compounds and coping with oxidative stress. The phase I family of enzymes (cytochrome P450) adds or exposes a hydroxyl group by hydrolysis, oxidation, or reduction reactions, which makes the compounds more hydrophobic (Torres *et al.*, 2008). Different types of enzymatic reactions for algae-mediated biodegradation/biotransformation of chemical contaminants include hydroxylation, carboxylation, oxidation, hydrogenation, glycosylation, demethylation, ring cleavage, decarboxylation, dehydroxylation, and bromination (Xiong *et al.*, 2018). Possible reactions and the proposed metabolism of ARV drugs in algae by enzyme cytochrome P450 are presented in Figure 2.6. While, the phase II family of enzymes (glutathione-S-transferase) helps to protect against oxidative damage (Ding *et al.*, 2017a). In algae, these enzymes have been reported to involve in cell responses such as biotransformation of endogenous organic compounds (Wang *et al.*, 2004, Foflonker *et al.*, 2016). Song *et al.* (2019) observed that biodegradation was the sole pathway for *Chlorella* sp. L38 to eliminate florfenicol when the initial concentration was 46 mg L^{-1}. Similarly, *Chlorella* sp. contributed approximately 97% to the total removal efficiency of thiamphenicol at an initial concentration of 46.2 mg L^{-1} (Song *et al.*, 2020). Alternatively, extracellular degradation is associated with the extracellular polymeric substances secreted by microalgae. These substances form a hydrated biofilm matrix and act as an external excretory system, where enzymes (extracellular) found within them metabolize or mineralize pharmaceutical contaminants (Song *et al.*, 2020). Furthermore,

EPS function as surfactants and emulsifiers, enhancing the bioavailability of PCs and contributing to their detoxification (Xiao and Zheng, 2016). Biosorption plays a important part in extracellular biodegradation, where PCs are initially trapped in the EPS on the surface of the algae walls through adsorption, followed by degradation by extracellular enzymes present in the EPS (Abdelfattah *et al.*, 2023, Xie *et al.*, 2020).

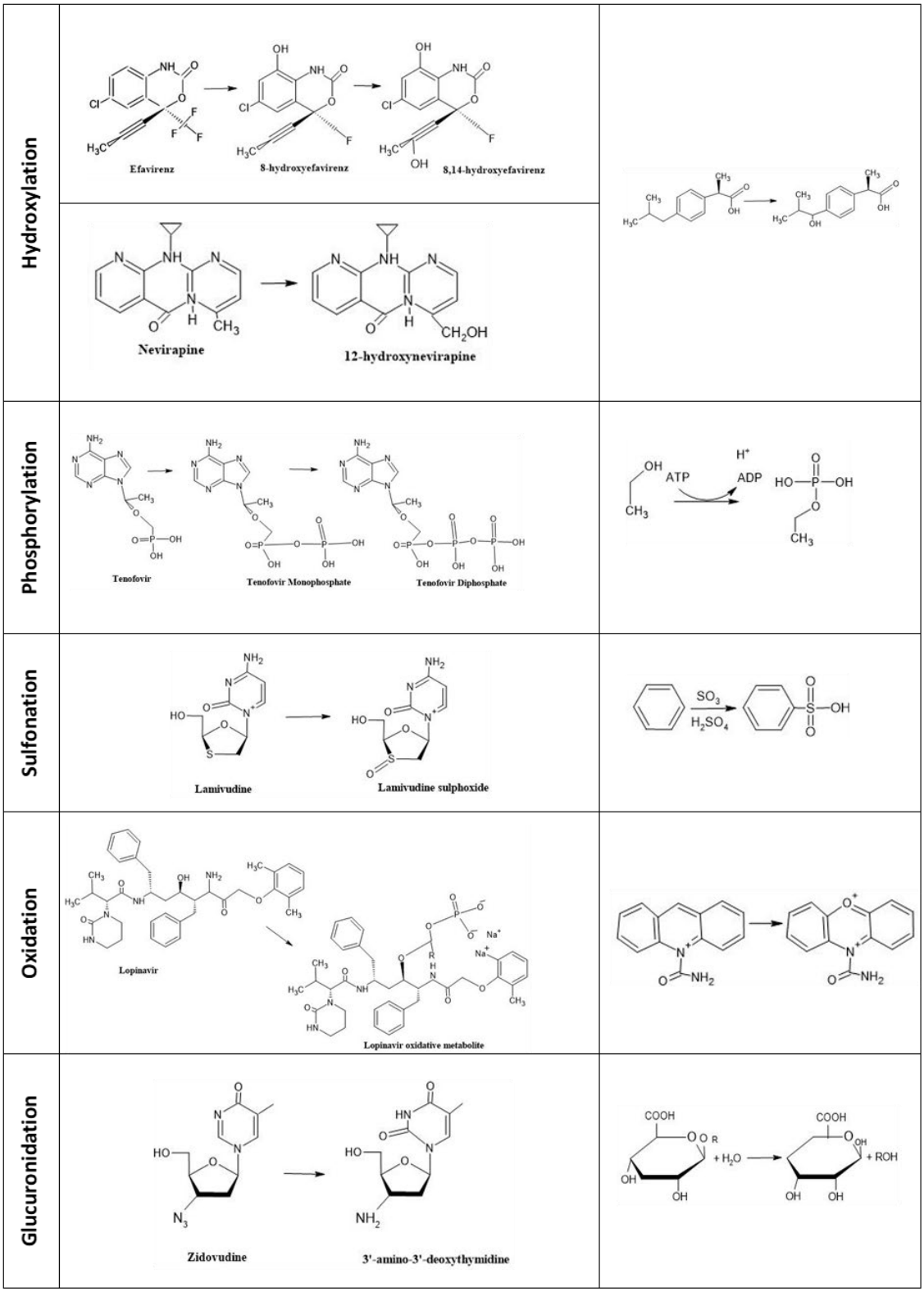


Figure 2.6 Possible reactions involving the metabolism of ARV drugs in algae by enzyme cytochrome P450 (Modified from Xiong *et al*., 2018).

Since these are the common mechanisms in algae for the removal of chemical contaminants and alleviation of oxidative stress, therefore, similar mechanisms can be postulated for algae-mediated ARV drugs removal. However, the metabolic potential as well as uptake kinetics for the removal of chemical contaminants, could vary in different algal species (Brain *et al.*, 2008). Moreover, limited information on this critical aspect necessitates future studies to explore the potential of algal systems in ARV drug removal in the environment, and to unravel possible mechanisms and metabolic responses of algal cells.

D. Photodegradation

The photodegradation of PCs involves both direct and indirect photolysis processes (Xiao and Zheng, 2016). A number of PCs present in the environment, such as tetracycline, are affected by sunlight, as the energy from absorbed photons breaks the chemical bonds of these contaminants. Indirect photodegradation also occurs, where PCs are eliminated or changed through the action of peroxyl radicals, free radicals and singlet oxygen produced during algal growth (Tian *et al.*, 2019). A study by Zhang *et al.* (2012) reported that microalgae act as photosensitizers in the photodegradation of norfloxacin. Generally, indirect photodegradation is quicker than direct photodegradation and contributes to the photolytic conversion of PCs (Xiong *et al.*, 2019). Various components in microalgae cultures impact indirect photodegradation. Tian *et al.* (2019) confirmed that extracellular organic matters produced by *C. vulgaris* were the major active substances that accelerate the photodegradation rate of chlortetracycline. Additionally, enzymes and chlorophyll act as photosensitizers, stimulating indirect biodegradation of PCs (Tian *et al.*, 2019). Humic constituents in microalgae also play a significant role in influencing the photodegradation of PCs (Tian *et al.*, 2019). These substances within microalgae cultures often work together to promote the indirect biodegradation of PCs. Several factors influence microalgae-induced photodegradation, including microalgae species, microalgae concentrations, type of PCs, light source and pH value (Wei *et al.*, 2021).

Even though the photodegradation activity of microalgae cultures for the removal of certain PCs are promising, the photodegradation products may exhibit higher toxicity to living organisms as when compared to the parent compounds. Chronic and acute toxicity studies have shown that photoproducts from naproxen demonstrate greater toxicity to the investigated

aquatic organisms (*B. calyciflorus*, *C. dubia*, and *T. platyurus*) (Fatta-Kassinos et al., 2011; Isidori *et al.*, 2005). Therefore, when applying photodegradation techniques, it is crucial to consider the characteristics of PCs to improve removal efficiency and prevent secondary pollution caused by photoproducts.

2.6.3 Cellular level response of microalgal cells to pharmaceuticals

Microalgae, as primary producers in aquatic ecosystems, face various environmental stressors that can lead to the creation of reactive oxygen species (ROS) (Vignaud *et al.*, 2023). When microalgae experience elevated levels of ROS, it can lead to oxidative stress, causing damage to crucial cellular components (Hamed *et al.*, 2017). However, algae have developed enzymatic antioxidant defence systems as a protective mechanism against ROS-induced damage. The antioxidant enzyme system in algae plays a crucial role in their adaptation and survival under changing environmental conditions (Rezayian *et al.*, 2019). By scavenging ROS, these enzymes protect vital cellular components, including lipids, proteins, and nucleic acids, from oxidative modification. The upregulation of antioxidant enzyme activity in response to stressors helps maintain cellular homeostasis and ensures the integrity of cellular functions, such as photosynthesis and respiration (Zandi and Schnug, 2022). Under normal physiological conditions, basal levels of antioxidant enzyme activity are maintained. However, exposure to PCs, high light intensity, heavy metals, temperature fluctuations, and nutrient imbalances can trigger an upregulation of enzyme activity (Nowicka, 2022). This adaptive response allows algae to cope with increased ROS production and minimize oxidative damage. The enzymatic antioxidant defence system in algae comprises various enzymes that play specific roles in scavenging and neutralizing ROS. These enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and ascorbate peroxidase (APX) (Singh *et al.*, 2021). These enzymes work together to detoxify ROS and prevent oxidative damage within the algal cells. Table 2.8 provides an overview of the enzymatic defence mechanisms employed by algae, highlighting the specific functions of these antioxidant enzymes.

In addition to the enzymatic defence system, microalgae also produce non-enzymatic antioxidants that work in conjunction with the enzymatic system to combat oxidative stress (Zandi and Schnug, 2022). Examples of non-enzymatic antioxidants synthesized by

microalgae include glutathione, ascorbate (vitamin C), and tocopherols (vitamin E) (Coulombier *et al.*, 2021). These non-enzymatic antioxidants act as electron donors and can directly scavenge and neutralize ROS, further enhancing the algae's defence against oxidative stress (Rezayian *et al.*, 2019). It is worth noting that microalgae possess the ability to respond to pollutants or environmental factors by upregulating the expression of antioxidant enzymes (Mojiri *et al.*, 2022). This adaptive response helps to increase the algae's defence mechanisms and minimize oxidative damage under stressful conditions. By activating their enzymatic antioxidant defence system, microalgae can enhance their survival and adaptability in a wide range of environmental conditions (Gauthier *et al.*, 2020). Overall, the enzymatic antioxidant defence system is a critical component of algae's response to oxidative stress. It enables them to counteract the harmful effects of ROS and maintain cellular integrity. The intricate interplay between enzymatic and non-enzymatic antioxidants provides microalgae with robust protection against oxidative stress, ensuring their ability to thrive in diverse ecological settings.

Table 2.8 The antioxidant enzymes and activity in algae.

Antioxidant enzyme	**Defence mechanism**	**Reference**
Superoxide dismutase	An SOD enzyme catalyzes the dismutation of superoxide radicals into O_2 and H_2O_2 as a first line of defence against oxidative stress.	(Rezayian *et al.*, 2019)
Catalase	The CAT enzyme is a tetrameric enzyme containing heme that converts H_2O_2 into H_2O and O_2.	(Janknegt *et al.*, 2007)
Glutathione peroxidase	The GPX enzyme decomposes H_2O_2 to H_2O.	
Ascorbate peroxidase	The APX is a heme enzyme that converts H_2O_2 to H_2O through electron transfer from ascorbate.	(Coulombier *et al.*, 2021)

H_2O_2 – hydrogen peroxidase; H_2O – Water; O_2 – Oxygen

Different algal species exhibit variations in their antioxidant enzyme systems, reflecting their adaptation to specific ecological niches and environmental conditions (Shilpi *et al.*, 2015). Genetic diversity in antioxidant enzymes contributes to the resilience and survival of algae in diverse habitats. Studies have identified specific isoforms and variants of antioxidant enzymes

in different algal species, highlighting the importance of enzyme diversity in coping with varying levels of oxidative stress. Understanding the antioxidant enzyme systems in algae, their activity modulation, and genetic diversity provides valuable insights into the mechanisms underlying algal adaptation and survival (Rajput *et al.*, 2021). In recent years, metabolomics has been instrumental in examining how algae cells react to contaminants present in wastewater (Nagarajan *et al*., 2022). Metabolites consist of cellular by-products produced through a range of biochemical reactions and are regulated by the activity of proteins and the expression of genes within an organism (Mo *et al*., 2023). These metabolites cover a wide array of molecules, each possessing its distinct function and importance. Within this group, primary metabolites such as sugars, amino acids, organic acids, and nucleotides serve as essential building blocks for crucial cellular processes (Maeda, 2019). Their levels reflect the final responses of biological systems to genetic or environmental alterations. Further research in this field can contribute to our understanding of the ecological resilience of algae and aid in the development of strategies to mitigate the impacts of pharmaceutical stress on algal communities.

2.6.4 Factors affecting algae-mediated pharmaceutical wastewater treatment

An algae-mediated wastewater treatment system can be affected by several factors. Some of the key factors are:

A. Light intensity

In order to grow and metabolically function, algae need sunlight for photosynthesis. Growth and activity of algae can be affected by light intensity and duration (Hena *et al*., 2021). Exposure to excessively high illumination levels has a detrimental effect on the photosystem II of chloroplasts in microalgae. This damage leads to a decrease in the metabolic activities of microalgae, resulting in reduced efficiency in the removal of pharmaceutical and personal care products (PPCPs) (Maltsev *et al*., 2021). Although the damaged photosystem II can be repaired over time, it takes longer for the cells to reach their exponential growth phase (Bashir *et al*., 2021). Consequently, this delay reduces the uptake efficiency of nutrients and the removal of micropollutants. It has been observed that the highest removal of nutrients and organic compounds occurs during the exponential growth phase of microalgae cells. Regarding removing PPCPs, a higher biomass of microalgae promote biodegradation and

sorption (Young *et al*., 2022). However, the mechanism of photolysis follows a slightly different pattern. Zhang *et al*. (2012), demonstrated a correlation between the biomass of *Chlorella vulgaris* and the photolysis of norfloxacin. They found that the photodegradation increased from 35.9% to 40.9% when the cell count increased from 1.7 × 106 cell/L to 1.7 × 107 cell/L within a 60-minute period. This increase in photodegradation is attributed to the production of hydroxyl radicals as photosensitizers by dissolved organic matter excreted by microalgae under illumination. However, a further increase in cell counts leads to self-shading effects, where the algal cells block the penetration of light, resulting in decreased photodegradation.

B. pH

The pH level of the solution is an essential factor that significantly influences the interactions between the adsorbent and adsorbate (Filali *et al*., 2021, Ahmed *et al*., 2015). It exerts its effects by affecting both the charge of algal cell surfaces and the speciation and ionization of pharmaceuticals. In a previous study conducted by de Godos *et al*. (2012), it was observed that controlling the pH within a range of 7.5 to 8 during cultivation has a profound impact. Specifically, this pH range facilitates the formation of tetracycline zwitterions, which are electrically neutral molecules with both positive and negative charges. The presence of these zwitterions enhances their affinity for adsorption onto algal surfaces. Therefore, maintaining a cultivation pH within the specified range is crucial for promoting the effective adsorption of pharmaceuticals onto algal surfaces.

C. Temperature

Microalgae's metabolic process and enzymatic activity are regulated by temperature. Algae are sensitive to temperature changes, and their growth and metabolism can be affected by variations in temperature. The optimal temperature for microalgae is reported in the range of 25–30 °C (Bamba *et al*., 2015). Elevated temperatures beyond the optimal range could have a detrimental impact on the chlorophyll-a content leading to a decrease in the photosynthesis process and ultimately affecting their growth (Singh and Singh, 2015). Consequently, high temperatures are not conducive to the biodegradation and sorption of PCs (Zhang *et al*., 2012).

However, it should be noted that high temperatures do promote indirect photolysis to some degree. For example, microalgae cells suffer injury when exposed to high temperatures, such as those exceeding 30 ˚C (Béchet *et al*., 2017) and as a result, they release low molecular organic acids into the surrounding culture media, which facilitate the indirect degradation of PCs through photolysis.

D. Hydraulic retention time (HRT)

A longer HRT allows for more efficient removal of pollutants by algae, because wastewater spends more time in the treatment system (Hena *et al*., 2021). Conversely, a longer HRT results in increased microalgae biomass through cell division, enhancing the potential for biodegradation and sorption of micropollutants by a larger number of cells. Matamoros *et al*. (2015), proposed that during warm or summer seasons, the HRT does not exert a significant influence. However, a notable disparity in the removal of PCs was observed during colder seasons when temperature was considered a limiting factor. A four-day HRT is deemed sufficient for the elimination of a wide range of PCs in HRAP. This HRT duration is comparatively longer than that employed by conventional WWTPs, which typically operate within a range of 12 to 24 hours (Hena *et al*., 2021).

E. Algal species

The selection of the appropriate microalgal strain is a crucial step in wastewater treatment, as it depends on the specific PCs present. The removal of certain PCs by microalgae is specific to the compound, while for others, it is specific to the species of microalgae (Li *et al*., 2022). Antibiotics, for example, generally exhibit compound-specific removal regardless of the microalgae used for phycoremediation. For instance, 7-amino cephalosporanic acid can be completely removed by different microalgae, such as *Chlorella* sp., *Chlamydomonas* sp., and *Mychonastes* sp. Different microalgae employ similar mechanisms (hydrolysis, photolysis, and adsorption) to remove 7-amino cephalosporanic acid (Guo *et al*., 2016). Similarly, diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), showed compound-specific behaviour with different species of *Chlorella* sp. Two different *Chlorella* species, namely *C. sorokiniana* and *C. vulgaris*, have demonstrated nearly equal removal rates of diclofenac (29%

and 21%, respectively) within a 9-day period (Escapa *et al*., 2016). On the other hand, species-specific compounds exhibit varying removal efficiencies with different microalgae. For example, the green microalgae *Nannochloris* sp. reportedly can completely remove triclosan (Escapa *et al*., 2016), while the cyanobacterium Microcystis aeruginosa only removed 46% from aqueous media within 7-8 days (Huang *et al*., 2016). Diclofenac also demonstrated species-specific behaviour with *Chlorella* sp. and *Scenedesmus obliquus*, showing removal rates of 21-29% and 79% within the same time frame (Escapa *et al*., 2016). The careful selection of the appropriate microalgal strain plays a vital role in achieving effective PC removal during wastewater treatment. This choice not only depends on the specific PC in question but also on the unique characteristics of the microalgal species employed. Such precision in selecting the ideal combination of PC and microalgal strain is fundamental in harnessing the full potential of algae-based systems for environmental remediation and wastewater treatment.

CHAPTER THREE: Isolation and screening of algal strains from wastewater treatment plants

3.1 Introduction

Microalgae are known for their rapid growth rates and high biomass production (Khan et al., 2018), which facilitates large-scale cultivation and efficient removal of PCs from wastewater, as larger algal biomass leads to increased adsorption and uptake capacity (Goh et al., 2023). Additionally, microalgae have the ability to assimilate and utilize nutrients present in wastewater, such as nitrogen and phosphorus (Abdel-Raouf et al., 2012). Microalgae, either as single cultures or mixed consortia, have gained significant attention as promising candidates for the removal of PCs from various wastewater sources (Abdelfattah *et al*., 2023). Their distinctive traits and metabolic capacities provide a sustainable and environmentally friendly approach of bioremediation (Silva *et al*., 2019). They have a high surface area-to-volume ratio, allowing for efficient adsorption of PCs onto their cell surfaces (Abdelfattah *et al*., 2023). Additionally, they possess transport mechanisms that enable the uptake and accumulation of various contaminants, including PCs, within their cells (Danouche *et al*., 2021). They also possess a wide range of metabolic pathways, including enzymatic systems, that enable the breakdown and transformation of diverse organic compounds (Amaro *et al*., 2023). This versatility allows them to metabolize and degrade PCs, thereby reducing their environmental persistence.

The selection of microalgae strains for PC removal depends on several factors, including their tolerance, growth rate, and specific metabolic capabilities (Hena *et al*., 2021). In the context of wastewater treatment, using microalgae strains specifically adapted to wastewater conditions provides certain advantages over freshwater strains (Sanchez Rizza *et al*., 2017, Ahiahonu *et al*., 2021). Microalgae strains isolated from wastewater environments are naturally adapted to the unique challenges and conditions present in wastewater. These strains have been exposed to and have developed tolerance to various pollutants, including PCs, making them more effective in removing these contaminants compared to freshwater strains. This chapter presents work that was aimed at isolating indigenous microalgal strains from wastewater treatment plants in KwaZulu-Natal, South Africa. Isolated strains were identified morphologically and measured for their growth rate and biomass content. Molecular techniques were employed for further identification of the selected microalgal strains. In

addition, microalgae were subjected to growth inhibition and viability tests in order to assess the toxicological concentration of NVP in the growth medium.

3.2 Materials and methods

3.2.1 Collection and processing of wastewater samples

Wastewater samples were collected from three WWTPs located in Durban, KwaZulu-Natal, South Africa, as indicated in Table 3.1. Samples were taken from three specific points within each WWTP: the raw influent, aeration tank, and pre-chlorinated effluent compartment. The sampling sites were characterized by assessing key physicochemical parameters. Parameters such as temperature, dissolved oxygen (DO), and pH were measured using a portable Yellow Springs Instrument (YSI 556 Multiprobe System). The wastewater samples were collected in 1 L autoclaved glass bottles and were securely capped to prevent sample leakage during transportation. The samples were promptly transported to the laboratory in a cooler box to maintain sample integrity.

Table 3.1 Wastewater treatment plants selected for the study.

WWTP location	**Sewage source**	**Treatment technology**
Isipingo	Domestic	Activated sludge (Conventional), trickling filter, anaerobic digester, and oxidation pond
Kingsburgh	Domestic	Extended aeration activated sludge
Shallcross	Domestic and industrial	Extended aeration activated sludge, oxidation pond

3.2.2 Enrichment, isolation, purification and maintenance of microalgal cultures

Wastewater samples were processed within 2-3 hrs after sampling. The samples were filtered through a Whatman no. 1 filter paper with a pore size of 11 µm to remove solid debris. Thereafter, the filtrate was diluted to 10^{-9} in BG-11 medium (Appendix three) Using spread plate method, 100 µL of diluted samples were transferred to agar plates containing BG-11 medium (Andersen and America, 2005). The inoculated plates were incubated at 25 °C, at a photon flux of approximately 120 µmol m^{-2} s^{-1} with a 16:8 h light dark cycle (Renuka *et al*., 2017). Single algal colonies obtained were thereafter streaked onto fresh agar plates containing BG-11. Axenic cultures were obtained by repeating the streak plate method. In order to maintain the purity of the isolated strains, stock cultures were sub-cultured onto BG-11 liquid medium and agar slants every month and were examined under light microscope. Algal strains were initially identified by morphological characteristics using light microscopy (Axiolab, Zeiss, Germany). Fig 3.1 illustrates the sampling and isolation protocol followed in this study.

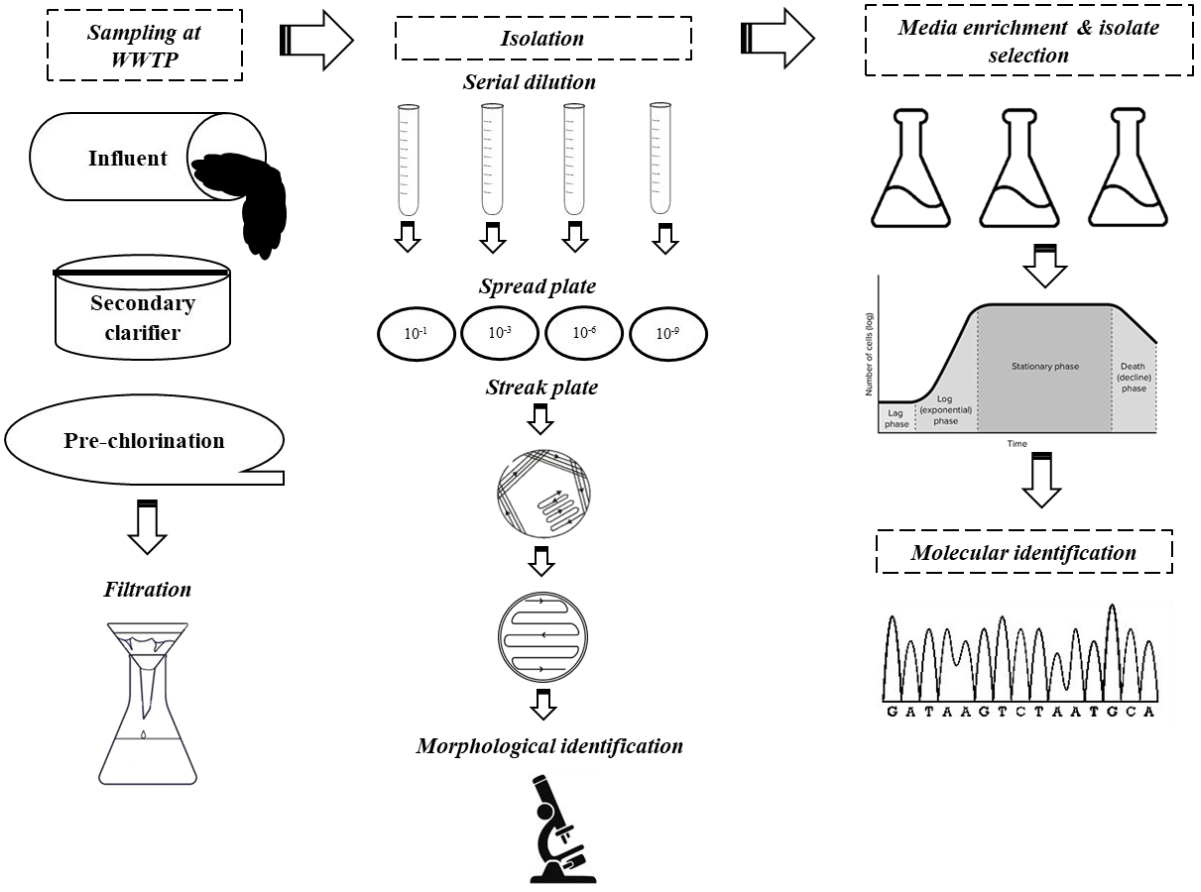


Figure 3.1 Schematic illustration of isolation, enrichment, and identification of microalgae from wastewater.

3.2.3 Microalgae growth analysis

To assess the growth of the pure microalgal isolates, each isolate was cultivated in 500 mL of BG-11 medium in 1L conical flasks, with each flask being inoculated with 10% of algal culture. All experiments were performed in triplicate. To evaluate the growth of the algal isolates, a spectral scan was conducted using a Thermo scientific (Multiskan Skyhigh) plate reader covering a wavelength of 200 to 800 nm, providing a comprehensive assessment of the light absorbance characteristics of the algal isolates. This scan was performed on each individual isolate to determine the optimal wavelength that would be used to assess its growth. The optical density was routinely assessed by collecting 1 ml of the microalgae culture and measuring the absorbance at 680 nm using a Jenway 7205 UV/Visible Spectrophotometer. To determine biomass productivity, 10 ml of a well-mixed culture was centrifuged at 15,000 rpm at 4 ºC for 10 min using a Hermle Z326K centrifuge. The supernatant was discarded, and the pellet was rinsed with distilled water. After removing excess water through centrifugation, the pellet was transferred to pre-weighed watch glasses and placed in an oven at 70 ºC overnight. The dry cell weight was then determined through gravimetric analysis using an analytical balance. The biomass productivity was determined according to Equation 3.1.

$$\textbf{Biomass productivity } (\textbf{mg}\boldsymbol{L^{-1}d^{-1}}) = \frac{\boldsymbol{Biomass\ (mg\ L^{-1})}}{\boldsymbol{Cultivation\ time}}$$

Equation 3.1 Biomass productivity.

where:

Biomass = Dry cell weight in mg L^{-1}

Cultivation time = Days

3.2.4 Molecular identification and phylogenetic analysis

3.2.4.1 Genomic DNA extraction

An aliquot (50 mL) of cultured cells was harvested in the late log phase by centrifugation and the cell pellets were washed twice using sterile deionized water in a sterile centrifuge tube. The genomic DNA was extracted from 50 mg microalgal biomass using ZR plant/seed DNA

kit (Zymo research Inc. Bohemia, New York, USA) following the manufacturer's instruction. Extracted DNA was quantified by Implen NanoPhotometer. Qualitative analysis of the extracted DNA was done using 1% agarose gel electrophoresis.

3.2.4.2 Polymerase chain reaction (PCR) and Sanger sequencing

The microalgal isolates were identified using the primers targeting 18S rRNA gene, Alg-AB1 forward (GGAGGATTAGGGTCCGATTCC) and Alg-TW4 reverse (CTTCCGTCAATTCCTTTAAG). OneTaq® 2x master mix with standard buffer was used in the PCR reaction mixture with 1 µl (20ng) genomic DNA ,1 µl (10 µM) each reverse and forward primers and 7 µl nuclease free water to make up a final volume of 20 µl. The optimised PCR conditions includes: 5 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30s, annealing at 50 °C for 30s, and extension at 72 °C for 1 min with a final extension of 10 min at 68 °C. The PCR products were separated by electrophoresis on 1% agarose gel stained with EZ-vision Bluelight DNA Dye. The positive amplicons were sequenced at Inqaba Biotechnical Industries (Pty) Ltd., South Africa. Sequence chromatogram analysis was performed using BLAST programme. To analyse phylogenetic analysis, nucleotide sequences obtained were aligned in CLUSTAL X and edited in BioEdit before being exported to MEGA 11. A phylogenetic tree was constructed using 1000 replicates by neighbour-joining analysis and alignment (Singh *et al.*, 2017).

3.2.5 Evaluation of the ecotoxicological effects of nevirapine on microalgal cells

The evaluation of the toxicological effects of NVP at various concentrations (0, 5, 10, 20, 40, 60, 80, 100 mg L^{-1}) supplemented in standard BG-11 medium on the growth of each microalgal species was conducted in accordance with the Organization for Economic Cooperation and Development (OECD) Guidelines for the testing of chemicals for a 96-hour period. Experiments were conducted in three replicates on a batch culture of exponentially growing microalgae. The growth response of *C. tenuitheca* MH176108.1 and *T. obliquus* MH307949.1 to varied concentrations of NVP was determined by taking daily measurements of OD at 680 nm using a microplate reader (Thermo Scientific Multiskan SkyHigh) for a period of 96 h. The viability of algal cells was evaluated using Evans blue staining at regular

intervals. A hemocytometer was used to determine the number of live (green) and dead (blue) cells. Viability was estimated based on the number of viable cells and compared with the control group (Nv *et al.*, 2017). As an endpoint determination, the growth rate of treated microalgae was compared with that of untreated microalgae to determine, whether growth rate inhibition occurred. Inhibitory concentrations were measured at 50% effect (IC_{50}) at which growth 50% reduction in growth occurred. The mean cell yields for the control group and the individual cell yield for microalgae exposed to NVP in each well were calculated. Growth inhibition percentages were then derived using the formula described in the equation (3.2) (Meng *et al.*, 2022).

$$I = [(R_C - R) \div R_C] \times 100$$

Equation 3.2 Percentage growth inhibition.

where:

I - the percent of growth inhibition of microalgae at each NVP concentration.

R_C - mean cell density (number mL^{-1}) for control microalgae.

R- cell density (number mL^{-1}) for each NVP concentration (OECD, 2006).

3.3 Results and discussion

3.3.1 Isolation and preliminary identification of microalgal strains

Table 3.3 provides an overview of the chemical and physical attributes of the wastewater used for isolating microalgae. The composition of nutrients in surrounding environments significantly influences the type of microalgae thrive in those habitats. The successful isolation of 11 green microalgal strains was achieved using the spread and streak plate technique from the collected wastewater samples (Table 3.2). The Isipingo wastewater treatment plant primarily receives domestic wastewater influent, which includes wastewater from the nearby hospital Prince Mshiyeni Memorial. At the Kingsburgh wastewater treatment plant, the influent consists mainly of domestic wastewater. Meanwhile, the Shallcross

wastewater treatment plant receives both domestic and industrial influent wastewater, with the additional proximity to the R. K. Khan hospital. The wastewater samples obtained from the influent (untreated wastewater) yielded the highest number of isolated strains in comparison to samples from the secondary clarifier and pre-chlorination tanks. From the 11 strains isolates studied, 6 were found in Kingsburgh, 4 from Isipingo, and 1 from the Shallcross WWTP. This finding highlights the ecological changes occurring within wastewater ecosystems, implying that influent samples could be considered as an ideal environment for the isolation of various algal strains. Table 3.3 suggests that influent samples could be deemed an optimal environment for the isolation of a diverse selection of algal strains, due to the rich nutrients (Nitrogen and phosphorous) and conditions conducive to algal growth present at this stage.

The preliminary identification of the algal isolates was based on detailed morphological observations (Fig. 3.4), following the well-established methodology outlined by Prescott in 1978. In wastewater environments, a diverse range of algae species can be found, each with its unique characteristics. Among these, *Scenedesmus* stands out as non-motile microalgae, often organized into distinct rows of 4, 8, 16, or 32 cells, and some feature distinguishing spines or bristles. In contrast, *Chlorococcum*, another prevalent species, consists of single-celled organisms with spherical to slightly elongated shapes, often forming irregular clusters or delicate layers on moist or submerged surfaces. These cells have minimal mucilage and typically house a solitary cup-shaped chloroplast with a single pyrenoid. *Chlorella* strains are single-celled, non-motile green algae, displaying spherical or ellipsoidal shapes, with cell diameters ranging from 2 to 12 µm, showing variability even within the same group. Furthermore, *Chlorococcales* contain cells with spherical, subspherical, or ellipsoidal shapes, each featuring a parietal, cup-shaped chloroplast located adjacent to the cross wall, housing a solitary pyrenoid. These various algae species play crucial roles in wastewater ecosystems, contributing to nutrient removal and overall ecosystem dynamics.

Table 3.2 Identification of isolated microalgal strains according to Prescott and Prescott (1978).

Isolate no.	WWTP	Sample source	Tentative identification
1	Kingsburgh	Influent	*Scenedesmus*
2	Kingsburgh	Influent	Chlorococcales
3	Shallcross	Influent	Chlorococcales
4	Kingsburgh	Influent	Chlorococcales
5	Isipingo	Effluent	*Scenedesmus*
6	Isipingo	Effluent	*Chlorococcum*
7	Kingsburgh	Influent	*Acutodesmus*
8	Kingsburgh	Influent	*Tetradesmus*
9	Kingsburgh	Secondary	Chlorococcales
10	Isipingo	Secondary	*Chlorella*
11	Isipingo	Influent	*Chlorella*

Figure 3.2 Isolation and cultivation of microalgal isolates from wastewater.

Table 3.3 Physical and chemical wastewater characteristics as at time of sampling.

Parameter	Wastewater Treatment Plant								
	Kingsburgh			**Isipingo**			**Shallcross**		
	Inf.	Sec.	Eff.	Inf.	Sec.	Eff.	Inf.	Sec.	Eff.
Temperature (°C)	21.44 ± 2.7	19.91 ± 3.1	19.63 ± 2.0	19.86 ± 2.0	20.20 ± 1.6	19.75 ± 2.3	22.21 ± 1.8	20.00 ± 2.2	20.08 ± 3.0
DO (mg L^{-1})	0.92 ± 0.3	1.30 ± 0.1	6.09 ± 0.4	0.67 ± 0.2	3.26 ± 0.1	1.86 ± 0.4	2.48 ± 0.3	1.43 ± 0.2	8.60 ± 0.3
pH	7.89 ± 0.2	7.37 ± 0.4	7.25 ± 0.1	7.64 ± 0.5	6.83 ± 0.3	7.00 ± 0.4	7.53 ± 0.6	7.22 ± 0.4	7.46 ± 0.2
Ammonia (mg L^{-1})	5.34 ± 6.2	18.66 ± 4.0	17.96 ± 3.1	14.93 ± 5.0	5.46 ± 7.2	7.84 ± 2.8	60.09 ± 7.1	1.71 ± 5.4	48.43 ± 6.5
TON (mg L^{-1})	0.09 ± 0.1	0.05 ± 0.4	0.04 ± 0.3	0.10 ± 1.2	5.02 ± 0.7	5.20 ± 0.4	0.09 ± 1.1	0.38 ± 2.3	0.07 ± 2.0
Phosphorus (mg L^{-1})	8.05 ± 1.8	0.70 ± 0.1	0.62 ± 0.2	5.82 ± 1.9	3.84 ± 2.5	4.49 ± 1.6	0.77 ± 2.1	0.97 ± 1.1	0.16 ± 0.5

Inf. - Influent; Sec. - Secondary; Eff. - Effluent; DO- Dissolved oxygen; TON- Total oxidised nitrogen

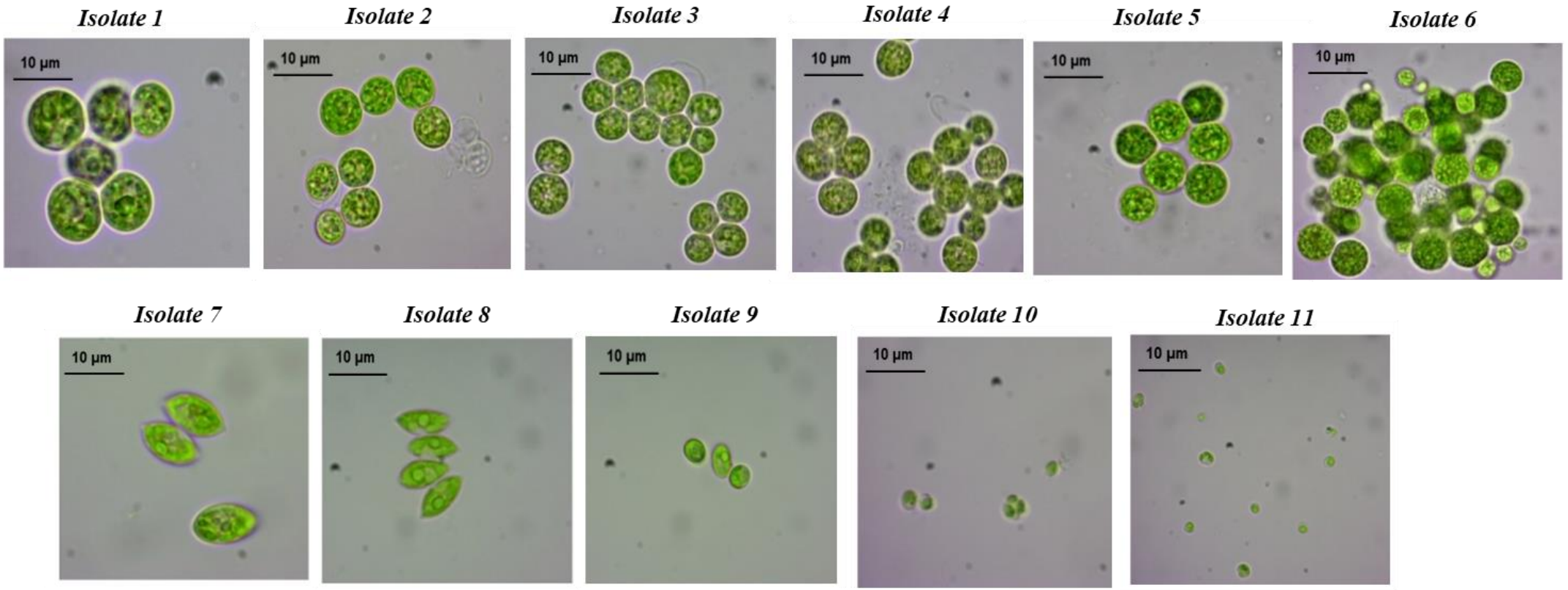


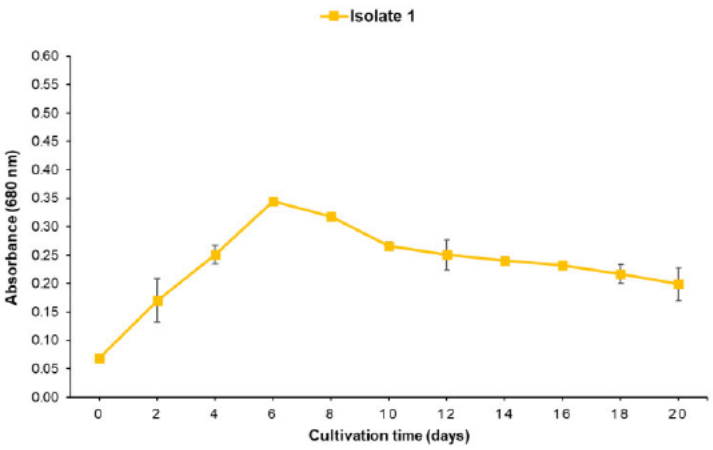
Figure 3.4 Microphotographs of the eleven microalgal strains isolated from wastewater treatment plants in KwaZulu-Natal.

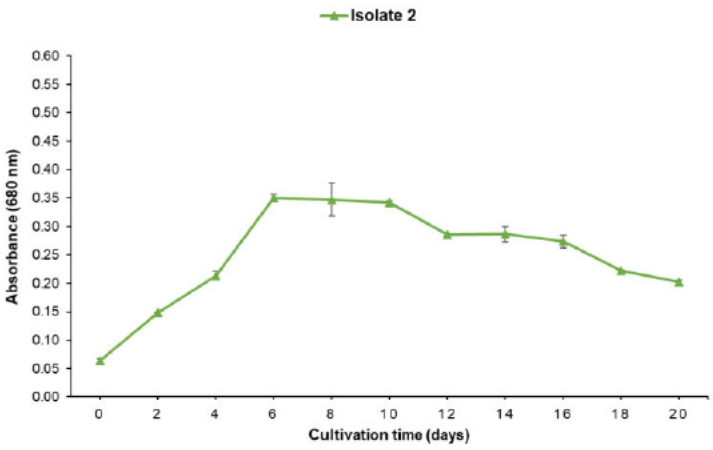
3.3.2 Evaluation of isolated microalgal strains

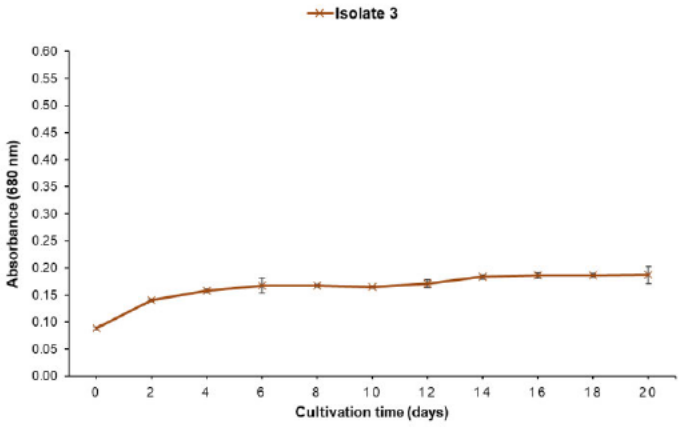
The selection of microalgal strains for NVP bioremediation involved a screening process, considering key factors such as growth rate, biomass production, and toxicity tests. This evaluation aimed to identify microalgae with the optimal combination of attributes necessary for effective NVP removal from the environment. Furthermore, toxicity testing was conducted to assess the cellular tolerance levels to varying concentrations of NVP.

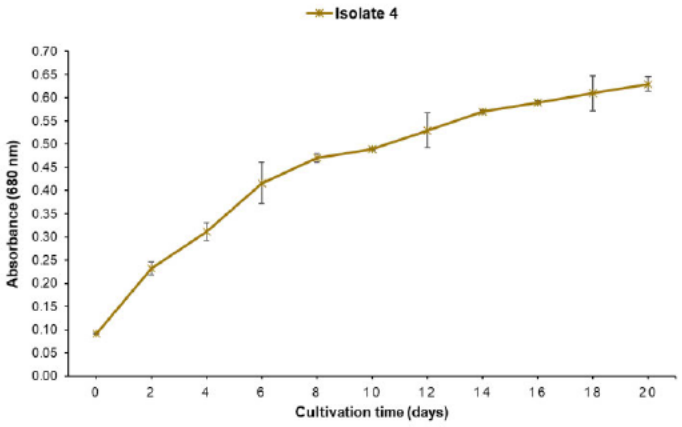
3.3.2.1 Growth profile and biomass production of isolated microalgal strains

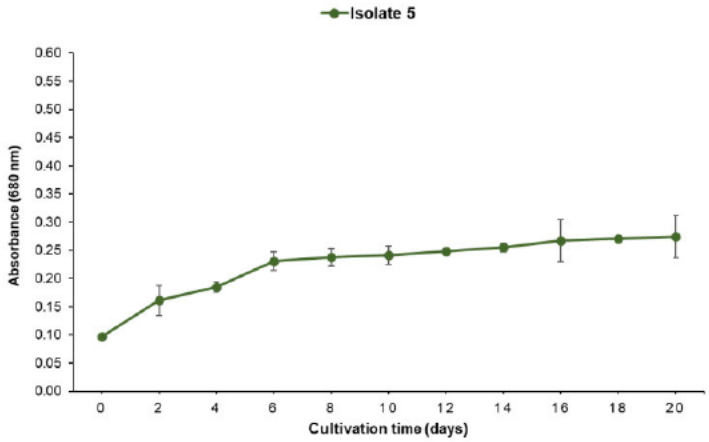
Most strains displayed a lag phase from 0-2 days except for isolates 2, 4, 8 and 9 when cultivated in BG-11 medium. The log phase was between day 2 and day 14, however, for few isolates (isolates 4, 8, 9, 10), the log phase of extended beyond day 14. Isolate 8 produced the highest biomass of 169.02 mg L^{-1} d^{-1} followed by isolate 4 with a biomass of 155.24 mg L^{-1} d^{-1} (Fig. 3.6). Isolate 9 and 11 have also shown moderate amount of biomass yields of respectively 148.12 mg L^{-1} d^{-1} and 107.41 mg L^{-1} d^{-1}. Isolates 4 and 8 which showed the fastest growth rates, were selected for ARV removal tests. For ARV drug removal studies, biomass production is one of the key criteria for strain selection. Higher algal biomass can help with remediation process in several ways. First, microalgae are capable of removing pollutants from water through biodegradation, bioadsorption and bioaccumulation (Goh *et al.*, 2023). An increase in biomass growth, would directly increase the adoption/absorption rate of nutrients from the water, including pollutants such as heavy metals and inorganic pollutants (Abdel-Raouf *et al.*, 2012). In addition, many microalgal species produce compounds such as pigments, lipids, proteins, and carbohydrates that have potential therapeutic benefits making them valuable resources for various applications in fields such as medicine, nutrition, and biotechnology (Ampofo and Abbey, 2022). Furthermore, this biomass can be harvested and processed to produce bioplastics, and other products, providing additional economic and environmental benefits (Onen Cinar *et al.*, 2020).

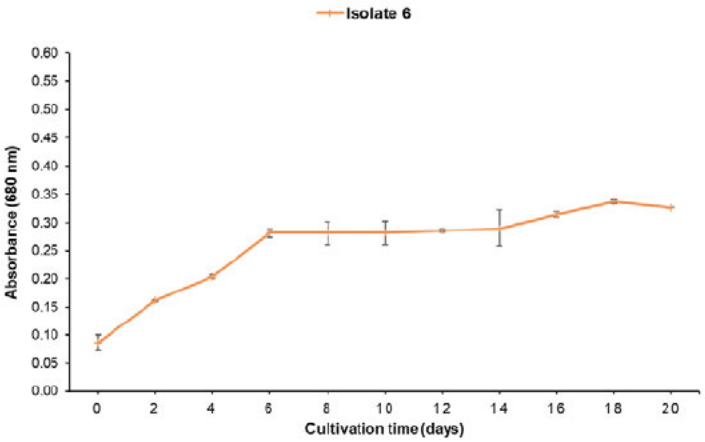

Isolate 1
Absorbance (680 nm)
Cultivation time (days)


Isolate 2
Absorbance (680 nm)
Cultivation time (days)


Isolate 3
Absorbance (680 nm)
Cultivation time (days)


Isolate 4
Absorbance (680 nm)
Cultivation time (days)


Isolate 5
Absorbance (680 nm)
Cultivation time (days)


Isolate 6
Absorbance (680 nm)
Cultivation time (days)

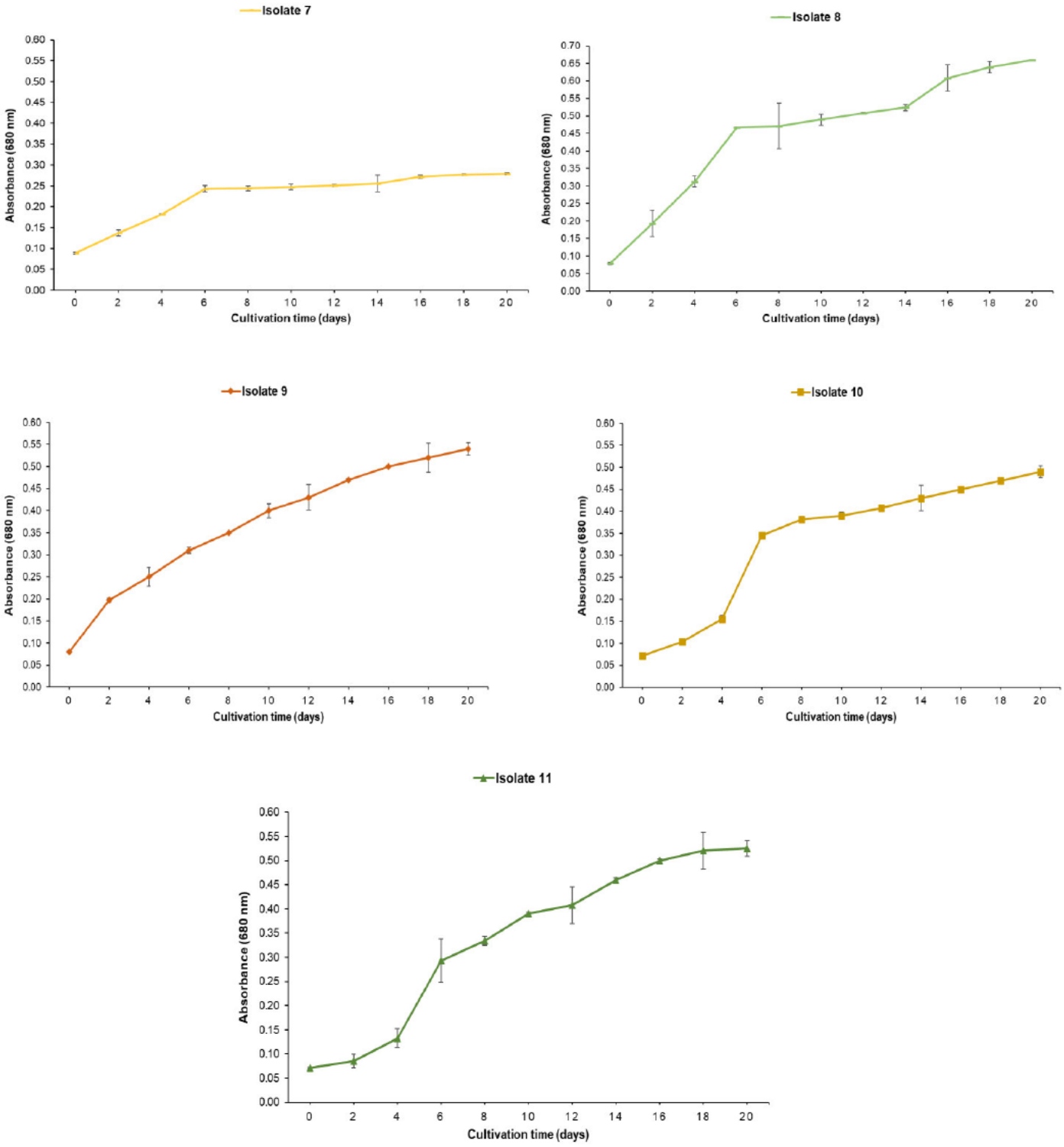


Figure 3.5 Growth curve analysis of 11 isolated microalgal strains.

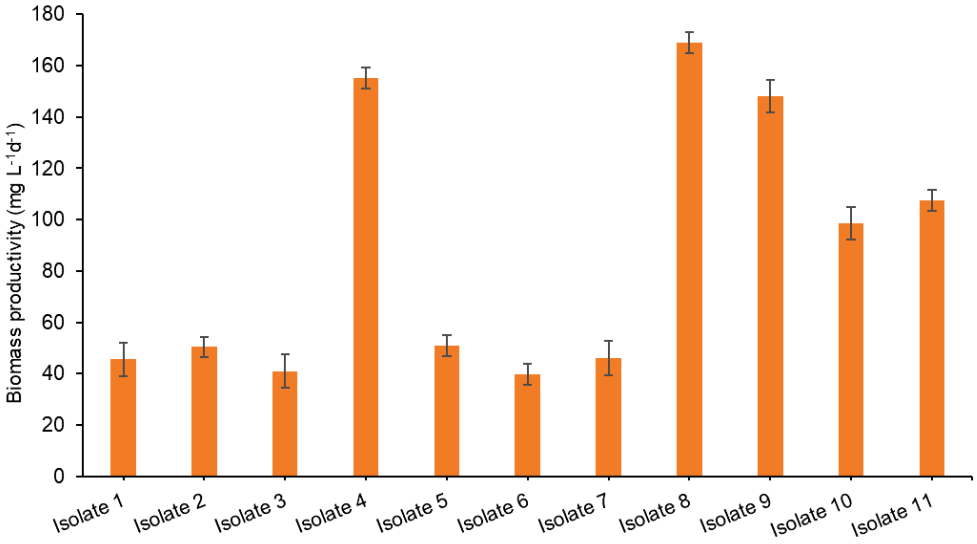


Figure 3.6 Biomass productivity of isolated microalgal strains (late log phase) in BG-11 medium.

3.3.3 Identification of selected strains by molecular and phylogenetic analysis

The two fastest growing isolates were further subjected to 18S rRNA sequencing and analysis. National Center for Biotechnology Information (NCBI) blast analysis and the results are shown 100% similarity and query cover to *Coelastrella tenuitheca* (Isolate 4). Isolate 8 demonstrated a similarity of 99.33% to *Tetradesmus obliquus*, another well-known microalgal species found in wastewater. The sequences were submitted to NCBI and the strain with a 100% similarity to *C. tenuitheca* was assigned the accession number MH176108.1, while the strain resembling *T. obliquus* received the accession number MH307949.1. The phylogenetic relationships of the identified microalgal strains, was conducted using maximum likelihood and neighbour joining algorithms (Figure 3.7). As depicted, *C. tenuitheca* (MH176108.1) has close similarity to *C. tenuitheca* MH176107.1; MK478814.1 and MH176106.1. All *Coelastrella* species have similar morphological characteristics such as round, unicellular or in few-celled aggregations (Goecke *et al*., 2020). The cells presented variable sizes, approximately 7 µm long and displayed a distinct singular pyrenoid. The cell wall exhibits a translucent appearance, and easy to distinguish a cup-shaped chloroplast (Goecke *et al*., 2020). *Tetradesmus obliquus* (MH307949.1) has close similarity to *T. obliquus* MK764915.1; MK764914.1 and MH651227.1. *Tetradesmus* species exhibited spindle-shaped cells that were elongated and lacked spines, featuring a smooth cell surface, and tapering obtuse ends. These

cells were organized in flat or curved coenobia, each composed of 4–8 individual cells. The cell size varied, measuring approximately 16 µm in length, with each cell containing a prominent pyrenoid and a sizable parietal chloroplast that extended across more than half of the cell's length (Sehgal *et al*., 2019). The taxonomic classification of the selected microalgal strains, detailed information can be found in Table 3.4. The table provides an overview of the strains' taxonomic affiliations, including their respective genus and species classifications. Overall, the identification of indigenous microalgal strains demonstrated a strong alignment between morphological characteristics and sequence-based phylogenetic analysis. Therefore, through a combined assessment of morphological and molecular criteria, the two strains chosen for this study were conclusively identified as *C. tenuitheca* MH176108.1 and *T. obliquus* MH307949.1.

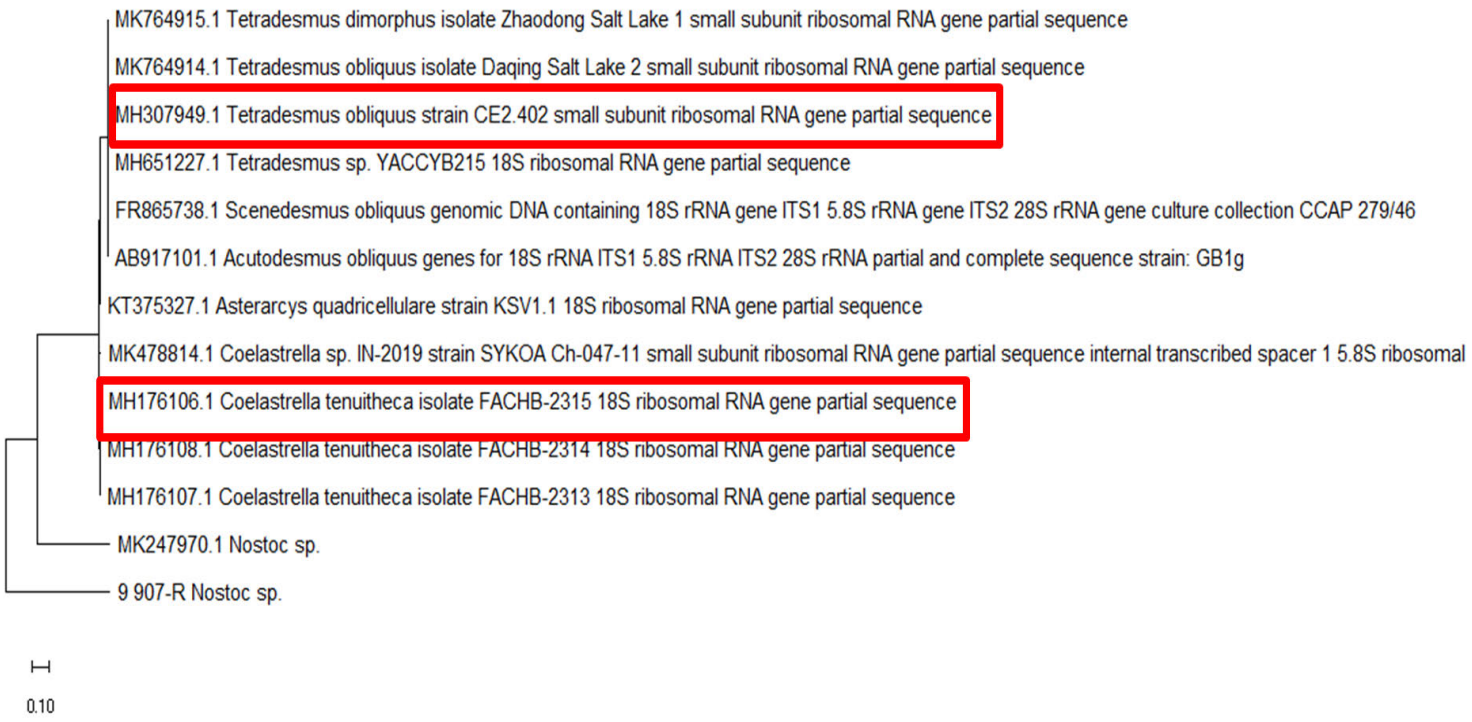


Figure 3.7 Phylogenetic tree depicts results of Neighbour-joining analysis (MEGA 11), *Coelastrella tenuitheca* (MH176105.1) and *Tetradesmus obliquus* (MH307949.1).

Table 3.4 Taxonomy of selected algal isolates.

	Taxonomic classification	
	Isolate 4	Isolate 8
Domain	Eukaryota	Eukaryota
Kingdom	Protista	Protista
Phylum	Chlorophyta	Chlorophyta
Class	Chlorophyceae	Chlorophyceae
Order	Sphaeropleales	Sphaeropleales
Family	Scenedesmaceae	Scenedesmaceae
Genus	*Coelastrella*	*Tetradesmus*
Species	*Coelastrella tenuitheca*	*Tetradesmus obliquus*

All information obtained from National Center for Biotechnology Information (accessed October 2023).

3.3.4 Evaluation of the ecotoxicological effects of nevirapine

The ecotoxicological effects of NVP were evaluated by exposing *C. tenuitheca* and *T. obliquus* to different concentrations of NVP (0–100 mg L^{-1}). Growth inhibition rate was used as a representative measure to determine the phytotoxicity of NVP on both microalgae species (Wang *et al.*, 2020). Toxicity data for NVP including 95% confidence interval (CI) based on growth as response variable is include in Table 3.5. Growth inhibition due to NVP treatments

was recorded and the 50% growth rate inhibitory concentrations (IC_{50}) were calculated during the period of 96 h for both the studied microalgal strains.

Figure 3.8 Batch tests conducted using microplates to evaluate the ecotoxicity of NVP on *C. tenuitheca* and *T. obliquus*.

The results revealed that *C. tenuitheca* was more prone to growth inhibition at NVP concentrations below 20 mg L^{-1} than *T. obliquus*. Nevirapine concentrations between 40 and 100 mg L^{-1} resulted in a gradual increase in growth inhibition of both strains (Fig. 3.9). The highest growth inhibition of *T. obliquus* after 96 h of exposure was observed in 80 and 100 mg L^{-1} NVP, resulting in 100% inhibition. Similarly, concentration of 40 mg L^{-1} NVP inhibited growth in *T. obliquus* by 97.95% and a concentration of 60 mg L^{-1} NVP inhibited growth by 98.98%, respectively. The growth of *C. tenuitheca* was not completely inhibited at concentrations of 5 mg L^{-1} to 100 mg L^{-1}, with 93.3% growth inhibition at 100 mg L^{-1} NVP. The difference in the effect of NVP on growth rates of *C. tenuitheca* and *T. obliquus* may be explained by the hormesis, which is characterized by a low-dose stimulation and a high-dose inhibition (Wang *et al.*, 2020).

Table 3.5 Inhibition of growth of *C. tenuitheca (A)* and *T. obliquus (B)*, and the calculated 96 h IC_{50} of nevirapine.

Species	NVP concentration (mg L^{-1})							96 h IC_{50}	95% CI	
	5	10	20	40	60	80	100		Lower limit	Upper limit
	Inhibition (%) of growth									
A	30	40	51.1	63.3	77.8	91.1	93.3	23.45	17.83	28.39
B	1.53	25.51	55.1	97.95	98.98	100	100	18.20	13.71	23.51

NVP- Nevirapine; IC- Inhibitory concentration; CI- Confidence interval; h- Hour

Despite the lack of studies that have investigated the molecular and cellular mechanisms behind hormetic dose-response relationships of ARV drugs, the phenomenon of hormesis has been well documented in various organisms, including microalgae for some chemicals (Ding *et al.*, 2017a, Ding *et al.*, 2017b, Nie *et al.*, 2013, Xiong *et al.*, 2017b). To further assess the toxicity of NVP on both microalgal strains, the IC_{50} effect was determined after exposure for 96 h. The IC_{50} is one of the most accepted endpoints for toxicity evaluation. This is defined as the concentration at which 50% of the growth rate of an organism is inhibited (Xiong *et al.*, 2017a). The calculated 96 h IC_{50} for NVP in *C. tenuitheca* and *T. obliquus* cultures in this study were 23.45 mg L^{-1} and 18.20 mg L^{-1}, respectively. The IC_{50} values of microalgal species to NVP have not been adequately investigated, however, Guo *et al.* (2015), reported an IC_{50} value > 43 mg L^{-1} with the exposure of NVP to *Pseudokirchneriella subcapitata* during a 72-h period. The results in this study demonstrated that *C. tenuitheca* is significantly more tolerant to NVP than *T. obliquus* as 100% inhibition was not achieved during the 96 h. In the present study, the microalgal species *C. tenuitheca* and *T. obliquus* were shown to tolerate higher concentrations of NVP and as a potential candidate for developing technologies for the remediation of NVP.

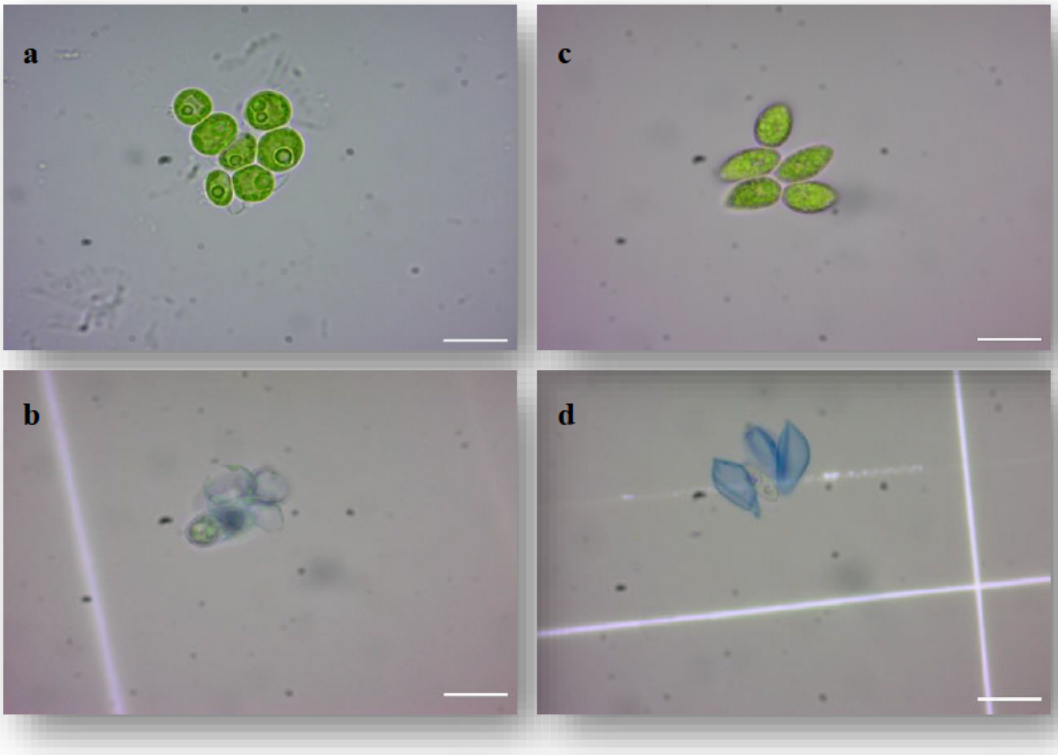


Figure 3.9 Micrographs **a**: *C. tenuitheca* (Live cells); **b**: *C. tenuitheca* (Dead cells); **c**: *T. obliquus* (Live cells); **d**: *T. obliquus* (Dead cells).

3.4 Conclusion

In this study, eleven isolated indigenous microalgal strains from municipal wastewater treatment plants. Different microalgal isolates showed varying growth rates and biomass productivity. Among these strains, *T. obliquus* displayed the highest biomass productivity, reaching 169.02 mg L^{-1} d^{-1}. Following closely was *C. tenutheica*, which exhibited a biomass productivity of 155. 24 mg L^{-1} d^{-1}. Toxicity tests of NVP showed that the growth of both species presented a hormesis dose-response phenomenon. *Coelastrella tenuitheca* revealed a higher IC_{50} (28.39), showing higher tolerance than *T. obliquus* during a 96-h period. The selection of these specific microalgal strains was based on their promising growth rates and productivity during screening. The best performing isolates were further explored their potential for removal of ARVs.

CHAPTER FOUR: Assessing the potential for nevirapine removal using *Coelastrella tenuitheca* and *Tetradesmus obliquus* in aqueous environment

Reddy, K., Renuka, N., Kumari, S., Ratha, S.K., Moodley, B., Pillay, K., Bux, F. (2023). Assessing the potential for nevirapine removal and its ecotoxicological effects on microalgae in an aqueous environment. *Environmental Pollution*, 120736.

4.1 Introduction

Antiretroviral drug therapy is administered daily in two-drug or three-drug fixed dose combinations (Meintjes *et al.*, 2017, Saag *et al.*, 2020). More than 5 million individuals in South Africa receive antiretroviral treatment (the highest rate in the world) (Horn *et al.*, 2022). In terms of treatment guidelines, NVP is listed among the non-nucleoside reverse transcriptase inhibitors for HIV-infected adolescents, adults, and most often pregnant women (Flynn *et al.*, 2018). After being administered, NVP is nearly 90% bioavailable, disintegrates after 45 h, and is excreted through the urine. The bioactivity of NVP, combined with its long half-life, allows it to persist in the environment for a long period of time (Yao *et al.*, 2021). The presence of NVP in wastewater at varying concentrations has been reported in a number of studies (Table 2.3), including WWTP influents and effluents. Most WWTPs are designed to remove suspended solids as well as pathogenic microorganisms, but none are specifically designed to remove PCs (Chandel *et al.*, 2022). The search for alternatives to conventional methods of pharmaceutical removal has led to interest in the use of microorganisms, i.e., bioremediation technology, for the treatment of wastewater as a result of their economic viability and environmental friendliness (Mohsenpour *et al.*, 2021). Microalgae can be used to remediate wastewater contaminated with pharmaceuticals in a way that is eco-friendly and does not produce secondary pollution as the biomass generated can be recycled and allows efficient nutrient reutilization (Elumalai and Saravanan, 2016, Musetsho *et al.*, 2021). In numerous studies, algae-based technologies have been found successful in removing various PCs. It was reported that *Nannochloris* sp. could remove 100% of triclosan (10 μg L^{-1}) after 7 days of exposure (Bai and Acharya, 2017) and *Scenedesmus obliquus* (modified algal biomass using alkaline solution) was able to remove 91% of tramadol (50 mg L^{-1}) after 45 min (Ali *et al.*, 2018a). Ding *et al.* (2020) showed that *Navicula* sp. could efficiently remove 4 PCs (atenolol, carbamazepine, ibuprofen and naproxen) (0.5 mg L^{-1}) with an efficiency of >90% after 21 days of exposure. According to their study, strain selection is crucial when using microalgae to remove PCs. Despite the successful removal of PCs by microalgae, concerns have been

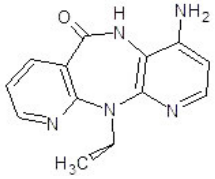
raised regarding its possible ecological effects on microalgae and other aquatic organisms. Although algae have been studied for their ability to remediate PCs, (Ding *et al.*, 2017a, Ding *et al.*, 2017b, Puerta *et al.*, 2020, Tong *et al.*, 2020, Xiong *et al.*, 2017b), little is known about the removal of ARV compounds by algae, the mechanisms involved. Hence, based upon the persistent nature of NVP in the environment, an objective was designed to assess the potential of microalgal monocultures of *C. tenuitheca* and *T. obliquus* for NVP removal from the aqueous environment under batch culture conditions. Additionally, the effects of NVP on the growth rate, chlorophyll content, physiology, and morphology of the selected microalgal strains were evaluated.

4.2 Materials and methods

4.2.1. Chemicals

The ARV compound, NVP used in this study was purchased from Sigma-Aldrich (Table 4.1). The chemical purity was ≥98%, which is within the HPLC grade and therefore, no further purification was performed. The stock and working solutions of NVP were prepared by dissolving in methanol (purity >99%).

Table 4.1 Structure and physicochemical properties of nevirapine.

Properties	
Structure	
Molecular formula	$C_{15}H_{14}N_4O$
pKa	2.8
Exact mass	266.117 g mol^{-1}
Water solubility	0.705 mg mL^{-1}

All information obtained from https://www.ncbi.nlm.nih.gov (accessed November 2023).

4.2.2 Evaluation of the nevirapine removal potential of the microalgal isolates

4.2.2.1. Experimental design

The NVP removal performance of the two microalgal species at different environmental concentrations was assessed for a period of 8 days. The isolates were inoculated in 1 L conical flasks containing 500 mL BG-11 medium (pH 7) at an initial optical density (OD) of 0.1 at 680 nm. The cultures were grown at the same culture conditions as mentioned in chapter 3, section 3.2.2. Nevirapine was added to BG-11 medium to achieve the desired concentration (0, 5, 10, 50, 100, 150, 200, 500, 1000, 1500, 2000 and 4000 ng L^{-1}). The range of concentrations was selected based on the environmental concentrations as reported in the literature (Table 2.3). The same concentration of NVP was added in the control group without algal inoculum to quantify NVP removal through photolysis process. All treatments were run in three replicates and the analysis was carried out at 2-day intervals.

4.2.2.2. Experimental analysis

4.2.2.2.1. Microalgal growth analysis

The growth of the isolates at different concentration of NVP was evaluated using an optical density spectrophotometer (DR6000, Hach) at a wavelength of 680 nm. As shown in equations (4.1), (4.2)), the values of OD_{680} were converted to dry cell weight (DCW) in (mg L^{-1}) based on the relationship between of OD_{680} and DCW.

Preliminary studies were conducted to determine OD_{680} as a function of dry weight.

$$\text{DCW of } Coelastrella\ tenuitheca = 2068.3 \cdot \text{OD680},\ R^2 = 0.99$$

Equation 4.1 Relationship between OD_{680} and DCW (*C. tenuitheca*).

$$\text{DCW of } Tetradesmus\ obliquus = 2409.4 \cdot \text{OD680},\ R^2 = 0.99$$

Equation 4.2 Relationship between OD_{680} and DCW (*T. obliquus*).

A calculation of the specific growth of the culture was performed using equation:

$$\mu = \frac{InN_{2\_}InN_0}{t_2 - t_0}$$

Equation 4.3 Specific growth rate (SGR).

Where, N_2 represents the DCW at time t_2 and N_0 denotes the DCW at time t_0 (day 0) (Wang *et al*., 2022a).

4.2.2.2.2. Chlorophyll content and photosynthetic performance

The effect of different concentrations of NVP on the photosynthetic performance and chlorophyll content of the selected microalgal strains was assessed based on the maximum quantum efficiency of photosystem II (PSII) (Fv/Fm) using a Dual PAM100 Chlorophyll Fluorometer. The rapid light curve was generated and the maximum quantum efficiency of PSII was calculated as reported by Ramanna *et al.* (2014) and relative electron transport (rETR) were calculated as described by White *et al.* (2011). Methanol extraction method was used to determine the total chlorophyll content (Bajguz and Piotrowska-Niczyporuk, 2013) following equation (4.4).

$$Total\ chlorophyll = 1.44\ (A_{665.2}) + 24.93\ (A_{652.4})$$

Equation 4.4 Total chlorophyll content.

4.2.2.2.3. Nevirapine removal

Nevirapine removal by microalgae was evaluated at regular intervals (2 days). A modified chromatographic method was developed based on the protocol described by Schoeman *et al.* (2015). Samples were drawn, centrifuged, and filtered (0.22 μm). The supernatant was used for NVP analysis on a Sciex Exion LC AD series liquid chromatography system coupled to a Sciex 5500+ triple quadrupole mass spectrometer (Promolab T/A Separations, South Africa). The mobile phase for liquid chromatography consisted of acidified water (CHROMASOLV™ LC-MS, Honeywell and 0.1% formic acid (LC-MS LiChropur™, 97.5–98.5%) and acidified

methanol (0.1% formic acid), namely solvents A and B, respectively. Chromatographic separations were achieved using a Shimadzu Shim-pack GISS C18 column (1.9 μm, 2.1 × 150 mm). The column oven temperature was maintained at 30 °C and the gradient elution was operated isocratically at 90% A: 10% B. The mobile phase flow rate was controlled at 0.25 mL min^{-1} and the sample injection volume was 1 μL. The Sciex 5500+ mass spectrometer (MS) was equipped with a Sciex Turbo V electrospray ionisation (ESI) source and NVP was detected in positive ionisation mode. The MS generated data was then processed using the Sciex OS data managing software and calibration graphs were plotted (Appendix four). These Multiple Reaction Monitoring (MRM) transitions were used to optimise the compound specific parameters on the MS. In order to evaluate the method's sensitivity both the limit of detection (2.06 ng mL^{-1}) and the limit of quantification (6.24 ng mL^{-1}) were calculated (Thomsen *et al.*, 2003). The total removal (P_t) of NVP was calculated using equation (4.5) (Xiong *et al.*, 2017c).

$$P_t(\%) = \frac{C_0 - C_t}{C_0} \times 100$$

Equation 4.5 Total removal percentage

Where, the initial concentration of NVP at time zero is represented by C_0, and the concentration at time t is represented by C_t.

4.2.2.2.4. Scanning electron microscopy

The morphological changes were assessed using scanning electron microscope (SEM) at the end (8 day) of the experiment. The harvested algal cells were concentrated by centrifugation at 2000×g for 1 min. The cells were fixed in 2.5% glutaraldehyde and were dehydrated by gradually increasing ethanol concentrations (up to 100% ethanol) in 10-min intervals with gentle centrifugation at each step (Michalak *et al.*, 2018). After dehydration, the samples were dropped onto nucleopore membrane, which was glued onto an aluminium stub. The samples

were then dried by dropping a small amount of hexamethyldisilazane onto each stub. Once dry, they were then carbon coated. The images were taken with the Tescan (MIRA 3) SEM.

4.2.3 Data analysis

The statistical analysis was performed on triplicate set of data using R statistical software. Levene's homogeneity test was used to compare differences between treatments based on a one-way ANOVA analysis. In graphs, SD (standard deviation) was shown as error bars. Duncan's multiple range test (DMRT) analysis was used to find out the significant treatments. Probit analysis was performed by SPSS 27 to determine IC_{50} and 95% confidence intervals (CI) for the toxicity experiments. Values were considered significant at a p value of <0.05 for all tests.

4.3 Results and discussion

4.3.1. Effect of nevirapine on microalgal growth and photosynthetic performance

4.3.1.1. Effect on microalgal growth

The effect of different concentrations of nevirapine (NVP) (0–4000 ng L^{-1}) supplemented in standard BG-11 medium on microalgae was examined during an 8-day cultivation period. The SGR and DCW were compared between the different treatment conditions. The growth curves of *C. tenuitheca* and *T. obliquus* in the presence of NVP and the corresponding controls are represented as values of absorbance versus time in Fig 4.1. In microalga *C. tenuitheca*, neither the growth was inhibited by the addition of different concentrations of NVP, nor were the lag and log phases significantly altered by NVP addition when compared to control cultures.

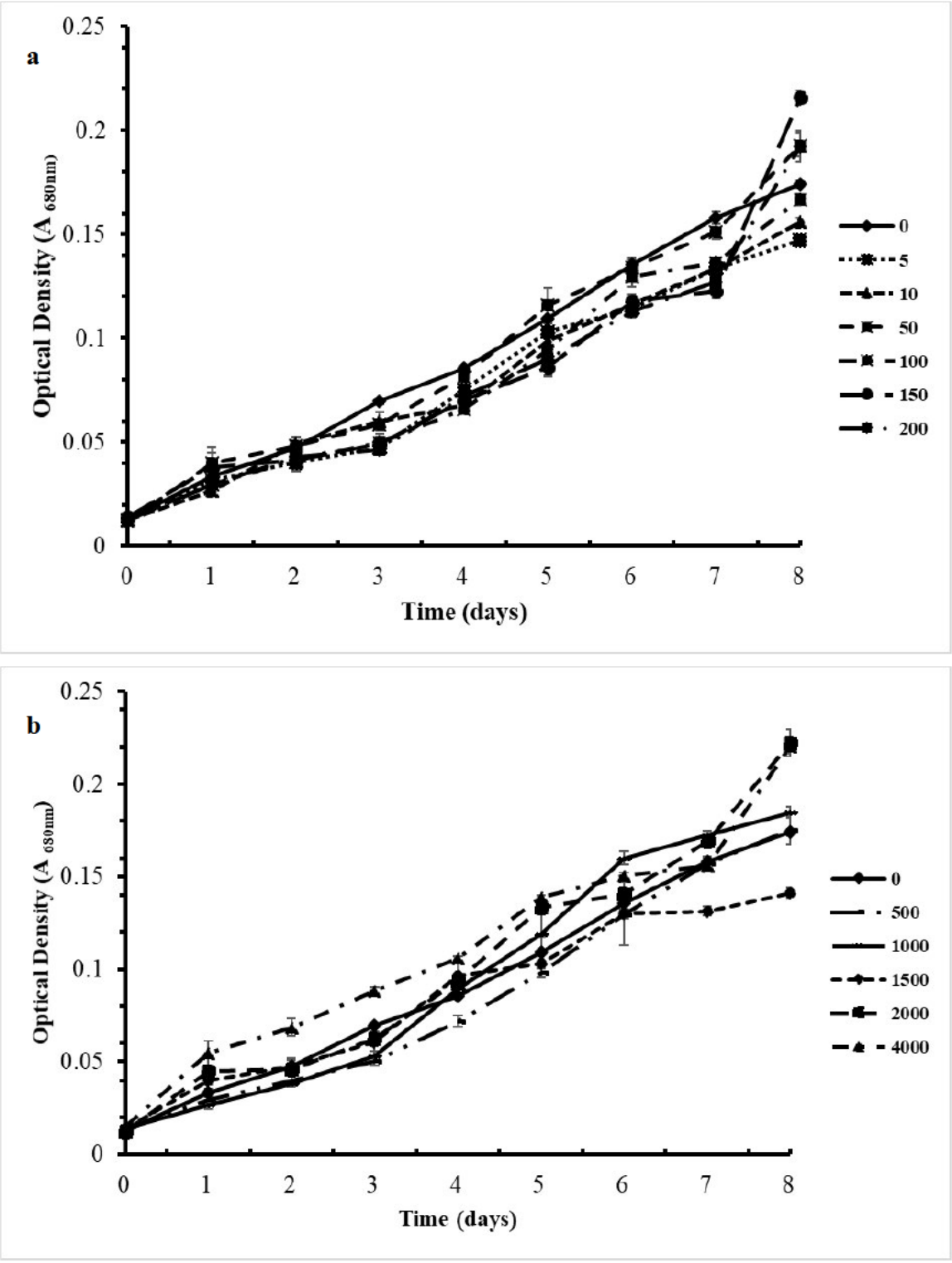


Figure 4.1 Effect of different nevirapine concentrations [0-200 ng L^{-1} (**a**) and 500-4000 ng L^{-1} (**b**)] on growth of *Tetradesmus obliquus* in BG-11 medium.

The DCW of *C. tenuitheca* in the control sample was 358.89 mg L^{-1} on day 8 of the experiment. Whereas the DCW of the NVP supplemented treatments ranged from 304.04 to 459.85 mg L^{-1}. It has been reported that the *Coelastrella* spp. are highly tolerant to heavy metals and pharmaceuticals (Gojkovic *et al.*, 2019, Plöhn *et al.*, 2021a). Gojkovic *et al.* (2019) reported that the presence of active pharmaceutical ingredients in the growth medium had no significant impact on the growth of *Coelastrella* spp. In the present study, NVP supplementation at lower concentrations (10–200 ng L^{-1}) resulted in a slight increase in DCW of *T. obliquus*. The highest DCW of 941.27 mg L^{-1} was obtained on day 8 (late log phase) at 50 ng L^{-1} NVP concentration. It was also evident that the addition of NVP at lower concentrations (up to 200 ng L^{-1}) stimulated the growth of *T. obliquus* as compared to the control (Fig 4.3). However, when NVP concentrations exceeded 200 ng L^{-1}, a lower concentration of DCW was obtained in *T. obliquus* than the control (296.36 mg L^{-1}). This may be explained by the fact that the drugs can act as a source of organic carbon at low concentrations (Escapa *et al.*, 2017). This phenomenon was also reported by Zhang *et al.* (2019), suggesting that the low doses of PCs could promote the growth of microalgae because of an inductive effect of pharmaceutically active compounds on the algal cell. On the other hand, high concentrations can adversely affect algal cell growth, as evidenced in this study.

Figure 4.2 Nevirapine supplemented in standard BG-11 medium on microalgae was examined during an 8-day cultivation period.

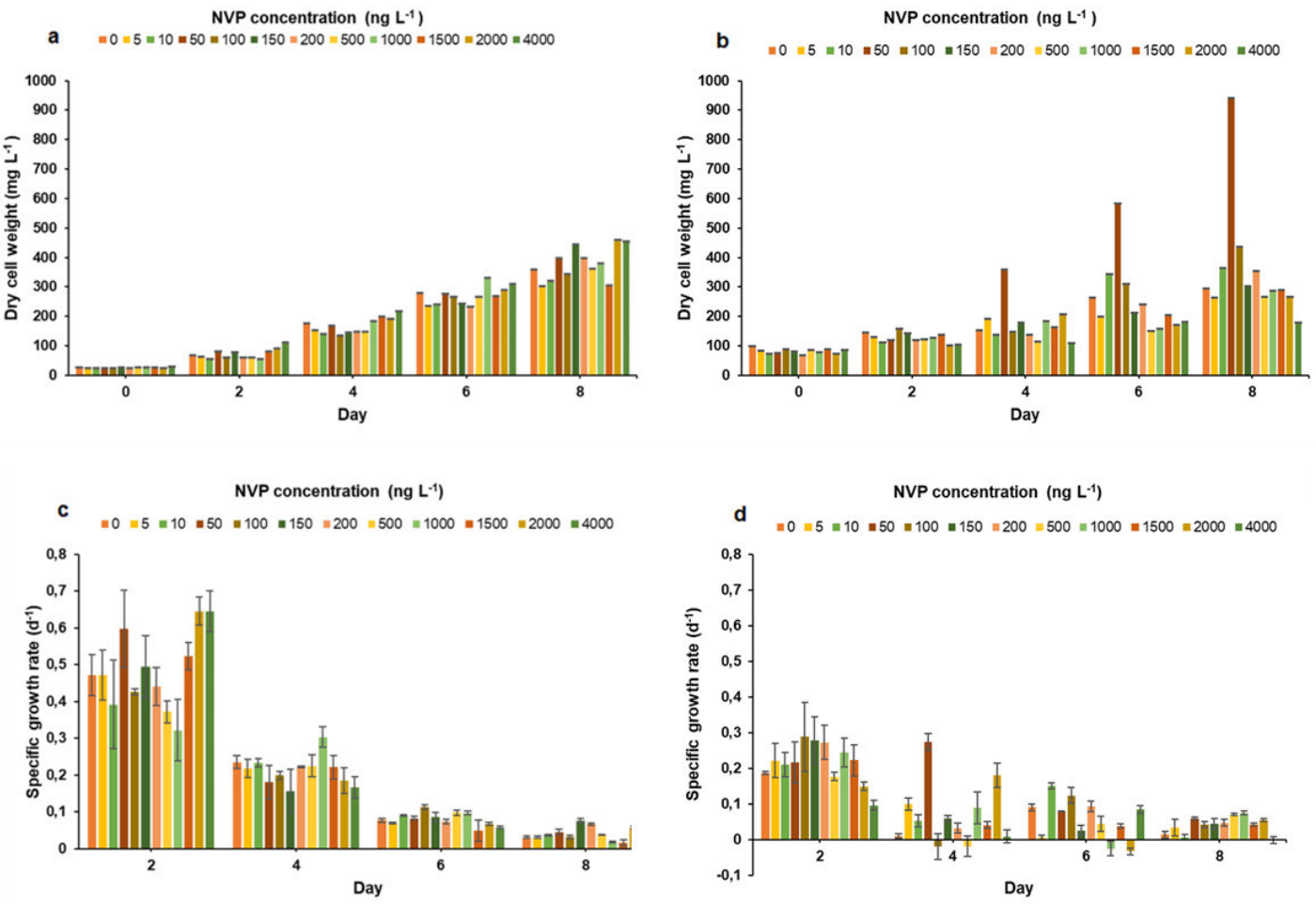


Figure 4.3 Dry cell weight and specific growth rate of *Coelastrella tenuitheca* (**a**, **c**) *Tetradesmus obliquus* (**b**, **d**) under different NVP concentrations during 8 days of cultivation.

The SGR of *C. tenuitheca* and *T. obliquus* during the 8 days of cultivation in different concentrations of NVP are illustrated in Fig. 4.3 (c) and (d). The SGR of *C. tenuitheca* was 5% greater than the control group on day 2 for concentrations above 1000 ng L^{-1}, thereafter, a gradual decrease in SGR was noted for all the treatments. However, on day 4, 6 and 8, the treatments (100, 150, and 200 ng L^{-1}) displayed a higher SGR compared to the control. A similar trend was noted for *T. obliquus*; where an increase of 97% in SGR was noted as compared to the control at 50 ng L^{-1}. The findings of the study were in coherence with a study by Escapa *et al.* (2017), demonstrating similar growth responses in microalgae on the addition of paracetamol in the culture medium. The SGR of *C. vulgaris* was 1.3 times higher on the dose addition of paracetamol (25 mg L^{-1}) as compared to the control (Santos *et al.*, 2017). Similarly, Tong *et al.* (2020), observed an increase of 20% in *Coelastrella* sp. growth in the

presence of tetracycline than the control. Thus, it is important to analyse the hormesis biphasic response of the PCs to the microalgal strains to evaluate their tolerance level.

4.3.2. Effect on photosynthetic performance of microalgae

Chlorophyll plays a key role in microalgal photosynthesis, including capturing light and transporting electrons (Wang *et al.*, 2022b). The changes in growth of microalgae during exposure to toxic compounds can be determined by chlorophyll biosynthesis (Xiong *et al.*, 2017c). In this study, cultures of *C. tenuitheca* and *T. obliquus* exposed to various NVP concentrations were assessed for changes in photosynthetic performance at late log phase (Table 4.2). The addition of NVP (up to 4000 ng L^{-1}) improved the chlorophyll content and photosynthetic performance of *C. tenuitheca*. In *C. tenuitheca*, the highest total chlorophyll content of 10.78 mg L^{-1} was observed at an NVP concentration of 1500 ng L^{-1}.

Table 4.2 The effect of NVP exposure on photosynthetic pigment content of *Coelastrella tenuitheca* and *Tetradesmus obliquus*.

NVP concentration (ng L^{-1})	**Total chlorophyll (mg L^{-1}) content in late log phase**	
	Coelastrella tenuitheca	***Tetradesmus obliquus***
0	5.29 ± 0.07	3.59 ± 0.03
5	6.41 ± 0.11	1.88 ± 0.02
10	6.17 ± 0.02	3.51 ± 0.01
50	5.79 ± 0.08	4.25 ± 0.04
100	8.29 ± 0.13	2.15 ± 0.01
150	8.23 ± 0.14	4.31 ± 0.03
200	3.83 ± 0.07	3.26 ± 0.02

500	6.54 ± 0.12	4.32 ± 0.05
1000	7.89 ± 0.10	2.85 ± 0.02
1500	10.78 ± 0.10	2.75 ± 0.02
2000	9.54 ± 0.11	2.53 ± 0.03
4000	5.34 ± 0.01	2.16 ± 0.01

Most of the treatments (5–150 ng L^{-1} and 500–2000 ng L^{-1}) in *C. tenuitheca* cultures displayed higher chlorophyll content than that of the control (5.29 mg L^{-1}). An increase in total chlorophyll content in *C. tenuitheca* is an indication of the protective mechanism of the culture to neutralize excess reactive oxygen species in the chloroplast for alleviating toxicity and maintaining stability of microalgal cells (Zhou *et al.,* 2021). *Tetradesmus obliquus,* however, revealed a decrease in chlorophyll content with increasing NVP concentrations (200–4000 ng L^{-1}) in the growth medium. A reduction in photosynthetic pigments is a common stress response to contaminants including PCs due to the toxicity, peroxidation of the thylakoid lipids and the degradation of PSII complexes (Xiong *et al*., 2016).

Microalgae photosynthetic electron transport rate can be measured non-invasively using pulse amplitude modulation (PAM) fluorometry. Photochemical energy is converted by PS II reaction centres by yielding fluorescent light, which is a direct measure of photochemical energy (Ramanna *et al.,* 2018). The fluorescence parameters (i.e. Fv/Fm) of chlorophyll are generally used to assess PSII activity since they are predominantly sensitive to stress (Wang *et al.,* 2020). In this study, the Fv/Fm values at the start of experimentation for microalgal strains showed values below 0.5. The highest Fv/Fm ratio of 0.693 and 0.669 was observed in *C. tenuitheca* and *T. obliquus* cultures at NVP concentrations of 50 ng L^{-1} and 1000 ng L^{-1} in the late log phase of growth, respectively, which was significantly higher than the control. It was observed that Fv/Fm increased with increasing NVP concentrations over time, showing that the addition of NVP in the medium did not affect the photosynthetic capacity of both the microalgae at the mid log (day 6) phase of growth (Fig. 4. 4a and b).

However, at day 8, a gradual decrease in Fv/Fm in *T. obliquus* was observed at NVP concentrations above 1000 ng L^{-1} when compared to that of lower concentration of NVP (50

ng L^{-1}). A previous study conducted by Wang *et al.* (2020), also observed the effect of non-steroidal anti-inflammatory drugs on the photosynthetic rate in *Scenedesmus obliquus*. The results obtained from their study showed a similar pattern of decrease in Fv/Fm ratio. They concluded that PCs could exert a significant toxic effect on the photosynthetic system of *S. obliquus* and inefficient cell assimilation may disrupt carbon fixation and adsorption, affecting microalgal photosynthetic efficiency. The Fv/Fm values of *C. tenuitheca* displayed more favourable findings, with no significant effect to the increasing concentrations of NVP at the mid log (day 6) and late log phase of growth (day 8). The photosynthetic efficiency of *C. tenuitheca* showed an improvement in Fv/Fm on day 4 ranging from 0.49 to 0.56 in the treatment groups as compared to 0.52 in the control. Additionally, an increase in exposure time (day 8) did not result in a decrease in the photosynthetic performance in *C. tenuitheca*, as the Fv/Fm values in the treatment groups remained within 0.66–0.69, which were either comparable or higher to the control (0.67). The highest Fv/Fm ratio of 0.693 was observed in a microalgal treatment supplemented with NVP concentration of 1000 ng L^{-1} at the late log phase. These results reflect the tolerance abilities of *C. tenuitheca* to NVP exposure at different environmental concentrations (up to 4000 ng L^{-1}).

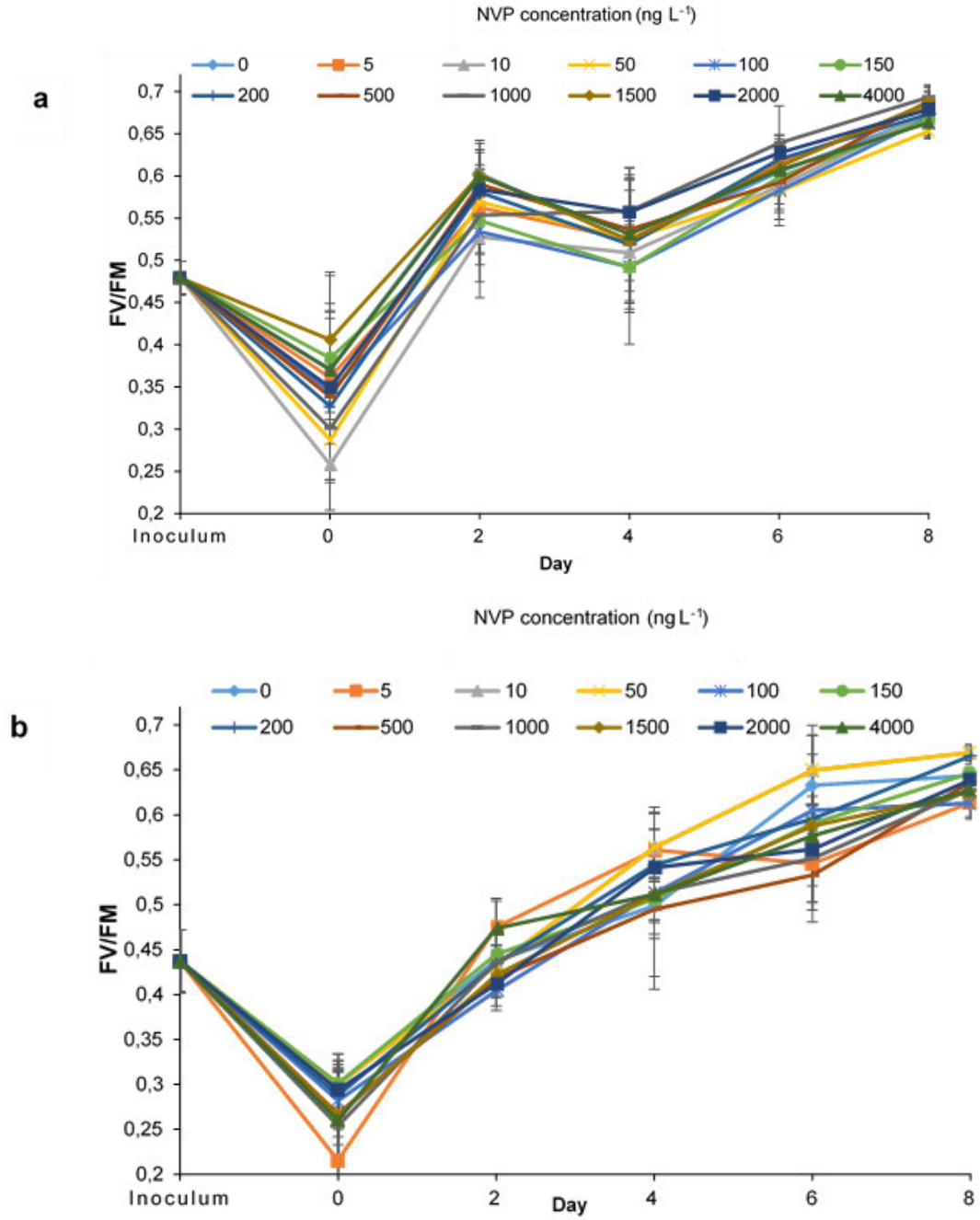


Figure 4.4 The maximum quantum yield of PS II (Fv/Fm) recorded in the treatments with different NVP concentrations in *Coelastrella tenuitheca* (**a**) and *Tetradesmus obliquus* (**b**) cultures during 8 days of cultivation.

The photosynthetic electron transport rate (rETR) was also evaluated at two-day intervals (Fig. 4.5 a and b). In light intensity, PSII's apparent electron transport efficiency is measured by the rETR (relative electron transfer rate) (Wang *et al.*, 2020). Relative electron transfer rate values increased with the addition of NVP over time, indicating that no inhibition of the electron transport process was observed. When *C. tenuitheca* and *T. obliquus* were grown in

standard BG-11 medium (control), the rETR values on day 8 were 51.43 ± 0.42 and 56.90 ± 4.43 respectively.

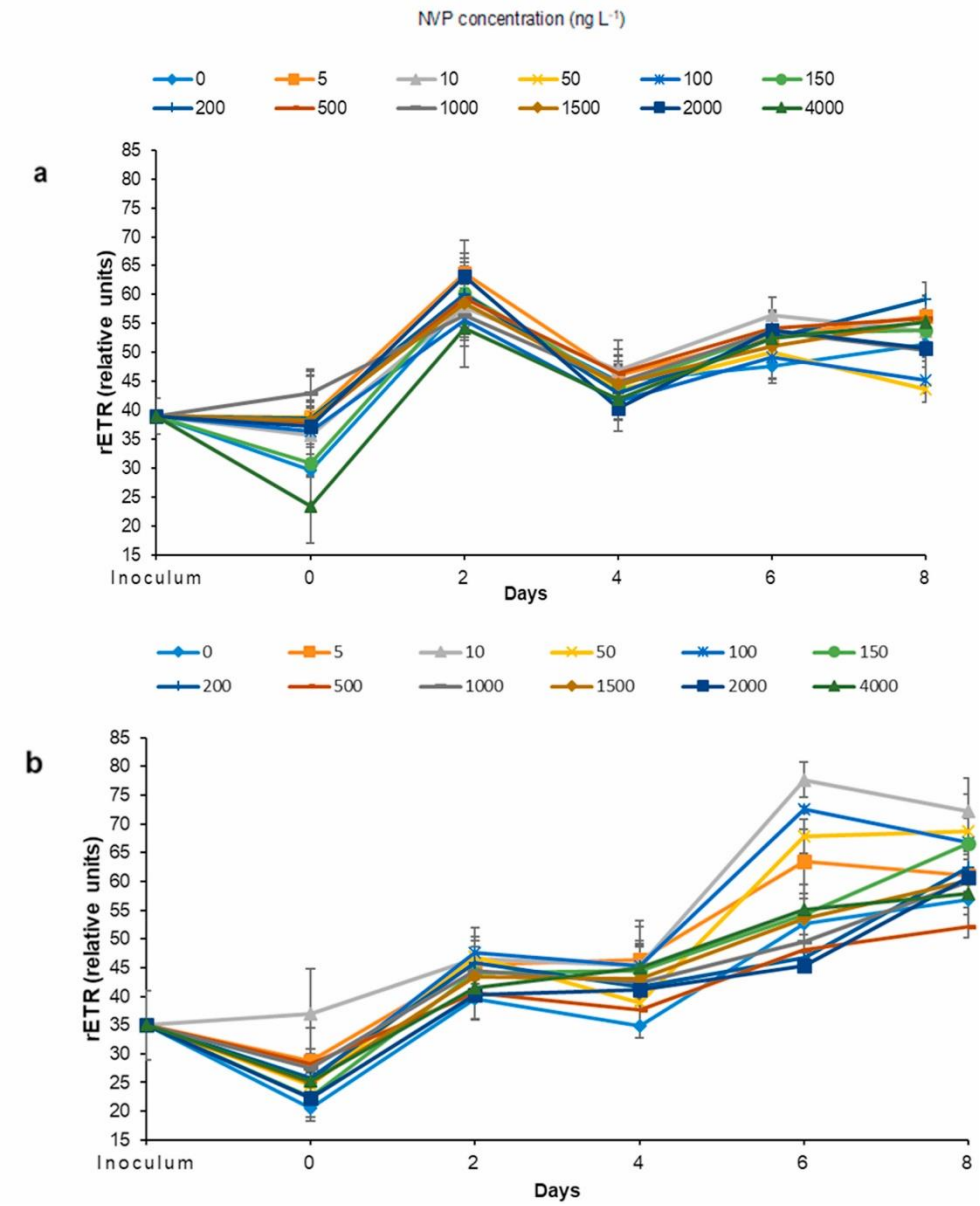


Figure 4.5 The electron transport rate (rETR (II)) in *Coelastrella tenuith*eca (**a**) and *Tetradesmus obliquus* (**b**) recorded under the different NVP concentrations during 8 days of cultivation.

However, when compared to the treatments with concentration range of 5–200 ng L^{-1} NVP, the rETR values were improved in the range of 53.7 ± 6.26 to 59.23 ± 6.26 and 61.07 ± 4.12 to 72.13 ± 5.84 for *C. tenuitheca* and *T. obliquus*, respectively. The photosynthetic performance of *C. tenuitheca* and *T. obliquus* was not hampered at NVP concentrations above 500 ng L^{-1}, which is evident from the higher rETR values. Thus, we can conclude that the

chlorophyll fluorescence parameters were not considerably changed, signifying that the PSII system was not greatly affected, and electron transfer was not significantly impaired in microalgae on exposure to environmental concentrations of NVP.

4.3.3. Removal of nevirapine by Coelastrella tenuitheca and Tetradesmus obliquus

In the present study, NVP removal efficiency of *C. tenuitheca* and *T. obliquus* was evaluated till late log phase. The concentrations of NVP decreased over time in the medium in the tested microalgal cultures (Fig. 4.6). Furthermore, <20% reduction of NVP was observed from photodegradation and/or volatilization in the control. While, the removal of NVP by *C. tenuitheca* and *T. obliquus* were 19.53–49.23% and 27.54–74.56%, respectively. A slight decrease in the NVP removal potential was observed with an increase in NVP concentration for microalgal species. The average NVP removal potential of *T. obliquus* was observed to be higher than that of *C. tenuitheca*. Specifically, on the 4th day (exponential growth phase), the removal of NVP at concentrations of 50, 100 and 150 ng L^{-1} by *T. obliquus* was 61.73, 53.89 and 42.50%, while that by *C. tenuitheca* was 25.85, 18.82 and 21.44%, respectively. As a result, it is hypothesized that *T. obliquus* has a higher NVP removal potential in aqueous environments. The Levene's homogeneity test was conducted to determine the significant differences between both microalgal strains for all treatments at a daily and accumulative removal rate resulting in p value < 0.001. This result was in accordance with a study conducted by Escapa *et al.* (2017). *Tetradesmus obliquus* had shown to have higher removal efficiencies of salicylic acid than using *C. vulgaris* in batch culture.

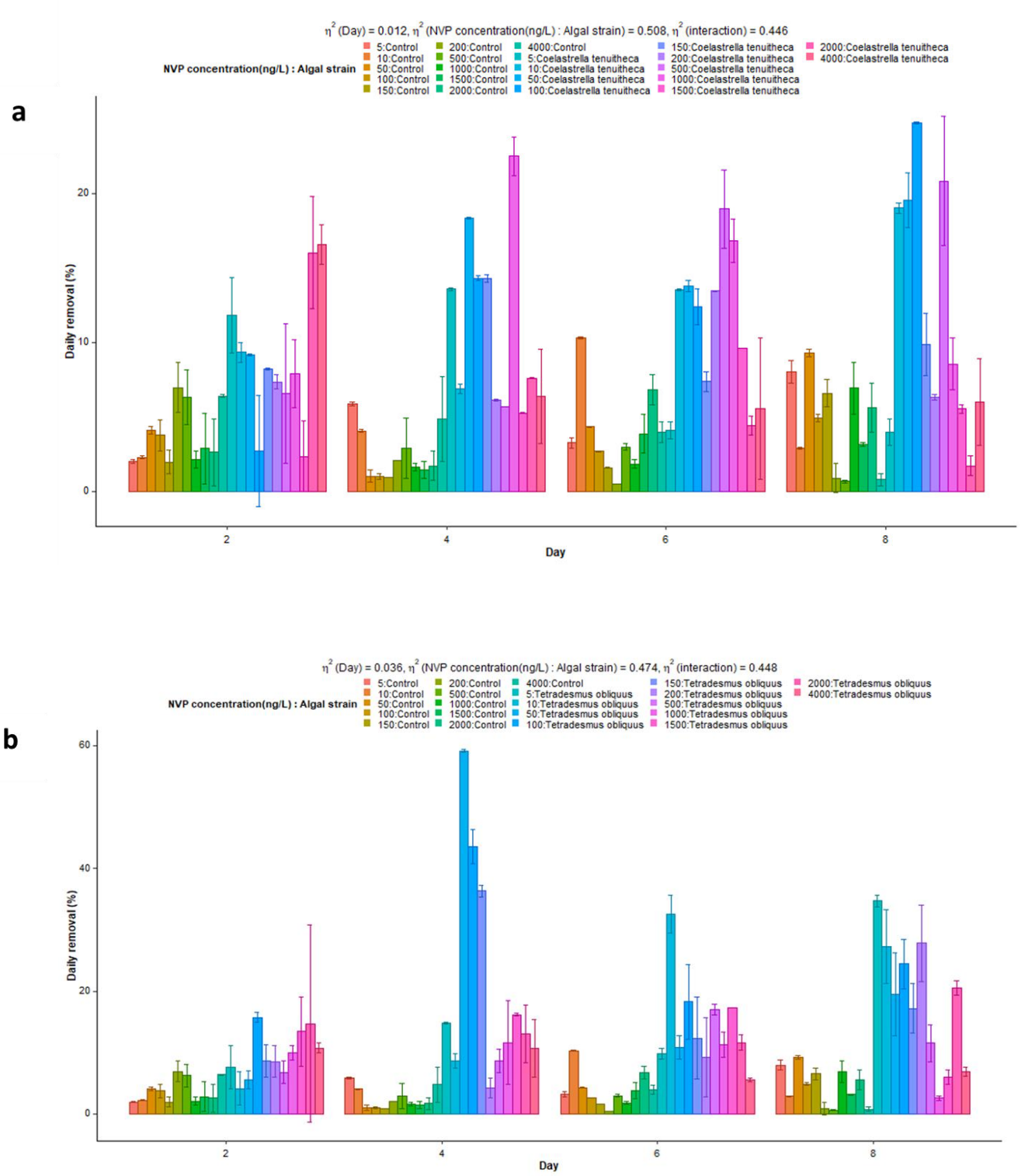


Figure 4.6 Removal of NVP in BG-11 medium by *Coelastrella tenuitheca* (**a**) and *Tetradesmus obliquus* (**b**) over a period of 8 days.

Due to the complexity of the structure and properties of ARV drugs, their removal mechanisms are presumed to include photolysis, adsorption, absorption, bioaccumulation, and biodegradation (Xiong *et al.*, 2018). However, the most likely removal mechanism of NVP could be through biosorption and bioadsorption, which generally take place over time during the algal growth phases (Bilal *et al.*, 2018, Li *et al.*, 2017). This is due to the presence of the positively charged amino group in NVP that makes the compound cationic. A recent study conducted by Hifney *et al.* (2021), evaluated the biosorption capacity of ketoprofen and diclofenac by living cells of *Chlorella* sp. They concluded that the number of biosorption sites available on the cell surface decreased over time, thus promoting the adsorption potential of the drugs. In an earlier study conducted by Peng *et al.* (2014), it was noted that increasing biomass concentrations may result in more adsorption of contaminants during the incubation of live algae.

The microalgal cell wall is negatively charged due to the dominant functional groups (Peng *et al.*, 2014), and thus bioadsorption can be more effective with PCs containing cationic groups that are actively attracted towards the microalgal surface through electrostatic interactions (Ayangbenro and Babalola, 2017). Papazi *et al.* (2019), described the comparative biodegradation of phenolic compounds by *S. obliquus* to determine the microalgal bioenergetic strategy. Their work demonstrated that microalgae manage their energy reserves rationally by investing them, either on growth or on the biodegradation of the toxic compounds. These conclusions can be directly related to the results in this study that microalgae may follow two distinct detoxification strategies; (1) The use of energy reserves to increase the biomass production, in the presence of low toxic compounds, which was demonstrated by *T. obliquus* and, (2) The microalgae invest more, or all of their energy reserves directly involved in the biodegradation of the toxic compounds, while less or no energy is invested in biomass increase, which was evident in the growth of *C. tenuitheca*. Thus, suggesting that selecting the appropriate strain of microalgae for the removal of pollutant compounds is a prerequisite and of utmost importance.

4.3.4. Effect of nevirapine on microalgal cell morphology

The morphology and the elemental surface composition of *C. tenuitheca* and *T. obliquus* surface was analysed using scanning electron microscopy (SEM) before and after (Day 8) NVP exposure (Fig. 4.7). The SEM micrographs of the control cultures of *C. tenuitheca* and *T. obliquus* reveal a smooth cell wall. The cells appeared to form ridges on the cell surface on NVP exposure, however, the cell shape of *C. tenuitheca* was observed to be distinctly intact, while *T. obliquus* displayed an irregular shape. Similarly, Bano *et al.* (2021) reported that the addition of estradiol (a steroid hormone) caused wrinkling and deformation in the cell wall, which was not seen in the control microalgal cells. The oxidative radicals generated by the pharmaceuticals can damage the lipid cell membrane of microalgae and damage its fluidity and integrity, thereby leading to membrane distortion (Ahmad *et al.*, 2022, Thiagarajan *et al.*, 2019). According to Thiagarajan *et al.* (2019), the exposure of n-TiO2 and tetracyclines to *Chlorella* sp. damaged the integrity of the algal cell wall and resulted in an irregular external structure.

In this study, the shape of *T. obliquus* displayed the greatest structural changes when exposed to 50 ng L^{-1} NVP, this could be attributed to the highest removal of NVP by this strain. Due to the complexity of the structure and properties of ARV drugs, the most likely removal mechanism of NVP could be through adsorption (Reddy *et al.*, 2021). This is due to the presence of the positively charged amino group in NVP that makes the compound cationic. The dominant functional groups such as carboxyl, phosphoryl, and amine groups on the algal cell wall contribute to the net negative charge (Peng *et al.*, 2014). Consequently, PCs containing cationic groups can be actively attracted towards the algal surface through electrostatic interactions (Ayangbenro and Babalola, 2017). Hence, it can be presumed that cationic ARV drugs would have a high affinity for the adsorption of negatively charged algal cell walls. Adsorption is an extracellular process that is influenced by the type of algal species and the hydrophobicity, functional groups, and chemical structure of the compounds (Ghernaout, 2014, Reddy *et al.*, 2021).

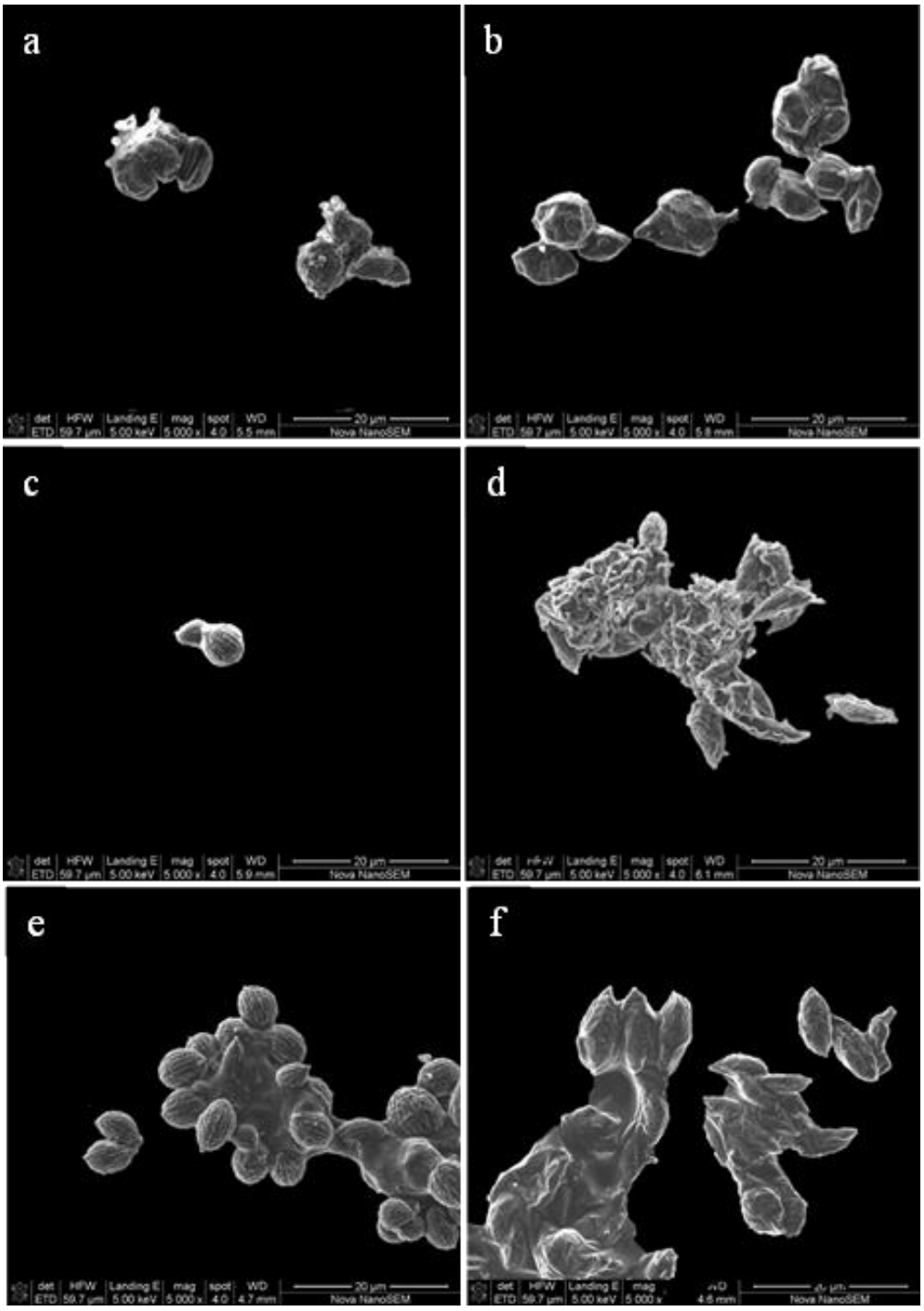


Figure 4.7 Scanning electron micrographs of *Coelastrella. tenuitheca* and *Tetradesmus obliquus* biomass before and after NVP biosorption. **a**. *C. tenuitheca* (control); **b**. *T. obliquus* (control); **c**. *C. tenuitheca* (50 ng L^{-1} NVP); **d**. *T. obliquus* (50 ng L^{-1} NVP); **e**. *C. tenuitheca* (4000 ng L^{-1} NVP); **f**. *T. obliquus* (4000 ng L^{-1} NVP).

4.4 Conclusion

Both microalgal species, *C. tenuitheca* and *T. obliquus* efficiently removed NVP at lower concentrations in an aqueous environment during the 8-day cultivation period. Microalgal growth and photosynthetic efficiency were not significantly impacted by environmental concentrations of NVP. A higher growth rate was observed in *T. obliquus* during NVP exposure compared to *C. tenuitheca*, which also displayed the highest NVP removal efficiency of 75%. No significant difference in chlorophyll fluorescence parameters in *C. tenuitheca* culture was observed. An increase in chlorophyll content at certain concentrations of NVP could have played a protective role in preventing oxidative damage. The obtained results extrapolate the use of microalgae for the bioremediation of ARV drugs in the aquatic environment, such as wastewater. It was observed that both species responded differently to NVP concentrations. Even though, *T. obliquus* showed high removal efficiency, *C. tenuitheca* could tolerate higher concentrations of NVP. Therefore, the results of this study suggest that NVP removal is highly species-specific, thus indicating the need for appropriate strain selection. The present study can be a basis for developing microalgae-based technologies for the treatment of ARV drugs in the aquatic environment. Further research can be directed to assess the biochemical and molecular mechanisms involved in the removal of NVP by microalgae cultivated in wastewater at different NVP concentrations. Moreover, in-depth investigations are needed to analyse the metabolites and degradation products in the microalgae based NVP removal process.

CHAPTER FIVE: Optimization of culture conditions for higher nevirapine removal by T*etradesmus obliquus* & determination of potential mechanisms of nevirapine remediation

5.1 Introduction

In microalgae, organic pollutants may serve as major substrates for cell development and trigger enzyme production for metabolism (Vo *et al.*, 2020a). For example, the cytochrome P450 enzymes are responsible for the transformation of parent compounds into less toxic molecules to make them more hydrophilic (Nguyen *et al.*, 2021, Liu *et al.*, 2021). Biodegradation/biotransformation of chemical pollutants by microalgae involves a variety of enzymatic reactions. Additionally, enzymes are produced in the antioxidant defence system (Superoxide dismutase, catalase, glutathione peroxidase and ascorbate peroxidase) to reduce toxic stress and cell damage (Singh *et al.*, 2021, Coulombier *et al.*, 2021). Although biodegradation appears promising, some pollutants cannot be utilized efficiently as carbon sources as they do not provide sufficient metabolic energy for microalgae (Baghour, 2019). Thus, carbon supplementation can be a useful strategy to improve the efficiency of microalgae for organic pollutants including PCs. During carbon supplementation such as glucose, saccharose, or acetate, an organic substrate (such as pharmaceuticals) is transformed by microalgae (Vo *et al.*, 2020a). In addition, microalgae have been studied extensively for their ability to remediate PCs under phototrophic conditions (Ding *et al.*, 2020, Zhang *et al.*, 2019, Larsen *et al.*, 2019, Lam *et al.*, 2022). In Chapter 3, it was found that *T. obliquus* was able to remove 74.56% NVP under the phototrophic mode of cultivation Nevertheless, the understanding of ARV drug removal under heterotrophic and mixotrophic culture conditions remains limited, and the biochemical and molecular mechanisms responsible for the removal and tolerance of ARV drug stress are yet to be explored. Therefore, this objective was aimed to evaluate the NVP removal potential of *T. obliquus* under autotrophic, heterotrophic and mixotrophic batch culture conditions. The effect of NVP on the growth rate of *T. obliquus* was evaluated. Moreover, metabolomic analysis and the expression of antioxidant genes *sod1*, *gpx1* and *cat2* were monitored during the mid and late log growth phases to examine the molecular level responses to NVP under different cultivation conditions.

5.2 Materials and methods

5.2.1 Microalgae culture conditions and NVP removal

The microalgal growth conditions of *T. obliquus* (MH307949.1) was described in chapter 4, section 4.2.2. *Tetradesmus obliquus* was evaluated for its NVP removal performance at the highest reported environmental concentration of NVP in South African wastewater effluent (4000 ng L^{-1}) (Ngumba *et al.*, 2020). At an initial optical density (OD) of 0.1 at 680 nm, the microalgal isolate *T. obliquus* was inoculated into 1 L conical flasks containing 500 mL BG-11 medium (pH 7) supplemented with NVP (4000 ng L^{-1}) and was cultivated for 8 days under three different growth modes. For autotrophic cultivation, the cultures were grown under standardized laboratory conditions at temperature as described in chapter 3, section 3.2.2. For mixotrophic and heterotrophic cultivation, glucose (6 g L^{-1}) was used as an organic carbon source, in the presence and absence of light, respectively. (Bentahar and Deschênes, 2022). To quantify the removal of NVP through photolysis, similar concentration of NVP (4000 ng L^{-1}) was supplemented in a control group without algal inoculum. All treatments were analysed in triplicates.

5.2.2 Measurement of cell density and specific growth rate

The growth of the isolates was evaluated by specific growth rate and dry cell weight according to the equation 4.2 and 4.3 (Chapter 4) (Wang *et al.*, 2022a).

5.2.3 Liquid Chromatography with tandem mass spectrometry

The removal mechanisms of ARV drugs by microalgae were evaluated following the predicted mechanisms (Xiong *et al.*, 2017a). A 2 mL aliquot of microalgal suspension was withdrawn at regular time intervals during cultivation (days 2, 4, 6, 8). The harvested cells were centrifuged for 10 min at 15,000 rpm (Digital Pro Haematocrit E-C14-H24P), and the supernatant was filtered using a 0.22 µm membrane filter. The filtrate was placed into sample vials and the residual concentrations of NVP in the aqueous medium were analysed. On the 8^{th} day of cultivation, 50 mL of each sample was recovered for the evaluation of the removal mechanisms (bioadsorption, bioaccumulation, and biodegradation) of NVP by *T. obliquus*.

The harvested microalgae cell pellets were suspended in distilled water (5 mL) and centrifuged at 15,000 rpm for 10 min. The supernatant was recovered for the analysis of the concentration of NVP adsorbed onto the cell surface (Xiong *et al.*, 2016). The residue cell pellets were suspended by adding 5 mL of dichloromethane: methanol (1:2 v/v) and sonicated for 1 h (40 kHz, 2.2 Kw). Thereafter, the supernatant was recovered by centrifugation at 15,000 rpm for 10 min and used to determine the concentration of bioaccumulated NVP from the lysed cells. Chromatography was performed in accordance with the previously described method in chapter 4, section 4.2.2.2.3. The calculation of biodegradation (P_b) of NVP in the *T. obliquus* culture was conducted using the following equation 5.1 (Xiong *et al.*, 2016).

$$P_b(\%) = \left(A_i - A_r - A_d - A_p - A_u\right)\frac{100}{A_i}$$

Equation 5.1 Mass balance

Where A_i is the initial concentration of NVP added to BG-11 medium, A_r is the residual quantity of NVP in the BG-11 medium, A_d is the amount of NVP adsorbed by the microalgal cells, A_p is the amount of NVP removed by photodegradation, and A_u is the uptake amount of NVP in the microalgal cells.

5.2.4 Untargeted analyses of NVP biotransformation products by high-resolution mass spectrometry

Nevirapine biotransformation products were analysed using mixotrophic samples on day 4 and 8 as these samples showed the highest NVP removal. Analyses were performed by Waters Cyclic Select coupled to a Waters UPLC (LC-MS) to identify the maximum of unknown degradation products. Sample were drawn on day 4 and 8 and extracted according to the method described in section 5.2.3. 0.5 µL of undiluted sample was injected on a Acquity UPLC HSS T3 column (1.8µm; 2.1 x 100mm). The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. A gradient method (Table 5.1) was used for separation of analytes. mzMine software was used to analyse the total ion chromatograms for all samples. Peaks that appeared in both the control and treated samples were disregarded and the unknown peaks were tabulated. The web-based software app ChemCalc (https://www.chemcalc.org/mf_finder/mfFinder_em_new) was then be used to calculate elemental composition possibilities using the accurate mass value of the compound from the spectra.

Table 5.1 Liquid chromatography gradient profile.

Time (min)	Flow	Rate	Mobile phase A (%)	Mobile phase B (%)
1	Initial	0.35	100	0
2	0.5	0.35	100	0
3	12	0.35	0	100
4	13	0.35	0	100
5	13.1	0.35	100	0
6	15	0.35	100	0

5.2.5 Fourier-transform infrared spectroscopy (FTIR) analysis of microalgae biomass

The cellular physiological responses were determined with FTIR micro-spectroscopy in the mid (Day 4) and late log (Day 8) phases of growth. A PerkinElmer Spectrum two instrument equipped with a diamond crystal iATR reflectance cell was used with a DTGS detector scanning over the wavenumber range of 4000–450 cm^{-1} at a resolution of 4 cm^{-1}. Algal liquid biomass was applied to the surface of the crystal and then pressed onto the crystal head. The

spectroscopic software spectrum (version 10 PerkinElmer, Germany) was used for recording the scans. A plot of the integrated area versus the percentage of the biochemical group (% DW) was used to select absorption bands (Mayers *et al.*, 2013).

5.2.6 Gene expression studies

5.2.6.1 Extraction of total RNA and synthesis of cDNA

Biomass was harvested by centrifugation at 4000 rpm at mid-log phase (day 4) and late log phase (day 8) of each cultivation period. Wet biomass of approximately 50 mg was ground in liquid nitrogen with a pestle and mortar. Thereafter, total RNA was extracted from the powdered sample using the QIAGEN Rneasy Mini Kit. The total RNA was evaluated by electrophoresis using 1.2% agarose gel and by spectrophotometric analysis (Implen NanoPhotometer®) to evaluate the quality and quantity respectively. The first-strand of cDNA was synthesised from the total extracted RNA, using a QuantiTect® Reverse Transcription Kit (Qiagen). The cDNA obtained was used as a template for the subsequent qPCR reactions (Singh *et al.*, 2017).

5.2.6.2 Real-time quantitative polymerase chain reaction (RT-PCR)

Real-time polymerase chain reaction was used to analyse the expression of the selected genes, superoxide dismutase (*sod1*), glutathione peroxidase (*gpx1*) and catalase (*cat2*). The 18S rRNA gene was used as a reference gene for the RT-PCR assay normalization (Table 5.2). The CFX96 TouchTM Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) was used for the RT-PCR assays. The reaction mix contained 12.5 µL SsoFast™ RT-PCR Supermix with EvaGreen (Bio-Rad USA), 0.4 µM forward and reverse primers and 20 ng template DNA in a final volume of 25 µL. Sterile Milli-Q water was used as a substitute for DNA template in RT-PCR assays used as a negative control. The RT-PCR protocol for the antioxidant genes consisted of initial denaturation for 10 min at 95 ℃, followed by 40 cycles of three steps consisting of 10 s at 95 ℃, 10 s at suitable annealing temperatures and 30 s at 72 ℃ and final elongation 10 min at 72 ℃ (Singh *et al.*, 2017). Optimized annealing temperature for each primer is mentioned in Table 5.2. The 2-ΔΔCT method was used to analyse the relative gene expression (Rao *et al.*, 2013). The data obtained represented the fold change (increase or decrease) of the target gene in the treated samples of *T. obliquus*

(autotrophic, mixotrophic and heterotrophic) compared to the control sample and the expression of the reference gene (18s rRNA) was used for normalization.

Table 5.2 Oligonucleotide sequences of primers used for relative gene expression studies.

Primer	Sequence (5'-3')	Optimized annealing temp. (°C)	Reference
18S rRNA	(F) CGGTCCGCCTATGGTGAGTA (R) CTCCGGTCCTACAGACCAACA	62	Gao et al., 2021
sod1	(F) ACG GCT CCC TGT CGA TCG A (R) CGC CAC GTC CGG CAG CGC GG	50	Wang et al., 2008
gpx1	(F) CGAAGCCGCACATGGTATAGT (R) TGCTCCAATCACGACCTATTTG	50	Wang et al., 2008
cat2	(F) GGAGGCTGCAGGAAAACTGA (R) ATTTCCAGCCTGGGCTACCT	50	Wang et al., 2008

F- Forward; R- Reverse, Temp- temperature (°C)

5.2.7 Metabolic Profiling of T. obliquus Response to NVP Using GC-MS-Based Untargeted Analysis

Metabolic changes in *T. obliquus* exposed to 4000 mg L^{-1} NVP were determined by untargeted metabolomics. Both control and treated *T. obliquus* (i.e with and without NVP) were assessed at Day 4, and 8 (mixotrophic growth) to evaluate the variation in metabolite levels during the algal growth phase. Samples (50ml) were frozen at – 80 °C for 24 h and were extracted in 80% methanol containing 2% formic acid (Lui *et al*., 2020). The algal cells were resuspended and vortexed for 60s, followed by 15 min of ultrasonication in a sonication bath. The mixture was centrifuged at 4˚C for 15 min at 10,000 x g. The supernatant was filtered through a 0.22µm filter. Metabolite analysis was performed on an Agilent 7890 GC coupled to a Leco Pegasus 4D (GCxGC-TOF-MS) mass spectrometer. A sample volume of 1 uL was injected in the injection port with a (Topaz liner, splitless 4mm x 6.5 x 78.5) using a Gerstel Multi-Purpose Auto sampler. The column used for analysis was Rxi 5 Sil MS, length 29.050 m of (30 m), the internal diameter 250 µm, and film thickness of 0.25 µm with a maximum temperature of 350 ˚C. Carrier gas was helium; front inlet type was split/splitless with a

splitless mode activated. The flow rate was 1.6 ml/min at constant flow while front inlet temperature was 180 ˚C. The initial oven temperature of 60 ˚C for maintained for 0.2 min and ramped at 20 ˚C /min to 180 ˚C for 3 min and finally ramped at 15 ˚C /min to 280 ˚C. Transfer line temperature was 295 ˚C. The acquisition delay of 3 min, mass range of between 35 to 800 m/z, with acquisition rate of 20 spectra/second, acquisition voltage at 1500 eV (300 eV offset) and electron energy (ionization energy) of -70 eV was used. The ion source temperature was 270 ˚C. The data processing methods used were as: The base line offset of 1 (just above the noise) was used, with a peak width of 4 secs and signal to noise (S/N) ratio of 10. The library search mode was normal and forward. The number of library hit was 10, while the minimum molecular weight allowed was 35 and maximum molecular weight was 800. Mass threshold was 50% and minimum similarity before name was assigned was 50%. The library used was Replib and mainlb from National Institute of Standards and Technology (NIST).

5.2.8 Data analysis

One-way analysis of variance and Kruskal-Wallis Test was used for the statistical analysis of the data by using SigmaXL® Version 9.0 software package. Results are presented as mean ± standard deviation. Differences among values were considered significant when p value was < 0.05. For metabolomic analysis, data sets were organized into a three-dimensional matrix, combining metabolites and samples, and were auto scaled for normalization. Metaboanalyst (v 5.0) was used for multivariate statistical analysis, which included one-way ANOVA to identify significantly changed metabolites. Additionally, principal component analysis was conducted to examine the clustering behaviour of crucial metabolites.

5.3 Results and discussion

5.3.1 Effect of NVP on microalgae growth in different cultivation modes

The growth curves of *T. obliquus* under different cultivation modes and the corresponding controls are represented in Fig. 5.2. It was observed that *T. obliquus* grew progressively in the presence of NVP under all the cultivation modes. All the treatments displayed a significant increase ($p < 0.05$) in DCW compared to their corresponding controls. As compared with

autotrophy and heterotrophy, mixotrophic cultivation of *T. obliquus* significantly improved biomass production. The highest DCW of 1503.47 mg L^{-1} and 1664.90 mg L^{-1} was obtained on day 8 in the mixotrophic conditions with and without NVP, respectively (Fig. 5.3a). While the lowest average DCW of 71.48 mg L^{-1} was observed in the presence of NVP under autotrophic cultivation mode, whilst the autotrophic control group without NVP obtained a DCW of 144.56 mg L^{-1}. Under heterotrophic growth, the addition of NVP enhanced the growth of *T. obliquus* (214.44 mg L^{-1}), which was 1.6 times higher than the control (130.91 mg L^{-1}) on day 8. As an organic carbon source, glucose demonstrated a positive effect on biomass production during mixotrophic and heterotrophic growth. It was further noted that the mixotrophic alga cells entered exponential phase after three days of cultivation, while autotrophic alga cells continued to grow slowly.

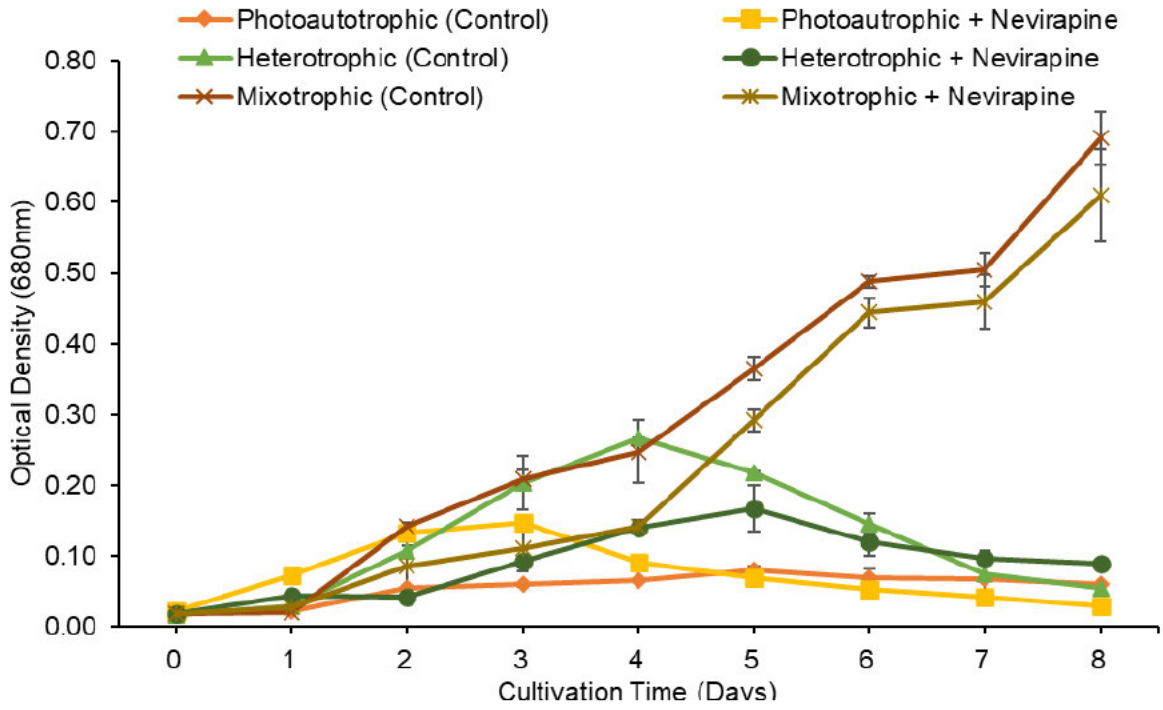


Figure 5.2 Effect of different cultivation conditions on growth of *Tetradesmus obliquus* in BG-11 medium supplemented with NVP.

The maximum SGR of *T. obliquus* was noted on day 2 for all the treatments (Fig. 5.3b). The highest SGR was observed in the mixotrophic control group (1.03 d^{-1}), followed by the mixotrophic cultures with NVP (0.89 d^{-1}). The lowest specific growth *T. obliquus* was found in the heterotrophic culture with NVP (0.43 d^{-1}) followed by the control (0.92 d^{-1}) under heterotrophic conditions. The Kruskal-Wallis test revealed a significant difference ($p=0.04$) in SGR values in mixotrophic growth compared to autotrophic cultivation, which was lower

in the treatments supplemented with NVP. A linear growth rate of (0.45 d^{-1}) was observed in autotrophic cultures (control), but an exponential growth rate of 0.88 d^{-1} was observed in mixotrophic cultures with NVP. It is consistent with the findings of Wang *et al*. (2022b) and Vo *et al*. (2020b), in which glucose as the carbon source improved microalgae growth in the presence of micropollutants. In microalgae, glucose is reported to produce more energy per mole than other carbon sources (xylose, rhamnose, fructose, sucrose, and galactose) (Zhan *et al.*, 2017, Gim *et al.*, 2014). In microalgae, the Embden-Meyerhof pathway (EMP) and pentose phosphate (PP) pathways are the only two pathways involved in the oxidative assimilation of glucose (aerobic glycolysis) (Sun *et al.*, 2018). Under dark conditions, glucose is primarily metabolized by microalgae via the PP pathway, whereas under light conditions, it is metabolized via the EMP pathway (Perez-Garcia *et al.*, 2011).

Under mixotrophy, microalgae gain an advantage as they can utilize both pathways during cultivation to maximize substrate utilization. In mixotrophic cultivation conditions, microalgae require light both to fix CO_2 through photosynthesis and to bind organic carbon, which makes light intensity and carbon critical for their growth and production (Zhan *et al.*, 2017). Earlier studies by Chojnacka and Noworyta (2004) and Subashchandrabose *et al*. 2013, have demonstrated that the mixotrophic cultivation of *Spirulina* sp. had the highest specific growth when compared to autotrophic growth. Also, the photoinhibition observed in the autotrophic culture was absent in the mixotrophic culture. This could be attributed to the protective influence of glucose or a shift in photoinhibitory light intensity (Chojnacka and Noworyta, 2004, Subashchandrabose *et al.*, 2013). Therefore, further research is needed to assess the effects of organic carbon sources and light intensity on the performance of microalgae.

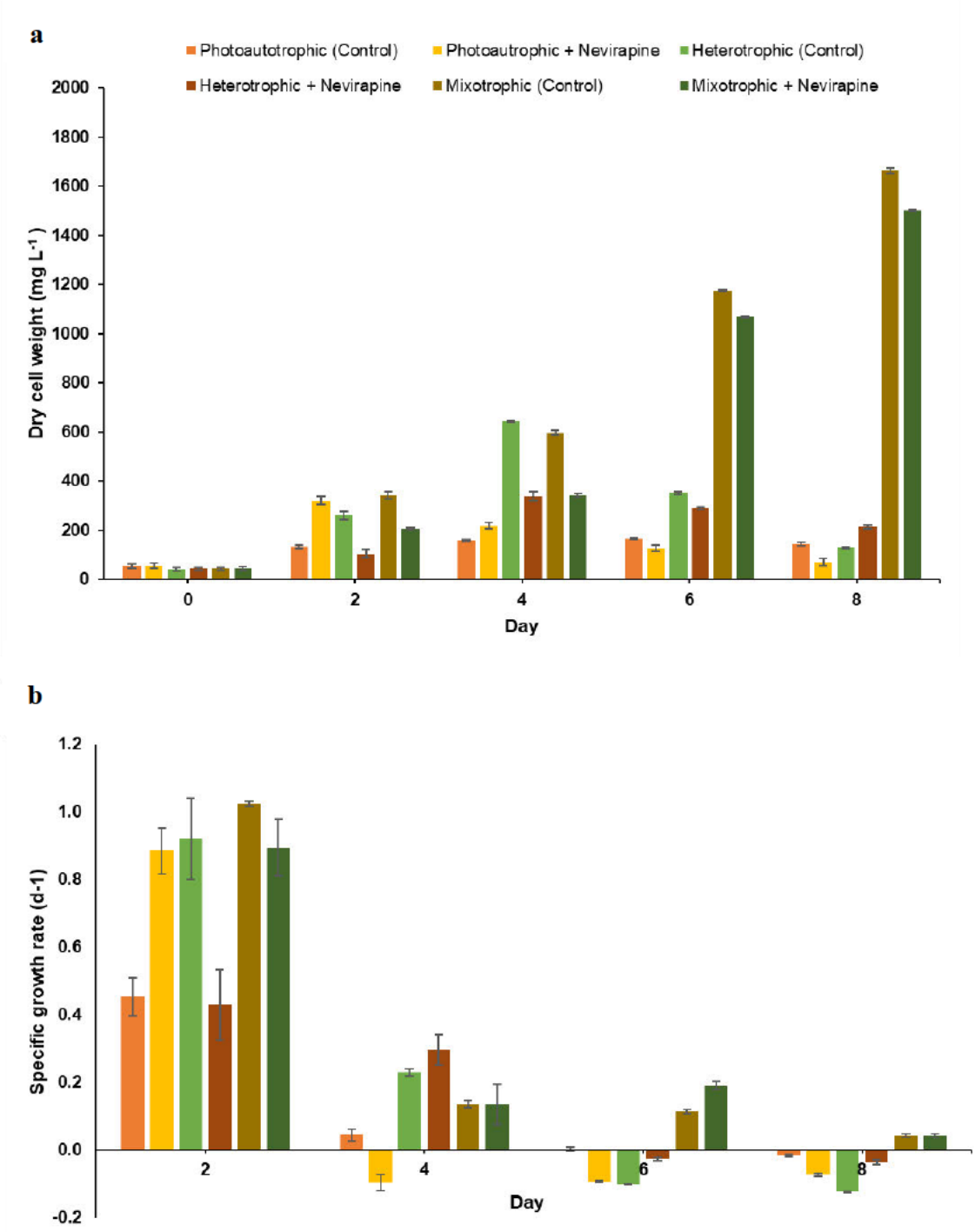


Figure 5.3 Dry cell weight (**a**) and specific growth rate (**b**) of *Tetradesmus obliquus* under different cultivation conditions during 8 days of cultivation.

5.3.2 NVP Removal by Tetradesmus obliquus under different modes of cultivation

The addition of readily available organic substrates, such as glucose or sodium acetate, has been reported to accelerate the microalgae-mediated biodegradation of pharmaceuticals (Wang *et al.*, 2022b). In this study, the NVP removal efficiency of *T. obliquus* was enhanced with the addition of glucose which is a frequently used co-substrate during mixotrophic cultivation of microalgae (Bentahar and Deschênes, 2022). The removal efficiency of NVP increased over time in all treatments (Fig. 5.5). It has been reported that supplementation of glucose could significantly accelerate the biodegradation of pharmaceuticals by acting as an electron donor for co-metabolism (Xiong *et al.*, 2018). In the present study, *T. obliquus* was found to be most effective at removing NVP under mixotrophic conditions (80.13%), followed by heterotrophic conditions (70.30%), demonstrating the potential role of glucose in enhancing NVP removal by microalgae. In a previous study, Xiong *et al.* (2020) have shown that the addition of sodium acetate significantly enhanced the removal efficiency of sulfamethoxazole by *Chlorella pyrenoidosa* (99.35%).

Figure 5.4 *Tetradesmus obliquus* cultivated in BG-11 growth medium supplemented with NVP in three cultivation modes.

Similar results were found for the removal efficiency of ciprofloxacin by *Chlamydomonas Mexicana*, whereby the addition of sodium acetate, removal increased from 13% to 56% (Xiong *et al.*, 2017d). Liang *et al.* (2019), evaluated the influence of acetate on the degradation of 26 pharmaceuticals. Based on their findings, acetate concentration was associated with both an improvement in removal efficiency, as well as regulating the transformation products and pathways. It has been reported that sugar-based carbon sources result in the highest

exopolysaccharides and enzyme concentrations in microalgae culture, as well as a greater pollutant removal efficiency (Vo *et al.* (2020a), Vo *et al.* (2020b).

However, some studies have also described that some carbon substrates can reduce removal efficiency of pollutants due to catabolite repression (Xiong *et al.*, 2017d, Xiong *et al.*, 2020). Thus, it is imperative to assess the effects of various carbon sources on the degradation of pharmaceuticals to achieve efficient bioremediation. The removal mechanisms observed in *T. obliquus* under different cultivation conditions including photodegradation is illustrated in Table 5.3. The mass balance results revealed that biodegradation was identified as the primary mechanism of removal, with a relatively low contribution from bioadsorption (2.39-3.36%) and bioaccumulation (0.55-0.87%).

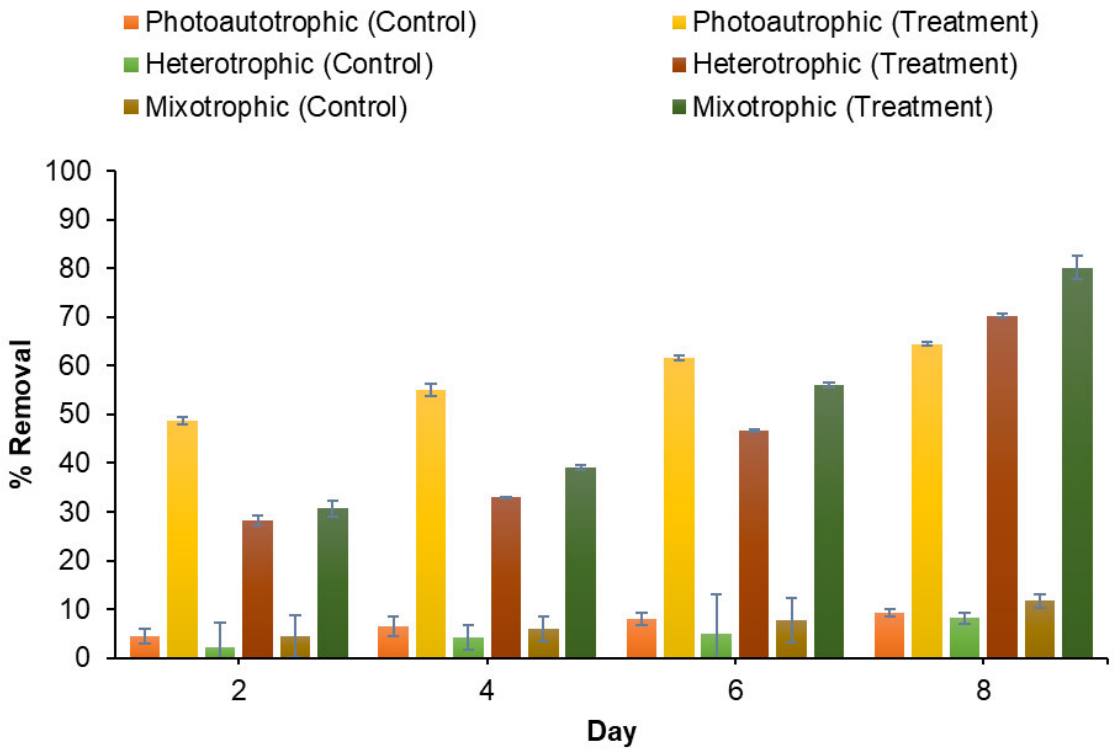


Figure 5.5 Removal of NVP (4000 ng L^{-1}) in BG-11 medium by *Tetradesmus obliquus* over a period of 8 days.

It has been previously reported that bioadsorption is more effective with PCs containing cationic groups that are actively attracted towards the algal surface through electrostatic interactions (Ayangbenro and Babalola, 2017). Therefore, it can be presumed that cationic NVP would have an affinity for the negatively charged algal cell wall. The n-octanol/water partition coefficient (Ko_w) value of any drug is an imperative parameter to determine their

bioaccumulation potential, which assists in determining their fate in living organisms (Reddy *et al.*, 2021). According to a study conducted by Dimitrov *et al.* (2019), compounds with log K_{ow} values lower than 4.5 are not readily bioaccumulative, this is evident in this study as NVP has a log K_{ow} value of 3.9. Therefore, the dominant route for NVP removal was found to be through biodegradation process. Similar observations were demonstrated in a study conducted by Wu *et al.* (2022), where biodegradation was the prevailing removal process of diclofenac, clarithromycin and benzotriazole in *Scenedesmus obliquus*.

Table 5.3 Mass balance of NVP (4000 ng L^{-1}) removal through different mechanisms by *T. obliquus* at the end of 8 days of cultivation.

Treatment	**Mass balance of NVP removal (%)**				
	Bioadsorption	**Bioaccumulation**	**Biodegradation**	**Abiotic**	**Total removal**
Autotrophic	2.39 ± 0.37	0.87 ± 0.31	82.74 ± 8.22	14 ± 2.23	**64.40 ± 2.11**
Heterotrophic	2.59 ± 0.58	0.55 ± 0.33	87.60 ± 3.71	9.25 ± 1.56	**70.30 ± 2.01**
Mixotrophic	3.36 ± 0.58	0.81 ± 0.18	80.60 ± 3.43	15.23 ± 5.70	**80.13 ± 6.28**

% - Percentage

5.3.3 Untargeted analyses of NVP biotransformation products by high-resolution mass spectrometry

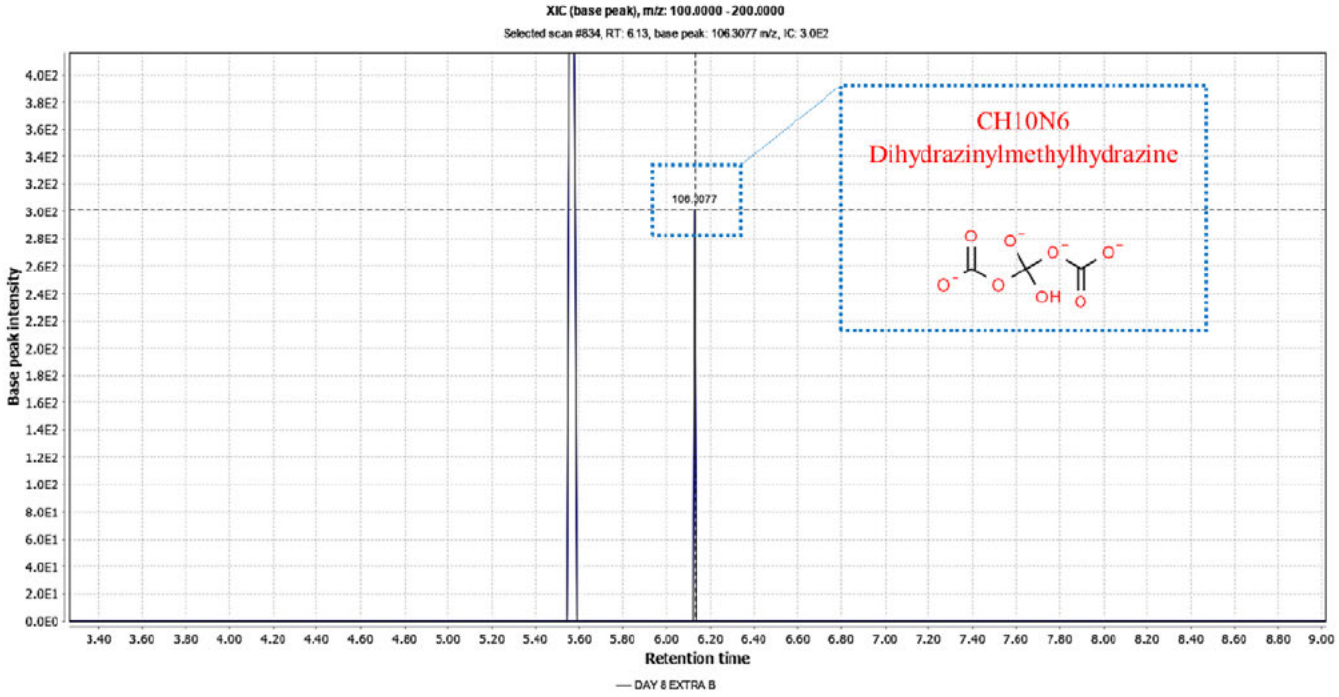
An investigation was carried out to explore the biotransformation products of NVP by *T. obliquus*. Over the course of 4 and 8 days of mixotrophic cultivation, a total of six distinct biotransformation products of NVP were detected in intracellular and extracellular samples using LC-MS, each characterized by its unique mass-to-ion ratio (m/z): 106.31, 164.92, 230.25, 274.28, 302.31, and 318.24.

The identified biotransformation products were tentatively assigned as follows:

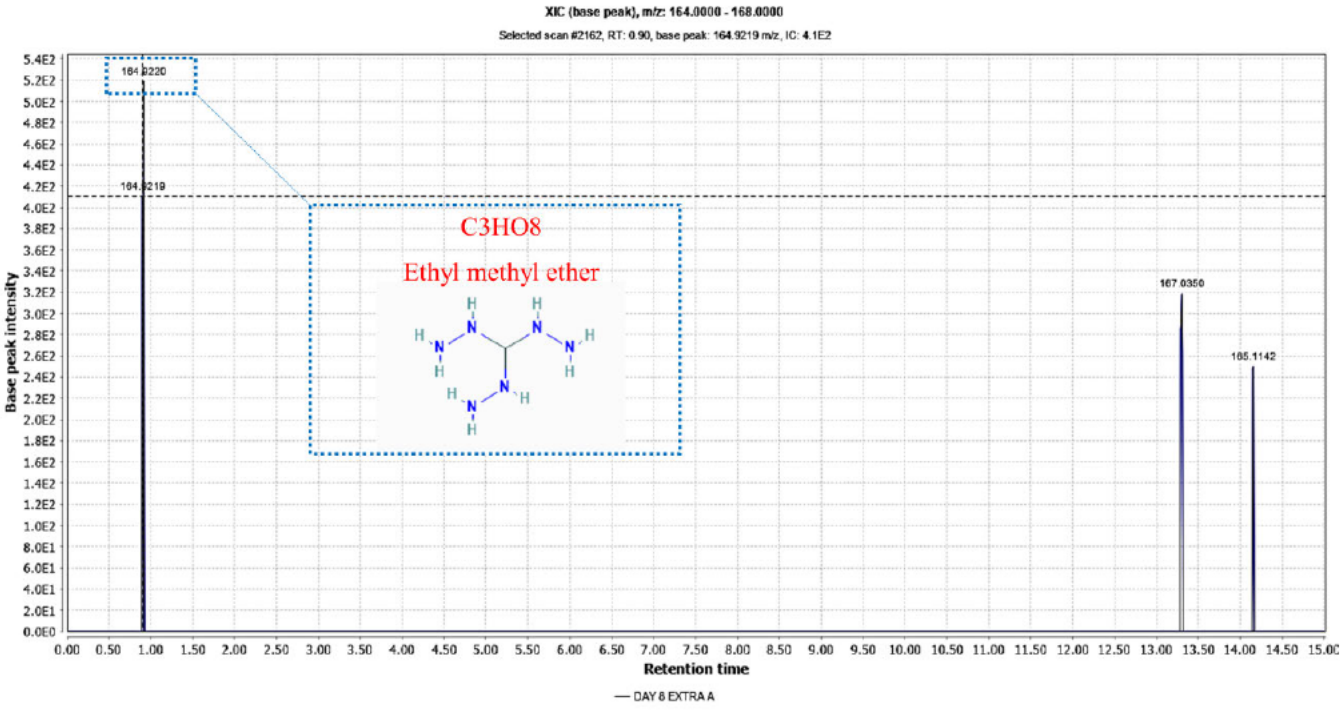
a) Dihydrazinylmethylhydrazine with an m/z value of 106.31 and a molecular weight (mol. wt.) of 106.13 (CH10N6).
b) Ethyl methyl ether with an m/z value of 164.92 and a mol. wt. of 165.04 (C3HO8).
c) Tris [2-(dimethyl amino) ethyl] amine with an m/z value of 230.25 and a mol. wt. of 230.39 (C12H30N4).
d) 2-[[Amino(ethyl)amino]methyl]-3-[amino(2,4,4-trimethylpentan-2-yl)amino]propan-1-ol with an m/z value of 274.28 and a mol. wt. of 274.44 (C14H34N4O).
e) 1,4-Butanediamine, N, N'-bis[3-(dimethylamino) propyl]-N, N'-dimethyl-O with an m/z value of 302.31 and a mol. wt. of 302.50 (C16H38N4O).
f) (2S)-4-(ethylamino)-1-[4-[[(2S)-4-(ethylamino)-2 hydroxy butyl] amino] butyl amino] butan-2-ol with an m/z value of 318.24 and a mol. wt. of 318.46 (C18H30N4O).

The process of biotransformation by algae involves the conversion of a chemical compound into different metabolites, known to be mediated by species-specific enzyme systems, such as cytochrome P450 (CYP450). These enzyme systems play a crucial role in the biotransformation of xenobiotics and the detoxification of pollutants, as documented in previous studies (Xiong *et al*., 2020; Torres *et al*., 2008). However, no prior studies have explored the intricate process of microalgal biotransformation when it comes to NVP. As a consequence of this research gap, it becomes imperative to embark on further investigations to comprehensively understand and evaluate the toxicity and precise mechanisms, transformation pathways that microalgae utilize during the biotransformation of NVP. These investigations are crucial not only for advancing our knowledge of microalgal biotransformation processes but also for potential applications in environmental detoxification.

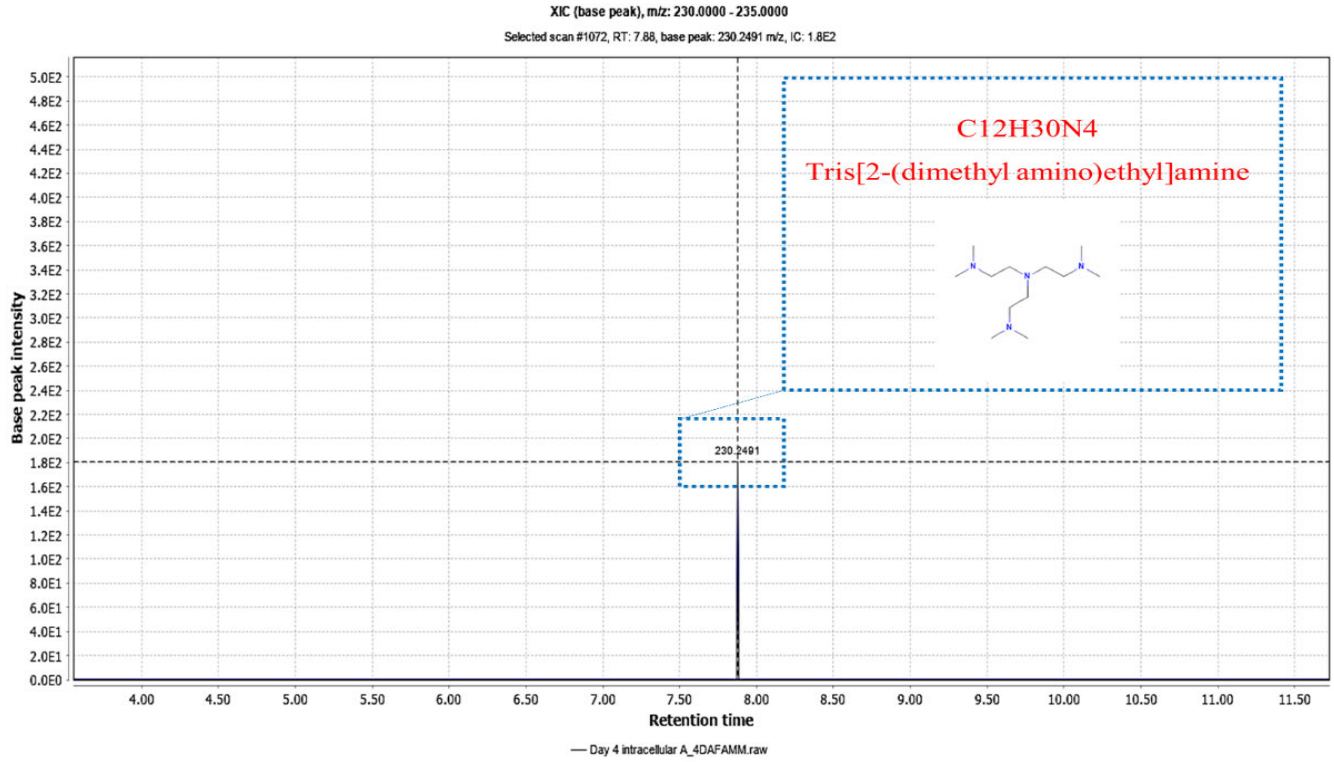
a


XIC (base peak), m/z: 100.0000 - 200.0000
Selected scan #834, RT: 6.13, base peak: 106.3077 m/z, IC: 3.0E2
106.3077
CH10N6
Dihydrazinylmethylhydrazine
Base peak intensity
Retention time
— DAY 8 EXTRA B

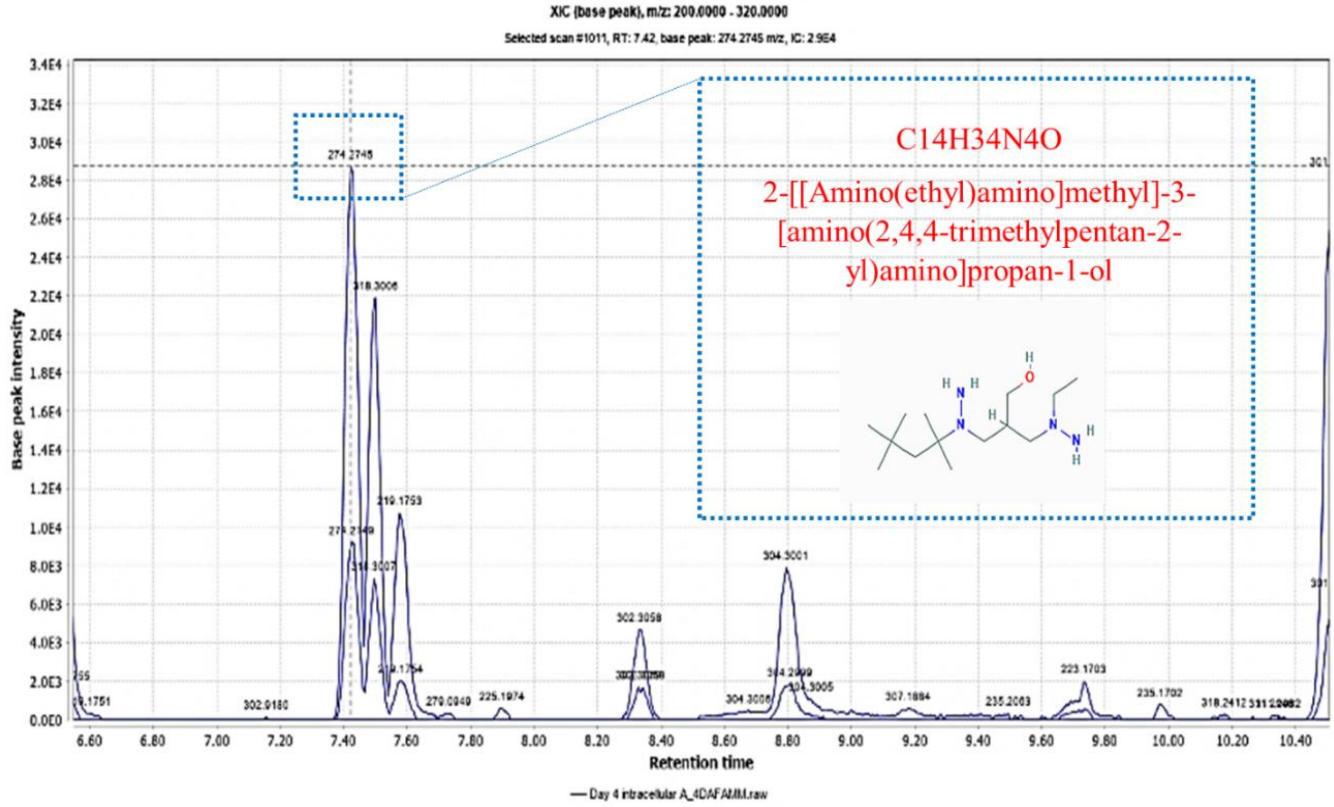
b


XIC (base peak), m/z: 164.0000 - 168.0000
Selected scan #2162, RT: 0.90, base peak: 164.9219 m/z, IC: 4.1E2
164.9220
164.9219
C3HO8
Ethyl methyl ether
167.0350
165.1142
Base peak intensity
Retention time
— DAY 8 EXTRA A

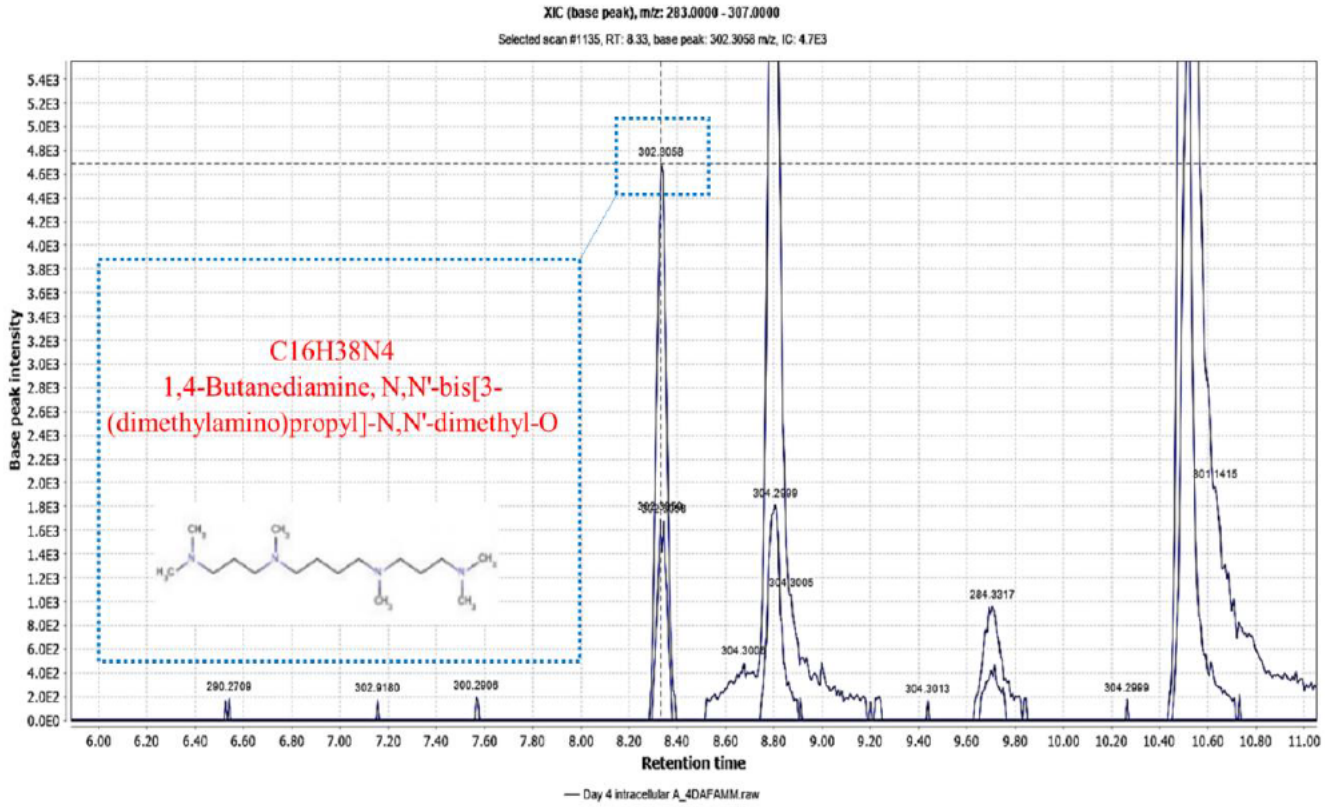
c


XIC (base peak), m/z: 230.0000 - 235.0000
Selected scan #1072, RT: 7.88, base peak: 230.2491 m/z, IC: 1.8E2
C12H30N4
Tris[2-(dimethyl amino)ethyl]amine
230.2491
Base peak intensity
Retention time
Day 4 intracellular A_4DAFAMM.raw

d


XIC (base peak), m/z: 200.0000 - 320.0000
Selected scan #1011, RT: 7.42, base peak: 274.2745 m/z, IC: 2.9E4
C14H34N4O
2-[[Amino(ethyl)amino]methyl]-3-[amino(2,4,4-trimethylpentan-2-yl)amino]propan-1-ol
274.2745
318.3006
219.1753
304.3001
302.3058
Base peak intensity
Retention time
Day 4 intracellular A_4DAFAMM.raw

e



f

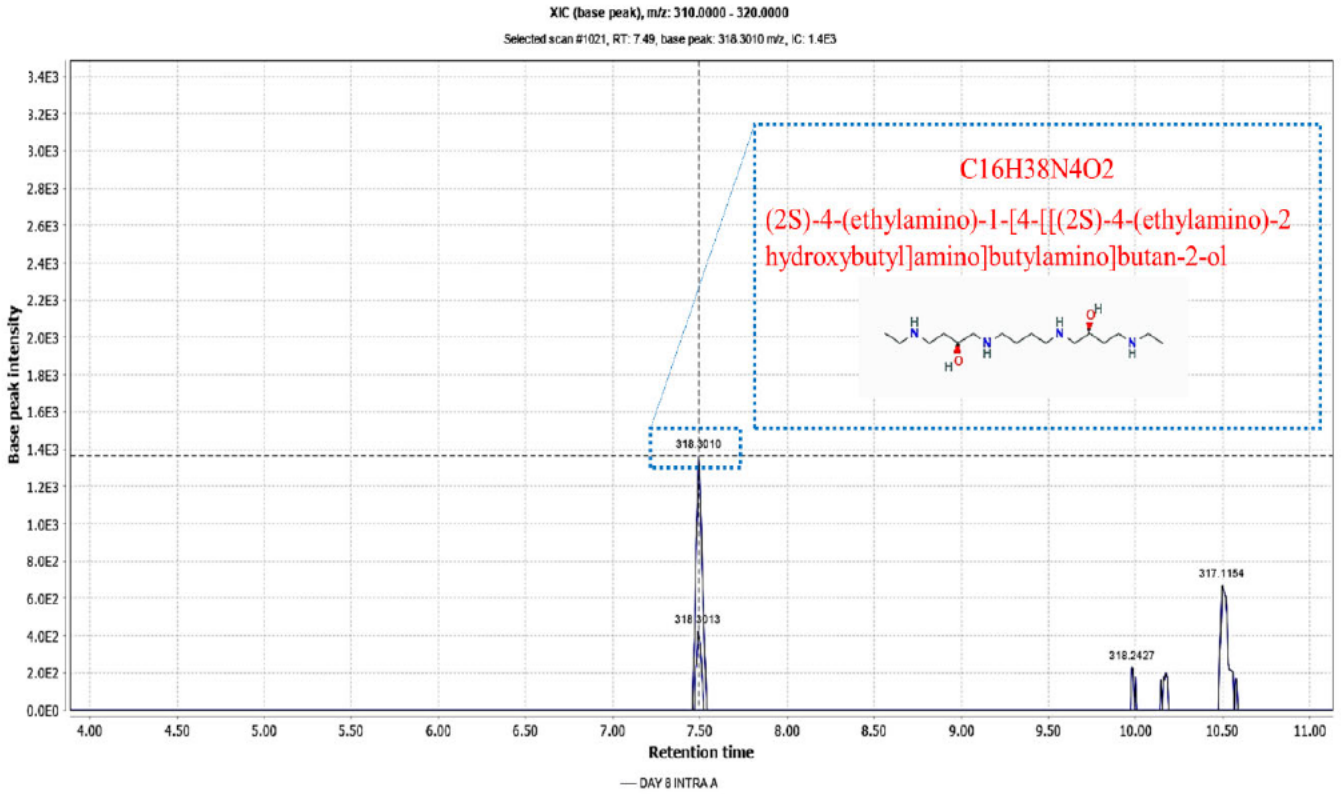
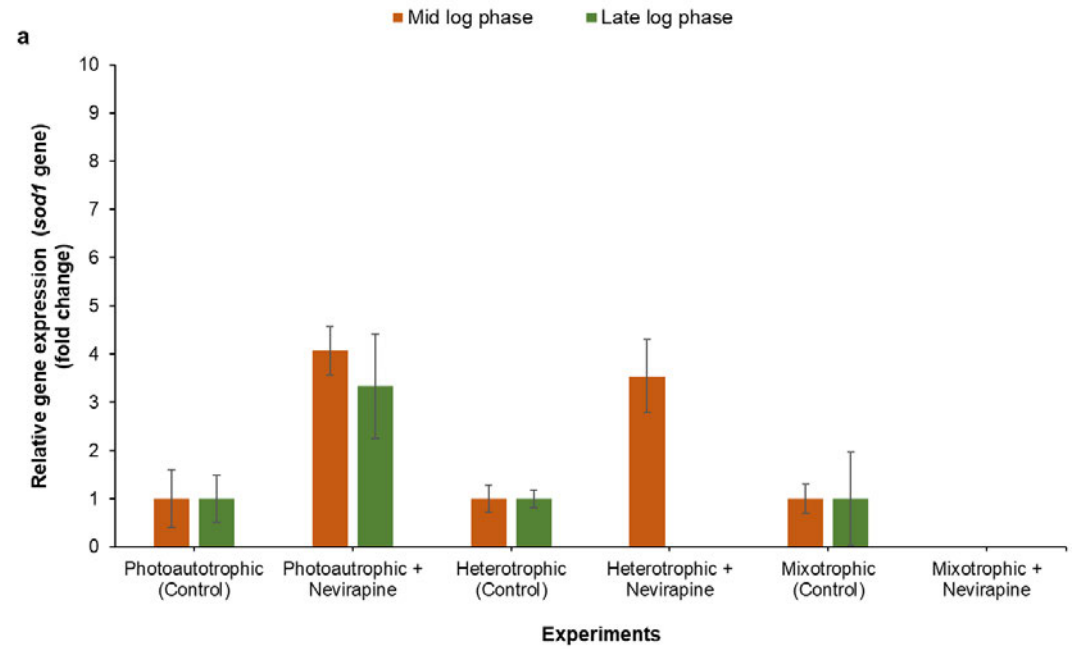


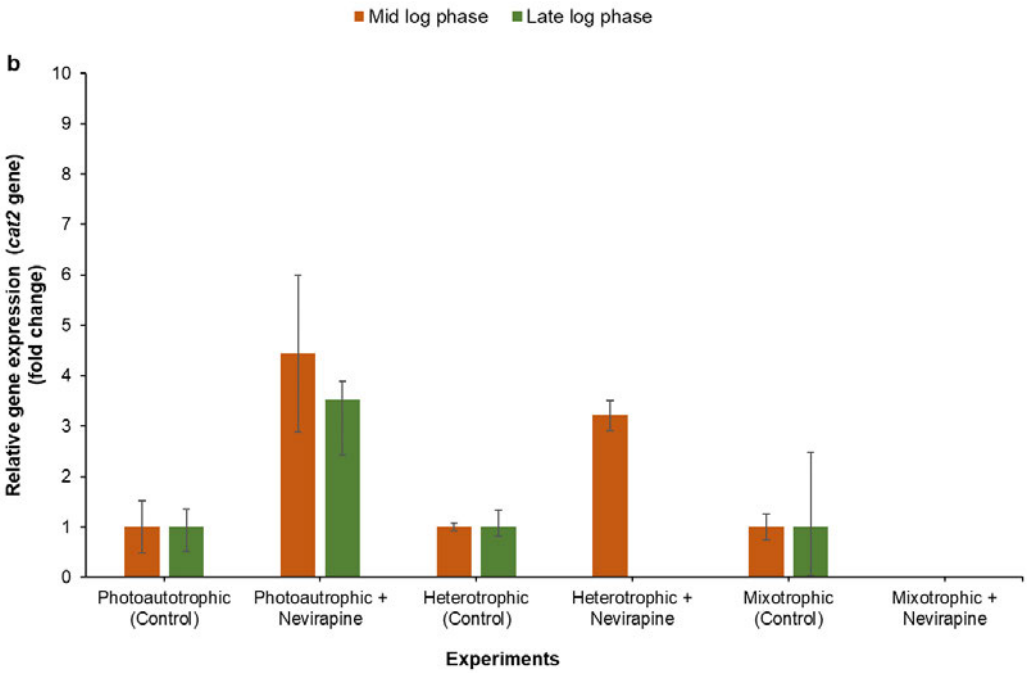
Figure 5.6 Base peak chromatograph for m/z 106.31 (a) ,164.92 (b), 230.25 (c), 274.28 (d), 302.31 (e) and 318.24 (f) with potential structures.

5.3.4 Effect of NVP stress on the gene expression of antioxidant enzymes in T. obliquus

The gene expression of antioxidant enzymes was measured in response to NVP stress in *T. obliquus* under different growth regimes. Among the key signalling molecules that regulate cellular growth and metabolism are ROS. Excessive cellular ROS may cause damage to the microbial membrane system (Rezayian *et al.*, 2019). Several enzymes, such as superoxide dismutase, glutathione peroxidase and catalase, can eliminate ROS-induced toxicity (Zandi and Schnug, 2022). When exposed to pharmaceuticals, microalgae can increase the activity of antioxidant enzymes to protect the photosynthetic process from hydrogen peroxide (H_2O_2) damage (Chen *et al.*, 2020). Reactive oxygen species can inactivate or change biomolecules, resulting in organelle dysfunction, altered cell structure, and mutations (Rezayian *et al.*, 2019). In this study, the gene expression of selected genes (*sod*1, *cat2* and *gpx1*) in *T. obliquus* was evaluated in the mid-log and late-log phases for all cultivation modes (Fig. 5.7). In order to determine the relative fold change in expression, microalgal cells grown in BG-11 medium without NVP supplementation were used as controls. The highest relative fold change of *sod1* was observed under autotrophic growth with NVP in the mid log phase of growth, which was 75.43% higher as compared to the control. There was, however, a decrease in *sod1* expression observed during mid log (99.98%) as well as late log (99.98%) stages of mixotrophic growth with NVP. The lowest (99.61%) relative fold change of *sod1* was noted in the heterotrophic cultures with NVP in the late log phase. The increased *sod1* activity is estimated to enhance the accumulation of H_2O_2, which is eliminated by CAT (Zhang *et al.*, 2022, Gauthier *et al.*, 2020). According to a previous report by Zhang *et al.* (2019), the increase in *sod*1, *cat2* and *gpx1* activity is explained by the defence mechanism in response to increased ROS concentrations, indicating that oxidative stress tolerance was initiated by excess O_2^- and H_2O_2 production. Thus, the activity of CAT activity is often coupled with SOD activity. The relative fold change of *cat2* displayed a similar trend for the expression of *sod1* gene. The highest fold increase of *cat2* (77.05%) was observed in *T. obliquus* grown with NVP under autotrophic conditions in the mid log growth phase as compared to the control. While the lowest *cat2* fold change was found in the heterotrophic cultures with NVP, resulting in a decrease (99.56%) in expression in the late log phase. A decrease in *cat2* expression was also observed in the mixotrophic growth of *T. obliquus* with NVP in both the mid (99.98%) and late log (99.97%) growth phases. The highest relative fold increase of *gpx1* (88.20%) was found under the heterotrophic growth of *T. obliquus* in the presence of NVP at the mid log phase. However, a decrease in *gpx1* gene expression was noted in heterotrophic growth (99.38%) at late log

phase, and mixotrophic growth resulting in ~99% decline in the expression for both mid and late log phases. This observation further supports the role of glucose supplementation in overcoming microalgal oxidative stress.





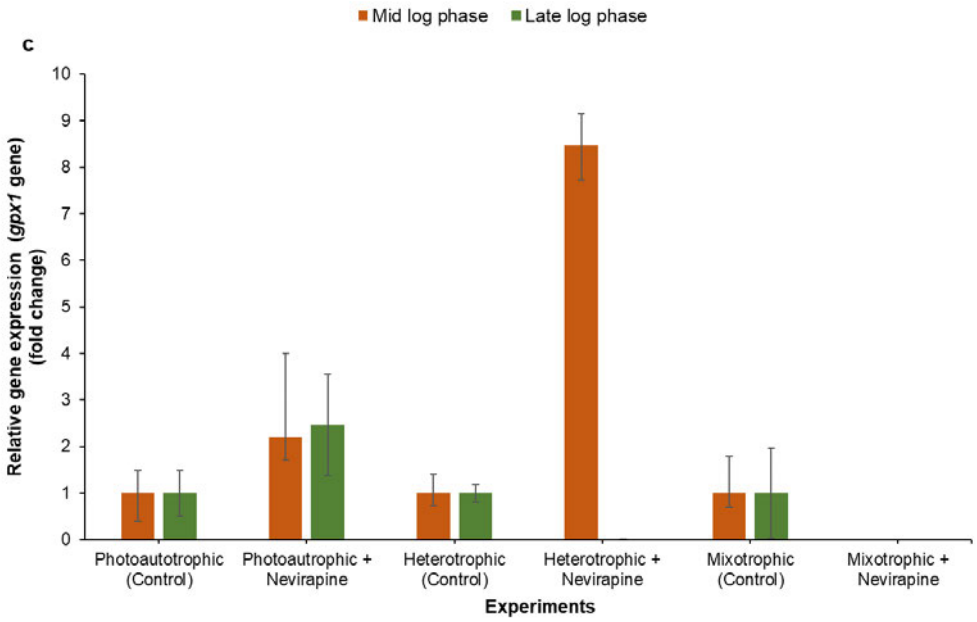


Figure 5.7 Effect of NVP on the relative expression of *sod1* (**a**), *cat2* (**b**), *gpx1* (**c**) gene at mid log phase and at late log phase in *T. obliquus*.

5.3.5 Effect of NVP on microalgal cells

Biosorption of pharmaceuticals is largely dependent on the functional groups present on the microalgae cell surface (Silva *et al.*, 2020). The microalgae exposed to NVP were analysed by FTIR to determine the changes in chemical composition based on previously published studies (Mohamed *et al.*, 2017). Under different cultivation modes, the spectra of *T obliquus* biomass during the mid and late log phases were analysed (Fig. 5.8) in order to monitor the stress induced by NVP on *T obliquus*, changes in microalgal biomolecules such as carbohydrates, proteins, and lipids were also assessed. The spectral scan was not comparable between the NVP-supplemented treatments and the control groups, exhibiting significant differences between them. The spectra showed stretching and bending vibration characteristics and revealed the adsorption of NVP on the chemical composition of the biomass. It is also possible to attribute the variations in peak intensities to changes in the biochemical composition of *T. obliquus* grown under different cultivation conditions. The distinct infrared transmittance spectral bends were presented at 3800-3000 cm^{-1}, which corresponds to the O-H stretching vibrations (water dissociation and non-dissociation) of bonds and N-H vibrations of proteins, or polypeptides. An earlier report by Kousha *et al.* (2013), observed changes in the FT-IR spectra of *Scenedesmus quadricauda* and *Chlorella vulgaris* biomasses after malachite green adsorption, which were attributed to the attachment

of this dye on –OH and –NH groups. Therefore, in the present work, it can be concluded that NVP adsorption on *T. obliquus* biomass was physical, although some interactions with –OH and –NH groups may have occurred, chemical bonding with biomass surface played a relatively minor role. Adsorption bands below 3000 cm^{-1} are related to C-H stretching vibrations, specifically CH_2 asymmetric and symmetric stretching vibrations of lipids. The absorption band at about 1650 cm^{-1} is in the range of 1900–1650 cm^{-1} where carbonyl groups usually show a strong band from esters and fatty acids (triacylglycerides). This region is significant for monitoring lipid accumulation in microalgal species (Grace *et al.*, 2020). Double bonds are found in the range 2000–1500 cm^{-1}, which is related to the amide groups of proteins (amide I and amide II). In Fig. 5.8, the region of 1630 cm^{-1}, represents the protein groups in *T. obliquus* which were much more prominent in heterotrophic and mixotrophic growth modes for both log and late log phase.

The region between 998 cm^{-1} and 1077 cm^{-1} was due to (C-O) stretches of polysaccharides from carbohydrate. These peaks were distinct in the heterotrophic and mixotrophic cultivation modes with and without NVP. Similarly, Ali *et al.* (2018), reported that tramadol adsorption did not only result in some slight shifting and intensity reduction but also caused bands to disappear when tramadol was bonded to alkaline modified biomass of *Scenedesmus obliquus*. According to them, modified algal biomass may adsorb tramadol through the interaction of O-H and C-O functional groups. Interestingly, a band appeared in the region of 950-1000 cm^{-1} in the mid log phase of growth of all cultivation modes with NVP, which is an indication of aromatic C-H rings that are found in NVP, which revealed the NVP bioadsorption potential of microalgae.

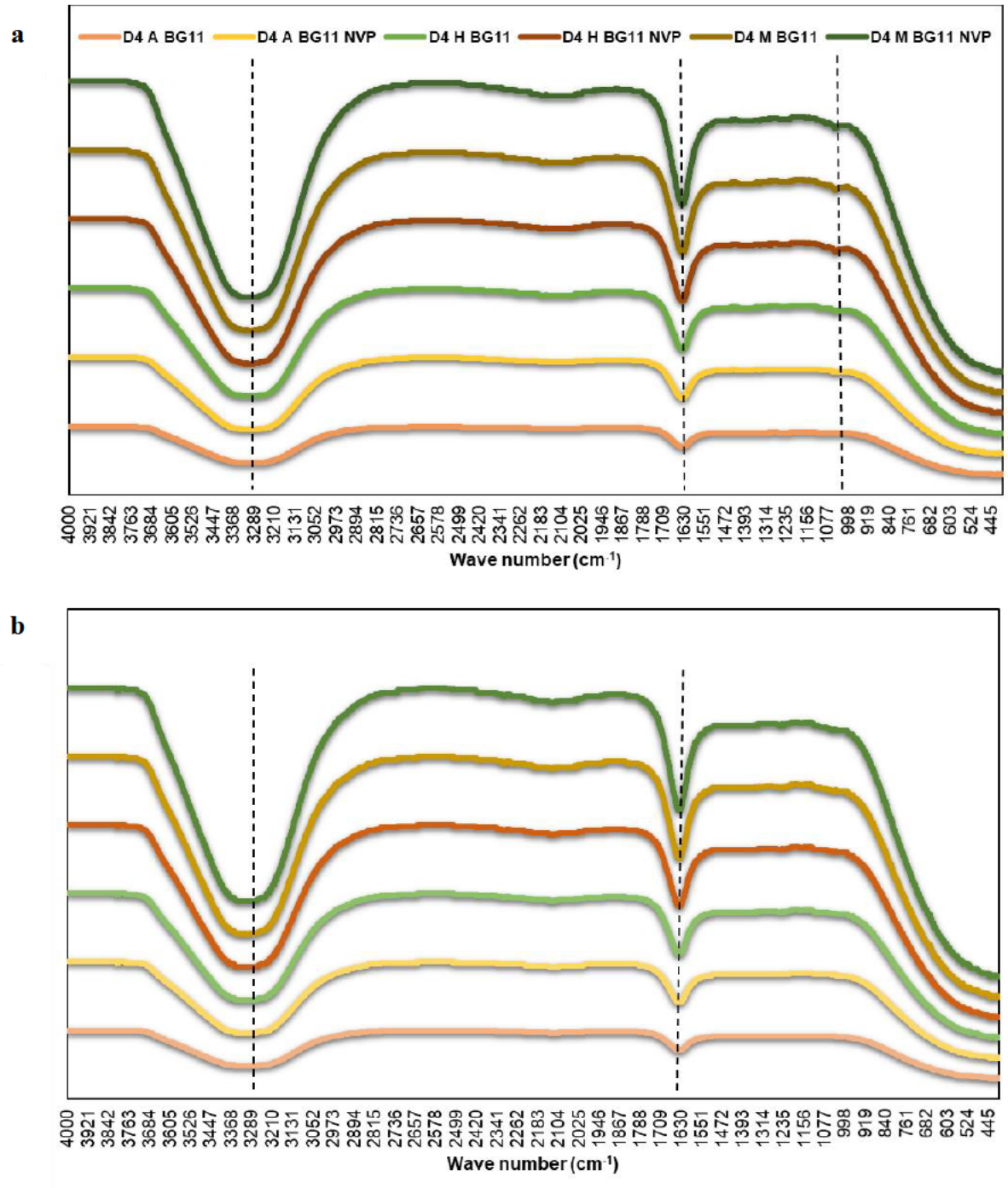


Figure 5.8 The fourier transform infrared spectra (FTIR) of *Tetrademus obliquus* cultivated in BG-11 medium supplemented with nevirapine on days 4 (**a**) and 8 (**b**) and their corresponding controls (autotrophic (A) , heterotrophic (H), mixotrophic (M)).

5.4 GC-MS untargetted metabolomics

Gas chromatography time-of-flight mass spectrometry is widely employed to investigate metabolic profiles at the cellular level in response to induced conditions (Sharma *et al*., 2023). This could effectively reveal various alterations in the metabolic pathways and molecular mechanisms of *T. obliquus* triggered by NVP. The representative GC-MS total ion chromatograms (TICs) of the control and NVP exposed samples are shown in Appendix eleven. The top 12 most significantly modulated metabolites in *T. obliquus* were analyzed in the presence of NVP and compared to the control grown in only BG-11 growth medium. Of these metabolites, the majority exhibited significant associations with algal carbohydrate, amino acid, and lipid metabolism. Heatmap analysis (Fig. 5.9) was used to visualize the expression of diverse metabolites in four samples collected on days 4, and 8 of the experiment. These samples included the control group without NVP and the treatment groups (i.e., samples supplemented with 4000 ng L^{-1} NVP during mixotrophic growth. Multivariate analysis in terms of PCA plots was used to limit the data to low dimensional space, wherein the discriminations in metabolite profiles across different groups can be modelled.

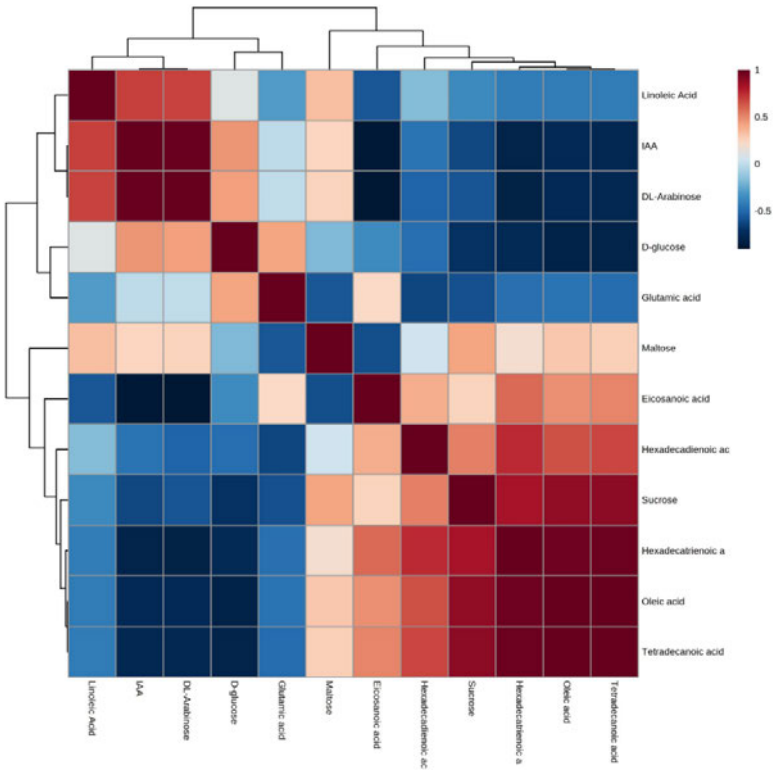


Figure 5.9 Clustering metabolites and samples depicted in a heatmap using the Euclidean distance and Ward clustering method. Control (Cnt_D4 and Cnt_D8) and NVP supplemented (T_D4 and T_D8) cells at day 4 and 8 during mixotrophic cultivation.

This observation implies that metabolomic compositions with similarities tend to group together, while those with distinct compositions tend to separate. The PCA results revealed the general structure of the obtained datasets, in which the two principal components showed a cumulative variance of 88.7%, with PC1 explaining 73% and PC2 explaining 15.7% of the total variance (Fig. 5.11). Furthermore, a clear data separation was evident among the control and treatment groups and the separation was more significant with increasing time. The results showed that the metabolic responses to NVP exposure were time dependent. To evaluate, the statistical significance of the applied multivariate models, a permutation test was performed (Fig. 5.12). From the permutation analysis (100 permutations), the significance of the power of these optimal models to predict the toxicity of NVP exposed *T. obliquus* and the control groups at different treatment days was found to be $p < 0.01$ (Fig. 5.12a and 5.12b). Variable Importance in Projection (VIP) scores were employed to identify the key metabolites that significantly contributed to variations in the metabolic profiles for each sample (Table 5.4). The relative abundance of metabolites is indicated by a coloured scale from blue to red representing the low and high, respectively as presented in Fig. 5.10. The VIP values of greater than 1 were identified as potential marker metabolites.

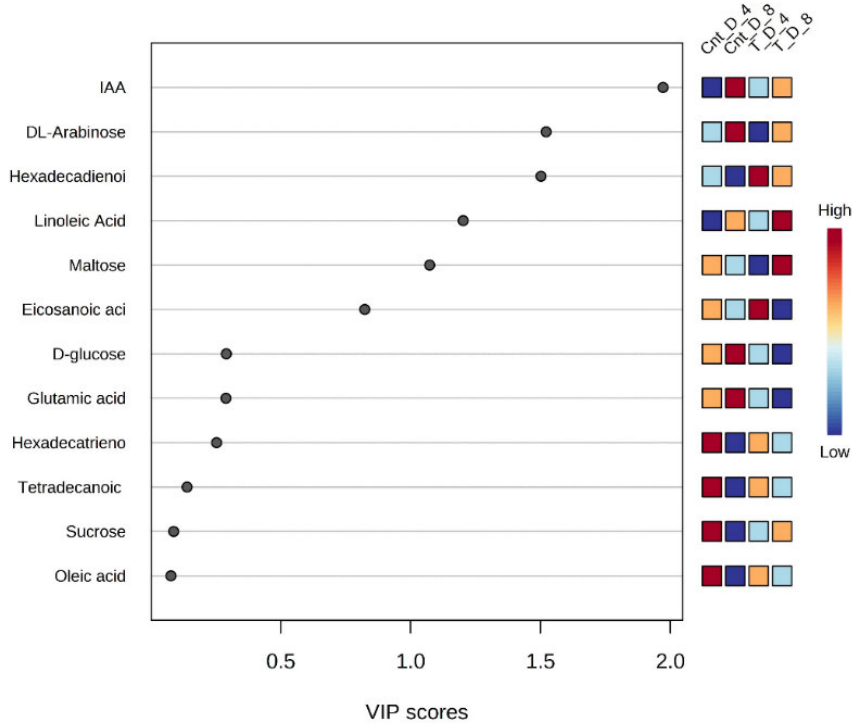


Figure 5.10. The metabolites were differentiated based on a Variable Importance in Projection (VIP) score in control (Cnt_D4 and Cnt_D8) and NVP supplemented (T_D4 and T_D8) cells at day 4 and 8 during mixotrophic cultivation.

Table 5.4 Metabolic differences responsible for distinguishing *T. obliquus* exposed to nevirapine from those in the control group.

ChEBI ID	Metabolite	VIP score	*f-value*	*p*-value	FDR
CHEBI:28822	Eicosanoic acid	2.065475	17.185	7.5702E-4	0.002271
CHEBI:22340	Hexadecatrienoic acid	1.2806	17.855	6.6419E-4	0.002271
CHEBI:77524	Hexadecadienoic acid	1.19305	8.149	0.0081	0.013617
CHEBI:28875	Tetradecanoic acid	1.005595	5.2821	0.026	0.035527
CHEBI:16196	Oleic acid	1.22682875	8.0691	0.0083	0.013617
CHEBI:17306	Maltose	1.10190625	27.119	1.5233E-4	9.1399E-4
CHEBI:16411	Indole acetic acid	1.14572	61.15	7.3764E-6	8.8516E-5
CHEBI:17992	Sucrose	1.44262875	7.8506	0.009	0.013617
CHEBI:22599	DL-Arabinose	1.42318875	4.8522	0.032	0.039514
CHEBI:17351	Linoleic Acid	1.403075	11.489	0.0028	0.0068533
CHEBI:18237	Glutamic acid	1.35306625	15.3	0.001	0.002
CHEBI:17634	D-glucose	0.1618825	9.065	0.004	0.0062

ChEBI ID- Entities of Biological Interest; VIP- Variable Importance in Projection; f-value -ratio of two variances; probability value; FDR- false discovery rate.

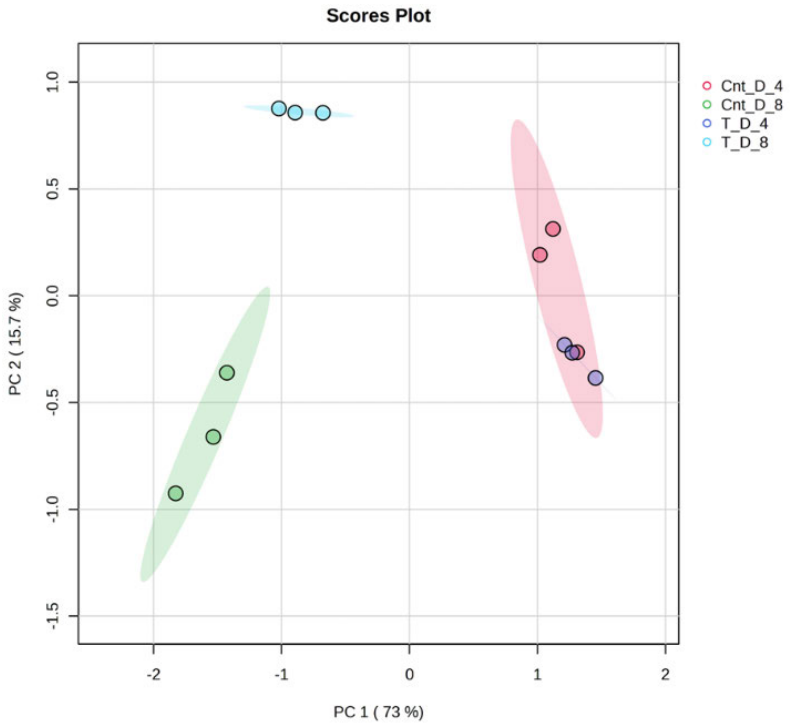


Figure 5.11. Principal component analysis, depicting expression of different metabolites in *T. obliquus* in control (Cnt_D_4 and Cnt_D_8) and NVP supplemented (T_D_4 and T_D_8) cells at day 4 and 8 during mixotrophic cultivation.

As depicted in Fig. 5.10, the hexadecatrienoic acid, hexadecanoic acid, linoleic acid and tetradecanoic acid contents was upregulated in the NVP supplemented (T_D4 and T_D8) cells, which demonstrates the influence of NVP on the fatty acid biosynthetic pathway. In algae cells, the primary sites for fatty acid synthesis reactions are situated within the plastid. The fatty acids utilized in the production of fats and oils primarily encompass oleic acid and linoleic acid (Zhang *et al*., 2018). Following synthesis, these free fatty acids are subsequently transported to the cytoplasm and further into the endoplasmic reticulum and other compartments involved in biosynthesis of triacylglycerols (TAG synthesis) (Zhang *et al*., 2018). Within algae, energy can be stored in the form of TAG, serving as a crucial carbon reserve and a significant factor contributing to survival in challenging environmental conditions. Thus microalgae, accumulate triacylglycerols when faced with induced stress (Gao *et al*., 2023). The sugar metabolites such as D-glucose and sucrose increased at day 4 in the NVP supplemented samples, and the controls remained at low representation. However, by day 8, the sugar levels were significantly lower compared to those in the control group, potentially indicating a reduction in carbohydrate biosynthesis in *T. obliquus* due to prolonged exposure of NVP. In Appendix twelve, the pathway impact analysis was displayed by all the matched pathways are represented as circles. The colour and size of each circle are determined

by the p-value and pathway impact value, respectively. The colour of the circle corresponds to the p-value, with darker colours indicating more statistically significant pathways ($p < 0.05$). The sensitivity and specificity of the different biomarkers were assessed through the use of a receiver operating characteristic curve (ROC), with performance quantified using the area under the ROC curve (AUC = 0.79-0.98) (Fig. 5.12c and Appendix thirteen).

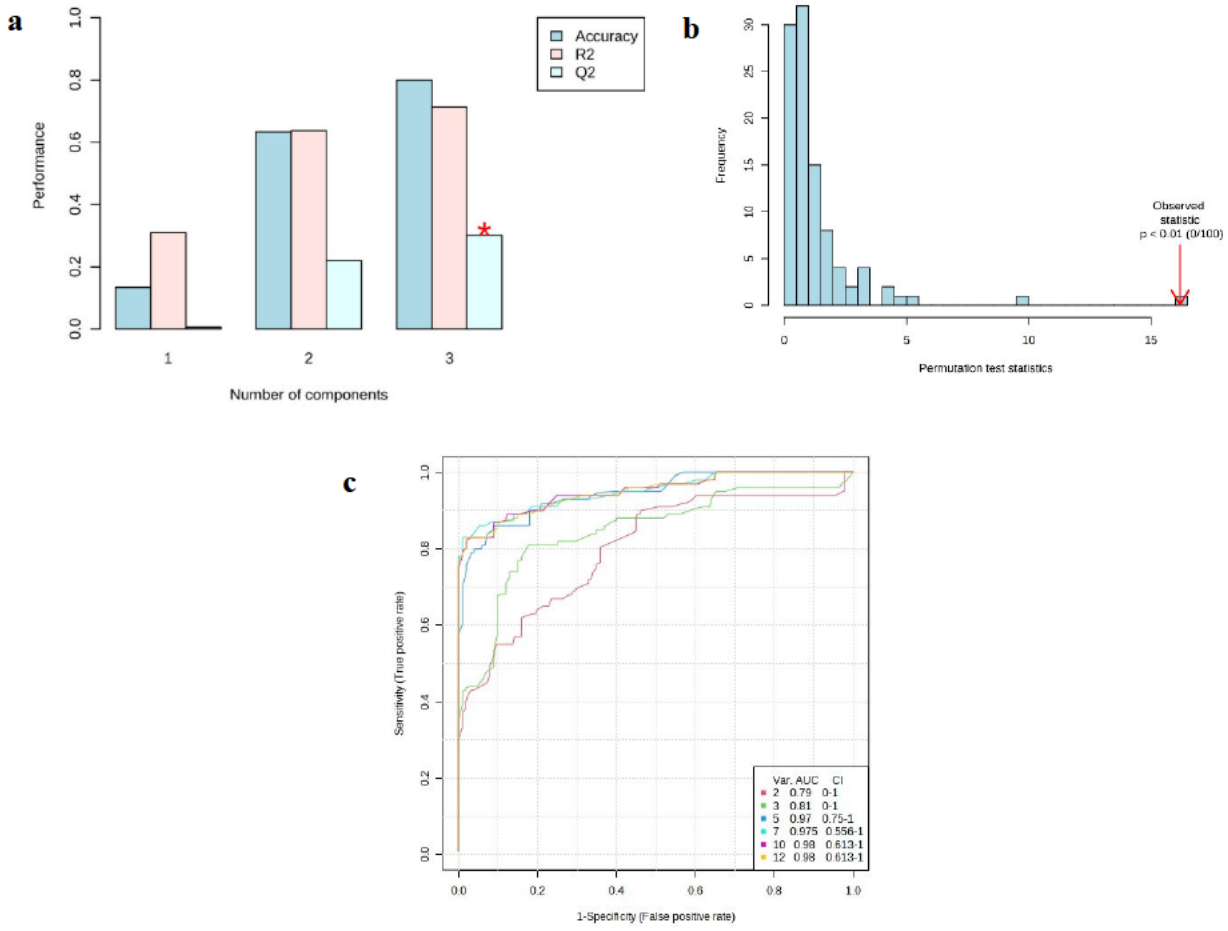


Figure 5.12 (a) Cross validation, **(b)** Permutation analysis of PLS-DA models derived from NVP exposed and controls of *Tetradesmus obliquus* biomass. **(c)** Predictive accuracy of the model separating NVP exposed, and control *Tetradesmus obliquus* summarised using ROC curve analysis. Area under the curve= 0.79-0.98.

5.5 Conclusion

In this study, the growth of *T. obliquus* was significantly improved by the addition of glucose as organic carbon source under mixotrophic conditions. The gene expression analysis showed the enhanced activities of SOD, CAT and GPX under autotrophic conditions, which promoted the defence capacity of *T. obliquus* for the oxidation stress induced by NVP. Under heterotrophic and mixotrophic growth conditions, the addition of glucose was found to reduce the toxic effects of NVP and decreased gene expression. Mixotrophic cultivation of *T. obliquus* enhanced NVP removal up to 80.30%, which was 15.90% higher than autotrophic NVP removal. It was further concluded that biodegradation was the main mechanism for the removal of NVP by *T. obliquus* during all modes of cultivation. Six proposed biotransformation products NVP were detected during mixotrophic growth. Fourier-transform infrared spectroscopy revealed that microalgae biomass before and after NVP biosorption displayed noteworthy shifts in absorption bands. Metabolomic analysis revealed the increase in activity of the fatty acid carbohydrate biosynthesis pathways was observed by *T. obliquus* in the presence of NVP. Thus, future research will focus on exploring the toxicity of transformation products and assessing how different carbon sources affect the removal of ARV drugs by algae.

CHAPTER SIX: General conclusions and recommendations

General conclusions

The purpose of this study was to isolate and screen microalgal isolates from wastewater capable of removing a selected ARV drug, as well as to evaluate the mechanism of ARV drug removal by the selected strain. The major conclusions of the study were as follows:

- Two potential green microalgae isolates, *C. tenuitheca* and *T. obliquus*, capable of removing ARV drugs, have been isolated from local wastewater treatment plants treating municipal wastewater. The strain selection was done mainly based on the growth rates, biomass productivity, and toleration to NVP level.
- The ecotoxicity assessment has demonstrated that the selected strains have high tolerance to NVP, with the calculated IC_{50} values for *C. tenuitheca* and *T. obliquus* were being 23.45 mg L^{-1} and 18.20 mg L^{-1}, respectively, which is higher than the typical concentration of NVP reported from wastewater treatment plants in South Africa.
- Both microalgae strains displayed varying NVP removal efficiencies (ranging from 19.53% to 74.56%) at different concentration of NVP (up to 4000 L^{-1}), with *T. obliquus* achieving the highest removal efficiency during the late log phase on day 8 in flasks supplemented with 50ng L^{-1} NVP.
- The most effective NVP drug removal occurred during mixotrophic growth, with a removal efficiency of 80.13% on day 8. In comparison, heterotrophic and autotrophic cultivation modes yielded slightly lower removal rates of 70.30% and 64.40%, respectively.
- Mass balance calculations revealed that biodegradation was the predominant mechanism of NVP removal, with only a relatively minor contribution from bioadsorption (2.39-3.36%) and bioaccumulation (0.55-0.87%).
- The potential transformation product analysis using untargeted liquid chromatography-time of flight mass spectrometry approach have revealed the formation of six potential biotransformation products during the biodegradation process, which require further investigation to assess their toxicity and identification.
- The analysis of microalgal cellular responses at the gene level demonstrated that the upregulation of antioxidant genes (*sod1*, *cat2* and *gpx1*) significantly enhanced the

organism's ability to defend against oxidative stress induced by NVP. Expression levels of these genes were significantly reduced during heterotrophic and mixotrophic growth, indicating that microalgae can overcome oxidative stress with glucose supplementation.

- Metabolomic analysis further indicated that *T. obliquus* exhibited increased activity in the fatty acid biosynthesis pathway and carbohydrate synthesis when exposed to NVP under mixotrophic growth conditions, demonstrating the microalgae's adaptability and metabolic adjustments in the presence of NVP.

Recommendations

- The transformation and degradation pathways of ARV drugs in wastewater-grown algal biomass require thorough investigation using advanced analytical techniques such as Nuclear magnetic resonance (NMR) and Gas Chromatography and Liquid Chromatography coupled with Mass Spectrometry.
- Laboratory-scale studies with synthetic wastewater need to be compared with real wastewater matrices to understand the potential of algae for ARV drugs removal in the real scenario.
- Monitoring factors like substrate ratio, temperature, light intensity, pH, dissolved oxygen, and hydraulic retention time is crucial for efficient ARV drug removal.
- The role of different carbon sources and other growth promoting substances in overcoming the oxidative stress caused by ARV drugs on algal cells requires further investigation in order to develop an optimal bioremediation strategy.

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APPENDIX

Appendix One. Global occurrence of ARV drugs in environmental samples.

Reference Data for Figure 2.2 and Figure 2.3 AFRICA				
Country	**ARV drug**	**Sample type**	**Concentration (ng L^{-1})**	**Reference**
South Africa	Emtricitabine	WWTP influent	300-3100	(Mlunguza *et al.*, 2020)
	Tenofovir	WWTP influent	100-250	
	Efavirenz	WWTP influent	1020- 26300	
	Emtricitabine	WWTP effluent	110-350	
	Tenofovir	WWTP effluent	<LOQ	
	Efavirenz	WWTP effluent	32700-37300	
	Tenofovir	Dam water	110	
South Africa	Efavirenz	WWTP influent	15400	(Mosekiemang *et al.*, 2019)
	Efavirenz	WWTP effluent	1930	
	Lamivudine	WWTP influent	20900	
	Lamivudine	WWTP effluent	<LOQ	
South Africa	Zidovudine	WWTP influent	6900-53000	(Abafe *et al.*, 2018)
	Zidovudine	WWTP effluent	87-500	
	Nevirapine	WWTP influent	670-2800	
	Nevirapine	WWTP effluent	540-1900	
	Efavirenz	WWTP influent	24000-34000	
	Efavirenz	WWTP effluent	20000-34000	
	Lopinavir	WWTP influent	1200-2500	

	Lopinavir	WWTP effluent	1900-3800	
	Ritonavir	WWTP influent	1600 -3200	
	Ritonavir	WWTP effluent	460-1500	
	Indinavir	WWTP influent	260-590	
	Indinavir	WWTP effluent	25-42	
	Darunavir	WWTP influent	69-43000	
	Darunavir	WWTP effluent	130-17000	
South Africa	Nevirapine	Dam water	55	(Rimayi *et al*., 2018)
	Efavirenz	Dam water	82	
	Nevirapine	River water	71	
	Efavirenz	River water	354	
	Emtricitabine	River water	13	
South Africa	Efavirenz	WWTP influent	5500-14000	(Schoeman *et al*., 2017)
	Nevirapine	WWTP effluent	92-473	
South Africa	Efavirenz	WWTP effluent	74.1	(Wood *et al*., 2015)
	Zidovudine	WWTP effluent	450-970	
	Lopinavir	WWTP effluent	130	
	Efavirenz	Surface water	>90.4	
	Lopinavir	Surface water	130-305	
	Ritonavir	Surface water	>90.4	
	Nevirapine	Surface water	130-1480	
	Tenofovir	Surface water	145-243	
	Lamivudine	Surface water	94.5-242	
	Zidovudine	Surface water	51.7-973	
	Tenofovir	Surface water	145-243	

South Africa	Efavirenz	WWTP influent	17400	(Schoeman *et al.*, 2015)
	Nevirapine	WWTP influent	2100	
	Nevirapine	WWTP effluent	350	
South Africa	Emtricitabine	Urine fertilizer	920	(Bischel *et al.*, 2015)
	Ritonavir	Urine fertilizer	4.6	
South Africa	Ribavirin	WWTP influent	19600	(Osunmakinde *et al.*, 2013)
	Ribavirin	WWTP effluent	42	
Kenya	Nevirapine	River water	33440	(K'Oreje *et al.*, 2012)
	Zidovudine	River water	18300	
	Lamivudine	River water	3150	
Kenya	Nevirapine	WWTP effluent	1357	(Ngumba *et al.*, 2016a)
	Zidovudine	WWTP effluent	513	
	Lamivudine	WWTP effluent	3985	
	Nevirapine	River water	4859	
	Zidovudine	River water	7684	
	Lamivudine	River water	5428	
Kenya	Nevirapine	WWTP influent	850-3300	(K'Oreje *et al.*, 2016)
	Nevirapine	WWTP effluent	1030-2080	
	Zidovudine	WWTP effluent	90-110	
ASIA				
Country	**ARV drug**	**Sample type**	**Concentration ($ng\ L^{-1}$)**	**Reference**
China	Stavudine	Dewatered sludge	<LOQ	(Peng *et al.*, 2014)

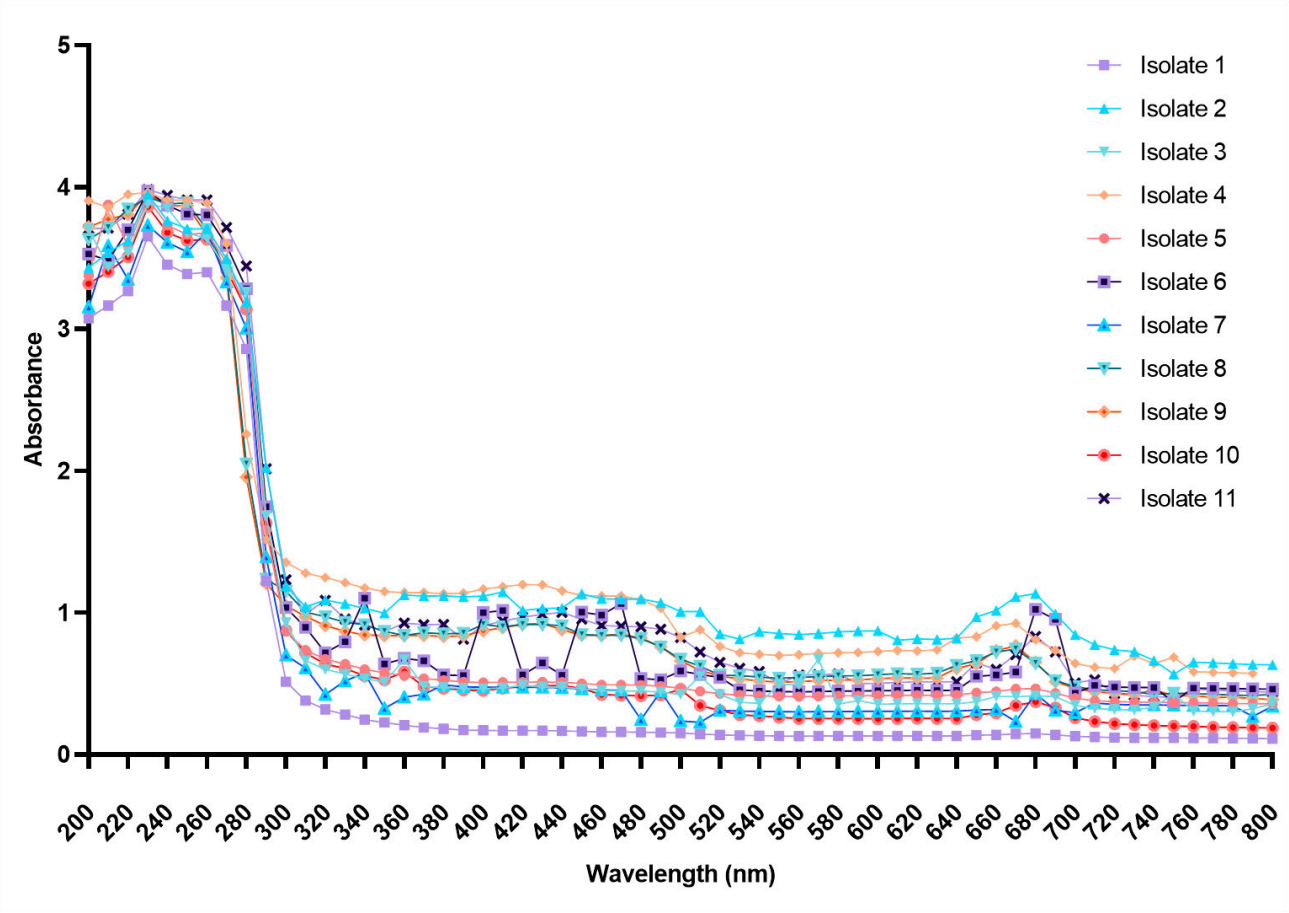
Japan	Valacyclovir	Hospital effluent	21	(Azuma *et al*., 2019)
EUROPE				
Country	**ARV drug**	**Sample type**	**Concentration ($ng\ L^{-1}$)**	**Reference**
Germany	Abacavir	WWTP effluent	10	(Boulard *et al*., 2018)
Germany	Zidovudine	WWTP effluent	57-180	(Funke *et al*., 2016)
	Abacavir	WWTP effluent	21	
Germany	Nevirapine	WWTP influent	21.8	(Prasse *et al*., 2010)
	Nevirapine	WWTP effluent	32.1	
	Ribavirin	WWTP influent	<LOQ	
	Ribavirin	WWTP effluent	<LOQ	
	Zidovudine	WWTP influent	380	
	Zidovudine	WWTP effluent	564	
	Zidovudine	Surface water	170	
	Abacavir	WWTP influent	225	
	Abacavir	WWTP effluent	<LOQ	
	Stavudine	WWTP influent	22.8	
	Stavudine	WWTP effluent	<LOQ	
	Lamivudine	WWTP influent	720	
	Lamivudine	WWTP effluent	<LOQ	
Poland	Darunavir	Tap water	3.4	(Giebultowicz *et al*., 2018)
	Darunavir	River water	169	
Greece	Atazanavir	WWTP effluent	<LOQ	(Ibanez *et al*., 2017)

Norway	Atazanavir	WWTP effluent	<LOQ	(Ferrando-Climent *et al*., 2016)
France	Abacavir	Estuary water	0.2-2.3	(Aminot *et al*., 2016)
	Lamivudine	Estuary water	2.6	
	Nevirapine	Estuary water	0.5	
	Ritonavir	Estuary water	0.2-0.5	
France	Abacavir	WWTP effluent	31-33	(Aminot *et al*., 2015)
	Abacavir	Upstream	0.7	
	Abacavir	Downstream	0.8-2.6	
	Indinavir	WWTP effluent	1.5	
	Lamivudine	WWTP effluent	6.5-44	
	Lamivudine	Downstream	4.1	
	Nevirapine	WWTP effluent	3.0-7.7	
	Nevirapine	Downstream	0.4-1.3	
	Ritonavir	WWTP effluent	53-155	
	Ritonavir	Upstream	0.2	
	Ritonavir	Downstream	2-12	
	Saquinavir	WWTP effluent	0.2	
	Zidovudine	WWTP effluent	154-191	
	Zidovudine	Downstream	33	
Finland	Zidovudine	WWTP effluent	22-37	(Ngumba *et al*., 2016b)
	Lamivudine	WWTP effluent	20	
Belgium	Lamivudine	WWTP influent	507	(Vergeynst *et al*., 2015)
Switzerland	Ritonavir	WWTP effluent	90	(Margot *et al*., 2013)

Switzerland	Ritonavir	Hospital effluent	108	(Kovalova *et al*., 2012)
NORTH AMERICA				
Country	**ARV drug**	**Sample type**	**Concentration (ng L^{-1})**	**Reference**
United states	Lamivudine	Drinking water	27.73	(Furlong *et al*., 2017)
United states	Lamivudine	Ground water	22.9	(Fisher *et al*., 2016)
	Nevirapine	Ground water	16.4-25.2	
United states	Abacavir	Landfill leachates	185	(Masoner *et al*., 2014)
	Lamivudine	Landfill leachates	355	
Canada	Tenofovir	Soil	-	(Al-Rajab *et al*., 2010)

*LOQ- Limit of quantification

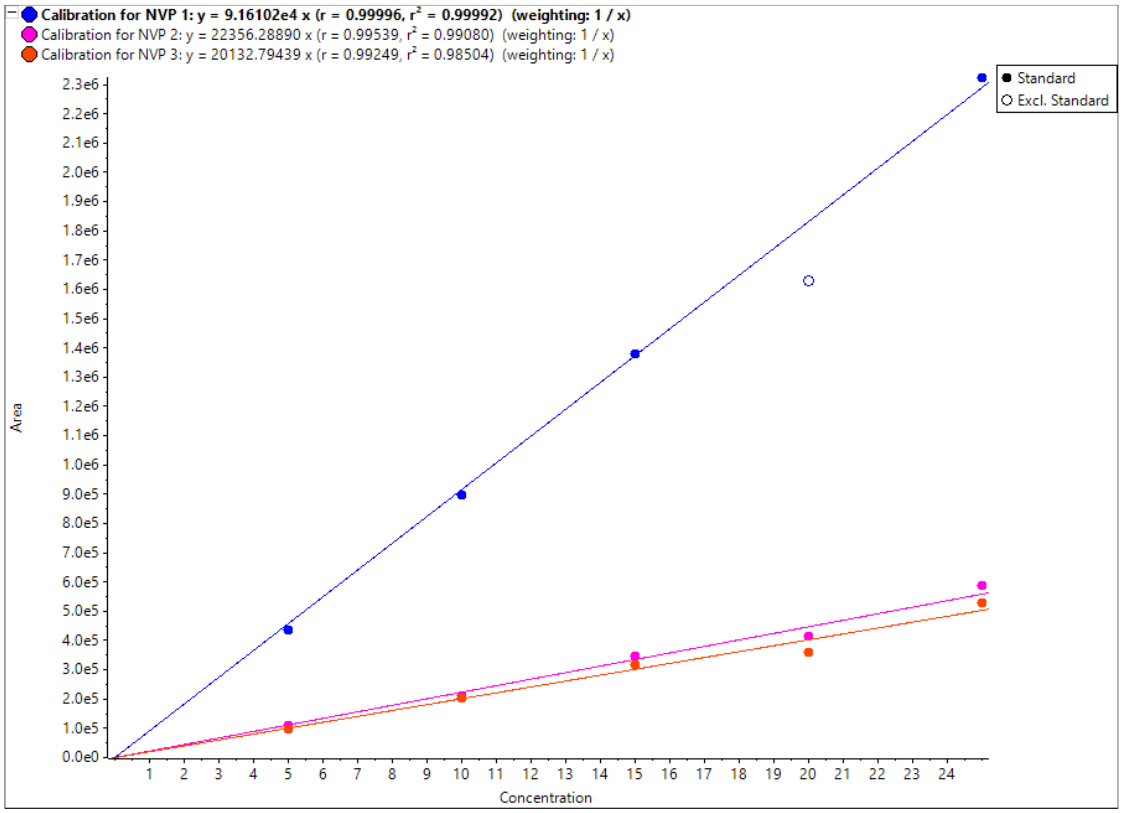
Appendix Two. Spectral scan of isolates 1-11 from 200-800 nm.



Appendix Three. Modified BG-11 medium recipe (Allen, 1968).

Solution	g L^{-1}
Stock 1	
Na_2Mg EDTA	0.1
Ferric ammonium citrate	0.6
Citric acid . $1H_2O$	0.6
$CaCl_2$. $2H_2O$	3.6
Stock 2	
$MgSO_4$. $7H_2O$	7.5
Stock 3	
K_2HPO_4 . $3H_2O$	4.0
or K_2HPO_4	3.05
Stock 4 (Microelements)	
H_3BO_3	2.86
$MnCl_2$. $4H_2O$	1.81
$ZnSO_4$. $7H_2O$	0.222
$CuSO_4$. $5H_2O$	0.079
$COCl_2$. $6H_2O$	0.050
$NaMoO_4$. $2H_2O$	0.391
or MoO_4 (85%)	0.018

Appendix Four. Standard curve of NVP (5-25 ng L^{-1}) generated by LC-MS/MS.

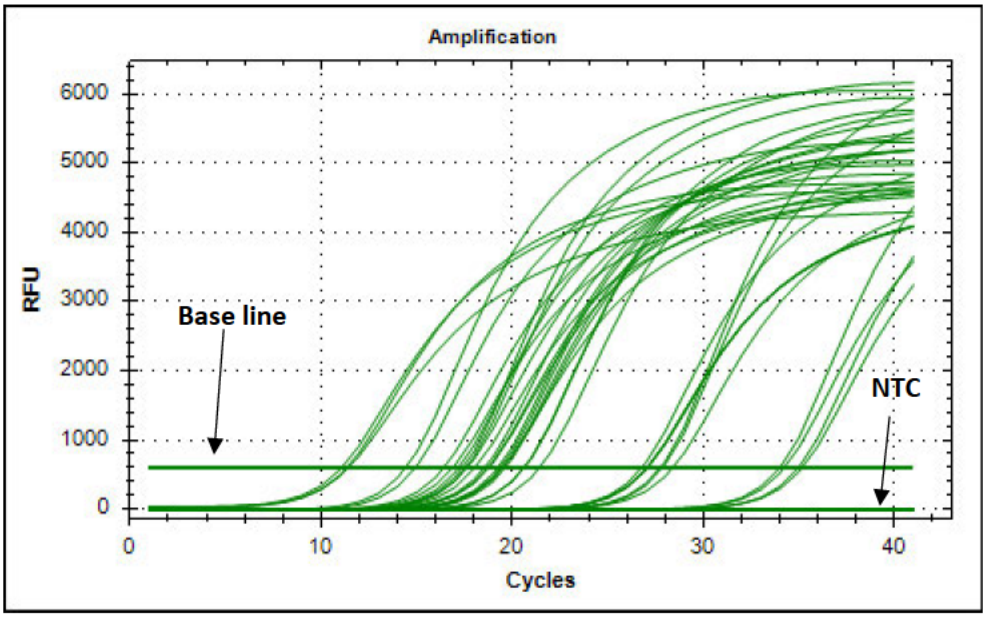


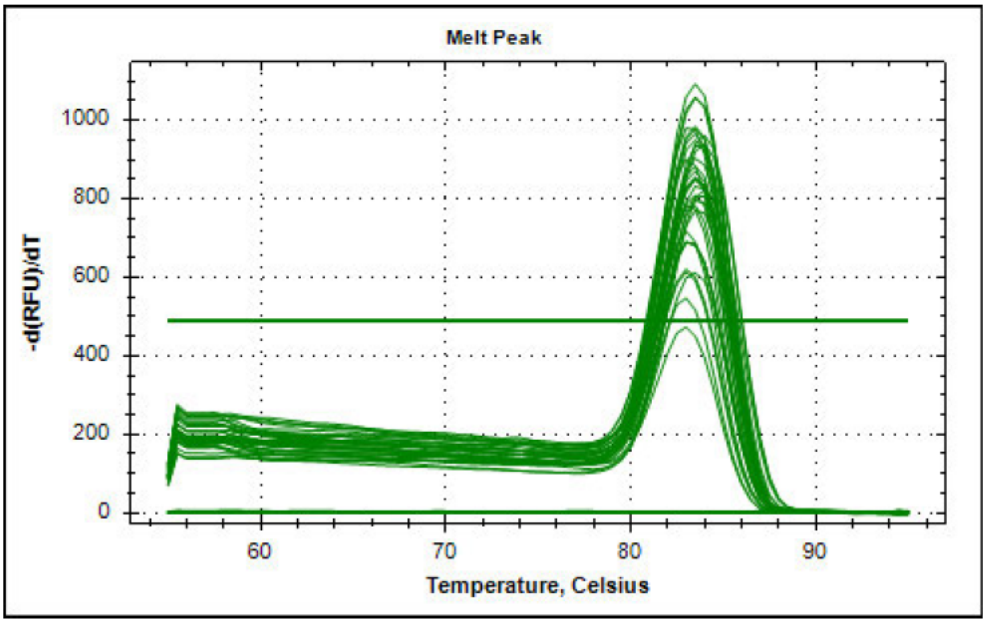
Appendix Five. The maximum quantum yield of PS II (Fv/Fm) recorded in the treatments with different NVP concentrations in *Coelastrella tenuitheca* and *Tetradesmus obliquus* cultures at the mid-log (day 6) and late log (day 8) phase of growth.

NVP concentration (ng L^{-1})	*Coelastrella tenuitheca*		*Tetradesmus obliquus*	
	6th day	**8th day**	**6th day**	**8th day**
0	$0.605 \pm 0.00^{ab*}$	0.672 ± 0.01^{def}	0.633 ± 0.03^{ab}	0.644 ± 0.01^{bc}
5	0.616 ± 0.03^{abc}	0.683 ± 0.02^{abcd}	0.546 ± 0.04^{c}	0.614 ± 0.02^{c}
10	0.588 ± 0.03^{bc}	0.675 ± 0.01^{cdef}	0.629 ± 0.05^{ab}	0.612 ± 0.01^{c}
50	0.581 ± 0.02^{c}	0.653 ± 0.01^{g}	0.650 ± 0.04^{ab}	0.669 ± 0.01^{a}
100	0.583 ± 0.04^{c}	0.669 ± 0.01^{def}	0.605 ± 0.03^{abc}	0.613 ± 0.02^{c}
150	0.598 ± 0.05^{abc}	0.667 ± 0.02^{ef}	0.590 ± 0.11^{abc}	0.647 ± 0.01^{abc}
200	0.620 ± 0.02^{abc}	0.673 ± 0.01^{cdef}	0.596 ± 0.02^{abc}	0.666 ± 0.01^{ab}
500	0.592 ± 0.03^{bc}	0.689 ± 0.02^{ab}	0.533 ± 0.04^{c}	0.637 ± 0.01^{bc}
1000	0.639 ± 0.04^{a}	0.693 ± 0.01^{a}	0.552 ± 0.03^{bc}	0.628 ± 0.01^{bc}
1500	0.611 ± 0.02^{abc}	0.687 ± 0.02^{ab}	0.588 ± 0.03^{abc}	0.626 ± 0.02^{bc}
2000	0.627 ± 0.02^{ab}	0.679 ± 0.02^{bcde}	0.562 ± 0.00^{bc}	0.639 ± 0.01^{c}
4000	0.607 ± 0.02^{abc}	0.663 ± 0.02^{fg}	0.577 ± 0.07^{abc}	0.628 ± 0.02^{bc}

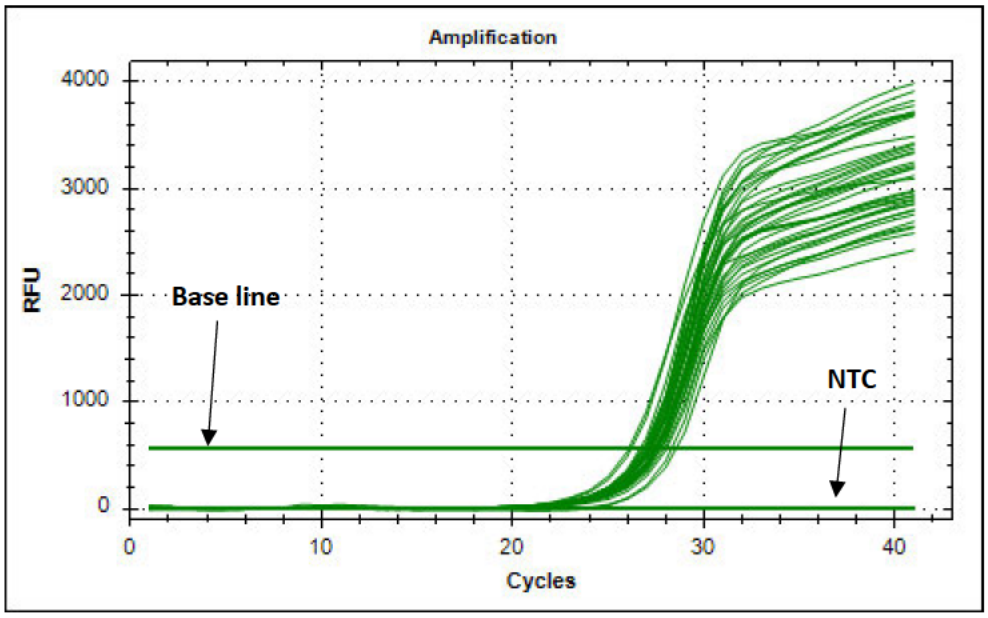
* Lowercase alphabets (a, b, c, etc.) represent DMRT (Duncan's Multiple Range Test) ranking, where ‘a’ represents the highest value ($p \leq 0.05$) and similar alphabets represent non-significant values.

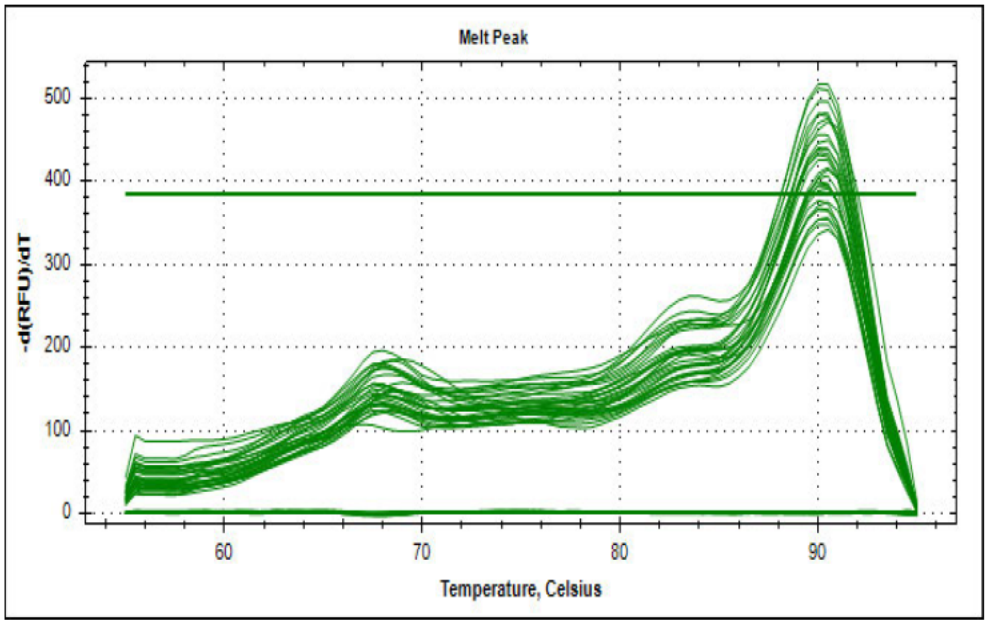
Appendix Six. Effect of various conditions on the amplification of *18S rRNA* gene in *T. obliquus*.



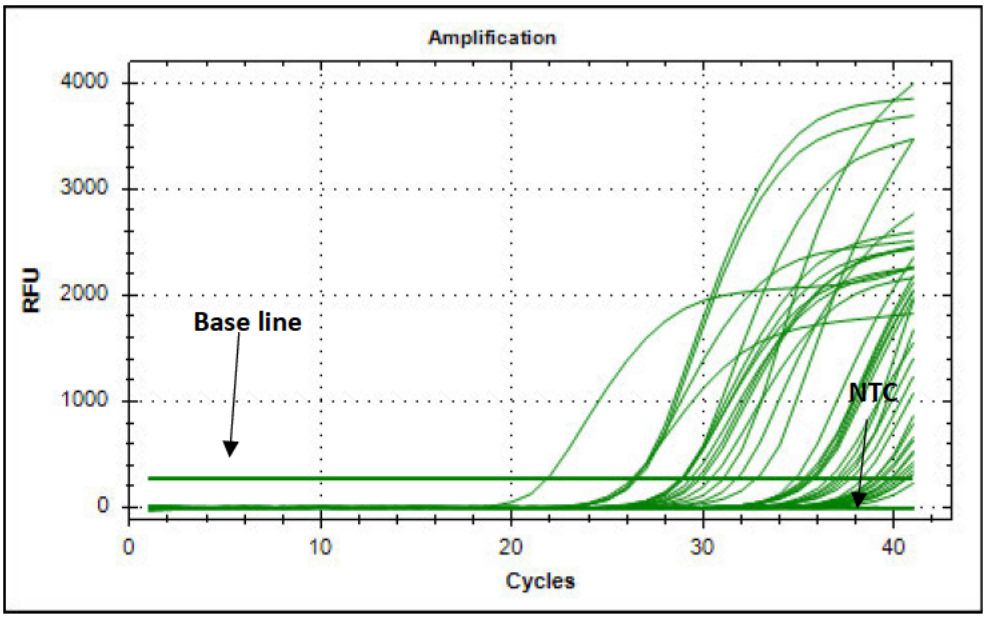


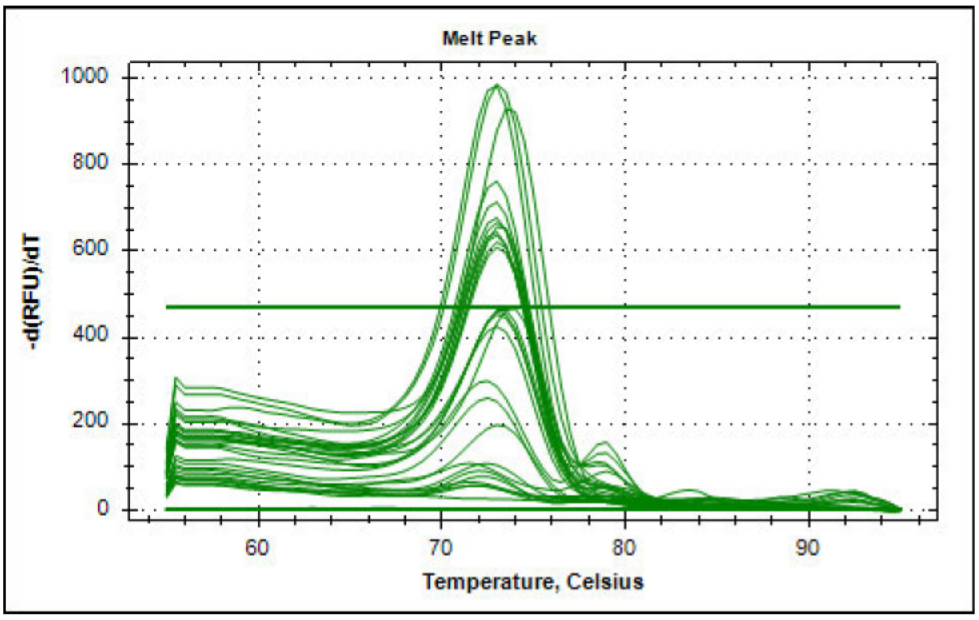
Appendix Seven. Effect of various conditions on the amplification of *sod1* gene.



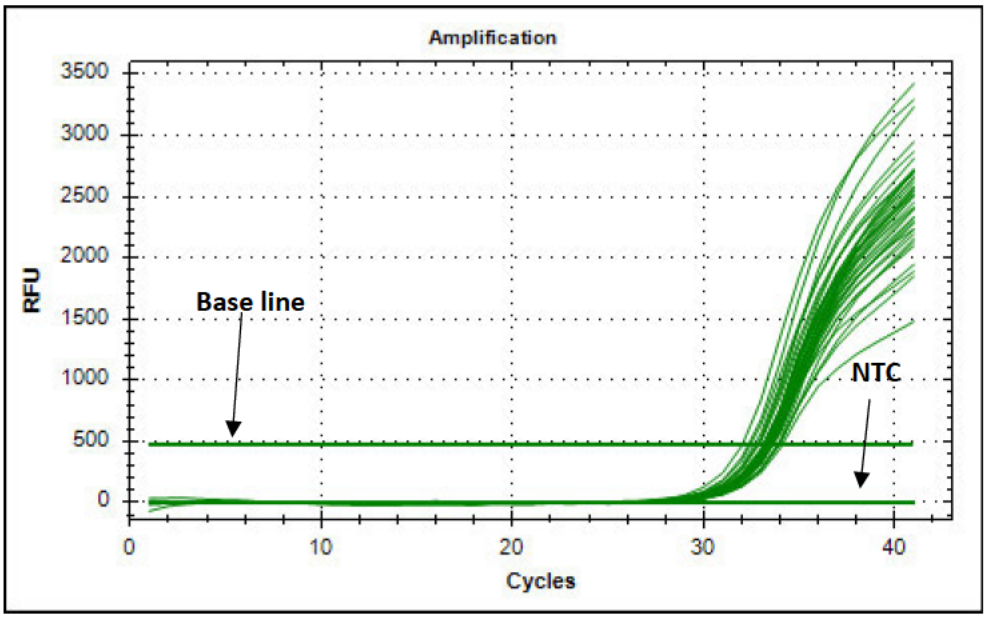


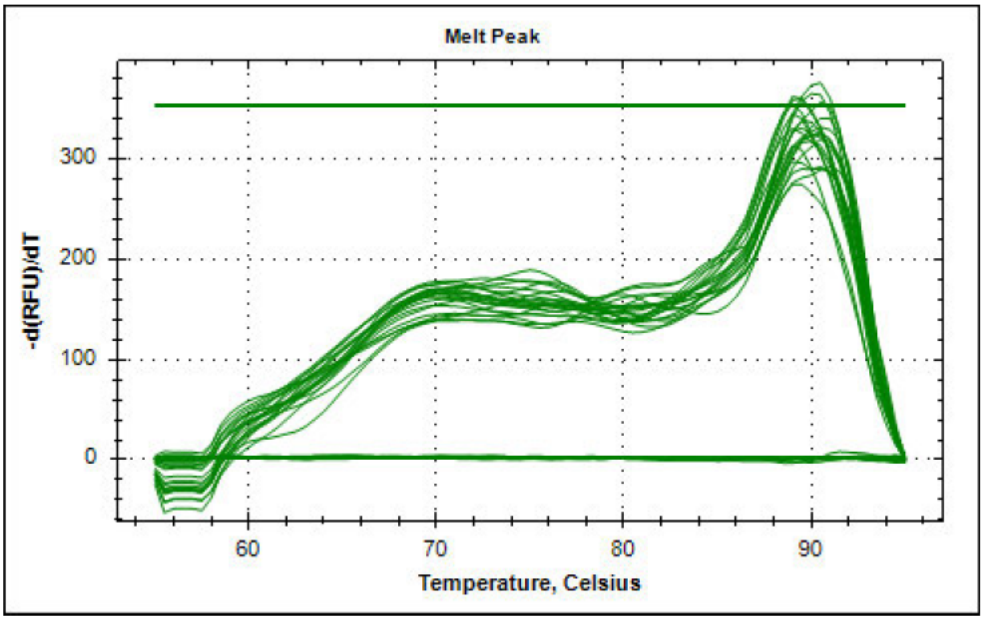
Appendix Eight. Effect of various conditions on the amplification of *cat2* gene.





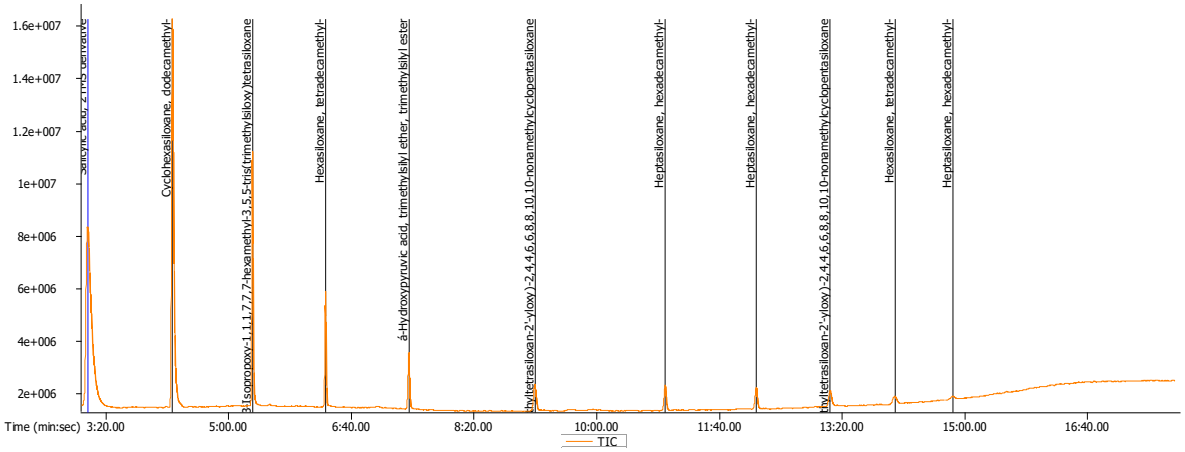
Appendix Nine. Effect of various conditions on the amplification of *gpx* gene.



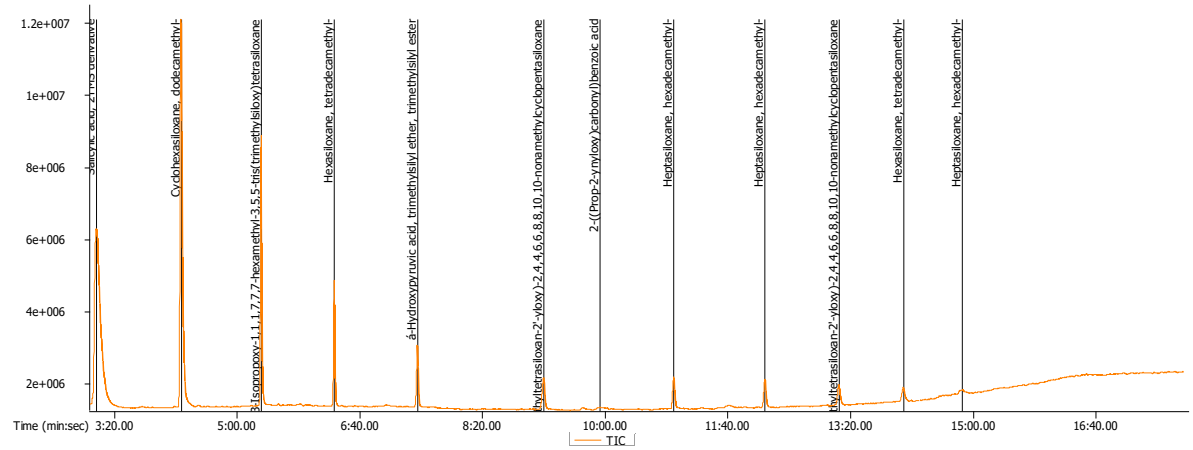


Appendix Ten. GC-MS total ion current chromatogram (TIC) of *T. obliquus* biomass obtained from (a) Control (Day 4); (b) Treated (Day 4); (c) Control (Day 8); (d) Treated (Day 8).

a



b



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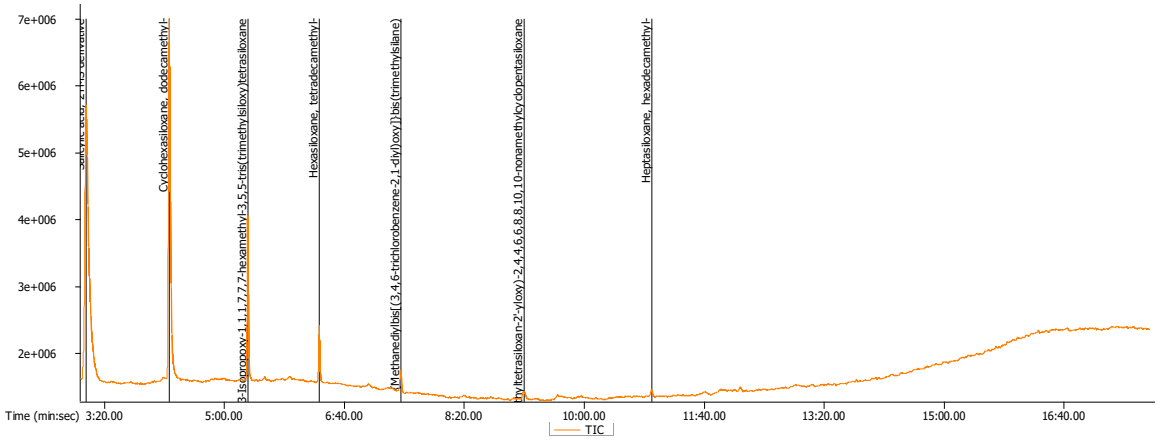

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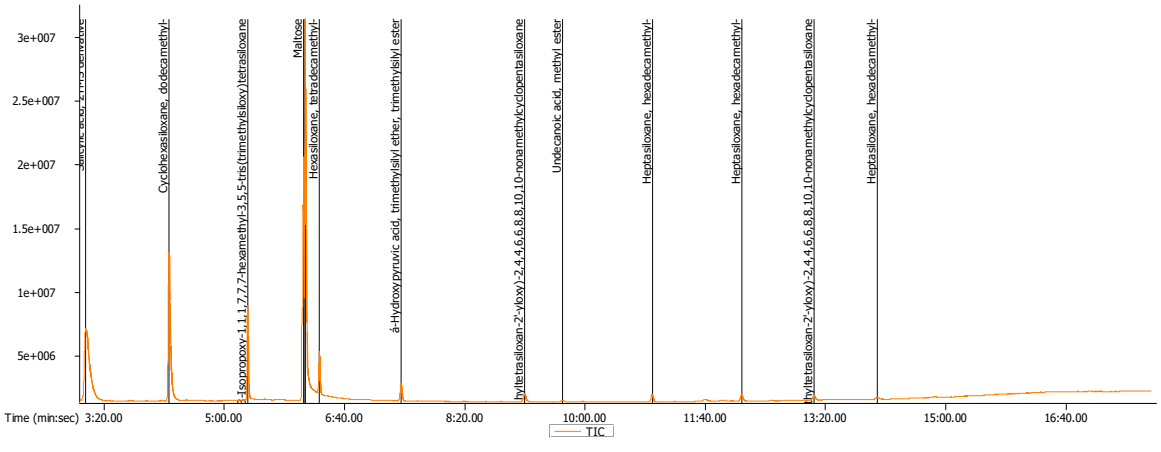
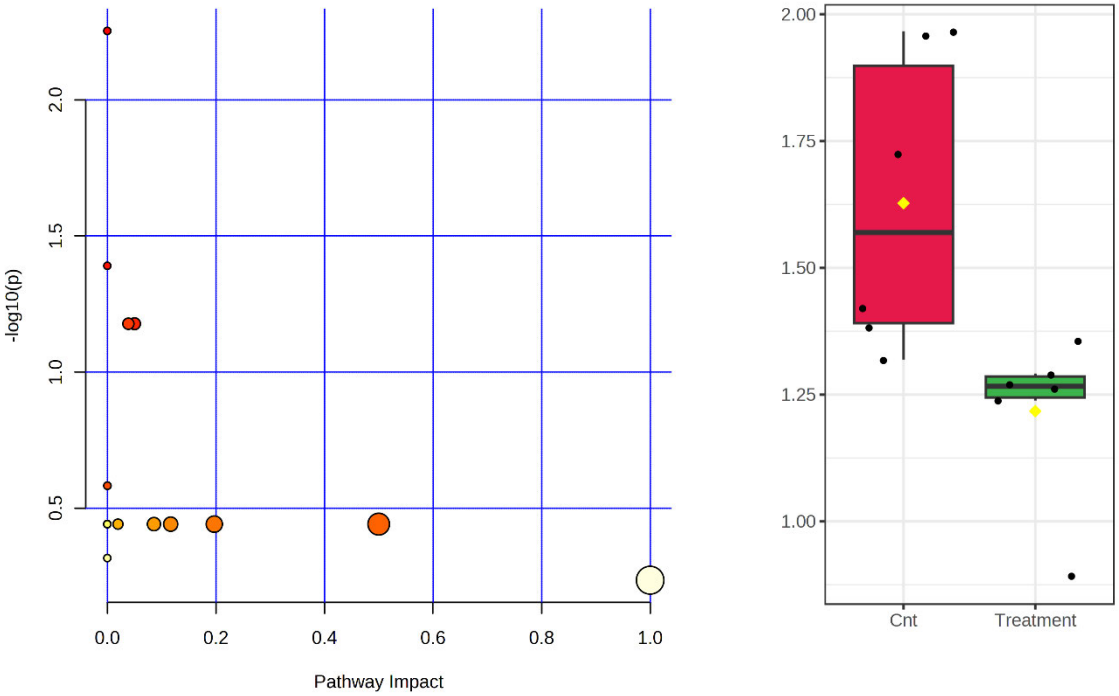
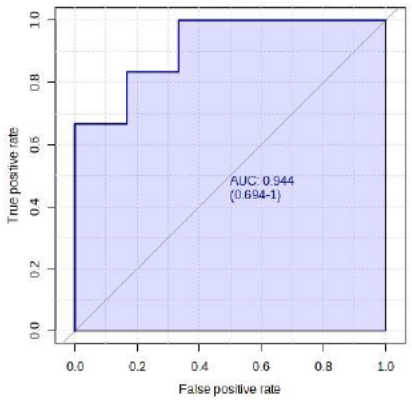
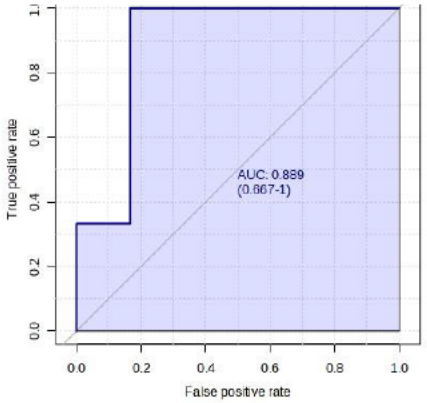


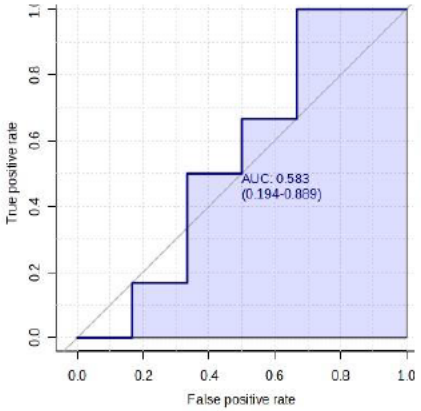
Appendix Eleven. Analysis of metabolic pathways for the 12 annotated metabolites.

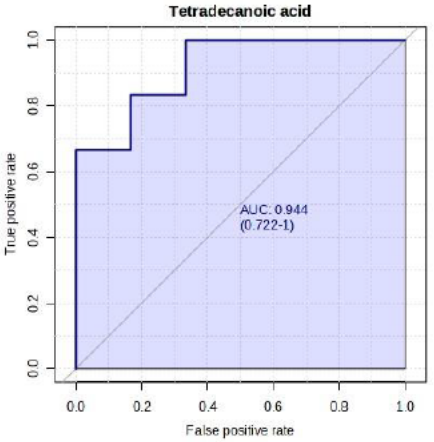


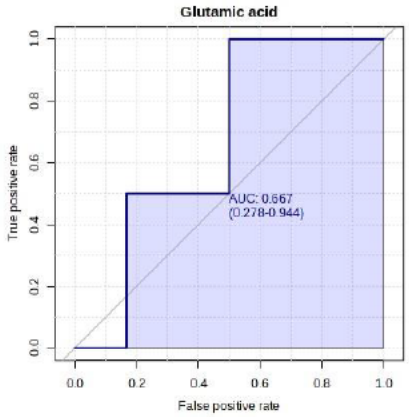
Appendix Twelve. Receiver operating characteristic curves.

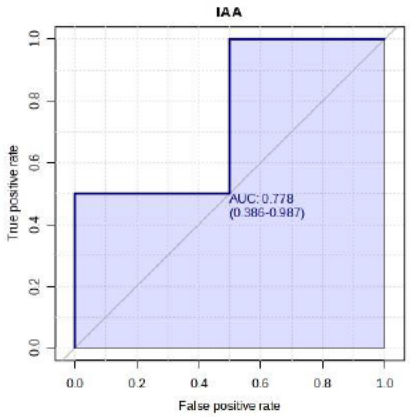


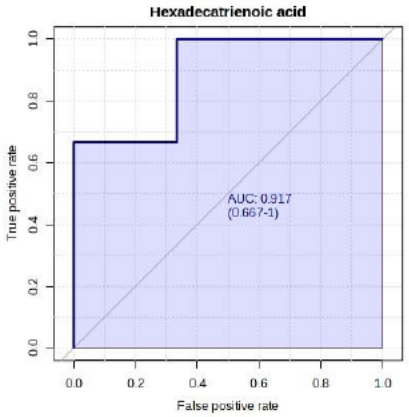


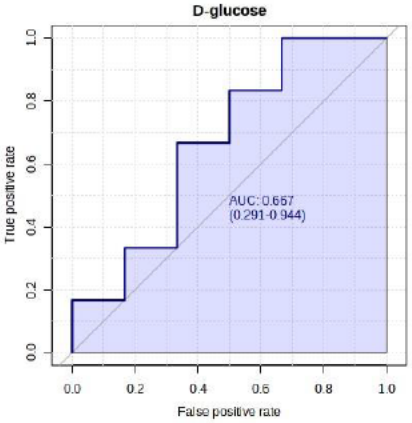


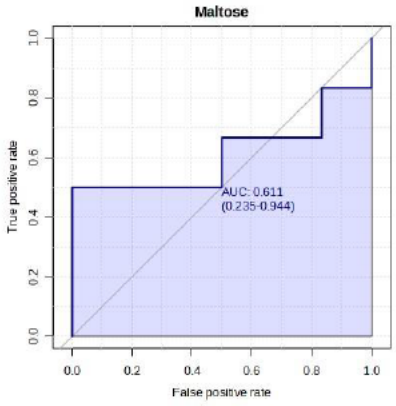


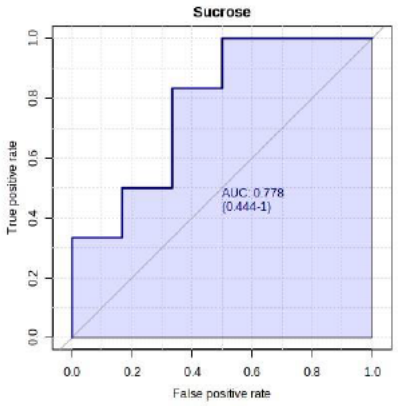












Appendix Thirteen. Publication: Algae-mediated processes for the treatment of antiretroviral drugs in wastewater: Prospects and challenges.

Chemosphere 280 (2021) 130674

Contents lists available at ScienceDirect

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journal homepage: www.elsevier.com/locate/chemosphere

Review

Algae-mediated processes for the treatment of antiretroviral drugs in wastewater: Prospects and challenges

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ABSTRACT

The prevalence of pharmaceuticals (PCs), especially antiretroviral (ARV) drugs in various aquatic ecosystems has been expansively reported, wherein wastewater treatment plants (WWTPs) are identified as the primary point source. Consequently, the occurrence, ecotoxicity and treatment of ARV drugs in WWTPs have drawn much attention in recent years. Numerous studies have shown that the widely employed activated sludge-based WWTPs are incapable of removing ARV drugs efficiently from wastewater. Recently, algae-based wastewater treatment processes have shown promising results in PCs removal from wastewater, either completely or partially, through different processes such as biosorption, bioaccumulation, and intra-/inter-cellular degradation. Algal species have also shown to tolerate high concentrations of ARV drugs than the reported concentrations in the environmental matrices. In this review, emphasis has been given on discussing the current status of the occurrence of ARV drugs in the aquatic environment and WWTPs. Besides, the current trends and future perspectives of PCs removal by algae are critically reviewed and discussed. The potential pathways and mechanisms of ARV drugs removal by algae have also been discussed.

1. Introduction

Global attention has been drawn in recent years to preserve freshwater resources due to the increased discharge of pollutants, especially emerging micropollutants (EMPs) to the aquatic environment (de Oliveira et al., 2020). Among different EMPs, the prevalence of pharmaceutical compounds/contaminants (PCs) has become a major threat to the environment (Majumder et al., 2019; Nnadozie et al., 2017; Rebello et al., 2018; UNODC, 2018). The commonly detected PCs in the aquatic environment include antibiotics, hormones, non-steroidal anti-inflammatory, analgesics and antiretroviral (ARV) drugs (Ebele et al., 2017; Hossain et al., 2018; Majumder et al., 2019). The increased occurrence of ARV drugs in different aquatic ecosystems has become a grave concern due to their toxic effects on aquatic organisms, and the induction of microbial drug resistance (Gaw et al., 2014).

ARV drugs are frequently used to treat human immunodeficiency virus (HIV), a global epidemic with its epicentre in South Africa (Tompsett, 2020). Moreover, South Africa has the largest antiretroviral therapy (ART) program in the world. Due to this, millions of people are accessing these drugs daily as long-term therapy for HIV. To date over 35 different ARV drugs are being used for HIV treatment (Mlunguza et al., 2020), with the most common being tenofovir (TDF), abacavir (ABC), lamivudine (3TC), nevirapine (NVP), efavirenz (EFV), and zidovudine (ZDV). Some of these drugs are used in combinations for better results (Ahmed et al., 2018; Russo et al., 2018). The annual rise in HIV infection rate over the years has led to a significant increase in the production and consumption of ARV drugs globally (Nannou et al., 2020). Presently, the use of ARV drugs has also been explored for the treatment of SARS-CoV-2, the new coronavirus (COVID-19) pandemic. Countries such as China and Japan have implemented clinical trials to test HIV medication such as lopinavir, ritonavir (RTV) and darunavir for COVID-19 treatment (amfAR, 2020; The Daily Briefing, 2020).

Studies have shown that these drugs may undergo metabolic reactions within the human body such as hydroxylation, cleavage or glucuronidation (Prasse, 2012). Pharmacokinetics studies have revealed that most of these drugs are not fully metabolized within the human body and are excreted *via* faeces and/or urine either in its original form or as transformation or degradation products (Bottoni et al., 2010; Dębska et al., 2004; Schoeman et al., 2017). In urban areas, these excreted products along with urine and faeces enter the sewage network

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[1] Karen Reddy and Nirmal Renuka are dual first authors and have equally contributed to the review article.

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Appendix Fourteen. Publication: Assessing the potential for nevirapine removal and its ecotoxicological effects on *Coelastrella tenuitheca* and *Tetradesmus obliquus* in aqueous.

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Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Assessing the potential for nevirapine removal and its ecotoxicological effects on *Coelastrella tenuitheca* and *Tetradesmus obliquus* in aqueous environment

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Microalgae

ABSTRACT

Remediation of the antiretroviral (ARV) drug, nevirapine (NVP) has attracted considerable scientific attention in recent years due to its frequent detection and persistence in aquatic environments and potential hazards to living organisms. Algae-based technologies have been emerging as an environmentally friendly option for the removal of pharmaceutical compounds, but their ARV drug removal potential has not been fully explored yet. This study aimed to explore the ecotoxicity and removal potential of NVP by two microalgal species, *Coelastrella tenuitheca* and *Tetradesmus obliquus*. Lower environmental concentrations (up to 200 ng L^{-1}) of NVP enhanced the microalgal growth, and the highest dry cell weight of 941.27 mg L^{-1} was obtained in *T. obliquus* at 50 ng L^{-1} NVP concentration. Both microalgae showed varying removal efficiencies (19.53–74.56%) when exposed to NVP concentration levels of up to 4000 ng L^{-1}. At the late log phase (day 8), *T. obliquus* removed the highest percentage of NVP (74.56%), while *C. tenuitheca* removed 48% at an initial NVP concentration of 50 ng L^{-1}. Photosynthetic efficiency (Fv/Fm and rETR) of the two microalgal species, however, was not affected by environmental concentrations of NVP (up to 4000 ng L^{-1}) at the mid log phase of growth. SEM analysis demonstrated that both algal species produced distinct ridges on their cell surfaces after NVP uptake. In the ecotoxicity study, the calculated IC_{50} values of NVP (0–100 mg L^{-1}) after 96 h of exposure were 23.45 mg L^{-1} (*C. tenuitheca*) and 18.20 mg L^{-1} (*T. obliquus*). The findings of the present study may contribute to a better understanding of the environmental hazards associated with NVP and the efficacy of microalgae in removing this pharmaceutical from aquatic environments.

1. Introduction

A worldwide pandemic of Human Immunodeficiency Virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS) began in the 1980s (Sharp and Hahn, 2011). Antiretroviral (ARV) drug therapy includes nucleoside and nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors, acyclic nucleoside phosphonate analogues and entry inhibitors, which are administered daily in two-drug or three-drug fixed dose combinations (Meintjes et al., 2017; Saag et al., 2020). Based on the recent data, the average number of people living with HIV/AIDS in South Africa is 7.9 million, and more than 5 million of them receive antiretroviral treatment (the highest rate in the world) (Horn et al., 2022). In South Africa, commonly used ARV drugs are didanosine, lamivudine, zidovudine, stavudine, efavirenz, lopinavir, nevirapine and ritonavir (Ncube et al., 2018). In terms of treatment guidelines, nevirapine (NVP) is listed among the non-nucleoside reverse transcriptase inhibitors for HIV-infected adolescents, adults, and most often pregnant women (Flynn et al., 2018). After being administered, NVP is nearly 90% bioavailable, disintegrates after 45 h, and is excreted through the urine. The bioactivity of NVP, combined with its long half-life, allows it to persist in the environment for a long period of time (Yao et al., 2021).

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Appendix Fifteen. Book chapter: Microbe-assisted bioremediation of pesticides from contaminated habitats: Current status and prospects.

Chapter 7

Microbe-Assisted Bioremediation of Pesticides from Contaminated Habitats

Current Status and Prospects

Karen Reddy,[1] *Shisy Jose*,[1] *Tufail Fayaz*,[2] *Nirmal Renuka*,[2,*] *Sachitra Kumar Ratha*,[3] *Sheena Kumari*[1] and *Faizal Bux*[1]

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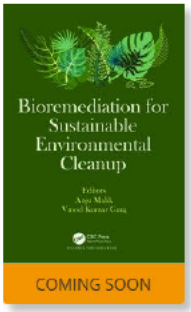
7.1 Introduction

The rapid growth of the global pesticide market, driven by widespread use of pesticides in agriculture and non-agriculture sectors, has led to the introduction of numerous pesticide residues into the environment (Mali et al. 2022). Pesticides are recalcitrant and non-biodegradable, thus when applied to farmlands, gardens and other vegetation, they often remain toxic for years (Gonçalves and Delabona 2022). They are also carcinogenic in nature and are banned in many countries due to the risk created by their presence in the environment (Singh et al. 2020). In addition to polluting soil and crops, they also pose a threat to ground water and other aquatic environments (Castelo-Grande et al. 2010, Lehmann et al. 2018). Most pesticides reach destinations other than their intended target, even though each is designed to eliminate a specific pest (Huang et al. 2018, Mali et al. 2022).

Several technologies have been developed and applied to contaminated sites to eliminate the adverse environmental effects of pesticides. These include, physical treatments (adsorption and percolator filters) and chemical treatments (advanced oxidation) (Satish et al. 2017). Even though these



PREVIEW BOOK

Canada, Germany, India, Pakistan, South Africa, the United Kingdom, and the United States of America, have contributed towards the chapters of this book.

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