Doctoral Thesis in Biotechnology

Wastewater treatment from pharmaceutical substances with filamentous fungi

BRIGITA DALECKA

Riga, Stockholm 2021

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Abstract

The ever-increasing concern about the widespread occurrence of pharmaceutical substances in the aquatic environment has been recognized as an emerging environmental issue as it can cause undesirable effects on the ecosystem and human health. The current wastewater treatment methods are not designed to treat municipal wastewater from the contamination of various pharmaceutical substances. As a result, pharmaceuticals can enter the environment and pose a threat to life forms. Therefore, it is important to enhance the classical wastewater treatment process in order to meet the challenges by advancing the technologies. Currently, the biological treatment method with filamentous fungi has been considered a promising, cost-effective, and environmentally friendly method for removing pharmaceutical substances from municipal wastewater.

The thesis "Wastewater treatment from pharmaceutical substances with filamentous fungi" demonstrates the potential application of fungi in removing pharmaceutical substances and their expedience to incorporate into the classical municipal wastewater treatment process. The investigation focused on selecting suitable fungal strains that could adapt without adjusting physico-chemical parameters and compete with the microbial community in the municipal wastewater. Further, the thesis investigated whether fungal strains could reduce nutrients and pharmaceutical substances in lab-scale and pilot-scale setup and the mechanisms of pharmaceutical substance removal.

The thesis consists of two main stages. In the first stage, the batch-scale experiments were carried out under laboratory conditions, finding out the most suitable fungal strains for the removal of pharmaceutical substances from wastewater. The results demonstrated that fungi compete with each other since higher removal efficiency was observed if the fungi were grown individually. Batch-scale experiments showed that *Trametes versicolor* a laboratory strain and *Aspergillus luchuensis* an environmental isolate from a municipal wastewater treatment plant – can be a promising strain for removing pharmaceutical substances in a non-sterile municipal wastewater treatment without the adjustment of pH level. Therefore, these strains were used for further study.

In the second stage, the pilot-scale system with a fungal fluidized bed pelleted bioreactor was developed. The results demonstrated that a high potential to remove phosphorus from municipal wastewater efficiently and successfully under a batch scale experiment with non-sterile municipal

wastewater while the results from the fluidized bed bioreactor did not demonstrate any significant decrease of phosphorus. Additionally, the fluidized pelleted bioreactor was optimized to perceive bioaugmentation as a strategy with the frequent addition of fungal biomass. The results from the optimization process showed that bioaugmentation is a relatively efficient approach to build on fungi in the fluidized pelleted bioreactor. Furthermore, the results from the AI-based platform with modeling study showed that optimization of bioaugmentation with fungi increases the removal efficiency of pharmaceutical substances from non-sterile municipal wastewater.

The author of this study showed that both the literature review and the results from the batch and pilot-scale experiments provided new knowledge that can be used for future investigations of wastewater treatment with fungi. The thesis will help to improve and better understand the possible application of fungi in the municipal wastewater treatment process.

The thesis is written in English and consists of 68 pages, 14 figures, 4 tables, and 133 literature sources were used for the development of the thesis.

Keywords
Filamentous fungi, municipal wastewater, pharmaceutical substances, fungal fluidized bed pelleted bioreactor, nutrients, diclofenac, ketoprofen, *Trametes versicolor*, *Aspergillus luchuensis*

Anotācija

Promocijas darba “Mikroskopisko sēņu izmantošana sadzīves notekūdeņu attīrīšanā no farmaceitiski aktīvajām vielām” ietvaros ir veikta mikroskopisko sēņu izpēte un pilnveidota alternatīva metode notekūdeņu attīrīšanai no farmaceitiski aktīvajām vielām.

Klasiskās notekūdeņu attīrīšanas metodes ne vienmēr var attīrīt sadzīves notekūdeņus no farmaceitiski aktīvo vielu piesārņojuma. Šī iemesla dēļ apkārtējā vidē var nonākt farmaceitiski aktīvās vielas un radīt apdraudējumu dzīvajiem organismiem. Lai novērstu farmaceitiski aktīvo vielu nokļūšanu apkārtējā vidē no sadzīves notekūdeņiem, nepieciešams meklēt jaunas un alternatīvas tehnoloģijas, lai uzlabotu gan jau esošās metodes, gan izstrādātu jaunas notekūdeņu attīrīšanas metodes. Kā viena no metodēm šī mērķa sasniegšanai var tikt izmantota bioloģiskā metode ar mikroskopiskajām sēnēm.

Promocijas darba pētījums ir sadalīts divās galvenajās daļās. Pirmajā daļā tika veikti laboratorijas mēroga eksperimenti, lai noskaidrotu atbilstošākās mikroskopiskās sēnes notekūdeņu attīrīšanai no farmaceitiski aktīvajām vielām. Promocijas darba gaitā tika noskaidrots, ka mikroskopisko sēņu augstākā efektivitāte farmaceitisko vielu attīrīšanā no notekūdeņiem tika sasniegta, ja sēnes tiek izmantotas atsevišķi, nevis kombinētas savā starpā. Tika noskaidrots, ka *Trametes veriocolor* un *Aspergillus luchuensis* ir vispiemērotākās mikroskopiskās sēnes notekūdeņu attīrīšanai no farmaceitiskajām vielām. Tāpēc šīs mikroskopiskās sēnes tika izmantotas promocijas darba otrajā daļā.

Promocijas darba otrajā daļā tika izstrādāts pilota mēroga bioreaktors. Šajā bioreaktorā tika pārbaudīta izvēlēto mikroskopisko sēņu efektivitāte farmaceitiski aktīvo vielu attīrīšanā no sadzīves notekūdeņiem. Darba gaitā tika pārbaudīta bioaugmentācija kā iespējamā metode, lai uzlabotu mikroskopisko sēņu efektivitāti farmaceitiski aktīvo vielu attīrīšanā no sadzīves notekūdeņiem. Tāpat darba gaitā tika pārbaudīta mikroskopisko sēņu spēja attīrīt notekūdeņus no fosfora un slāpekļa savienojumiem. Tika noskaidrots, ka mikroskopiskās sēnes spēj samazināt kopējo fosfora daudzumu sadzīves notekūdeņos. Tāpat iegūtie rezultāti parādīja, ka mikroskopiskās sēnes spēj sadzīves notekūdeņus attīrīt no farmaceitiski aktīvo vielu piesārņojuma un bioagmentācijas izmantošana var kalpot kā efektīva metode, lai uzturētu nepārtrauktu bioreaktora darbību ar mikroskopiskajām sēnēm.

Promocijas darbs ir uzrakstīts angļu valodā un satur 68 lappuses, 14 attēlus, 4 tabulas, un 133 literatūras avoti tika izmantoti promocijas darba izstrādē.

Atslēgas vārdi
Mikroskopiskās sēnes, sadzīves notekūdeņi, farmaceitiskās vielas, mikroskopisko sēņu bioreaktors, diklofenaks, ketoprofens, *Trametes versicolor*, *Aspergillus luchuensis*

Annotering

Den ständigt ökande oron om läkemedelsresters vida spridning i den akvatiska miljön är ett globalt erkänt växande problem, då dessa substanser orsakar oönskade effekter på människa och miljö. Dagens avloppsvattenreningsmetoder är inte designade för att rena vattnet från läkemedelsrester, vilket resulterar i att dessa substanser sprids och riskerar orsaka skada.

På grund av detta är det viktigt att förbättra den konventionella reningsprocessen för att möta framtidens krav på läkemedelsrening. En lovande teknik för att åstadkomma denna förbättring är biologisk rening med hjälp av filamentösa mikrosvampar, vilken visat sig vara både kostnadseffektiv och miljövänlig. Syftet med denna avhandling var således att undersöka möjligheten att tillämpa mikrosvampar för att rena vatten från läkemedelsrester, men också hur väl en sådan lösning skulle kunna passa in i ett konventionellt reningsverk.

Arbetet fokuserade på att hitta lämpliga stammar av mikrosvampar som kunde anpassa sig till miljön i en konventionell avloppsvattenreningsprocess utan att konkurrera med den aktiva mikrobiologiska floran, eller att behöva förändra rådande fysikalisk-kemiska parametrar. Vidare undersöktes hur väl lämpliga kandidater av mikrosvampar reducerade näringsämnen och läkemedelsrester, samt dess mekanismer, både i labbskale- och pilotskaleförsök.

Avhandlingen består av två delar i vilken den första behandlar batch-experiment i labbskala som genomfördes i labbmiljö där lämpliga stammar av mikrosvampar utvärderades efter dess förmåga att rena vatten från läkemedelsrester. Resultaten visade att svamparna konkurrerar med varandra då högre reduktion observerades i de fall svamparna odlades individuellt. Två lovande kandidater att använda i en icke-steril miljö utan pH-justering var Trametes versicolor, en laboratoriestamm, samt Aspergillus luchuensis, ett isolat från ett kommunalt avloppsreningsverk. Dessa två kandidater användes i avhandlingens andra del, vilken behandlar utvecklingen av en FPB-bioreaktor (fluidiserad pelletbädd-bioreaktor) i pilot-skala. Resultaten visade en hög potential för att effektivt minska halten fosfor i icke-sterilt vatten från ett kommunalt avloppsreningsverk.

Vidare optimerades bioreaktorn med avseende på bioaugmentering, genom kontinuerlig tillförsel av svamp-biomassa. Resultaten från optimeringen visade att bioaugmentering är en effektiv metod för att snabbt bygga upp biomassa i bioreaktorn. En modelerings-studie med hjälp av en

AI-baserad plattform visade dessutom att optimeringen av bioaugmentering ökade systemets effektivitet för att minska halten läkemedelsrester från icke-sterilt avloppsvatten.

Författaren av den här avhandlingen har därmed visat att litteraturstudien samt resultaten från experimenten bidragit till ny kunskap som kan användas i framtida forskning om avloppsvattenrening med hjälp av mikrosvampar. Det här arbetet kommer förbättra och utöka förståelsen för hur mikrosvampar kan appliceras i kommunala vattenreningsprocesser.
Avhandlingen är skriven på engelska och består av 68 sidor, 14 figurer, 4 tabeller, samt 133 litteratur-referenser.

Nyckelord
Filamentösa svampar, kommunalt avloppsvatten, läkemedelsrester, bioreaktor, näringsämnen, diklofenak, ketoprofen, *Trametes versicolor*, *Aspergillus luchuensis*

List of appended papers

Paper I

Dalecka B., Juhna T. Rajarao G. K. Constructive use of filamentous fungi to remove pharmaceutical substances from wastewater. Journal of Water Process Engineering, 33, 2020.

Paper II

Dalecka B., Oskarsson C., Juhna T. Rajarao G. K. Isolation of fungal strains from municipal wastewater for the removal of pharmaceutical substances. Water, 12, 524, 2020.

Paper III

Dalecka B., Strods M., Rajarao G. K., Juhna T. Removal of total phosphorus, ammonia nitrogen and organic carbon from non-sterile municipal wastewater with Trametes versicolor and Aspergillus luchuensis. Microbiological Research, 241, 2020.

Paper IV

Dalecka B., Strods M., Cacivkins P., Ziverte E., Rajarao G. K., Juhna T. Bioaugmentation with fungi: An emerging strategy for removing pharmaceutical substances in wastewater treatment process by fluidized bed pelleted bioreactor. Chemosphere, 2021 (under revision).

Contributions to papers

Paper I

Design and performed the experiments, analyzed the data and wrote the manuscript.

Paper II

Design the experiments, analyzed the data and wrote the manuscript.

Paper III

Design and performed the experiments, analyzed the data and wrote the manuscript.

Paper IV

Design and performed the experiments, analyzed the data and wrote the manuscript.

Table of Contents

1. Introduction

Water is a valuable resource, crucial to all living organisms and multiple human activities, e.g., domestic use, agriculture, and industry (Besha et al., 2017). Therefore, the urge for sustainable development, including a more circular use of water sources, and the resource inefficiency of current wastewater treatment practices have driven a paradigm shift within the scientific community with regard to wastewater solutions (Kehrein et al., 2020). Furthermore, wastewater treatment has always been one of the core problems of environmental protection as wastewater may contain a variety of hazardous substances (Lu et al., 2016).

The conventional wastewater treatment plant is typically designed to remove high concentrations of mostly biodegradable organic matter and nutrients (Margot et al., 2015). However, hazardous substances such as antibiotics, pesticides, personal care products, and pharmaceuticals are not removed completely and can pose a threat to water resources and aquatic organisms (Cruz-Morató et al., 2013). Additionally, the release of these substances has become an increasing concern over recent years and new advanced treatment technologies should be optimized and developed (Yamashita and Yamamoto-Ikemoto, 2014). Generally, advanced removal methods for pharmaceutical substances can be divided into three categories: physical, chemical, and biological methods (Wang and Wang, 2016). Among these categories, the biological method by fungi has attracted relatively high interest from researchers (Espinosa-Ortiz et al., 2016).

Over the past decade, biological treatment of municipal wastewater with white-rot fungi has proven to be a good candidate to remove pharmaceutical substances (Mir-Tutusaus et al., 2018). For instance, batch-scale experiments have already demonstrated the fungi's ability to secrete relatively large amounts of enzymes, and capacity to degrade a wide range of environmental pollutants, from dyes to pharmaceutical substances, heavy metals, trace organic and endocrine-system disrupting contaminants (Asif et al., 2017; Lucas et al., 2016; More et al., 2010; Stenholm et al., 2018). Furthermore, previous studies have also shown that fungi can use biosorption as a strategy for pharmaceutical substance removal (Cruz-Morató et al., 2014; Legorreta-Castañeda et al., 2020). However, there are still many unanswered

questions regarding industrial, full-scale application of fungi in wastewater treatment (Espinosa-Ortiz et al., 2016). Two of the main scientific questions are how the use of different fungal strains could affect the removal efficiency of pharmaceutical substances and how fungi would compete with other microorganisms in sewage wastewater. A previous study from Gros et. al. (2014) found that bacteria and fungi can show a positive synergistic effect whereby the production of enzymes from both microorganisms might increase the removal efficiency of pharmaceuticals in sewage wastewater treatment (Gros et al., 2014). Further research, however, needs to be done to confirm this hypothesis. Moreover, operating conditions like temperature and pH level might play a key role, especially on enzyme production and functional activity (Gao et al., 2010). Thus, the research needs to determine if a fungal approach for the removal of pharmaceutical substances is efficient and feasible in municipal wastewater treatment applications. Fungal systems have been regarded as cost-effective solutions for pharmaceutical substances removal (Viancelli et al., 2020), however, the application cost strongly depends on several factors: the cost of inoculum and biomass production, the requirements for operating conditions (e.g., pH level maintenance), need for additional unit processes, and hydraulic retention time among other systems (Mir-Tutusaus et al., 2018).

This study focused on studying the fungi and their potential to remove pharmaceutical substances from municipal wastewater without adjusting the initial pH level correction.

Figure 1. presents how the research work was performed for the thesis. The thesis outline was as follows:

First, a **theoretical background** of the studied concept is presented, i.e., fungi and their ability to use extracellular enzyme systems and biosorption as a mechanism for pharmaceutical substance removal. The main steps of the conventional wastewater treatment process are described and compared with advanced treatment technologies. The possible application of fungal treatment is discussed. The summary of materials and methods and results are reported and explained.

Paper I investigated the potential of five globally distributed fungal strains - *T. versicolor*, *I. lacteus*, *P. ostreatus*, *T. reesei,* and *F. solani* - to remove pharmaceutical substances from municipal wastewater under non-sterile conditions. In this paper, the pH level, the effect of carriers, the removal efficiency, and the enzymatic laccase activity were examined for each strain separately and for mixed cultures in batch-scale experiments over defined

time periods. Furthermore, a biosorption experiment was performed to better understand fungal removal mechanisms for pharmaceutical substances.

In **Paper II**, the main focus was to study the isolation of fungal strains from municipal wastewater and to test their ability to remove pharmaceutical substances. To achieve this, fungi were isolated from municipal wastewater and cultivated in the presence of selected pharmaceuticals. This study also investigated the effect of the pH level on the removal efficiency.

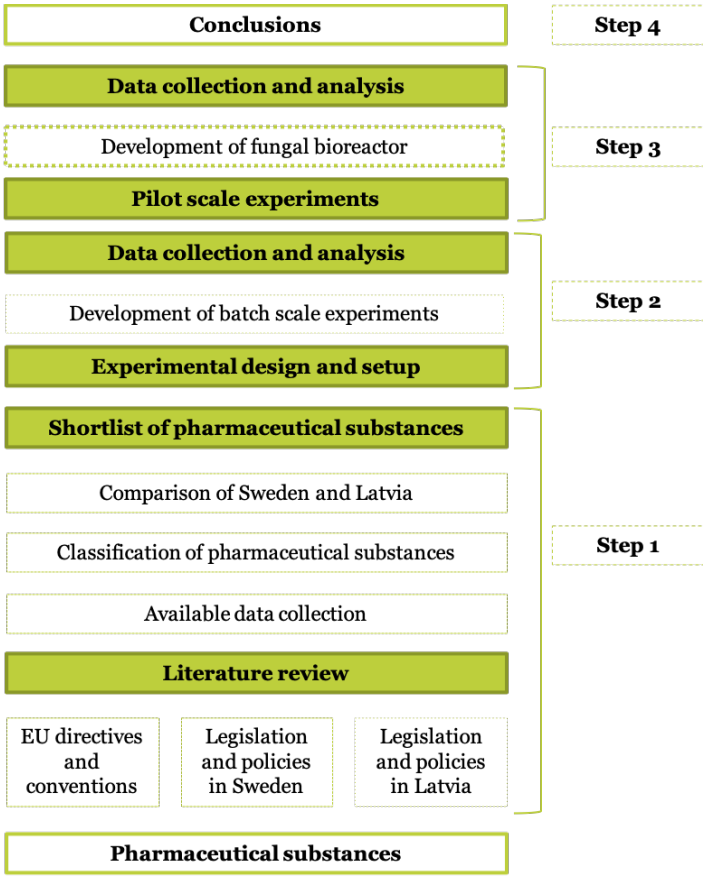

Figure 1. Conceptual scheme of a thesis outline.

In **Paper III**, the total phosphorus (P), ammonia nitrogen (NH_4-N), and the total organic carbon (TOC) removal from non-sterile municipal wastewater of two fungi, *T. versicolor* as a laboratory strain and *A. luchuensis* as an environmental isolate from **Paper II**, was investigated. In this study, a fungal-fluidized bed pelleted bioreactor was designed in which both fungal

cultures were incubated, and the data of nutrient removal was collected in order to compare the nutrient removal efficiency from the batch-scale to the bioreactor.

Paper IV combined all the obtained results from the previous papers and examined bioaugmentation as a strategy for successful operation of fungal-fluidized, bed pelleted bioreactor systems for continuous, long-term pharmaceutical substance removal from municipal wastewater with *T. versicolor* and *A. luchuensis*.

Finally, the **Conclusions and future outlook** summarize the obtained results and conclusions and discuss the practical implementation of the presented approach. The future perspectives of fungi application in the wastewater treatment system and possible costs are presented and discussed.

2. Theoretical background and the relevance of the study

2.1. Pharmaceutical substances removal in the conventional wastewater treatment system

The removal of pharmaceuticals from municipal wastewater has become an emerging worldwide concern due to the ability of these substances to cause eutrophication in surface water and increase the negative risk on aquatic organisms (Molins-Delgado et al., 2016; Yamashita and Yamamoto-Ikemoto, 2014). Conventional wastewater treatment plants worldwide use technology for organic pollutant removal where the wastewater is disinfected, and with low or to near zero impact waste (i.e., N_{tot} < 5 mg/L, P_{tot} < 1mg/L, TOC < 10 mg/L) (Batstone et al., 2015; Li et al., 2019; Mook et al., 2012).

The conventional wastewater treatment process typically starts with a pre-treatment step for removing coarse materials and sands (Figure 2). Subsequently, the settleable substances are mechanically removed in primary treatment. This is followed by secondary treatment, which removes organic contaminants such as ammonia nitrogen and phosphorus through biological treatment with activated sludge. Finally, biological solids are separated in a secondary clarifier and the effluent can be discharged into the surface water (Rajasulochana and Preethy, 2016).

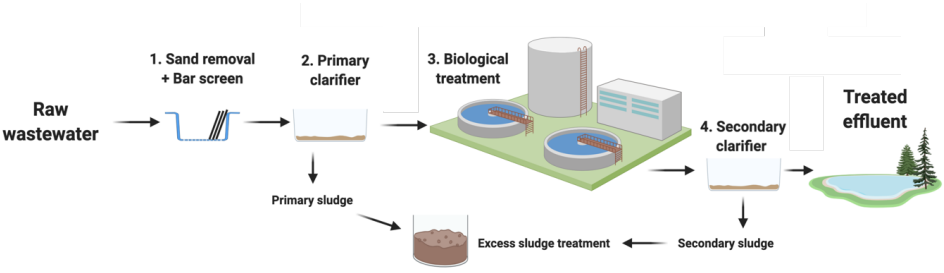

Figure 2. Conceptual scheme of a conventional wastewater treatment plant with biological treatment (created with BioRender.com).

Previous studies have already shown that most of the hazardous substances, including pharmaceuticals, are not effectively removed by conventional biological treatment (Mir-Tutusaus et al., 2017; Yang et al., 2013). Effective wastewater treatment for pharmaceutical substances is a challenge due to the enormous volume, complexity, and hazardous nature of such contaminants (Pal, 2018). High human consumption of pharmaceuticals has led to a concomitant concern observing presence of their compounds in the environment because a large proportion of these therapeutic compounds

cannot be assimilated and metabolized by the human body, thus are excreted via feces and urine and into municipal wastewater treatment systems (Tiwari et al., 2017). For instance, Ternes (1998) detected the presence of more than 30 pharmaceutical compounds in the effluent of a conventional wastewater treatment plant, confirming that classical biological treatment is not effective (Ternes, 1998). Carball et al. (2004) investigated the fate of eight pharmaceutical compounds and three hormones in a municipal wastewater treatment plant. The results showed that the removal efficiency of the targeted compounds during primary treatment was in the range of 20–50 %; however, the removal efficiency of secondary treatment by activated sludge process increased and varied from 30 to 70 % (Carballa et al., 2004). Zhang and Zhou (2008) demonstrated that six estrogen-balance disrupting compounds such as bisphenol A (BPA), diethylstilbestrol (DES), 17a-ethynylestradiol (EE2), 17b-estradiol (E2), estriol (E3), and estrone (E1) could be detected in the wastewater effluent after classical treatment (Zhang and Zhou, 2008). Other studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, ketoprofen, and ibuprofen can be detected in the wastewater effluent in relatively high concentrations, which means they have not been effectively removed from wastewater (Wang and Wang, 2016; Yang et al., 2017). Moreover, some polar chemical compounds such as nonylphenols and perfluoroalkyl substances can be even formed from precursor compounds during conventional wastewater treatment processes. (Loos et al., 2013).

During conventional wastewater treatment processes, pharmaceutical compounds are mainly removed by sorption and biodegradation process. Sorption of pharmaceuticals occurs due to the hydrophobic interaction of the aliphatic and aromatic groups, to lipid molecules of sludge or to the cell membrane of microorganisms, and due to electrostatic interactions of positively charged compounds to negatively charged microbes and sludge. Consequently, sorption depends on the values of the log K_{ow} (octanol-water coefficient), the log K_d (sludge adsorption coefficient), and the log K_a (acid dissociation constant) (Vieno and Sillanpää, 2014). Most pharmaceuticals have low K_d, therefore, sorption, compared to biodegradation, is the minor removal pathway for most pharmaceutical compounds (<10 %) (Ternes, 1998; Tiwari et al., 2017). The biodegradation of pharmaceutical residues in the wastewater treatment process occurs by two principal mechanisms, i.e., either by co-metabolism, in which pharmaceutical substances are degraded by enzymes secreted by microorganisms present in sewage sludge, or by sole substrate degradation, in which targeted compounds are the sole carbon and energy source for microorganisms (Tiwari et al., 2017). However, the complete

degradation pathway and microbial catabolic enzyme involvement and efficiency in the degradation process are still largely unknown (Tiwari et al., 2017). Thus, the need to improve the conventional biological wastewater treatment for pharmaceutical substances and nutrient removal has attracted more and more attention in recent years (Rajasulochana and Preethy, 2016). Advanced treatment processes, such as activated carbon adsorption, advanced oxidation processes, nanofiltration, reverse osmosis, membrane bioreactors, and biological treatment by algae and fungi can be considered alternatives to classical wastewater treatment, achieving higher and more consistent efficiency in terms of pharmaceutical substance removal (Chowdhary et al., 2018; Luo et al., 2014; Naghdi et al., 2018).

2.2. Environmental risks posed by the presence of pharmaceutical substances in wastewater

Although a multitude of pharmaceutical substances have been present in wastewater for decades, only recently their levels in the environment have begun to be quantified, acknowledged as potentially unsafe to the ecosystem, and suspected to have direct toxicity to certain aquatic organisms (Pal et al., 2014). For instance, a previous study has shown that antibiotics at the concentrations found in the environment may contribute to the appearance of antibiotic-resistant genes in bacteria (Kraemer et al., 2019). Also, the feminization of fish and mussels as intersex and reproductive disruption has been observed in several rivers downstream of wastewater treatment plant outfalls, likely related to the release of estrogenic endocrine disruptors (Kidd et al., 2007; Pal et al., 2014; Tran and Gin, 2017). These anthropogenic substances, often addressed as micropollutants, are commonly present in water sources at trace concentrations, ranging from a few ng/L to several μg/L (Loos et al., 2013). The low concentration and diversity of hazardous substances not only complicate the associated detection and analysis procedures but also create challenges for water and wastewater treatment processes (Zuloaga et al., 2012).

Unfortunately, many hazardous substances, including pharmaceutical compounds, are not completely removed by conventional wastewater treatment plants, and consequently, they have been detected in effluents, surface waters, and, less frequently, in-ground and drinking water all over the world raising an important question related to human health, ecology, and economic impacts (Mailler et al., 2015). Thus, further research is needed in order to explore and better understand the legislation and policy in the

wastewater field and how to improve the existing wastewater treatment systems for pharmaceutical substances removal (Mir-Tutusaus et al., 2018; Söderberg, 2016).

2.3. Occurrence of pharmaceutical compounds in municipal wastewater: comparison of the current situation and legislation in Latvia and Sweden

Water legislation in every country pays considerable attention to the legal regulations of the use and protection of water resources against pollution (Preisner et al., 2020). Since 2000, the implementation of the Water Framework Directive (WFD) (2000/60/EC) in all EU countries, including Latvia and Sweden, provides a seemingly logical answer to longstanding externalities and coordination problems in water protection and management (Cantinho et al., 2016; Söderberg, 2016).

The Water Framework Directive 2000/60/EC (WFD) is widely accepted as the most substantial and ambitious piece of European environmental legislation to date (Voulvoulis et al., 2017). The main aim of the WFD is to establish a framework for the protection of European waters in order for Member States to reach "good status" objectives for water bodies throughout the EU (Directive 2000/60/EC, 2000). The last upgrade of the WFD (EC 2013, Annex I) lists forty-five priority pollutants and 14 substances in the watch list (a.k.a., priority hazardous substances), whose dissemination into the environment must cease (priority hazardous substances) or be reduced in order to meet the Environmental Quality Standards (EQS) (EC 2013, Annex II) (Directive 2013/39/EU, 2013; Kõrgmaa et al., 2020). While the priority list contains mainly heavy metals and phenolic substances, the watch list details emerging sources of water contamination, from antibiotic and fungicide pharmaceutics (Cortes et al., 2020; European Commission, 2020) (Table 1.).

The watch list under WFD is a mechanism for obtaining high-quality monitoring data on emerging pollutants that may pose a significant risk to the aquatic environment (Cortes et al., 2020; Schröder et al., 2016). During the last update in 2018, the substances diclofenac, oxadiazon, 2,6-di-tert-butyl-4-methylphenol, triallate, and 2-ethylhexyl-4-methoxycinnamate were removed from the watch list, while metaflumizone, amoxicillin, and ciprofloxacin were identified as suitable candidates and were included in the watch list (Cortes et al., 2020; European Commission, 2020; Schröder et al., 2016; Tiedeken et al., 2017). However, the work on developing the list of priority substances should

be continued in order to identify and monitor the most harmful pharmaceutical substances being released into the aquatic environment. Therefore, it is very important to monitor and develop a ranking system to prioritize pharmaceutically active compounds by considering the following four criteria: (a) occurrence (prevalence, frequency of detection), (b) highest percentages of excretion, (c) removal in wastewater treatment plants, and (d) ecological effects (bioaccumulation, ecotoxicity) (Schröder et al., 2016).

Table 1. The watch list of substances for EU-wide monitoring as set by Directive 2008/1005/EC.

Pharmaceutical substance	Molecular formula	Classification
Metaflumizone	$C_{24}H_{16}F_6N_4O_2$	insecticide
Amoxicillin	$C_{16}H_{19}N_3O_5S$	antibiotic
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	
Trimethoprim	$C_{14}H_{18}N_4O_3$	
Venlafaxine and O-desmethylvenlafaxine	$C_{17}H_{27}NO_2$ and $C_{16}H_{25}NO_2$	antidepressant
Clotrimazole	$C_{22}H_{17}ClN_2$	azole (fungicide)
Fluconazole	$C_{13}H_{12}F_2N_6O$	
Imazalil	$C_{14}H_{14}Cl_2N_2O$	
Ipconazole	$C_{18}H_{24}ClN_3O$	
Metconazole	$C_{17}H_{22}ClN_3O$	
Miconazole	$C_{18}H_{14}Cl_4N_2O$	
Penconazole	$C_{13}H_{15}Cl_2N_3$	
Prochloraz	$C_{15}H_{16}Cl_3N_3O_2$	
Tebuconazole	$C_{16}H_{22}ClN_3O$	
Tetraconazole	$C_{13}H_{11}Cl_2F_4N_3O$	
Dimoxystrobin	$C_{19}H_{22}N_2O_3$	Pesticide
Famoxadone	$C_{22}H_{18}N_2O_4$	

Monitoring data plays a key role in implementing the WFD and other legislation (Anna et al., 2016; Reinholds et al., 2017). Up to now, the Swedish experience demonstrates that well-designed and financially-supported surface water monitoring can be used to understand and manage a range of stressors and societal concerns. In 1967, the Swedish Environmental Protection Agency (SEPA) was established and until today the Swedish national surface water monitoring program comprises a large number of lakes and streams to meet the information demands from the WFD, UN-ECE LRTAP, OSPAR, and HELCOM conventions and the national environmental goals (Fölster et al., 2014). The results of a recent compilation of available measurements of pharmaceutical residues in wastewater comprising all

Swedish reports and surveys, show that more than 70 different hazardous substances have been observed in the influent municipal wastewater with median concentrations of a few ng/L to approximately 100 μg/L (Baresel et al., 2015). For instance, the concentration closest to the effect concentrations in Swedish recipients are for endocrine disruptors, such as ethinyl estradiol and estriol, as well as some tranquilizers and antidepressants, such as oxazepam and fluoxetine.

In Latvia, the monitoring of hazardous substances has been implemented only recently. The monitoring and the list of hazardous substances were adopted from the WFD and implemented by Cabinet Regulation No.34 on discharge of polluting substances into water (Ministru kabineta noteikumi Nr.34, 2002). The detection and treatment of hazardous compounds have provoked increasing concern in the wastewater treatment field, particularly because of the absence of clear requirements for hazardous substance analysis methodology and a well-organized long-term monitoring system (Ministru kabineta noteikumi Nr.34, 2002; Verlicchi et al., 2012). Furthermore, the data on the concentrations of hazardous substances, including pharmaceutical compounds, in the water environment is limited. Previous research (Muter et al., 2017; Reinholds et al., 2017) have mainly analyzed pharmaceuticals in municipal wastewater at the Daugavgriva wastewater treatment plant. The results from Reinholds et al. (2017) have shown that the predominant compounds of the analyzed substances in municipal wastewater were caffeine and acetaminophen, ranging between the levels of 7.6 – 11.4 ng/L and 810–11.4 ng/L respectively. Meanwhile, Muter et al. (2017) detected 21 pharmaceutical compounds in municipal wastewater samples with concentrations ranging from 13.2 ng/L to 51.9 ng/L. The majority of the detected pharmaceutical compounds with concentrations above 1000 ng/L belong to the group of nonsteroidal anti-inflammatory drugs (NSAIDs), e.g., acetaminophen, naproxen, ibuprofen, diclofenac, and ciprofloxacin (Muter et al., 2017).

Overall, the WFD and its watch list could have played a greater role in delivering coherent and sustainable water management, especially in Latvia (Voulvoulis et al., 2017). Despite the implementation of strict legal standards concerning nutrient loads within wastewater discharges in all of the European Union (EU) Member States, including Latvia and Sweden, good ecological and chemical water status was not achieved by 2020 (Petrie et al., 2015; Preisner et al., 2020). One of the main reasons for this situation is the imperfections of the legislative tools regarding the standardization of wastewater quality and the methodology of determining the conditions for wastewater introduction

between EU countries (Gardner et al., 2012; Preisner et al., 2020). Thus, it is necessary for the EU to find a common strategy for regulating and legislating hazardous substances. These substances require strict regulation, monitoring, and control since the majority of all significant water bodies, lakes, and streams are shared between European countries (Anna et al., 2016; Luo et al., 2014; Söderberg, 2016). The occurrence of pharmaceutical compounds in untreated wastewater varies across countries and particular wastewater treatment plants. Furthermore, it is impossible to monitor all hazardous substances in production and use, and science-based strategies for prioritization are essential (Anna et al., 2016). Accordingly, model experiments to investigate the behavior of pharmaceutical compounds during the wastewater treatment process are needed (Muter et al., 2017). Also, further studies to test alternative treatment methods are needed to determine the fate and removal of pharmaceutical substances, and the possible implementation of these methods into the conventional wastewater treatment system (Petrie et al., 2015).

2.4. Advanced treatment processes on pharmaceutical substance removal from wastewater

Pharmaceutical substances have been widely detected in wastewater effluents, thus, the need to investigate and develop advanced treatment methods has been addressed more frequently over the past decade (Rajasulochana and Preethy, 2016). Several advanced treatment systems, including ultrafiltration, reverse osmosis, ozonation, advanced oxidation processes, and activated carbon adsorption, have been used for the effective removal of pharmaceutical substances (Figure 3) (Yang et al., 2017).

Membrane filtration processes, such as nanofiltration and reverse osmosis, can be promising advanced wastewater treatment methods in terms of pharmaceutical substance removal. The membrane filtration is a barrier that separates two phases from each other by restricting the movement of components through it in a selective style (Ezugbe and Rathilal, 2020). However, the removal efficiency for pharmaceutical substances is relatively low because membrane pore sizes are considerably larger than pharmaceutical molecules (Watkinson et al., 2007). Furthermore, the previous studies have shown that complete pharmaceutical removal requires post-treatment with other methods, e.g., ozonation and advanced oxidation (Høibye et al., 2008).

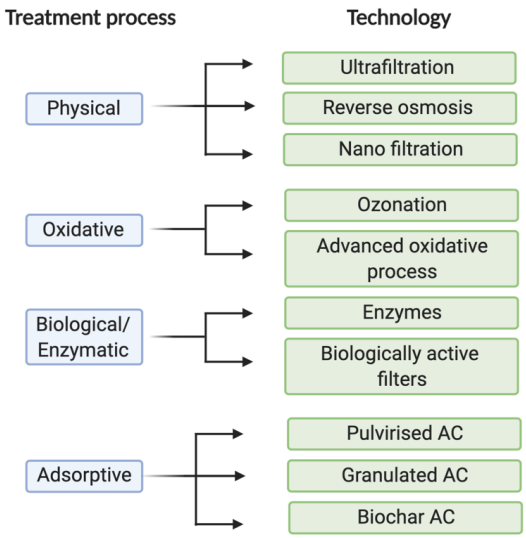

Figure 3. Schematic characterization of advanced treatment technologies for wastewater (adapted by the Swedish Environmental Protection Agency (Naturvårdsverket, 2017), (created with BioRender.com)

Ozonation and advanced oxidation treatment have recently emerged as an important class of alternative technologies for the oxidation and destruction of a wide range of pharmaceutical compounds in wastewater (Ikehata et al., 2006; Wei et al., 2017). The advanced oxidation treatment is characterized by a variety of radical reactions that involve combinations of chemical agents (e.g., ozone (O_3), hydrogen peroxide (H_2O_2), transition metals, and metal oxides) and auxiliary energy sources (e.g., ultraviolet-visible (UV-Vis) radiation, electronic current, gamma radiation, and ultrasound) (Ikehata et al., 2006). Ozonation and advanced oxidation treatment are particularly appropriate for treating municipal and industrial wastewaters containing bio-refractory and/or toxic organic pollutants such as pesticides, surfactants, and other pharmaceuticals (Margot et al., 2015). Furthermore, ozone treatment is often employed for pathogenic microorganism reduction (Hollender et al., 2009; Ikehata et al., 2006). However, the use of ozonation and advanced oxidation treatment methods can generate toxic byproducts, leading to a negative impact on the environment, and thus creating the need for appropriate additional post-treatment (Völker et al., 2019). Consequently, toxicity removal is a crucial aspect of these methods' application and performance for wastewater treatment systems (Hollender et al., 2009).

Activated carbon (AC) is defined as a carbonaceous solid with high micropores volume, well-developed surface area, and high adsorptive capacity (Pezoti et al., 2016). Thus, this adsorption technique is a relatively attractive method for reducing the amount of pharmaceutical substances in wastewater (Wong et al., 2018). Currently, researchers are focusing on the development of activated carbon from relatively cheap materials such as shells, coals, woods, and lignin to replace costly commercial activated carbon (Demirbas, 2009). Besides this, researchers also intend to search for various methods to improve the performance of activated carbon treatment in terms of removing pharmaceuticals from wastewater, e.g., by adding ultrasonic irradiation and ammonia activation as a post-treatment step (Guo et al., 2017; Wong et al., 2018). Although activated carbon has shown relatively high pharmaceutical removal efficiency in wastewater, there are still scientific questions regarding this method and its application to wastewater treatment systems. The improvement of adsorption performance by modification of adsorbent, utilization of composite adsorbents, binary and multicomponent adsorption, treatment of real effluents, fixed-bed studies, and enhancement of regeneration need to be investigated (Ahmed, 2017).

Overall, membrane filtration, ozonation, and activated carbon adsorption are promising technologies and particularly suited for removing pharmaceutical substances from wastewater (Wong et al., 2018; Zhang and Zhou, 2008). However, the high operating costs and the possible formation of by-products are still limiting factors to implement these methods as an alternative to current wastewater treatment processes (Wong et al., 2018; Yunlong et al., 2014). As a result, there has been a growing interest in adopting biological treatment methods with fungi because of their ability to produce enzymes which degrade pharmaceutical substances, relatively low operation costs, energy-efficiency, and valuable end-products that can be used for energy production or as fertilizers (Espinosa-Ortiz et al., 2016; Mir-Tutusaus et al., 2016; Sankaran et al., 2010).

2.5. Selection of fungi for pharmaceutical removal from wastewater

Previous researchers have pointed out that fungal treatment of wastewater is a promising technology due to the unspecific enzymatic system which is able to degrade a wide range of pharmaceuticals even at very low concentrations (Lucas et al., 2018; Shreve et al., 2016; Stenholm et al., 2018; Wesenberg et al., 2003). Therefore, fungi might play an important role in the biodegradation

of pharmaceutical compounds in wastewater (Sankaran et al., 2010). The earliest documented research on fungi in wastewater was conducted by Curtis (1969). Curtis examined different fungal species commonly present in domestic wastewater and their effects on wastewater treatment (Curtis, 1969). Nowadays, many studies have been performed focusing on the fungal treatment of pharmaceuticals under batch-scale experiments, indicating relatively good removal values, especially when using the white-rot fungi (Lucas et al., 2016; Sankaran et al., 2010). The concept for the development of environmentally friendly wastewater treatment technology using white-rot fungi and their enzyme systems was proposed in 1980s (Bumpus and Aust, 1987).

The white-rot fungi (WRF) is not a taxonomical grouping, but rather a collection of fungal species such as basidiomycetes and some relevant species including *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Trametes versicolor, Ganoderma lucidum,* and *Irpex lacteus* that are able to degrade lignin (Dashtban et al., 2010). WRF can secrete three main classes of lignin modifying enzymes: lignin peroxidases (LiPs), manganese-dependent peroxidases (MnPs), and laccase (Reddy, 1995). Although WRF can potentially secrete all three groups of enzymes, a particular strain may not secrete all of them. For instance, *T. versicolor* has been associated with all three enzymes, however, Yang et. al. (2013) have shown that strain ATCC 7731 can secrete mostly laccase (Yang et al., 2013). Thus, the removal of pharmaceutical substances by fungal strains varies widely from one pharmaceutical compound to another. Furthermore, Yang et al. (2013) have stated that physicochemical properties of the target molecules appear to be a key reason for such variation. For instance, some pharmaceutical substances are easily adsorbed due to their high hydrophobicity, some have molecular features that render them readily biodegradable by fungi, while others are resistant to the fungi enzyme system due to certain features of their molecular structure (Yang et al., 2013). Thus, it is stated that there can be two main removal mechanisms for pharmaceutical substances from wastewater by WRF: biosorption and biodegradation by enzyme systems (Figure 4) (Lu et al., 2016; Lucas et al., 2018).

Although several investigations have been carried out to select the most appropriate strains for wastewater treatment to remove pharmaceutical substances with high or specific biodegradation performance, there is still a need for further work (Gao et al., 2010). Factors including the medium composition, pH, concentration of spore suspensions, incubation duration, and the mixing speed of the incubator, have a significant influence on the

mycelium growth and enzyme production (Sankaran et al., 2010; Silva et al., 2019). Furthermore, enzyme production by fungi is strongly affected by many operation parameters such as time of cultivation, stationary or submerged cultures, organic or inorganic compound concentrations, inducer concentration, aeration, and degradation or activation by protease (Viswanath et al., 2014). Therefore, screening of fungal species and their variations is important for selecting suitable enzyme-producing organisms that will work under non-sterile wastewater conditions for pharmaceutical substance removal (Viswanath et al., 2014).

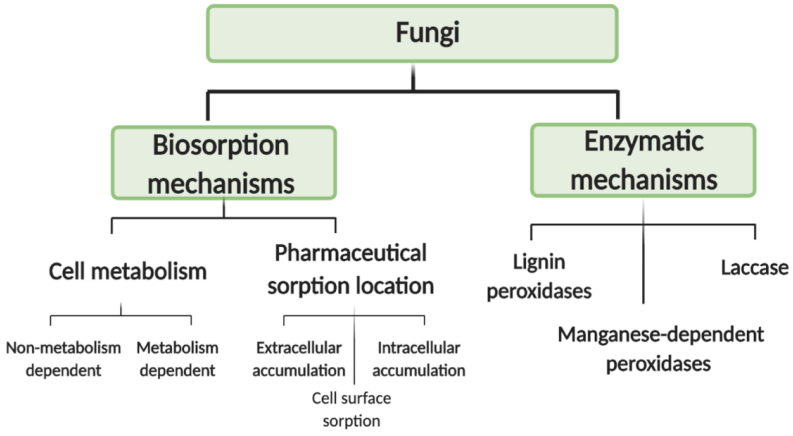

Figure 4. Schematic characterization of fungal removal mechanisms for pharmaceutical substances (created with BioRender.com)

Fungi are screened for their enzyme production on solid media containing colored indicator compounds that facilitate the visual detection of laccase production or with liquid cultivations monitored with enzyme activity measurements (Madadi and Abbas, 2017). Currently, the strains that have been named as most promising for pharmaceutical removal from wastewater are *Trametas versicolor*, *Bjerkandera adusta*, *Irpex lacteus*, *Pleurotus ostreatus*, *Pycnoporus cinnabatinus*, *Dichotomitus squalenes*, *Phanerochaete chrysosporium, Trichoderma reesei* (Yang et al., 2013). Furthermore, for pharmaceutical removal from wastewater, Guest and Smith (2007) have suggested using fungi that are naturally available in a municipal wastewater treatment plant due to their adaptation to the environmental and operation conditions (Guest and Smith, 2002). Thus, determination of the removal potential of pharmaceutical substances in residential wastewater fungi is an important task in developing industrial process applications in

order to accomplish the long-term goal of pharmaceutical removal (Silva et al., 2019) (Figure 5).

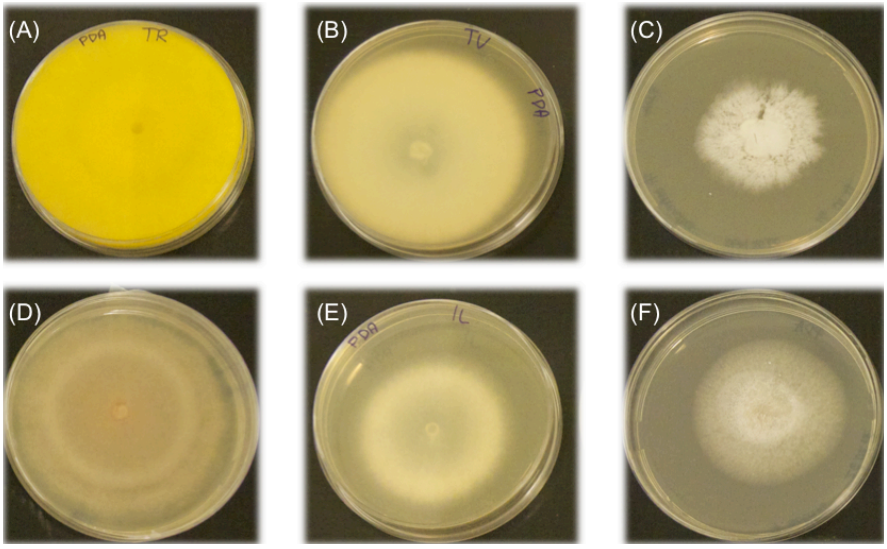

Figure 5. The selected fungi for this study **(A)** *Trichoderma reesei* DSM 768; **(B)** *Trametes versicolor* DSM 6401; **(C)** *Pleurotus ostreatus* DSM 1020; **(D)** *Bjerkendera adusta* DSM 23426; **(E)** *Irpex lacteus* IBB 104; **(F)** *Fusarium solani* (wastewater isolate from a pharmaceutical wastewater treatment plant)

Despite all the potentialities of WRF and the extent amount of promising studies about fungi ability to remove pharmaceuticals from wastewater, fungal systems are not being commonly applied at an industrial scale (More et al., 2010). One of the main reasons for this is the fungi's need for nutrient addition, for instance, some WRF need an additional assimilable carbon source for growth and survival while wastewater usually does not have nutrients like glucose, which is the main carbon source for *T. versicolor* growth, biological activity and enzyme production (Mir-Tutusaus et al., 2018; Stadlmair et al., 2018). Other limiting factors are the competition with microorganisms and the requirement of relatively long hydraulic retention time (i.e., 1 – 3 days for pharmaceutical removal) (Mir-Tutusaus et al., 2018; Zahmatkesh et al., 2016). However, researchers have proposed a wide range of alternatives for dealing with these limitations. For instance, for reducing the competition with bacteria, the reduction of the pH level and the immobilization of fungi can restrain the growth of diverse bacteria and other competitive microorganisms (Espinosa-Ortiz et al., 2016; Mir-Tutusaus et al., 2018).

Overall, certain WRF species are reported to have several notable advantages in order to improve the conventional wastewater treatment system (Zahmatkesh et al., 2016). Use of fungi can increase the degradability and dewaterability of wastewater treatment (More et al., 2010). Because of the high adsorption capacity, enzyme systems, easy solid-liquid separation, relatively good adverse resistance, and broad degradation ability, WRF make fungi excellent candidates for wastewater treatment from pharmaceutical substances (Lu et al., 2016). However, the possible fungal application in a real wastewater treatment plant is not well discussed, established, and further investigations from laboratory work under batch experiments by a series of pilot-scale studies with municipal wastewater are necessary for future application at industrial scale. (Yang et al., 2013).

2.6. State of art of fungal application to wastewater treatment for the removal of pharmaceutical substances

Due to their ability to secret a non-specific extracellular enzymatic complex during their secondary metabolism and use the biosorption process, fungi have the unique aptitude to remove organic and inorganic pollutants, including pharmaceuticals (Espinosa-Ortiz et al., 2016). Therefore, over the past decade, there has been growing interest to integrate fungal bioreactor into the wastewater treatment system (Cruz del Álamo et al., 2020; Freitas et al., 2009; Mir-Tutusaus et al., 2019; Negi et al., 2020). However, compared to the number of studies investigating the impact of selected dissolved wastewater constituents under sterile batch-scale experiments, only a few attempts to assess fungal treatment for the removal of pharmaceutical substances from non-sterile municipal wastewater system can be found (Asif et al., 2017; Mir-Tutusaus et al., 2019). Furthermore, most commonly, bacteria are used in bioreactors for the treatment of wastewater, whereas the use of fungi has received much less attention (Espinosa-Ortiz et al., 2016). However, compared to bacteria, fungi have the advantage of being able to grow on a medium of relatively low pH, nitrogen content, and temperature (Sankaran et al., 2010). These properties can give fungi an advantage to grow over other organisms in adverse conditions (More et al., 2010). Therefore, the effect on wastewater treatment systems by fungi implementation should be investigated (Cecconet et al., 2017). For example, how fungal bioreactor implementation in wastewater treatment system can change the load of nutrients and pH level and how these changes later can impact and

complement the next treatment steps, especially biological process, of conventional wastewater treatment systems.

2.6.1. Fungal bioreactor

Fungal bioreactors are advantageous due to the rich source of degrading enzymes produced by fungi as well as their ability to withstand harsh conditions, especially fluctuating pollutant loads, low pH, and tolerance to low nutrient concentrations (Espinosa-Ortiz et al., 2016). Thus, fungal bioreactors might be a feasible approach not only for pharmaceutical substance removal but also to improve the classical biological treatment for wastewater in terms of reducing loads of nutrients such as P and NH_4-N (Millan et al., 2000). Asif et al. (2017) has demonstrated that the removal efficiency of pharmaceutical substances by fungi mainly depends on the pH and temperature of wastewater as they impact the stability and catalytic efficiency of enzymes (Asif et al., 2017). Moreover, as fungi are eukaryotes and grow more slowly than bacteria, the latter can outperform fungi in the competition for the substrate from wastewater, causing bacterial colonization and damage on fungal mycelium (Borchert and Libra, 2001; Yang et al., 2013). Sterilization of wastewater is not a cost-efficient or optimal option for wastewater treatment (Mir-Tutusaus et al., 2019). Thus far, this issue is one of the main drawbacks for implementing and introducing fungal reactors into the wastewater treatment system (Espinosa-Ortiz et al., 2016). The ultimate utility and application of fungi at pilot and full-scale use is still lacking, however, and first extensive laboratory examination followed by a series of pilot-scale studies are needed for future industrial application and optimization.

One of the most commonly used reactors for the fungal treatment of wastewater is the fluidized bed bioreactor (Andrews, 1988; Espinosa-Ortiz et al., 2016). The use of a fluidized bed bioreactor for wastewater treatment offers many advantages such as a compact bioreactor size due to a short hydraulic retention time, long biomass retention on the carriers, a high conversion rate due to fully mixed conditions, and, consequently, high mass transfer rates, no channeling of flow, dilution on an influent concentration due to a recycle flow (Li et al., 2010; Moreira et al., 1996; Özkaya et al., 2019) (Figure 6).

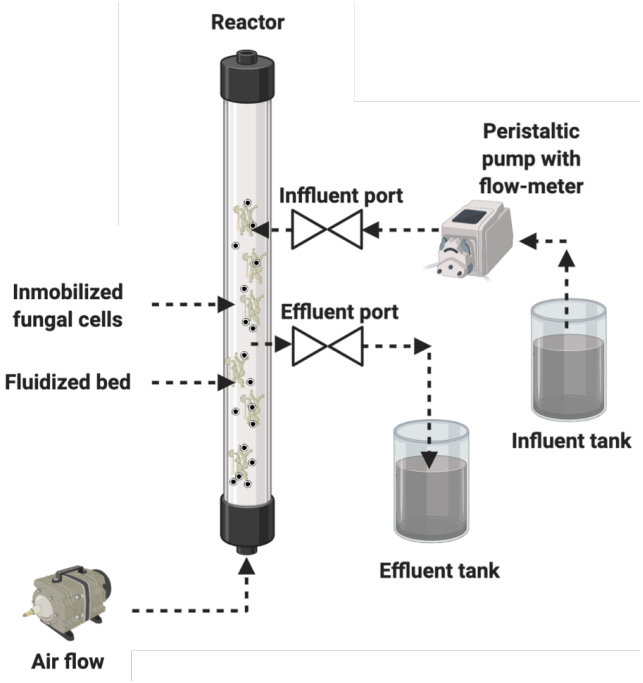

Figure 6. The schematic diagram of the fluidized-bed bioreactor system (adapted from Xiao- Ming et al. (2010), (created with BioRender.com)

Therefore, the fungal bioreactor is widely applied in the field of environmental engineering for many purposes, including the minimization of organic compound load in the treatment process of different wastewater types (Özkaya et al., 2019). However, when a fungal treatment process is scaled up to a bioreactor, aeration and agitation may change when compared to a batch experiment. Thus, fungal biomass may respond differently to the mechanical and oxidative stress, and fungal metabolic activity may change in a fluidized bed pelleted bioreactor (Spina et al., 2014).

2.6.2. The application of the fungal bioreactor in wastewater treatment system

The application of the fungal bioreactor in a large-scale wastewater treatment plant for improving pharmaceutical substances removal is not well established. Municipal wastewater is rich in easily degradable organics that may interfere in the fungal enzymatic degradation of pharmaceutical compounds. In such cases, the enzymatic and biosorption process of fungi

could be used as a pre-treatment for enhanced pharmaceutical removal (Asif et al., 2017). However, the most widely used wastewater treatment technology is the conventional wastewater treatment by activated sludge, in which aerobic microorganisms metabolize the organic fraction, including P and NH_4-N, present in the wastewater under constant oxygen supply (Kehrein et al., 2020). Therefore, the use of a fungal bioreactor as a pre-treatment step for pharmaceutical removal might cause a shortage of nutrients that might be needed to successfully continue wastewater treatment by activated sludge. A feasible alternative might be fungal post-treatment of the effluents containing organic pharmaceuticals (Mir-Tutusaus et al., 2018). However, the fungal-based bioreactor has not been transferred from laboratory scale to industrial level treatment of municipal wastewater. The process needs to be developed further to achieve technical and economic feasibility of fungal treatment. The optimization of chemo-physical parameters (e.g., nutrient addition, pH) and technological features (e.g., carrier selection, design of proper reactor configuration) allow setting up whole-cell fungal treatment in the wastewater treatment system, synergistically working with the existing techniques to reduce the concentration and toxicity of pharmaceutical substances (Purchase, 2016).

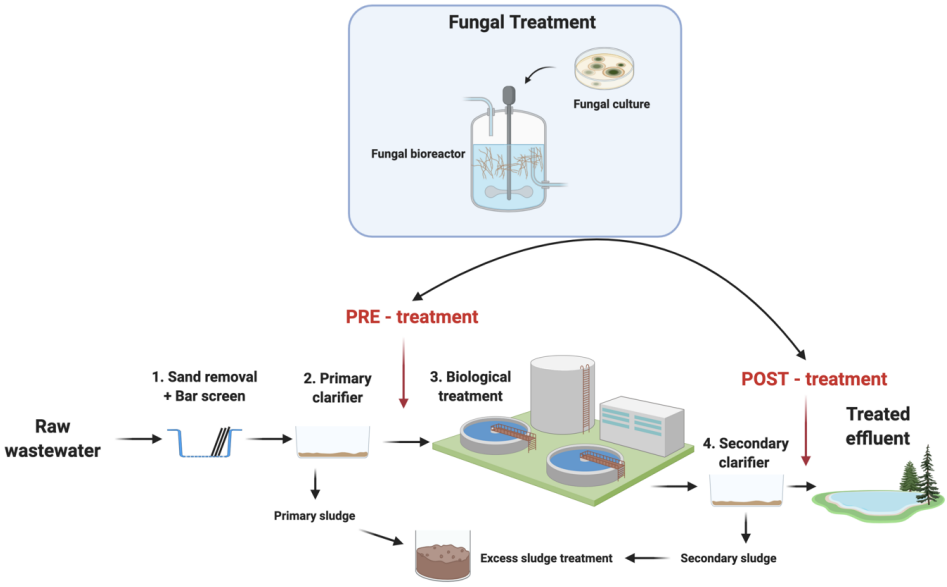

Figure 7. Scheme of fungal application in conventional wastewater treatment system (created with BioRender.com)

So far, the authors of the previous studies (Djelal and Amrane, 2013; Lacina et al., 2003; Ortega-Clemente et al., 2009) believe that there are at least two possible ways to apply the fungal bioreactor in the wastewater treatment system for pharmaceutical substances removal: (i) to encourage fungi growth *in situ* on an organic substrate present in the wastewater as a pre-treatment step, or (ii) to cultivate them separately and then dose in the process (bioaugmentation) as a post-treatment step (Figure 7).

Overall, the best strategy for fungi application in wastewater plants will depend on the wastewater to be treated, the final use of the treated wastewater, and consequently the cost of the treatment (Mir-Tutusaus et al., 2018). Further research on post-treatment technology, as a polishing step, to remove the pharmaceutical matter should be addressed, along with the possibility of using the treated effluent as irrigation water (Espinosa-Ortiz et al., 2016).

3. Present investigation

3.1. The main tasks of the present investigation

The main goal of the study was to investigate the potential of filamentous fungi to remove pharmaceutical substances from municipal wastewater under non-sterile conditions without pH correction. Therefore, the main scientific question to address in the study was: *Can filamentous fungi remove pharmaceutical substances from non-sterile municipal wastewater without pH correction?* According to this question, various tasks were set to accomplish this study:

- To identify the most commonly observed pharmaceutical substances in municipal wastewater.
- To investigate the efficiency of individual and mixed fungal cultures to remove pharmaceutical substances from municipal wastewater under non-sterile conditions.
- To examine fungal removal mechanisms, biosorption, and biodegradation by laccase enzyme of pharmaceutical substances.
- To evaluate the effect of fungal biomass on removal efficiency of pharmaceutical substances using carriers as a strategy.
- To isolate fungi from municipal wastewater and test their ability to remove pharmaceutical substances.
- To study the removal efficiency of total phosphorus, ammonia nitrogen, and the total organic carbon by fungi from municipal wastewater.
- To design possible application and optimization of a fungal fluidized bed pelleted bioreactor for municipal wastewater treatment for the removal of pharmaceutical substances.
- To investigate the potential of bioaugmentation as a strategy for fungal treatment in a fluidized bed pelleted bioreactor for municipal wastewater treatment for the removal of pharmaceutical substances and nutrients.
- To evaluate the cost associated with a fungal treatment in a fluidized bed pelleted bioreactor and compare it to classical and advanced treatment methods.

To reach the goal and complete the tasks, experiments were carried out and results reported in four scientific research papers.

There are still many unanswered questions regarding the full-scale application of fungi in the wastewater treatment process. One of the questions is how the use of fungal cultures, both individual or mixed, could affect the removal efficiency of pharmaceutical substances. Another question to address

is how fungi compete with other microorganisms under non-sterile conditions. Moreover, the operating conditions might play a key role, especially in the production of fungal enzymes. Therefore, the main objective of **Paper I** was to investigate the potential of five globally distributed fungal strains - *T. versicolor*, *I. lacteus*, *P. ostreatus*, *T. reesei*, and *F. solani* - to remove pharmaceutical substances from municipal wastewater under non-sterile batch-scale experiments. In this paper, the effect of pH level and Kaldnes K1 carriers on the removal efficiency and the enzymatic laccase activity were examined for each strain separately and in mixed cultures in batch-scale experiments for a certain period of time. The effect of non-sterile municipal wastewater using fungal biofilm carriers K1 with the most promising strain *T. versicolor* was tested to assess the potential of fungal treatment for the removal of pharmaceutical substances. Additionally, the difference in initial inoculum for fungal cultures was analyzed in order to determine the removal efficiency in non-sterile municipal wastewater. Finally, the biosorption experiment was done to better understand fungal removal mechanisms of pharmaceutical substances.

In **Paper II**, the main focus was to investigate the isolation of fungal strains from municipal wastewater and to test their ability to remove pharmaceutical substances. To achieve this, fungal isolates were cultivated on a synthetic wastewater media in the presence of selected pharmaceuticals. In this paper, the effect of the pH level on removal efficiency was studied. The most promising isolate was further identified and analyzed in non-sterile municipal wastewater. Finally, a biosorption experiment was conducted with the isolate, and enzyme activity was measured to better understand the removal mechanisms of pharmaceutical substances. All results of the fungal isolate were compared to *T. versicolor* to evaluate the potential of an isolated fungal strain and the advantages of its application in wastewater treatment to remove pharmaceutical substances.

In **Paper III**, the total phosphorus (P), ammonia nitrogen (NH_4-N), and the total organic carbon (TOC) removal from non-sterile municipal wastewater by two fungal species, *T. versicolor* as the most promising strain from **Paper I** and *A. luchuensis* as wastewater isolate from **Paper II**, was investigated. The removal efficiency of P, NH_4-N, and TOC was studied and compared taking into consideration the aspect of process design possible application and optimization of a fungal fluidized bed pelleted bioreactor. The investigation consisted of two phases. First, an observation of results was done under a batch-scale experiment with *T. versicolor* and *A. luchuensis*. During this phase, the removal of P, NH_4-N, and TOC were analyzed. In the second

phase, the fungal fluidized bed pelleted bioreactor was designed and both fungal cultures were incubated in reactors allowing collecting the data of P, NH_4-N, and TOC removal in order to compare the nutrient removal efficiency from the batch-scale to the bioreactor. Finally, to better understand the removal mechanism of nutrients and fungal interaction with natural microorganisms in municipal wastewater, the pH value, laccase enzyme activity, and quantification of total bacteria were determined.

In **Paper IV**, the bioaugmentation as a strategy for the fungal approach for removal of pharmaceutical substances from wastewater treatment was tested in the fungal fluidized bed pelleted bioreactor. The fungal fluidized bed pelleted bioreactor was optimized from **Paper III** and both fungal cultures, *T. versicolor* and *A. luchuensis*, were incubated in reactors allowing collecting the data of total phosphorus, ammonia, nitrate and nitrite, and total organic carbon removal. To better understand the removal mechanism of nutrients and fungal interaction and adaption with the microbial community in municipal wastewater, the pH value, laccase enzyme activity, and quantification of total bacteria were determined. Additionally, the removal efficiency of pharmaceutical substances such as diclofenac, ketoprofen, carbamazepine, ibuprofen, sulfamethoxazole, and metoprolol was tested and analyzed.

3.2. Summary of materials and methods

3.2.1. Fungal strains

In **Paper I**, the white-rot fungus *Trichoderma reesei* DSM 768, *Trametes versicolor* DSM 6401, *Pleurotus ostreatus* DSM 1020 from Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Culture (Germany), *Irpex lacteus* IBB 104 from Parker H. Petit Institute for Bioengineering and Bioscience (Georgia), and *Fusarium solani* as an isolate from a pharmaceutical wastewater treatment plant were used.

As **Paper II** investigated the isolation of fungal strains from municipal wastewater and the isolate ability to remove pharmaceutical substances, only *T. versicolor* from the microorganism collection was used in this study to compare the removal efficiency.

In **Papers III** and **IV**, the isolate *A. luchuensis* from **Paper II** and *T. versicolor* from **Paper I** were used as the most promising strains for wastewater treatment under non-sterile conditions in the pilot-scale test with fungal fluidized bed pelleted bioreactor.

3.2.2. Selection of pharmaceutical substances

A literature study for the selection of pharmaceutical substances was conducted. The peer-reviewed literature was the main source of information and data about pharmaceutical substances. The literature search also included the European, Swedish, and Latvian legislation on hazardous substances and surface water monitoring under the Water Frame Directive (Directive 2013/39/EU, 2013).

Table 2. The shortlist of 15 pharmaceutical substances and their concentration and removal efficiency by a conventional wastewater treatment plant (WWTP) in influent and effluent from Latvia and Sweden

Pharmaceutical substance	Concentration		Removal efficiency (%)	Reference
	Influent	Effluent		
Diclofenac	from 0.16 µg/L to 1275 ng/L	from 0.12 µg/L to ~ 80 ng/L	from 1 % to 60 %, also observed negative effect on removal	(Baresel et al., 2015; Directive 2013/39/EU, 2013; Falås et al., 2012; Fölster et al., 2014; Hallgren and Wallberg, 2015; Hering et al., 2010; Kårelid et al., 2017; Kosjek et al., 2012; Luo et al., 2014; Muter et al., 2017; Naturvårdsverket, 2017; Nr.34, 2002; Peng et al., 2012; Poikāne and Kadiķe, 2011; Reinholds et al., 2017; Schröder et al., 2016; Tiedeken et al., 2017; UNESCO and HELCOM, 2017; Wernersson, 2012)
Ketoprofen	from 10 to 329 ng/L	< 10 ng/L	~ 60 %	
Carbamazepine	from 10 to 245 ng/L	from 21 to 832 ng/L	< 40 % and also observed negative effect on removal - 80 %	
Ibuprofen	from < 0.004 to 2232 ng/L	from 55 µg/L to 100 ng/L	from 40 to 80 %	
Sulfa-methoxazole	from 10 to 173 ng/L	from 10 to 57 ng/L	20 - 80 %	
Metoprolol	from 0.002 to 967 ng/L	from 0.003 to ~ 100 ng/L	from 3 to 80 %	
Citalopram	from 4 to 89 ng/L	From 33.8 to 173 ng/L	78 %	
Erythromycin	from 3 to 13.2 ng/L	from ~ 10 to 182 ng/L	> 50 %	
Fluoxetine	385 ng/L	from 2 to 11 ng/L	-50 % (negative effect on removal)	
Naproxen	from 10 to 3998 ng/L	< 10 ng/L	80 - 90 %	
Oxazepam	from 185 to 7 ng/L	from 30 to 633 ng/L	< 20 % and also observed negative effect on removal	
Ciprofloxacin	from 80 to 1264 ng/L	from 80 to 630 ng/L	30 - 89 %	
Fluconazole	from 132 to 3 ng/L	from 89 to 190 ng/L	< 10 %	
Sertraline	from 8 to 0.4 ng/L	from 10 to 49 ng/L	67 %	
Tramadol	from 709 to 21 ng/L	from 48 to 256 ng/L	3 %	

Literature was collected using the keyword search function in Elsevier database Scopus, focusing on publications and legislation reports starting from 2012. Several keyword combinations were used to search the title, abstract, and keywords, including:

- "Hazardous substances", "Pharmaceutical substances", "Heavy metals", "Veterinary pharmaceuticals", "Pesticides", "Chlorinated organic substances", "Organic substances", "Wastewater treatment by fungi", "Wastewater and pharmaceutical substances";
- Combination of keywords "EU directive", "water" and "priority list of hazardous substances"; "EU directive", "water" and "watch list of hazardous substances"; "EU directive", "water" and "priority substances"; "Monitoring", "Hazardous substances" and "Sweden", "Latvia"; "Report", "Hazardous substances" and "Sweden", "Latvia".

After the literature study, the shortlist of 15 pharmaceutical substances was made (Table 2). The shortlist presents the most reported pharmaceuticals in wastewater in Sweden and Latvia.

Table 3. Physio-chemical properties of the selected pharmaceutical substances

Pharmaceutical	Molecular formula	Functional group	Log K_d	Classification	Reference	Paper
Diclofenac	$C_{14}H_{11}C_{12}NO_2$	amine, chlorine, carboxylic (–COOH)	2.7	nonsteroidal anti-inflammatory drug (NSAIDs)	(Baresel et al., 2015; Carballa et al., 2004; Kramer et al., 2018; Martín et al., 2012; Maurer et al., 2007; Taheran et al., 2016; Ternes, 1998; Wang and Wang, 2016; Zahmatkesh et al., 2017)	Paper I,II and IV
Ketoprofen	$C_{16}H_{14}O_3$	Carboxylic acids and salts, ketones and ketals	2.4	nonsteroidal anti-inflammatory drug (NSAIDs)		Paper I and IV
Carbamazepine	$C_{15}H_{12}N_2O$	heterocycles	1.15	anticonvulsants		Paper II and IV
Ibuprofen	$C_{13}H_{18}O_2$	alkane substituents and a carboxylic acid	2.1	nonsteroidal anti-inflammatory drug (NSAIDs)		Paper II and IV
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	amines and amine salts, heterocycles, sulfur containing groups	1.04	nonsteroidal antibacterial-inflammatory drug (NSAIDs)		Paper II and IV
Metoprolol	$C_{15}H_{25}NO_3$	Alcohols and phenols, amine salts, ethers and oxides	2.4	antihypertensives		Paper IV

In this thesis, diclofenac, ketoprofen, carbamazepine, ibuprofen, sulfamethoxazole, and metoprolol were selected as model compounds due to the high levels of consumption in Latvia and Sweden, and as the most reported and detected compounds in the wastewater treatment plant influent and effluents with relevant low removal efficiency by conventional wastewater treatment (Table 3). Furthermore, these compounds contain different functional groups that might provide new insight into the removal efficiency by fungi. For the experimental setup, the standard solutions of the selected pharmaceutical substance provided by Sigma-Aldrich (Germany) were prepared separately, according to the manufacturer's solubility guidelines, and used in further experimental setups for investigating the removal efficiency by fungi.

3.2.3. Wastewater

In **Paper I**, the synthetic wastewater medium was used in order to test the synergetic effect of fungal strains and the removal of diclofenac and ketoprofen. Also, the removal of diclofenac with the most promising strain *T. versicolor* was tested in non-sterile municipal wastewater. The inlet wastewater was provided by the Henriksdal wastewater treatment plant (Stockholm, Sweden). The inlet wastewater was taken directly from the entry tank. The same synthetic and municipal wastewater were used also in **Paper II** for fungi isolation and batch-scale experiments.

In **Papers III** and **IV**, the municipal wastewater sample for a batch experiment was collected from the Henriksdal wastewater treatment plant, while for the pilot-scale test the municipal wastewater sample was provided by the Daugavgriva wastewater treatment plant (Riga, Latvia). Both inlet wastewater samples were taken directly from an entry tank.

3.2.4. Synergistic effect

In **Paper I**, the synergetic effect of selected fungal strains was investigated by growing each fungal strain separately (viz. individual experiment); mixed cultures were investigated by different combinations with selected fungi strains. The combination of *T. reesei*, *I. lacteus* and *T. versicolor*, and the combination of *F. solani* and *P. ostreatus* (viz. mixed experiment) were compared.

3.2.5. Removal of pharmaceuticals and nutrients by fungi

The removal efficiency of pharmaceutical substances (**Papers I** and **II**) and nutrients (**Paper III**) by fungal strains under batch-scale experiment was evaluated under sterile and non-sterile conditions, using 100 ml flasks filled with 25 ml of wastewater medium, pharmaceutical substance, and the fungal inoculum or suspension. The scheme of the experimental setup can be seen in Figure 8.

In **Paper III**, the fungal biomass was cultivated in the potato dextrose media, incubated in a shaking incubator for 5 days. To achieve a higher initial concentration of the selected fungi, in **Papers I, III,** and **IV**, the Kaldnes K1 carriers (AnnoxKaldness, France; diameter 9.1 mm) were used for biofilm formation. After growing, the fungal biomass was separated from the media and added to a non-sterile municipal wastewater sample.

In all three papers, additional samples were incubated in a shaker incubator for a time period of 72 h. Further, an additional investigation of pharmaceuticals and nutrient removal was done, and samples were taken every 3 h for up to 72 h.

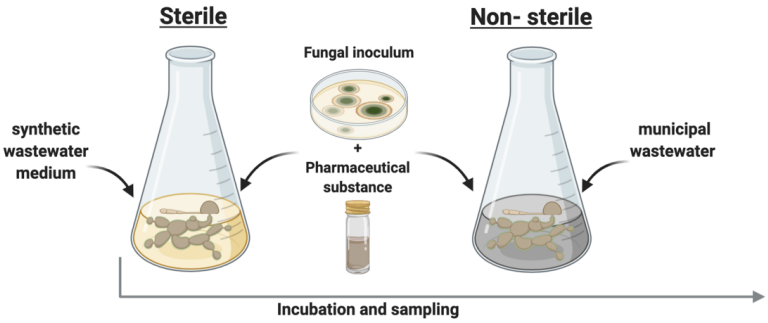

Figure 8. Batch experiment with fungi (created with BioRender.com)

3.2.6. Biosorption test

To study the removal mechanisms of pharmaceutical substances by fungi, the biosorption test was performed in **Papers I** and **II**. The fungal biomass was cultivated in potato dextrose medium and incubated in a shaking incubator for a period of 5 days (Figure 9).

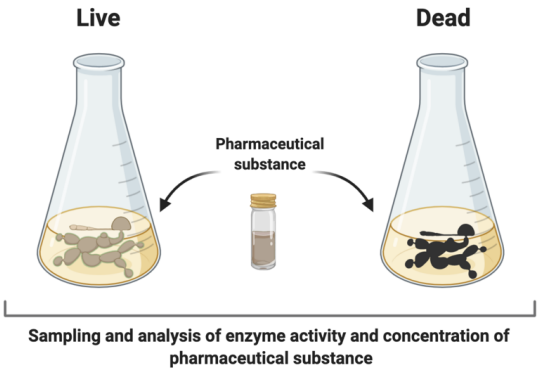

Figure 9. Biosorption test with fungi (created with BioRender.com)

After incubation, half of the flasks were double autoclaved to establish a heat-killed control (dead fungal cells). Diclofenac was then added to the flasks of live and dead fungal cells. Samples of diclofenac and enzyme activity of laccase analysis were taken every 3 h for up to 24 h.

3.2.7. Bioreactor configuration and operating conditions

In **Paper III,** a fungal fluidized bed pelleted bioreactor was designed, consisting of a reactor, biomass tank for bioaugmentation, a feed peristaltic pump with a flow meter, air supply with a flow regulator, and an effluent tank (Figure 10).

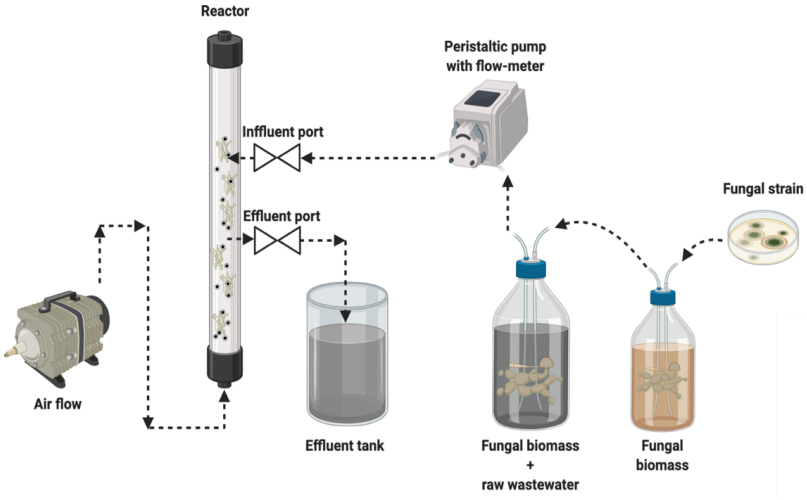

Figure 10. A scheme of a fungal fluidized bed pelleted bioreactor (created with BioRender.com)

The reactor consisted of a 2 L cylindrical plastic column with a working volume of 1.25 L. The up-flow velocity in the reactor was settled according to an experimental plan, i.e., approx. 1.08 mL/min or 0.11 mL/min where fungal biomass was maintained fluidized by air pulses generated by an air supply. In the beginning, the column was sterilized and filled with 1.25 L of municipal wastewater and 100 g of wet fungal biomass on Kaldnes K1 carriers. **Paper III** mainly studied the removal efficiency of nutrients. Therefore, after one week of adoption, the amount of fungal biomass (25 g wet biomass per 100 mL), harvested from 250 mL of a potato dextrose media without carriers for a 5 day cultivation period, was weighted and washed with deionized water. After washing, the wet biomass was homogenized with 250 mL non-sterile wastewater and added to the reactor. Before the fungal biomass adjustment, 250 mL of wastewater from the fungal fluidized bed pelleted bioreactor was removed through the effluent port and poured out in an effluent tank. Additionally, a negative control, i.e., a reactor with carriers without fungal biomass, was prepared in order to conclusively establish a link to the nutrient removal induced by fungi.

In **Paper IV**, the main focus was to optimize the fungal fluidized bed pelleted bioreactor through bioaugmentation and investigate the removal efficiency of pharmaceutical substances. After three weeks of incubation, the pharmaceuticals were added to the bioreactor and the removal efficiency was evaluated. Throughout the experiment, the bioaugmentation strategy was tested, i.e., the amount of two fungal biomasses of 50g and 10 g were added to the reactor three times per week. Before the fungal biomass adjustment, 250 mL of wastewater from the fungal fluidized bed pelleted bioreactor was removed through the effluent port and poured out in an effluent tank. Additionally, a negative control, i.e., a reactor with carriers without fungal biomass, was prepared to compare the experimental results in order to completely establish a link to the nutrient removal induced by fungi.

In **Papers III** and **IV**, the samples were taken from the fungal fluidized bed pelleted bioreactor effluent port before (B) the adjustment of fresh non-sterile wastewater and after (A) the adjustment of 250 mL fresh non-sterile municipal wastewater.

3.2.8. Analytical methods

In most of the papers, the pH value, the laccase activity, and the concentration of pharmaceuticals were analyzed. The pH measurements to monitor pH

changes were performed using universal pH-indicator strips. The laccase activity was measured spectrophotometrically, using the standardized procedure of the enzymatic assay for laccase by Sigma-Aldrich (Germany). The concentration of pharmaceutical substances was measured using HPLC. In **Papers III** and **IV**, a Hach-Lange (Germany) spectrophotometric system and kits were used to determine the concentrations of P, NH_4-N, and TOC; a direct counting method using DAPI was used to obtain the total microorganism count in the municipal wastewater sample.

3.3. Results

3.3.1. Growth effect and synergistic effect of fungi

In **Paper I**, the growth and interaction of selected fungal strains were tested by a classical cultivation method with and without the presence of pharmaceutical substances. The results indicated that all the fungal strains could grow in the presence of pharmaceuticals diclofenac and ketoprofen. When the cultures were grown together, the formation of the inhibition zone among the tested strains was observed. Therefore, in further study, the removal efficiency of diclofenac and ketoprofen was investigated by applying the fungi individually and mixed in the liquid media of synthetic wastewater. The results from the synergistic study showed that the individual growth strategy to remove pharmaceutical compounds not only excluded competition and inhibition among the strains, but also increased the efficiency of pharmaceutical substance removal. However, Mir-Tutusaus et al. (2018) believe that the results may not be as significant as they could be if the fungi were grown under non-sterile wastewater conditions with a variable microbial community in municipal wastewater (Mir-Tutusaus et al., 2018). Therefore, to examine the capability to remove diclofenac in non-sterile municipal wastewater by the selected fungal strains - *T. versicolor*, *I. lacteus*, *F. solani*, and *P. ostreatus* - was evaluated individually.

3.3.2. Removal of pharmaceuticals by fungi in municipal wastewater

In **Paper I**, the removal of the diclofenac was initially examined by using each fungal strain separately (individual cultures). Furthermore, all batch-scale experiments were investigated under non-sterile conditions using inlet municipal wastewater.

The results from the batch-scale experiment demonstrated that the initial concentration of a fungal inoculum was relatively low. The fungi might

have been outcompeted by bacteria, *Archaea,* or other fungal species from the wastewater. Thus, the initial fungal concentration of the biomass was increased in further experiments using 9.1 mm diameter Kaldnes K1 carriers (Figure 11).

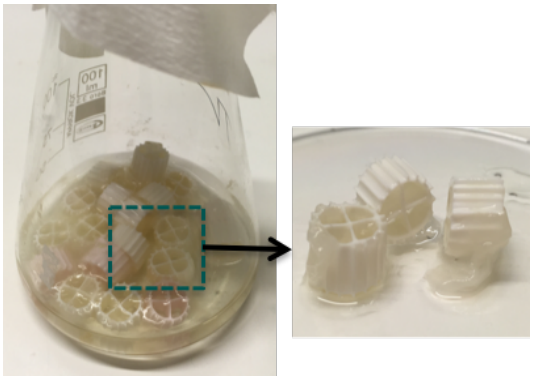

Figure 11. Fungal growth on Kaldnes K1 carriers

The results demonstrated that all fungal cultures were able to completely remove (> 99.9 %) diclofenac after 3 days of incubation (Figure 12). However, a longer incubation time demonstrated a release of diclofenac back to wastewater from all fungal strains except for *T. versicolor* (Figure 12 B). These results might be explained by the use of different removal mechanisms of the tested fungi. Therefore, the removal mechanisms of *T. versicolor* were further investigated and analyzed using the biosorption test.

The results of the biosorption test in **Paper I** indicated that *T. versicolor* used both mechanisms - enzyme activity and biosorption - to remove diclofenac. The observation of the biosorption test showed that laccase was responsible for removing ~ 20 % of diclofenac, while the *T. versicolor* live biomass could remove ~ 80 % of diclofenac.

Overall, **Paper I** have shown the potential of five fungal strains to remove ketoprofen and diclofenac individually and in mixed cultures. The experiments with synthetic wastewater media showed that the fungi compete with each other, since higher removal efficiency was observed if the fungi were grown individually. The capability of the selected fungal strains to remove diclofenac in a non-sterile municipal wastewater sample using carriers suggested a feasible approach for the wastewater treatment process. The results show that *T. versicolor* could fully (> 99.9 %) remove diclofenac after a 3-h long incubation period in non-sterile municipal wastewater. Moreover, *T. versicolor* demonstrated an ability to use both mechanisms - enzyme

activity and biosorption - to remove diclofenac. Therefore, *T. versicolor* could be a promising candidate to remove pharmaceutical substances in the wastewater treatment process.

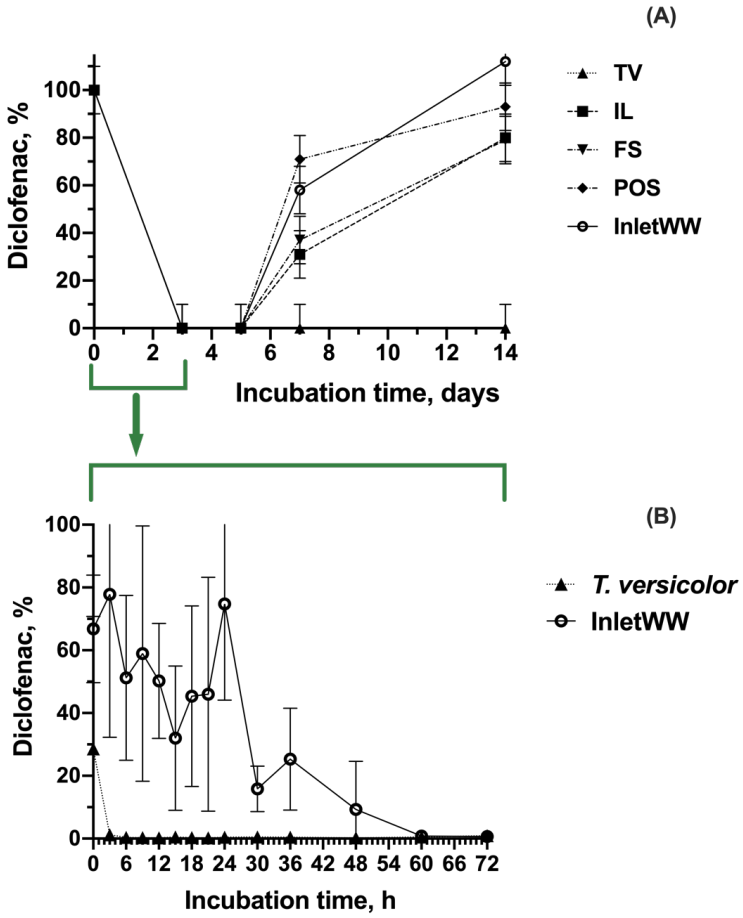

Figure 12. (A) The removal efficiency (%) of diclofenac from non-sterile municipal wastewater with fungal biofilm on K1 carriers of *T. versicolor* (TV), *I. lacteus* (IL), *F. solani* (FS), and *P. ostreatus* (POS), and negative control without the addition of fungal biomass (InletWW); **(B)** Removal efficiency (%) derived from the additional research of diclofenac removal within 3 days with *T. versicolor* in non-sterile municipal wastewater with fungal biofilm on K1 carriers compared to negative control without the addition of fungal biomass (InletWW)

3.3.3. Isolation of fungal strains from municipal wastewater for the removal of pharmaceutical substances

In **Paper II**, the municipal wastewater was used for fungi isolation. The isolated fungi were grown on potato dextrose agar in the presence of all the

selected pharmaceutical compounds, such as carbamazepine, diclofenac, ibuprofen, and sulfamethoxazole. Based on growth efficiency, a total of seven fungal isolates were used for further study to investigate their ability to remove pharmaceutical substances in synthetic wastewater media, and the results were compared to *T. versicolor* in order to see the differences in removal efficiency. The pH effect on removal efficiency was also investigated.

The results from **Paper II** indicated difficulties to obtain relatively high removal efficiency (> 80 %) for carbamazepine by the tested isolates. The results from diclofenac removal showed that one of the fungal isolates could completely (> 99.9 %) remove this pharmaceutical after 6 days of incubation at pH 5.5, while complete reduction of diclofenac at pH 6.3 was obtained after 10 days of incubation. The same result was observed for *T. versicolor*. Therefore, further in **Paper II**, the removal efficiency for diclofenac in non-sterile municipal wastewater with the most promising isolate was examined and compared with *T. versicolor*. The most promising isolate was identified as *Aspergillus luchuensis*.

When evaluating the diclofenac removal efficiency after fungal treatments at various pH values from a non-sterile municipal wastewater sample, it can be stated that the fungal isolate of *A. luchuensis* can remove > 95 % of diclofenac at both pH values for the entire incubation time (Figure 13 A, B).

In contrast, *T. versicolor* demonstrated a relatively slower diclofenac removal efficiency at pH 7.8 after 24 h of incubation time, compared to pH 5.5 at which diclofenac was completely removed (> 99.9 %) after 3 h of incubation period. In order to better understand the removal mechanisms of *A. luchuensis*, the enzyme activity of laccase was measured and the biosorption test was done establish the relationship between the enzyme activity, fungal biomass, and the removal efficiency for diclofenac (Figure 13 C, D).

The results indicated that there was no enzyme activity detected with *A. luchuensis* and wastewater without a fungal inoculum, while *T. versicolor* produced laccase for the entire incubation period for both non-sterile municipal wastewater samples with different pH values. Furthermore, the live biomass of *A. luchuensis* did not present any laccase enzyme activity throughout the incubation period. Moreover, *A. luchuensis* showed complete removal (> 99.9 %) of diclofenac for both live and dead fungal biomass, immediately after the biomass adjustment, indicating that the removal of diclofenac could be due to the biosorption mechanism.

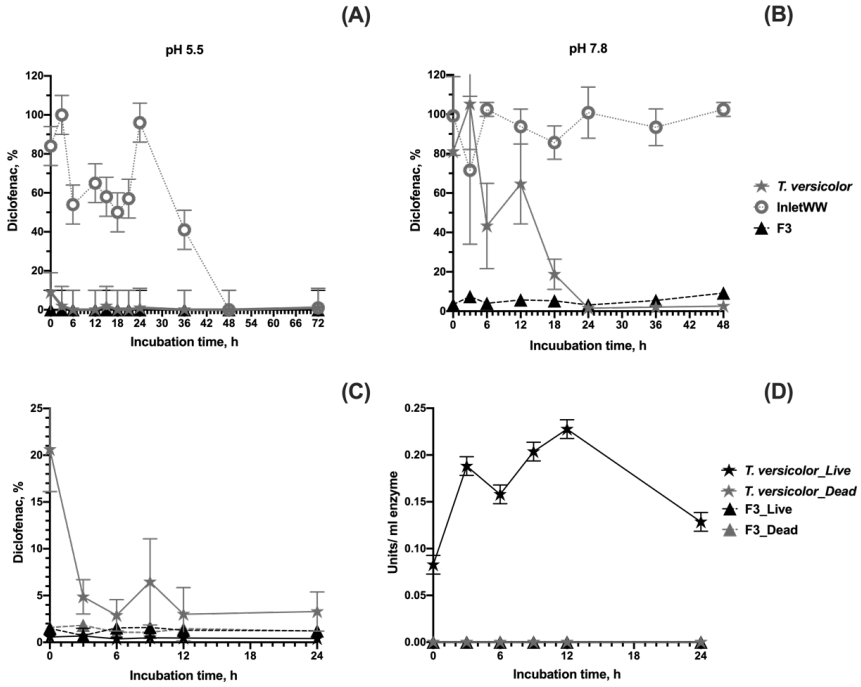

Figure 13. The removal efficiency of the isolate *A. luchuensis* (F3) and *T. versicolor* (%) for diclofenac at **(A)** pH 5.5 and **(B)** pH 7.8 from non-sterile municipal wastewater compared to a negative control without the addition of fungal biomass (InletWW); **(C)** The removal efficiency (%) of diclofenac from the biosorption test of live and dead fungal biomass by the isolate *A. luchuensis* (F3) and *T. versicolor*; **(D)** Enzyme activity of laccase from the biosorption test of live and dead fungal biomass by the isolate *A. luchuensis* (F3), compared to *T. versicolor*

Overall, these results from **Paper II** have shown that *A. luchuensis* has a higher removal efficiency in a non-sterile wastewater sample without a pH correction than *T. versicolor*. Thus, *A. luchuensis* has a high potential for use in industrial wastewater treatment due to minimized specific pH requirements. Therefore, in the further study in **Papers III** and **IV**, these two strains were selected and tested in a fluidized bed pelleted bioreactor for removal of nutrients and pharmaceutical substances.

3.3.4. Removal of total phosphorus, ammonia nitrogen, and organic carbon from non-sterile municipal wastewater under batch scale experiment

In **Paper III**, the nutrient removal efficiency and the effect of pH on nutrient removal by two fungi - *T. versicolor* as a laboratory strain from **Paper I** and

A. luchuensis isolate from a municipal wastewater treatment plant from **Paper II** - were studied and compared for nutrient reduction in non-sterile municipal wastewater under a batch and pilot-scale experiment. The insight into the potential of fungi to remove not only pharmaceutical substances but also nutrients can help better understand the utility of developing a fungal treatment technology for contaminant removal (Mook et al., 2012).

The results in **Paper III** from the batch scale experiment indicated that both fungi were able to remove phosphorus (P) in non-sterile municipal wastewater without/with a pH adjustment. The results of ammonia nitrogen (NH_4-N) removal by *T. versicolor* without pH adjustment showed an increase of NH_4-N concentration immediately after the incubation was started. The same tendency was observed with *A. luchuensis*. On the contrary, the results for *T. versicolor* and *A. luchuensis* with pH adjustment showed relatively small changes in NH_4-N concentration throughout the incubation time. Therefore, both fungi had no direct effect on NH_4-N reduction in municipal wastewater, i.e., it is believed that both fungi did not use NH_4-N in their metabolic pathway to reduce the nitrogen concentration in the wastewater. However, a further investigation is required to better understand the fungal role in the NH_4-N reduction in municipal wastewater. When evaluating the total organic carbon (TOC) removal efficiency after both fungal treatments, it can be stated that *T. versicolor* and *A. luchuensis* can reduce TOC concertation after a 72 hour incubation period with a pH level adjustment to 5.5 for wastewater. In contrast, the results of *T. versicolor* and *A. luchuensis* without a pH level adjustment showed diverse changes in the TOC concentration throughout the incubation period of 72 h. It can be concluded that the pH value adjustment might stabilize the TOC removal process by fungi, while wastewater without a pH value adjustment showed an unsteady reduction of TOC for the entire incubation time of 72 h.

Overall, the results from **Paper III** under batch scale experiments with non-sterile wastewater demonstrated that both fungi can remove P from wastewater, and the pH value had a significant effect on N and TOC concentrations. According to these results, both fungi were further investigated in a fluidized bed pelleted bioreactor and the removal efficiency of nutrients was analyzed.

3.3.5. Removal of total phosphorus, ammonia nitrogen, and organic carbon in a fluidized pelleted bioreactor from non-sterile municipal wastewater

Once the results from the batch experiments achieved a relatively good success in the P reduction by fungal treatment and showed that the pH adjustment to 5.5 helped to stabilize the N and TOC reduction process, the removal analysis was further tested in a fluidized bed pelleted bioreactor.

The results in **Paper III** from a fluidized pelleted bioreactor showed that both fungi were able to reduce more than 80 % of P by the end of the incubation period. However, there was no statistically significant difference regarding the P reduction efficiency between the fungi and the negative control. The result of the NH_4-N concertation did not show any changes for both fungi until the end of the incubation period. Finally, the results for TOC demonstrated that after 15 days of incubation, the TOC had been reduced by 35 %. Overall, the results of a fluidized bed bioreactor demonstrated different tendencies on nutrient removal, using *T. versicolor* and *A. luchuensis* compared to a batch experiment. Therefore, in **Paper IV**, the fluidized pelleted bioreactor was optimized using bioaugmentation as a strategy to add fresh fungal biomass.

3.3.6. The bioaugmentation effect on the removal efficiency of pharmaceutical substances in a fluidized pelleted bioreactor

In order to gain a better understanding of how bioaugmentation affects the removal efficiency, two wet biomasses of 10 and 50 g for *T. versicolor* and *A. luchunesis* were tested in **Paper IV**. The results in Figure 13 demonstrate the removal efficiency of *T. versicolor* and *A. luchunesis* for diclofenac, ketoprofen, carbamazepine, ibuprofen, sulfamethoxazole, and metoprolol.

Results from both fungi showed that adjustment of 50 g of wet biomass demonstrated relatively high removal efficiency (> 90 %) for ketoprofen and metoprolol (Figure 14, B and F) compared to the adjustment of 10 g of wet biomass after 3 hours of incubation time. At the same time, relatively low removal efficiency for both bioaugmentation strategies (> 40 %) was detected for diclofenac, carbamazepine, ibuprofen, and sulfamethoxazole.

The results from **Paper IV** showed that bioaugmentation can be a promising strategy to optimize and operate the fungal bioreactor. Also, the results indicated that the fungi have a variety of strategies on how to counteract pharmaceutical substances.

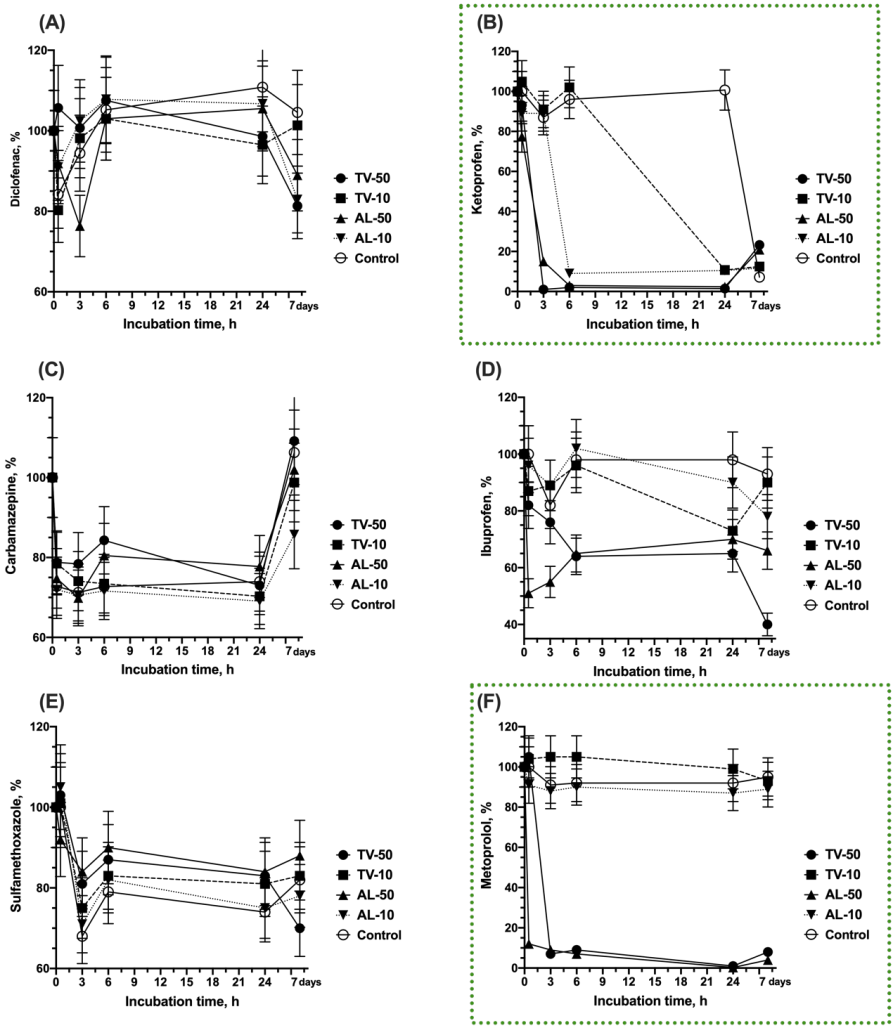

Figure 14. The bioaugmentation effect on removal efficiency of pharmaceutical substances (%) **(A)** diclofenac **(B)** ketoprofen **(C)** carbamazepine **(D)** ibuprofen **(E)** sulfamethoxazole, and **(F)** metoprolol by fungi *T. versicolor* (TV) and *A. luchuensis* (AL) with the adjustment of 10 and 50 g wet biomass

3.3.7. Cost evaluation of fungal treatment

In **Papers III** and **IV**, the results showed that with bioaugmentation it is possible to maintain domination of fungi over bacteria without a pH adjustment and effectively remove nutrients and pharmaceutical substances such as metoprolol and ketoprofen. However, it does require additional

expenses, including for an extra source of the organic substrate to cultivate fungi. In **Paper III**, the author has estimated the costs based on the current average market prices in Europe. All estimated fungal treatment costs (EUR/m^3) include the cost of fungal growth and operation in a fluidized bed pelleted bioreactor (Table 4).

Table 4. The average price for different wastewater treatment technologies and the cost of the studied fungal treatment by *T. versicolor* and *A. luchuensis*

Wastewater Treatment Technology	Cost, EUR/m^3	Reference
Fungal Treatment	from 200 to 2000	This study
Coagulant-Flocculant	from 0.35 to 8.5	(Pelendridou et al., 2014; Yoo, 2018)
Membrane-Based Treatment	from 2	(Rongwong et al., 2018)
Conventional Biological Treatment	from 0.036 to 1	(Hansen et al., 2007)

According to the literature (Hansen et al., 2007; Pelendridou et al., 2014; Rongwong et al., 2018; Yoo, 2018) the cost of typical wastewater treatment technologies are as follows: 0.35–8.5 EUR/m^3 for coagulation-flocculation process, from 2 EUR/m^3 for membrane-based technologies, from 0.035 to 1 EUR/m^3 for conventional biological treatment, while the growth and operation costs of fungal treatment vary from 200 to 2000 EUR/m^3.

The cost of fungal treatment highly depends on fungal growth requirements (temperature, incubation time, electricity of shaking, composition of media). Thus, the cost of the fungal treatment presented here is among the highest reported in the literature, contradicting the hypothesis that fungal treatment can be a cost-effective treatment technology. However, fungal treatment still has a high potential to be an environmentally friendly and sustainable wastewater treatment method not only considering the nutrient load perspective but also for pharmaceutical substances removal and complementary to the conventional biological step (Mir-Tutusaus et al., 2018). Furthermore, fungi might give valuable benefit in the long term by enhancing nitrogen removal, recovering value-added products from fungal biomass, and fine-tuning the wastewater treatment process, thereby reducing the cost of the wastewater treatment process (Sankaran et al., 2010).

4. Conclusions and future outlook

The objective of this thesis was to investigate the potential of filamentous fungi to remove pharmaceutical substances from municipal wastewater under non-sterile conditions without pH level correction. To achieve the goal of this thesis, the literature study was done and filamentous fungi potential for wastewater treatment from pharmaceutical substances were examined.

In **Paper I**, the potential of five fungal strains to remove ketoprofen and diclofenac individually and in mixed cultures was investigated. The experiments with synthetic wastewater media showed that the fungi compete with each other since higher removal efficiency was observed if the fungi were grown individually. The results showed that *T. versicolor* could fully (> 99.9 %) remove diclofenac after a 3-hr long incubation period in non-sterile municipal wastewater. Moreover, *T. versicolor* demonstrated an ability to use both mechanisms, namely, enzyme activity and biosorption, to remove diclofenac. Therefore, *T. versicolor* proved to be a promising candidate for the removal of pharmaceutical substances through wastewater treatment process and was further used in **Papers II**, **III**, and **IV**.

Paper II demonstrated the ability of fungal isolates to grow in the presence of pharmaceuticals such as carbamazepine, diclofenac, ibuprofen, and sulfamethoxazole. The results indicated that *A. luchuensis* is a promising candidate to remove diclofenac from wastewater with a typical pH of 7.8, compared to *T. versicolor,* which has shown a relatively higher removal efficiency at pH 5.5. As batch scale experiments from **Papers I** and **II** showed that both fungi, i.e., a laboratory strain of *T. versicolor* and an environmental isolate of *A. luchuensis* from a municipal wastewater treatment plant, might be feasible for wastewater treatment from pharmaceutical substances. Therefore, these strains were used in further study of **Papers III** and **IV.** These papers investigated the efficiency of removing nutrients and pharmaceuticals in fluidized bed bioreactor after fungal treatment.

In **Paper III**, a fluidized bed bioreactor was designed and the efficiency of removing nutrients was tested. During this study, the fungi have demonstrated a high potential to remove phosphorus from municipal wastewater efficiently and successfully under a batch scale experiment, while the results from the fluidized bed bioreactor did not demonstrate a significant decrease in nutrients. Therefore, in **Paper IV**, the fluidized pelleted bioreactor was optimized and the bioaugmentation as a strategy to add fresh fungal biomass was used.

The main benefit of pharmaceutical treatment by fungi is that it is a biological treatment and does not require specific use of chemicals (Pointing, 2001). The author of this study believes that there might be two possible practical applications for this method:

1. Fungal pre-treatment for industrial wastewater where specific pharmaceuticals need to be removed;
2. Fungal post-treatment for pharmaceutical substances removal at the end of the municipal wastewater treatment system.

However, the results derived from this study showed that fungal growth in non-sterile conditions in the bioreactor is limited, and the removal efficiency decreased compared to results from batch-scale experiments. Sterilization of wastewater is not a cost-efficient and suitable option for wastewater treatment by fungi (Espinosa-Ortiz et al., 2016; Ferreira et al., 2020; Mir-Tutusaus et al., 2019). Therefore, in future studies development of a symbiotic fungal–bacterial/ fungi-algae consortium for the removal of pharmaceuticals from non-sterile wastewater can be investigated (Muradov et al., 2015; Wei et al., 2018). Further research is needed to better understand the operational challenges and requirements for full-scale applications (Mir-Tutusaus et al., 2018). In **Paper IV**, the fungal bioreactor has a relatively high concentration of added fungal biomass, inhibiting the fresh biomass adaption and growth in the bioreactor. Therefore, the process of how to efficiently remove the used biomass has to be implemented in the bioreactor. Furthermore, *T. versicolor* and *A. luchuensis* are fungi that are able to produce spores (Benson et al., 2019; Hong et al., 2013). Thus, the strategy of what to do with fungal biomass after wastewater treatment needs to be discussed and developed (Sankaran et al., 2010). Furthermore, this study showed that the fungi can use two mechanisms for removing pharmaceutical substances – enzyme activity and biosorption. As fungi can use biosorption for pharmaceutical substance removal, the biomass can be relatively rich in removed pharmaceutical substances (Jureczko, 2018). Additionally, the strategy of what to do with this biomass needs to be discussed and the possible impact on the environment also needs to be investigated.

Finally, the full-scale application of fungal treatment for wastewater does not exist at the moment. According to researcher Mir-Tutusaus, developments in fungal treatment direction depend on overcoming several shortcomings, namely: (1) maintaining a stable activity of the fungal pellets over prolonged periods of time and (2) preserving good performance in non-sterile conditions (Mir-Tutusaus et al., 2019). Already, the fungal treatment costs highly depend on fungal growth requirements (temperature, incubation

time, the electricity of shaking, the composition of media) (Mir-Tutusaus et al., 2018; Sankaran et al., 2010). Thus, the cost of the fungal treatment presented in **Paper III** is among the highest reported in the literature, contradicting the hypothesis that the fungal treatment can be a cost-effective treatment technology (Arikan et al., 2019; Lu et al., 2016; Wang and Wang, 2016). However, the author of this study believes that fungal treatment still has a high potential to be an environmentally friendly and sustainable treatment method for wastewater treatment not only considering the nutrient load perspective but also for pharmaceutical substance removal. Furthermore, the fungal biomass after treatment can be used as a source for valuable byproducts, therefore covering the incurred costs of growth (Sankaran et al., 2010).

Overall, the results from this thesis approve the scientific question derived from this thesis, i.e., filamentous fungi can remove pharmaceutical substances such as diclofenac and ketoprofen from non-sterile municipal wastewater. The observed results from this study give a relevant and useful knowledge for future investigations and help to improve the fungal application in real wastewater treatment systems. For instance, the investigation of the synergetic study showed that selected fungi have relatively higher removal efficiency for pharmaceutical substances if they are incubated individually instead of mixed cultures of fungal strains. The results from this study also give insight into a better understanding of the effectiveness of fungal treatment and the mechanisms behind the removal of pharmaceuticals. However, improvements still need to be developed for full-scale application of fungi in the wastewater treatment process, addressing such aspects as the optimal operation conditions for fungal growth and adaption in fluidized bed bioreactor to efficiently remove nutrients and pharmaceutical substances from wastewater.

In the future, researchers need to cooperate with stakeholders and governments to set up strategies and processes on how to implement innovations and optimize the existing methods for wastewater treatment. According to Global Goals for Sustainable Development, this study mainly overlaps with Goal 6. The main aim of Goal 6 (called *Clean water and sanitation*) is to ensure the availability and sustainable management of water and sanitation for all people. One of the main objectives of Goal 6 is to improve water quality and wastewater treatment. Therefore, this study goes hand in hand with the objective of this goal. As this goal proposes that by 2030 the release of chemicals and hazardous substances needs to be minimized, the

author thinks that the results derived from this thesis can be used to advance fungal treatment for pharmaceutical substance removal from wastewater.

To the best of the author's knowledge, this is the first study to test pharmaceutical and nutrient removal from non-sterile municipal wastewater in a fluidized bed pelleted bioreactor using bioaugmentation as a strategy for operating the fungal bioreactor treatment system. The author sees this work as a valuable result which can be used as a first step to improve the fungal technology for municipal wastewater treatment and gain strong insights and knowledge of the application of fungi in municipal wastewater treatment systems.

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PAPER I

Constructive use of filamentous fungi to remove pharmaceutical substances from wastewater

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Constructive use of filamentous fungi to remove pharmaceutical substances from wastewater

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ABSTRACT

The removal efficiency of diclofenac and ketoprofen for five fungal strains was examined. The synergistic effect between selected fungal strains using synthetic wastewater media was studied using both individual and mixed strains. The mixed cultures had no additional effect in reducing diclofenac and ketoprofen. In contrary, fungi removed pharmaceutical substances faster when growing individually. The results showed that *T. versicolor* monoculture removed diclofenac completely (> 99.9 %) in non-sterile municipal wastewater within three hours of incubation and continued to show complete reduction within 14 days using fungal biofilm carriers. The mechanism of removal was proposed based on the laccase enzyme production and biosorption study which revealed that *T. versicolor* could used in both processes. Additionally, this study demonstrated that *T. versicolor* could be stable in the biofilm carriers, and thus able to compete with other microorganisms in wastewater to be a promising candidate to remove diclofenac from municipal wastewater during the treatment process.

1. Introduction

Over the last few decades, the widespread occurrence of hazardous substances such as pharmaceuticals, personal care products, pesticides and endocrine-disrupting chemicals in the aquatic environment has been recognized as an emerging environmental issue as it can cause undesirable effects on the ecosystem and human health [1,2]. The main source of these compounds is effluent from municipal wastewater treatment plants. The conventional treatment plant is commonly designed to remove high concentrations, mostly biodegradable organic matter and nutrients using mainly bacteria [3,4]. Thus, most of the hazardous substances are not effectively removed [5,6]; consequently, may have negative effects on human health and aquatic ecosystem [7]. For instance, extensive use of antibiotics and antimicrobial agents can result in development of antimicrobial resistance genes in bacteria [2]. Furthermore, a previous study has demonstrated that diclofenac has negative effect on marine organisms such as mussels [8].

Although advanced treatment technologies, such as ozonation, advanced oxidation, membrane processes and adsorption by activated carbon, are considered as a promising alternative to remove hazardous substances from wastewater [9], high operating costs and formation of by-products are still limiting factors to implement the advanced treatment technologies as alternative to an effective wastewater treatment process [10]. Therefore, fungi have been considered as a promising alternative to advanced treatment methods to remove hazardous substances from wastewater [11].

Fungi can use hazardous substances as a secondary substrate due to their ability to produce nonspecific enzymes (e.g., ligninolytic extracellular oxidative enzymes), which are able to degrade a wide range of hazardous substances even at very low concentrations [1,5,12,13]. Previous studies have shown that it is attainable to achieve high removal efficiency applying biodegradation of many hazardous compounds. For instance, *Fusarium solani* was able to degrade the emerging water pollutants like di(2-ethylhexyl)phthalate (DEHP), fluoranthene, and aminomethylphosphonic acid (AMPA) in a batch-scale experiment in a synthetic media for fungi [14]. Likewise, several studies have demonstrated high removal efficiency of hazardous compounds as progesterone, atenolol, ibuprofen, diclofenac, and ketoprofen by *Trametes versicolor, Pleurotus ostreatus, Trichoderma reesei*, and *Irpex lacteus* [15–18] in laboratory-scale experiments under sterile conditions. Based on these investigations, it has been proposed that these fungi might be suitable candidates to remove hazardous substances. However, most of experiments with fungal cultures both in batch and continuous reactors for hazardous substances removal, have been tested by individual

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strains and cultured in specific nutrient media or synthetic wastewater [19]. Therefore, there are still many unanswered questions regarding a full-scale application of fungi in the wastewater treatment process. One of the questions is how the use of fungal culture could affect the removal efficiency of hazardous substances, and how it would compete with other microorganisms under non-sterile conditions. Moreover, the operating conditions might play a key role, especially on enzyme activity. For instance, previous studies have demonstrated that the use of biofilm carriers not only improve fungal growth and production of enzyme activity but also increase the removal efficiency of hazardous substances [20–22]. Thus, further research is needed to determine if the approach, using fungi, can be applied for the wastewater treatment process in order to better understand their ability to grow, produce enzymes and remove hazardous substances in municipal wastewater under non-sterile conditions.

The main objective of this study was to investigate the potential of five worldwide-distributed fungal strains - *T. versicolor*, *I. lacteus*, *P. ostreatus*, *T. reesei*, and *F. solani* - to remove hazardous substances from municipal wastewater under non-sterile conditions. In this work, the pH level, the effect of carriers, the removal efficiency and the enzymatic laccase activity were examined for each strain separately and in mixed cultures in batch-scale experiments for a certain period of time. To the best of our knowledge, this is the first study where the synergetic effects have been tested. Furthermore, the effect of non-sterile municipal wastewater using fungal biofilm carriers K1 with the most promising strains was tested to assess the potential of fungal treatment to remove hazardous substances. Additionally, the difference of initial inoculum for fungal cultures was analyzed in order to determine the removal efficiency in non-sterile wastewater. Finally, the biosorption experiment was done to better understand fungal removal mechanisms for pharmaceutical substances.

2. Materials and methods

2.1. Fungal strains

The fungal strains - *Trichoderma reesei* DSM 768, *Trametes versicolor* DSM 6401, and *Pleurotus ostreatus* DSM 1020 - were obtained from DSMZ Culture Collection (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell cultures, Germany). *Irpex lacteus* IBB 104 (Parker H. Petit Institute for Bioengineering and Bioscience, Georgia) and *Sordariomycetes* filamentous fungus, *Fusarium solani* (an environmental isolate from a pharmaceutical wastewater treatment plant located in Riga, Latvia) were used in this study. All cultures were grown and maintained on the potato dextrose agar medium (PDA; Oxoid, United Kingdom).

2.2. Chemicals

High purity grade (> 90 %) ketoprofen and diclofenac were purchased from Sigma-Aldrich (Germany) (Table 1). The standard solutions (at the concentration of 5 mg l^{-1}) of diclofenac sodium salt and ketoprofen were prepared according to the manufacturer's solubility guidelines.

2.3. Preparation of synthetic wastewater medium

The synthetic wastewater medium (0.8 g l^{-1} KH_2PO_4; 0.2 g l^{-1} K_2HPO_4; 0.5 g l^{-1} $MgSO_4$; 0.2 g l^{-1} yeast extract (Oxoid, United Kingdom); pH 5.5) was prepared as broth medium and 1.5 % agar was added to prepare a solid medium. The medium was autoclaved at 121 °C for 15 min. The prepared media was stored at 4 °C before the experimental setup started.

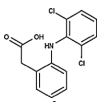
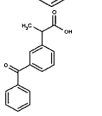
2.4. Synergistic effect of selected fungal strains

The synergetic effect of selected fungal strains was investigated by growing each fungal strain separately (viz. individual experiment); mixed cultures were investigated by different combinations with selected fungi strains, e.g., the combination of *T. reesei*, *I. lacteus* and *T. versicolor,* and combination of *F. solani* and *P. ostreatus* (viz. mixed experiment). Plastic petri dishes (90 mm inner diameter) containing about 25 ml synthetic wastewater media with and without diclofenac and ketoprofen (the final concentration of 5 mg l^{-1} of both selected chemicals) were used in pre-screening experiments. The pharmaceutical compounds were added immediately after synthetic wastewater media was poured into dishes. After inoculation, fungi were incubated at 25 °C for 14 days. The fungal growth and interaction (inhibition or antagonistic activity) among strains were estimated qualitatively by taking images of mycelia and interaction zone on the agar plate with digital photo camera (Kodak, USA).

2.5. Removal of diclofenac and ketoprofen by fungal strains in a synthetic wastewater medium

The removal of pharmaceutical substances by fungal strains was evaluated under sterile conditions, using 100 ml flasks (glass bottles; Glassco, United Kingdom) filled with 25 ml of synthetic wastewater medium, pharmaceutical substances (the final concentration of 5 mg l^{-1} of selected chemical), and the fungal inoculum (pre-cultivated fungal agar plug; r ≈ 2 mm). In addition, two negative controls - a control without pharmaceutical compounds and a control without adding fungi - were prepared to compare that the observations of the experiments could be completely linked to the removal of pharmaceutical substances by fungi. Finally, flasks were closed with cotton stoppers and incubated in a shaking incubator (150 rpm) at 25 °C for a period of 14 days. The pharmaceutical's concentration, the laccase activity, and the pH level were periodically determined after days 0, 3, 7, and 14. All the experiments were carried out in duplicate or triplicate.

Table 1
Physical-chemical properties of the selected hazardous substances.

Compound name	Molecular formula	Structure	Functional group	Classification	Reference
Diclofenac	$C_{14}H_{11}C_{12}NO_2$		amine, chlorine, carboxylic (–COOH)	nonsteroidal anti-inflammatory drug (NSAIDs)	[23]
Ketoprofen	$C_{16}H_{14}O_3$		keton (-CO), carboxylic (–COOH), methyl (-CH_3)	nonsteroidal anti-inflammatory drug (NSAIDs)	[24]

2.6. Removal of diclofenac in municipal wastewater

Removal of pharmaceutical substance in municipal wastewater was conducted, using selected fungal strains. All the experiments were performed under non-sterile conditions, using 100 ml flasks filled with 25 ml of inlet wastewater, pharmaceutical substances (the final concentration of 5 mg l^{-1} of selected chemical), and 100 µl of fungal suspension. The fungal suspension was prepared on a PD agar plate after fungal incubation for 7 days. After adding 5 ml of sterile water on agar, the concentration of spore density was calculated, using the Bürker chamber method. The inlet wastewater was provided by R&D facility Hammarby Sjöstadsverk (Stockholm, Sweden). The inlet wastewater was taken directly from the entry tank with the following composition: chemical oxygen demand (COD) 500–700 mg l^{-1}, N_{tot} 40–50 mg l^{-1}, P_{tot} 4,0–5,0 mg l^{-1}, pH 7.8. After collection of wastewater, the pH was corrected to 5.5. Likewise, two additional negative controls - a control without chemical compounds and a control without fungi - were prepared to compare the experiment observations so they could be completely linked to the bioremoval induced by fungi. Finally, flasks were closed with cotton stoppers and incubated in a shaker incubator (150 rpm) at 25 °C for a time period of 14 days. All the experiments were carried out in duplicate or triplicate.

2.7. Effect of fungal biofilm in removing diclofenac

To achieve a higher initial concentration of selected fungal strains, the Kaldnes K1 carriers (AnoxKaldnes, France; diameter 9.1 mm) were used for biofilm formation (one carrier unit per 1 ml) [25]. After growing biomass on carriers with PD broth media (Oxoid, United Kingdom) for 5 days, the fungal biomass was separated, added to a non-sterile municipal wastewater sample, and experiments were performed as mentioned in the 2.6 paragraph. For all the experiments, the cultures were withdrawn on days 3, 7, 10 and 14 of incubation. Further, the additional investigation of diclofenac removal was done for 72 h, and samples were taken at every 3 for up to - 72 h. All samples were filtered and collected the culture filtrate for further analysis of the pharmaceutical's concentration, the laccase enzyme activity, and the pH level. After 3 days of incubation, all samples were also mounted on clean glass slides and observed under the light microscope (40x; Leica, Germany). All the experiments were carried out in duplicate or triplicate.

2.8. Biosorption experiment

The fungal biomass was cultivated in potato dextrose broth (PDB, Oxoid, United Kingdom) and incubated in a shaking incubator (50 rpm) at 25 °C for a period of 5 days. After incubation, a half of the flasks were double autoclaved to establish a heat killed control (dead fungal cells). Diclofenac was then added to the flasks of live and dead fungal cells to a concentration of 2.5 mg l^{-1}. Samples of diclofenac and enzyme activity of laccase analysis were taken every 3 h for up to - 24 h. The experiment was carried out in duplicate.

2.9. Analytical procedures

2.9.1. pH measurements

The pH measurements to monitor pH changes were performed during the batch experiments using universal pH-indicator strips (pH 0–14; Merck KGaA, Germany).

2.9.2. Enzyme activity

The laccase activity was measured spectrophotometrically, using the standardized procedure of the enzymatic assay for laccase by Sigma-Aldrich (Germany). Briefly, each enzyme assay was measured in a 4 ml cuvette with a total reaction mixture volume of 3 ml. Deionized water was used to attain the volume of the reaction mixture where necessary. The test reaction mixture for laccase consisted of a 2.2 ml of 100 mM potassium phosphate buffer (KH_2PO_2, pH 6.0), 0.5 ml of laccase from *T. versicolor* (crude powder, ≥ 50 units mg^{-1} solids; Sigma-Aldrich, Germany) and 0.3 ml of 0.216 mM syringaldazine solution ($C_{18}H_{20}N_2O_6$; Sigma-Aldrich, Germany). After mixing, the results were recorded as an increase in 530 nm for approximately 10 min. The detection limit of laccase activity was 0.01 U ml $enzyme^{-1}$. All results were calculated and analyzed using MS Excel 2013. Each sample was measured in triplicates.

2.9.3. HPLC analysis

Four different HPLC analysis controls were made for each matrix in order to avoid of a false result that could be detected in a case of an instrumental error. As a negative control, a matrix without any chemical compounds was used; as a positive control a matrix combined with diclofenac and ketoprofen was used. Also, a positive control with a matrix and a mixture of two compounds was prepared. The concentration of diclofenac and ketoprofen was measured in all culture filtrates and control samples by the HPLC system of Alliance 2695 Separations Module (Waters, USA) using the 2996 Photodiode Array detector (Water, USA). Chromatographic separation was achieved using the Nova-Pak C_{18} column (4 µm, 3.9 × 300; Water, USA) with flow of 0.5 ml min^{-1}. The mobile phase was 70 % v/v methanol and a 30 % v/v 20 mM phosphate buffer (pH 2.5) (Sigma-Aldrich, Germany). All results were analyzed using Empower 3 Chromatography Data Software (Water, USA). For a *t*-test (two-tailed distribution; significance level ≤ 0.05) and a data analysis MS Excel 2013 was used.

3. Results and discussion

3.1. Growth effect of selected fungal strains

During the initial study, the growth and interaction of selected fungal strains were tested by a classical cultivation method with and without pharmaceutical substances. The combinations of fungi were selected based on the previous study [26] where these fungi demonstrated the most appropriate ability to grow in a sterile wastewater agar media. In this study, diclofenac and ketoprofen were selected as model compounds due to high levels of consumption in various European countries and their appearance in the wastewater treatment plant effluents [27]. Furthermore, these compounds contain different functional groups that might provide a new insight into the removal efficiency by fungi.

The results indicated that all fungal strains could grow in the presence of pharmaceuticals similar to control strains (without pharmaceutical substances) (Supplementary Fig. S1). When the cultures grown together, the formation of the inhibition zone among the tested strains was observed (Fig. 1). For instance, results of the interaction test demonstrated an inhibition zone formation among of *T. reesei*, *I. lacteus* and *T. versicolor* (Fig. 1A). The same was observed in the case of *F. solani* and *P. ostreatus* strains (Fig. 1B). Hence it would be an interest of the research to find out whether these fungal strains could remove pharmaceutical substances by applying mixed cultures or culturing them individually in liquid media of synthetic wastewater.

The fungal strains of *T. reesei*, *T. versicolor*, *I. lacteus*, *F. solani*, and *P. ostreatus* were investigated in the individual experiment to test their capability to remove ketoprofen and diclofenac in a synthetic wastewater medium. Although previous studies have shown that the maximum concentrations of diclofenac and ketoprofen in municipal wastewater vary from 0.44 to7.1 µg l^{-1} for diclofenac, and 0.004 to 8.56 µg l^{-1} for ketoprofen [10,28], relatively higher concentrations, e.g., mg l^{-1}, of these substances can be found in the industrial wastewater and can be acknowledged as a potential ecological risk [29]. Therefore, the concentration of 5 mg l^{-1} was selected and investigated for both substances.

The results showed that only *T. versicolor* was able to reduce more than 80 % of ketoprofen after 21 days of incubation, while other fungal

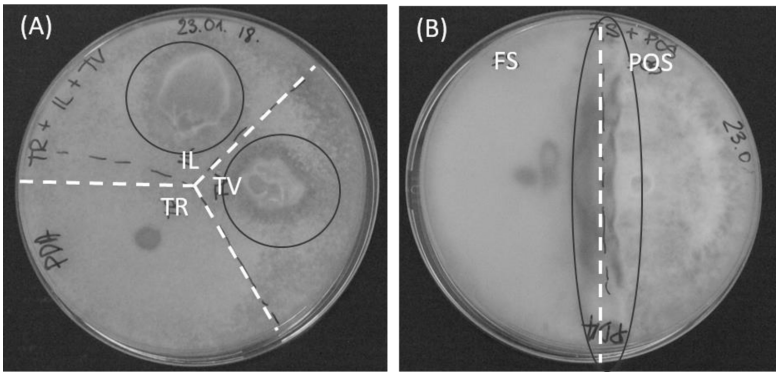


Fig. 1. The result derived from the interaction test by a classical cultivation method of (A) *T. reesei*, *I. lacteus* and *T. versicol* after 14 days of incubation; (B) *F. solani* and *P. ostreatus* after 14 days of incubation.

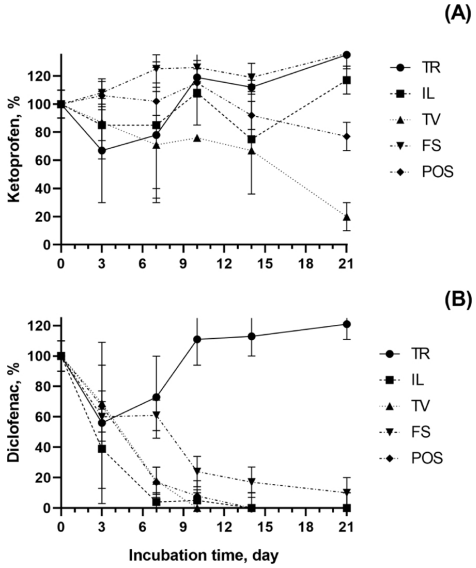


Fig. 2. Removal efficiency (%) of ketoprofen (A) and diclofenac (B) obtained from individual experiments of *T. reesei* (TR), *I. lacteus* (IL), *T. versicolor* (TV), *F. solani* (FS) and *P. ostreatus* (POS).

strains did not indicate any reduction of ketoprofen after 7 days of incubation (Fig. 2A). Furthermore, results from the individual experiment of *T. reseei*, *I. lacteus*, *F. solani* and *P. ostreatus* showed an increase of ketoprofen concentration (Fig. 2A). For instance, the HPLC peak profile of the *T. reseei* has shown a double peak formation close to the control peak after 21 days of incubation, which presumably has caused misleading calculated results of the analyzed substance's removal (Supplementary Fig. 2A). These results may indicate the formation of by-products from the degraded chemical compounds as described earlier [14,30]. Therefore, the analytical method to analyze by-products with the HPLC system needs to be improved in additional studies.

In contrast to ketoprofen, fungal strains of *T. versicolor*, *I. lacteus*, and *P. ostreatus* showed a higher removal efficiency of diclofenac. For instance, the results derived from the individual experiment of *T. versicolor*, *I. lacteus* and *P. ostreatus* have shown the ability to remove more than 80 % of diclofenac, while, *F. solani* and *T. reseei* were able to remove only 40 % of diclofenac within 7 days of incubation (Fig. 2B). However, a longer incubation period of *F. solani* indicated a maximum reduction of 90 % of diclofenac on day 21 while *T. reseei* demonstrated significantly the same concentration as of the beginning of this experiment. As the HPLC peak profile of *T. reseei* did not demonstrate any extra peak formation (Supplementary Fig. 2B), it has been observed that this strain started to release this compound back to synthetic wastewater after 3 days of incubation. It might be explain by the sorption as the strategy for pharmaceutical substance removal [31]. Overall, the obtained results of the individual cultures showed that selected strains, especially *T. versicolor, I. lacteus*, *P. ostreatus* and *F. solani*, are promising candidates for ketoprofen and diclofenac removal. Therefore, the synergetic effect of fungi was investigated in the further study.

3.2. Synergistic effect of selected fungal strains

The synergetic effect of mixed cultures from *T. reesei* in combination with *I. lacteus* and *T. versicolor* was investigated. When *T. reesei* in the mix with *I. lacteus* and *T. versicolor* was cultivated for 3 days, the reduction of ketoprofen and diclofenac was observed (Fig. 3). For example, *T. reesei* in the mix with *I. lacteus* demonstrated up to 60 % reduction of both pharmaceutical substances after 3 days of incubation. However, results of the HPLC analysis for a longer incubation period of *T. reesei* in the mix with *I. lacteus* and *T. versicolor* did not show any chemical compound reduction. Furthermore, results of the HPLC peak profile from a mixed experiment of *T. reesei* combined with *I. lacteus* and *T. versicolor* after 7 days of incubation, demonstrated an extra HPLC peak formation causing an increase in the concentration of ketoprofen (Supplementary Fig. S3). It might be explained by fungal cell-surface sorption as hazardous substances occur due to physicochemical interaction between the substance and the functional groups which are present on the fungal cell surface. This type of biosorption is relatively rapid and can be reversible [32].

The Fig. 3 presents the ketoprofen and diclofenac removal results, using *I. lacteus* in the combination with *T. versicolor*. *I. lacteus* in a combination with *T. versicolor* were able to reduce ketoprofen for more than 50 % after 3 days of incubation until the end of the incubation period of 14 days (Fig. 3A). Furthermore, after 14 days of incubation based on the individual combination of *I. lacteus,* the results have shown a 25 % removal rate of ketoprofen while *T. versicolor* could remove up to 40 % of the substance. Thus, the removal efficiency in a mixed culture with *T. versicolor* and *I. lacteus* was relatively higher compared to individual cultures (Figs. 2A and 3 A).

On the contrary, the combination of *I. lacteus* and *T. versicolor* was

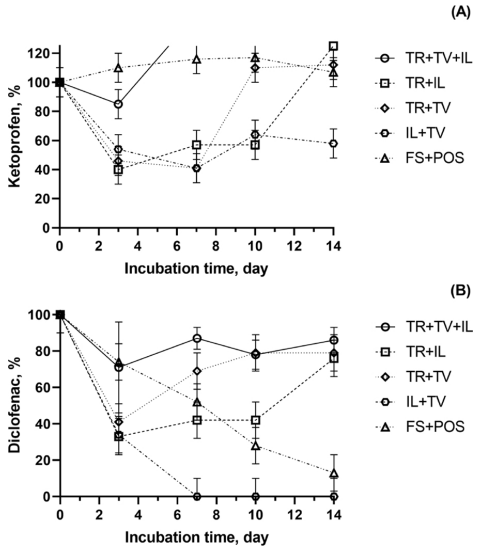


Fig. 3. Removal efficiency (%) of ketoprofen (A) and diclofenac (B) as obtained from mixed experiments of *T. reesei* (TR) with *I. lacteus* (IL), and *T. versicolor* (TV); *F. solani* (FS) with *P. ostreatus* (POS) simultaneously.

able to completely (> 99.9 %) remove diclofenac concentration after 7 days of incubation (Fig. 3B). Results showed that there was no statistically significant difference ($P > 0.05$) between removal efficiency if the fungi were grown separately or when compared to the mixed cultures (Figs. 2B and 3 B). For instance, after 7 days of individual incubation of *I. lacteus* and *T. versicolor,* they demonstrated more than 90 % removal of diclofenac. Furthermore, in all three combinations, the analysis of the HPLC did not display any extra peak formation comparing to the control HPLC peak of diclofenac. Therefore, results from mixed cultures had no extra effect in reducing diclofenac in the sample. Furthermore, the results of the mixed experiment of *T. reesei* in a combination with *I. lacteus* and *T. versicolor*, demonstrated a negative synergetic effect in removing diclofenac and ketoprofen using *T. reesei* on *I. lacteus* and *T. versicolor*. For instance, the combination of *I. lacteus* and *T. versicolor* completely removed (> 99.9 %) diclofenac while *T. reesei* in the combination with *T. versicolor* and/or *I. lacteus* was not able to remove more than 20 % of diclofenac after 14 days of incubation (Fig. 3B).

The synergistic relationship effect of removing ketoprofen and diclofenac between *F. solani* and *P. ostreatus* was also investigated (see Fig. 3A and B). These strains were mixed together based on the previous study [26] where both tested cultures separately demonstrated a slower growth rate in a sterile wastewater agar media comparing to *T. reesei*, *I. lacteus*, and *T. versicolor*.

The results of ketoprofen reduction results, using mixed cultures of *F. solani* and *P. ostreatus,* demonstrated a similar tendency as previously derived results, i.e., there was a difficulty with the ketoprofen analysis, utilizing the HPLC technique. For instance, all samples demonstrated an increase of ketoprofen while the reduction was observed only for an individual bioassay of *P. ostreatus* after 21 days of incubation (see Figs. 2A and 3 A). On the contrary, the combination of *F. solani* and *P. ostreatus* showed > 90 % removal of diclofenac after 14 days of incubation (Fig. 3B). Furthermore, the results from individual *P. ostreatus* culture demonstrated a faster removal efficiency of diclofenac; this compound was > 99.9 % removed after 14 days of incubation comparing with the mixed cultures where 80 % of diclofenac was removed after 14 days of incubation (Figs. 2B and 3 B).

Overall, the results of the synergistic study demonstrated the ability to remove pharmaceutical substances with fungal strains. In most cases the results showed that fungi can remove pharmaceutical substances faster if they grow individually. The possible reason to reduce pharmaceutical substances in mixed cultures may be due to the competition and growth inhibition between the fungal strains as observed in this study (Fig. 1). Fig. 1 shows that the growth of mixed fungal cultures can visibly observed on an agar plate, emphasizing the inhibition of growth. Though *T. reesei* out-compete the growth against *I. lacteus* and *T. vesicolor*, the reduction of pharmaceutical substances was lower compared to the *I. lacteus* and *T. versicolor* mixed culture. There was an inhibition zone observed around *I. lacteus* and *T. vesicolor*. Mixed cultures of *F. solani* and *P. ostreatus* showed a difference at the meeting zone of these two cultures; this might cause an effect in reducing pharmaceutical substances. Therefore, further experiments were focused on individual species rather than mixed.

The removal of pharmaceutical substances by fungal strains varies widely from one compound to another. Yang et al. [6] have stated that physicochemical properties of the target molecules appear to be a key reason for such variation. For instance, some hazardous substances are easily biosorbed due to their high hydrophobicity; some have molecular features that render them readily biodegradable by fungi, while others are resistant to the fungi enzyme system due to certain features of their molecular structure [6]. For instance, ketoprofen is a nonsteroidal anti-inflammatory drug which belongs to the substituted 2-phenyl|propionic acids group [24]. Its structural formula demonstrates functional groups of methyl, keton and carboxylic acid group (Table 1). The presence of electron withdrawing functional groups such as amide (−CONR2), halogen (–X), nitro (–NO2) and carboxylic (−COOH) group generates an electron deficiency and thus renders the compounds less susceptible to oxidative catabolism [6]. Therefore, the appearance of the carboxylic acid group in the ketoprofen structure might explain the difficulties to remove this compound for investigated fungal strains. For instance, previous studies have shown that ketoprofen is not completely removed in most cases of wastewater treatment [33]. Additionally, Palli et al. [16] have demonstrated that heat-killed biomass of *T. versicolor* can remove only 15% of ketoprofen. Moreover, Marco-Urrea et al. [15] proved that the enzyme system of *T. versicolor* is not involved in ketoprofen degradation. Therefore, the removal of ketoprofen required further investigation.

In contrast to ketoprofen, which has functional groups of methyl, keton and carboxylic acid groups, diclofenac is an anti-inflammatory drug with one functional group - hydroxyl. The hydroxyl group has been reported as the first electron-donating group for diclofenac [6]. Therefore, diclofenac can be efficiently degraded by a fungal treatment. Furthermore, several studies have presented similar results of diclofenac removal by *T. versicol* and *I. lacteus* [1,6]. For instance, Lucas et al. [1] have demonstrated high removal efficiency of diclofenac by *T. versicolor* and *I. lacteus* in batch experiments indicating the contribution of the sorption processes. However, most of these studies have been conducted on an autoclaved soil or on a synthetic media [1,34].

In this study, the laccase enzyme activity was measured to better understand the relationship between the enzyme activity and the reduction of diclofenac. The activity of the laccase enzyme was observed only when fungi were incubated separately (Supplementary Table S1). Therefore, the results of the interaction tests and laccase activity confirm the assumption that the fungal strains are able to remove diclofenac with a higher degree of efficiency if they are incubated separately and are not mixed. This might be explained by fungal interaction with each other on enzyme production. However, further investigation has to be carried out.

The obtained results are similar and comparable with the results from the previous study conducted by Vasiliadou et al. [18]; the study examined the efficiency of two white-rot fungi, *T. versicolor,* and *G. lucidum*. Vasiliadou et al. [18] demonstrated the simultaneous use of both strains, indicating a significant decrease in removal efficiencies of

tested hazardous substances, e. g., carbamazepine, and sulpiride. Therefore, the individual growth strategy to remove pharmaceutical compounds does not only exclude competition and inhibition among the strains but also increases the efficiency of pharmaceutical substances removal mechanisms, e.g., extracellular enzyme production for compound degradation. Furthermore, the effect of the pH level might also play an important role in terms of the fungal metabolisms activity to produce enzymes and affect the morphology of the fungal cell structure [35]. Therefore, the pH was monitored during the incubation time. However, measuring the pH level in all samples, no changes were observed during the incubation time (data not shown).

The results from this study have demonstrated the potential to remove diclofenac efficiently and successfully under sterile conditions by selected fungi. However, results could not be as significant as they would be if fungi would be grown under real wastewater conditions with the variable microbial community in municipal wastewater [36]. For this reason, in the further work the capability to remove diclofenac in non-sterile municipal wastewater by the selected fungal strains - *T. versicolor*, *I. lacteus*, *F. solani*, *and P. ostreatus* - was evaluated.

3.3. Removal of pharmaceuticals by fungi in municipal wastewater

Based on the previous results derived from the synergetic effect investigation conducted among the fungal strains, the removal of the diclofenac was initially examined by using each fungal strain separately (individual cultures). Furthermore, all experiments were investigated under non-sterile conditions using inlet wastewater (from a municipal wastewater treatment plant) as a fungal culture medium.

The overall removal rate of diclofenac by selected fungi grown in municipal wastewater for 14 incubation days is shown in the Fig. 4A. After 10 days of incubation, the results demonstrated less than 30 % removal of diclofenac for all tested fungal strains. As compared to the previous results from the synthetic wastewater (e.g., Fig. 2B, the total removal of diclofenac by *T. versicolor* was achieved after 10 days of incubation), the removal efficiency has been decreased more than three times (i.e., from > 99.9 % to < 30 %, respectively).

This can be regarded a certain interaction of the tested fungal strain with other microbial community from the non-sterile wastewater sample. In systems of mixed populations, such as bacteria and protozoa, the interaction that often occurs is a competition for specific compounds [18]. Due to the competition among microorganisms, it is possible to decrease the biological removal of substances, e. g., pharmaceutical compounds [37]. Moreover, the preliminary study on the growth of fungal strains in sterile inlet wastewater showed very limited/poor growth (data not shown). This could be due to a low concentration and composition of specific compounds in inlet wastewater. Furthermore, in the present study, the initial concentration of a fungal inoculum was relatively low. Therefore, the fungi might have been outcompeted by *Bacteria*, *Archaea* and other fungal species from wastewater [38]. Thus, the initial fungal concentration of biomass was increased in the further experiment.

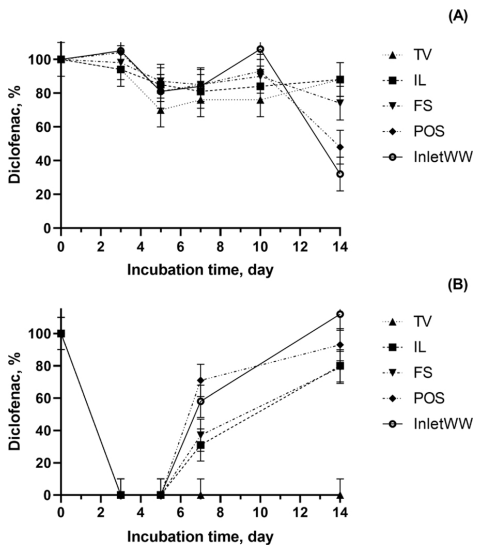


Fig. 4. (A) Removal efficiency (%) of diclofenac from non-sterile municipal wastewater with selected fungal strains; (B) removal efficiency (%) of diclofenac from non-sterile municipal wastewater with fungal biofilm on K1 carriers.

3.4. Effect of diclofenac removal by fungal biofilm on K1 carriers

In order to achieve a higher initial concentration of selected fungal strains, the K1 carriers for biofilm formation were used. As shown in Fig. 4B, all fungal cultures were able to completely remove (> 99.9 %) diclofenac after 3 days of incubation. This observation is in line with recent studies [20,39] that have also suggested to use the biofilm carriers for fungal mycelium immobilization in order to increase the laccase production and biomass growth. For instance, Spina et al. [39] showed that *B. adusta* produced a higher enzyme activity if fungal biomass was immobilized on the carrier. However, a longer incubation time demonstrated a release of the diclofenac from all fungal strains back to wastewater, except for *T. versicolor*. The obtained results might be explained by the use of different removal mechanisms of tested fungi. For instance, Vasiliadou et al. [18] have shown that fungi might use biosorption and/or secretion of enzymes to remove pharmaceutical substances. Furthermore, Lucas et al. [1] have demonstrated that sorption of the active fungal biomass might not be the same as the sorption of inactive (dead) biomass due to the structural changes of fungal biomass. In this study, the laccase enzyme activity measurements showed that *T. versicolor* has a two times higher laccase activity on day 3, compared to other fungal strains when grown in an inlet of non-sterile municipal wastewater (Supplementary Table S2). Thus, it might be possible that *T. versicolor* could use an enzyme process to degrade diclofenac since it has been removed completely. Therefore, an additional investigation of diclofenac removal by *T. versicolor* was done for 3 days of incubation time.

The result of diclofenac removal by *T. versicolor* has been presented in Fig. 5A. The result showed that diclofenac has been completely (> 99.9 %) removed after 3 h of incubation, while inlet wastewater without fungal inoculum of *T. versicolor* could remove 99.9 % of diclofenac after 60 h of incubation. Furthermore, the measurement of the laccase activity showed a constant and immediate increase after incubation of *T. versicolor* which lasted up to 15 -hs; no enzyme activity was observed from inlet wastewater (Fig. 5B). The difference in longer removal times compared to *T. versicolor* might be explained due some additional time requirements for microorganim's adoption, for instance, formatting granules and decreasing the pH level as a strategy for diclofenac bioabsorption. However, more investigations should be conducted to better understand wastewater biodiversity of microorganisms and the potential of pharmaceutical biosorption with biomass. Additionally, the results of *T. versicolor* also indicated more than a 80 % removal rate immediately after adding diclofenac. Therefore, the role of biosorption and production of the laccase enzyme was investigated in the further study in order to better understand the removal mechanisms by *T. versicolor*.

3.5. Removal efficiency and its mechanisms of T. versicolor

The results of the biosorption experiment showed that live cells of *T. versicolor* could remove > 99.9 % of diclofenac once incubation

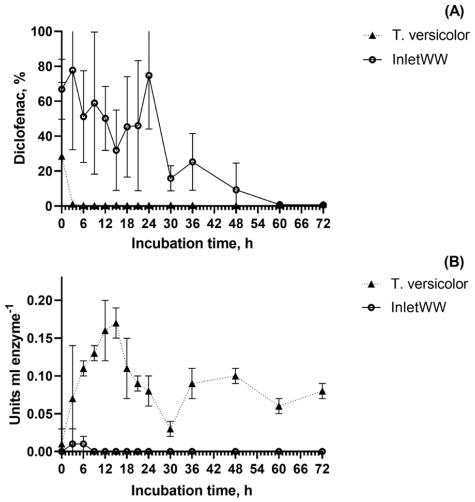


Fig. 5. (A) Removal efficiency (%) derived from the additional research of diclofenac removal within 3 days with *T. versicolor* in non-sterile municipal wastewater with fungal biofilm on K1 carriers; (B) Laccase enzyme activity (U ml enzyme^{-1}) derived from the additional research of diclofenac removal within 3 days by *T. versicolor* in municipal wastewater with fungal biofilm on K1 carriers.

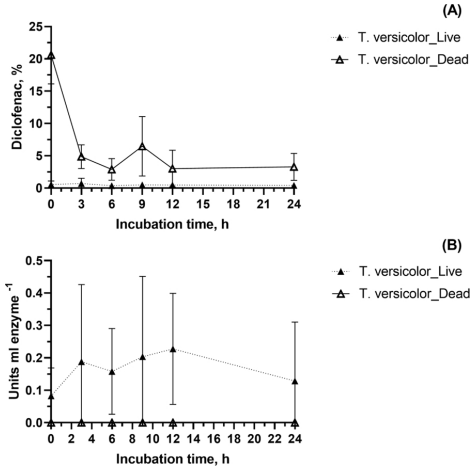


Fig. 6. (A) Removal efficiency (%) of diclofenac derived from the biosorption experiment with *T. versicolor;* (B) Laccase enzyme activity (U ml enzyme^{-1}) derived from the biosorption experiment with *T. versicolor*.

commences. At the same time, the results indicated that dead fungal cells of *T. versicolor* could not completely remove diclofenac, respectively not more than 95 % within a 24 -hs long incubation period (Fig. 6A). Furthermore, the measurement of laccase showed a constant concentration of the enzyme activity throughout the entire incubation time for live *T. versicolor* cells while no enzyme activity was observed in the case of dead *T. versicolor* cells (Fig. 6B). Therefore, results of the biosorption experiment indicated that *T. versicolor* uses both mechanisms - enzyme activity and biosorption - to remove diclofenac. Moreover, the observation of this biosorption experiment showed that laccase was responsible for removing ~ 20 % of diclofenac while the *T. versicolor* biomass could remove ~ 80 % of diclofenac (Fig. 6A). Previous studies of batch experiments have reported similar results for *T. versicolor*. For instance, Stenholm et al. [40] have indicated a 99.9 % diclofenac removal rate after a 4 h incubation period under non-sterile conditions. However, enzymatic reactions contributed to an approximate drop of < 0.5 %.

4. Conclusions

This study has showed the potential of five fungal strains to remove ketoprofen and diclofenac individually and in mixed cultures. Experiments with synthetic wastewater media showed that fungi compete with each other since higher removal efficiency was observed if the fungi was grown individually. The capability of selected fungal strains to remove diclofenac in a non-sterile municipal wastewater sample using carriers showed a promising approach for the wastewater treatment process. The results show that *T. versicolor* could fully (> 99.9 %) remove diclofenac after a 3 -hs long incubation period in non-sterile municipal wastewater. Moreover, *T. versicolor* demonstrated an ability to use both mechanisms - enzyme activity and biosorption - to remove diclofenac. Therefore, *T. versicolor* could be a promising candidate to remove pharmaceutical substances by wastewater treatment process.

Declaration of Competing Interest

We have no conflicts of interest to disclose.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jwpe.2019.100992.

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PAPER II

Isolation of fungal strains from municipal wastewater for the removal of pharmaceutical substances

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Isolation of Fungal Strains from Municipal Wastewater for the Removal of Pharmaceutical Substances

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Abstract: Fungi have been shown to be promising candidates to be used in removal of pharmaceutical compounds during wastewater treatment processes. However, fungal growth, including removal efficiency, can be affected by several factors, such as temperature and the pH. The ability of fungal isolates to grow in the presence of carbamazepine, diclofenac, ibuprofen, and sulfamethoxazole was tested. Removal efficiency results indicated that a fungal isolate of *Aspergillus luchuensis* can completely (>99.9%) remove diclofenac from a synthetic wastewater media without a pH correction within 10 days of incubation. Furthermore, the results of the biosorption test for *A. luchuensis* indicate that this isolate uses the biosorption mechanism as a strategy to remove diclofenac. Finally, the results demonstrate that *A. luchuensis* can remove >98% of diclofenac in non-sterile wastewater without a pH correction immediately after biomass inoculation on biofilm carriers while *Trametes versicolor* requires an incubation period of at least 24 h to completely remove diclofenac. Therefore, this isolate is a promising candidate for use in removal of pharmaceutical compounds from wastewater with typical pH 7.8, minimizing a requirement of the pH correction.

Keywords: wastewater; fungi; *Trametes versicolor*; *Aspergillus luchuensis*; diclofenac; carbamazepine

1. Introduction

Conventional wastewater treatment technologies cannot always remove pharmaceutical substances efficiently [1]. In such cases, the pharmaceuticals are released into the environment where they negatively affect living organisms. [2,3]. Alternative technologies used in methods to remove pharmaceutical substances from wastewater such as advanced oxidation, UV disinfection, ozonation, and granular activated carbon progressing still cause concerns around by-product formation and the cost of energy and chemical consumption [4–6]. Therefore, research into biological treatments to remove pharmaceutical substances using fungi might be an attractive topic, with an aim to develop effective and environmentally friendly wastewater treatment technology.

White-rot fungi have shown to be good candidates to remove pharmaceutical substances from wastewater [7]. They are able to degrade a wide variety of pharmaceuticals due to their enzymatic processes. Furthermore, fungi can use a biosorption strategy to counteract the effects of pharmaceuticals because of their specific cell wall composition, formed from chitosan and chitin [8,9]. In addition, *Trametes versicolor* can metabolize and integrate some pharmaceuticals, such as benzophenone-3 and diclofenac, into amino acids of fungi [10,11]. However, the growth of specific fungal strains can be affected by several factors such as the pH, temperature, concentration of inhibitory substances,

and nutrients [12]. Therefore, fungi isolated from municipal wastewater treatment plants are more likely to be adapted to the environmental and operating conditions [13]. Thus, determination of the removal potential of residential wastewater fungi is an important task in developing industrial process applications in order to accomplish the long-term goal of pharmaceutical removal. Furthermore, when using low-cost materials such as fungi, advantages includes a low capital investment, relatively simple operations, low operating costs, and the lack of degradation by-products [14,15].

The main focus of this research was to investigate the isolation of fungal strains from municipal wastewater, and to test their ability to remove pharmaceutical substances. To achieve this, fungal isolates were isolated and cultivated on a synthetic wastewater media in the presence of selected pharmaceuticals. During this study, the effect of the pH on removal efficiency was studied. The most promising isolate was further identified and analyzed in non-sterile municipal wastewater. Finally, a biosorption experiment was conducted with the isolate, and enzyme activity was measured to better understand the removal mechanisms of pharmaceutical substances. All results of fungal isolate were compared to *T. versicolor* to evaluate the potential of an isolated fungal strain and the advantages of its application in wastewater treatment to remove pharmaceutical substances.

2. Materials and Methods

2.1. Fungi, Pharmaceuticals Substances and Wastewater

The white-rot fungus of *Trametes versicolor* DSM 6401 was obtained from the culture collection (Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures, Brunswick, Germany) and used as a control strain. The standard solutions at the concentration of 5 mg/L of carbamazepine (CAR), diclofenac (DIC), ibuprofen (IBU), and sulfamethoxazole (SUL) (Sigma-Aldrich, Taufkirchen, Germany) were prepared separately, according to the manufacturer's solubility guidelines. The inlet of municipal wastewater was provided by the Henriksdal wastewater treatment plant (Stockholm, Sweden). The inlet wastewater (InletWW) was taken directly from an entry tank with the following composition: COD 500–700 mg/L, N_{tot} 40–50 mg/L, P_{tot} 4.0–5.0 mg/L.

2.2. Isolation and Selection of Fungi from Municipal Wastewater

Potato dextrose (PD) agar (Oxoid, Cheshire, UK), in the presence of CAR, DIC, IBU, and SUL, was used for fungal isolation from the InletWW. The pharmaceutical compound with the final concentration 5 mg/L of selected chemical was added immediately after the synthetic wastewater media was poured into dishes. Briefly, the wastewater sample of 100 µL was spread on the plate and incubated for 7 days at 25 °C. After incubation, the isolates obtained were re-streaked on the new agar plate to isolate ten pure cultures for further experiments, and the colony size (%) of isolated fungi was determined in triplicate. Shortly, the fungal growth among strains was estimated qualitatively by taking images of the mycelia on the agar plate with a digital photo camera (Kodak, Rochester, NY, USA) and manually by measuring the colony size with a ruler (mm). The diameter of agar plate was accepted to be 100 % (90 mm). Thus, the colony size (%) was calculated using the formula: colony size (%) = (colony size (mm) × 100)/90 (mm).

For a t-test (two-tailed distribution; significance level ≤ 0.05) and data analysis, MS Excel 2013 was used.

2.3. Removal of Pharmaceutical Substances in Synthetic and Municipal Wastewater Media

The removal efficiency of DIC and CAR by fungal isolates was evaluated under sterile conditions, adjusting the pH of 5.5 and 6.3 (1 M HCl acid). Municipal wastewater (InletWW) was used as a negative control.

Firstly, a 250 mL glass flask was filled with 50 mL of a sterile synthetic wastewater medium (0.2 g K_2HPO_4, 0.8 g KH_2PO_4, 0.5 g $MgSO_4$, 0.2 g yeast extract in 1 L distilled water), pharmaceutical substance (the final concentration 5 mg/L of a selected chemical), and fungal inoculum (re-streaking on

a new agar plate, r = 20 mm). Finally, flasks were incubated in a shaking incubator (150 rpm) for a period of 10 days at 25 °C. The pharmaceuticals concentrations were periodically determined after incubation periods of 0, 3, 5, 8, and 10 days.

The removal efficiency of pharmaceutical substances from non-sterile municipal wastewater was conducted, using the most promising fungal isolate. Briefly, all experiments were performed under non-sterile conditions, as mentioned earlier. To achieve a higher initial concentration of fungal biomass, the K1 carrier units for a biofilm formation were used (one carrier unit per 1 mL) [16]. After growing biomass on carriers with a PD broth medium (Oxoid, United Kingdom) for 5 days, the fungal biomass was separated and added to non-sterile municipal wastewater. Finally, an additional investigation of the pharmaceutical removal efficiency under non-sterile conditions was performed for 48 or 72 h, and samples were taken at 0, 3, 6, 9, 12, 15, 18, 21, 24, 36, 48, and 72 h. All samples were filtered for further high-performance liquid chromatography (HPLC) and laccase activity analysis. *T. versicolor* was used as a positive control. All experiments were carried out in duplicate or triplicate.

2.4. Biosorption Test

Fungal biomass of the isolate was cultivated in the PD media (Oxoid, United Kingdom), incubating in a shaking incubator (50 rpm) for 5 days at 25 °C. Subsequently, half of the flasks were double autoclaved for 15 min at 121 °C to establish a heat-killed control (dead fungal cells). After autoclaving, the pharmaceutical substance was added to the live and dead fungal biomass of a concentration at 2.5 mg/L. Two additional negative controls—a control without chemical compounds and a control without fungi—were prepared to compare the experimental observations in order to be completely linked to the bioremoval induced by fungi. Finally, the samples for the HPLC and laccase activity analysis were taken at 0, 3, 6, 9, 12 and 24 h. The experiment was carried out in duplicate [17].

2.5. Analytical Procedure of Enzyme Activity and HPLC

The activity of laccase was measured spectrophotometrically, using the standardized procedure of the enzymatic assay for laccase by Sigma-Aldrich (Germany). Shortly, the test reaction consisted of 2.2 mL of a 100 mM potassium phosphate buffer (KH_2PO_2, pH 6.0), 0.5 mL of laccase from *T. versicolor* (crude powder, ≥50 units/mg solids, Sigma-Aldrich) and 0.3 mL of a 0.216 mM syringaldazine solution ($C_{18}H_{20}N_2O_6$, Sigma-Aldrich). After the reaction, absorbance changes were measured for 10 min at 530 nm. The measurements were carried out in triplicate.

The concentration of pharmaceuticals was measured by the HPLC system of an Alliance 2695 Separation Module (Waters, Milford, MA, USA) using 2996 Photodiode Array detector (Waters, Milford, MA, USA). Chromatographic separation was achieved with the Nova-Pak C_{18} column (4 µm, 3.9 × 300; Water, USA) using a flow of 0.5 mL/min. The mobile phase was 70% *v/v* methanol and a 30% *v/v* 20 mM phosphate buffer (pH 2.5) (Sigma-Aldrich, Darmstadt, Germany). All results were analyzed, using Empower 3 Chromatography Data Software (Water, USA).

2.6. Identification of Fungal Isolates

Pure culture of an isolated fungal strain was sent for molecular-based identification to the Fungal Biodiversity center of the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands).

3. Results and Discussion

3.1. Isolation of Fungal Strains

From the inlet wastewater sample, ten fungal colonies were randomly selected and isolated to pure culture (Figure 1a). Isolated fungi were grown on PD agar in the presence of all selected pharmaceutical compounds, such as carbamazepine (CAR), diclofenac (DIC), ibuprofen (IBU), and sulfamethoxazole (SUL). Based on growth efficiency, three fungal isolates, F8, F9, and F10, were excluded from further analysis, due to the slow growth during the incubation period of 7 days (data not shown). Meanwhile,

the fungal strain F4 showed the highest growth on PD agar with/without pharmaceutical substances after 7 days of incubation (Figure 1a,b). Moreover, there was no statistically significant difference among fungal isolates F1, F3, F5, F6, and F7 ($p > 0.05$) when colony sizes of the fungi growth efficiency, on PD agar with or without pharmaceuticals, were compared. However, fungal isolates F2 and F4 demonstrated a relatively higher growth efficiency when the fungi were grown on PD agar without pharmaceuticals after 7 days of incubation.

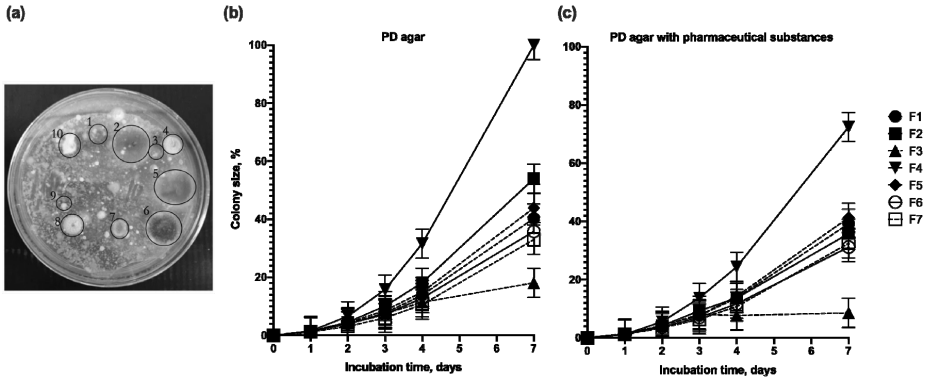

Figure 1. (**a**) Fungal isolates from municipal wastewater on a potato dextrose (PD) agar plate in the presence of carbamazepine (CAR), diclofenac (DIC), ibuprofen (IBU), and sulfamethoxazole (SUL); (**b**) Colony size (%) of isolated fungi on PD agar without pharmaceutical substances during the incubation period of 7 days; (**c**) The colony size (%) of isolated fungi on PD agar with pharmaceutical substances during the incubation period of 7 days.

In this study, the compounds selected cover a wide range of chemical structures. For instance, DIC has an active group of chlorine, while CAR has a group of amine. Meantime, SUL has a combination of active groups in their molecular structure. This variety of chemical structures might inhibit fungal growth in the presence of chemical compounds [8]. Therefore, this might explain why different colony sizes were observed in this study for each of the ten isolates.

Overall, a total of seven fungal isolates was used for further study to investigate their ability to remove pharmaceutical substances.

3.2. Removal of Pharmaceuticals by Fungal Isolates in Synthetic Wastewater Media

In this study, DIC and CAR were selected as model compounds, due to high consumption levels in various European countries and their appearance in wastewater treatment plant effluents [18]. The results in Figure 2 indicate the efficiency of removal by *T. versicolor* for DIC and CAR, comparing to fungal isolates F1, F2, F3, F4, F5, F6, and F7 in a synthetic wastewater media at a pH of 5.5. Results for fungal strains F4, F6, and F7 showed a relatively high removal efficiency (>80%) for CAR while *T. versicolor* removed <20% of this substance after 3 days of the incubation (Figure 2a). At the same time, fungal isolates F3 and F4 were able to remove >80% of DIC after 3 days of incubation while *T. versicolor* demonstrated complete removal (>99.9%) of DIC after 5 days of incubation (Figure 2b). Finally, fungal isolates F1, F2, and F5 showed a relatively low removal efficiency (<20%) of both pharmaceuticals throughout the incubation time. Therefore, the fungal isolates F3, F4, F6, and F7 were selected for a further investigation, e.g., to find out how the pH affects the removal efficiency of these strains.

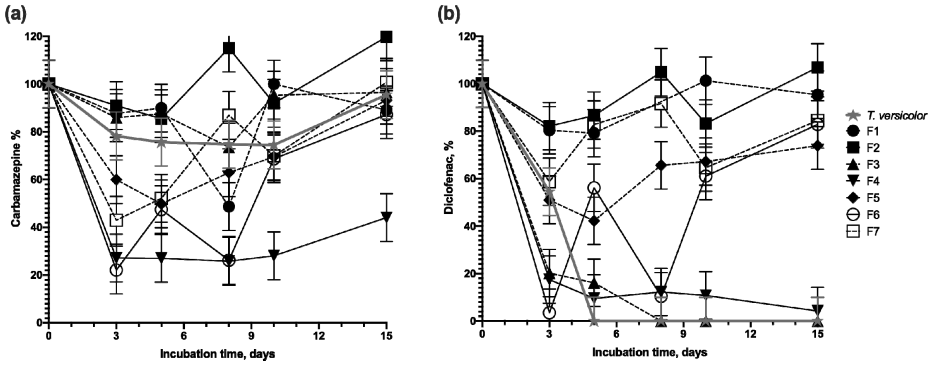

Figure 2. The removal efficiency (%) of CAR (**a**) and DIC (**b**) from a synthetic wastewater media of selected fungal isolates compared to *T. versicolor*.

3.3. pH Effect on Removal Efficiency

Previous studies have reported that the pH level has been determined to be one of the most important factors for enzyme production in fungi [19]. Additionally, the pH of 5.5 has been reported as the most relevant pH value for white-rot fungi growth and enzyme production, especially for *T. versicolor* [19] while the pH of 6.3 presents a natural pH value of synthetic wastewater media. Therefore, during this study, two pH values were selected, 5.5 and 6.3 respectively.

The results of Figure 3 showed the pH effect on CAR and DIC removal efficiency. The results of CAR indicated a relatively low (<20%) removal efficiency for both pH values of fungal strains F4, F6, and F7; there was no statistical difference ($p < 0.05$). However, a fungal isolate F3 and *T. versicolor* showed >25% removal of CAR at the pH 6.3, while no removal activity was obtained at the pH 5.5 after 3 days of incubation (Figure 3a,b). Overall, the results indicated difficulties to obtain relatively high removal efficiency (>80%) from CAR by tested isolates. Therefore, a further investigation is required to understand the removal mechanisms for this compound.

The results were derived to measure the DIC removal efficiency, showing that the fungal isolate F3 could completely (>99.9%) remove this pharmaceutical after 6 days of incubation at pH 5.5, while complete reduction of DIC at pH 6.3 was obtained after 10 days of incubation. Furthermore, the same result was observed for *T. versicolor* (Figure 3c,d). Overall, the fungal isolate of F3 has demonstrated the potential to remove DIC efficiently and successfully under sterile conditions. Thus, further in this work, the removal efficiency for DIC in non-sterile municipal wastewater with the isolate F3 was examined.

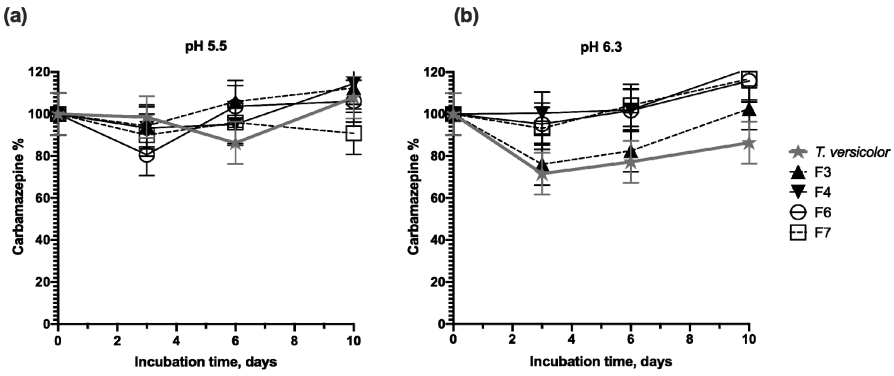

Figure 3. *Cont.*

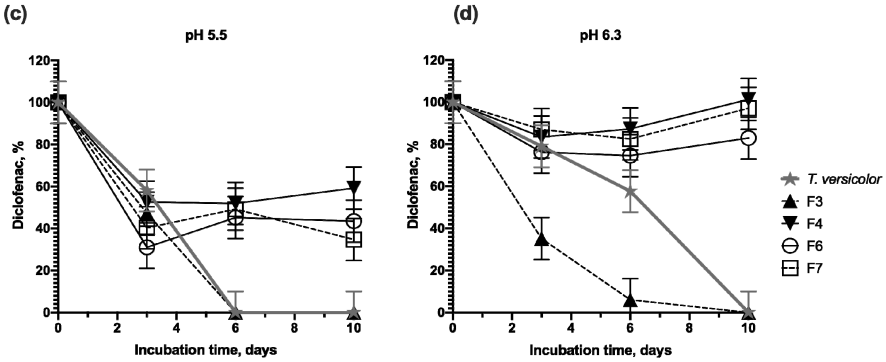

Figure 3. The removal efficiency (%) of CAR at (**a**) the pH 5.5 and (**b**) the pH 6.3 from synthetic wastewater media; The removal efficiency (%) of DIC at (**c**) the pH 5.5 and (**d**) the pH 6.3 from a synthetic wastewater media.

3.4. Removal of Pharmaceuticals by Fungal Isolates in Municipal Wastewater

Over the last decade, most studies on fungi have been conducted on an autoclaved media [14,20,21]. Therefore, the results were not always relevant to situations where fungi are applied to raw wastewater conditions [1]. Accordingly, biomass of the isolate F3 was transferred to non-sterile wastewater and the removal efficiency of DIC was analyzed. Based on previous results from this study, the pH of municipal wastewater was corrected from 7.8 to 5.5. However, the effect of a pH of 7.8 on the removal efficiency was also investigated to see the isolate potential for applications in wastewater treatment processes without a pH correction.

Figure 4 demonstrates the removal efficiency for DIC of the isolate F3 from non-sterile municipal wastewater, compared to *T. versicolor* and municipal wastewater (InletWW) as a negative control without a selected fungal strain.

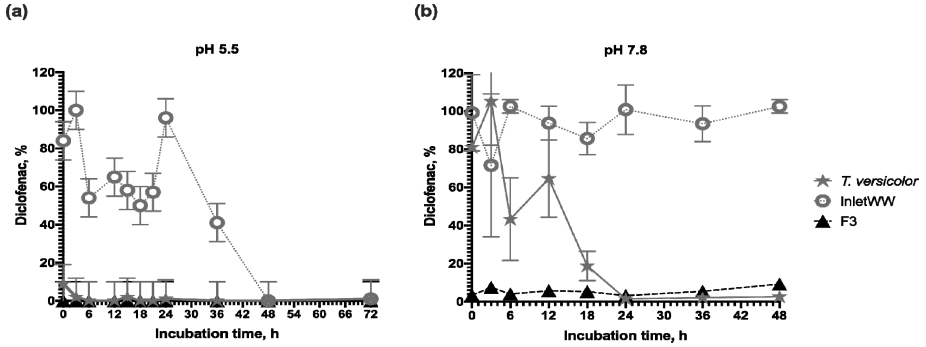

Figure 4. The removal efficiency (%) of DIC at (**a**) the pH 5.5 and (**b**) the pH 7.8 from non-sterile municipal wastewater.

When evaluating the DIC removal efficiency after fungal treatments at various pH values for a non-sterile wastewater sample, it can be stated that the isolate F3 can remove >95% of DIC for both pH values for the entire incubation time; there was no statistically significant difference between removal efficiency for both pH values ($p > 0.05$). In contrast, *T. versicolor* demonstrated a relatively slower DIC removal efficiency at the pH 7.8 after 24 h of the incubation time, compared to the pH 5.5 when DIC was completely removed (>99.9%) after 3 h of an incubation period. Furthermore, non-sterile

wastewater (InletWW) used as a negative control showed an ability to remove DIC at pH 5.5 after 48 h of the incubation time while no reduction was observed at pH 7.8. Furthermore, Figure 5a shows the pH changes during the incubation time with the isolate F3 where the pH was decreased from 7.8 to 3.5 for both samples immediately after the incubation started. This might be explained by the biological metabolism where the acetic acid and alkali might be produced to assimilate nutrients [22,23]. However, further investigation needs to be conducted to better understand the metabolism behavior of the isolate F3. At the same time, wastewater with *T. versicolor* demonstrated a reduction of the pH level from 7.8 to 5.5 after 24 h of the incubation period, when the total removal of DIC was also accomplished (Figures 4a and 5a). Similar results have been observed in a previous study of *T. versicolor* where the maximal removal efficiency for DIC was observed at the pH 5 [24]. Meanwhile, wastewater without a fungal inoculum as a negative control did not show any pH changes throughout the incubation time (data not shown).

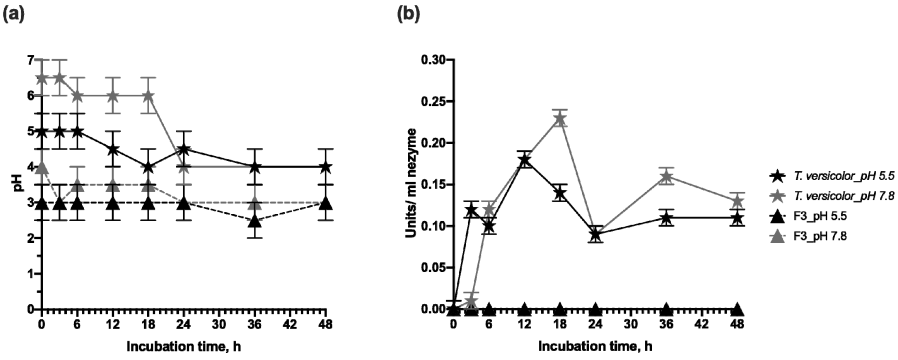

Figure 5. (**a**) pH changes in non-sterile municipal wastewater during the incubation period; (**b**) enzyme activity of laccase in non-sterile municipal wastewater during the incubation period.

Fungi have a variety of strategies to counteract pharmaceutical compounds, e.g., enzymatic processes such as biosorption as well as biotransformation and biodegradation mediated by enzymatic systems [25]. Therefore, in order to better understand the removal mechanisms for the isolate F3, the enzyme activity of laccase was measured to better understand the relationship between the enzyme activity and the removal efficiency for DIC. Laccase has been reported as an enzyme that can degrade a variety of pharmaceutical compounds, e.g., *T. versicolor* has shown an ability to remove ibuprofen and carbamazepine with the laccase enzyme [14].

The results indicated that there was no enzyme activity detected for the isolate F3 and wastewater without a fungal inoculum, while *T. versicolor* produced laccase for the entire incubation period for both non-sterile municipal wastewater samples with different pH values (Figure 5b). Furthermore, the live biomass of the isolate F3 did not present any laccase enzyme activity throughout the incubation period (Figure 6b). Moreover, the isolate F3 showed complete removal (>99.9%) of DIC for both live and dead fungal biomass, immediately after the biomass adjustment (Figure 6a), indicating that the removal of DIC could be due to the biosorption mechanism. In contrast, the results of the biosorption test indicated that *T. versicolor* used both strategies—biosorption and enzyme production—to remove DIC (Figure 6a,b). Finally, the results from negative controls with DIC did not show any DIC removal throughout the incubation period in the PD broth. Therefore, the results obtained could be induced by fungi (data not shown).

Finally, the isolate F3 was identified as *Aspergillus luchuensis*. Overall, these results have shown that *A. luchuensis* has a higher removal efficiency in a non-sterile wastewater sample without a pH correction than *T. versicolor*. Thus, *A. luchuensis* has a high potential for use in industrial wastewater treatment due to minimized specific pH requirements. Furthermore, to the best of our knowledge,

this is the first study where *A. luchuensis* has been reported as a promising strain for wastewater treatment in order to remove pharmaceutical substances. However, the requirements for nutrients and temperature need to be investigated; the impact of the treatment cost should be evaluated in further studies.

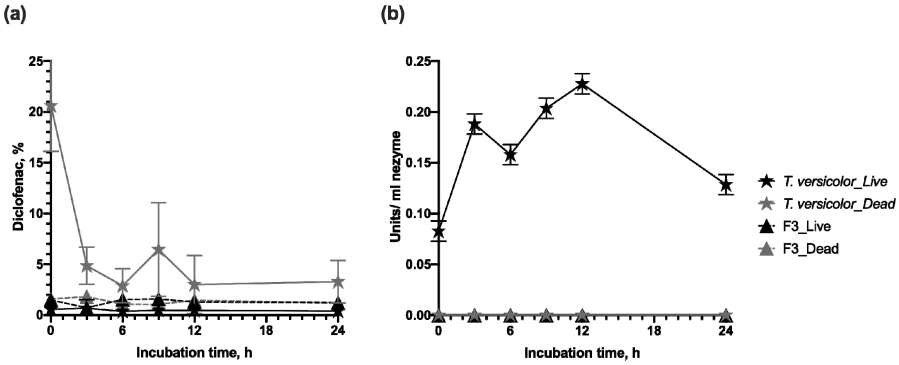

Figure 6. (**a**) The removal efficiency (%) of DIC from the biosorption test of live and dead fungal biomass by the isolate F3 and *T. versicolor*; (**b**) Enzyme activity of laccase from the biosorption test of live and dead fungal biomass by the isolate F3, compared to *T. versicolor*.

4. Conclusions

This study of fungi isolated from municipal wastewater demonstrated isolates' ability to grow in the presence of pharmaceuticals such as CAR, DIC, IBU, and SUL. A high removal efficiency rate was observed in the fungal isolate *Aspergillus luchuensis*, where complete (>99.9%) removal of DIC was observed in a synthetic wastewater media after 10 days of the incubation period, while >98% of DIC was removed in non-sterile wastewater without a pH correction. Isolates showed a lower removal efficiency of CAR compared to DIC. The removal mechanism for DIC of *A. luchuensis* is proposed to be a biosorption strategy. Overall, the results indicated *A. luchuensis* is a promising candidate to remove DIC from wastewater with a typical pH of 7.8, compared to *T. versicolor* which has shown a relatively higher removal efficiency at pH 5.5.

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PAPER III

Removal of total phosphorus, ammonia nitrogen and organic carbon from non-sterile municipal wastewater with Trametes versicolor and Aspergillus luchuensis

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Removal of total phosphorus, ammonia nitrogen and organic carbon from non-sterile municipal wastewater with *Trametes versicolor* and *Aspergillus luchuensis*

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ABSTRACT

Discharge of organic load from treated wastewater may cause environmental eutrophication. Recently, fungi have gained much attention due to their removal of pharmaceutical substances by enzymatic degradation and adsorption. However, the fungal effect in removing nutrients is less investigated. Therefore, two fungal species, the white-rot fungus *T. versicolor* as a laboratory strain and the mold *A. luchuensis* as an environmental isolate from the municipal wastewater treatment plant, were studied to determine the fungal potential for phosphorus, nitrogen, and the total organic carbon removal from municipal wastewater, carrying out a batch scale experiment to a fluidized bed pelleted bioreactor. During the batch scale experiment, the total removal (99.9 %) of phosphorus by *T. versicolor* was attained after a 6 h-long incubation period while the maximal removal efficiency (99.9 %) for phosphorus from *A. luchuensis* was gained after an incubation period of 24 h. Furthermore, both fungi showed that the pH adjustment to 5.5 kept the concentration of nitrogen constant and stabilized the total organic carbon reduction process for the entire incubation period. The results from the fluidized bed bioreactor demonstrated opposite tendencies on a nutrient removal comparing to a batch experiment where no significant effect on phosphorus, nitrogen, and total organics carbon reduction was observed. The obtained results from this study of batch and fluidized bed bioreactor experiments are a promising starting point for a successful fungal treatment optimization and application to wastewater treatment.

1. Introduction

White-rot fungi mainly have been studied for the removal of micropollutants as emerging concerns from wastewater throughout the last decade (Bulkan et al., 2020; Mir-Tutusaus et al., 2018). However, the removal of organic load and nutrients by fungi have been less investigated under non-sterile wastewater (Vasiliadou et al., 2016). Thus far, Shoun et al. (1992) have shown that a wide variety of fungi can perform denitrification (Shoun et al., 1992); Sankaran et al. (2010) have demonstrated the nitrogen source for fungi can be nitrates, nitrites, ammonium or organic nitrogen substances such as a yeast extract and peptone, depending on the type of fungi (Sankaran et al., 2010). Chemical precipitation, e.g., struvite precipitation, and biological assimilation by microorganisms, including fungi, are two major technologies used to remove P from municipal wastewater (Ye et al., 2015). In contrast to chemical precipitation, the fungal removal of phosphorus is considered as a more environmentally favorable and less expensive technology to remove and recover P from wastewater (He et al., 2019). Compared with bacteria, filamentous fungi have the advantage of being easy to harvest due to the mycelium growth and greater resistance to toxic and inhibitory compounds (Guest and Smith, 2007; Ye et al., 2015). Thus, filamentous fungi might be a promising candidates not only for micropollutant removal, but also to improve the classical biological treatment for wastewater to reduce the concentration of nutrients (Millan et al., 2000). However, several questions should be investigated in order to use fungi at full-scale

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bioreactor, e.g., the pH effect on fungal growth; the need of fungal bioaugmentation in order to find optimal conditions of natural microorganisms interaction with fungi.

Trametes versicolor from the white rot fungi class has been shown as a promising candidate for removing micropollutants in wastewater treatment due fungal ability to use multiple biochemical and physical reactions to breakdown intermolecular bonds, demethylation, hydroxylation, dichlorinations, and the opening the aromatic rings (Freitas et al., 2009). Furthermore, all of these transformations are developed together to be combined with the enzyme system and the ability of adsorption, deposition and ion exchange (Dalecka et al., 2020a,b; Pedroza-Rodríguez and Rodríguez-Vázquez, 2013). However, most of the studies investigated the removal of pharmaceutical substances from municipal wastewater using laboratory strains from culture collections, including *T. versicolor* (He et al., 2019; Hultberg and Bodin, 2017; Spina et al., 2012). Therefore, Guest and Smith (2007) have suggested using fungi that are naturally available in a municipal wastewater treatment plant due to their adaptation to the environmental and operation conditions. For instance, recently *Aspergillus luchuensis,* - as an environmental isolate from a municipal wastewater treatment plant recently - has been showed as a promising candidate for a pharmaceutical substances removal from non-sterile wastewater (Dalecka et al., 2020a,b). Moreover, the experiments on both fungal strains have been performed in a batch-scale while only few works have examined the *T. versicolor's* potential of wastewater treatment under non-sterile conditions, using a bioreactor (Dalecka et al., 2020a,b; Pezzella et al., 2017). However, the removal efficiency can be affected by interaction with microbial community from the non-sterile wastewater (Dalecka et al., 2020a,b). Therefore, in terms of a fungi application to full scale wastewater treatment systems, more research is encouraged in this field (Mook et al., 2012).

In this paper, the investigation of the total phosphorus (P), ammonia nitrogen (NH_4-N), and the total organic carbon (TOC) removal from non-sterile municipal wastewater of two fungal species, *T. versicolor* as a laboratory strain and *A. luchuensis* as an environmental isolate, was done. The removal efficiency of P, NH_4-N, and TOC was studied and compared taking into consideration the aspect of process design possible application and optimization of a fungal fluidized bed pelleted bioreactor. The investigation consisted of two phases. First, an observation of results were done under a batch-scale experiment with *T. versicolor* and *A. luchuensis*. During this phase, the removal of P, NH_4-N, and TOC was analyzed. In the second phase, the fungal fluidized bed pelleted bioreactor was designed and both fungal cultures were incubated in reactors allowing collecting the data of P, NH_4-N, and TOC removal in order to compare the nutrient removal efficiency from the batch-scale to the bioreactor. Furthermore, to better understand the removal mechanism of nutrients and fungal interaction with natural microorganisms in municipal wastewater, the pH value, laccase enzyme activity, and quantification of total bacteria were determined. To the best of our knowledge, this is the first study where the nutrient removal of P, NH_4-N and TOC has been tested in a fluidized bed pelleted bioreactor, using *T. versicolor* and *A. luchuensis*.

2. Materials and methods

2.1. Fungal species

The fungal species – the white-rot fungus *Trametes versicolor* (L.) Lloyd strain DSM 6401 (Leibniz Institute DSMZ–-German Collection of Microorganisms and Cell Cultures, Brunswick, Germany) and the mold *Aspergillus luchuensis* (current name: *Aspergillus awamori* Nakaz.; an environmental isolate from a municipal wastewater treatment plant located in Stockholm, Sweden) were used in this study. The fungi were selected based on previous studies (Dalecka et al., 2020a,b) where *T. versicolor* and *A. luchuensis* have demonstrated a high potential to remove micropollutants.

2.2. Municipal wastewater samples

A municipal wastewater sample for a batch experiment was collected from the Henriksdal wastewater treatment plant (Stockholm, Sweden). The inlet wastewater was taken directly from an entry tank with the following composition: COD 500–700 mg/L, N_{tot} 40–50 mg/L, P_{tot} 4.0–5.0 mg/L, pH 7.6 – 7.7. For the pilot scale test the municipal wastewater sample was provided by the Daugavgriva wastewater treatment plant (Riga, Latvia). The inlet wastewater sample was directly taken from an entry tank with the following composition: COD 500–700 mg/L, N_{tot} 40–50 mg/L, P_{tot} 4.0–5.0 mg/L, pH 7.5 – 7.6.

2.3. Batch experiment

Fungal biomass was cultivated in the potato dextrose (PD) media (Oxoid, United Kingdom), incubating in a shaking incubator (50 rpm) for 5 days at 25 °C. To achieve a higher initial concentration of the selected fungi, the Kaldnes K1 carriers (AnoxKaldnes, France; diameter 9.1 mm) were used for a biofilm formation (one carrier unit per 1 mL) (Andersson et al., 2008). After growing, the fungal biomass was separated from the PD media and added to a non-sterile municipal wastewater sample without/with pH adjusting to 5.5 (1 M HCl acid). Additional samples were incubated in a shaker incubator (50 rpm) at 25 °C for a time period of 72 h. Further, an additional investigation of the P, NH_4-N, and TOC removal was done, and samples were taken every 3 for up to 72 h. All samples were filtered through a 0.22 μm membrane (Sartorius Stedim Biotech, Germany) and collected as culture filtrates for a further analysis of the P, NH_4-N, and TOC concentration, the laccase enzyme activity, and the pH level. Two additional negative controls – a control without carriers and a control without fungi – were prepared to compare the experimental results in order to be completely certain that the bioremoval of nutrients was induced by fungi. All experiments were carried out in duplicate or triplicate.

2.4. Bioreactor configuration and operating conditions

A fungal fluidized bed pelleted bioreactor was designed, consisting of a reactor, biomass tank for bioaugmentation, a feed peristaltic pump with flow-meter, air supply with flow regulator, and an effluent tank (Fig. 1). The reactor consisted of a 2 L cylindrical plastic column with working volume of 1.25 L. The up-flow velocity in the reactor was settled according to an experimental plan, i.e., approx. 1.08 mL/min or 0.11 mL/min where fungal biomass was maintained fluidized by air pulses generated by an air supply. At the beginning, the column was sterilized and filled with a 1.25 L synthetic wastewater medium (0.8 g/L KH_2PO_4; 0.2 g/L K_2HPO_4; 0.5 g/L $MgSO_4$; 0.2 g/L yeast extract (Oxoid, United Kingdom) and wet fungal biomass on Kaldnes K1 carriers (one carriers unit per 1 mL). After one week of adoption, the next amount of fungal biomass (25 g wet biomass per 100 mL) - harvested from 250 mL of a PD broth without carriers at least for 7 days cultivation period - was weighted and washed with deionized water. After washing, the wet biomass was homogenized with 250 mL non-sterile wastewater and added to the reactor. Before the fungal biomass adjustment, 250 mL of wastewater from the fungal fluidized bed pelleted bioreactor was removed through the effluent port and poured out in an effluent tank. Additionally, a negative control – a reactor with carriers without fungal biomass – was prepared to compare the experimental results in order to be completely establish a link to the nutrient removal induced by fungi. Samples were taken from the fungal fluidized bed pelleted bioreactor effluent port before (B) the adjustment of fresh non-sterile wastewater and after (A) the adjustment of

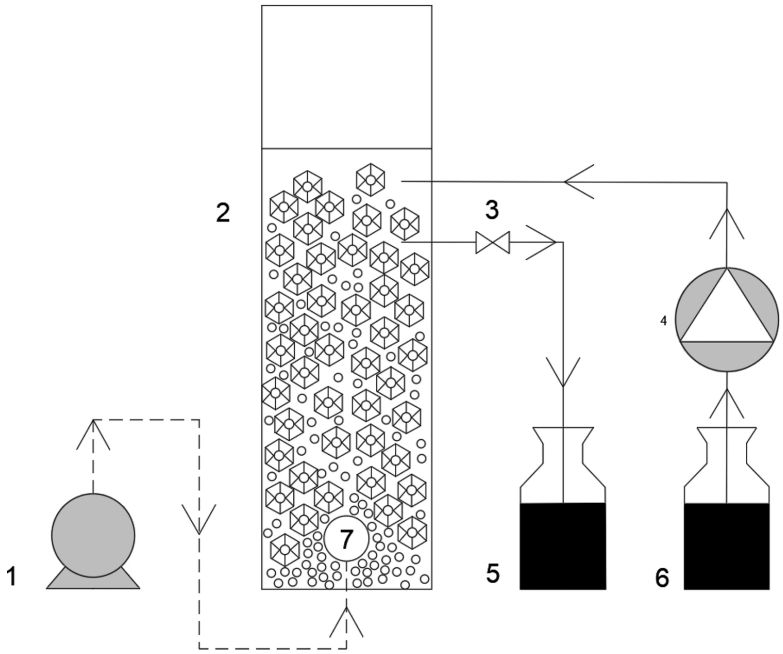

Fig. 1. A scheme of a fungal fluidized bed pelleted bioreactor. (1) Air flow; (2) Reactor; (3) Effluent port; (4) Peristaltic pump and flow-meter; (5) Effluent tank; (6) Fungal biomass tank; (7) Reactor with carriers.

250 mL fresh non-sterile municipal wastewater. All experiments were carried out in duplicate.

2.5. Analytical methods

2.5.1. P, NH_4-N, and TOC analysis

The Hach-Lange (Germany) spectrophotometric system and kits were used to determine the standardized procedure of PO_4^--P (total phosphorus; LCK 349; 0.05–1.5 mg/L), NH_4^--N (total ammoniacal nitrogen; LCK 304,; 0.015–2 mg/L), and TOC (total organic carbon; LCK 385; 3–30 mg/L). The determination of total P in a wastewater sample, using cuvette tests, is based on the reaction between phosphate ions and molybdate ions, as well as, subsequent reduction by ascorbic acid. The determination of total ammonical N is based on the ammonium ions reaction with hypochlorite ions and salicylate ions in the presence of sodium nitroprusside as a catalyst to form indophenol blue at the pH 12.6. The determination of the total TOC consists of a two-stage process. First, the total inorganic carbon is expelled with the help of the TOC-X5 shaker. Thus, the TOC is oxidized to carbon dioxide (CO_2). The CO_2 passes through a membrane into the indicator cuvette, where it causes a color change to occur, which is evaluated with a spectrophotometric system.

2.5.2. Quantification of bacteria

A direct counting method, using DAPI (4′,6-diamidino-2-phenylindole), was applied to obtain the total bacterial count in the wastewater sample (Zafiriou and Farrington, 1980). Shortly, a respective volume of sample was filtered onto a 25-mm-diameter filter (a pore size: 0.2 μm; Whatman, Germany). The sample was fixed with 3–4 % (v/v) formaldehyde and stained with 10 μg/mL DAPI for 10 min. A cell number was obtained by counting 20 random fields of view with an epifluorescence microscope (Leica DMLP, Germany), combined with a 50-W power supply, mercury lamp, and filter sets for DAPI (Ex.: 340/380 nm; Em: > 425 nm nm).

2.5.3. Enzymatic activity and pH measurements

The laccase activity was measured spectrophotometrically, using the standardized procedure of the enzymatic assay by Sigma-Aldrich (Germany). In brief, the test reaction contained 2.2 mL of a 100 mM potassium phosphate buffer (KH_2PO_2, pH 6.0), 0.5 mL of laccase from *T. versicolor* (crude powder, ≥50 units/mg solids, Sigma-Aldrich) and 0.3 mL of a 0.216 mM syringaldazine solution ($C_{18}H_{20}N_2O_6$, Sigma-Aldrich). The absorbance changes were measured for 10 min at 530 nm. The measurements were carried out in triplicate.

The pH level was measured during the batch and reactor experiments in every sampling by using the universal pH-indicator strips (pH 0–14; Merck KGaA, Germany).

3. Results and discussion

3.1. Nutrient removal in a batch experiment and the effect on pH

Over the last decades, it is stated that fungi, which have been isolated from wastewater treatment plants are more likely to be adopted to the natural environment, minimizing the operating conditions and costs (Guest and Smith, 2007). Therefore, during the initial study, the nutrient removal efficiency and the effect of pH on nutrient removal by two fungi - *T. versicolor* a laboratory strain and *A. luchuensis* an environmental isolate from a municipal wastewater treatment plant - were studied and compared to nutrient reduction in non-sterile municipal wastewater under a batch and pilot-scale experiment. The fungi were selected based on previous studies (Dalecka et al., 2020a,b) where *T. versicolor* and *A. luchuensis* have demonstrated a high potential to remove micropollutants as diclofenac and carbamazepine, during the wastewater treatment. However, there is still limited research about these fungi and their ability to remove nutrients from municipal wastewater under non-sterile conditions. The insight in the fungal potential to remove not only pharmaceuticals but also nutrients from municipal wastewater,

could also help to better understand the fungal long-term goal of developing incorporation of fungal treatment technology (Mook et al., 2012).

3.1.1. Phosphorus removal

Experimental results which are shown in the Fig. 2, reflect the effect on the pH level of the P removal under the batch-scale test which has undergone a 72 h-long incubation period, using *T. versicolor* and *A. luchuensis*. The control was prepared with carriers as a no-biomass addition sample to confirm that the P removal performance occurred by fungi only. The results indicated that both fungi were able to remove P in non-sterile municipal wastewater without/with a pH adjustment. For instance, *T. versicolor* was able to reduce P from 2.6 mg/mL to 0.7 mg/mL immediately after the incubation was started without a pH adjustment (Fig. 2 a). The total removal (99.9 %) of P by *T. versicolor* was reached after 6 and 12 h of incubation period without/with a pH level adjustment, respectively (Fig. 2 a and b). On the contrary, the total removal (99.9 %) of P with *A. luchuensis* was gained after a 24 h-long incubation period for both pH values (Fig. 2 a and b). At the same time, the P removal from the control was relatively slow compared to *T. versicolor* and *A. luchuensis*. For example, the P removal from the control without a pH level adjustment was reduced from 2.6 mg/mL to 0.5 mg/mL after a 48 h of incubation period (Fig. 2 a) while the control with a pH level adjustment could reduce P from 2.7 mg/mL to 0.1 mg/mL after a 36 h of incubation period (Fig. 2 b). Therefore, the removal efficiency with the fungal biomass adjustment showed a higher and faster removal efficiency compared with the control, i.e., a 6 h incubation period for *T. versicolor* and a 24 h of incubation period for *A. luchuensis*. Finally, the results indicated that there was no statistically significant effect ($p > 0.05$) on a pH adjustment to increase the P removal efficiency for selected fungi. Furthermore, the results did not indicate that the P was release back in municipal wastewater for the entire incubation period.

Similarly, researchers who have previously conducted research on the P removal from municipal wastewater under batch experiments, have also presented a fungal ability to reduce P where the removal efficiency has varied from 12 to 100 % (Hultberg and Bodin, 2017; Sankaran et al., 2010; Ye et al., 2015). For example, Hultberg and Bodin (2017) investigated the pH effect on the P removal by using fungi in synthetic brewery wastewater (Hultberg and Bodin, 2017). In addition, results showed there was no significant effect on the pH level. However, *T. versicolor* was able to reduce P by 28 % only. In the cited study the selected synthetic wastewater contained approx. a 20 times higher P concentration (60 mg/mL, sterile) compared to the present study (3 mg/mL, non-sterile). Ye et al. (2015) have claimed that possible mechanisms for P removal by fungi, including *T. versicolor*, might be adsorption. Therefore, the adsorption capacity of P for the selected fungus might be reached in the cited study. In this study, there is no release of P observed till 72 h. Thus, there might be involved other mechanism of P removal, e.g., cellular growth of fungi. Overall, in the future application, phosphorus reduction by fungi might play an important role in a wastewater treatment system and reduction mechanisms. Most of the conventional treatment process uses chemical precipitation to remove P which require addition of chemicals and sedimentation steps (Ye et al., 2015). The P reduction in this study showed a potential application of fungi in wastewater treatment process. Therefore, fungal capacity should be investigated (Hultberg and Bodin, 2017).

3.1.2. Removal of ammonia nitrogen

The Fig. 3 indicates the removal efficiency of NH_4-N by *T. versicolor* and *A. luchuensis* comparing the results from wastewater without/with an adjustment of pH value. Results from *T. versicolor* without the adjustment of pH value showed an increase of NH_4-N concentration from 0.25 mg/mL to 2.3 mg/mL immediately after the incubation was started (Fig. 3 a). The same tendency was observed from *A. luchuensis* where the NH_4-N concentration presented an increase from 0.2 mg/mL to 1.4 mg/mL (Fig. 3 a). On the contrary, the results of *T. versicolor* and *A. luchuensis* with adjustment of pH value showed relatively small changes in NH_4-N concentration throughout the incubation time, i.e., from 1.8 mg/mL to 2.7 mg/mL (Fig. 3 b). Similarly, the concentration of NH_4-N for the control stayed at a relatively low level (> 0.6 mg/mL) until the end of the incubation period of 72 h for both pH values (Fig. 3 a and b). In this study, the NH_4-N increment might be explained by the effect of the pH value in municipal wastewater.

According to Mook (2012) et al., there are two forms of NH_4-N in wastewater, free ammonia (NH_3) and ammonium ion (NH_4^+) - which are reversible (Mook et al., 2012). The composition ratio of NH_3 to NH_4^+ mainly depends on the pH value in wastewater (Luo et al., 2015). The higher the pH value, the higher proportion of NH_3 - conversely, the ammonium ion proportion is higher at a lower pH value (Luo et al., 2015; Rezagama et al., 2017). Furthermore, the previous studies have shown *T. versicolor* and *A. luchuensis* have the ability to decrease the pH level to 5 immediately after the fungal biomass incubation in wastewater (Dalecka et al., 2020a,b). Therefore, this might explains the increment of the ammonia nitrogen concentration in municipal wastewater without a pH adjustment for both fungi (Fig. 3 a). The same tendency has also been presented by Biplob et al. (2011) where the NH_4-N removal increased linearly with the raise of the pH level, indicating the importance of the pH for a system stability of wastewater treatment (Biplob et al., 2011).

On the contrary, the wastewater with the pH adjustment showed a

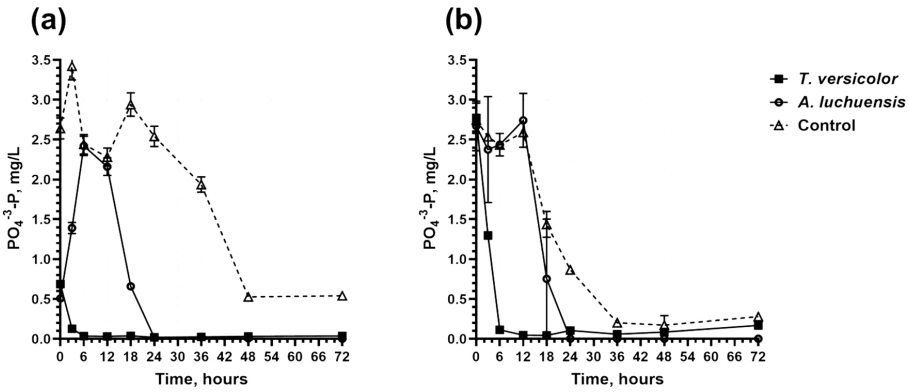

Fig. 2. P reduction from non-sterile wastewater by *T. versicolor* and A. *luchuensis* in a batch test (a) without a pH adjustment (b) with a pH adjustment to 5.5.

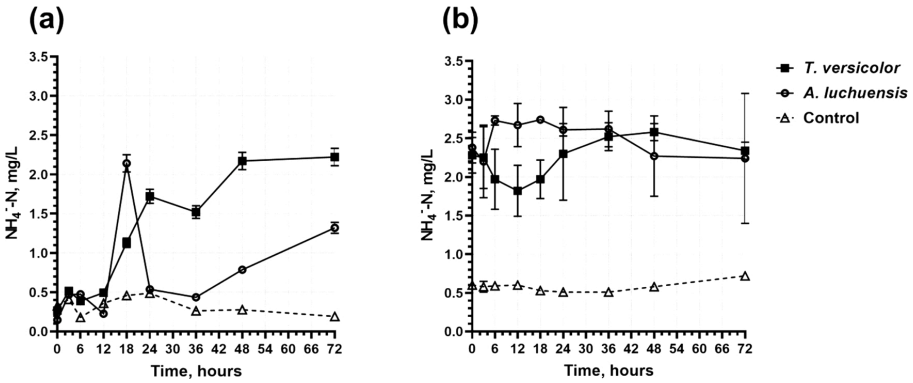

Fig. 3. The ammonia nitrogen reduction from non-sterile wastewater by *T. versicolor* and A. *luchuensis* in a batch test (a) without a pH adjustment (b) with a pH adjustment to 5.5.

more stable concentration of NH_4-N throughout the entire incubation time due of the pH adjustment to 5.5. Thus, the fungal biomass had no direct effect on NH_4-N reduction in municipal wastewater, i.e., it is believed that both fungi did not use NH_4-N in their metabolic pathway to reduce the nitrogen concentration in wastewater. However, a further investigation is required to better understand the fungal role in the NH_4-N reduction in municipal wastewater.

3.1.3. Removal of total organic carbon

Results of the Fig. 4 demonstrate the removal of TOC by *T. versicolor* and *A. luchuensis* from non-sterile municipal wastewater, compared to a control without fungal biomass as a negative control. When evaluating the TOC removal efficiency after a fungal treatment, it can be stated that *T. versicolor* and *A. luchuensis* can reduce TOC from $>$ 3000 mg/mL to $<$2100 mg/mL after a 72 h of incubation period with a pH level adjustment for wastewater (Fig. 4 b). In contrast, the results of *T. versicolor* and *A. luchuensis* without a pH level adjustment showed diverse changes in the TOC concentration throughout the incubation period of 72 h (Fig. 4 a). For instance, both fungi showed the TOC reduction until 12 h of incubation and started to decrease after 18 h of incubation time. In the meantime, the control demonstrated relatively small changes in the TOC concentration reduction throughout the entire incubation time of 72 h for both pH values (Fig. 4 a and b). Therefore, the pH value adjustment might stabilize the TOC removal process for fungi while wastewater without a pH value adjustment showed an unsteady reduction of TOC for the entire incubation time of 72 h. The same tendency of TOC reduction in wastewater was observed by Kim et al. (2004). The cited research investigated *T. versicolor* and a membrane filtration potential of TOC removal from dye wastewater. Results showed that the TOC reduction by *T. versicolor* was relatively low ($<$ 5 % from the starting concentration of TOC). Furthermore, the TOC removal was mainly caused by membrane filtration (Kim et al., 2004). Thus, the results of this study showed that selected fungi might have a less significant effect on the TOC reduction from municipal wastewater compared to the control. The inconsistency of the TOC reduction may require more time to adapt the fungi within the wastewater microbial community. However, the adjustment of the pH was able to keep the TOC removal more stable and constant for the entire incubation time.

Finally, to better understand the fungal mechanisms behind the nutrient removal of P, NH_4-N, and TOC by selected fungi, the pH and laccase activity were also monitored in this study. Fig. 5 demonstrates the pH changes and laccase enzyme activity derived from the batch experiment for the entire incubation time. The results showed that *T. versicolor* and *A. luchuensis* decrease the pH value immediately when the incubation was started and kept the pH value around 5 for the entire incubation time (Fig. 5 a and b) while the control without a fungal biomass adjustment presented a pH value at 6.5−7.5. Furthermore, the laccase activity was observed for white-rot fungus *T. versicolor* only. Laccases occur in many white-rot fungi together with lignin-peroxidases, manganese-peroxidases and further degrading agents

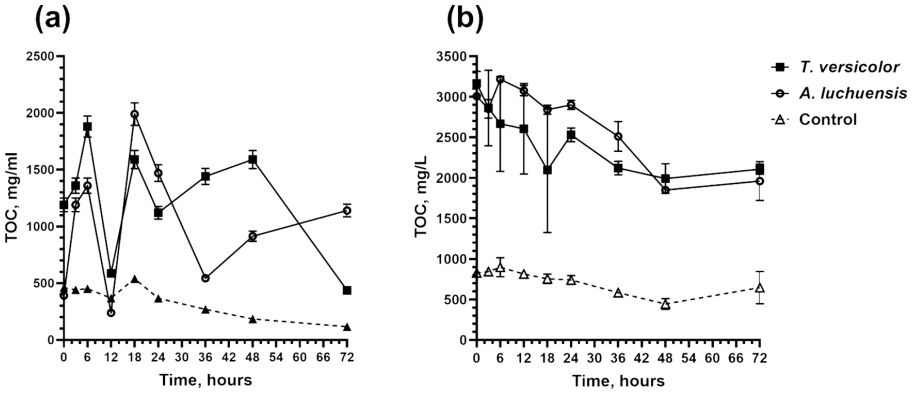

Fig. 4. TOC reduction from non-sterile wastewater by *T. versicolor* and A. *luchuensis* in a batch test (a) without a pH adjustment (b) with a pH adjustment to 5.5.

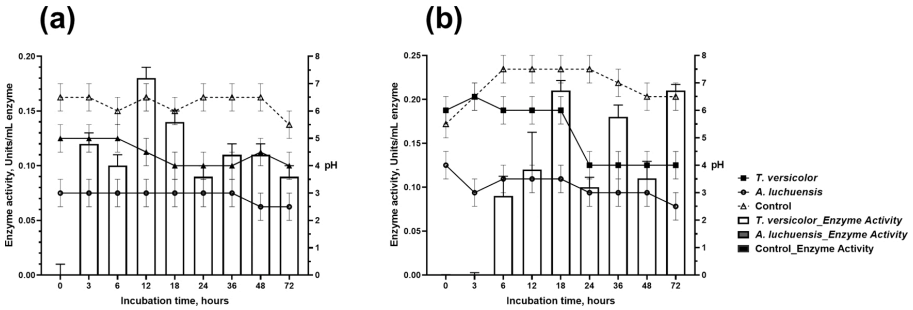

Fig. 5. A laccase enzyme activity (U/mL enzyme) and pH changes derived from a batch experiment (a) without a pH adjustment (b) with a pH adjustment to 5.5.

(Naghdi et al., 2018). However, laccases were also found in some other fungi like molds such as Aspergillus spp. which only discolor wood on its surface (Ramos et al., 2011). Therefore, it is believed that the mold *A. luchuensis* for the P removal used biosorption while *T. versicolor* - both mechanisms, i.e., biosorption and metabolism mechanisms. Moreover, the obtained results of the pH demonstrated that the pH value had a significant effect on N and TOC concentrations. However, results demonstrated that there is no need to adjust the pH value to 5.5 in non-sterile municipal wastewater because of the ability of fungi to decrease and keep stable the pH value to 5 naturally.

3.2. Nutrient removal in a fluidized bed pelleted bioreactor and an effect on flow

Once the results from the batch experiments achieved a relatively good success in the P reduction by fungal treatment and showed that the pH adjustment to 5.5 helped to stabilize the N and TOC reduction process, the removal analysis was further tested in a fluidized bed pelleted bioreactor.

The fluidized bed bioreactor is one of the most commonly used reactors for fungal treatment of wastewater (Andrews, 1988; Espinosa-Ortiz et al., 2016). The use of a fluidized bed bioreactor for wastewater treatment offers many advantages such as a compact bioreactor size due to a short hydraulic retention time, long biomass retention on the carriers, a high conversion rate due to fully mixed conditions, and consequently high mass transfer rates, no channeling of flow, dilution on an influent concentration due to a recycle flow (Moreira et al., 1996; Özkaya et al., 2019). Therefore, the fungal bioreactor is widely applied in the environmental engineering field for many purposes, including to minimize the organic compound load for the treatment process of different wastewater types (Özkaya et al., 2019). However, when a process is scaled up to a bioreactor, aeration and agitation may change when compared to a batch experiment. Thus, fungal biomass may responds differently to the mechanical and oxidative stress and fungal metabolic activity may change in a fluidized bed pelleted bioreactor (Spina et al., 2014). In this study, the removal efficiency of P, NH_4-N, and TOC, using *T. versicolor* and *A. luchuensis* was studied and analyzed. Furthermore, two up-flow velocity rates −1.08 L/min as maximal permissible flow for peristaltic pump and 0.11 L/min as 10 times lower flow compare to maximal permissible flow - were selected and tested in order to find the best optimal conditions for fungal adaption and growth in a fluidized bed pelleted bioreactor. The optimization of the flow can result in a continuously high density production of enzymes and a biomass formation in a bioreactor (Musoni et al., 2015).

3.2.1. Removal of P, NH_4-N, and TOC

Initially, the fluidized bed pelleted bioreactor was designed of three identical bioreactors (Fig. 1). Two of the bioreactors were used for each fungal species separately while the third bioreactor was used as a negative control without an addition of fungal biomass in order to compare the reduction of P, NH_4-N, and TOC between the selected fungi and exclude the interference on the nutrient reduction of any other microorganisms present in municipal wastewater. A sampling was done before (B) the adjustment of fresh non-sterile wastewater and after (A) the adjustment of 250 mL fresh non-sterile municipal wastewater in order to compare the changes in the nutrient load and total bacteria count throughout the entire incubation period.

The P removal profiles for both up-flow velocity rates, using fluidized bed bioreactors with *T. versicolor* and *A. luchuensis* are shown in the Fig. 6 (a and b). The results presented that both fungi were able to reduce more than 80 % of P until the end of the incubation period for both up-flow velocity rates. However, there was no statistically significant difference on the P reduction efficiency between fungi and the negative control ($p > 0.05$). Therefore, the results showed that there was no effect on the fungal adjustment on the P reduction, using a fluidized bed bioreactor, compared to results of batch experiments (Fig. 2).

The result of the NH_4-N concentration with up-flow velocity rates 1.08 L/min did not show any changes for both fungi until the 15th days of incubation period while the results of the negative control demonstrated an increase of the NH_4-N concentration for the entire incubation period (Fig. 6 c). On the contrary, the results from fluidized bed bioreactors with up-flow velocity rate of 0.11 L/min, indicated relatively small changes in the NH_4-N concentration for the entire incubation time (including the negative control) (Fig. 6 d).

Finally, the Fig. 6 (e and f) presents the TOC reduction results of *T. versicolor* and *A. luchuensi,* comparing to the negative control without an adjustment of fungal biomass. The results demonstrated that TOC has been reduced from 700 mg/L to > 250 mg/L after 15 days of the incubation period for both up-flow velocity rates. Furthermore, there was no statistically significant difference among both fungi and the negative control ($p > 0.05$) when the TOC concentration for both up-flow velocity rates after 15 days of the incubation time, were compared. Overall, the results of a fluidized bed bioreactor demonstrated different tendencies on the nutrient removal, using *T. versicolor* and *A. luchuensis* compared to a batch experiment. For example, the batch experiment showed a significant effect on the P reduction by *T. versicolor* and *A. luchuensis* compared to a negative control while there was no significant effect on P reduction by fungi in a fluidized bed bioreactor. One of the main problem to achieve a successful bioreactor performance in stable conditions with fungi is related to limiting hyphal growth, as well as, avoiding diffusional restrictions (Moreira et al., 1996). In bioreactor, the excessive growth of fungi provokes operational problems, i.e., growth back along the nutrient feed and sampling lines, decrease in the treatment efficiency due to increase of viscosity and mass transfer limitations (Moreira et al., 1996). The previously mentioned factors cause practical and technical difficulties in culturing fungi. Therefore in this study, the ability to control the fungal growth and regulate hyphal extension,

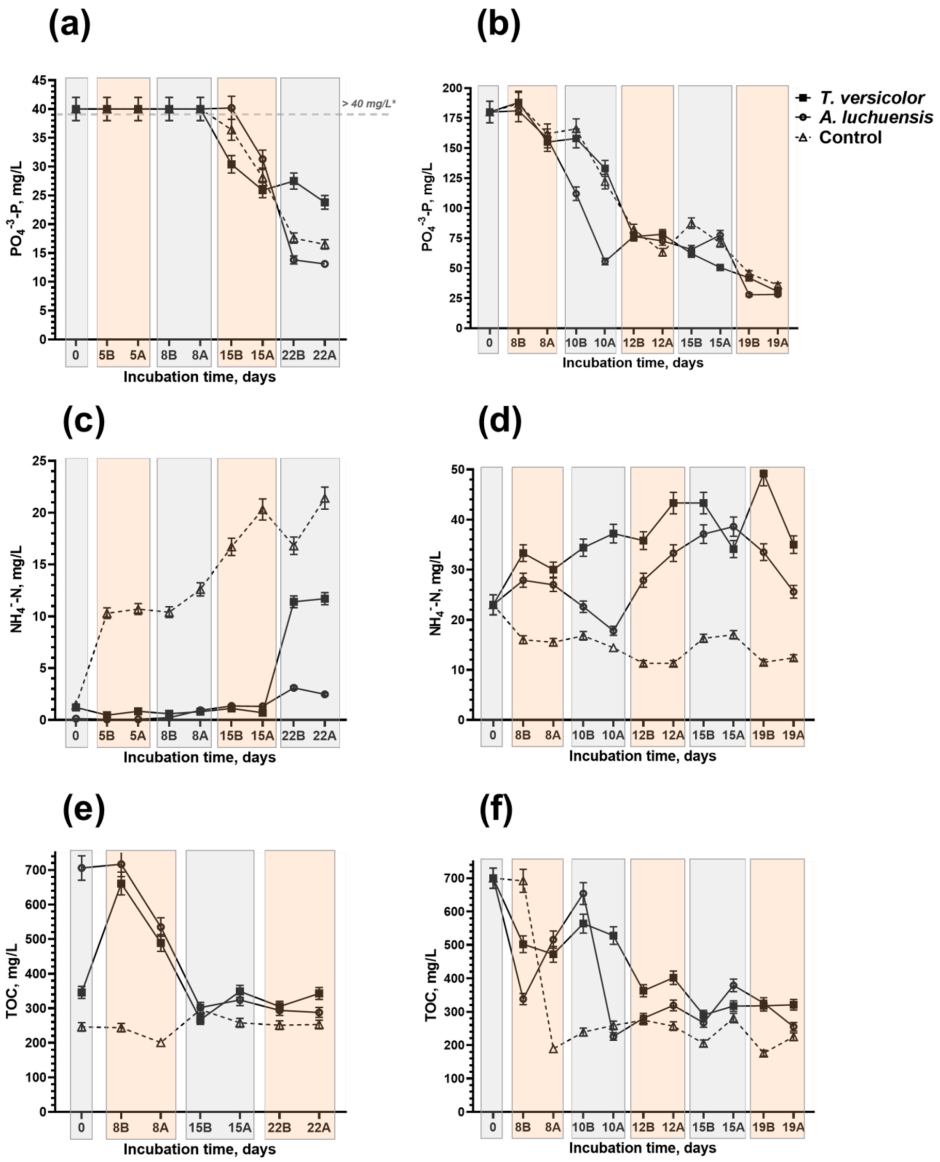

Fig. 6. A nutrient removal from non-sterile wastewater by *T. versicolor* and A. *luchuensis* in a fluidized bed pelleted bioreactor before (B) the adjustment of fresh non-sterile wastewater and after (A) the adjustment of 250 mL fresh non-sterile municipal wastewater. (a) P removal with a flow of 1.08 L/min (b) P removal with a flow of 0.11 L/min; (c) NH_4-N removal with a flow of 1.08 L/min; (d) NH_4-N removal with a flow of 0.11 L/min; (e) TOC removal with a flow of 1.08 L/min; (f) TOC removal with a flow of 0.11 L/min.

biofilm formation, and interaction around the carriers became difficult and required improvements. Furthermore, the results from a fluidized bed bioreactor showed the P/N ratio 1:5 and 1:7 for a batch and fluidized bed bioreactor, respectively. The difference in the nutrient load might be explained by wastewater sampling from two different wastewater treatment plants and the use of synthetic wastewater in a fluidized bed bioreactor at the beginning of the incubation time. The synthetic wastewater was used to better adopt the fungal biomass in fluidized bed bioreactor conditions (Sankaran et al., 2010). Additionally, the changes in the nutrient load were caused by an adjustment of fresh non-sterile municipal wastewater. Therefore, the removal of P, NH_4-N, and TOC was relatively slower compared to the batch-scale experiment.

In this study, the results have also demonstrated that a sufficient and regular fungal biomass augmentation (< 20 g wet biomass per 100 mL) to non-sterile wastewater in a bioreactor helped to adjust the pH level lower than > 5. Therefore, the natural growth of microorganisms was limited and fungi were able to reduce P (Fig. 7 a). Moreover, the ability to decrease the pH level by *T. versicolor* and *A. luchuensis* may

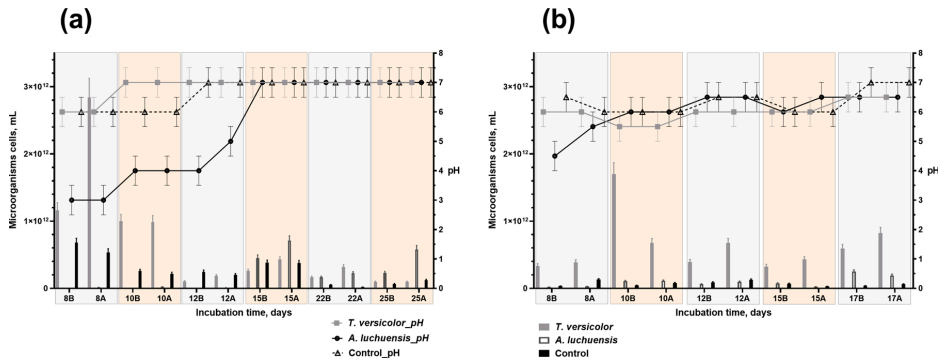

Fig. 7. A number of microorganism's cell and pH changes in a fluidized bed pelleted bioreactor with (a) a flow of 1.08 L/min; (b) a flow of 0.11 L/min. The total bacteria count was obtained before (B) the adjustment of fresh non-sterile wastewater and after (A) the adjustment of 250 mL fresh non-sterile municipal wastewater.

demonstrate an advantage to minimize the cost of the pH adjustment. Therefore, a preliminary analysis was performed to evaluate the cost associated with a fungal treatment in a fluidized bed pelleted bioreactor and compared to classical treatment methods.

3.2.2. Cost evaluation of fungal treatment

Due to the ability to apply the secretion of an extracellular non-specific enzymatic complex during their secondary metabolism, fungi have the unique ability to degrade the bulky, heterogeneous and recalcitrant polymers (Espinosa-Ortiz et al., 2016). This potential can be used to remove xenobiotics and micropollutants from wastewaters (Dalecka et al., 2020a,b; Naghdi et al., 2018). Thus, in the last decade there has been growing interest to integrate fungal bioreactors into the wastewater plants (Cruz del Álamo et al., 2020; Freitas et al., 2009; Mir-Tutusaus et al., 2019; Negi et al., 2020). The authors of this study believe that there are at least two possible ways to apply fungi at the WWTP: (i) to encourage fungi growth in situ on an organic substrate present in the wastewater, or (ii) to cultivate them separately and then dose in the process (bioaugmentation). In this study authors examined the second option. This study showed that with bioaugmentation is possible to maintain domination of fungi over bacteria without a pH adjustment and effectively remove P, NH_4-N, and TOC (Fig. 7). However, it does require an additional costs, including an extra source of the organic substrate to cultivate fungi. Here, the authors have estimated costs based on the current average market prices in Europe. All estimated fungal treatment costs (EUR/m^3) include the cost of fungal growth and operation in a fluidized bed pelleted bioreactor (Table 1).

According to the literature (Hansen et al., 2007; Pelendridou et al., 2014; Rongwong et al., 2018; Yoo, 2018), the cost of typical treatment technologies such as a coagulation-flocculation process for wastewater treatment, is in the range from 0.35–8.5 EUR/m^3; for membrane-based technologies - from 2 EUR/m^3; for conventional biological treatment - from 0.035 to 1 EUR/m^3 while the fungal treatment growth and operation costs may vary from 200 to 2000 EUR/m^3. The fungal treatment costs highly depend on fungal growth requirements (temperature, incubation time, electricity of shaking, composition of media). Thus, the cost of the fungal treatment presented here is among the highest reported in the literature, declaiming the hypothesis that the fungal treatment can be a cost-effective treatment technology. However, the fungal treatment still has a high potential to be an environmentally friendly and sustainable treatment method for wastewater treatment not only considering the nutrient load perspective, but also for micropollutant removal (Mir-Tutusaus et al., 2018). Furthermore, the fungal biomass after treatment can be used as a source for valuable byproducts therefore covering the incurred costs of growth (Sankaran et al., 2010).

Table 1
The average price for different wastewater treatment technologies and the cost of the studied fungal treatment by *T. versicolor* and *A. luchuensis*.

Wastewater Treatment Technology	Cost, EUR/m^3	Reference
Fungal Treatment	From 200 to 2000	This study
Coagulant-Flocculant	From 0.35 to 8.5	(Pelendridou et al., 2014; Yoo, 2018)
Membrane-Based Treatment	From 2	(Rongwong et al., 2018)
Conventional Biological Treatment	From 0.036 to 1	(Hansen et al., 2007)

4. Conclusions

In this study, a batch scale experiments using *T. versicolor* and *A. luchuensis* were performed for non-sterile municipal wastewater and the pH effect on the P, NH_4-N, and TOC reduction was analyzed. Additionally, the fluidized bed bioreactor was designed and removal efficiency was tested. Although, bacteria are still the preferred microorganisms to be used in bioreactors for the treatment of municipal wastewater, during this study, fungi have demonstrated a high potential to remove phosphorus from municipal wastewater efficiently and successfully under a batch scale experiment. In the further work, optimization and development of fluidized bed bioreactor operations, using fungi, should be investigated and evaluated.

Author contributions

T.J. and G.K.R. devised the project, its main conceptual idea, and proof outline. B.D. designed and carried out experiments. T.J. and M.S. designed the concept of the fluidized bed bioreactor. B.D. wrote the manuscript with support from M.S., T.J., and G.K.R. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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PAPER IV

Bioaugmentation with fungi: An emerging strategy for removing pharmaceutical substances in wastewater treatment process by fluidized bed pelleted bioreactor

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Bioaugmentation with fungi: An emerging strategy for removing pharmaceutical substances in wastewater treatment process by fluidized bed pelleted bioreactor

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Abstract

Fungi have been shown to be effective candidates to remove pharmaceuticals. However, the pilot-scale application mainly deals with the strict growth conditions and competition between microbial community from wastewater. Thus, the bioaugmentation for removing the pharmaceuticals in municipal wastewater by *T. versicolor* and *A. luchuensis* using fluidized bed pelleted bioreactor was studied. To find the optimal performance of bioaugmentation two periodical biomass volume, 10 and 50 g per 1.25 L bioreactor, were tested. The removal of nutrients, pharmaceutical substances and changes in the pH value, laccase activity, and total microorganisms were analyzed. The results showed that bioaugmentation has an effect in removing NH_4-N and lower accumulation of NO_3-N, whereas PO_4^{-3}-P and TOC did not show significant effect on the reduction of nutrient load. The results of removal efficiency for diclofenac, carbamazepine, and sulfamethoxazole showed that there was no significant effect on the removal while the highest removal efficiency (> 90 %) for ibuprofen, ketoprofen, and metoprolol was achieved by both fungi with periodical addition with 50 g of biomass after an incubation time of 3 hours. Finally, the data analysis with AI-based experimental design indicated that *A. luchuensis* can be a promising fungus for pharmaceutical removal and implies as a promising approach for optimization of fluidized bed pelleted bioreactor. Overall, the obtained results and the use of an AI-based platform are a promising approach for optimization and operation of fluidized bed pelleted bioreactor.

Keywords: Fungi, bioaugmentation, pharmaceutical substances, wastewater

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1. Introduction

Filamentous fungi are eukaryotic, heterotrophic, and achlorophyllous microorganisms which have the ability to produce organic acids, intro- and extracellular proteins, including a large variety of enzymes (Espinosa-Ortiz et al., 2016; Lacina et al., 2003). For example, white-rot fungi can produce three types of lignin modifying enzymes, i.e., lignin peroxidase, manganese-dependent peroxidase, and laccase (Gao et al., 2010). These enzymes can be used to degrade hazardous pollutants like pesticides, personal care products, and pharmaceutical substances (Sankaran et al., 2010). Furthermore, among the enzymatic systems, fungi can also use biosorption as a mechanism to remove hazardous compounds (Asif et al., 2017). Therefore, fungi have been shown to be effective for hazardous compounds removal, especially for pharmaceutical substances due to fungi' capacity to transform most of the compounds with their enzymatic systems and biosorption mechanisms (Badia-Fabregat et al., 2015; Mir-Tutusaus et al., 2016).

The primary source of pharmaceutical contaminants in the aquatic environment is known to be through wastewater treatment plant effluents due to inefficient removal of pharmaceutical compounds by conventional wastewater treatment plants (Mir-Tutusaus et al., 2016). Previous studies have shown that pharmaceuticals can cause a negative effect on aquatic organisms (Cecconet et al., 2017; Yang et al., 2013). For instance, the synthetic steroid hormone 17a-ethynylestradiol, which is widely used in the contraceptive pill, can impose detrimental endocrine-disrupting effects on

fish and other aquatic organisms at concentrations as low as several ng/L while diclofenac, as one of the most widely detected drug in wastewater, has demonstrated a negative effect on embryo development on mussels (Balbi et al., 2018; Palli et al., 2017; Yang et al., 2013). In recent years, special attention has been focused on biological treatment by fungi as a promising method to remove pharmaceutical substances from wastewater (Cecconet et al., 2017; More et al., 2010). For instance, previous studies have demonstrated *Trametes versicolor* and *Aspergillus luchuensis* relatively high potential (> 95 %) to remove pharmaceutical substances like diclofenac and ketoprofen (Cecconet et al., 2017; Brigita Dalecka et al., 2020; Brigita; Dalecka et al., 2020; Stenholm et al., 2018). Therefore, the use of fungi to remove pharmaceutical substances from wastewater can be a good alternative to traditional wastewater treatment technologies (Espinosa-Ortiz et al., 2016). However, most of the research on the fungal application for wastewater treatment has so far focused mainly on assessing the removal efficiency in sterile batch-scale tests (Cecconet et al., 2017; Espinosa-Ortiz et al., 2016; More et al., 2010; Nguyen et al., 2014). Thus, more research needs to be done in order to develop a full-scale application for fungi in wastewater treatment to remove pharmaceutical substances (Asif et al., 2017).

So far, the application of fungal treatment in full-scale is not executed at the moment (Mir-Tutusaus et al., 2019). According to Mir-Tutusaus et al. (2019) development of fungal wastewater treatment mainly depends on overcoming several shortcomings, i.e., (i) maintaining a stable activity of the fungal biomass over prolonged periods of time and (ii) preserving good

performance in non-sterile conditions. Additionally, a major limitation of fungi in wastewater treatment under non-sterile conditions is its sensitivity to biological process operation (Gao et al., 2010). The pilot-scale application in fungal bioreactors mainly deals with the strict growth conditions required for most fungi (e.g., pH and temperature) and competition between microbial community from wastewater (Hu et al., 2020). In order to increase the metabolic activity for fungi, the bioaugmentation as the process of adding selected microorganism in a bioreactor for wastewater treatment to remove pharmaceutical substances can be used (Li et al., 2015). Lately, bioaugmentation for fungi has been pointed out as a promising strategy for solving practical problems in the installation of municipal wastewater treatment plants as well as to improve the efficiency of pharmaceutical substances removal (Shah, 2017). Thus, extensive essential laboratory works on bioaugmentation tests followed by a series of pilot-scale studies with fungal bioreactors are needed for future application in industrial process (More et al., 2010).

The main objective of the present work was to evaluate the effect on bioaugmentation with fungi for removing the pharmaceutical substances in municipal wastewater using fluidized bed pelleted bioreactor. Two fungal species, *T. versicolor as* a laboratory strain and *A. luchuensis* as a wastewater isolate was used in this study. The investigation consisted of two stages. First, observation of results from bioaugmentation with two periodical biomass addition, 10 and 50 g per each bioreactor of 1.25 L, was analyzed and compare from tests in fluidized bed pelleted bioreactor. In the second stage,

the removal of diclofenac, ketoprofen, carbamazepine, ibuprofen, sulfamethoxazole, and metoprolol were analyzed. To better understand the removal efficiency of nutrients and fungal interaction with microbial community in municipal wastewater, the removal of NH_4-N, NO_3-N, NO_2-N, P_4-^{3}-P, and TOC were also studied. Additionally, the pH value, laccase activity, and quantification of total microorganisms were investigated. Finally, in order to determine the optimal operation conditions of bioaugmentation and fungal adaption in fluidized bed pelleted bioreactor, the artificial intelligence (AI) based platform was used to analyze the data and model the optimal process of fungal treatment for pharmaceutical substances removal in fluidized bed pelleted bioreactor. To the best of our knowledge, this is the first study where the bioaugmentation with *T. versicolor* and *A. luchuensis* has been tested and the obtained data analyzed with AI-based platform.

2. Materials and methods

2.1. Fungal strains

Trametes versicolor DSM 6401 (TV; Leibniz Institute DSMZ–-German Collection of Microorganisms and Cell Cultures, Brunswick, Germany) and *Aspergillus luchuensis* (AL; an isolate from a municipal wastewater treatment plant located in Stockholm, Sweden) were used in this study. The fungal cultures were grown in potato dextrose agar (Oxoid, United Kingdom) for regular maintenance and grown in potato dextrose broth (Oxoid, United Kingdom) for bioaugmentation experimental setup.

2.2. Chemicals

High purity grade (> 98 %) diclofenac (DIC), ketoprofen (KET), carbamazepine (CAR), ibuprofen (IBU), sulfamethoxazole (SULF), and metoprolol (MET) were purchased from Sigma-Aldrich (Germany) (Table 1). The standard solutions at the concentration of 50 mg/L were prepared according to the manufacturer's solubility guidelines.

2.3. Municipal wastewater samples

The municipal wastewater sample was provided by the Daugavgriva wastewater treatment plant (Riga, Latvia). The inlet wastewater sample was directly taken from an entry tank after the screen with the following composition: COD 500–700 mg/L, N_{tot} 40–50 mg/L, P_{tot} 4.0–5.0 mg/L, pH 7.5 – 7.6.

2.4. Bioaugmentation and operating conditions of bioreactor

A fungal fluidized bed pelleted bioreactor was designed, consisting of five columns, biomass tank for bioaugmentation, a feed peristaltic pump with flow-meter, air supply with flow regulator, and an effluent tank (Figure 1). The bioreactor consisted of a 2 L cylindrical plastic column with working volume of 1.25 L. The up-flow velocity in the reactor was settled according to an experimental plan, i.e., approx. 0.11 mL/min where fungal biomass was maintained fluidized by air pulses generated by an air supply. At the beginning, the column was filled with a 1.25 L of non-sterile municipal inlet wastewater and 100 g of wet fungal biomass on Kaldnes K1 carriers (one carrier unit per 1 ml) was added. After three days of adoption, the next batch of fungal biomass was added, i.e., 10 and 50 g, respectively. Before the addition, the biomass was grown in potato dextrose broth at 120 rpm for 25° C without carriers for 5 days cultivation period - was weighted and washed with 300 mL of deionized water. Before the fungal biomass addition with 250 mL of fresh non-sterile municipal wastewater, 250 mL of wastewater from the fungal fluidized bed pelleted bioreactor was removed through the effluent port and poured out in an effluent tank. The biomass from the effluent port was not measured (weighed) hence the biomass added to the reactor was referred as “addition of biomass”. Additionally, a negative control – a bioreactor with carriers without fungal biomass – was prepared to compare the experimental results in order to completely establish a link to the nutrient, pH level and pharmaceutical substances removal enhanced by fungi. Samples were taken from the fungal fluidized bed pelleted bioreactor effluent port before (B) the addition of fresh non-sterile wastewater with

fungal biomass and after (A) the addition of 250 mL fresh non-sterile municipal wastewater for analyze the pH level, total microorganisms count and nutrient level of PO_4^{-3}-P, NH_4-N, NO_3-N, NO_2-N, and TOC. The bioaugmentation of addition of fungal biomass was repeated every third day of incubation period for 4 weeks in order to maintain relatively constant biomass concentration in the bioreactors.

2.5. Addition of pharmaceutical substances and sampling

After 4 weeks of bioaugmentation, the mixture of pharmaceutical substances of DIC, KET, CAR, IBU, SULF, and MET with final concentration of 50 mg/L were added to all five columns in fluidized bed pelleted bioreactor after 24 h of the final addition of 250 mL fresh non-sterile municipal wastewater and fungal biomass. An additional investigation of the removal efficiency of pharmaceutical substances was performed for 1 week and samples were taken at 0, 0.5, 3, 6, 24 h and 7 days of incubation time. All samples were prepared for analysis, i.e., samples were filtered for further nutrient, high-performance liquid chromatography (UHPLC) and laccase activity analysis.

2.6. Analytical methods

2.6.1. Pharmaceutical substances analysis with UHPLC

Four different controls were made for experimental setup in order to avoid of a false result that could be detected in a case of an instrumental error. As a negative control, a wastewater without any pharmaceutical substance was used; as a positive control a wastewater combined with all selected pharmaceutical separately was prepared. Also, a positive control with a

wastewater and a mixture of all pharmaceutical compounds was analyzed. The concentration of pharmaceutical substances was measured in all controls and samples by UHPLC system of Perkin Elmer (USA) using the UV/VIS UHPLC detector. Chromatographic separation was achieved using the Acquity UPLC BEH C_{18} column (1,7 µm, 2,1 × 100 mm; Water, USA) with flow of 0,3 ml/min. The mobile phase was 90 % v/v acetonitrile (Fisher Scietific, United Kingdom) and 10 % v/v deionized water. All results were analyzed using Perkin Elmer UHPLC Data Software (USA).

2.6.2. Nutrient analysis

The Hach-Lange (Germany) spectrophotometric system with kits were used to determine the standardized procedure of PO_4^{-3}-P (total phosphorus - LCK 349 with 0.05 – 1.5 mg/L), NH_4—N, NO_3-N, and NO_2-N (total ammoniacal nitrogen - LCK 304 0.015 with 2 mg/L; nitrate - LCK339 with 1 – 60 mg/L; nitrite - LCK342 with 0.6 – 6 mg/L), TOC (total organic carbon - LCK 385 with 3 – 30 mg/L). The determination of total PO_4^{-3}-P in a wastewater sample, using cuvette tests, is based on the reaction between phosphate ions and molybdate ions, as well as, subsequent reduction by ascorbic acid. The determination of total NH_4—N is based on the ammonium ions reaction with hypochlorite ions and salicylate ions in the presence of sodium nitroprusside as a catalyst to form indophenol blue at the pH 12.6. The determination of nitrate is based on the color reaction with sulphuric and phosphoric acids with 2.6-dimethylphenol forming 4-nitro-2.6-dimethylphenol while nitrites is based on the color reaction with primary aromatic amines in acidic solution to form diazonium salts. The determination of the total TOC consists of a two-

stage process. First, the total inorganic carbon is expelled with the help of the TOC-X5 shaker. Thus, the TOC is oxidized to carbon dioxide (CO_2). The CO_2 passes through a membrane into the indicator cuvette, where it causes a color change to occur, which is evaluated with a spectrophotometric system.

2.6.3. Quantification of microorganisms

A direct counting method, using DAPI (4',6-diamidino-2-phenylindole), was applied to obtain the total microorganism count in the wastewater sample (Zafiriou and Farrington, 1980). Shortly, a respective volume of sample was filtered onto a 25-mm-diameter filter (a pore size: 0.2 μm; Whatman, Germany). The sample was fixed with 3-4 % (v/v) formaldehyde and stained with 10 μg/mL DAPI for 10 min. A cell number was obtained by counting 20 random fields of view with an epifluorescence microscope (Leica DMLP, Germany), combined with a 50-W power supply, mercury lamp, and filter sets for DAPI (Ex.: 340/380 nm; Em: > 425 nm nm).

2.6.4. Enzymatic activity and pH measurements

The laccase activity was measured spectrophotometrically, using the standardized procedure of the enzymatic assay by Sigma-Aldrich (Germany). In brief, the test reaction contained 2.2 mL of a 100 mM potassium phosphate buffer (KH_2PO_2, pH 6.0), 0.5 mL of laccase from *T. versicolor* (crude powder, ≥50 units/mg solids, Sigma-Aldrich) and 0.3 mL of a 0.216 mM syringaldazine solution ($C_{18}H_{20}N_2O_6$, Sigma-Aldrich). The absorbance changes were measured for 10 min at 530 nm. The measurements were carried out in triplicate. The pH level was measured during the batch and

reactor experiments in every sampling by using the universal pH-indicator strips (pH 0 – 14; Merck KGaA, Germany).

2.6.5. Data analysis by AI based platform

In this study, the data were analyzed with software xT smart_DoE (2019, Latvia) which is artificial intelligence (AI) web-based platform. After all data collection from experiment with fluidized bed pelleted bioreactor, the AI based platform was used to compare the results and perform the optimization in fungal bioreactor. In the context of this research, the problem in AI based platform with obtained data was defined in terms of its key variables (amount of fungal biomass, type of fungi), objectives (time series of pH, the number of total microorganism's cells, the changes in concentration of total PO_4^{-3}—P, NH_4—N, NO_3-N, NO_2-N, TOC, and pharmaceutical substances), and objectives as well as existing data points (the performed bioreactor experiments including the constant addition of selected fungal biomass, incubation time, and time series measurements of objectives). The software was then used to produce suggestions based on said relevant information. The format in which the AI software generates suggestions and models is called regions of interest (ROIs). ROIs are calculated by employing a set of Monte Carlo simulations of the generative active learning model, which maps problem input (variable) data distribution to problem output (objective) data distribution and tries to optimize given problem in terms of novel variable configurations. Finally, the ROIs are presented as a heatmap displaying relevant problem space regions in form of a distribution of predicted probabilities (i.e., red meaning high probability, white – low probability). The

higher the predicted probability displayed in the ROIs heatmap, the higher the chance the obtained data from said region will contain improved solutions that better meet the set multi-objective criteria. Furthermore, as more data are accumulated in the software, the ROIs begin to produce better approximations of the problem itself.

3. Results and discussion

3.1. Bioaugmentation effect on fungal growth in fluidized bed pelleted bioreactor

Many alternative physico-chemical and biological methods have been suggested to solve the problem with wastewater treatment to remove pharmaceutical substances were rather challenging both to be technically and economically successful (Dhouib et al., 2006). Additionally, fungi have been shown as an effective treatment technology due to their bioremediation capabilities to produce enzymes and biosorption characteristics to remove the pharmaceutical substances (Mir-Tutusaus et al., 2018). In contrast to the significant number of reports and studies demonstrating the excellent capacity of fungi to degrade pharmaceutical substances in small-scale, sterile batch tests, far fewer studies have been conducted in continuous bioreactors (Yang et al., 2013). Pilot- and full-scale applications are essential to evaluate the effectiveness and economy of fungal technology (Gao et al., 2010).

Although different reactors based on fungi have been designed and tested for the degradation of various hazardous substances from wastewater, their implementation at full-scale still needs to overcome several problems, e. g., bacteria contamination (Hu et al., 2020). Bioaugmentation has been pointed out as a promising strategy to improve bioreactor performance (Ferreira et al., 2020; Li et al., 2015; Shah, 2017). The main goals of the use of bioaugmentation are the possibility to increase the density of desirable microorganisms from wastewater and achieve efficient removal of

pharmaceutical substances (Hailei et al., 2017; Shah, 2017). Thus, the main tasks of the experimental setup were to investigate the effect on bioaugmentation for nutrient reduction and find the optimal performance of fluidized bed pelleted bioreactor for fungi.

To reach this goal, bioaugmentation experimental setup with periodical addition of 10 and 50 g of wet biomass from two fungal strains - *T. versicolor* a laboratory strain and *A. luchuensis* a wastewater isolate, was studied to investigate the effect on bioreactor performance. *T. versicolor* and *A. luchuensis* were selected for this study based on results from previous studies (Brigita Dalecka et al., 2020; Brigita; Dalecka et al., 2020) where these fungi have demonstrated a high removal efficiency of diclofenac and carbamazepine from non-sterile wastewater under batch-scale experiments. In order to better understand the performance of fluidized bed pelleted bioreactor, the pH level, laccase activity, and total microorganism cell number changes were observed for all incubation time of 28 days. The nutrient removal of total phosphorus (PO_4^{-3}—P), ammoniacal nitrogen (NH_4—N), nitrate (NO_3-N), nitrite (NO_2-N), and organic carbon (TOC) were analyzed.

3.1.1. Nutrient removal in fluidized bed pelleted bioreactor

The results from Fig. 2 present the removal efficiency of total NH_4-N, NO_3-N, NO_2-N, PO_4^{-3}-P, and TOC for an incubation time of 28 days. Results from *T. versicolor* and *A. luchuensis* with periodical addition of 10 and 50 g wet

biomass showed a decrease of NH_4—N from approx. 50 mg/L to < 1 mg/L after the incubation time of 26 days and more than 80 % reduction was seen at day 12. However, the negative control (i.e., bioreactor without the addition with fungal biomass) demonstrated the same removal efficiency of NH_4—N for all incubation time of 4 weeks (Fig. 2 A) indicating that there is no significant effect on NH_4-N removal by fungal bioaugmentation. On the contrary, the results of NO_3-N from *T. versicolor* and *A. luchuensis* with the periodical addition of 10 g wet biomass demonstrated the increase from < 1 mg/L to > 25 mg/L until the end of incubation time for 28 days (Fig. 2 B). On the other hand, the higher volume of biomass addition showed a decrease in NO_3-N concentration. Similarly, the concentration of NO_2-N an increase after incubation time of 26 days was observed from *T. versicolor* with the constant addition of 10 and 50 g wet biomass whereas *A. luchuensis* showed high reduction irrespective of biomass concentration (Fig. 2 C) illustrating the possible difference in the metabolic mechanisms and the effect on bioaugmentation in nitrogen removal from wastewater compare negative control.

According to literature (Wiesmann, 1994), the nitrogen can be dissolved in many kinds of wastewaters as NH_4-N, NO_3-N, and NO_2-N. During biological degradation, the organic nitrogen is transformed into ammonia (NH_4-N) where it can be oxidized to nitrite (NO_2-N) and later to nitrate (NO_3-N) by microorganisms (i.e., by nitrification and denitrification processes) (Wiesmann, 1994). Thus far, previous studies have shown that not only bacteria but also a wide variety of fungi can be involved in denitrification and

nitrification processes using nitrates, nitrites, and ammonium as a nutrient source (Sankaran et al., 2010; Shoun et al., 1992). Additionally, the results of this study demonstrate that *T. versicolor* and *A. luchuensis* has an effect on NO_3-N reduction from wastewater. This might be explained by pH and total microorganisms number changes during the incubation time (Fig. 3). The composition ratio of NH_4-N to NO_3-N mainly depends on the pH value in the wastewater (Luo et al., 2015; Mook et al., 2012), i.e., the higher the pH value, the higher proportion of NH_3-N - conversely, the NH_4-N proportion is higher at a lower pH value. The results showed that the addition of fungi biomass decreased and stabilized the total microorganism cells number in wastewater compared to negative control (Fig. 3 A, B, and E). Thus, the nitrification and denitrification processes were impacted by both fungi which might explain the difference of the NO_3-N and NO_2-N concentrations in wastewater compared to the control bioreactor.

Experimental results which are shown in Fig. 2 (C and D), reflect PO_4^{-3}-P and TOC removal. The results indicated that both fungi with periodical addition of 10 and 50 g wet biomass compared to negative control did not have a significant effect on PO_4^{-3}-P and TOC removal. Therefore, the results showed that there was no effect on fungal bioaugmentation on PO_4^{-3}-P and TOC reduction from municipal wastewater in fluidized bed pelleted bioreactor.

Overall, the removal of the organic loading and nutrients by fungi has not deeply studied under non-sterile wastewater conditions while lately carbon and nitrogen sources availability during bioreactor operation is reported as

one of the main limiting factors for fungi growth (Cruz del Álamo et al., 2020). Therefore, the finding of the optimal C:N:P ratio can play an important role in fungal wastewater treatment optimization and operation in fluidized bed pelleted bioreactor. Thus, the obtained results from this study were analyzed with AI web-based platform in order to indicate the optimal conditions for fungi in fluidized bed pelleted bioreactor compare to negative control based on the results from nutrient removal and changes in pH and total microorganism cell numbers for all incubation time of 4 weeks.

3.1.2. Data analysis with AI-based platform

In this study, the software xT smart_DoE as AI-based platform was used in order to analyze the obtained data during the bioaugmentation for incubation time of 28 days. The AI-based platform can be used in order to design, model, and optimize the experimental setup and pilot-scale tests. The main advantage of the AI-based platform is the ability to perform optimization in experimental setup and generate suggestions based on already obtained data from previous experiments. While there are several conventional design of experiments software tools (e.g., MODDE, Design Expert, Minitab, OriginLab, JMP), their lack at providing user friendly framework for optimizing multidimensional objectives that are time-series based is still missing. The software xT smart_DoE as AI-based platform for experiment analysis and optimization uses advanced search techniques and iterative Monte Carlo simulations to generate experiment suggestions that have high probability of satisfying not only multiple scalar objectives but also very complex time-

series, spatial or space-time-based objectives. Additionally, AI-based software supports time-series based variables for continuous control of bioprocesses.

The data analysis with an AI-based platform using ROI heatmap is presented in Fig. 4A. The ROI heatmap shows that that satisfactory operation for nutrient removal in fluidized bed pelleted bioreactor was achieved with *A. luchuensis* with addition of 10 g wet biomass while *T. versicolor* with addition 10 and 50 g of wet biomass ROI heatmap did not display as a promising fungal strain for bioreactor operation to reduce nutrients (Fig. 4 A). Furthermore, the AI-based platform highlighted that the negative control without fungal addition also is a promising strategy for reducing the nutrients and operate the bioreactor indicating that conventional biological wastewater treatment method is efficient for nutrient removal from municipal wastewater (Machineni, 2019). However, the main objective of using fungi for wastewater treatment is not focused to reduce the nutrient loads in this study rather improve the removal of pharmaceutical substances. Therefore, the removal of pharmaceuticals diclofenac (DIC), ketoprofen (KET), carbamazepine (CAR), ibuprofen (IBU), sulfamethoxazole (SULF), and metoprolol (MET) by *T. versisolor* and *A. luchuensis* were tested and analyzed in further study.

3.2. Pharmaceutical removal in fluidized bed pelleted bioreactor

The overall removal of DIC, KET, CAR, IBU, SUL, and MET by *T. versicolor* and *A. luchuensis* in fluidized bed pelleted bioreactor after final addition of wet biomass for both fungi is presented in Fig. 5. During the pharmaceutical removal, the reduction of nutrient loads was also analyzed (Fig. 6).

The results showed that there was no significant effect on DIC, CAR, and SULF reduction compared to the negative control (Fig. 5A, C, and E) for all incubation time of 7 days. For instance, the results of DIC removal demonstrated that there was no removal observed after an incubation time of 6 hours for both fungi and negative control (Fig. 5A). A similar tendency was observed for CAR and SULF when less than < 30 % of the removal was observed for both fungi and negative control (Fig. 5C and E) indicating that there was no effect on fungal addition for DIC, CAR, and SULF removal from wastewater.

The results of IBU removal have been presented in Fig. 5D. The results showed that *T. versicolor* and *A. luchuensis* with periodical addition with 50 g of wet biomass had relatively higher removal efficiency (> 30 % reduction from starting concentration) compared to the negative control and *T. versicolor* and *A. luchuensis* with periodical addition with 10 g of wet biomass after an incubation time of 6 hours. Furthermore, the results of KET and MET demonstrated the same tendency, i.e., the highest removal efficiency (> 90

%) was achieved with *T. versicolor* and *A. luchuensis* with periodical addition with 50 g of wet biomass (Fig. 5B and F) after an incubation time of 3 hours. The data analysis with AI-based platform presented the same tendency, i. e., *T. versicolor* and *A. luchuensis* with periodical addition with 50 g of wet biomass is the most promising strategy for removing the pharmaceuticals such as IBU, KET, and MET in fluidized bed pelleted bioreactor (Fig. 4B). Finally, the results of nutrient removal demonstrated a similar tendency to compare the results from the bioaugmentation investigation of incubation time for 4 weeks. For instance, *A. luchuenesis* showed reduction of nitrogen at 50 g biomass addition compare to control while TOC concentration increases because of the addition of pharmaceutical substances (Fig. 6). Thus, it can be stated that addition of fungi is not inhibiting the nutrient reduction and these results could be a promising indication that fungi could efficiently incorporate with microbial community in wastewater treatment process.

The results of pharmaceutical reduction from this study illustrated that the difference in removal efficiency by *T. versicolor* and *A. luchunesis*, can vary widely from one compound to another. Furthermore, the results showed that significantly higher periodical addition of fungal biomass, i.e., 50 g instead of 10 g, increases the removal efficiency for IBU, KET, and MET. During this study, the enzymatic activity of laccase in fluidized bed pelleted bioreactor was no detected for *T. versicolor* and *A. luchuensis* for both periodical addition of fungal biomass for all incubation time of 5 weeks. Therefore, it is believed that the main mechanism for removing the pharmaceuticals by *T. versicolor* and *A. luchuensis* was biosorption.

Sorption in wastewater treatment plant processes can be reasonably predicted by using the solid-water distribution coefficient (Kd value) (Ternes et al., 2004). If the Kd value of a pharmaceutical substance is relatively low, this substance is expected to occur mainly in the wastewater liquid phase and will not be sorbed to the microorganisms biomass (Baresel et al., 2015). The Kd values for all selected pharmaceuticals can be found in Table 1. The Kd value higher than > 2 has been reported for DIC, KET, IBU, and MET while the Kd value around 1 has been reported for CAR and SULF. Thus, the removal efficiency derived from this study did not correlate directly with Kd values. For instance, DIC has the highest Kd value (i. e., 2.7) compared to other pharmaceutical substances, however, the results from removal of DIC did not show any reduction (Fig. 5 A). Therefore, the results from this study highlight that the removal of pharmaceutical substances by fungi varies widely from one substance to another and physicochemical properties of the target molecule (i.e., functional group of the substance), enzymatic degradation, and interaction to fungal biomass are the main reasons for such variation (Yang et al., 2013).

Sorption processes in fungi can be presented as both absorption (entry of pharmaceuticals inside the biomass) and adsorption (adhesion of pharmaceutical to the biomass surface) (Lucas et al., 2018). So far, sorption processes in fungal treatment have been studied for textile dyes, personal care products, and some specific pharmaceuticals however, sorption evaluation was done indirectly (Lucas et al., 2018). Thus, in the future study,

the removal efficiency of pharmaceutical substances needs to be analyzed in both, wastewater and fungal biomass phase in order to better understand the role of the sorption and biodegradation mechanisms.

Finally, the data analysis with AI-based platform indicated that *A. luchuensis* can be a promising fungus for pharmaceutical removal and the optimal addition of the periodical biomass varies from 10 to 50 g of wet biomass while *T. versicolor* was not highlighted as a promising fungus for future study.

Contrary to the results derived from this study, the high removal efficiency for pharmaceutical removal by *T. versicolor* using fluidized bed pelleted bioreactor was achieved by Cruz-Morato et al (2013), for instance, the total removal of CAR was reached of incubation time for 2-3 days. However, during Cruz-Morato et al (2013) investigation, glucose and nitrogen were continuously supplied in a bioreactor in order to increase *T. versicolor* enzymatic activity (Cruz-Morató et al., 2013). Also, Hu et al. (2020) have shown *T. versicolor* as one of the most efficient fungus for degrading pharmaceuticals (Hu et al., 2020). However, *T. versicolor* compare to *A. luchuensis* uses enzymatic systems, including laccase for removing the pharmaceutical substances (Brigita Dalecka et al., 2020). As the laccase activity was not detected in this study, it is believed that the main mechanism for removing the pharmaceuticals was sorption. Therefore, the crucial step for applying *T. versicolor* and increase the metabolic activity for

removing the pharmaceutical substances from municipal wastewater is to find the optimal performance of fluidized bed pelleted bioreactor.

Overall, this is the first study where bioaugmentation with *A. luchuensis* for pharmaceutical removal in fluidized bed pelleted bioreactor has been reported as a promising biological treatment method. Further investigation using an AI-based platform needs to be used in order to reach high performance for the bioreactor operation and implementation in the wastewater treatment systems.

Conclusions

The effect on bioaugmentation for *T. versicolor* and *A. luchuensis* for removing the pharmaceutical substances from municipal wastewater has been tested and analyzed. Additionally, bioaugmentation with fungi has an effect on the reduction of nutrients in fluidized bed pelleted bioreactor. The removal of diclofenac, carbamazepine, and sulfamethoxazole demonstrated that there was no significant effect on the removal efficiency compared to control while the highest removal efficiency (> 90 %) for ibuprofen, ketoprofen, and metoprolol was gained by *T. versicolor* and *A. luchuensis* with periodical addition with 50 g of biomass after an incubation time of 3 hours. This is the first study where data analysis with an AI-based platform has been used. The data analysis indicated that *A. luchuensis* can be promising fungus for pharmaceutical removal and the optimal addition of the periodical biomass varies from 10 to 50 g of biomass. Overall, the obtained results and the use of an AI-based platform can be a promising approach for successful fungal optimization and operation in fluidized bed pelleted bioreactor for pharmaceutical substances removal.

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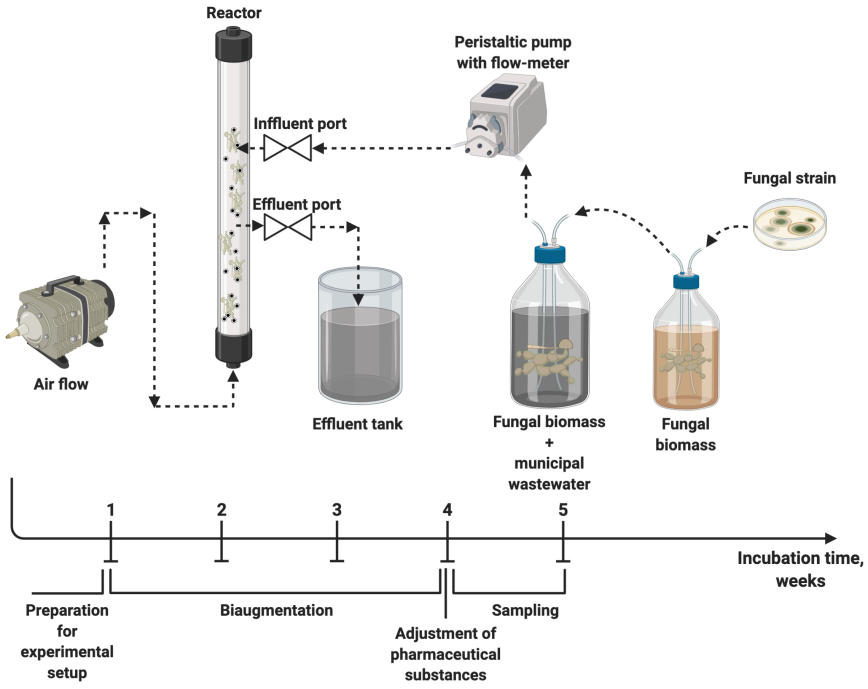

Figure 1. A conceptual scheme of a fungal fluidized bed pelleted bioreactor and experimental setup (created with BioRender.com).

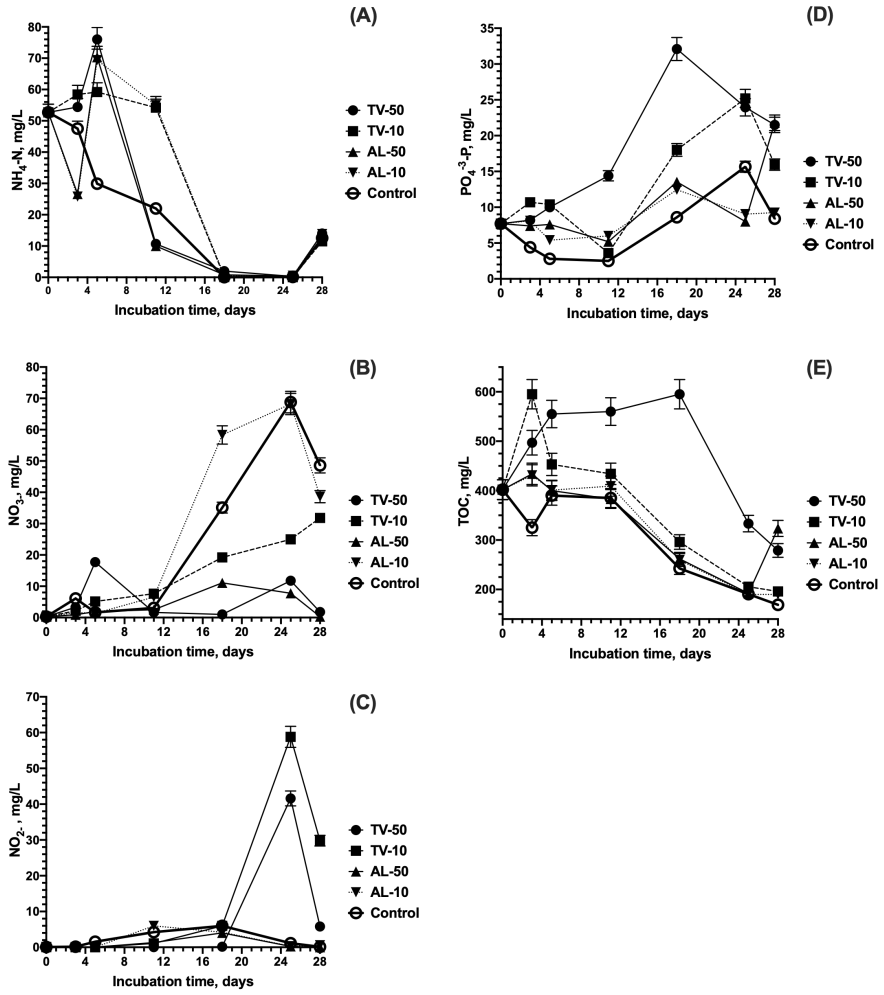

Figure 2. The reduction of **(A)** NH_4-N, **(B)** NO_3-N, **(C)** NO_2-N, **(D)** PO_4^{-3}-N, and **(E)** TOC by *T. versicolor* (TV) and *A. luchuensis* (AL) in fluidized bed pelleted bioreactor.

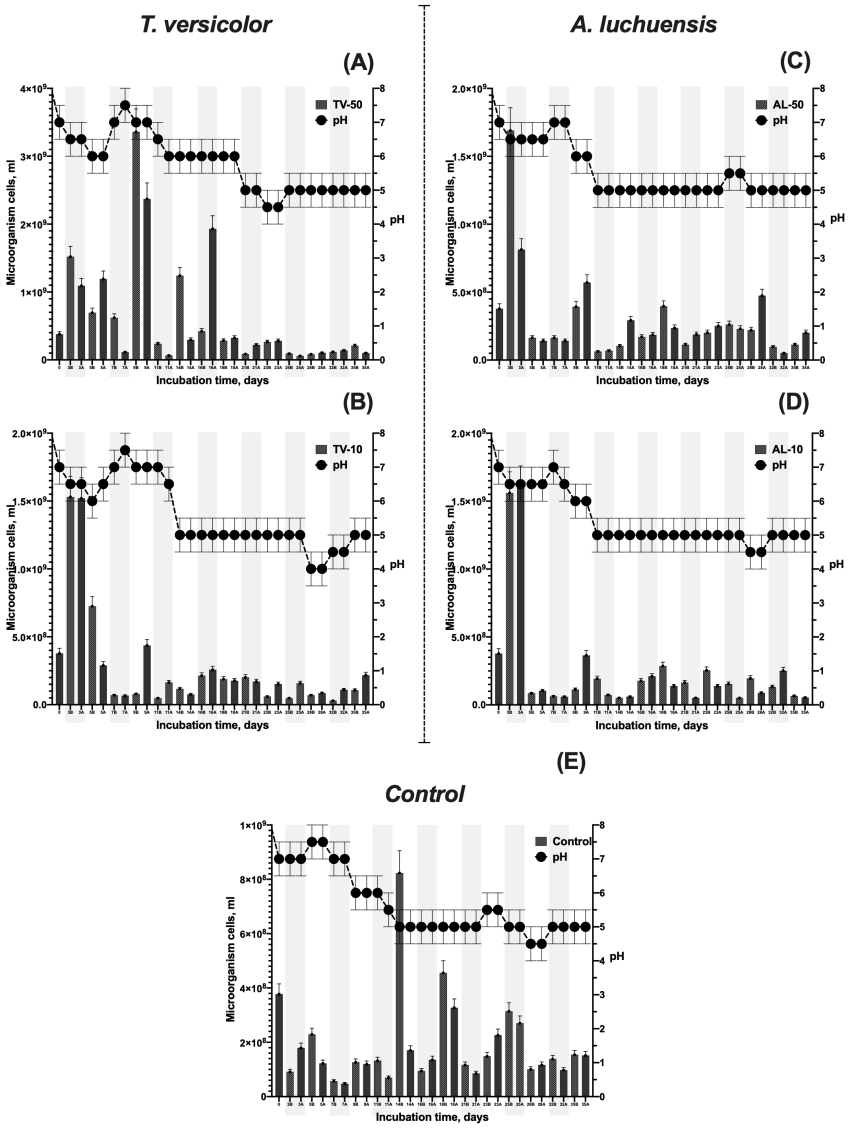

Figure 3. Bioaugmentation effect on total number of microorganism's cells and pH in a fluidized bed pelleted bioreactor with addition of **(A)** 50 g of *T. versicolor* wet biomass; **(B)** 10 g of *T. versicolor* wet biomass; **(C)** 50 g of *A. luchuensis* wet biomass; **(D)** 10 g of *A. luchuensis* wet biomass; **(E)** negative control column without fungal addition. The total microorganisms count and pH was obtained before (B) the addition of fresh non-sterile wastewater and after (A) the addition of 250 ml fresh non-sterile municipal wastewater.

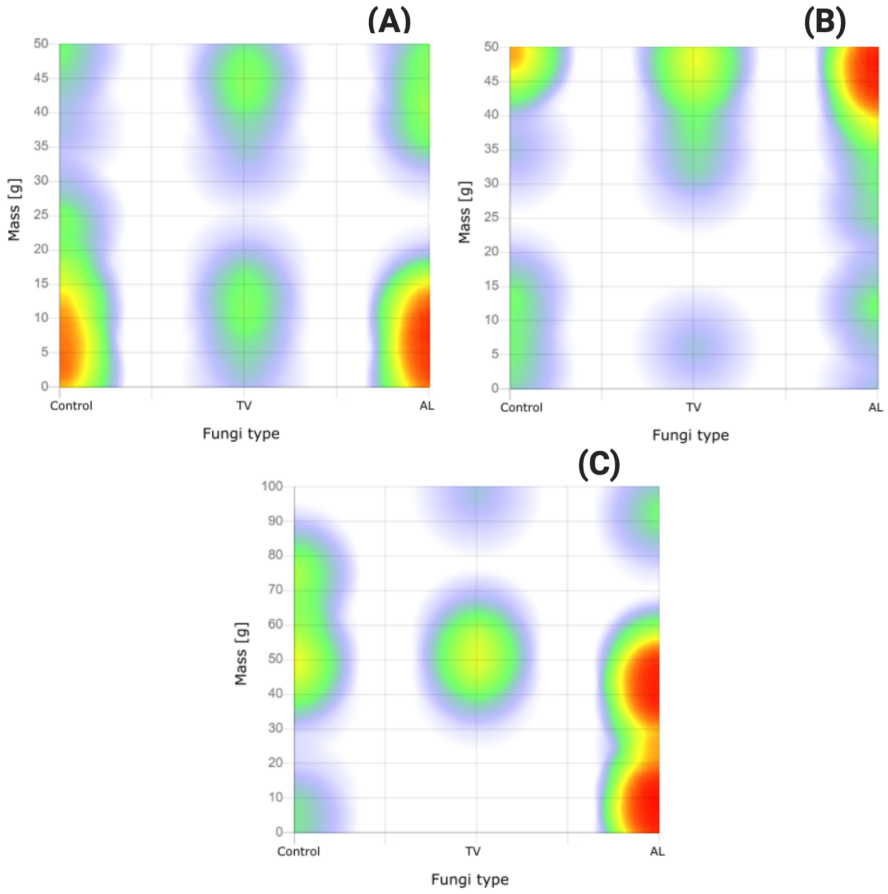

Figure 4. AI based platform analysis and comparison of ROIs for *T. versicolor* (TV), *A. luchuensis* (AL), and negative control without fungal biomass addition **(A)** after bioaugmentation; **(B)** after pharmaceutical removal; **(C)** after all incubation time of 35 days in order to model the optimal biomass addition and selection of fungi for fungal bed pelleted bioreactor optimal operation to remove pharmaceutical substances from wastewater (red - high, green – medium, blue – low, white - zero probability).

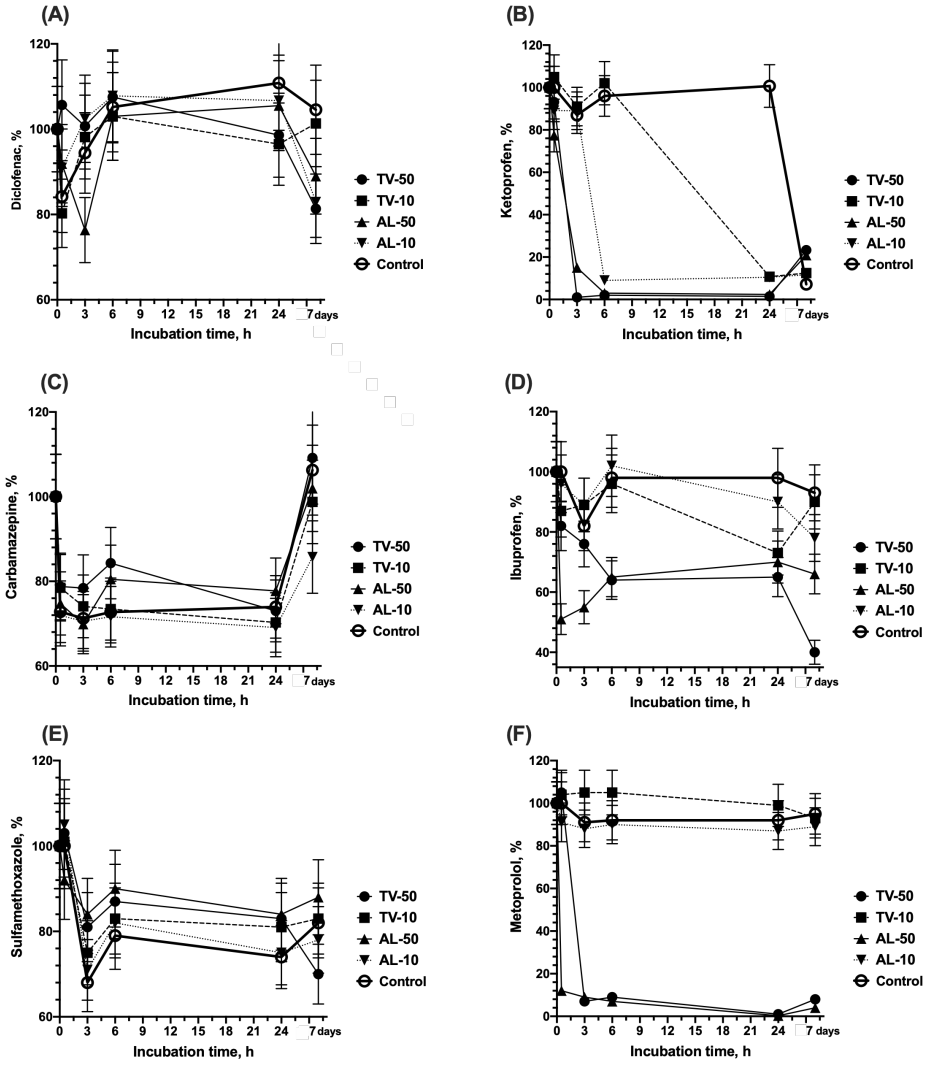

Figure 5. The removal efficiency (%) in fluidized bed pelleted bioreactor for incubation time of 7 days of pharmaceutical substances **(A)** diclofenac, **(B)** ketoprofen, **(C)** carbamazepine, **(D)** ibuprofen, **(E)** sulfamethoxazole, and **(F)** metoprolol by *T. versicolor* (TV) and *A. luchuensis* (AL) after 24 h of the final addition of 10 and 50 g wet biomass.

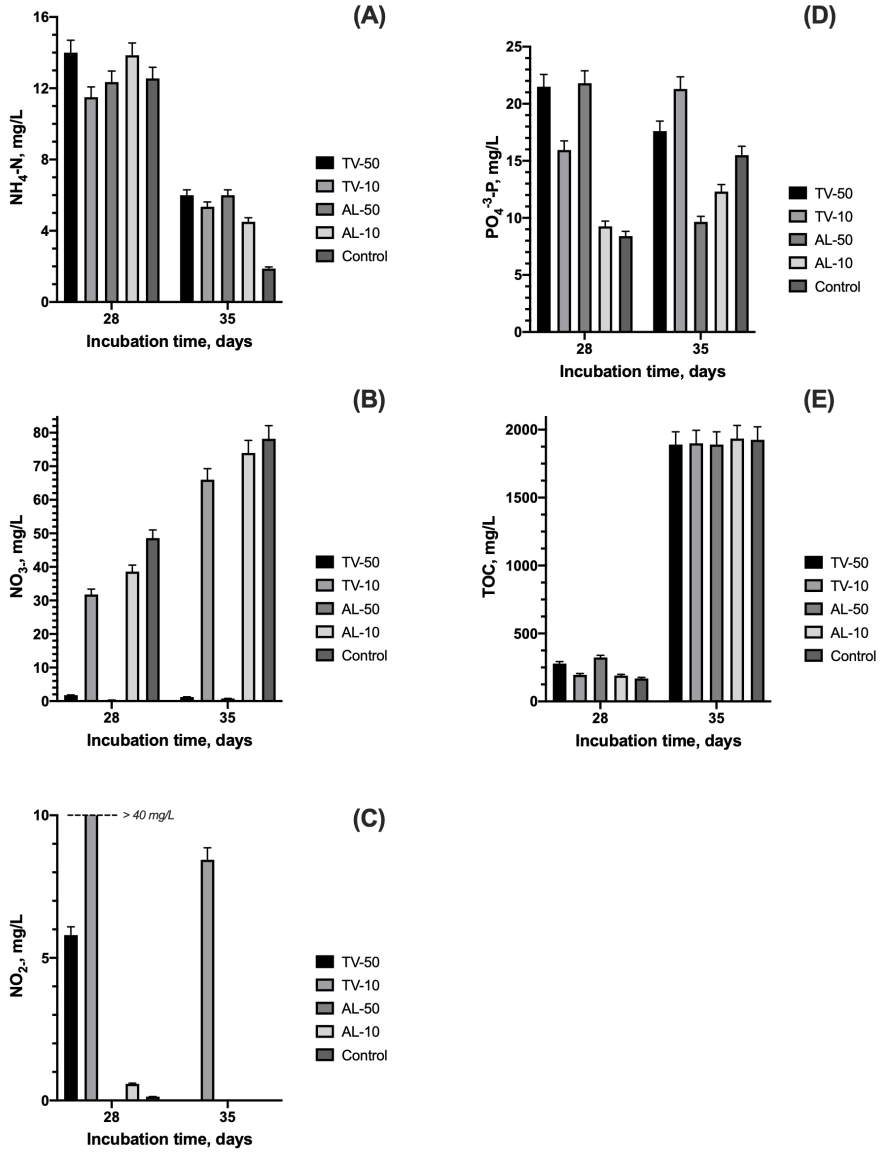

Figure 6. The reduction of **(A)** NH_4-N, **(B)** NO_3-N, **(C)** NO_2-N; **(D)** PO_4^{-3}-P; and **(E)** TOC by *T. versicolor* (TV) and *A. luchuensis* (AL) in fluidized bed pelleted bioreactor after addition of pharmaceutical substances and bioaugmentation.