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Fungal-based wastewater treatment: pharmaceutical removal and nutrient release from a pellet system

Malin Hultberg [a,*], Tsz Yung (Patrick) Wong [b], Bent Speksnijder [b], Oksana Golovko [b]

[a] Department of Biosystems and Technology, Swedish University of Agricultural Sciences (SLU), Alnarp, 234 56, Sweden
[b] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences (SLU), 750 07, Uppsala, Sweden

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ABSTRACT

In this study, we evaluated the use of fungal pellets produced from commercially available mushroom spawn of two white-rot fungi (*Pleurotus ostreatus* and *Trametes versicolor*) for removing pharmaceuticals from municipal wastewater. The impact of the treatment on 33 pharmaceuticals in wastewater sampled from various process stages, including after primary and secondary clarification and tertiary treated effluent, was evaluated. Treatments were tested for up to 72 h, with monitoring of pharmaceutical concentrations, laccase activity, and nutrient levels. High removal efficiencies, exceeding 90 %, were achieved within 24 h of treatment, including for several pharmaceuticals prioritized under the revised Urban Wastewater Treatment Directive. Removal performance remained high in the wastewater collected after primary clarification, demonstrating that the spawn-based fungal pellets remained functional in this complex wastewater matrix. This finding is of interest as the treatment resulted in increased levels of organic carbon and total phosphorus, highlighting the need for downstream management or process integration. Integration early in the treatment process, coupled with strategies for biomass valorization and nutrient control, could enhance its application in municipal wastewater management. Our results support the feasibility of fungal pellet treatment as a sustainable option for pharmaceutical removal. Further research is needed to address transformation products, economic viability, and large-scale deployment.

1. Introduction

Water is fundamental to all life on Earth, yet its quality is increasingly compromised by the combined pressures of climate change, population growth, and intensive chemical use. While conventional wastewater treatment plants (WWTPs) are highly effective at removing biodegradable organic matter and plant nutrients such as nitrogen and phosphorus, they exhibit limited and variable removal efficiencies for complex organic micropollutants—most notably pharmaceuticals (Golovko et al., 2021) Reflecting growing environmental and public health concerns, the recently revised Urban Wastewater Treatment Directive mandates an 80 % reduction of several high-priority pharmaceuticals (EU, 2024), underscoring the urgent need to develop and implement complementary treatment strategies.

Bioremediation has emerged as a promising, sustainable, and cost-effective approach for the removal of persistent organic pollutants from wastewater (Mohammadi et al., 2022; Unuofin et al., 2019; Arregui et al., 2019; Sá et al., 2022). Among biological systems, white-rot fungi (WRF) have garnered particular attention due to their exceptional ability to degrade lignocellulosic materials. This capability is largely attributed to their secretion of nonspecific oxidative enzymes, such as laccases and peroxidases, which can break down a wide range of recalcitrant organic molecules, including pharmaceuticals (Zhou and Fan, 2021).

From an application standpoint, several strategies have been explored to harness the enzymatic potential of WRF for water treatment. One approach involves the direct use of isolated fungal enzymes, although this method is often limited by high extraction costs and rapid enzyme deactivation in complex water matrices (Arregui et al., 2019; Sodhi et al., 2024). Alternatively, spent mushroom substrate from commercial mushroom cultivation has been investigated as a low-cost, circular source of ligninolytic enzymes (Yang et al., 2021; Ghose et al., 2024). This approach has the advantage of valorising a waste source; however, fast washout of the enzymes has been demonstrated (Hultberg and Golovko, 2024). The use of a growing fungal culture, producing the enzymes *in-situ*, has been explored in several studies (Zhou and Fan,

* Corresponding authors.
E-mail address: malin.hultberg@slu.se (M. Hultberg).

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2021). The challenge, however, is fungal survival and growth. Most WRF are aerobic terrestrial organisms, and optimization is required to enhance their enzyme production in unfamiliar habitat, as submerged in wastewater.

When grown under submerged conditions, some filamentous fungi form pellets composed of a mycelial network. This type of fungal pellet has been highlighted as an interesting strategy for water treatment (Pereira et al., 2021; Negi and Das, 2023; Zhang et al., 2024) and has benefits in terms of removal of the biomass after treatment compared to systems based on dispersed microbial growth (Ferreira et al., 2020). The pellets can be composed purely of mycelium, or a co-substrate can be included as a carrier (Gutiérrez-Quirós et al., 2024). In a previous study, it was demonstrated that mushroom spawn, the starting material used in mushroom production, could be used as a fungal inoculum to produce pellets in water and to obtain laccase production *in-situ* (Hewage et al., 2025). The use of spawn, a well-known and commercially available product (Balan et al., 2022), offers a straight-forward and applied approach for inoculation of a viable fungal culture into water. Kraft lignin, a waste product from the production of pulp, was observed to be a suitable inducer of laccase activity in these spawn-based pellets, with lignin accumulating in fungal mycelium growing out of the spawn (Hewage et al., 2025).

The aim of this study was to evaluate the use of fungal pellets produced from commercially available mushroom spawn of two white-rot fungi (*Pleurotus ostreatus* and *Trametes versicolor*) for removing pharmaceuticals from municipal wastewater. The performance of the spawn-based pellet was assessed in wastewater collected from three stages of treatment: after primary clarification, after secondary clarification, and in tertiary-treated effluent. Additionally, this study evaluated the impact on nutrients and organic load of the fungal treatments. This aspect is considerably less studied compared to the impact of fungal treatments on organic micropollutants despite that removal of plant nutrients and biodegradable organic matter is indispensable at the WWTPs. To assess the feasibility of integrating fungal pellets treatment into existing wastewater treatment frameworks this knowledge is essential.

While both *P. ostreatus* and T. *versicolor* are well-known ligninolytic fungi and fungal pellet formation is a well-established concept, the novelty of this study lies in the development and validation of a spawn-based inoculation approach for real wastewater treatment. Commercial mushroom spawn is a standardized and scalable material, but its use as a direct inoculum for wastewater bioremediation has not been comprehensively evaluated under non-sterile, complex matrices representing different stages of municipal treatment. By using real wastewater without spiking or sterilization, this study bridges the gap between laboratory findings and practical application. The investigation focuses on demonstrating that viable fungal pellets can be generated directly from commercial spawn, that these pellets remain enzymatically active in real wastewater, and that they sustain broad-spectrum pharmaceutical removal. Thus, the novelty lies primarily in aspects related to operational simplicity, system resilience, and scalability rather than in introducing new fungal strains or pelletization techniques. To provide a comparative understanding, both *P. ostreatus* and *T. versicolor* were tested under identical conditions. The two species were studied individually, and no interaction or co-cultivation effects were investigated in the present work.

2. Materials and methods

2.1. Wastewater

Wastewater samples were collected from two municipal WWTPs. The WWTPs had similar processes including an initial separation step, primary clarification, activated sludge, secondary clarification, and precipitation of phosphates with $FeCl_3$ before the release of the wastewater to the catchment. In the first experiment, effluent wastewater was collected from a municipal WWTP treating 200 000 population equivalents (PE). In the second experiment, wastewater was collected after the primary clarification and after the secondary clarification from a municipal WWTP treating 120 000 PE. The samples from both locations were collected in 1 L high-density polyethylene containers pre-washed with methanol. Samples were transported cold, stored at 4 °C until use in the experiments, which were performed within one week.

2.2. Production of spawn-based fungal pellets

Grain spawn of the WRF *P. ostreatus* M2191 and *T. versicolor* M9911 were obtained from Mycelia BVBA, Belgium. The spawn of these two WRF was produced with the same recipe and at the same time (personal communication, Magda Verfaillie, CEO Mycelia BVBA, Belgium). The fungal pellets were then developed in wastewater, based on grain spawn (40 g/L fresh weight), and lignin (4 g/L of kraft lignin, Sigma-Aldrich 370,959) was added. The production of pellets is described in detail in Hewage et al. (2025).

2.3. Experimental set-up

The experiments were performed in Erlenmeyer glass flasks on a horizontal orbital shaker (VWR, Advanced 5000 Shaker, Radnor, PA, USA) operating at 100 rpm at room temperature (20–22 °C). In the first experiment, spawn of *P. ostreatus* or *T. versicolor* and lignin were added to wastewater effluent as described above (Fig. S1). After three days (72 h), the liquid phase and the solid phase (the fungal pellets with accumulated lignin) were separated by filtration using a nylon filter (mesh size 800 μm). In the second experiment, spawn of *P. ostreatus* and lignin were added to two different municipal wastewater samples, collected directly post-primary clarification and post-secondary clarification. Samples were taken after 24 h, 48 h, and 72 h, and the liquid and solid phases were separated as described above. For each experiment, control samples of wastewater were prepared without fungal pellets. Control samples were analyzed immediately (0 h) to determine the initial pharmaceutical concentrations. The controls were not incubated over time, as the objective was to assess the removal efficiency of fungal treatment relative to the untreated wastewater baseline.

The samples were analysed for pharmaceuticals, pH, chemical oxygen demand (COD) or total organic carbon (TOC), total nitrogen (TN), ammonium (NH_4—N), total phosphorus (TP), and laccase activity. The analyses are described below. Analysis of laccase activity, pH, COD or TOC, TN, NH_4—N, and TP was performed directly on the liquid samples. For the pharmaceutical analysis, the samples were frozen (−20 °C) until analysis, which was performed within one week. The harvested fungal pellets were freeze-dried before the extraction of pharmaceuticals.

2.4. Analysis

2.4.1. Organic pollutant analysis

Standards, reagents, and chemicals: Reference standards were purchased from Sigma-Aldrich (Sweden), 33 compounds were analyzed including eight compounds, which are relevant in the revised Urban Wastewater Treatment Directive. The analyzed compounds are described in the supplementary material (Table S1). Isotopically labelled internal standards were purchased from Wellington laboratories (Canada), Teknolab AB (Kungsbacka, Sweden), Sigma-Aldrich, and Toronto Research Chemicals (Toronto, Canada). All analytical standards were of high analytical grade (>95 %). The studied pharmaceuticals were selected for analysis based on occurrence and distribution in the aquatic environment and production and consumption patterns (Malnes et al., 2022).

Water samples (200 mL) were extracted by solid-phase extraction (SPE) using Oasis HLB-cartridges (6 mL, 60 mg, 30 μm, Waters Oasis, MA, USA) according to the procedure described by Sörengård et al. (2019). In brief, aliquots of 200 mL for each water sample were transferred to pre-rinsed (methanol) 1-L PP bottles. Each sample was spiked

with 20 ng of the internal standards mixture. All SPE cartridges were pre-conditioned with 6 mL methanol followed by 6 mL Millipore water by gravity. The samples were loaded onto the SPE reservoirs and loaded on the SPE cartridges at a rate of approximately one droplet per second. The SPE cartridges were dried and subsequently eluted two times with 4 mL methanol into 15 mL PP tubes (Corning™). All eluted samples were evaporated under a gentle stream of nitrogen gas to a volume of 0.5 mL. The extracts were then transferred to 1.5 mL auto-injector glass vials (Eppendorf, Germany), and the walls of the PP tubes were rinsed three times with 200 μL methanol, which were transferred to the same vials. The extracts were fortified with 0.5 mL Millipore water and vortexed for 30 s before injection.

For the first experiment, the freeze-dried fungal pellets were extracted using a previously validated method published elsewhere (Kodešová et al., 2019). Of the 33 target compounds, only 5 were detected in the pellet samples. Therefore, in the second experiment, the analyses were focused exclusively on the liquid phase.

All samples were analyzed using a DIONEX UltiMate 3000 ultra-performance liquid chromatograph (UPLC) system (Thermo Scientific, Waltham, MA, USA) coupled to a triple quadrupole mass spectrometer (MS/MS) (TSQ Quantiva, Thermo Fischer Scientific, Waltham, MA, USA). The data were evaluated with Tracefinder 4.1 (Thermo Fischer Scientific, MA, USA).

The calculated removal efficiency of the compound in the aqueous phase during the experiment was calculated using the following equation:

$$\text{Removal efficiency } (\%) = \left(\frac{(C_0 - C)}{C_0}\right) X\ 100\%$$

where C_0 corresponds to the initial concentration of compound (ng/L), and C represents the residual amount of compound (ng/L).

2.4.2. Analysis of nutrients and organic load

The Hach (https://se.hach.com/) spectrophotometric system (DR6000) and LCK-kits were used to determine the impact of nutrients (TN, NH_4–N, TP) and organic load (COD/TOC).

2.4.3. Enzyme activity

Laccase activity was determined in the wastewater by detecting the oxidation product 2,6-dimethoxyphenol (DMP), as described by Parenti et al. (2013). The reaction mixture contained 0.45 mL of sample, with appropriate dilution, and 0.5 mL of 10 mM DMP in 100 mM acetate buffer (pH 5). Absorbance was measured at 468 nm, and one unit (U) of enzyme activity was defined as the formation of 1 μmol of product per min. Manganese peroxidase (MnP) activity was initiated by the addition of H_2O_2, as described by Field et al. (1996), with peroxidase activity corrected for background laccase activity.

2.4.4. Statistical analysis

Both experiments had three replicates per treatment. Statistical analyses were carried out using Minitab version 2020, and data were tested for significant differences ($p < 0.05$) using ANOVA and Tukey's post-hoc test, and *t*-test. Values presented are mean ± standard deviation (std).

3. Results and discussion

3.1. Impact of treatment with fungal pellets on pharmaceuticals in municipal wastewater

In this study, we used real municipal wastewater, without spiking, and all analysed pharmaceuticals were detected above the limit of quantification. In the first experiment, the total concentration of the 33 pharmaceuticals in the effluent wastewater, prior to fungal treatment, was 16.4 ± 2.0 μg/L. The dominant therapeutic classes included diuretics, antihypertensive agents, and non-steroidal anti-inflammatory drugs (NSAIDs) (Fig. 1).

Treatment with WRF pellets, of *P. ostreatus* or *T. versicolor*, for 72 h under shaking conditions, resulted in substantial reductions in pharmaceutical concentrations. Mean removal efficiencies were 91 ± 1 % for *P. ostreatus* and 87 ± 1 % for *T. versicolor*, with no statistically significant difference between the two treatments. The fungal species exhibited broadly similar removal profiles across therapeutic classes (Fig. 1), suggesting their comparable efficiency. It is important to note that these values refer to the disappearance of the parent compounds. Transformation products (TPs) were not analyzed in this study, which represents a limitation when evaluating the overall environmental safety of the fungal treatment. Previous studies have shown that certain fungal-mediated degradation processes can yield intermediate products with altered, and sometimes increased, toxicity (Jaén-Gil et al. 2019; Maculewicz et al., 2022). Therefore, while the observed reductions in parent pharmaceutical concentrations indicate strong degradative capacity, the environmental benefit of the treatment must be confirmed through identification and toxicity assessment of TPs. Future work will apply high-resolution mass spectrometry coupled with bioassays to characterize TPs and evaluate the net toxicity of treated effluents. It should also be pointed out that although both fungi showed broad-spectrum removal, a few species-specific differences were observed for select compounds (Table S3). *P. ostreatus* achieved significantly higher removal of the compounds hydrochlorothiazide and oxazepam, with hydrochlorothiazide being removed by 84 % while oxazepam was reduced below its detection limit. The significant difference between the species in removal of diuretics (Fig. 1) is due to the higher removal of hydrochlorothiazide by *P. ostreatus*. In the treatment with *T. versicolor,* the removal of hydrochlorothiazide was approximately 70 % compared to the control. On the other hand, *T. versicolor* demonstrated significantly higher removal of valsartan with concentration of this compound being below detection limit after treatment. Still, in the *P. ostreatus* treatment, valsartan was also removed to a high extent (82±9 %). Variations in the degradation spectrum of different WRF has been highlighted in the study of Kózka (2023). As discussed in that study, these differences suggest the potential for species-specific optimization depending on the pharmaceutical composition of the wastewater. Additionally, a combination of WRF species is an option. This is exemplified in the study by Vasiliadou et al. (2016), which demonstrated that *T. versicolor* and *Ganoderma lucidum* individually removed several pharmaceuticals, and their combined use further enhanced the removal of more persistent compounds up to 41 %.

Laccase activity in the treated wastewater was considerably higher in the treatment with *P. ostreatus* pellets (550 U/L) compared to *T. versicolor* (300 U/L) (Table 3). Minor activity of MnP (< 10 U/L) was detected only in the treatment with *T. versicolor*. Laccase-based biocatalytic systems have been shown to effectively remove highly persistent pharmaceuticals, including carbamazepine (Ferrari et al., 2003), diclofenac (Younes et al., 2019; Hultberg et al., 2020), and different estrogen hormones (Lloret et al., 2010). Laccases remove pharmaceuticals through their strong oxidative potential, which facilitates a one-electron oxidation process (Mehra et al., 2018). During this process, one electron is removed from substrate molecule (either pharmaceutical or mediator) to the laccase active site and form a highly reactive radical (Aza and Camarero, 2023). The pharmaceutical radical can be degraded by multiple pathways, including oxidative coupling, oxidative cleavage, bond breakage, oxygenation (such as formation of hydroxyl and carbonyl), demethylation, dehalogenation (Kozka et al., 2020; Schultz et al., 2001; Singh et al., 2025). Alternatively, the mediator radical can act as an electron shuttle from laccase to pharmaceutical, transferring the oxidizing power of laccase to other persistent pollutants (Asif et al., 2017).

While the high laccase activity measured in the aqueous phase indicates that oxidative enzymatic degradation played a central role in pharmaceutical removal, the results cannot establish a direct causal relationship. Other ligninolytic enzymes, such as peroxidases, may have

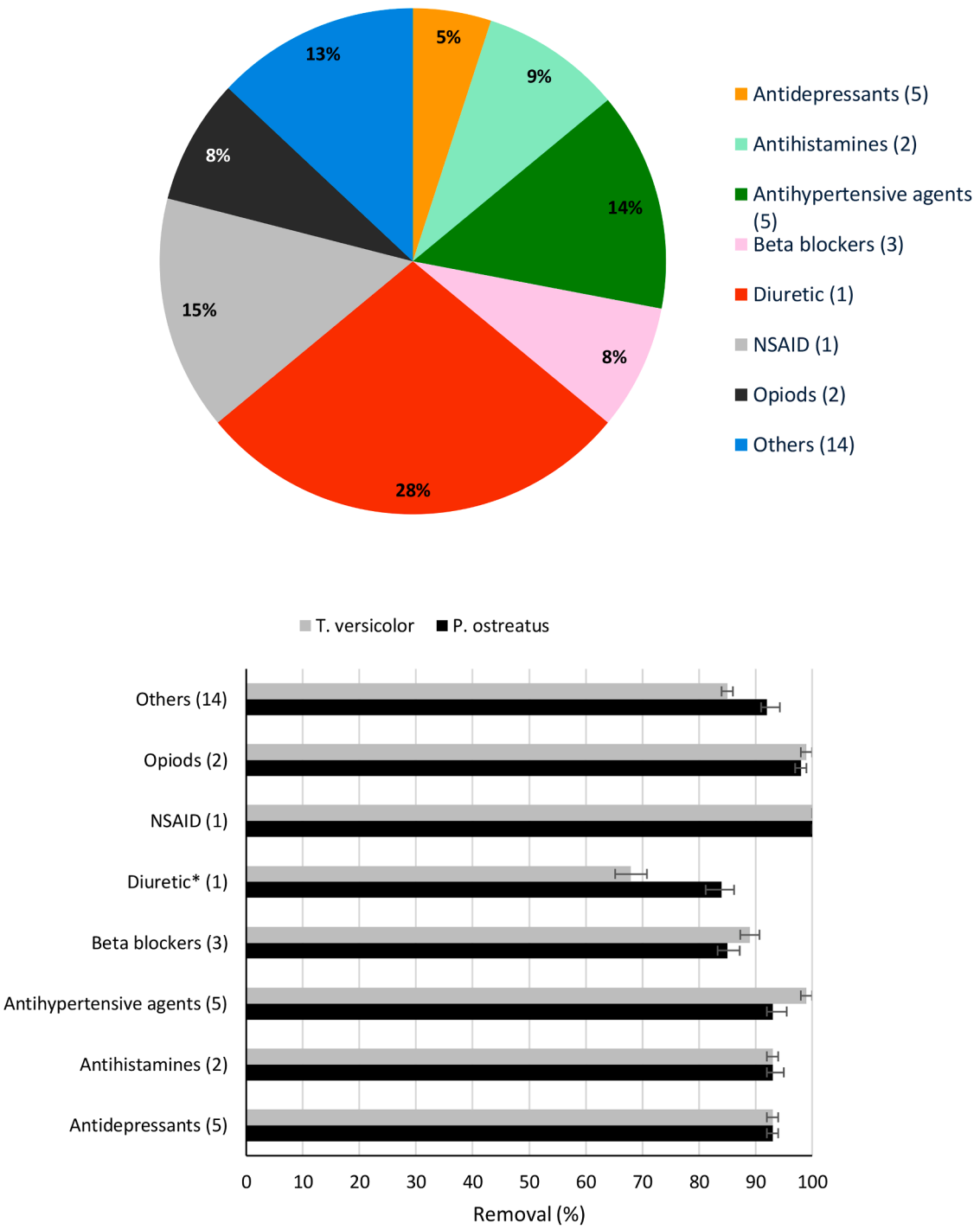

Fig. 1. Dominant therapeutic classes in municipal effluent wastewater and their removal (%) after 72-hour treatment with fungal pellets of *Pleurotus ostreatus* or *Trametes versicolor*. The asterisk (*) indicate significant difference ($p < 0.05$) between the species in degradation of the therapeutic class. Values in parentheses indicate the number of compounds per group (see Table S2).

contributed to the degradation. For example, Wen et al. (2009) demonstrated the degradation of two antibiotics mediated by manganese peroxidase and lignin peroxidase. In comparison to laccase, peroxidases require hydrogen peroxide as a substrate to perform the oxidation–reduction reaction. Manganese peroxidase specifically oxidises Mn^{2+} into Mn^{3+} using the ferric heme group. Mn^{3+} is consequently used as a non-specific redox mediator, creating phenolic, amino and non-phenolic aromatic radicals, promoting the degradation of persistent pharmaceuticals (Saikia et al., 2023). Lignin peroxidase functions in a similar fashion, however it utilises a small molecule, such as veratryl alcohol, as a redox mediator instead of Mn^{3+} (Janusz et al., 2017). In addition, non-enzymatic processes, including radical-mediated oxidation or limited sorption–desorption dynamics, may also influence observed removal patterns. Future studies are needed to quantify the relative contribution of different mechanisms.

Fungal pellets recovered after treatment weighed (dry weight) 24.7 ± 0.3 g/L (*P. ostreatus*) and 23.2 ± 0.5 g/L (*T. versicolor*). Upon extraction, five pharmaceuticals were detected in the freeze-dried biomass of both treatments, with fexofenadine concentrations significantly higher in *T. versicolor* pellets. This may reflect differences in

sorption capacity or intracellular degradation between the species. Sorption to the pellets accounted for 8.3 ± 2.0 % (*P. ostreatus*) and 11.7 ± 2.7 % (*T. versicolor*) of total pharmaceutical removal. While sorption to biomass was a minor contributor overall, it played a key role for specific compounds; for instance, 84 % of fexofenadine and 73 % of cetirizine removal in the *T. versicolor* treatment was attributed to pellet-associated removal (Table 1). From an environmental management perspective, the ability to capture and remove residual pharmaceuticals within fungal biomass opens pathways for sustainable end-of-life handling. Harvested pellets could be subjected to pyrolysis for biochar production, aligning with circular economy principles by converting biomass waste into a value-added product.

A second experiment assessed fungal treatment efficiency on municipal wastewater sampled after the primary and secondary clarification steps. The composition of therapeutic classes remained largely consistent across the sampling sites (Fig. 2). The total pharmaceutical load of the untreated wastewaters was 27.0 ± 3.5 μg /L (post-primary clarification) and 23.1 ± 0.8 μg/L (post-secondary clarification).

Given the high removal observed after 72 h in the first experiment, temporal dynamics were assessed in the second experiment by sampling at 24, 48, and 72 h. Results showed that most of the pharmaceutical removal occurred within the first 24 h, with no significant improvement from extended incubation (Fig. 3). After 24 h, removal efficiency was 92 ± 2 % in primary-clarified wastewater and 93 ± 1 % in secondary-clarified wastewater. Laccase activity was slightly lower in primary wastewater, likely due to elevated organic loads and microbial competition (Espinosa-Ortiz et al., 2016). Nonetheless, removal performance remained high, demonstrating that the spawn-based fungal pellets remained functional in this complex wastewater matrix. As shown in Fig. 3, laccase activity did not exhibit statistically significant changes between 48 and 72 h in the second experiment. These findings are consistent with previous studies on this species reporting stable enzyme activity over time in batch bioreactors (Kózka et al., 2020).

Of the 33 pharmaceuticals, 8 are prioritized under the revised Urban Wastewater Treatment Directive (EU, 2024): hydrochlorothiazide, metoprolol, carbamazepine, citalopram, diclofenac, clarithromycin, venlafaxine, and irbesartan. These were detected at comparable concentrations across all wastewater types, underlining their persistence and environmental relevance (Table 2). With the exception for carbamazepine, removal exceeded 80 % for these compounds following 24-hour treatment with pellets of *P. ostreatus* (Fig. 4). Carbamazepine was removed by 63 % in the wastewater collected after the primary clarification step and 76 ± 3 % in the wastewater collected after the secondary clarification step. Besides this compound, the removal efficiencies of the prioritized pharmaceuticals were similar in the different wastewaters. Prolonged treatment offered only a small improvement (Fig. 4), confirming the practicality of a 24-hour treatment window.

3.2. Impact of treatment with fungal pellets on nutrients and organic load of municipal wastewater

Numerous studies (Espinosa-Ortiz et al., 2016; Torán et al., 2017; Cruz-Morató et al., 2014; Hu et al., 2021) have investigated fungal pellet reactors for treating various wastewaters (e.g., municipal wastewater, hospital effluents), evaluating the removal of organic contaminants under both sterile and non-sterile conditions. However, these studies often did not describe or omitted information on nutrient levels and organic loads, which are critical factors influencing treatment performance. Still, for nutrient-rich wastewaters from the food industry, i.e. breweries or dairies, the use of fungal treatment has been put forward as an option for nutrient removal (Bansfield et al., 2024; Timm et al., 2024). This would allow for a circular approach where nutrients in the water are consumed and used for the production of fungal biomass, which is easily harvested and have a wide range of different usages (Sar et al., 2024).

Nutrient removal after fungal treatment have also been demonstrated in municipal wastewater when the fungi is introduced as a washed mycelium (Dalecka et al., 2020). In the present study, the fungi were inoculated on a nutrient-rich plant material (grain) and lignin was added to stimulate laccase production. Thus, a considerable amount of nutrients was added together with the fungus. This is reflected in our finding of a high impact of the fungal treatment on COD in the effluent wastewater with an increase from 20–30 mg/L up to >1000 mg/L (Table 3). This increase is most likely mainly explained by the presence of lignin and its residues in the water and is an evident disadvantage considering treatment of effluent wastewater.

Also, exudates from the grain spawn, which will be partly degraded during fungal growth, will impact COD levels. Exudates from the grain spawn can also be expected to increase nitrogen and phosphorus levels in the water, which agrees with the finding of an impact on TN and TP after treatment (Table 3). TP increased significantly in both treatments, with a very high increase observed for the treatment with *T. versicolor*. Considering that the spawn of *P. ostreatus* and *T. versicolor* were produced using the same recipe, the difference in TP is probably related to variances in how the fungal species degrade the grain during their colonization. Overall, the treatment with the *P. ostreatus* is favourable in regard to the lower impact of TP in the wastewater. Additionally, the NH_4—N concentrations showed a significant increase after treatment with *T. versicolor* and, on the other hand, a significant decrease after treatment with *P. ostreatus* (Table 3). An interpretation could be that NH_4—N was consumed during fungal growth, indicating that *P. ostreatus* grew better in the effluent wastewater compared to *T. versicolor*.

Still, the fact remains that the impact on organic load and plant nutrients by the fungal treatment was considerable in the first experiment. This implies that additional treatment must be applied after removal of the fungal pellet, which would increase treatment costs and time substantially. We therefore applied fungal treatment to

Table 1
Five of the analysed pharmaceuticals were detected in the fungal pellets after harvest and their concentrations (ng/g dry weight (dw)) are presented below. Removal (%) of the pellets (Pellets) corresponds to the amount detected in the pellets compared to the amount removed from the wastewater for the specific compound. The unit for comparison is 1 L and the weight of pellets were 24.7 ± 0.3 g/L (dw) for *Pleurotus ostreatus* and 23.2 ± 0.5 g/L (dw) for *Trametes versicolor*. Total removal (%) corresponds to the removal after treatment of the specific compound, based on analysis of the wastewater before and after harvest. Mean±std, $n = 3$.

Pharmaceutical	*Pleurotus ostreatus*			*Trametes versicolor*		
	Concentration (ng/g dw)	Removal (%)		Concentration (ng/g dw)	Removal (%)	
		Pellets	Total		Pellets	Total
Metoprolol	15.6 ± 4.2a*	54±12	91±2	15.0 ± 1.0a	50±5	88±1
Cetirizine	20.7 ± 3.5a	53±12	92±3	34.3 ± 8.5a	84±11	92±1
Bicalutamide	5.4 ± 2.0a	36±21	95±1	7.3 ± 1.4a	44±4	91±2
Bisoprolol	5.3 ± 2.0a	62±24	94±1	6.5 ± 1.4a	73±16	92±1
Fexofenadine	2.5 ± 0.0a	14±1	96±1	6.6 ± 0.9b	32±4	97±1
Sum	49.6 ± 5.5a	44±4	93±1	70.8 ± 9.4a	60±8	92±1

*Values followed by different letters indicate significant difference between the fungal species ($p \leq 0.05$).

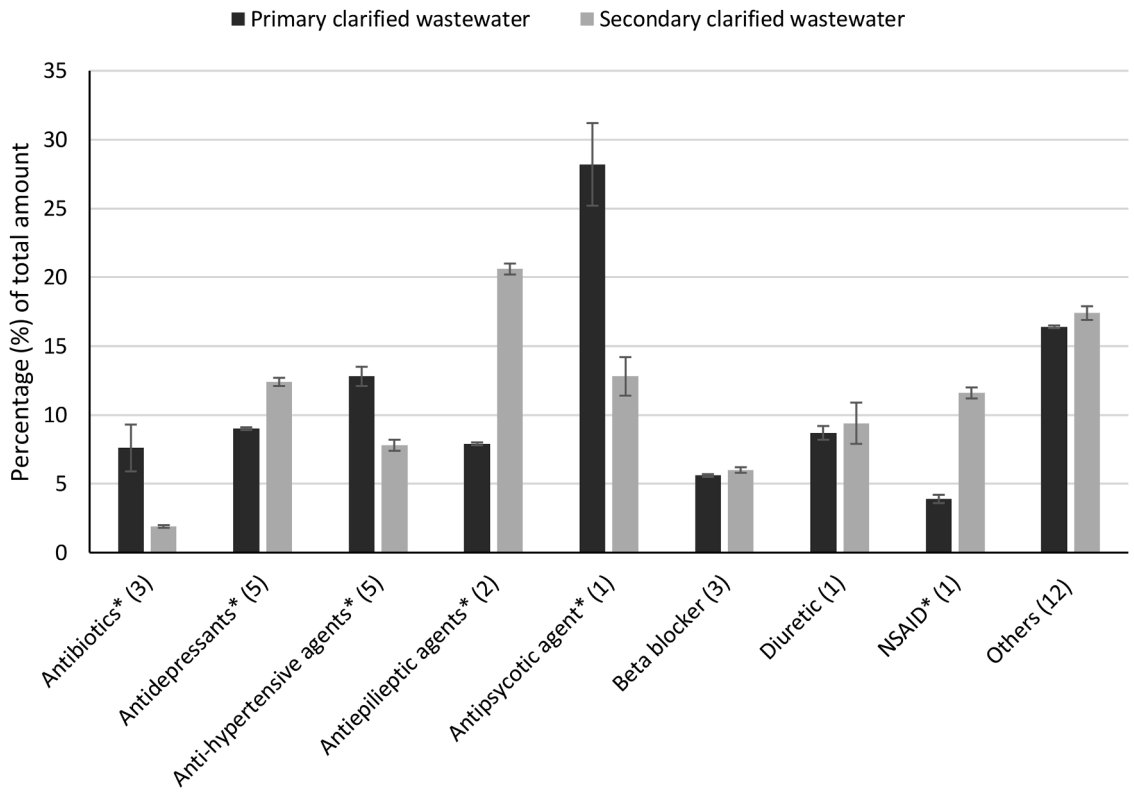

Fig. 2. Dominant therapeutic classes in municipal wastewater collected after primary and secondary clarification. The asterisk (*) indicate significant difference ($p < 0.05$) in concentration of the therapeutic class between the wastewaters. Numbers in parentheses indicate the number of compounds in each group (see Table S2). Values represent mean ± std ($n = 3$).

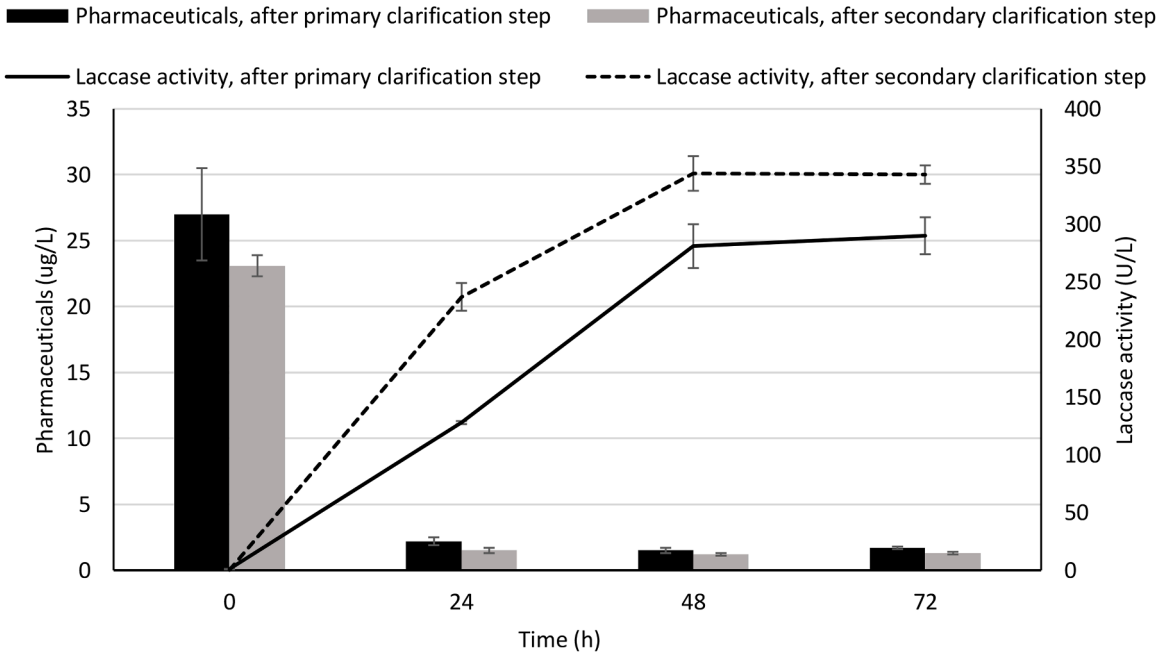

Fig. 3. Time-course of pharmaceutical concentration (columns) and laccase activity (lines) in wastewater collected after primary and secondary clarification. Most removal occurred within 24 h. Values represent mean ± std ($n = 3$).

wastewaters collected from earlier steps in the treatment process. This exposed the used fungus, *P. ostreatus,* to more challenging environments and, consequently, lower laccase activity (300 and 350 U/L) were recorded compared to the first experiment (550 U/L), see Fig. 3 and Table 3. However, similar treatment efficiency considering pharmaceutical removal was observed in the experiments. In the second experiment, TOC was analysed instead of COD to decrease the production of toxic waste in the laboratory. In a regulatory context, a COD/TOC ratio of 3 is commonly applied (EEA, 2024). Using this ratio for conversion, the increase in organic load added by the treatment is

Table 2
Initial concentrations (ng/L) of pharmaceuticals, highlighted in the revised Urban Wastewater Treatment Directive, in the different wastewaters included in this study. Mean±std, $n = 3$.

Pharmaceutical	After primary clarification step	After secondary clarification step	Effluent wastewater
Hydrochlorothiazide	2333±208	2167±306	4533±723
Metoprolol	1063±118	1100±0	790±44
Carbamazepine	340±26	330±17	230±17
Citalopram	593±68	590±45	133±12
Diclofenac	1047±92	2667±58	2443±1657
Clarithromycin	121±27	35±1	32±3
Venlafaxine	607±60	736±31	403±6
Irbesartan	257±12	153±6	85±9

comparable between the experiments, being in the range of 230–300 mg/L of TOC (Tables 3 and 4).

In wastewater collected after primary clarification, 24 h of treatment resulted in increased TOC by approximately 80 %. Although the increase was substantial, a considerable amount could be removed during the subsequent active sludge process. Interestingly, TOC in this wastewater decreased overtime (Table 4). This decrease is likely due to absorption by particles in the water followed by precipitation. Potentially also degradation of organic carbon due to intense microbial activity might have a role. TN was not impacted by the treatment in the wastewater collected after the primary clarification. The same mechanisms, intense microbial activity leading to denitrification, and also adsorption to particles followed by precipitation, can be considered as an explanation for this finding. On the other hand, a considerable increase was observed in TP in treated wastewater collected after both primary and secondary clarification, approximately 2–3 mg/L (Table 4). Compared to the impact on TP observed in the first experiment, a larger increase in the TP concentration was observed in the second experiment. An explanation might be that different batches of spawn were used in the experiments. This may lead to variances in phosphor content of the grain and in the age of the spawn, affecting its structural integrity and thereby its release of phosphorus.

Table 3
Impact of spawn-based pellets on organic load (chemical oxygen demand (COD)), total nitrogen (TN), ammonium (NH_4–N) and total phosphorous (TP) after three days (72 h) of treatment of effluent municipal wastewater. Also, the laccase activity (U/L) in the water is presented (Lac). The control represents the initial value before pellets treatment. The white-rot fungi used in the experiment were *Pleurotus ostreatus* and *Trametes versicolor*. Mean±std, $n = 3$.

Treatment	Lac (U/L)	pH	COD (mg/L)	TN (mg/L)	NH_4–N (mg/L)	TP (mg/L)
Control	0.1 ± 0.0	7.6 ± 0.01	31.6 ± 2.3a*	27.1 ± 0.6a	21.7 ± 0.2a	0.07 ±0.01a
P. ostreatus	552 ±14	6.9 ± 0.03	1005 ±28b	25.3 ± 0.7a	<0.015b	0.7 ± 0.06b
T. versicolor	299 ±81	6.0 ± 0.03	1360 ±90c	76.8 ± 7.3b	33.2 ± 5.5c	40.3 ± 0.7c

*Values within columns followed by different letters indicate significant difference ($p \leq 0.05$).

3.3. Practical implications and future directions

This study demonstrates that spawn-based fungal pellets obtained from WRF can serve as effective biocatalysts for the removal of a wide range of pharmaceutical contaminants from municipal wastewater. Pharmaceutical removal rates exceeding 90 % were achieved within 24 h, including for compounds prioritized in the revised Urban Wastewater Treatment Directive (EU, 2024). These findings underscore the potential of fungal-based treatment as a complementary polishing step within existing wastewater treatment frameworks. A comprehensive perspective on the use of fungal pellets in wastewater treatment is provided in the review by Espinosa-Ortiz et al. (2016), summarizing pellet formation mechanisms, reactor designs, pollutant removal applications, and operational challenges, and highlighting their potential for practical implementation. It provides a good example of how green technology could be implemented for pollution removal. The findings from the present study add practical advantages from an operational standpoint. Development of fungal pellets based on commercially available mushroom spawn simplifies inoculum production, and the solid pellet formation facilitates easy recovery via coarse filtration. Moreover, the fungal biomass enriched with sorbed pharmaceuticals could be

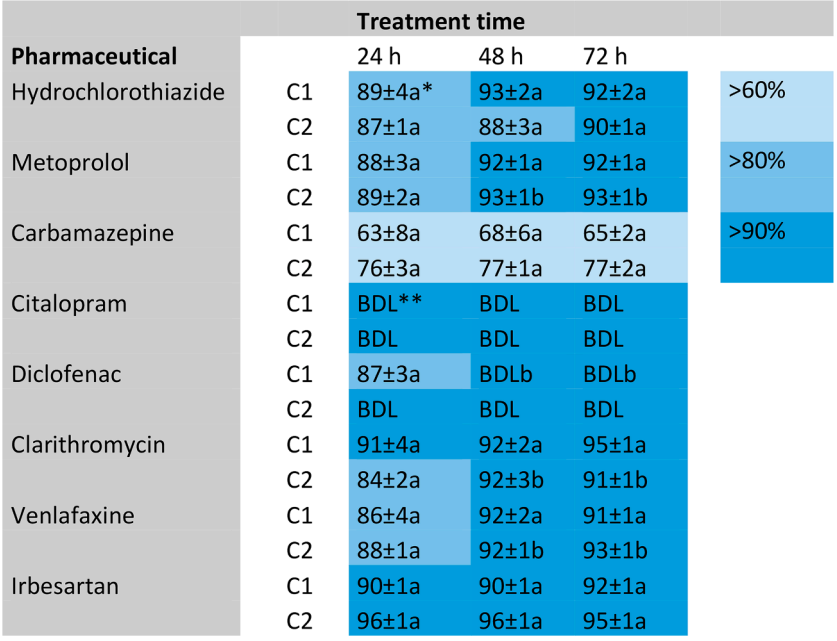

Fig. 4. Relative removal (%) of priority pharmaceuticals highlighted in the revised Urban Wastewater Treatment Directive by treatment with *P. ostreatus* in wastewater sampled after primary clarification (C1) and secondary clarification (C2). Values represent mean ± std ($n = 3$). *Significance letters summarise the comparison between time points within a compound and clarification type ** BDL - Below detection limit.

Table 4
Impact of spawn-based pellets of *Pleurotus ostreatus* on organic load (total organic carbon, TOC), total nitrogen (TN) and total phosphorous (TP) over time in municipal wastewater collected after primary and secondary clarification. Mean±std, $n = 3$.

Wastewater	Time (h)	pH	TOC (mg/L)	TN (mg/L)	TP (mg/L)
Primary clarification	0	7.4 ± 0.1	281±5a*	60.9 ± 1.5ab	5.2 ± 0.4a
	24	7.0 ± 0.1	508±60b	65.5 ± 5.6a	8.5 ± 0.9b
	48	7.2 ± 0.1	373±39a	45.9 ± 5.2c	8.2 ± 1.1b
	72	7.4 ± 0.1	313±13a	53±2.3bc	8.6 ± 0.7b
Secondary clarification	0	6.8 ± 0.1	7.7 ± 0.9a	7.9 ± 0.5a	0.3 ± 0.05a
	24	6.6 ± 0.02	281±25b	19.3 ± 1b	2.4 ± 0.3b
	48	6.8 ± 0.01	259±14b	17.6 ± 2.1b	2.3 ± 0.1b
	72	7.1 ± 0.1	263±21b	19.3 ± 1.9b	2.7 ± 0.2b

*Values within columns for each wastewater followed by different letters indicate significant difference ($p \leq 0.05$).

thermochemically converted to biochar, aligning the process with circular economy principles and mitigating risks associated with pharmaceutical re-release. While a detailed comparison with advanced treatment technologies such as ozonation or activated carbon adsorption was beyond the scope of this study, it is worth noting that fungal-based treatment offers a biologically driven alternative that could complement existing advanced oxidation or adsorption processes.

However, the study also highlights important challenges that must be addressed for large-scale implementation. One of the primary limitations observed was the substantial increase in organic load (measured as COD or TOC) and phosphorus levels resulting from the addition of grain-based spawn and lignin. This secondary pollution could complicate downstream treatment requirements, particularly when fungal treatment is applied at the tertiary stage. Consequently, integration of fungal treatment upstream—tentatively after primary clarification—appears more feasible. In the earlier treatment stages, the higher microbial load and nutrient content did not impair removal efficacy, and the resulting increase in organic load may be partially mitigated in subsequent treatment processes.

Species-specific differences in nutrient release and pharmaceutical removal also merit further investigation. For instance, *P. ostreatus* showed better removal of NH_4–N and lower increase of phosphorus compared to *T. versicolor*, suggesting the potential for fungal species optimization based on specific water quality targets. Additionally, the presence of TPs and their potential ecotoxicological impacts were not assessed in this study and should be a priority for future research to ensure the overall safety and effectiveness of fungal bioremediation.

While the fungal pellet system presents several features consistent with a low-energy and sustainable treatment option—such as operation under ambient temperature and minimal chemical or mechanical inputs—it should be emphasized that this conclusion is qualitative. No direct measurements of energy consumption or operational costs were performed in the present study. The short hydraulic retention time (24 h) and passive pellet formation indicate a process with potentially lower energy requirements than conventional advanced oxidation or membrane systems, but this assumption requires quantitative validation. A comprehensive life-cycle assessment will be necessary to confirm the true environmental benefits and cost-effectiveness of large-scale implementation.

4. Conclusion

This study demonstrates the feasibility of using spawn-based fungal pellets derived from *P. ostreatus* and *T. versicolor* as an effective and operationally simple biotechnological approach for the removal of pharmaceuticals from municipal wastewater. The fungal treatments achieved broad-spectrum pharmaceutical removal exceeding 90 % within 24 h across different stages of wastewater treatment, including compounds prioritized in the revised Urban Wastewater Treatment Directive. The use of commercial mushroom spawn as an inoculum provides a standardized, scalable, and readily available material, facilitating practical implementation and bridging the gap between laboratory-scale findings and real-world applications.

While the observed removal efficiencies underscore the strong degradative capacity of the system, the study also highlights several operational challenges that must be addressed prior to full-scale application. The addition of nutrient-rich grain spawn and lignin significantly increased the organic and phosphorus loads in treated effluents, indicating that post-treatment polishing or upstream integration (e.g., after primary clarification) would be necessary to avoid secondary pollution. Furthermore, although enzymatic activity, particularly laccase, was closely associated with pharmaceutical degradation, the relative contributions of other oxidative enzymes and non-enzymatic mechanisms warrant further investigation. Future research should focus on minimizing nutrient release, identifying and characterizing TPs, and evaluating process sustainability through life-cycle and techno-economic assessments.

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CRediT authorship contribution statement

Malin Hultberg: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Tsz Yung (Patrick) Wong:** Writing – review & editing, Investigation. **Bent Speksnijder:** Writing – review & editing, Investigation. **Oksana Golovko:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2025.100440.

Data availability

Data will be made available on request.

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