Biodiversity restoration and conservation of inland water ecosystems for environmental and human well-being

BioReset

2020 - 2021 Joint Call
Joint COFUND Call on "Conservation and restoration of degraded ecosystems and their biodiversity, including a focus on aquatic systems"
BiodivRestore-406

Deliverable 2.2.2

Targeted suspect screening list of transformation products, which can be potentially formed during mycoremediation with white-rot fungi

Lead Beneficiary	Work package	Delivery month
UVIGO (WP 2 lead) SLU (Task 2.2)	2 (task 2.2)	18

Table of contents

1. Executive Summary 3
2. Task description 3
3. Developed activities 3
4. Results 5
5. Associated indicators 7

1. Executive Summary

Pollution is threatening the biodiversity of inland waters that are vital to society and the future of the Earth. A major source of this pollution are effluent discharges from wastewater treatment plants (WWTPs). Treatment processes used in WWTPs do not efficiently remove emerging contaminants, such as pharmaceuticals and microplastics, which lead to health hazards to non-target species, including humans. This polluting source limits the conservation and restoration of freshwater systems. At the same, there is a need for strategies for up-scaling restoration solutions and for rapid and simple to use methodologies to assess conservation and restoration progress, i.e. assessment strategies anticipating the success of conservation/remediation measures in suitable timescales, ensuring reliable data comparison over time and space, and guiding intervention measures. Thus, the **BIORESET** project proposes to advance treatment processes (chemical, physical, biological and their combination) to promote ecosystem recovery and conservation and to develop assessment strategies.

Deliverable 2.2.2 is intended to develop a suspect screening list of transformation products, which potentially can be formed during mycoremediation with white-rot fungi. Additional information regarding communication and academic works within the work package is outlined in this report.

2. Task description

Work Package (WP) 2 regards improving the effectiveness and upscaling of wastewater treatments for removal of pharmaceuticals in water. Within this, Task 2.2 focus on bioremediation with white-rot fungi (mycoremediation). This task has focused on: i) construction of laccase-producing biomaterials (D. 2.2.1) and, as reported here, ii) Targeted suspect screening list of transformation products, which can be potentially formed during mycoremediation with white-rot fungi **(D. 2.2.2.)**.
Bioremediation based on fungi is particularly promising in the decontamination of WW from EC because of the nonspecific nature of the ligninolytic enzymatic system, which is able to degrade a wide range of EC. The formation of transformation products (TPs) from pharmaceuticals has emerged as a critical environmental challenge, as TPs may exhibit enhanced toxicity compared to their parent compounds. The primary objective of pharmaceutical degradation processes is the removal of active pharmaceutical ingredients; however, advanced degradation strategies must account for the generation of TPs to ensure comprehensive removal of all potential contaminants (1, 2). A key aspect of environmental risk assessment is the characterization of TPs, including those generated through bioremediation methods, such as fungal degradation. Investigating the nature of these TPs is essential for optimizing post-treatment processes to mitigate residual toxicity. Pharmaceuticals often persist in the environment due to their prolonged half-lives, leading to bioaccumulation and potential adverse effects on both human health and ecosystems, even at trace concentrations. Increasing concern surrounds the transformation of pharmaceuticals into TPs, which may introduce novel toxicological profiles that differ from those of the parent compounds.

WP2 Task 2.2 team members
The Team members in WP2 Task 2.2 are detailed as follows:

Name	Organization	Role
Malin Hultberg	SLU	Task coordinator
Oksana Golovko	SLU	Researcher

3. Developed activities

The white-rot fungus *Pleurotus ostreatus* was cultivated in flasks with Kraft lignin to induce laccase production. After 48 hours, fungal pellets were formed, consisting of grain, mycelium, and lignin. Laccase activity was

measured using a colorimetric assay. Grain spawn of the white-rot fungal species *Pleurotus ostreatus* M2191 was obtained from Mycelia BVBA, Belgium. The grain spawn was added to sterile distilled water (40 g/L wet weight) cultivated in Erlenmeyer glass flasks on a horizontal orbital shaker (VWR, Advanced 5000 Shaker, Radnor, PA, USA) operated at 100 rpm at room temperature (20-22 °C). Kraft lignin (Sigma-Aldrich 370959) was added at start in a concentration of 5 g/L (dry weight, dw) in order to induce the laccase production. After 48 hours of incubation on the shaker, fungal pellets with production of laccase had developed. The pellets were composed of grain, mycelium of *P. ostreatus* growing out from the grain and lignin. The later compound accumulated in the pellets resulting in a brown pellet and a translucent water phase. The laccase activity was determined colorimetrically by detecting the oxidation product 2,6-dimethoxyphenol (DMP, ε468=49,600 M^{-1} cm^{-1}), as described by Parenti et al. (2013). The reaction mixture contained 0.45 mL of diluted sample 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 formation of 1 µmol of product per min.

Experimental set-up

This experiment aimed to assess the impact of fungal treatment on three pharmaceuticals (clarithromycin, sertraline, and venlafaxine) considering removal as well as possible formation of transformation products. After 24 hours, the liquid and solid phases were separated for analysis. The targeted pharmaceuticals were added as individual compounds and as a mixture of all three compounds. A nominal concentration of 100 µg/L of the individual compounds were aimed for. After the pharmaceuticals had been exposed to fungal treatment for 24 hours, the liquid phase and the solid phase (the pellets) were separated by centrifugation at 3000 g for 15 min. The samples were immediately stored in a freezer at -20 °C before analysis, which was performed within one week. The solid samples were lyophilized before analysis.

Analysis

Chemical analysis: Target chemical analysis.

For the chemical analysis, reference standards for 3 target pharmaceuticals (clarithromycin, sertraline and venlafaxine) were purchased from Sigma-Aldrich (Sweden). The physicochemical properties of these compounds are presented in Table 1. The compounds were selected according to information found in the literature on their occurrence in aquatic environments and on high human use and consumption worldwide. Isotopically labeled internal standards were purchased from Wellington Laboratories (Canada) and Toronto Research Chemicals (Toronto, Canada). All analytical standards were of high analytical grade (>95%).

Samples of the solid phase were extracted by a validated in-house method based on previous studies (Kodešová et al. 2019).

Water samples were filtered through 0.2 µm regenerated cellulose filters and 1 mL aliquots were used for analysis. Spiking was performed by adding 10 ng of internal standard to 1 mL of filtered sample.

All samples were analyzed using a DIONEX UltiMate 3000 ultra-high pressure liquid chromatography (UPLC) system (Thermo Scientific, Waltham, MA, USA) coupled to a triple quadrupole mass spectrometer (MS/MS) (TSQ QUANTIVA, Thermo SCIENTIFIC, Waltham, MA, USA). An Acquity UPLC BEH-C18 column (Waters, 100 mm × 2.1 i.d., 1.7 µm particle size from Waters Corporation, Manchester, UK) was used. The injection volume was 10 µL for all samples. Heated electrospray ionization (H-ESI) was used to ionize the target compound. The mobile phase consisted of MQ with 5 mM ammonium acetate and acetonitrile. The flow rate was 0.5 mL/min and run time was 15 min.

Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA) was used for optimizing the instrumental methods and running samples. The data obtained were evaluated using TraceFinderTM 3.3. software (Thermo Fisher). No target compounds were detected in method blanks and control samples.

Chemical analysis: Transformation products.

The highest nominal concentration extracts, controls (0 µg/L), and blanks were analyzed for transformation product (TP) identification using a Vanquish Binary Pump H with a UHPLC system coupled to a Q-Exactive Focus Orbitrap mass spectrometer. A preliminary suspect screening was conducted using a curated list of known

transformation products (TPs) derived from sertraline, venlafaxine, and clarithromycin. This list included TPs identified in literature, reported in ShinyTPs, or predicted in-silico using Biotransformer, Table 2.
The screening was performed using Compound Discoverer and MS-Dial. To identify potential formation of structurally related compounds, the characteristic masses of the parent compounds were examined across different MS2 windows.
Additionally, characteristic neutral losses, commonly observed under positive ionization, were searched to aid in filtering detected features. Finally, halogenated isotopic patterns were analyzed to identify potential TPs of sertraline, given its halogenated structure.
This approach aimed to comprehensively screen for transformation products while using known structural and isotopic characteristics to enhance detection and verification of new compounds.

4. Results

Fungal treatment and enzyme production: After 48 hours of incubation, laccase activity was recorded at 341±21 U/L, with no significant change (360±31 U/L) observed after an additional 24-hour growth and pharmaceutical exposure. No laccase activity was detected in the positive controls, and no significant differences were found in the biomass weight across treatments (average of 1.3±0.03 g dw per replicate, equivalent to 25.0 g dw of fungal pellets per liter).

Pharmaceutical removal: The removal efficiency of targeted pharmaceuticals in both the water and solid phases after 24 hours of fungal treatment was quantified. Clarithromycin and venlafaxine exhibited approximately 60% reduction in concentration in both individual and mixture treatments within the solid phase. Sertraline showed the highest reduction among the tested pharmaceuticals for both treatments, Table 1.

Table 1. The concentration of the pharmaceuticals clarithromycin, sertraline and venlafaxine after 24 hours of treatment with fungal pellets compared to the untreated control. The pharmaceuticals were applied both individually (ind) and as a mixture (mix). The concentration was determined both the water phase and in the solid phase (fungal pellets). The control treatment was composed of water phase only. Mean±std, n=3

Pharmaceutical	Fungal Treatment		Control
	Water phase (µg/L)	Solid phase (µg/kg)	Water phase (µg/L)
Clarithromycin (ind)	23.0±2.6	553.3±20.8	97.3±13.6
Sertraline (ind)	b.d.*	146.7±15.3	63.0±8.7
Venlafaxine (ind)	18.3±2.9	490.0±88.9	78.7±7.0
Clarithromycin (mix)	26.6±10.4	453.3±72.3	93.0±20.0
Sertralin (mix)	3.3±2.1	115.3±17.5	56.0±13.1
Venlafaxin (mix)	22.3±8.4	522.2±83.3	80.7±12.3

*b.d. below detection limit

Identification of transformation products
The aim of this study was to investigate the formation of transformation products (TPs) during mycoremediation using white-rot fungi. White-rot fungi play a crucial role in the biodegradation of pharmaceuticals in the environment, serving as an essential component of biotic decomposition. Biodegradation represents a critical pathway for the removal of pharmaceuticals and other potential pollutants, but this process can lead to the formation of TPs. The extent of biodegradation, and consequently the formation of TPs, may vary significantly depending on the specific pharmaceutical compound.
Both the parent compounds and their TPs can contribute substantially to ecological risks. It is now widely acknowledged that the environmental risk assessment of pharmaceuticals should not be limited to the parent compounds alone. TPs, in particular, may exhibit distinct physicochemical properties, mechanisms of action, and environmental behaviors that necessitate thorough toxicological evaluation.
Furthermore, the combination of TPs and parent compounds can result in complex joint-toxic effects, including synergistic or antagonistic interactions, which may significantly influence the overall toxicity profile.

In this study, we evaluated the formation of TPs from individual and mixed pharmaceuticals: clarithromycin, venlafaxine, and sertraline. Notably, in 2020, the venlafaxine metabolite, O-desmethylvenlafaxine, was added to the European Union's 3rd Watch List (Commission Implementing Decision (EU) 2020/1161), highlighting the growing recognition of the need to monitor not only parent compounds but also their metabolites in environmental waters, especially for pharmaceuticals of this class.
Based on our preliminary results only the different isomers of hydroxy-sertraline were confirmed as true transformation products (TPs) formed during the treatment process. The remaining TPs detected likely originated from the standard mix, possibly due to degradation over time.
The low spiking concentrations may have been insufficient to generate a significant number of TPs; however, it is anticipated that higher concentrations would likely result in greater TP formation.
The absence of detectable TPs for clarithromycin and venlafaxine, despite comprehensive screening, suggests the efficiency of the treatment process in degrading newly formed compounds, underscoring the effectiveness of the data treatment methods.
In a previous study (3), we investigated the uptake of perfluoroalkyl substances (PFASs), pharmaceuticals, and parabens by edible oyster mushrooms (*Pleurotus ostreatus*) cultivated on a spiked growth substrate. The results demonstrated that *P. ostreatus* is capable of absorbing certain micropollutants (MPs), with a higher uptake efficiency observed for PFASs compared to parabens and pharmaceuticals. Furthermore, the findings suggest that oyster mushrooms possess the potential to degrade pharmaceuticals and parabens. However, despite this degradation potential, no transformation products (TPs) were detected during the study (unpublished data).

Table 2. Suspect list for Clarithromycin, Sertraline and Venlafaxine.

TP name	Exact mass	m/z POS	Parent	Rt parent	Source	Molec Form.
Clarithromycin	747.4769	748.48417	Clarithromycin	8.72	Parent	C38H69NO13
Clarithromycin TP1	764.4796	765.487425	Clarithromycin	8.72	Biotransformer	C38H70NO14
Clarithromycin N-oxide	763.4718	764.4790819	Clarithromycin	8.72	Pak Choi/ShinyTPs/Biotransformer	C38H69NO14
Clarithromycin TP3	745.4612	746.469025	Clarithromycin	8.72	Biotransformer	C38H67NO13
Clarithromycin TP4	733.46124	734.469065	Clarithromycin	8.72	Biotransformer	C37H67NO13
Clarithromycin TP5	590.3666	591.374425	Clarithromycin	8.72	Biotransformer	C30H54O11
Clarithromycin TP6	589.3825	590.390325	Clarithromycin	8.72	Biotransformer	C30H55NO10
Clarithromycin TP7	588.3509	589.358725	Clarithromycin	8.72	Biotransformer	C30H52O11
Clarithromycin TP8	587.3669	588.374725	Clarithromycin	8.72	Biotransformer	C30H53NO10
Clarithromycin TP9	176.1048	177.112625	Clarithromycin	8.72	Biotransformer	C8H16O4
Clarithromycin TP10	175.1208	176.128625	Clarithromycin	8.72	Biotransformer	C8H17NO3
Clarithromycin TP11	174.0892	175.097025	Clarithromycin	8.72	Biotransformer	C8H14O4
Clarithromycin TP12	173.1051	174.112925	Clarithromycin	8.72	Biotransformer	C8H15NO3
Sertraline	305.0738	306.08108	Sertraline	8.63	Parent	C17H17Cl2N
Sertraline TP1	337.0636	338.071425	Sertraline	8.63	Biotransformer	C17H17Cl2NO2
Acetylation of sertraline	333.0687	334.0757	Sertraline	8.63	Pak Choi	C18H17NOCl2
4-hydroxy sertraline (two isomers)	321.0687	322.075996	Sertraline	8.63	Pak Choi	C17H17Cl2NO
N-methylsertraline	319.0895	320.0967	Sertraline	8.63	Pak Choi	C18H19Cl2N

Sertraline oxime	305.0374	306.0446958	Sertraline	8.63	Pak Choi	C16H13Cl2NO
Norsertraline	291.0582	292.065	Sertraline	8.63	Pak Choi/ShinyTPs	C16H15Cl2N
Tetralone sertraline	290.0265	291.0338	Sertraline	8.63	Pak Choi	C16H12Cl2O
Sertraline TP3	287.1076	288.115425	Sertraline	8.63	Biotransformer	C17H18ClNO
Sertraline TP2	271.1127	272.120525	Sertraline	8.63	Biotransformer	C17H18ClN
Venlafaxine	277.2042	278.21146	Venlafaxine	7.26	Parent	C17H27NO2
Venlafaxine N-oxide	293.1991	294.2066	Venlafaxine	7.26	ShinyTPs	C17H27NO3
N-Desvenlafaxine	263.1885	264.1958	Venlafaxine	7.26	ShinyTPs	C16H25NO2
O-Desvenlafaxine	263.1885	264.1958	Venlafaxine	7.26	ShinyTPs	C16H25NO2
TP 2 Venlafaxine	263.1885	264.1958	Venlafaxine	7.26	https://doi.org/10.1016/j.chemosphere.2021.130148	C16H25NO2
TP 1 Venlafaxine	259.1936	260.2002	Venlafaxine	7.26	https://doi.org/10.1016/j.chemosphere.2021.130147	C17H25NO
N,N-Didesvenlafaxine	249.1728	250.18016	Venlafaxine	7.26	ShinyTPs	C15H23NO2
N,O-Didesvenlafaxine	249.1728	250.18016	Venlafaxine	7.26	ShinyTPs	C15H23NO2
Venlafaxine TP5	248.1412	249.149025	Venlafaxine	7.26	Biotransformer	C15H20O3
TP 3 Venlafaxine	245.1776	246.1851	Venlafaxine	7.26	https://doi.org/10.1016/j.chemosphere.2021.130149	C16H23NO
P4 Venlafaxine	157.1831	158.19	Venlafaxine	7.26	https://doi.org/10.1016/j.chemosphere.2021.130153	C10H23N
TP 4 Venlafaxine	157.183	158.19	Venlafaxine	7.26	https://doi.org/10.1016/j.chemosphere.2021.130150	C10H23N
P3 Venlafaxine	157.1466	158.154	Venlafaxine	7.26	https://doi.org/10.1016/j.chemosphere.2021.130152	C9H19NO
P2 Venlafaxine	153.1517	154.1586	Venlafaxine	7.26	https://doi.org/10.1016/j.chemosphere.2021.130151	C10H19N

References:
1. Parezanović GŠ, Lalic-Popovic M, Golocorbin-Kon S, Vasovic V, Milijašević B, Al-Salami H, et al. Environmental Transformation of Pharmaceutical Formulations: A Scientific Review. Arch Environ Contam Toxicol. 2019;77(2):155-61.
2. Maculewicz J, Kowalska D, Świacka K, Toński M, Stepnowski P, Białk-Bielińska A, et al. Transformation products of pharmaceuticals in the environment: Their fate, (eco)toxicity and bioaccumulation potential. Sci Total Environ. 2022;802:149916.
3. Golovko, O., Kaczmarek, M., Asp, H., Bergstrand, K-J., Ahrens, L., Hultberg, M. 2022. Uptake of perfluoroalkyl substances, pharmaceuticals, and parabens by oyster mushrooms (*Pleurotus ostreatus*) and exposure risk in human consumption. Chemosphere 291 132898

5. Associated indicators

Publications

Hultberg M, Asp H, Bergstrand KJB, Golovko O (2023) Production of oyster mushroom (*Pleurotus ostreatus*) on sawdust supplemented with anaerobic digestate. Waste Management 155: 1-7 https://www.sciencedirect.com/science/article/pii/S0956053X22005116

Hultberg M, Golovko O (2024) Use of sawdust for production of ligninolytic enzymes by white-rot fungi and pharmaceutical removal. Bioprocess Biosyst Eng. https://doi.org/10.1007/s00449-024-02976-8

Manuscripts in preparation: (3)

Fungal pellets for pharmaceutical removal: Treatment efficiency and environmental impacts

Impact of treatment with fungal pellets on quality of effluent municipal wastewater
Fungal pellets, pharmaceuticals and transformation products

Communications

Golovko, O., Hultberg, M. 2024. Fungal biomass in wastewater: a strategy for pharmaceutical removal. SETAC Europe 34rd Annual Meeting, 5-9 May, Seville, Spain. Poster

Golovko, O., Hultberg, M. 2023. Green Bioremediation with White-rot Fungi. SETAC Europe 33 Annual Meeting. 30th of April to 4th of May. Dublin, Ireland. Poster

Outreach

BioReset project presented at ”BranschDag Trädgård”, SLU Alnarp, Sweden, 23 Nov 2023; at ”Borgeby Fältdagar”, Borgeby, Sweden, 29-30 June 2022

Grants awarded

The fungal pellets concept was awarded the SLU Alnarp and Sparbanken Scania Innovation Award in 2023

Student thesis

Inoka Sanjeewani Ranamukha Hewage. Impact of nitrogen source on laccase activity in water by spawn pellets of *Pleurotus ostreatus* (oyster mushroom) and applications for water treatment. Presented 17.06.24 SLU, Alnarp, Sweden https://stud.epsilon.slu.se/20506/ (master thesis)

Frida Lövnäs. Potential for increasing laccase activity in white-rot fungi *Pleurotus ostreatus* in aquatic cultivation. Presented 16.08.24 SLU, Alnarp, Sweden https://stud.epsilon.slu.se/20531/ (bachelor thesis)