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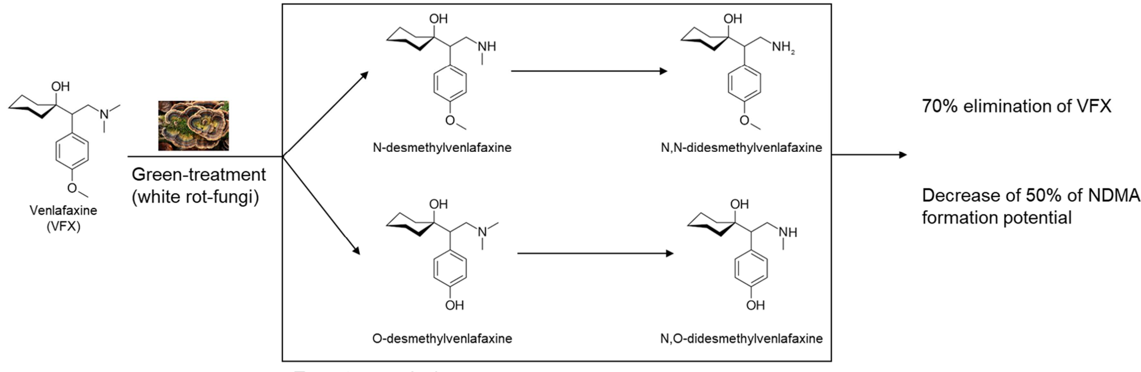

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1 # Fungal biodegradation of the *N*-nitrosodimethylamine
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16

17 **Abstract**

18 Antidepressant drugs such as Venlafaxine (VFX) and *O*-desmethylvenlafaxine (ODMVFX) are
19 emerging contaminants that are commonly detected in aquatic environments, since
20 conventional wastewater treatment plants are unable to completely remove them. They can be
21 precursors of hazardous by-products, such as the carcinogenic *N*-nitrosodimethylamine
22 (NDMA), generated upon water chlorination, as they contain the dimethylamino moiety,
23 necessary for the formation of NDMA. In this study, the capability of three white rot fungi
24 (*Trametes versicolor*, *Ganoderma lucidum* and *Pleurotus ostreatus*) to remove both
25 antidepressants from water and to decrease NDMA formation potential was investigated.
26 Furthermore, transformation by-products (TPs) generated along the treatment process were
27 elucidated and also correlated with their NDMA formation potential.

28 Very promising results were obtained for *T. versicolor* and *G. lucidum*, both being able to
29 remove up to 100 % of ODMVFX. In the case of VFX, which is very recalcitrant to conventional
30 wastewater treatment, a 70 % of removal was achieved by *T. versicolor*, along with a reduction
31 in NDMA formation potential, thus decreasing the associated problems for human health and
32 the environment. However, the NDMA formation potential remained practically constant during
33 treatment with *G. lucidum* despite of the equally high VFX removal (70 %). This difference was
34 attributed to the generation of different TPs during both fungal treatments. For example, *G.*
35 *lucidum* generated more ODMVFX, which actually has a higher NDMA formation potential than
36 the parent compound itself.

37

38 Promising results of the bioremediation of venlafaxine and *O*-desmethylvenlafaxine
39 antidepressants, with removals of 70 % and decrease of 50 % of the NDMA formation potential.

40

41 Keywords: Transformation by-products; psychiatric drugs; fungal treatment; carcinogenic *N*-
42 nitrosodimethylamine (NDMA); contaminated water; potential human health and environmental
43 problems

44 ## 1. Introduction

45 Venlafaxine (VFX) is an antidepressant drug that acts as a serotonin-norepinephrine reuptake
46 inhibitor (SNaRI) (García-Galán et al., 2016a). This antidepressant is widely used for the
47 treatment of major depressive disorders, generalized anxiety disorder, panic disorder and social
48 phobia. During the last years, *O*-desmethylvenlafaxine (ODMVFX), one of its main human
49 metabolites, has been also commercialized as a new SNaRI (CIMA, 2012; García-Galán et al.,
50 2016a). The presence of these compounds in the environment is related to their low removal
51 percentages in conventional wastewater treatment plants (WWTPs) (García-Galán et al.,
52 2016a), but also to the transformation of VFX into ODMVFX during degradation processes, both
53 at the WWTP and in the aquatic environment (Aymerich et al., 2016; Writer et al., 2013). For
54 example, VFX has been detected at concentrations of 13 – 1914 ng/L in hospital effluent
55 (Santos et al., 2013), between 68 and 268 ng/L in urban WWTP influents from Portugal and
56 between 184 and 322 ng/L in the corresponding WWTP effluents (Santos et al., 2013), ranged
57 from 176 to 215 ng/L in WWTP effluents in Canada (Lajeunesse et al., 2008), and reached
58 concentrations as high as 211 ng/L and 2190 ng/L in WWTP effluents from Spain (Aymerich et
59 al., 2016) and USA (Schultz and Furlong, 2008) respectively. The elimination of VFX by
60 conventional WWTP treatments is thus considered inefficient, with removal percentages
61 between 20 % (Aymerich et al., 2016) and 30 % (Collado et al., 2014; Gros et al., 2012) in
62 Spain, 7 % in USA (Schultz and Furlong, 2008), or even with negative removal ratios in WWTPs
63 from Portugal (Santos et al., 2013). Because of this, VFX can also be found in river waters,
64 although at lower levels, ranging from 12.9 to 45.9 ng/L in Canada (Lajeunesse et al., 2008),
65 below 33 ng/L in Spain (Aymerich et al., 2016) and 1310 ng/L in USA (Schultz and Furlong,
66 2008). ODMVFX concentrations in influents and effluents have been reported between 274 and
67 346 ng/L and from 223 to 330 ng/L, respectively, at levels usually higher than VFX (176 to 215
68 ng/L) (Lajeunesse et al., 2008). Removals achieved in WWTPs for ODMVFX ranged between 5
69 and 19 % whereas concentration values reported in receiving surface waters ranged between
70 21.0 and 68.7 ng/L. These values are, again, higher than those observed for VFX in the same
71 rivers (Lajeunesse et al., 2008).

72 Therefore, new advance wastewater treatment technologies are needed to enhance the
73 removal of such recalcitrant compounds. Some authors studied the removal of VFX and
74 ODMVFX by advanced oxidation processes (AOPs) (García-Galán et al., 2016a; Giannakis et
75 al., 2017; Lambropoulou et al., 2017; Pawar et al., 2012), commonly applied as tertiary
76 treatments (Oller et al., 2011). However, AOPs usually imply the use of high chemical dosages
77 and high energy consumption (Pérez-González et al., 2012) and thus they are not considered
78 sustainable cost-effective technologies. Hence, an environmental-friendly alternative treatment
79 based on the potential of fungal biodegradation is proposed in this work for the removal of VFX
80 and ODMVFX from contaminated waters. Within this study, three white rot fungi (WRF) are
81 assessed as potential biodegradation candidates, including *Trametes versicolor*, *Ganoderma*
82 *lucidum* and *Pleurotus ostreatus*. All fungi used are part of the Basidiomycota division and the

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83 Agaricomycetes class, but while *T. versicolor*, *G. lucidum* belong to Polyporales order, *P.*
84 *ostreatus* belongs to Agaricales. All fungal species have been previously used for biodegrading
85 micropollutants in contaminated water due to their potential enzymatic system, able to degrade
86 recalcitrant anthropogenic compounds (Covino et al., 2016; Marco-Urrea et al., 2009). In fact,
87 they all have been successfully used for the elimination of certain pharmaceuticals (Badia-
88 Fabregat et al., 2015; Cruz-Morató et al., 2014; García-Galán et al., 2011; Marco-Urrea et al.,
89 2010; Marco-Urrea et al., 2009; Palli et al., 2017; Rodríguez-Rodríguez et al., 2012) with overall
90 removal percentages of up to 83 % in the optimum conditions (Cruz-Morató et al., 2014).
91 Removal of other organic contaminants with *T. versicolor* such as anticancer drugs (Ferrando-
92 Climent et al., 2015), personal care products (Rodríguez-Rodríguez et al., 2012), endocrine
93 disrupting compounds (Cruz-Morató et al., 2014; Llorca et al., 2017) and polybrominated flame
94 retardants (Rodríguez-Rodríguez et al., 2012), among others have been studied. Laccase,
95 among other extracellular enzymes, is one of the most characteristic WRF extracellular
96 enzymes and it has been proved able to degrade many pharmaceuticals (such as diclofenac,
97 naproxen, ketoprofen (Taheran et al., 2016)). The activity of this enzyme was analyzed in this
98 study as an indicator of fungal activity, although its correlation between its activity and VFX or
99 ODMVFX degradation could not be demonstrated.

100 Generation of any possible transformation by-product (TP) from target pollutants during
101 treatment processes should be assessed since these by-products can sometimes be more toxic
102 than their parent compounds (García-Galán et al., 2011). Furthermore, VFX and ODMVFX were
103 pointed out as precursors of *N*-nitrosodimethylamine (NDMA) (Farré et al., 2016), a by-product
104 that is generated during disinfection treatment employing chlorine-based disinfectants (Shen
105 and Andrews, 2011). NDMA is a disinfection by-product of the disinfection of wastewater and
106 drinking water at treatment plants that use chloramines and is classified as "2B carcinogen -
107 reasonably anticipated to be a human carcinogen" by the United States Environmental
108 Protection Agency (EPA, 2008). The United States Office of Environmental Health Hazard
109 Assessment has issued a public health goal of 3 ng/L for NDMA (2006) and this compound has
110 been added in the third Contaminant Candidate List for further evaluation in the United States
111 Environmental Protection Agency's regulatory determination process. In general, the removal of
112 precursors in raw waters is easier to achieve than the removal of NDMA itself, as it is a small
113 polar molecule that overcomes many different barriers, including reverse osmosis membranes
114 (Fujioka et al., 2013). Previous works investigated the fate of NDMA precursors through
115 different barriers used for water reclamation such as microfiltration followed by reverse osmosis
116 (Farré et al., 2011a; Fujioka et al., 2013; Sato et al., 2014; Sgroi et al., 2015), ozone followed by
117 biological carbon filtration (Farré et al., 2011b; Gerrity et al., 2014) and membrane bioreactors
118 (Farré et al., 2016). However, there are no previous studies investigating the potential of green
119 biodegradation technologies such as the ones based on fungi with capability to degrade NDMA
120 precursors and, thus, to decrease any related human health problem.

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121 In this context, the main objectives of the present work are i) the evaluation of the potential of
122 three fungal species for the elimination of VFX and ODMVFX from water; ii) the characterization
123 of TPs generated during the biodegradation process; and iii) the evaluation of NDMA formation
124 potential before and after fungal treatment. To the author's knowledge, this is the first time that
125 the generation of VFX TPs after fungal treatment was evaluated, including information about
126 potential precursors of NDMA.

127 2. Materials and methods

128 2.1. Chemicals, fungal biomass and synthetic media

129 VFX standard was purchased from Sigma-Aldrich while ODMVFX and *N*-desmethylvenlafaxine
130 (NDMVFX) were purchased from Toronto Research. Labelled sulfamethoxazole was used as
131 internal standard (Fluka - Buchs, Switzerland). NDMA (5000 µg/mL in methanol) had a purity of
132 >99.9 % (from Supelco). Deuterated d_6-NDMA was used as internal standard (Sigma-Aldrich).
133 For solid-phase microextraction, NaCl (ACS, ISO, Reag, Sharlau) was used. For the NDMA
134 formation potential test NH_4Cl (>99.5 %, Sigma-Aldrich), NaOH (ACS, ISO. Reag, Sharlau) and
135 NaClO (reagent grade, available chlorine ≥4 %, Sigma-Aldrich) were used. KH_2PO_4 KH_2PO_4
136 (>99 %, Sigma) and $Na_2HPO_4{\cdot}2H_2O$ (>99 %, Sigma) were used as buffer (pH7). Na_2SO_3
137 (>98%, Sigma) was used to quench the chloramines. Commercial DPD (*N*,*N*-diethyl-*p*-
138 phenylenediamine) test kits (LCK310, Hach Lange) were used for the analysis of free and total
139 chlorine using a Hach DR2800 spectrophotometer. The calibration mixture used for high
140 resolution mass spectrometry purposes was supplied by Thermo Fisher Scientific (LTQ ESI
141 Positive Ion Calibration Solution and ESI Negative Ion Calibration Solution).

142 All the solvents used during the studies were of high purity grade. High-performance-liquid-
143 chromatography (HPLC) grade methanol, acetonitrile and water (Lichrosolv®) were supplied by
144 Merck (Darmstadt, Germany). Formic acid 98 % was provided by Merck (Darmstadt, Germany).
145 HPLC-high resolution mass spectrometry grade acetonitrile and water (Lichrosolv®) were
146 supplied by Thermo Fisher Scientific.

147 Three different white rot fungi (WRF) were used in this work: *Trametes versicolor* (ATCC
148 #42530 strain), *Ganoderma lucidum* (FP-58537-Sp strain) and *Pleurotus ostreatus* (NCBI
149 KJ020935 (Palli et al., 2014)). All fungi were maintained and subcultured on 2 % malt extract
150 agar (MEA) petri plates (pH 4.5) at 25 ºC. Pellet production was performed for all fungi following
151 the same procedure described by Font et al. (Font et al., 2003). The pellets obtained by this
152 process were washed with sterile deionized water and kept in a 0.8 % NaCl solution at 4ºC until
153 use.

154 Synthetic medium used in the experiments was composed of 12 g/L of glucose, 3.3 g/L of
155 ammonium tartrate, 1.2 g/L of 2,2-dimethylsuccinate buffer, 1 mL of a micronutrient solution and

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156 10 mL of a macronutrient solution from Kirk medium (Kirk et al., 1978). The pH of the medium
157 was adjusted to 4.5 with NaOH and HCl solutions.

158 **2.2. Degradation experiments in synthetic medium**

159 In order to test the feasibility of fungal treatment for the degradation of VFX and ODMVFX, the
160 experiments were carried out with synthetic medium under controlled conditions.

161 Degradation experiments were performed in triplicate in 250 mL Erlenmeyer flasks with either
162 VFX or ODMVFX at 5 mg/L, spiking an appropriate volume of the corresponding pharmaceutical
163 methanol stock solution (1000 mg/L) into a sterile synthetic media. Although the concentrations
164 selected for the experiments were higher than those reported in effluent WWTPs, they were
165 chosen in order to: i) test the capabilities of degradation with fungi with high amounts of
166 compounds; ii) obtain detectable concentrations of any possible transformation product; and iii)
167 in order to be able to determine the NDMA formation potential for any possible transformation
168 product detected during bioremediation.

169 Sterile conditions were obtained by sterilizing the medium at 121 ºC during 30 min. Each flask
170 was inoculated with mycelial pellets approximately equivalent to 3.5 g/L dry weight. Liquid
171 samples were taken at 0, 3, 7, 10 and 15 days and were subsequently centrifuged in glass vials
172 to remove any biomass fragments or suspended solids.

173 Abiotic (same conditions as described above but without biomass), biotic (same conditions but
174 without VFX nor ODMVFX) and inactivated biomass (same conditions but with heat-inactivated
175 biomass) controls were also performed in triplicate to measure the potential effects of
176 physicochemical processes in pharmaceutical concentration, VFX and ODMVFX toxicological
177 effects on fungal biomass, and pharmaceutical sorption processes on biomass (as well as
178 NDMA precursor generation by fungal metabolism), respectively.

179 Experiments were carried out at room temperature, controlled and maintained along
180 experiments at 25 ºC. The pH was not controlled but 1.2 g/L of 2,2-dimethylsuccinate buffer was
181 added to the medium and pH was adjusted at 4.5 before the sterilization.

182 **2.3. NDMA formation potential tests**

183 The NDMA formation potential test was adapted from the protocol described by Mitch et al.
184 (2003). Briefly, it consists of adding chlroamines at high concentration to the water samples and
185 let them react for seven days. To this aim, chloramine was prepared at pH 8 and was added to
186 a 10 mM phosphate buffered sample to obtain a final concentration of 140 mg/L Cl_2. Formation
187 potential tests were carried out for each triplicate experimental sample. During the formation
188 potential test samples were stored in the dark and at ambient conditions (T=21±1 ^{o}C). After this
189 time, chloramines were quenched with 2.5 g of sodium sulfite. NDMA formation potential tests

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190 were also performed to abiotic blanks and inactivated biomass control blanks. NDMA formation
191 potential tests of VFX, NDMVFX and ODMVFX were performed by adding the same
192 concentration of chloramine to a 10 mM phosphate buffered to individual solutions of 2 mg/L of
193 the specific compound each.

194 **2.4. Analytical methods**

195 2.4.1. Evaluation of fungal performance

196 Glucose consumption was monitored during the experiment as an indicator of fungal activity
197 and to assess the possible mechanisms of VFX and ODMVFX biodegradation. In addition,
198 Laccase activity was monitored thorough the experiments since it is one of the most
199 characteristic extracellular enzymes of white rot fungi, which has proved to be able to degrade
200 many pharmaceuticals (Taheran et al., 2016).

201 Glucose concentration was measured using an YSI 2700 SELECT enzymatic analyzer (Yellow
202 Spring Instruments) according to instrument specifications for glucose analysis
203 (Marshal_SCIENTIFIC). The quantifiable concentration ranged from 0 and 10 g/L with a
204 precision of ±0.04 g/L. Laccase activity was analyzed using 2,6-dimetoxyphenol (DMP) reagent
205 as previously described (Badia-Fabregat et al., 2016).

206 In addition, initial biomass was estimated from the wet weight of pellets and the wet and dry
207 weight ratio. This was obtained through real measurements of the wet and dry weight values of
208 a sample of the same inoculum. The final biomass dry weight was determined directly by
209 dehydrating the mycelial mass at 105 ºC to a constant weight.

210 2.4.2. Chemical analysis of VFX, ODMVFX and related transformation by-products

211 The identification of any possible transformation by-product was carried out using a liquid
212 chromatography system coupled to a hybrid linear ion trap – high resolution mass spectrometer
213 LTQ Orbitrap (LC-LTQ Orbitrap). 10 µL were directly injected in an Aria TLX-1 chromatographic
214 system (Thermo Fisher Scientific) used for separation purposes. This system comprised a PAL
215 autosampler and two mixing quaternary pumps (eluting pump and loading pump). The entire
216 system was controlled via Aria software, version 1.6, under the Xcalibur 2.2 software. The
217 compounds were separated in a Hypersil GOLD analytical column (50 × 2.1; 3 µm; Thermo
218 Fisher Scientific, Franklin, MA) according to Llorca et *al.* (2017).

219 The chromatograph was coupled to a hybrid linear ion trap-Fourier Transform Mass
220 Spectrometry Orbitrap analyzer (LTQ-OrbitrapVelos™, Thermo Fisher Scientific) equipped with
221 a diverter valve (used in order to divert to waste unwanted portions of chromatographic runs)
222 and an Electrospary Ionization source (ESI). More detailed information can be seen elsewhere
223 (Llorca et al., 2017).

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224 Data processing was carried out with SIEVE 2.0 software (Thermo Scientific) in order to perform
225 the chromatographic peak deconvolution and ExactFinder 2.5 software (Thermo Scientific) for
226 identification purposes of selected compounds and any possible transformation product.

227 The compounds VFX, ODMVX and NDMVFX were quantified in the samples by LC-MS/MS
228 using the corresponding pure standards. A Thermo Scientific EQuan MAX Plus
229 chromatographic system (Thermo Fisher Scientific; Industriestrasse, Switzerland) was used for
230 separation purposes. The system was equipped with a Hypersil GOLD analytical column (50 ×
231 2.1; 1.9 µm; Thermo Fisher Scientific, Franklin, MA) working with the same gradient conditions
232 as described for the analysis by means of LC-LTQ Orbitrap described elsewhere (Llorca et al.,
233 2017).

234 The chromatographic system was coupled to a TSQ Vantage triple quadrupole mass
235 spectrometer analyser (Thermo Fisher Scientific, San Jose, USA), equipped with a Turbo Ion
236 Spray source. The ionization of the compounds was under positive mode. The acquisition was
237 performed in selected reaction monitoring mode (SRM) to obtain enough identification points,
238 with two transitions per compound: 278>**260**, 58 for VFX; 264>**107**, 58 for ODMVFX; and
239 264>**121**, 44 for NDMVFX (in bold the quantification transitions). The method limits of
240 quantification were 0.05 µg/L for VFX; 0.52 µg/L for ODMVFX; and 0.1 µg/L for NDMVFX,
241 comparable to previous published works (García-Galán et al., 2016b).

242 2.4.3. NDMA analysis

243 NDMA formation potential tests were performed with the samples taken during the
244 biodegradation experiments. Fungi are usually involved in the production and secretion of
245 metabolites, some of them antibacterial or antifungal (Rai and Tidke, 2005; Wessjohann et al.,
246 2013), therefore, blank NDMA formation potentials of the fungi solution without the drug (biotic
247 control) were also done at the same sampling times to subtract additional potential excreted
248 NDMA precursors. Sampling times for NDMA analysis were 0, 3, 7 and 10 days. Each sample
249 was analyzed in duplicates. The analytical method was developed based on Grebel et al. (2006)
250 and it is described elsewhere (Mamo et al., 2016). GC-QqQ analysis was performed by Trace
251 GC Ultra as chromatograph equipped with a TriPlus™ autosampler coupled to a TSQ Quantum
252 triple quadrupole mass spectrometer system (Thermo Fisher Scientific).

253 Mass spectrometric ionization was carried out in electron impact (EI) ionization mode with an EI
254 voltage of 70 eV and a source temperature of 250 ^{o}C. Detection was performed in Selected
255 Reaction Monitoring (SRM) mode. NDMA was monitored by using the *m/z* 74 parent ion and 42
256 and 43 daughter ions as quantification and qualification transitions, respectively. The internal
257 standard d_6-NDMA was quantified by using the *m/z* 80 parent ion and *m/z* 46 daughter ion.
258 Acquired data were processed by TracerFinder EFS 3.1 software. The method limit of
259 quantification was 50 ng/L.

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3. Results and Discussion

3.1. Biomass, glucose consumption and laccase activity

Based on the biomass measurements at the end of the experiment we can conclude that biomass concentration was maintained or slightly increased in most of the cases (Figure S1). An exception was found for *P. ostreatus* in the ODMVFX experiment, where biomass increased to approximately 60 % (dry weight, data not shown). In the case of ODMVFX experiment, glucose concentration dropped to almost zero after 10 days of experiment for all species (Figure S2). The same happened with *T. versicolor* and *G. lucidum* during VFX degradation experiments. However, in that case *P. ostreatus* presented a slower consumption of glucose and a minimum of 7.6 g/L glucose remained unconsumed at the end of the experiment (initial concentration of 12 g/L glucose). This slower decrease rate in carbon source could be explained by a lower metabolism (the use of *P. ostreatus* older fungal biomass in VFX experiment could explain the differences when compared to the ODMVFX experiment) which could have impacted its capability to remove VFX (more details in section 3.2).

Laccase activity showed great differences among assayed fungi as described in Figure S3. *T. versicolor* showed considerably high laccase activity, reaching a maximum of 167 U/L in the case of VFX degradation both at day 3 and at day 15. In the case ODMVFX degradation, high laccase activity could be observed during the first and last days of the experiment although activity peaks were slightly lower. These maxima in the laccase activity, at the beginning and at the end of the experiment, could be explained by changes in carbon content and carbon/nitrogen concentrations ratio, as pointed out by previous studies where certain carbon/nitrogen ratios or a depletion of carbon source could promote laccase production (Hailei et al., 2009; Mikiashvili et al., 2005). In contrast, laccase activity levels of *P. ostreatus* reached a maximum of 19 U/L in day 15 of the ODMVFX removal experiment. The reason of such a low laccase activity may be explained by agitation/aeration rates higher than those needed by these species to achieve optimal production levels of laccase. This excessive agitation could lead to laccase hydrolysis due to an induction in external proteases production (Tinoco et al., 2011). In the same way, *G. lucidum* laccase activity was negligible, probably because experimental conditions were not optimized for laccase induction with this specific fungi (Manavalan et al., 2013). Hence, any potential removal or biodegradation phenomena in the experiments with *G. lucidum*, is less probable to be caused by laccase oxidation mechanisms.

3.2. Elimination efficiency for VFX and ODMVFX by *T. versicolor*, *G. lucidum* and *P. ostreatus*

VFX and ODMVFX removal efficiency for the three fungal treatments assayed was calculated according to equation 1. The main results are summarized in Figure 1.

$$\%Removal = 100 - \frac{Experimental\ Concentration\ (t)}{Initial\ Concentration\ (t0)} \times 100$$ **Equation 1**

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296 Control experiments showed that VFX removal by physicochemical processes was negligible.
297 No sorption onto flasks was observed while sorption onto fungi was low (Figure 1A). In
298 particular, the sorption of VFX onto *T. versicolor* fungi seemed to be less than 20 % after 15
299 days of experiment whereas percentages of sorption seemed to be less than 10 % for the other
300 two species of fungi. On the other hand, the removal percentages for VFX were *ca.* 70 % for *G.*
301 *lucidum* and *T. versicolor,* while just a 25 % removal was observed for *P.ostreatus* after 15 days
302 of treatment. In all cases, the highest removal was achieved during the first 7 days (near 55 %
303 for *G. lucidum* and *T. versicolor* and close to 20 % for *P. ostreatus*) coinciding with the period
304 where glucose was still available in solution. After this time, the removal rate decreased until the
305 end of the experiment as it can be appreciated in Figure 1A. Previous works related to the
306 biodegradation of VFX in batch experiments (VFX spiked at 30 µg/L) showed an elimination ca.
307 50 % and even 100 % but only after 90 days of aerobic activated sludge treatment and under
308 anaerobic conditions respectively (Gasser et al., 2012), or between 7 % and 30 % in WWTPs
309 with conventional treatments (Aymerich et al., 2016; Collado et al., 2014; Gros et al., 2012;
310 Schultz and Furlong, 2008).

311 ODMVFX was completely removed after 3 days in the case of *P. ostreatus* and *T. versicolor* and
312 after 7 days in the case of *G. lucidum* (Figure 1B). In this sense, the three species can be
313 considered highly effective for the removal of this drug. The elimination of ODMVFX can be
314 entirely attributed to biological processes since no physicochemical processes (such as sorption
315 or abiotic degradation) were noticed during the control experiments. Gasser et al. (Gasser et al.,
316 2012) also investigated the removal of ODMVFX by activated sludge under aerobic and
317 anaerobic conditions at a spiked concentration of 25 µg/L and observed 100 % removal after 60
318 days of experiment under aerobic conditions, while this percentage was almost negligible under
319 anaerobic conditions (Gasser et al., 2012), or between 5 and 19 % in conventional WWTPs
320 (Lajeunesse et al., 2008).

321 **3.3. Identification of VFX and ODMVFX transformation by-products during fungal** 322 **biodegradation**

323 Table 1 summarizes the main TPs detected during the degradation experiments of VFX and
324 ODMVFX. Figures 2 and 3 show VFX and ODMVF levels respectively along with their
325 corresponding TPs during the experimental time.

326 In the case of VFX, best results were obtained with samples from the experiments using *T.*
327 *versicolor* and *G. lucidum.* Degradation of VFX with *P. ostreatus* was negligible and, therefore,
328 the TPs of this process were not investigated. The main TPs postulated for VFX were ODMVFX
329 and NDMVFX, both of them detected after two days of experimental time (Figure 2).
330 Additionally, *N,N*-didesmethylvenlafaxine (NNDMVFX) appeared after 3 days of treatment time
331 with *T. versicolor* and after 10 days with *G. lucidum*. The generation of O- and NDMVFX could
332 potentially be regioselective or regiospecific since more NDMVFX is detected at the end of the
333 experiments (Figure 2 and Table S1). The concentration of ODMVFX reached a maximum of

334 1.3 µg/L at day 3 during the *T. versicolor* degradation experiment to subsequently disappear by
335 day 15. On the other hand, the case of *G. lucidum,* ODMVF concentration increased with time
336 to a maximum concentration of 3.2 µg/L by day 15. The concentration of NDMVFX reached a
337 maximum of 21.5 µg/L after 3 days during the *T. versicolor* degradation experiment. Afterwards,
338 the TP was degraded by the fungal activity, leaving 14.3 µg/L at the end of the experiment. In
339 the case of *G. lucidum,* NDMVFX was detected after 3 days of experiment and its concentration
340 gradually increased until the end of the experiment (till 30.8 µg/L). In this context, we can
341 conclude that *T. versicolor* is more efficient for the elimination of VFX and its TPs (NDMVFX,
342 ODMVFX) than *G. lucidum*. In the case of the ODMVFX generation, the demethylation of the
343 methoxy group of VFX could be due to the activity of a non-heme iron-dependent demethylase
344 enzyme LigX from lignin metabolism (Bugg et al., 2011; Sonoki et al., 2000) present in *T.*
345 *versicolor* (Paice et al., 1993) or from other demethylase enzymes like, for example, from the
346 group of enzymes conforming the cytochrome P450 (Taheran et al., 2016). On the other hand,
347 demethylation of the dimethylamino group of VFX, which lead to the formation of NDMVFX, is
348 likely to be attributed to the activity of enzymes related to *N*-demethylation metabolic pathway
349 from fungi, similar to human liver pathways (Jollow, 2012). Gasser et al. (2012) also identified
350 NDMVFX as a degradation by-product of VFX as well as ODMVFX and *N,O*-
351 didesmethylvenlafaxine (NODMVFX) during the aerobic degradation of VFX in wastewaters with
352 activated sludge. Nonetheless, in this case the presence of ODMVFX could not be only related
353 to the degradation of VFX since ODMVX, as a human metabolite of VFX, was already present
354 in the raw wastewater at the beginning of the experiments. In contrast, high regioselectivity was
355 observed for the experiments carried out under anaerobic conditions as the authors detected
356 the generation of ODMVFX at higher amounts than NDMVFX.

357 Degradation experiments for ODMVFX in waters with *T. versicolor*, *G. lucidum* and *P. ostreatus*
358 denoted the presence of one TP: NODMVFX (Table 1 and Figure 3). It is suspected that the
359 generation of NODMVFX was due to the activity of enzymes related to the *N*-demethylation, the
360 same process implied in the amino demethylation of VFX.

361 Finally, the detected TPs within this work, *N*- and *O*- desmethyl products from VFX and *N*,*O*-
362 didesmethyl from ODMVFX do not have higher toxicity effects than parent compounds (VFX
363 and ODMVFX) for bioluminescent bacteria, as it has been reported for those compounds in the
364 literature (García-Galán et al., 2016a; Lambropoulou et al., 2017).

365 **3.4. NDMA formation potentials**

366 Both VFX and ODMVFX are potential NDMA precursors as they contain the dimethylamino
367 moiety, which in presence of chloramine generates NDMA (Farré et al., 2016). Moreover, the
368 capability to generate NDMA upon disinfection deepens not only on the presence of this moiety,
369 but also the steric hindrance and electronic distribution of the molecule (Farré et al., 2012;
370 Radjenovic et al., 2012). Therefore, it is interesting to evaluate how the NDMA formation
371 potential evolves during a biodegradation process where the parent compound is removed but

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372 other TPs, still containing the active moiety, are formed as in the present study. As degradation
373 of VFX by *P.ostreatus* was negligible, this fungus was not considered in this part of the study.
374 Therefore, only degradation by *T. versicolor*, *G. lucidum* was investigated.

375 Preliminary experiments to evaluate the NDMA formation potential yield of VFX and the
376 metabolites were done and the results are shown in Text S1 from SI. Also, NDMA formation
377 potential test for live controls were performed to investigate if precursors of NDMA were
378 released due to the own fungi metabolism. This information is also shown in Text S2 from SI.

379 Results showing the NDMA formation potential evolution for the degradation of VFX with both,
380 *T. versicolor* and *G. lucidum* fungi are plotted in Figure 4. This figure shows both, the results
381 from the experimental NDMA formation potential test performed directly on the samples taken
382 during the degradation experiments (i.e., experimental values), and the results theoretically
383 derived from the VFX and ODMVFX concentrations measured in the samples (i.e. formation
384 potential yield of 0.53±0.01 % and 1.19±0.02 % for VFX and ODMVFX, respectively).

385 During the treatment with *T. versicolor* fungi, NDMA formation potential continuously decreased
386 (from 1220±50 ng/L to 620±30 ng/L), whereas during degradation with *G. lucidum* fungi it
387 remained practically constant (from 965±45 ng/L to 990±90 ng/L). The different NDMA formation
388 potential profiles obtained in the two fungal experiments contrast with VFX´s similar degradation
389 kinetic (Figure 1). The reason for this difference could be attributed to the generation of different
390 TPs, with different NDMA formation potential, during both experiments. In fact, a higher
391 formation of NDMVFX could be observed in *T. versicolor* in comparison to *G. lucidum*
392 experiments (Table S1). However, NDMVFX does not contain the dimethylamino group from the
393 parent compound VFX. This dimethylamino group is the responsible for the NDMA formation
394 and hence no NDMA formation would be expected from this TP. On the other hand, VFX´s
395 metabolite ODMVFX still contains the dimethylamine moiety attached and has a higher NDMA
396 formation yield than VFX. However, the higher formation of ODMVFX observed during
397 biodegradation with *G. lucidum* fungi could not alone explain the increase in NDMA formation
398 potential measured (up to day 7) in comparison to the results obtained for the *T. versicolor* fungi
399 (Figure 4), as the concentration of this TP is low (maximum concentration measured is 3.2 µg/L
400 that corresponds to 11 ng/L of NDMA according the calculated 1.19±0.02 % NDMA formation
401 yield). In fact, the NDMA formation potential measured experimentally during treatment with *G.*
402 *lucidum* fungi was greater than the value that could theoretically be calculated from the
403 concentration of the remaining parent compound and the generated by-products, ODMVFX in
404 particular. That means that other TPs are likely to be form during the treatment, and although
405 they could not be identified, contribute to the NDMA formation potential. Interestingly, the
406 difference between the experimental and theoretical NDMA formation potential was negligible in
407 the case of *T. versicolor,* which means that probably the entire potential for NDMA formation
408 could be explained by the presence of the remaining parent compound VFX and ODMFX
409 generated.

410 ## 4. Conclusions

411 Three WRF species were tested for the elimination of VFX and ODMVFX antidepressants drugs
412 from contaminated water. Results show that VFX and ODMVFX removal rates are highly
413 dependent on glucose concentration while laccase does not play an essential role compared to
414 previous experiments where laccase extracellular enzyme of WRF has been able to degrade
415 many pharmaceuticals. The experiments conclude that *T. versicolor* and *G. lucidum* were able
416 to remove up to 70 % of VFX in 15 days, whereas *P. ostreatus* was only able to remove 25 %
417 during the same time. In contrast, all three fungi achieved a removal of 100 % of ODMVFX,
418 though *T. versicolor* and *P. ostreatus* did it within 3 days of experiment whereas *G. lucidum*
419 needed 6 days. The main detected TPs are coming from the demethylation of the
420 dimethylamino and methoxy moieties for VFX, being two of these TPs identified (ODMVFX and
421 NDMVFX) and one tentatively identified (*N,N*-didesmethylvenlafaxine). In the case of ODMVFX,
422 *N,O*-didesmethylvenlafaxine was tentatively identified.

423 The evaluation of NDMA formation potential along the degradation experiments of VFX denoted
424 that *G. lucidum* has higher NDMA formation potential than *T. versicolor,* although both of them
425 have similar removal efficiency. This cannot be solely attributed to the detected TPs but also to
426 the formation of other unknowns' TPs that may still contain the dimethylamino moiety in the
427 molecule, which is responsible for NDMA formation upon chloramination. These results highlight
428 the necessity not only to evaluate removal efficiencies of new and conventional
429 decontamination processes but also to investigate the generation of transformation by-products
430 and their ecotoxicological and human health implications such as, in this case, the potential to
431 generate NDMA upon disinfection.

432 Finally, in the near future experiments with those fungal treatments will be tested in real batch
433 experiments with real waste water as it has been successfully done with other pharmaceutical
434 compounds (Badia-Fabregat et al., 2015; Badia-Fabregat et al., 2016; Llorca et al., 2017; Mir-
435 Tutusaus et al., 2017).

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Table 1: Identified and postulated transformation by-products during biodegradation experiments with fungi. The error of the tentative identified compounds was always between ±4 ppm.

Proposed TP	**biodegradation process**	**tr (min)**	**Exact mass (m/z)**	**Proposed structure for neutral formula**
	VFX			
ODMVFX	*T. versicolor* *G. lucidum*	7.21	$[M+H]^+$ 264.1958056	Molecular formula: $C_{16}H_{25}O_2N$ DBE: 4.5
NDMVFX	*T. versicolor* *G. lucidum*	7.91	$[M+H]^+$ 264.1958056	Molecular formula: $C_{16}H_{25}O_2N$ DBE: 4.5
N,N-didesmethylvenlafaxine	*T. versicolor* *G. lucidum*	7.74	$[M+H]^+$ 250.1801555	Molecular formula: $C_{15}H_{23}O_2N$ DBE: 4.5
	ODMVFX			
N,O-didesmethylvenlafaxine	*P. ostreatus* *T. versicolor* *G. lucidum*	6.51	$[M+H]^+$ 250.1801555	Molecular formula: $C_{15}H_{23}O_2N$ DBE: 4.5

Compounds detected under positive ionization mode with ESI

DBE: double bound equivalents

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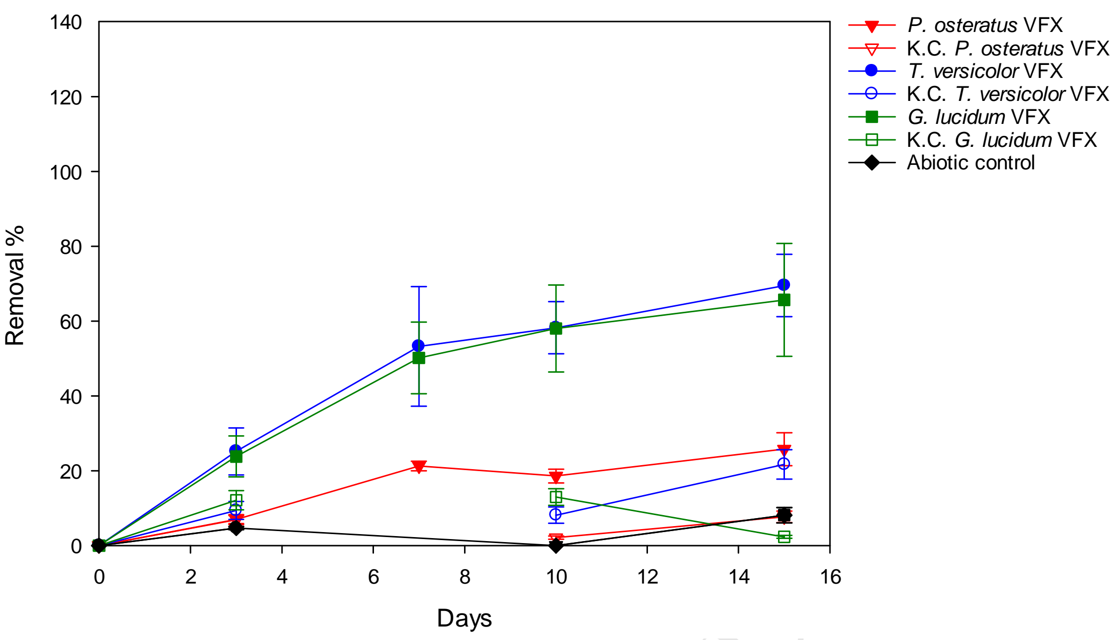

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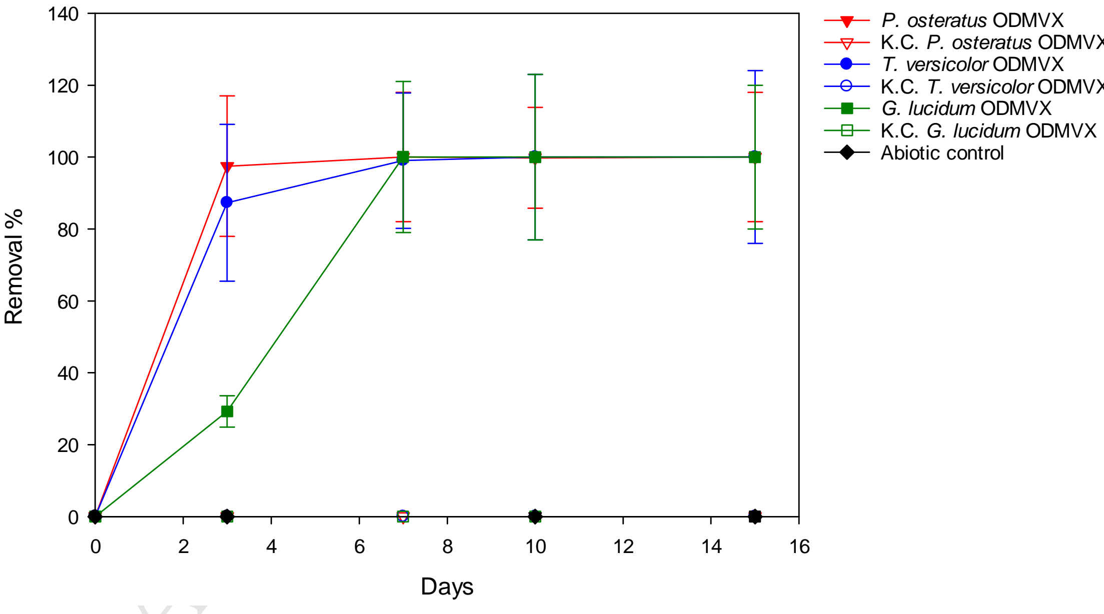

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640 **Figure 1:** Removal percentage of (A) VFX and (B) ODMVFX with *P.ostreatus* (red inverted
641 triangles ▼), *T. versicolor* (blue circles ●) and *G. lucidum* (green squares ■). Abiotic control
642 (black rhombi in dashed line ◆) refers to removal by physicochemical processes without fungi
643 and inactivated biomass control (KC) (empty dots in dashed lines, ▽, O and ☐ respectively,
644 colored according to the fungus) refers to removal by sorption phenomena onto fungi species.

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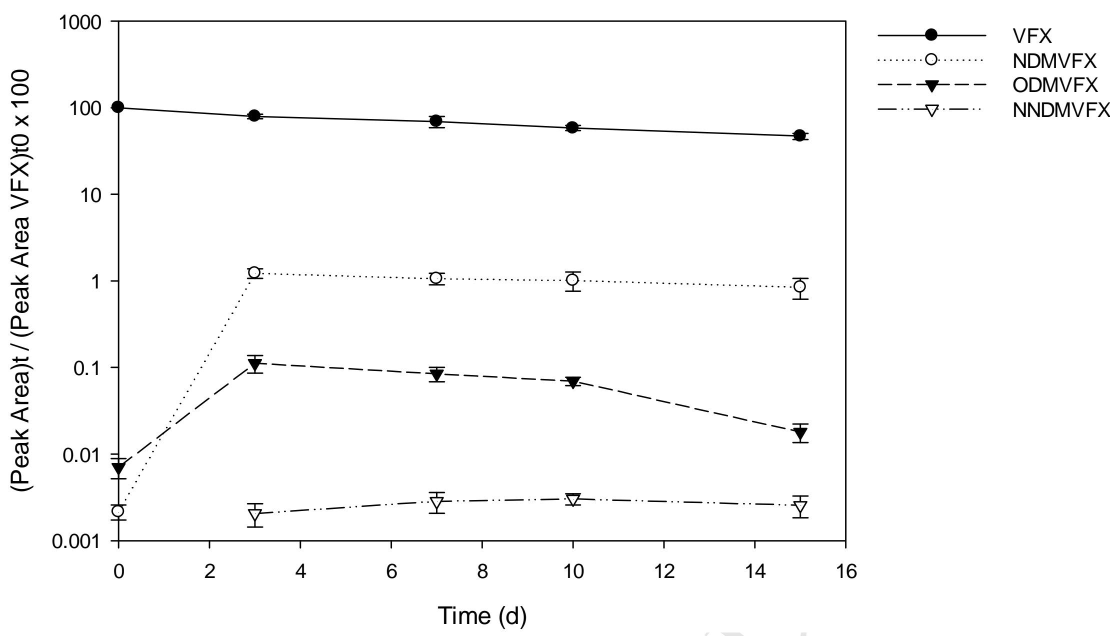
A) VFX with *T. versicolor*



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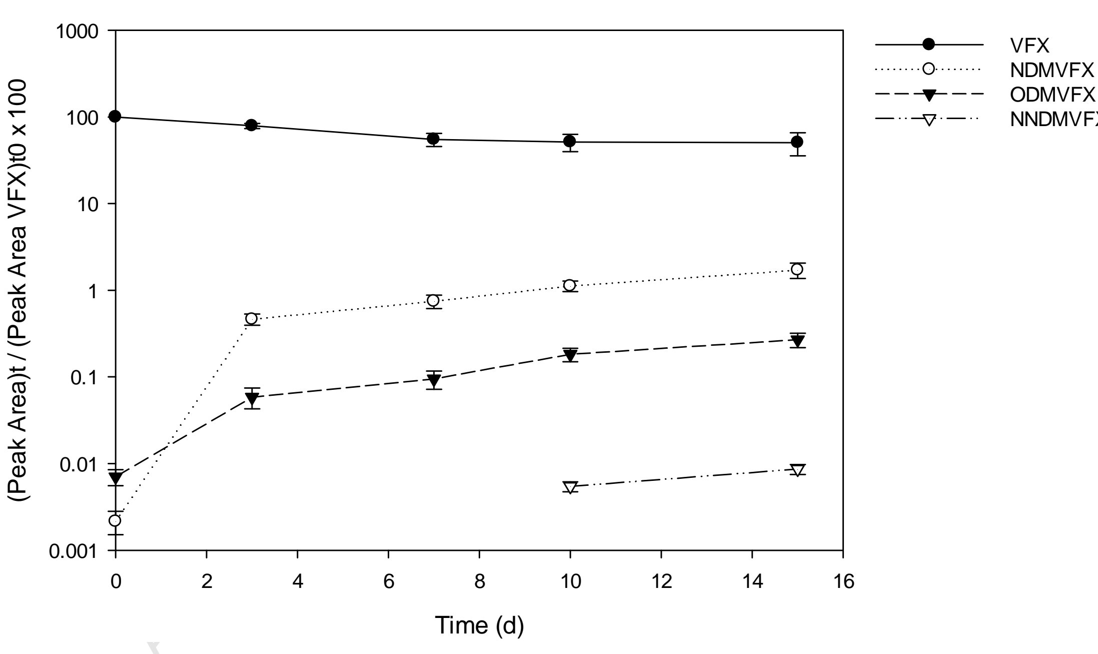
B) VFX with *G. lucidum*



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650 **Figure 2:** transformation by-products identified or postulated during removal treatments of VFX
651 with *T. versicolor* (A) and *G. lucidum* (B). The results are expressed as the log of the percentage
652 of generated peak area in the chromatogram at time t *vs.* the peak area in the chromatogram of
653 VFX at time 0. Compounds are represented as follows: VFX (●),NDMVFX (O), ODMVFX (▼),
654 NNDMVFX (▽).

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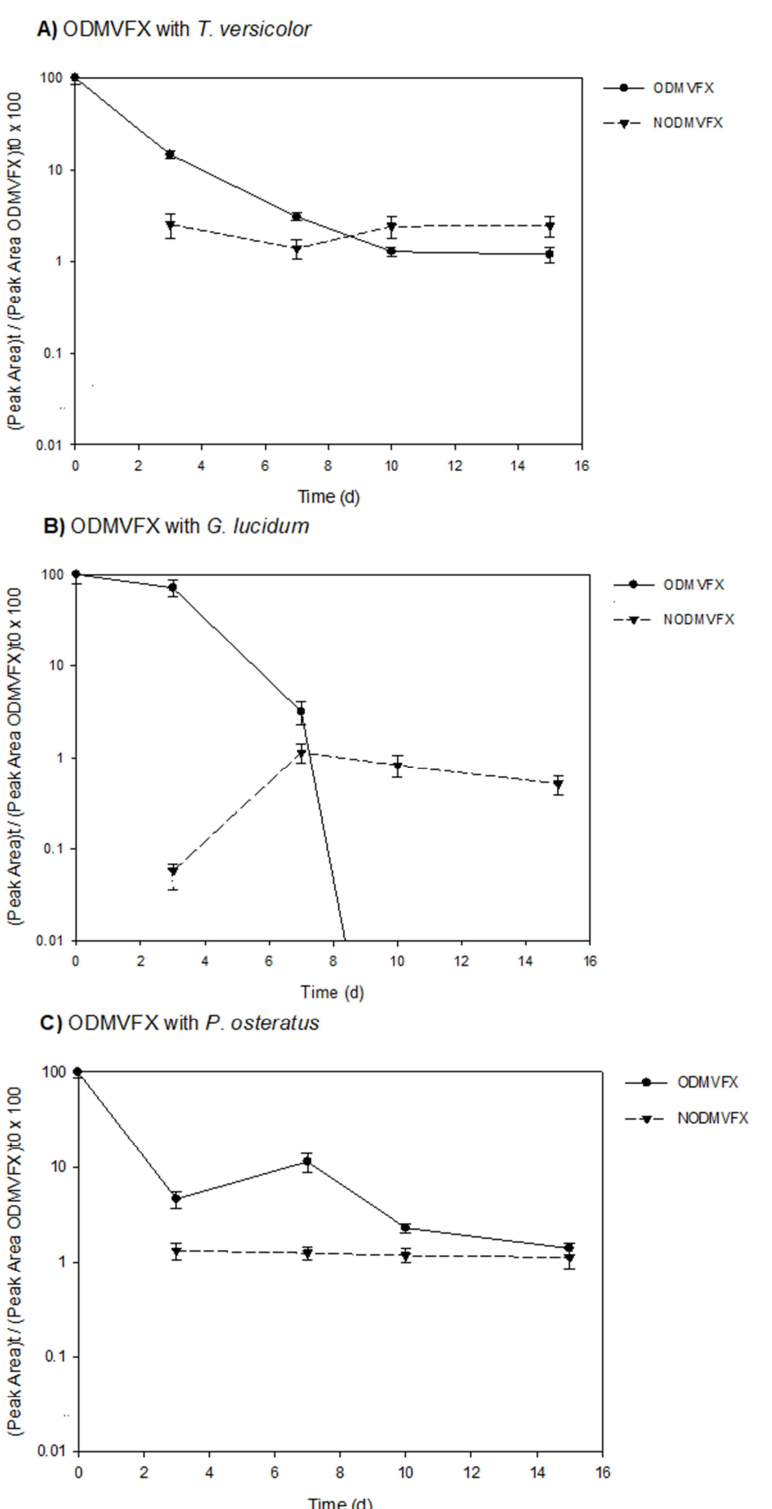

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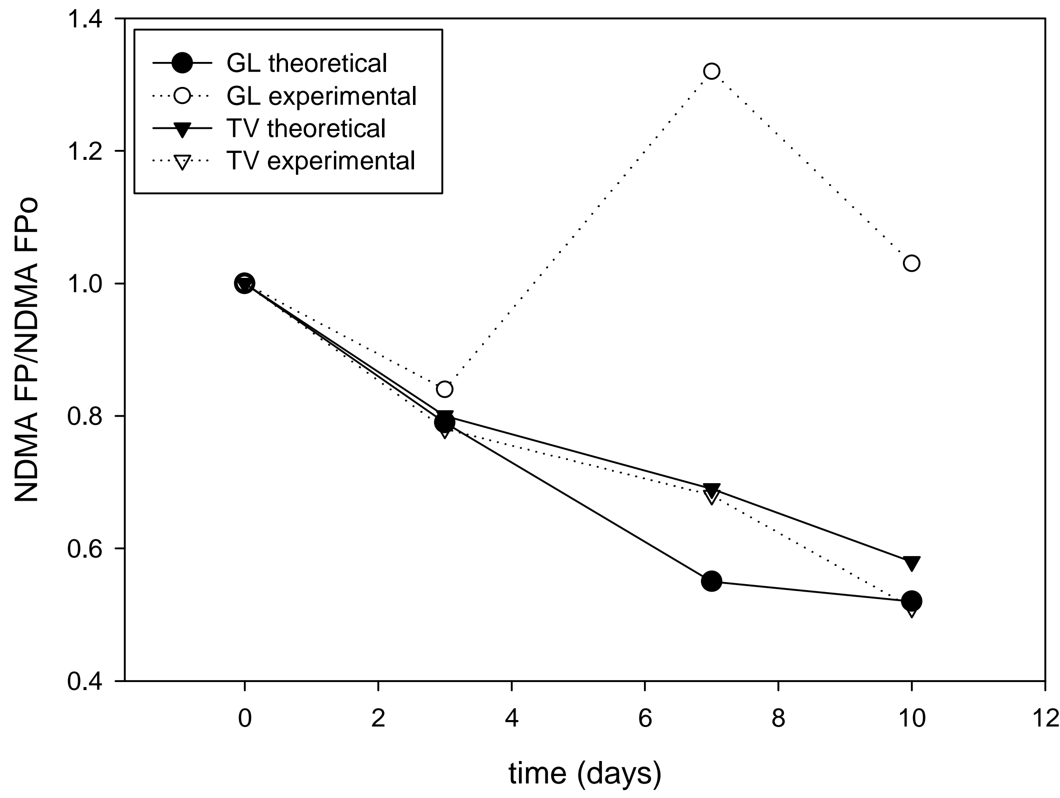
657 **Figure 3:** transformation by-products identified or postulated during removal treatments of
658 ODMVFX with *T. versicolor* (A), *G. lucidum* (B) and *P. ostreatus* (C). The results are expressed
659 as the log of the percentage of generated peak area in the chromatogram at time t *vs.* the peak
660 area in the chromatogram of ODMVFX at time 0. Compounds are represented as follows:
661 ODMVFX (●),NODMVFX (▼).



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663 **Figure 4:** Experimental values correspond to the NDMA formation potential of the samples
664 taken during *G. lucidum* and *T. versicolor* experiments of VFX degradation after subtracting the
665 values obtained for the blank experiments. The NDMA formation potential experimental
666 conditions are: [NH_2Cl]= 140 mg/L, pH8 (10 mM phosphate buffered sample), T=21±1°C and 7
667 days contact time.. Theoretical values correspond to the NDMA formation that can be calculated
668 from the concentration of VFX and ODMVFX measured in the samples using the NDMA
669 formation yields of the individual compounds (0.53±0.01% and 1.19±0.02% for VFX and
670 ODMVFX).

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673 # Supporting Information

674 ## Fungal biodegradation of the NDMA precursors
675 ## venlafaxine and *O*-desmethylvenlafaxine in water

676 Marta Llorca [1,2,a], Francesc Castellet [3,a], María-José Farré [1], Adrián Jaén-Gil [1], Maira
677 Martínez-Alonso [4], Sara Rodríguez-Mozaz [1,*], Montserrat Sarrà [3], Damià Barceló [1,2]

678

679

680 **S1**

681 Before investigating the changes on NDMA formation potential during the fungal degradation
682 experiments, NDMA formation potential tests of the commercial standards VFX, NDMVFX and
683 ODMVFX were performed. The NDMA formation potential yield corresponds to the maximum
684 amount of NDMA on a molecular basis that can be formed from the disinfection of certain
685 compound with chloramines. Results showed a NDMA formation potential of 0.53±0.01%,
686 1.19±0.02% and <0.02% for VFX, ODMVFX and NDMVFX respectively. The higher NDMA
687 formation potential observed for ODMVFX in comparison to the parent compound VFX could be
688 explained by the increased electron density in the vicinity of the dimethylamino moiety induced
689 by hydroxylation of the aromatic ring and facilitates NDMA formation as previously observed
690 (Farré et al., 2012). On the other hand, NDMVFX has no NDMA formation potential due to the
691 loss of the dimethylamino moiety of the molecule that is responsible of NDMA formation during
692 the reaction with chloramines.

693 **S2**

694 As degradation of VFX by *P.ostreatus* was negligible, this fungus was not considered in this part
695 of the study. Therefore, only degradation by *T. versicolor*, *G. lucidum* was investigated. Results
696 for live control showed that in fact, NDMA formation potential slightly increased in both cases.
697 NDMA formation potential increased from 100±62 to 340±32 ng/L and from 136±6 to 267±76
698 ng/L for *T. versicolor* and *G. lucidum* control solutions, respectively (error corresponds to the
699 standard deviation of three experimental replicates), probably due to metabolites generated by
700 the fungi itself. These results were subtracted from the concentration of NDMA formed during
701 the formation potential tests of the degradation experiments with VFX in order to evaluate the
702 real change of NDMA FP due to the drug degradation.

703

704 **Table S1:** concentrations of *N*-desemthylvenlafaxine and *O*-desemthylvenlafaxine identified
705 during biodegradation of venlafaxine with *T. versicolor* and *G. lucidum*.

Days	NDMVFX				ODMVFX			
	T. versicolor		*G. lucidum*		*T. versicolor*		*G. lucidum*	
	Conc. (µg/L)	%RSD	Conc. (µg/L)	%RSD	Conc. (µg/L)	%RSD	Conc. (µg/L)	%RSD
0	0	0	0	0	0	0	0	0
3	21.5	5	11.4	6	1.31	15	0.66	23
7	18.4	4	15.3	11	0.98	21	1.10	14
10	17.4	5	19.6	28	0.79	17	2.18	16
15	14.3	9	30.8	16	0.16	11	3.23	19

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707 %RSD: percentage of relative standard deviation

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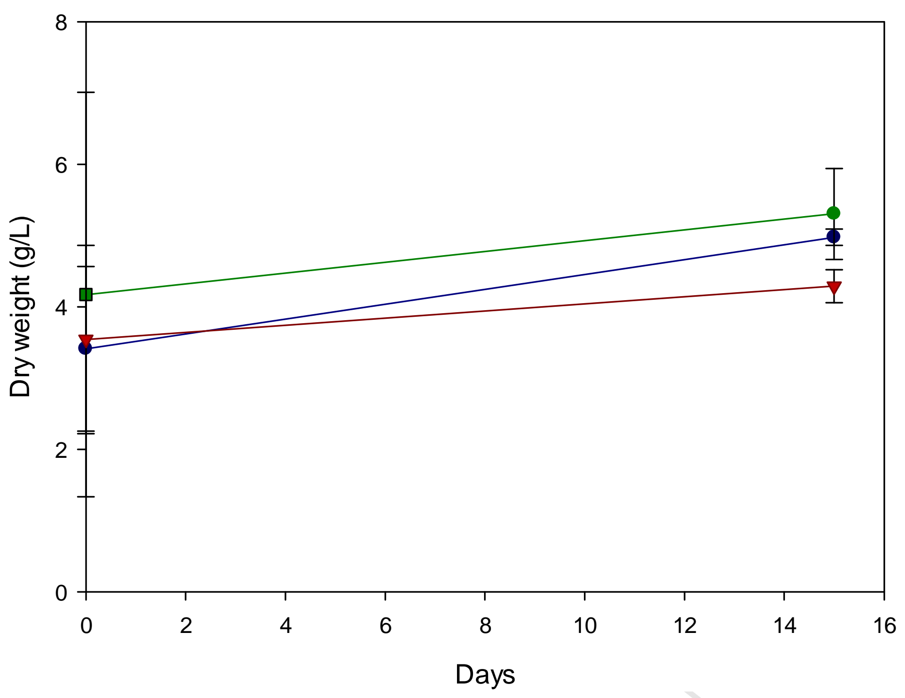

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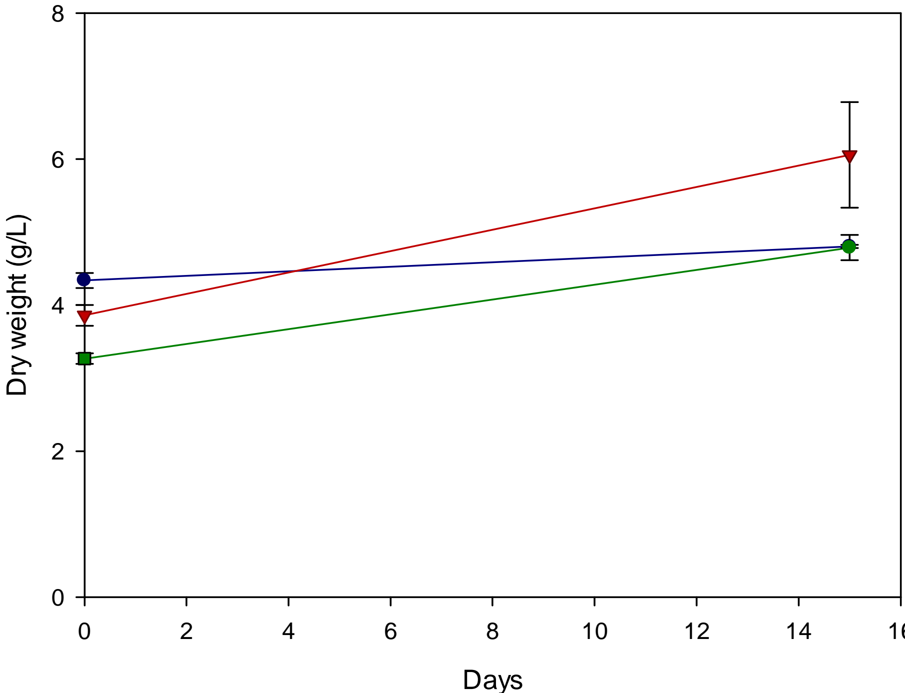
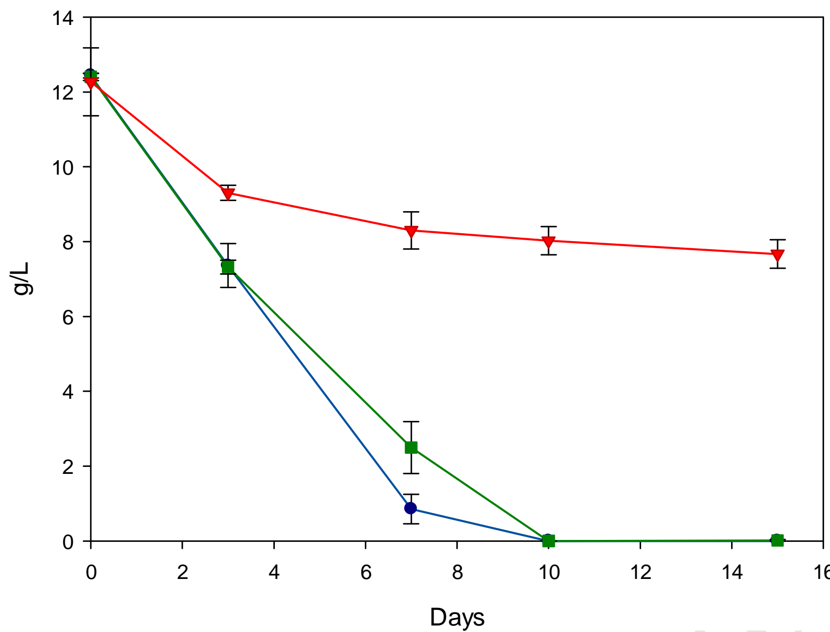


712 **Figure S1.** Dry biomass weight during the two degradation experiments. VFX (Figure S1A) and
713 ODMVFX (Figure S1B). The three fungal species are represented as follows: *P.ostreatus* (red
714 inverted triangles ▼), *T. versicolor* (blue circles ●) and *G. lucidum* (green squares ■).



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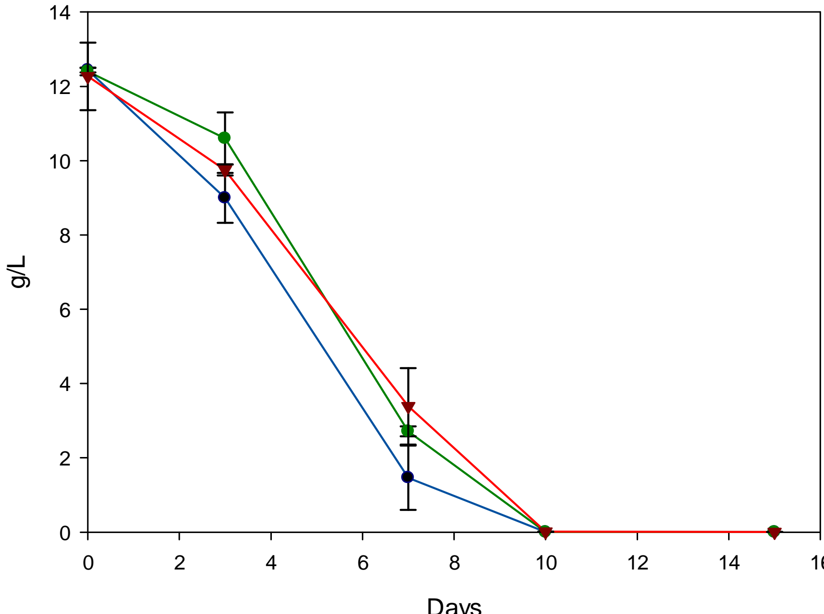

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717 **Figure S2:** Glucose consumption during the two degradation experiments. VFX (Figure S2A)
718 and ODMVFX (Figure S2B). The three fungal species are represented as follows: *P.ostreatus*
719 (red inverted triangles ▼), *T. versicolor* (blue circles ●) and *G. lucidum* (green squares ■).

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A) Venlafaxine

B) O-desmehtylvenlafaxine

Figure S3. Laccase activity during the two degradation experiments: VFX (Figure S3A) and ODMVFX (Figure S3B). The three fungal species are represented as follows: *P.ostreatus* (red inverted triangles ▼), *T. versicolor* (blue circles ●) and *G. lucidum* (green squares ■).

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Highlights

- Fungal bioremediation of antidepressants venlafaxine and *O*-desmethylvenlafaxine
- Study of transformation by-products and evaluation of NDMA formation potential
- Removal of antidepressants between 70 and 100 % by *T. versicolor* and *G. lucidum*
- Reduction of NDMA formation potential