Exploring the degradation capability of *Trametes versicolor* on selected hydrophobic pesticides through setting sights simultaneously on culture broth and biological matrix

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1. Introduction

Over the last few decades, micropollutants such as pesticides, pharmaceutically active compounds, personal care products, industrial chemicals and their by-products had been introduced into aquatic and terrestrial environments due to anthropogenic activities, evoking global concerns even in small traces (Mir-Tutusaus et al., 2018; Bilal et al., 2019a; Bilal et al., 2019b; Bilal et al., 2020). Particularly, pesticides usage shows a sustainable growing tendency because of the exponential increase in human population that actually further stresses the necessity for augmenting food production. Although substantial improvements have been obtained in crop yields by controlling pests, weeds and disease, the risk of environmental pollution resulted from pesticides drifting among hydrosphere, lithosphere and biosphere could not be ignorable (Carvalho, 2017; Ullah et al., 2018; Bilal et al., 2019b). Virtually, pesticides are considered as the main trigger of environmental deterioration, owing to their persistence, recalcitrance and multi-faceted toxicity.

Hydrophobic pesticides, such as chlorpyrifos, dicofol, and cypermethrin usually demonstrate better persistence because of their lower bioaccessibility. Specifically, chlorpyrifos and dicofol correspond to organophosphorus and organochlorine pesticides, respectively, while cypermethrin belongs to the family of synthetic pyrethroids. All of them are used extensively throughout the world in public health, agricultural, and domestic applications since they are highly effective at low doses. However, accumulating evidence has shown that the occurrence of those contaminants in environment is posing a severe threat to human as well as other non-target organism in several

aspects (Burr, 2014; Koshlukova and Reed, 2014; Aznar-Alemany et al., 2017; Carvalho, 2017; Ullah et al., 2018; Bilal et al., 2019b). Besides, they have been restricted by the Stockholm Convention and classified as possible human carcinogens by the Environmental Protection Agency (EPA) of USA. Hence, although some proper managements or strategies should be practiced to minimize release amount (Ullah et al., 2018), seeking for elimination techniques targeting such pollutants from environment is strongly encouraged and urgent.

Compared with chemical and physical methods, friendly to environment, efficient and low-cost features are the major advantages of biodegradation, which is widely applied in pesticide elimination (Mir-Tutusaus et al., 2018). Indeed, different microorganisms have been reported harbor the capacity to degrade chlorpyrifos (Singh et al., 2004; Aswathi et al., 2019), dicofol (Osman et al., 2008; Lu et al., 2019), and cypermethrin (Deng et al., 2015; Tang et al., 2018), among which degradation pathways has also been proposed (Singh et al., 2004; Deng et al., 2015; Tang et al., 2018). However, limited attention has been paid to white-rot fungi (WRF) that are metabolically versatile and are capable of degrading a wide spectrum of xenobiotics due to their nonspecific lignin-degrading enzymes (Mir-Tutusaus et al., 2018; Bilal et al., 2019b). On the other hand, in any case, pesticide degradation effectiveness was basically evaluated according to the difference in concentration of culture medium between initial and final (Chen et al., 2012; Deng et al., 2015; Tang et al., 2018). No study so far has paid attention to the sorption from fungal biomass by means of direct measurement of these compounds in the solid phase, namely biological matrix.

The purpose of the present study was to explore the capacity of WRF *Trametes versicolor* to degrade selected hydrophobic pesticides corresponded to different families, and contributions from either degradation or adsorption were thoroughly assessed through mass balance evaluation.

Meanwhile, metabolites of chlorpyrifos and dicofol were identified because this part of work for cypermethrin has been already done in previous research (Mir-Tutusaus et al., 2014).

2. Materials and Methods

2.1. Microorganisms and media

T.versicolor ATCC 42530 was acquired from American Type Culture Collection, and maintained by subculturing every 30 d on 2% (w/v) malt extract plates (pH 4.5) at 25 °C. *T.versicolor* blended mycelial suspension and pellets were prepared as the method of Blánquez et al. (2004).

Defined medium consisted of (per liter) glucose 8 g, 3.3 g ammonium tartrate, 1.68 g dimethyl succinate, 10 mL micronutrients and 100 mL macronutrients (Kirk et al., 1978). pH was adjusted to 4.5.

2.2. Chemicals and reagents

Analytical standards (purity, > 99%) of chlorpyrifos, dicofol and cypermethrin were purchased from Sigma-Aldrich (Barcelona, Spain). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade. Stock solutions (5 mg mL^{-1}) of pesticides were prepared in methanol and stored at – 20 °C.

2.3. Degradation experiments

Degradation experiments were conducted in 500 mL Erlenmeyer flasks containing 100 mL of fresh defined medium with mixed pesticides at 5 μg L^{-1}. Briefly, pellets were transferred into as inoculum, thereby achieving a concentration of approximately 3.3 g dry weight (DW) L^{-1}. Then, the cultures were incubated at 25 °C under shaken (135 rpm) and dark condition for 14 d. Abiotic (uninoculated) as well as heat-killed culture (121 °C, 30 min) were used as controls, and triplicate

replications were set for each experiment. Samples were taken at specific intervals during degradation by totally sacrificing each flask culture. Pellets and culture medium were separated firstly through 0.45 μm glass microfiber filter (GF/A), and liquid phase was centrifuged (17, 700 × g, 15 min). Afterward, 100 μL of deuterated standards pesticides (d_{10}-chlorpyrifos, d_{10}-chlorfenvinphos and phenoxy-d_5-fenvalerate) solution (0.5 μg mL^{-1}, dissolved in methanol), used as internal standards for quantification purposes, was added to obtained supernatant up to a final volume of 100 mL, followed by transferring into amber vial. Solid samples, namely the fugal pellets, were kept in aluminum foil. Both of them were stored at – 20 °C before subsequent pretreatments as follows:

(1) Liquid samples: based on Feo et al. (2010), 30 mL of sample was ultrasonically extracted for 15 min. Then, 1 mL of chloroform was added and the samples were centrifuged (2, 200 × g, 5 min) after 5 min. The organic phase was recovered and the aqueous phase was washed once more with 1 mL chloroform. The organic phases were pooled and evaporated to dryness with nitrogen. The residue was reconstituted with 50 μL ethyl acetate and then subjected to Gas chromatography-tandem mass spectrometry (GC-MS-MS) analysis (Feo et al., 2011).

(2) Fungal pellet samples: the freeze-dried samples were mixed with 15 ng of internal standards mentioned above and they were left overnight at 4 °C. Then samples were extracted by pressurized liquid extraction, using a 350 ASE system (Dionex, USA). Fungal pellet samples and 2 g of Florisil were loaded into an ASE extraction cell (22 mL) previously filled with 6 g of Florisil. After that, the cell was completely filled with hydromatrix. Hexane and dichloromethane (1:1, v/v) were used as extraction solvent. Temperature and pressure were settled at 100 °C and 1650 psi, respectively. After 2 cycles of 10 min extraction, extracts were evaporated with nitrogen

to dryness and the residue was reconstituted with 50 μL ethyl acetate prior to GC-MS-MS analysis.

2.4. Metabolites identification

Given high degradation rate and the different properties of their probable metabolites, research on transformation products (TPs) were performed individually. In brief, pellets were carefully transferred into 100 mL of fresh define medium separately spiked each pollutant at final concentration of 1 mg L^{-1} and then incubated under same conditions described in section 2.3 for 7 d. Abiotic was prepared as control and each experiment was conducted in triplicate. Samples were taken at specific intervals during incubation by filtrating with 0.45 μm glass microfiber filter. 20 mL of filtrate was mixed with 50 μL of internal standard methanol solution (d_{10}-chlorpyrifos or d_7-oxadiazon, 100 mg L^{-1}), followed by adding filtrate up to 50 mL. Afterward, samples were kept at – 20 °C prior to analysis.

2.5. Analytical methods

2.5.1. Biomass quantification

Biomass were determined by the dry weights of mycelia, which were obtained through filtering with glass microfiber filter (0.45 μm), followed by drying at 100 °C to constant weight.

2.5.2 Glucose

Samples were filtrated with nylon filter (0.45 μm) and then measured by biochemistry analyzer (2700 select, Yellow Springs Instrument, USA).

2.5.3. Laccase

Laccase activity was assayed through the oxidation of 2,6-dymetoxyphenol (DMP) by the enzyme as described elsewhere (Wariishi et al., 1992). Activity units per liter (UA L^{-1}) are defined

as the amount of DMP in μM which are oxidized per minute.

2.5.4. Pesticide analyses

Residual pesticide concentrations were determined through GC-MS-MS (7890B/7000C, Agilent, USA) equipped with a DB-5MS capillary column (30 m × 0.25 mm, 0.25 μm). The operating conditions were as follows: the column was held initially at a temperature of 80 °C for 2 min, then raised at 25 °C min^{-1} to 180 °C for 6 min, at 5 °C min^{-1} to 240 °C for 5 min, at 10 °C min^{-1} to 280 °C for 5 min, and finally at 30 °C min^{-1} to 325 for 2 min. The temperature corresponding to the transfer line and the ionization source were 300 °C and 280 °C, respectively. The collision energy was 70 eV. The injection volume was 2 μL at 280 °C. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}.

2.5.5. Metabolite analyses

The sample was subjected to separation by an Acquity ultra performance liquid chromatography (UHPLC) system (Waters, USA) equipped with a Purospher STAR RP-18 endacapped (2 μm) Hibar HR 150-2.1 UHPL column (Merck, Germany). Methanol and water both containing 0.1% formic acid (v/v) were used as mobile phases A and B, respectively. A gradient elution program was started with 80% (v/v) B from 0 min to 1 min, decreasing to 5% at 8 min and held until 13 min. Then, the percentage of B was further reverted to 80% by 13.5 min and maintained it until 15 min. The follow rate was 200 μL min^{-1} with an injection volume of 10 μL. The UHPLC system was coupled to a hybrid quadrupole-Orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific, USA) equipped with a heated-electrospray ionization source HESI II, which was operated in positive ionization mode under the following conditions: spray voltage, + 3.0 kV; sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary units; sweep gas, 2 arbitrary units;

capillary temperature, 350 ℃ and vaporizer temperature, 300 ℃. Nitrogen (> 99.98%) was employed as sheath, auxiliary and sweep gas. The mass spectrometer performed a Fourier Transform Mass Spectrometry (FTMS) scan event of 50-700 m/z at a resolution of 70, 000 and a subsequent MS/MS scan event acquired at a resolution of 35, 000. Xcalibur software (Thermo Fisher Scientific, USA) was employed for instrumental control and data processing.

In the case of post-acquisition MS data processing, the total ion current (TIC) chromatograms acquired at different sampling time were compared using Compound Discoverer (Thermo Fisher Scientific, USA), which allows performing differential analysis among selected sets of samples comparing simultaneously thousands of MS spectra, to identify all potential TPs. Accurate mass of the potential TPs was then extracted in Xcalibur to confirm their presence. Identification of the potential TPs was based on their accurate mass, mass error, molecular formula and degree of unsaturation of the parent ion and product ions.

2.5.6. Data analysis

Degradation percentage was calculated using the following equation:

$$\text{Degradation percentage}(\%) = \frac{M_0 - M}{M_0} \times 100$$

where M_0 corresponds to initial amount of contaminant (ng), and C represents the residual amount of contaminant (ng).

The mean and standard deviation (SD) of data were calculated and subjected to analysis of variance (ANOVA). Statistical significance was determined using SPSS V22.0.

3. Results and discussion

3.1. Degradation of mixed pesticides

Taking into account that pesticides detected from environment are usually present in the range

of ng L^{-1} to μg L^{-1} (Zheng et al., 2016; Moreno-González and Leon, 2017; Tang et al. 2018), degradation experiments were running at 5 μg L^{-1}, along which time-course in glucose consumption and laccase activity were also investigated. As shown in Figure 1, glucose was totally utilized after 7 d incubation. With respect to laccase, a continuous increase in activity was observed within 7 d, thereby achieving the maximum value at 48 AU L^{-1} and then maintained at this constant level until 14 d, although the elimination occurred largely before first sampling time. Similar performances were observed in different treatments using *T.versicorlor* (Blánquez et al., 2004; Mir-Tutusaus et al., 2014).

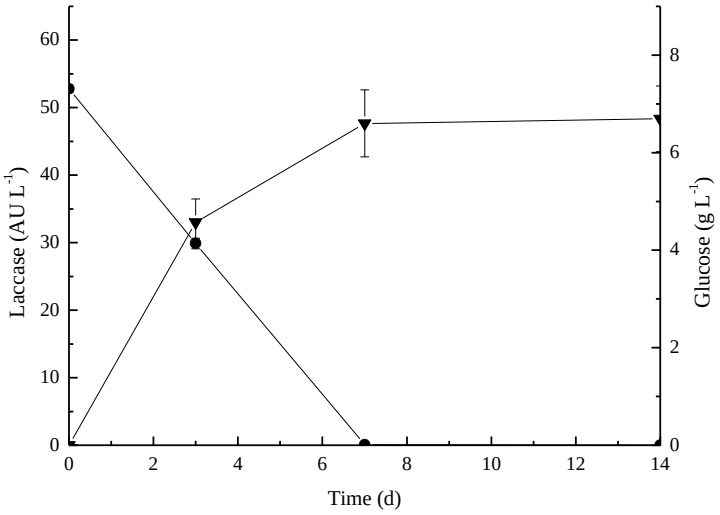

Figure 1 Profiles of laccase and glucose during pesticides degradation by *T.versicolor*. *Filled inverted triangles*, laccase; *filled circles*, glucose. Values are means of three replicates with standard deviation.

On another side, the pesticide residues in liquid phase were determined in the beginning (Figure 2) as most cases did (Chen et al., 2012; Deng et al., 2015; Tang et al., 2018), showing that the pollutants in abiotic control maintained at relatively constant levels, although the final concentration of cypermethrin was slightly less than initial. Besides, no statistical differences were observed along treatment. Conversely, considerable removal of added chlorpyrifos, dicofol and

cypermethrin occurred in both experimental and killed control flasks, by which it is not easy to determine whether degradation occurred. Therefore, focus was switched to biological matrix, in order to seek for the explanation for this phenomenon. Interestingly, results indicated that pesticides in killed control accumulated along incubation and then essentially achieved a stabilisation ($p < 0.05$), whereas much less amounts of pollutants were detected when it comes to experimental treatment, and it displayed an apparent decreasing tendency ($p < 0.05$). These profiles correspond to the time-course glucose consuming and laccase activity, because no increase in laccase was detected since 7 d, which perhaps means laccase was involved in this particular biochemical process. But intracellular enzymes (Mir-Tutusaus et al., 2018) should also be further investigated. Nevertheless, it's feasible to suppose that adsorption not only played an important role but also happened in the first place during pesticides elimination by *T.versicolor*, which corroborates that observed by Blánquez et al. (2004). Accordingly, decolorization process was started by the initial adsorption of the dye, which was then absorbed and degraded as the result of enzyme, followed by final release of metabolites. And it could also explain why the results were expressed as C/C_0. Because this physical adsorption process occurred immediately once the pollutant was added, resulting in the initial concentration was not lower than 5 μg L^{-1}. Simultaneously, in respect of liquid phase, more efficient elimination towards dicofol and cypermethrin were observed in killed control than in experimental treatment. The reasonable explanation is that active metabolic processes somehow negatively affect adsorption (Fomina and Gadd, 2014).

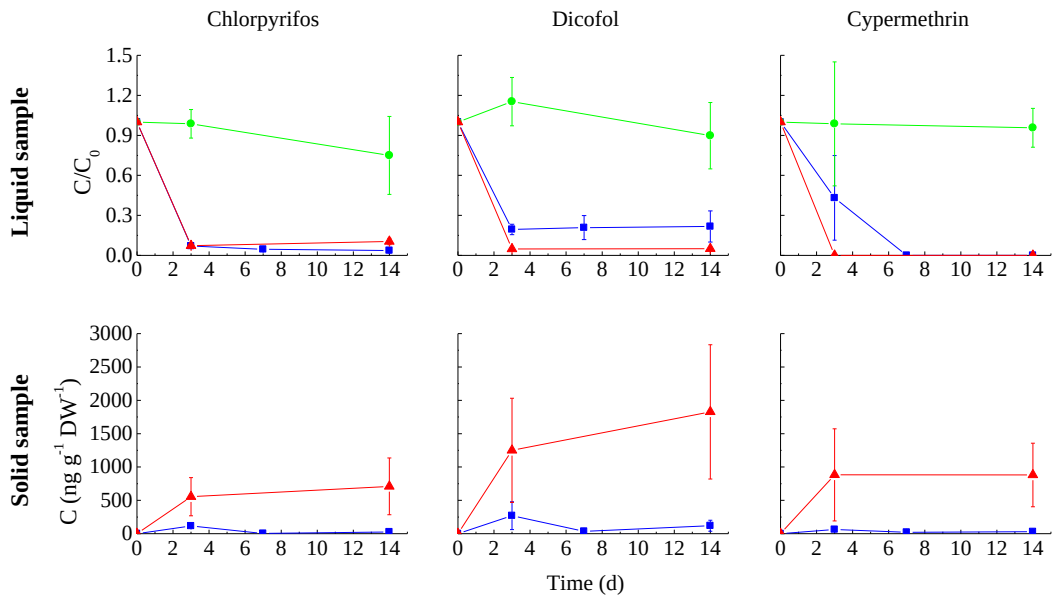

Figure 2 Pesticides degradation by *T.versicolor*. C represents the residual concentration of pesticide in sample (μg L^{-1}), and C_0 corresponds to the initial concentration of pesticide in sample (μg L^{-1}); *Blue lines with filled squares*, experimental; *red lines with filled triangles*, killed control; *green lines with filled circles*, abiotic. Values are means of three replicates with standard deviation Values are means of three replicates with standard deviation.

In order to ascertain the contributions from either biodegradation or sorption, by which the selected pesticides were eliminated, mass balances during incubation were proposed and the results are summarized in Table 1. Clearly, pesticides were effectively removed in experimental sets, but most of them were still remained in the killed control, especially dicofol, demonstrating even more amounts than initial. The most probable reason for this fact could be the systematic error, likely the inoculum differences in terms of dry weight, which could also explain the high standard deviations between samples. Lucas et al (2018) evaluated the contribution of sorption process in the elimination of pharmaceuticals during the fungal treatment of wastewater, from which a mean value as 7% was obtained. By contrast, much higher average adsorption was observed in present study, yielding more than 90%, probably because of that the selected pesticides possess higher hydrophobicities (Fomina and Gadd, 2014). Anyway, there is no doubt that *T.versicolor* demonstrated efficient capabilities in degrading chlorpyrifos, dicofol and

cypermethrin at concentrations in the range of μg L^{-1}, resulting in 94.7%, 87.9% and 93.1% of removal, respectively.

Table 1 Mass balance profile of pesticides in Erlenmeyer flasks according to the residues in both liquid and solid phase

Pesticide	Set up	Amounts (ng)			
		Time (d)			
		0	3	7	14
Chlorpyrifos	Experimental	235.24 ± 18.75*	45.62 ± 4.18*	6.08 ± 0.63*	12.55 ± 1.83*
	Killed control	312.75 ± 80.76	194.82 ± 77.41	ND	249.30 ± 98.47
Dicofol	Experimental	335.68 ± 95.41*	90.54 ± 56.07*	12.64 ± 2.31*	40.75 ± 19.11*
	Killed control	417.98 ± 135.30	412.90 ± 209.72	ND	603.26 ± 235.28
Cypermethrin	Experimental	144.64 ± 17.34*	38.67 ± 4.20*	6.92 ± 0.84*	9.98 ± 0.95*
	Killed control	371.71 ± 64.79	291.17 ± 185.91	ND	290.73 ± 111.27

Note: Means and standard deviation of triplicate are shown; ND, not data; *statistically different compared along the time ($p < 0.05$)

Although there are numerous literatures documenting degradation of selected compounds by microorganisms (Chen et al., 2012; Deng et al., 2015; Tang et al., 2018; Aswathi et al., 2019; Lu et al., 2019), few sight have been thrown into adsorption attributed to biological matrix. Because it's involved in elimination process at different degree, especially remarkable in the cases of hydrophobic xenobiotics (Margot et al., 2015; Liu et al., 2019). To address this gap, this present work is actually first time to evaluate degradation capability of *T.versicolor* on degradation chlorpyriphos, cypermethrin and dicofol based on analysis of residual concentrations both in culture medium and biological matrix combined with mass balance evaluation. So, it essentially offered a strong proof and better understanding of the elimination mechanism. In the meantime, it also worth to emphasis that comparing the obtained efficacy with previous studies is not that feasible. Because not only should the differences in term of substrate concentration be taken into consideration, but also culture conditions such as temperature, pH and medium can be effect factors. Anyway, this particular species displays promising potential in real bioremediation.

3.2. Metabolites of chlorpyrifos and dicofol by T.versicolor

Owing to pesticides removals contributed from both degradation and adsorption took place so fast, higher concentration was applied into metabolites identification experiments, in order to catch the TPs more easily. As summarized in Table 2, three compounds were captured and identified as metabolites within 7 d incubation according to the results of UHPLC-MS/MS analysis, of which benzaldehyde is first time to be reported as TP of dicofol. In addition, the compounds 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid and 3-phenoxybenzoic acid have been identified as TPs of cypermethrin using *T.versicolor* by Mir-Tutusaus *et al* (2014).

Table 2 Chromatographic characteristics of the TPs of chlorpyrifos and dicofol by *T.versicolor*

Pesticide	Identified TP	Nominal mass	Retention time (min)	Measured mass (m/z)	Mass error (ppm)	Molecular formula	RDB
Chlorpyrifos	O,O-diethyl thiophosphate	171	4.21	171.0239	– 0.280	$C_4H_{12}O_3PS$	– 0.5
		115		114.9616	2.716	H_4O_3PS	– 0.5
		143		142.9928	1.134	$C_2H_8O_3PS$	– 0.5
		97		96.9510	2.238	H_2O_2PS	0.5
		81		80.9741	6.089	H_2O_3P	0.5
	diethyl phosphate	155	3.10	155.0467	– 0.528	$C_4H_{12}O_4P$	– 0.5
		127		127.0156	1.325	$C_2H_8O_4P$	– 0.5
		99		98.9846	4.732	H_4O_4P	– 0.5
		81		80.9740	4.360	H_2O_3P	0.5
Dicofol	benzaldehyde	107	7.47	107.0495	3.537	C_7H_7O	4.5
		91		91.0547	5.307	C_7H_7	4.5
		81		81.0341	8.005	C_5H_5O	3.5
		79		79.0548	7.630	C_6H_7	3.5
		77		77.0392	8.350	C_6H_5	4.5

Note: Molecular formulas were calculated on the basis of their accurate mass measurements and the observed isotopic patterns. Unsaturation degree was expressed as double bound equivalents (RDB).

So apparently, hydrolyzation occurred in degradation process of chlorpyrifos and cypermethrin, keeping in line with most cases described to date (Singh et al., 2004; Tang et al., 2018; Aswathi et al., 2019; Deng et al., 2015). And it is always the first reaction which also play an irreplaceable

role in detoxification of other organophosphorus and pyrethroids (Sogorb and Vilanova, 2002; Singh and Walker, 2006). On the other side, considering our result and previous findings (Bumpus and Aust, 1987; Bumpus et al., 1993), we speculated that dichlorination occurred firstly during dissipation process of dicofol, transforming it into 2,2-dichloro-1,1-bis-(4-chlorophenyl)-ethanol (FW-152). FW-152 was then subject to successive reductive dichlorinations and oxidative cleavage, resulting in formation of 4,4'-dichlorobenzophenone, which was further degraded into benzaldehyde through ring cleavage reaction probably because of the role of lignin-degrading system (Bumpus and Aust, 1987).

4. Conclusions

Measuring target compounds in liquid and solid phases simultaneously is a worthwhile strategy to illustrate the elimination mechanism of pollutants. Both biodegradation and adsorption were considerably involved in removal of chlorpyrifos, dicofol and cypermethrin. *T.versicolor* demonstrated ideal degradation capability on those compounds, showing as 94.7%, 87.9% and 93.1% removal percentage respectively. Our findings suggest that the particular microorganism is useful for bioremediation of hydrophobic pesticides contaminated environments. Meanwhile, the related metabolites were identified, indicating that hydrolyzation, dichlorination and oxidation played important roles within degradation process.

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Conflict of interest

We declare that no conflict of interest exists in the submission of this manuscript.

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