Analyzing the Degradation of Fipronil Insecticide via *Trametes versicolor*

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With massive urbanization continuing to spread across the globe and new chemicals, pesticides, and pharmaceutical compounds being developed at a blistering pace that exceeds safe regulation, our concerns in regards to urban stormwater runoff have since shifted towards more problematic pollutants that current wastewater treatment plants cannot properly remove. One of these emerging pollutants is an urban-use pesticide known as fipronil {5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile} which has recently become prevalent in water systems near urban areas due to its low solubility in soils and the longevity of its similarly-toxic metabolites.[1,2] With this, there is a pressing need to develop new water treatment methods that can degrade or remove trace organic compounds like fipronil from freshwater systems in a sustainable, cost-effective, and environmentally-conscious manner that traditional water treatment plants cannot capacitate.

Over the past two decades, the lignin-degrading properties of various white rot fungi (WRF) have been shown to be effective in degrading trace organic compounds in both soil and aquatic environments.[3] Much like the application of microbial digestion in wastewater treatment processes, the degrading properties of fungi may be vital in developing new methods to remove emerging trace organic pollutants from wastewater, particularly in urban stormwater runoff. However, due to the complexities of fungal degradation processes, field applications of various species of fungi have yet to be researched to the extent of other microbial processes. Previous experiments at Stanford have shown that the WRF species, *Trametes versicolor*, can degrade fipronil more efficiently than other WRF species in aqueous environments (Unpublished). Currently there have been no studies on the ability of *T. versicolor* to degrade fipronil in non-sterile environments. Additionally, it is still unclear which fungal enzyme complexes are instrumental in the degradation of fipronil. As such, the aim of this research is to gain a better understanding on what conditions are necessary for *T. versicolor* fungi to effectively degrade fipronil.

Enzyme Studies. Previous Stanford WRF studies have shown that *T. versicolor* fungi can degrade fipronil in aqueous environments. Previous studies also indicated that extracellular enzymes were less important in the degradation of trace organic pollutants than intracellular enzymes, specifically the P450 complex enzymes found within *T. versicolor*.[3] To better understand the metabolic processes at play, an experiment was designed to isolate the fungal enzymes necessary for fipronil degradation. *T. versicolor* was incubated for seven days in glass bottles in 2% malt extract media (MEM) and spiked to a concentration of about 10 mg/L fipronil. Two treatment groups were exposed to high (5mM) and low (0.5mM) doses of 1-aminobenzotriazole (ABT) which inhibited the P450 enzyme complexes. It was later determined that the 5mM concentration was too high for the batch studies, as it prevented the fungus from growing adequately.

The bottles were then individually sampled at 0, 0.25, 1, 3, and 7 days and concentrations of fipronil and fipronil-sulfone were quantified via HPLC-UV. The results from this initial experiment were inconclusive due to the fact that the negative fipronil control did not remain at a constant concentration, decreasing dramatically over the course of the seven-day study. To assess the cause of this unforeseen effect, a separate fipronil control study was performed. One of the most common degradation pathways of fipronil is through photolysis, though the half-life duration varies widely depending on the physical state of fipronil and the environment it inhabits.[4] To prohibit this from occurring, the negative controls were incubated in dark amber glass bottles in MEM and stormwater media, respectively. As a result, the aforementioned change in methods allowed the fipronil concentration in MEM to remain statistically constant, though the fipronil concentrations in the synthetic stormwater were still inconsistent with how fipronil is expected to act in these bench studies (Figure 1). In future studies, a lower concentration of fipronil (approximately less than 2mg/L) should be used to ensure a uniform, stable concentration.

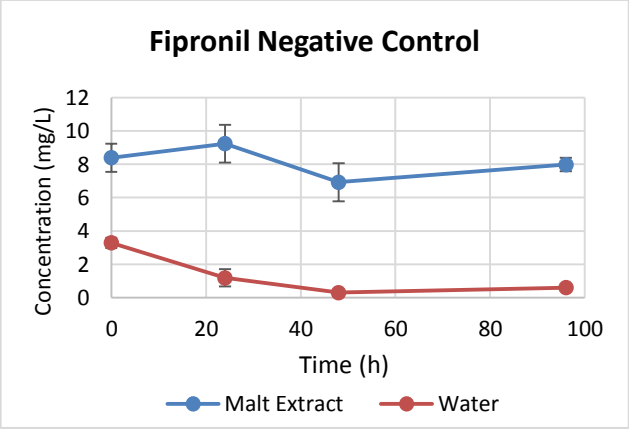

Figure 1. While fipronil concentrations remained fairly constant in MEM, fipronil does not appear to be as stable or directly measurable in stormwater.

A second enzyme study was then developed using amber bottles, three constituents (negative control, positive control, and treatment with 0.5 mM ABT), and improved procedural methods to ensure the dissolution of fipronil in MEM and accurate spiking concentrations. These methodical changes yielded more reliable results than the first enzyme study, with the negative control remaining at a statistically constant concentration (Figure 2). Our results show that the positive control and the *T. versicolor* treated with ABT exhibit similar degradation rates, indicating that fipronil degradation is not solely reliant on P450 enzyme complexes. However, trace amounts of fipronil-sulfone (a metabolite of fipronil produced by oxidative degradation) were found in the positive control ("Fipronil w/ T.V."). Fipronil-sulfone was not detected in the ABT-treated constituent. This discovery suggests that P450 complexes may necessitate a step in fipronil degradation, specifically the oxidation of fipronil into fipronil-sulfone.

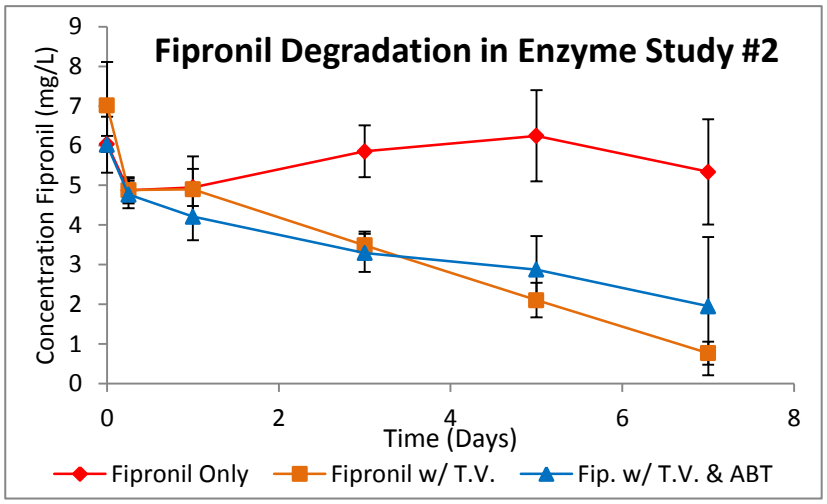

Figure 2. Fipronil exposed to *T. versicolor* gradually degrades over time. Addition of ABT does not markedly affect degradation patterns, but may prevent fipronil-sulfone metabolites from being produced (not shown on graph).

Mesocosm Study. This experiment was developed in order to determine the fungi's ability to thrive in different controlled environments. In this study, malt extract media was replaced with woodchips and stormwater media. Glass bottles were prepared and incubated at room temperature over the course of seven days to compare the fungi's ability to grow on sterile and non-sterile woodchips, as well as its ability to grow in a low-carbon environment (synthetic stormwater alone). Fipronil was spiked in all constituents, though the majority of the samples had non-detectable amounts of fipronil remaining after the seven-day incubation due to the high detection limit of HPLC-UV. Qualitative observations suggest that *T. versicolor* grows best in a sterile woodchip-stormwater matrix. A visual comparison of sterile and non-sterile environments shows that microbial nutrient competition is a major factor when attempting to properly introduce *T. versicolor* in new environments (Figure 3).

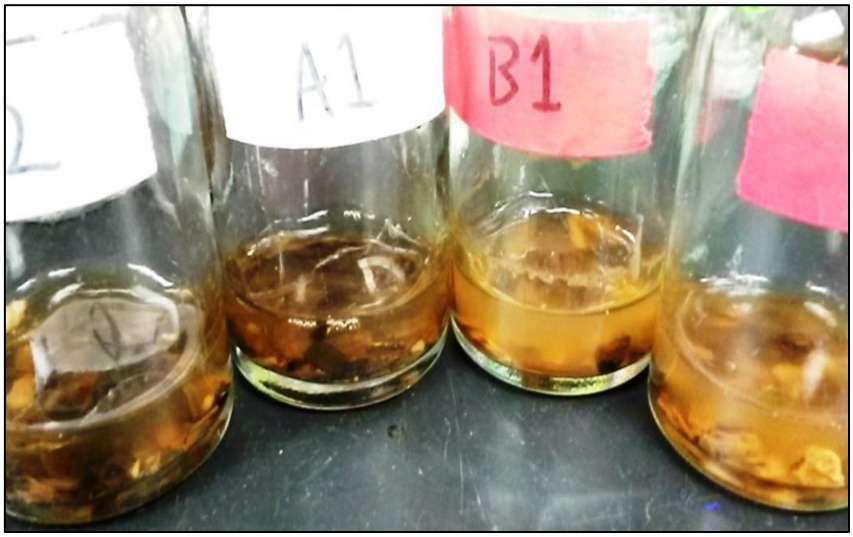

Figure 3. Sterile media (left) remains clear and allows T. versicolor to thrive while non-sterile media (right) becomes murky and causes other microbes to take over.

References

(1) Gan, J.; Bondarenko, S.; Oki, L.; Haver, D.; Li, J. X. Occurrence of fipronil and its biologically active derivatives in urban residential runoff. *Environ. Sci. Technol.* **2012**, *46* (3), 1489–1495.

(2) Weston, D. P.; Lydy, M. J. Toxicity of the insecticide fipronil and its degradates to benthic macroinvertebrates of urban streams. *Environ. Sci. Technol.* **2014**, *48* (2), 1290–1297.

(3) Bending, G. D.; Friloux, M.; Walker, A. Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. *FEMS Microbiol. Lett.* **2002**, *212* (1), 59–63.

(4) Gunasekara, A. S.; Truong, T.; Goh, K. S.; Spurlock, F.; Tjeerdema, R. S. Environmental fate and toxicology of fipronil. *J. Pestic. Sci.* **2007**, *32* (3), 189–199.