Fungal Remediation of Nematocyte with Fluopyram Active Ingredient by *P. frequantans* and its Mortality Effect on *D. magna*

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Abstract

In this study, to understand the bioremediation efficiency of *Penicilium frequantans* soil fungus used as a useful tool for bioremediate a fluopyram nematocide with important environmental parameters such as chemical oxygen demand (COD) and total organic carbon (TOC) studied. Additionally, mortality tests were performed for treated and untreated media with *D. magna* to gain additional knowledge about the toxic effect of this pesticide after bioremediation step. According to the results of the study, *P. frequantans* performed high COD and TOC reduction rates of fluopyron nematocide as 90 % for both parameters in 6 and 7 days respectively. This means *P. frequantans* is a suitable tool for bioremediating this kind of nematocides. According to the *D. magna* mortality test results, TOC and COD values of pesticide dereeased, mortality rates increased by the reason of chemical structure and side products of pesticide.

Keywords: Bioremediator, Chemical oxygen demand, *Daphnia magna, Penicillium frequantans*, Total organic carbon.

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INTRODUCTION

Increasing population, rapid industrialization and advancing technology create a pollution burden, especially in terrestrial and aquatic ecosystems, and this creates negative effects on living life. Disposal of pesticide-containing untreated water from agricultural fields greatly affects the receiving environments near these areas (Luka et al., 2018). In order to eliminate environmental problems caused by organic chemicals, many researchers have preferred physical, chemical or biological treatment methods. Although physical and chemical methods are initially effective, they can also produce toxic secondary metabolites after purification. Bioremediation, one of the biological methods, can effectively prevent these problems (Saravanan et al., 2023).

In agricultural activities, weeds, pests and diseases cause the yield and quality of agricultural products to deteriorate. According to statistics, approximately 30% of agricultural products are damaged every year due to various harmful factors (Kučić Grgić et al., 2019). As a result of this situation, the use of pesticides becomes inevitable. However, as a result of the excessive use of these pesticides, the chemicals in the pesticide not only accumulate in crops but also remain in agricultural soils, affecting soil fertility and indirectly microbial communities (Satapute and Kaliwal, 2016). Pesticides move both vertically and horizontally in the soil. Pesticides leaking into receiving environments and groundwater also pollute water resources, and as a result, aquatic invertebrates and fish are negatively affected (Dwivedi et al., 2011).

Bioremediation refers to the application of microorganisms and other microbial-derived products for the degradation or reduction of various organic pollutants or their toxic effects (Priyadarshanee and Das, 2021). The basic concept of bioremediation is that microorganisms can use pollutants as a source of carbon and/or nitrogen and completely mineralize them into carbon dioxide and water, or at least convert them into non-toxic secondary decomposition products (Shabbir et al., 2018). Therefore, bioremediation is an ideal, environmentally friendly and inexpensive treatment method for the removal of environmental pollutants.

Fluopyram (CAS No.: 658066–35–4) is a nematicide developed by Bayer CropScience (Fought et al., 2009). This pesticide has been used as a promising nonfumigant nematicide in many high value crops including fruits and vegetables. As a result of its widespread use in recent years, its ecotoxicological effects have caused great concern. The low vapor pressure and volatility of fluopyram lead to high adsorption in soil and cause serious damage to microbial communities (Chawla et al., 2018). Zhang et al. (2014) observed that fluopyram had negative impacts on the activity of soil microorganisms and altered the structure and function of the microbial community.

In toxicological experiments, *Daphnia magna* is widely utilized as a model and important organism in the water ecosystem. It possesses several characteristics that make it suitable for research purposes, including ease of reproduction, high sensitivity to environmental stimuli, diverse phenotypes, and ease of treatment and handling (Hiruta and Tochinai, 2014).

In this study, fluopyrom nematocide was prepared at recommended concentrations in agricultural areas and a study was conducted on the bioremediation activities of *P. frequantans* fungus. In addition, a mortality test was performed on *D. magna* in environments where bioremediation activities were carried out, and the difference in mortality between treated and untreated environments was revealed.

MATERIALS and METHODS

Preparing *P. frequantans*

P. frequantans pure fungi cultures used in the study are already available in Munzur University Environmental Engineering laboratories. These cultures were isolated from an agricultural area in Istanbul before.

Pure fungal cultures were first taken from the petri dishes, scraped into the broth medium with a diameter of 1 cm, and added under sterile conditions according to the methods specified in Cruikshank (1972). These cultures were transferred to broth media and placed in a 27°C incubator and multiplied and enriched at 160 rpm.

Preparing Pesticide Solutions

Fluopyram nematocyte was obtained from a commercial company in Tunceli Province, and COD and TOC values were calculated taking into account the amount of active ingredient. Fluopyrom used in the experimental stages was prepared in 250 ml conical flasks at the concentrations recommended for use by farmers: 250, 500, 750 and 1000 ppm.

Bioremediation of Fluopyram

To investigate the bioremediation activities of *P. frequantans* on fluopyram on the basis of COD and TOC, firstly the Chemical Oxygen Demand (COD) and Total Organic Carbon (TOC) values in the enriched environment in which *P. frequantans* was found were measured. All of the experimental studies were performed triplicate and average value of these replicants were used. While the open relux method specified in standard method 5220B was used for COD analysis in the study; in TOC analyses, the standard method high temperature combustion method 5310A was preferred with Shimadzu TOC-V device.

For COD assays DR860 HACH spectrophotometer, the closed-reflux colorimetric method was used to calculate the COD concentration. The test values are the average of three readings, and the measurement discrepancies were found to be less than 0.02. The effectiveness of COD and TOC elimination discovered using Eq (1) and Eq (2)

$$COD\ removal\ (\%) = \frac{(COD_{initial} - COD_{final})}{COD_{initial}} \times 100 \qquad (1)$$

$$TOC\ removal\ (\%) = \frac{(TOC_{initial} - TOC_{final})}{TOC_{initial}} \times 100 \qquad (2)$$

COD_{initial} and COD_{final} (mg/L) are the values of initial COD and COD after bioremediation process at time *t*, respectively.
TOC_{initial} and TOC_{final} (mg/L) are the values of initial TOC and TOC after bioremediation process at time *t*, respectively.

As a result of COD and TOC measurements made every 24 hours, when a value close to the application concentration of fluopyram in the agricultural field was reached, 1 ml of *P. frequantans* (Each ml of mushroom contains approximately 10^6 colony-forming individuals) was started to be added to the fluopyram media for bioremediation activities. The samples taken every 24 hours were filtered through 0.45µm filter paper, and bioremediation studies were followed by COD and TOC analyzes on these filtrates. In order to inhibit the activity of fungal colonies during this process, one drop of freshly prepared 1N H_2SO_4 was added to the filtrates.

Mortality on *Daphnia magna*

D. magna organisms were obtained from Fırat University Fisheries Engineering Laboratories and species determinations were made in advance. *D. magna* individuals were kept in a 120-litre aquarium for a period of one month to adapt to laboratory conditions acclimated to a 16:8-hour light: dark cycle at a temperature of 16–18°C (±1) at laboratory. During the adaptation period of *D. magna*, the daily mortality rate was determined to be less than 10%. *D. magna* was fed periodically once a day with a mixture of dry spirulina powder and baker's yeast (*Saccharomyces cerevisiae*), and the aquarium water was regularly aerated with an air pump. Additionally, 25% of the water has been renewed at a rate of 1/7.

To evaluate mortality rates, nine different experimental groups were created. In this context, 350 mL of filtered water was taken from all application groups (control, 250, 500, 750 and 1000 ppm treated and untreated media at the same concentrations) and placed in polycarbonate containers filled with natural living water (NLW) on the 11^{th} day. The water temperature in each container was adjusted to 20°C (±1) by air conditioning, and changes in water temperature were checked regularly. There are 10 first stage juvenile *D. magna* individuals in each container. Test organisms were not fed throughout the experiment and a 16:8-hour light: dark photoperiod was maintained. Three repetitions were made for each experimental group. The number of dead daphnia in each container was counted after 24, 48, and 72 hours. At the end of the test period, that is, after 72 hours, mortality rates were calculated as percentages in each experimental group (Babu et al., 2015).

RESULTS and DISCUSSION

Bioremediation Studies Results on the basis of COD and TOC

In the study, the bioremediation activity results based on Chemical Oxygen Demand (COD) and Total Organic Carbon (TOC) shown by *P. frequantans* on Fluopyram nematocytes at different concentrations are shown in Figures 1 and 3 on a concentration basis; on the basis of removal efficiency, it is shown in Figures 2 and 4.

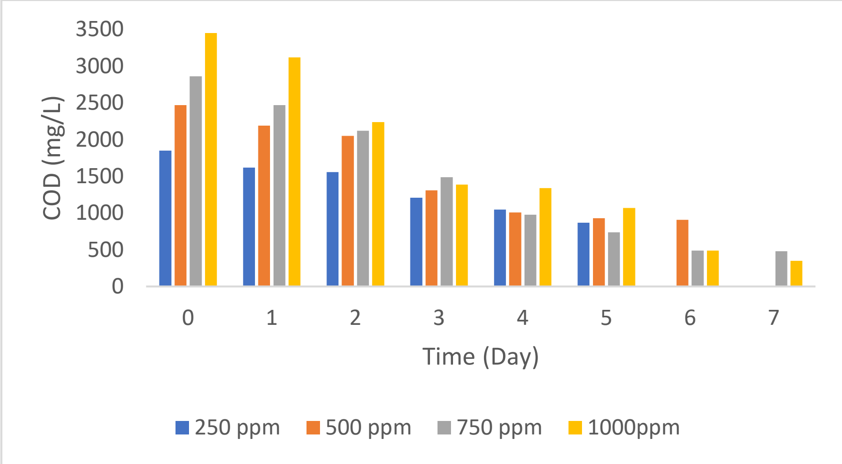

Figure 1. COD decrease based on concentration *by P. Frequantans*

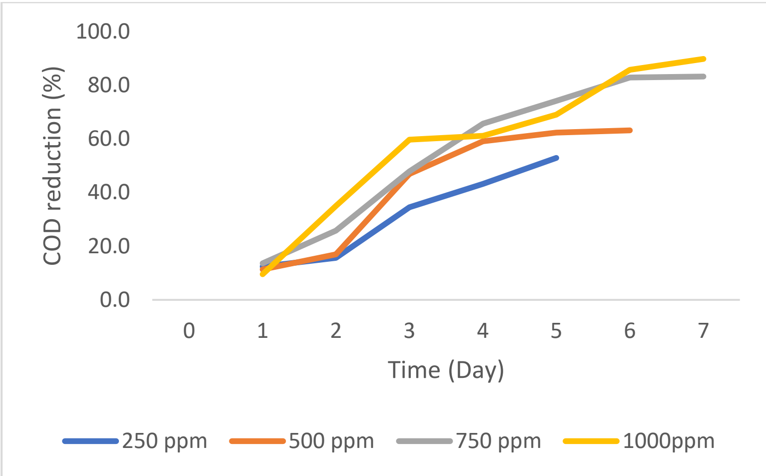

Figure 2. COD reduction efficiency by *P. Frequantans*

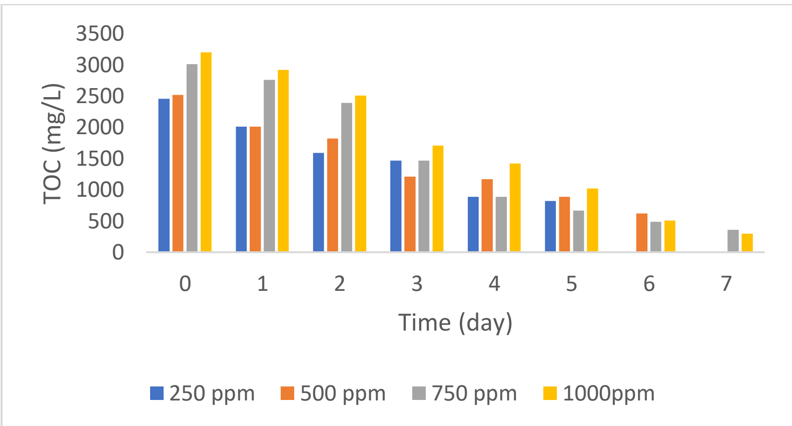

Figure 3. TOC decrease based on concentration by *P. frequantans*

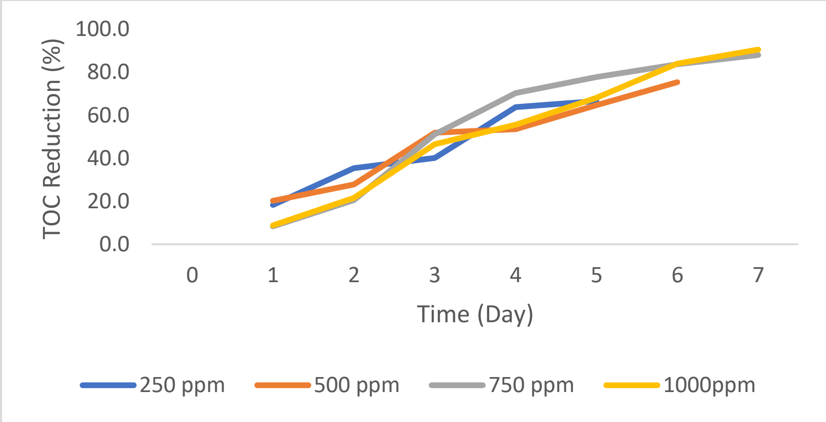

Figure 4. TOC reduction efficiency by *P. frequentans*

According to the results of the removal performance of *P. frequantans* on nematocyte with fluopyrom active ingredient on COD basis, the COD value, which had an initial value of 1850 ppm, was reduced to 870 ppm with 53% at the end of 5 days in a 250 ppm environment It is seen that the COD value, which has 2470 ppm value in the 500 ppm environment, decreased to 910 ppm, which means a decrease of 63.2% at the end of 6. days.

The initial COD value, which was 2860 ppm in the 750 ppm environment, decreased to 480 ppm by 83.2% at the end of 7 days, while the highest removal efficiency was achieved with a decrease of 89.9% in the 1000 ppm environment. With this decrease, it is seen that the initial value of 3450 ppm has been reduced to 350 ppm.

TOC measurements were also made in the filtrate samples taken for COD measurements. When the decreases in this parameter are examined; at the end of 5 days in a 250 ppm environment, the initial TOC value of 2460 ppm decreased by 66.7% to 820 ppm; the TOC value, which had an initial value of 2520 ppm in a 500 ppm environment, decreased to 620 ppm with a decrease of 75.4% at the end of 6 days; and it was determined that the TOC value, which initial value was 3010 ppm in a 750 ppm environment, decreased to 360 ppm with a decrease of 88% at the end of 7 days. The highest removal efficiency on a TOC basis, 90.6%, was observed in the 1000 ppm environment at the end of 7 days. While the initial TOC value in this environment was 3200 ppm, it decreased to 300 ppm.

Various soil fungi are used for the bioremediation of different xenobiotics such as pesticides, polyaromatic hydrocarbons and heavy metals. Immobilized cells may show high performance in bioremediation and may also have an increased tolerance to toxic substances. Moreover, these microorganisms can show effective degradation and good stability (Aneez et al., 2011). Chikhi et al. (2016)' Streptomyces spp. It was determined in a study using immobilized microbial cells that they could remove xylene in the range of 90-99%. Additionally, a higher microbial population was determined to show a higher xylene degradation rate in immobilized cells than in free cells. Saez et al. (2012) also reported that the removal of lindane using immobilized Streptomyces cells was more effective than free cells in all support matrices tested. Similarly, the removal of diazinon, an insecticide, from liquid media resulted in the elimination of Streptomyces spp. species, which were found to increase by up to 20% when immobilized (Briceño et al., 2015).

Pesticide applications applied to plant roots can negatively affect plants by affecting the bacteria and fungi in the phyllosphere layer of the soil. Xu et al. (2020) also determined that S-metolachlor application (10 mg/kg soil, 14 days) reduced the diversity in the wheat phyllosphere and changed the composition of the microbial community. Azospirillum and Herbaspirillum have been found to reduce pesticide residues by 73.9% and approximately 99.7%, respectively. This indicates that S-metolachlor may cause changes in the phyllosphere microbial composition and reduce the presence of beneficial nitrogen-fixing bacteria in plants, thereby impairing plant nutrient absorption.

Zhao et al. (2020) applied the herbicides R-imazethapyr and S-imazethapyr at a concentration of 40 µg/L (soil) to *Arabidopsis thaliana*. An analysis of leaf layer microbial alpha and beta diversities revealed an increase in these indices, indicating an effect of this enantiomeric herbicide on the structure of the bacterial community. At the family level, the effects of pesticide treatments on bacterial abundance differed. The abundance of the dominant Pseudomonadaceae was decreased by 22% and 41% after R-imazethapyr and S-imazethapyr treatments, respectively, compared to that in the control group.

Endophytic microorganisms also directly affect pesticide bioremediation. Nasrollahi et al. (2020) suggested that the endophytic bacteria *B. altitudinis* DB26-R and *B. subtilis* subsp. inaquosorum B6-L could biodegrade 58.52% and 51.32% of the initial concentrations of diazinon (20 ppm), respectively.

Mortality results

The mortality rates at the end of the 96-hour period seems that treated and untreated environments at 250 ppm are 70% and 55%, respectively. In treated and untreated environments at 500 ppm, these rates increase to 80% and 50%, respectively. Mortality rates in treated and untreated environments at 750 ppm are 90% and 35%, respectively. Finally, in 1000 ppm treated and untreated environments, these rates were determined to be 85% and 45%, respectively. According to these results, the highest mortality rate was seen in the 750 ppm application group, purified by 90%; the lowest mortality rate as 35%, was detected in the 750 ppm untreated application group. Due to increasing concentrations, mortality rates were observed to be higher in treated environments (Figure 5). This may be thought to be due to the toxic effects of by-products that emerge after the pesticides are broken down. The fact that no mortality rate was observed in the control group indicates that the living conditions and population health of the test organisms used in the experiment are intact.

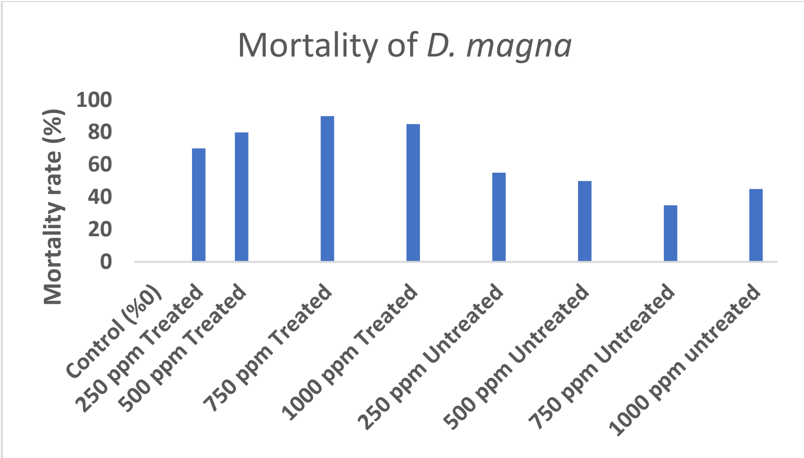

Figure 5. Mortality rates (%) of treated and untreated environments on *D. magna*

Daphnia's response to toxic substances varies depending on the level of contamination and duration of exposure (Ren et al., 2009). Juveniles of Daphnia have been reported to be more sensitive to toxic pollutants than adults (Hanazato, 1991). Daphnia cannot directly purify incoming wastewater, however, theoretically, it has been stated that Daphnia may experience growth inhibition as a result of oxygen depletion (Pous et al., 2020). Therefore, 10-15 days old *D. magna* was used in this study.

Williams et al. (2000) also stated that the large-scale use of herbicides makes it necessary to evaluate their toxic potential on non-target species. It is known that Daphnia species are more sensitive to toxic chemicals than other creatures. This sensitivity is associated with increased sensitivity of the relevant sodium channel, small body size, and changes in metabolic rate (Korkmaz et al., 2021).

The results of mortality tests showed that especially the *S. pseudosanguinis* bacterial isolate was highly effective in the detoxification of herbicides. The non-biodegradability of pyroxasulfone may lead to increased mortality in environments with high removal efficiency. Aghoghovwia and Izah (2018) reported that environmental toxicants can affect various organs or systems of *D. magna*. Colomer et al. (2019) stated that *D magna* can survive longer when exposed to foreign substances than in calm conditions when food concentrations do not limit their biodegradation capacity.

CONCLUSIONS

This is the first study on the use of the *P. frequantans* fungus obtained from agricultural soils in the bioremediation of the Fluopyram nematocyte and the subsequent reflections of its toxic effects on *D. magna* in the untreated and treated pesticide environments. When recent literature was examined, it was seen that the microorganisms used for bioremediation activities were mostly microorganisms isolated from the activated sludge of pesticide producing industries. What distinguishes this study from others is that the *P. frequantans* fungus was isolated directly from an agricultural field and used in the bioremediation study.

The nematocyte with the active ingredient fluopyram used in the study is a highly preferred product, especially in recent years. It offers a biological treatment method recommendation for agricultural lands to farmers who are considering using this product to rehabilitate their agricultural lands. In addition, the opinion has emerged that bioremediation activities can be examined with COD and TOC parameters instead of high-cost chromatographic methods to monitor this bioremediation process. The results reveal that the *P. frequantans* has the capacity to successfully eliminate fluopyrome nematocyst. Therefore, the use of this fungus is recommended. According to the results obtained from mortality studies on *D. magna*, it is thought that the by-products, namely metabolites, of the pesticide may have a toxic effect on *D. magna.* This means that, considering the fact that agricultural lands are exposed to pesticides, the sensitivity, adaptation and bioremediation capacity of such microorganisms to pesticides may be naturally high.

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