RESEARCH ARTICLE
Eurasian J Bio Chem Sci, 7(2):171-177, 2024
https://doi.org/10.46239/ejbcs.1564696

EJBCS

Eurasian Journal of Biological and Chemical Sciences

Journal homepage: www.dergipark.org.tr/ejbcs

Effect of different azinphos-ethyl and azinphos-methyl concentrations on *Tetradesmus obliquus* growth in culture conditions

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Received : 11/10/2024
Accepted : 23/12/2024

To Cite / Atıf için: Soylu EN, Temizel B. 2024. Effect of different azinphos-ethyl and azinphos-methyl concentrations on *Tetradesmus obliquus* growth in culture conditions. Eurasian J Bio Chem Sci, 7(2):171-177 https://doi.org/10.46239/ejbcs.1564696

Abstract: Azinphos methyl (S-3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-ylmethyl O,O-dimethyl phosphorodithioate) and Azinphos ethyl (S-3,4-dihydro-4 - oxo-1,2,3-benzotriazin-3-ylmethyl O,O-diethyl phosphorodithioate) are two phosphorus-containing pesticides. These pollutants are widely used as agricultural pesticides and acaricides and are used as broad-spectrum pesticides. It is assumed that these insecticides are carried out of the soil by rain, flood and snow water and pollute rivers, lakes and seawater. The aim of the study is to determine the effects of different concentrations of azinphos-ethyl and azinphos-methyl on the growth of green algae isolated from streams under culture conditions. The aim of this study was to determine the change in algal growth as a function of increasing the concentration of these pesticides in the algal cultures of *Tetradesmus obliquus*. In addition, pH and conductivity measurements were carried out on the control group and on the cultures after dosing. In this study, based on the counts carried out in the cultures, it was found that the number of species has decreased over time, but there was no significant decrease.

Keywords: Algal growth, Azinphosethyl, Azinphosmethyl, stream, pesticide, greenalgae

Farklı azinfos-etil ve azinfos-metil konsantrasyonlarının Tetradesmus obliquus'un kültür koşullarında büyümesi üzerindeki etkisi

Özet: Azinfos metil (S-3,4-dihidro-4-okso-1,2,3-benzotriazin-3-ilmetil O,O-dimetil fosforoditiyoat) ve azinfos etil (S-3,4-dihidro-4 - okso-1, 2,3-benzotriazin-3-ilmetil O,O-dietil fosforoditiyoat) iki fosfor içeren pestisitlerdir. Bu kirleticiler tarımsal pestisit ve akarisit olarak yaygın olarak kullanılır, geniş spektrumlu pestisit olarak kullanılır. Bu insektisitin yağmur, sel ve kar sularıyla topraktan sürüklenerek nehirleri, gölleri ve deniz sularını kirlettiği düşünülmektedir. Çalışmanın amacı, kültür koşulları altında akarsulardan izole edilen yeşil alglerin büyümesi üzerine farklı azinfos-etil ve azinfos-metil konsantrasyonlarının etkisini belirlemektir. Bu araştırma ile *Tetradesmus obliquus* alg kültürlerinde bu pestisitlerin konsantrasyonunun artışına bağlı olarak alg büyümesinin değişimi belirlenmeye çalışılmıştır. Ayrıca çalışma sırasında kontrol grubu ve kültürlerin dozlama sonrası pH ve iletkenlik ölçümleri yapılmıştır. Bu çalışmada kültürlerde yapılan sayım sonucunda tür sayısının zamanla azaldığı ancak çok önemli bir düşüş olmadığı belirlenmiştir.

Anahtar Kelimeler: Alg büyümesi, Azinfosetil, Azinfosmetil, dere, pestisit, yeşil alg

1. Introduction

Various pesticides are widely used in agriculture to protect against all kinds of pests that reduce the yield of products and prevent their development. It is known that the pesticides used in these activities mix with irrigation or rainwater and run back into lakes or rivers, leading to the accumulation and death of living organisms at every stage of the food chain (Amdur et al. 1991).

Pesticides that pollute aquatic systems have serious ecological consequences such as the death of some aquatic organisms, the deterioration of species composition and the alteration of the ecosystem. Therefore, the study of the effects of pesticides on aquatic organisms is becoming increasingly important (Peterson et al. 1994).

Among the living groups in the aquatic environment that are most affected by the mixing of agricultural pesticides

with surface waters are the phytoplankton organisms that form the first step of the food chain. Microalgae are a widely used living group in pesticide bioassays to determine the effects of pollutants on ecosystems. Algae are sensitive to most pollutants and are used as bioassay organisms to determine the effects of chemical substances in the aquatic environment. There are many studies that have been conducted on microalgae to determine the toxic effects of herbicides and various industrial chemicals (Abdel-Hamid 1996, Djomo et al. 2004, Geyer et al. 1985, Lu et al. 2001, Ma et al. 2003, McFeters et al. 1983, Moreno-Garrido et al. 2001, Moreno-Garrido et al. 2003, Sáez et al. 2001, Sabater and Carrasco 2001, Sabater et al. 2002, Shehata et al. 1984, Soylu and Temizel 2023, Wong 2000).

Azinphosmethyl (S-3,4 - dihydro-4- oxo-1,2,3-benzo triazin-3-ylmethyl O,O-dimethylphosphorodithioate) and azinphos ethyl (S-3,4 - dihydro-4- oxo-1,2,3-benzotriazin-3-ylmethyl O,O-diethylphosphorodithioate) are two phosphorus containing pesticides. These pollutants are widely used as agricultural insecticides and acaricides, which are used as broad-spectrum pesticides. Like many other insecticides, these chemicals are compounds that are considered potential pollutants to surface and groundwater, even though they are used in agriculture worldwide. It is believed that these insecticides are washed out of the soil with rain, flood and snow water and pollute rivers, lakes and marine waters. The aim of the research is to determine the effects of pesticides on algae growth by applying different doses of pesticides to microalgae species to be isolated from streams.

The aim of this study is to show the effects of azinphos-methyl and azinphos-ethyl, insecticides used for agricultural purposes in the streams of Giresun province, on the phytoplanktonic algae species prevalent in the streams and to create a scientific database with the data obtained, which can be used for subsequent planning studies. In this study, new data will be obtained in this field by using organisms such as *Tetradesmus obliquus* (Turpin) Kütz. from the Chloropyhta division, which are dominant in the waters of our country.

Studies investigating the effects of pesticides on freshwater algae have mostly used green algae such as *Chlorella*, *Chlamydomonas* and *Scenedesmus* (Tadros et al. 1994). The aim of this study was to determine the toxic effects of azinphos ethyl and azinphos methyl on the microalgae *Tetradesmus obliquus* (Turpin) Kützing isolated from natural waters. The development of the species to be cultivated in cultures treated with 1 mg/l, 1.1 mg/l, 1.2 mg/l, 1.4 mg/l azinphos-methyl and in cultures treated with 0.1 mg/l, 0.5 mg/l, 1 mg/l, 1.5 mg/l, 2 mg/l azinphos-ethyl is compared with the development of the species in cultures without these pesticides. The determination of the toxic effects of these herbicides on the growth rate of *Tetradesmus obliquus* and the comparison of their toxic effects are of great importance for aquatic ecosystems. Repetition and continuity of such studies are very important to reveal the effects of pesticide exposure on aquatic ecosystems and especially on algae (Öterler 2009).

2. Materials and Method

2.1. Description of the Research Area and Sampling Stations

Green algae were isolated and analysed from water samples taken from the Aksu stream in the province of Giresun in the eastern Black Sea region. The Aksu stream is about 60 km long and 100 m wide. Its average depth is about 3 metres. The map of the study area is shown in Figure 1.

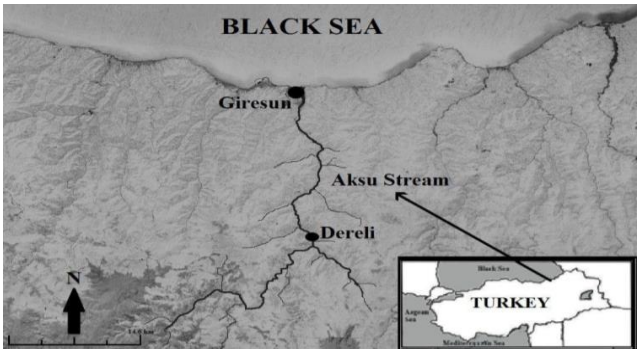

Fig. 1 Map of study area

2.2. Algological Features

2.2.1. Sampling

Water samples were taken from the surface (0-20 cm) at the stations of the streams to be determined. To identify planktonic algae, 1-liter water samples were filtered through Whatman GF/A filter papers and preliminary preparations were made by scraping the algae collected on the surface of the filter paper with a coverslip and covering them in water or 10% glycerol solution. These preliminary preparations were examined under a research microscope and the algae identified. The works of Krammer and Lange-Bertalot, (1991a), Krammer and Lange-Bertalot, (1991b), Krammer and Lange-Bertalot, (1999a) and Krammer and Lange-Bertalot, (1999b) were used in the description of algae.

2.2.2. Culture Conditions

The culture isolation was carried out in BG 11 medium from water samples from streams.

The algae were isolated and cultured from water samples from the Aksu stream. For the preservation of the cultures, the climate chamber was set to conditions suitable for algae (Hong et al. 2008, Lockert et al. 2006, Sabater and Carrosa 2001).

100 ml cultures were grown under defined climatic conditions (International Standard ISO-8692:1989) up to 4000-5000 cells/ml.

2.2.3. Cell Count

The determination of the number of cells in a given volume is used for counting chambers. In our study, cell counts were performed with a Sedgewick-Rafter counting chamber. The Sedgewick-Rafter counting chamber

consists of a total of 1000 squares, 50 columns and 20 rows. The volume of each square is 1 µl. The evaluation is carried out by random counting (LeGresley and McDermott 2010).

For the continuity of the algae cultures, 100 mL of culture medium was prepared in 250 mL bottles (ISO-8692 1989).

In the experiments, 4 different doses of azinphos-methyl (1 mg/l, 1.1 mg/l, 1.2 mg/l, 1.4 mg/l) and 5 different doses of azinphos-ethyl (0.1 mg/l, 0.5 mg/l, 1 mg/l, 1.5 mg/l, 1.5 mg/l, 2 mg/l) were applied to the algae cultivated in 100 ml flasks, which were prepared in accordance with similar studies (İbrahim et al. 2014, Öterler and Albay 2016).

The experiments were conducted in 2 different phases, which were initiated by treating the cultures with pesticides at predetermined concentrations approximately 5 days after entering the exponential growth phase. In the first phase, the cells of the pesticide-treated samples were counted with a Sedgewick-Rafter counting chamber after 0, 24, 48, 72 and 96 hours for 5 days. Subsequently, the spectrophotometric growth rates and absorbance values of the pesticide-treated samples were measured at 500, 665 and 750 nm wavelength and chlorophyll-*a* calculations were performed (Nusch 1980).

In addition, pH and conductivity measurements of the control group and the cultures after dosing were carried out during the study.

2.2.4. Pesticide Analysis

For the species *Tetradesmus obliquus*, large quantities of precultures were established and their development regularly monitored. When the algal culture had reached a sufficient number of cells and entered the rapid growth phase (between days 15-21), 100 ml of the cell culture was divided into 250 ml vials under sterile conditions.

At 0 h, 20 ml of culture samples were taken from each experimental set under sterile conditions and the absorbance values were measured at 500, 665 and 750 nm to determine the spectral growth rates. The conductivity and pH were then measured and the cells counted. The chlorophyll values were measured the next day.

The experiments were carried out with doses according to the ISO 8692 and OECD 201 standards (ISO 8692, 2012; OECD, 2014) after 0 hours, 24 hours, 48 hours, 72 hours and 96 hours (ISO 8692, 2012; OECD, 2014).

3. Results

To determine the effects of Azinphos methyl and Azinphos ethyl on the growth of *Tetradesmus obliquus*, one of the green algae isolated from the Aksu stream, 1 mg/l, 1.1 mg/l, 1.2 mg/l, 1.4 mg/l Azinphos methyl and 0.1 mg/l, 0.5 mg/l, 1 mg/l, 1.5 mg/l, 2 mg/l Azinphos ethyl were used under culture conditions. In order to determine the effects of the pesticides added to the cultures, a comparison was made with the control group without pesticides. The aim of this study was to determine the change in algae growth in the cultures by increasing the concentrations of azinphos methyl and azinphos ethyl. As a result of the study, it was found that the number of organisms did not decrease significantly by counting in the cultures, but the doses determined over time had a negative effect on algae growth.

At low pesticide applications, all pesticides induced the formation of chlorophyll-*a* and increased photosynthesis during the first 24 hours. However, there was no specific increase in absorbance and cell number measured spectrophotometrically over time. However, development slowed down after 48, 72 and 96 hours.

The pH and conductivity values of the samples were measured at 0 o'clock and the cells were counted. Then the remaining portion in the beaker was filtered through Whatman GF/C filter papers and chlorophyll-a determinations were performed by absorbance measurements at 500 nm, 680 nm and 750 nm wavelength. These measurements were repeated every day at the same time. The last measurement was taken at the 96th hour. Each dose was applied in 3 repetitions.

For azinphos-methyl, the lowest cell count in the cultures was determined at hour 0 with $2.07x10^6$ cells/ml at a dose of 1.4 mg/l. After 24 hours, the lowest cell count was determined to be $2x10^6$ cells/ml at a dose of 1.2 mg/l. After 48 hours, the lowest cell count was determined to be 1.71 $x10^6$ cells/ml at a dose of 1.4 mg/l. After 72 hours, the lowest cell count was determined to be 1.48 $x10^6$ cells/ml at a dose of 1 mg/l. Finally, after 96 hours, the lowest cell count was determined to be 1.006 $x10^6$ cells/ml at a dose of 1 mg/l (Figure 2).

In the second step, the same procedures were repeated for azinphos ethyl. The cultures that had reached the rapid growth phase (day 15-25) were divided into subcultures and 5 days after entering the exponential growth phase, 5 different doses of azinphos ethyl (0.1 mg/L, 0.5 mg/L, 1 mg/L, 1.5 mg/L and 2 mg/L) were applied to the cultures to determine the effect of the pesticide on algal growth.

For azinfos ethyl, the lowest cell count in cultures was determined after 0 hours with $1.855x10^6$ cells/ml at a dose of 0.5 mg/l. The lowest cell count after 24 hours was determined to be $2.06x10^6$ cells/ml at a dose of 1.5 mg/l. The lowest cell count after 48 hours was determined to be 1.77 $x10^6$ cells/ml at a dose of 1.5 mg/l. The lowest cell count after 72 hours was determined to be 1.94 $x10^6$ cells/mL in the control group. Finally, the lowest cell count after 96 hours was determined to be 1.65 $x10^6$ cells/mL at a dose of 1.4 mg/L (Figure 3). A decrease in the number of organisms was observed with increasing dose. A decrease in the number of organisms was observed particularly after the 24th hour when the dose was increased.

As a result of the experiments, the lowest pH value for azinphosethyl in the control group was measured at the beginning of the experiment (time 0) at 6.7, and the highest pH value was measured at a dose of 2 mg/l after 48 hours at 8.1 (Figure 5). The lowest pH value for azinphos-

methyl was measured at the beginning of the experiment (time 0) at a dose of 1.4 mg/l at 6.1, and the highest pH value was measured in the control group after 24 hours at 7.6 (Figure 4).

The lowest conductivity value (µS/cm) for azinphosethyl was measured in the control group at the 24th hour with 195 µS/cm; the highest conductivity values were measured in the control group at the 96th hour and at a dose of 1 mg/l with 285 µS/cm (Figure 7). For azinphos-methyl, the lowest conductivity value of 1140 µS/cm was measured in the control group at the 48th hour, while the highest conductivity value of 1955 µS/cm was measured at a dose of 1.1 mg/l at the 24th hour (Figure 6). It was found that the conductivity values generally did not vary greatly over time in all dose groups.

According to the test results, the highest chlorophyll-*a* for azinphos ethyl was measured at 3.97 µg/l after 0 hours in the control group, and the lowest chlorophyll-a was measured at 0.74 µg/l after 96 hours (Figure 9). The highest chlorophyll-*a* for azinphos-methyl in the control group was measured at 0.78 µg/l after 0 hours; the lowest chlorophyll-*a* was measured at 0.03 µg/l after 96 hours (Figure 8). As a result of our studies, it was found that chlorophyll-*a* levels in *Tetradesmus* cultures containing azinphos-ethyl increased in the first 24 hours at almost all doses and then decreased; while chlorophyll-*a* levels in *Tetradesmus* cultures containing azinphos-methyl increased in the first 48 hours at a dose of 1 mg/l and then decreased.

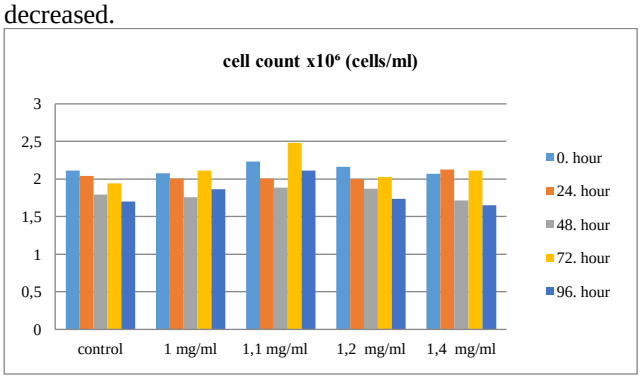

Fig. 2 Time-dependent change in the cell count, determined by counting in the control group and different doses of azinphos methyl

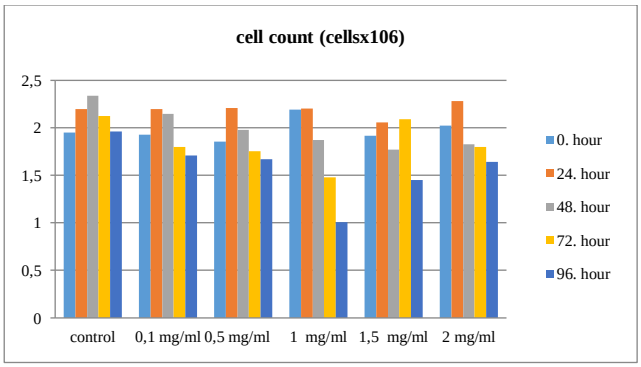

Fig. 3 Time-dependent change in the cell count, determined by counting in the control group and different doses of azinphos ethyl

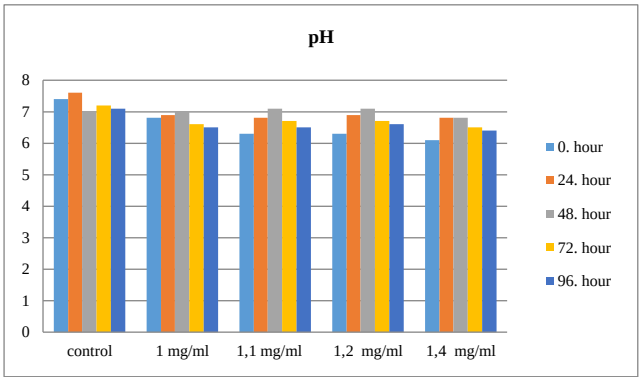

Fig. 4 pH change in the control group and different azinphos methyl doses

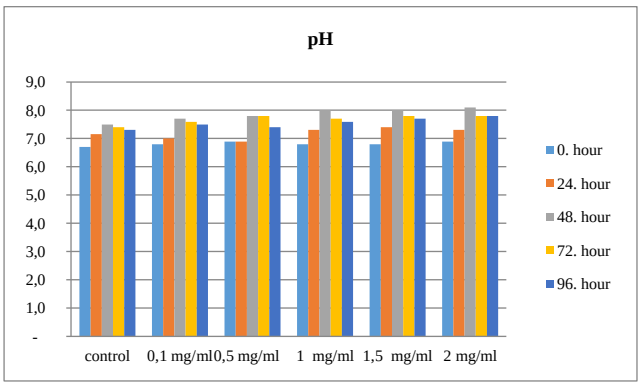

Fig. 5 pH change in the control group and different azinphos ethyl doses

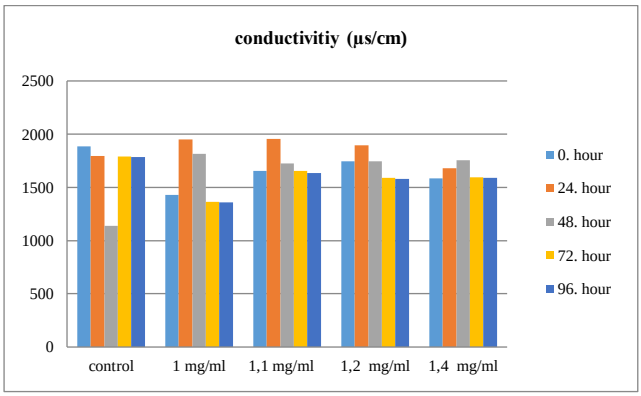

Fig. 6 Change in conductivity in the control group and different doses of azinphos methyl

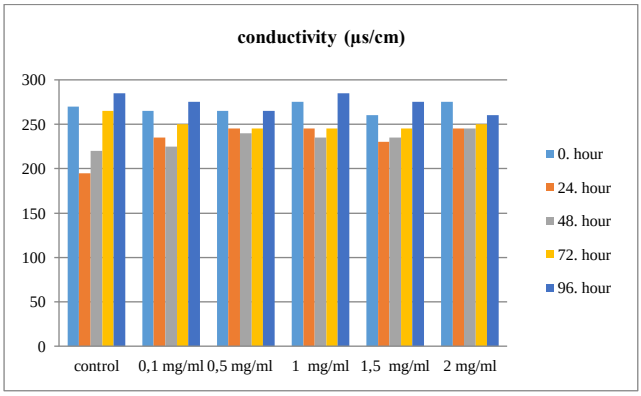

Fig. 7 Change in conductivity in the control group and different doses of azinphos ethyl

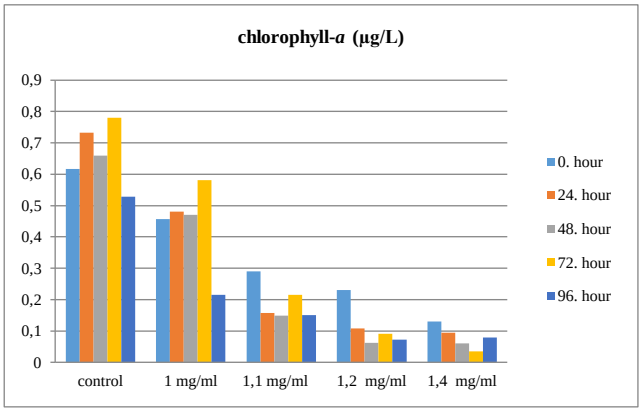

Fig. 8 Change in chlorophyll-*a* in the control group and different doses of azinphos-methyl

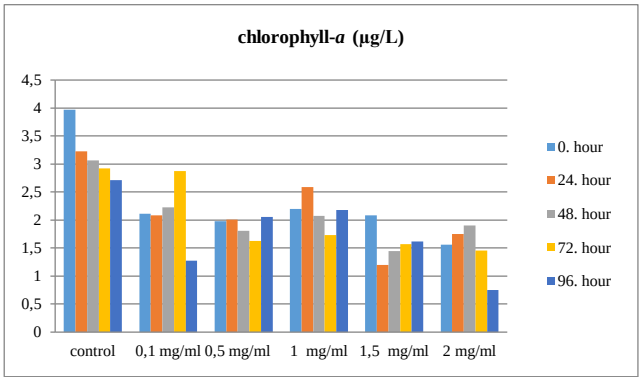

Fig. 9 Change in chlorophyll-*a* in the control group and different doses of azinphos-ethyl

4. Discussion

Pesticides are substances that humans have used for many years to affect the environment, food sources and plants. A pesticide can be a chemical substance, a biological agent such as a virus or bacterium, an antimicrobial agent, a disinfectant, or another agent (Kaya 1996). The primary target of most herbicides used as pesticides against plant pests is the light reactions of photosynthesis. Therefore, herbicides used in agriculture interfere with the light reactions of photosynthesis. Chlorophyll is one of the most important photosynthetic pigments found in green algae. It is one of the most commonly used parameters for assessing the effects of pesticides on algal growth. Some herbicides inhibit photosynthesis and the synthesis of carotenoids. Some inhibit photosynthesis (Sikka and Pramer 1968), others inhibit fatty acid formation and thus cell division and growth (Couderchet and Boger 1993). Pesticides such as colchicine prevent chromosome segregation by inhibiting tubule formation (Fedtke 1982). As in similar studies, the chlorophyll-*a* content decreased over time in our experiments, as shown in Figure 8 and Figure 9 (Nie et al. 2002; Ou et al. 2003).

A small proportion (~0.1%) of the pesticides used for pest control reaches the target organism. The remainder enters the aquatic environment via leakage or surface runoff and poses a serious threat to other aquatic organisms. Many studies have shown that the accumulation of pesticides is also very harmful to non-target organisms (Baruah et al. 2024, Damalas and Eleftherohorinos 2011, Sanoja-López et al. 2023). Microalgae, which are primary producers and form the basis of the aquatic food web, are the most notable group among aquatic non-target organisms (Mofeed and Mosleh, 2013). They are responsible for about 60% of the total oxygen production in water (Castro et al. 2022) and play an important role in nutrient cycling (Sabater and Carrasco, 2001; Källqvist and Svenson, 2003). Pesticides that accumulate in algae can reach higher trophic levels through trophic interactions and accumulate toxins in the tissues of zooplankton and other herbivores that eat these algae (Meng et al. 2022).

Algae, which impair soil fertility and are the main food for fish in water bodies, are widespread in nature. The accumulation of pesticides in water, which are widely used worldwide, has a significant negative impact on algae. Any negative impact on the microalgae community can affect the structural balance of the entire ecosystem (Martinez et al. 2015, Villem, 2011). Therefore, it is very important to investigate the harmful effects of pollutants on microalgae (Neury-Ormanni et al. 2020). Recently, studies investigating the effects of pesticides on algal cultures have expanded. In their study investigating the effects of five different organophosphate pesticides on the growth of *Chlorella vulgaris*, a green microalgae, Öterler and Albay (2016) found that at a lower pesticide concentration, the pesticide degenerated like a nutrient and was taken up by the algal cells, whereupon the toxic effect of the pesticide limited the growth of the algae. In our study, the amount of algae increased for a while after the addition of the pesticide and then decreased (Figures 2 and 3). Therefore, this situation is parallel to our study as in many other studies.

In a similar study investigating the effects of Azinphos methyl and Azinphos ethyl on the growth of the marine green alga *Tetraselmis suecica*, it was found that these chemicals had an inhibitory effect on algal growth after 96 hours (Vagi et al. 2005). In another study investigating the time-dependent change in cell number in an Aphanizomenon aphanizomenoides culture to which azinphos ethyl was added, it was found that azinphos ethyl had a limiting effect on algal growth and caused a relative decrease by the end of 24 hours (Oğuz 2009).

5. Conclusion

Changes in the community structure of algal species in the aquatic environment and the response of these species to environmental pollutants are very important for the ecosystem. Pesticide residues that accumulate in living organisms over time, even in very small amounts, have negative effects on living organisms and the environment.

Experiments have shown that pesticides cause structural changes in non-target organisms. Efforts should therefore be made to reduce the use of synthetic agrochemicals. Organic farming with the use of biopesticides/bioherbicides can help to reduce pesticide exposure.

In addition to biomonitoring, it is also very important to conduct research that provides early warning of pesticide contamination in aquatic systems. This and many other studies show that the accumulation of pesticides in water over time has a negative impact on algae growth. Although no rapid changes and declines can be observed, it is clear that the accumulation of pesticides over time has a negative impact on aquatic organisms. New projects should be developed to address the negative effects of pesticide use on the environment and human health, and water resources should be protected from pesticide pollution.

In order to predict future ecological scenarios, it is important to understand how pollutants affect algal communities and thus the aquatic ecosystem. Monitoring programs should be established to observe the long-term effects of pesticides on algal ecosystems, and how exposure to pesticides affects algal communities should be investigated. Knowledge of how different species interact and respond as a community will lead to a more comprehensive understanding of ecosystem dynamics, which will be of great benefit in the development of environmental policies. Knowing about the interaction between pesticides and microbial-algal communities can help us understand how ecosystems work more broadly.

Studies that consider the social and ecological aspects of pesticide use and understanding the relationships between ecological processes and human activities, as well as the use of sustainable and socially responsible pesticide management techniques, will further advance the sustainable management of aquatic ecosystems.

Acknowledgements

This paper was presented in 7th International Eurasion Conference on Biological and Chemical Sciences, 02-04 October 2024, Ankara-Türkiye and the authors would like to thank Giresun University Scientific Research Projects (BAP) unit for their contribution to this research (Project No: FEN-BAP-A-150219-07).

Authors' contributions:

ENS: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

BT: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Conflict of interest disclosure:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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