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Possible Effects of Pesticide Washout on Microalgae Growth

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Abstract

Aquatic ecosystems are threatened by various anthropogenic activities, including those exacerbated by pesticides leaching from agricultural lands. Although legislation and regulations regarding pesticides aim to eliminate the risk of eutrophication and pollution, only a few studies have examined the impact of these substances on non-target organisms, such as microalgae, which are highly involved in biogeochemical cycles and critical for ecosystem integrity. We studied the effect of the agricultural insecticide Teppeki based on flonicamid, the fungicide Ortiva with azoxystrobin, and the herbicide Basar with (S)-metolachlor on the green microalga *Chlorella vulgaris* and the cyanobacterium *Synechococcus leopoliensis*. Ortiva and Basar were more toxic at lower doses than Teppeki, with (S)-metolachlor demonstrating the most instantaneous and potent inhibition. Half maximum effective concentration (EC_{50}) values confirmed the strong inhibitory effect of the herbicide on both strains on days 3 and 8, and highlight the differing temporal responses, especially for Ortiva. This observed pattern of toxicity is consistent with pulse-amplitude-modulated fluorescence measurements of photosystem II, which indicate that both species are more sensitive to (S)-metolachlor and azoxystrobin than to flonicamid. We claim that the side effects of pesticides on non-target organisms must be given more attention. It is well established that herbicides can impair photosynthetic organisms such as microalgae, but pesticides targeting other pests can also cause adverse effects on these communities. Such unwanted side effects are directly related not only to the reduction of biodiversity, but also to human health.

Keywords: microalgae; agricultural runoff; pesticide; non-target organisms

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1. Introduction

The widespread use of pesticides started thousands of years ago, when the development of agriculture and settlements brought household pests, making pest control increasingly necessary. When applied appropriately, pesticides are effective in controlling invasive species, insects, and microorganisms—including bacteria and fungi—as well as rodents, thereby contributing to the management or suppression of pest populations. Powdered sulfur was the first recorded insecticide used by the Sumerians more than 4500 years ago to control insects and related pests. Using combinations of arsenic and mercury, the ancient Chinese also developed insecticides 3200 years ago to control lice [1,2].

At the end of the nineteenth century, grapes were treated with a Bordeaux mixture of calcium hydroxide and copper sulfate with water to fight the mold [3]. Later in the twentieth century, farmers sought to control insects with long-term chemicals, and started to use

chlorinated organic pesticides, such as toxaphene, aldrin, dichlorodiphenyltrichloroethane (DDT), and benzene hexachloride (BCH). These compounds developed in the 1930s and gained popularity in the 1950s and 1960s [4] and were subsequently applied in the control of insect-borne diseases, most notably malaria. By 1955, nearly 90% of pesticides used in U.S. agriculture were synthetic organic compounds, as highlighted in a 1993 National Research Council Book. Moreover, DDT was already found in 334 crops by 1961. Phenoxy herbicides, such as ethylene dithiocarbamates (EBDC) and dicarboximide fungicides, were also widely used during this period. However, as early as the late 1950s, DDT was assumed to be a threat to health and the environment [4,5].

The most widely known pesticides today are divided into the following categories: insecticides, herbicides, rodenticides, and fungicides. Less recognized but widely used ones include biocides, plant growth regulators (defoliants), disinfectants (antifoulants), and pool treatments (algicides) [6].

Active Ingredient Composition

We focused on three pesticide categories: an insecticide under the Teppeki trademark, a fungicide under the Ortiva trademark, and an herbicide under the Basar trademark (Table 1, Figure 1).

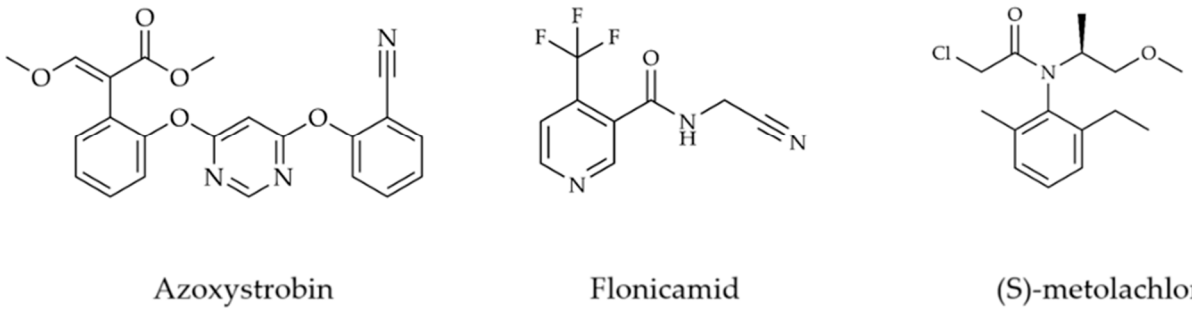

Figure 1. Molecular structures of the tested active substances of pesticides drawn using ChemDraw 25.0.2 (PerkinElmer, Waltham, MA, USA, 2024).

The insecticide Teppeki is commonly used to control and kill insects such as aphids. The product is applied to various crops, including apples, pumpkins, watermelons, quinces, potatoes, cherries, and tobacco, as well as tree-like and soft-stemmed ornamental plants in the field and during reproduction. Even at low doses, it reduces the spread of plant pathogens such as viruses by affecting the previously mentioned aphid vectors [7–9]. Flonicamid is the active ingredient of Teppeki. Hancock and Morita [8] described the unique activity of flonicamid, which demonstrated effects different from those typical of other neonicotinoids. It was found that flonicamid leads to a complete cessation of feeding, a decrease in honeydew and salivation production, and changes in the behavior of aphids. Examples include photosensitivity, irregular foot movements, altered reactions to straightening, and uncoordinated movements. A recent study conducted by Zhu et al., [10] showed that flonicamid could affect inward-rectifying potassium channels, which are essential for homeostasis and other physiological functions. However, the effect on the metabolic activity of insects and the exact metabolic pathways remains to be studied. Flonicamid increases liver enzyme activity in mammals, altering liver metabolism and contributing to weight loss and morphological changes [11]. Studies also showed that flonicamid undergoes metabolic transformation through the hydrolysis of functional groups, including –CN and –CONH [12]. Further research is needed to clarify its full metabolic effects, primarily on non-target organisms.

Table 1. Pesticides used in this report include the name of the crop protection product, the composition of the active ingredients, the product type, the chemical formula, the CAS number, the molecular weight, and the license holder [13–15].

Plant Protection Product	Active Ingredient	Active Ingredient in the Product	Agent	Chemical Formula	CAS-Number	Active Ingredient Molecular Weight
Teppeki® 50 WG	Flonicamid	≥50%–≤80% *v*/*v*	Insecticide	$C_9H_6F_3N_3O$	158062-67-0	229.2 g·mol^{-1}
Ortiva®	Azoxystrobin	≥20%–<25% *v*/*v*	Fungicide	$C_{22}H_{17}N_3O_5$	131860-33-8	403.4 g·mol^{-1}
Basar® 960 EC	(S)-metolachlor	86.5% *w*/*w*	Herbicide	$C_{15}H_{22}ClNO_2$	87392-12-9	283.8 g·mol^{-1}

The fungicide Ortiva effectively kills parasitic fungi and their spores on various vegetables, including broccoli, onions, kale, cauliflower, and ornamental plants, as well as cucumbers, peppers, tomatoes, and cabbage [16]. The active substance of Ortiva, azoxystrobin, is a strobilurin-based fungicide that inhibits mitochondrial respiration by blocking the cytochrome bc1 complex (complex III) in the electron transport chain. Thus, ATP synthesis is blocked, which subsequently leads to the depletion of energy resources and the death of fungal cells [17]. Azoxystrobin primarily acts on fungi, but it can also interact with plants, animals, and aquatic flora and fauna in various ways [18]. While plants can neutralize azoxystrobin more effectively, excessive exposure can still inhibit photosynthesis and potentially disrupt mitochondrial function due to chromosomal abnormalities [19]. It can also cause oxidative stress and energy depletion in aquatic organisms. A striking example of this is that the 48 h LC50 level for *Daphnia magna* is 190 µg L^{-1}; for *Mysidopsis bahia*, the 96 h LC50 level is 56 µg L^{-1} [20]. Studies have also shown that azoxystrobin can cause neurotoxicity, metabolic disorders, and oxidative stress in gastropods and mammals [21–23].

(S)-metolachlor was produced under the trademark Basar until legislation was enacted, according to which EU member states were ordered to revoke their permits for the active substance by 23 April 2024. This herbicide proved effective in controlling unwanted weeds in the cultivation of sweet corn, corn kernels, and sunflowers [24,25]. As highlighted in a 2016 study by Rose et al., 2016 [26], herbicides based on the pesticides triazine, phenyl urea, and (phenyl)amides can block binding sites where catalytic reactions with quinones occur in photosystem II (PSII), which can cause increased formation of reactive oxygen species and damage to cell membranes, proteins, and nucleic acids such as DNA. In the case of (S)-metolachlor, this mechanism is manifested in the inhibition of elongases and gibberellin pathway intermediates, such as geranylgeranyl pyrophosphate cyclase (GGPP), which play a crucial role in regulating various developmental processes of grasses. (S)-metolachlor is a chloroacetamide-type herbicide, and studies have also shown that in multiple plants, including rice, *Arabidopsis thaliana*, and even leek seedlings, chloroacetamides affect the synthesis of very-long-chain fatty acids (VLCFAs) and inhibit the elongation of Acyl-CoA, which leads to plant damage. Possible death occurs due to severe suppression of the growth of their structural and functional parts, such as shoots and roots [27–29].

In addition to their direct effects on pest physiology, the abovementioned fungicides, herbicides, and pesticides significantly contribute to the deterioration of aquatic and terrestrial ecosystems, posing a serious threat to human and animal health. Point- and non-point-based water pollution in agriculture and urban areas is exacerbated by the widespread use of synthetic pesticides and industrial organic compounds. Point sources of such pesticide contamination include, for example, improper storage, disposal, or accidental spills of pesticides [30], while non-point sources of pollution include (wind) erosion and runoff. As 45% of the population is concentrated in watersheds, the problem of eutroph-

ication of estuaries and coastal ecosystems is crucial, as they are remarkably threatened by the growth of agriculture, cities, and industry, which increases nutrient load [31,32]. As the population increases, which entails further development and urbanization, the natural vegetation landscape is being progressively replaced by impermeable surfaces. These surfaces significantly reduce water drainage into the soil, for example, during the construction of new roads, houses, parking lots, and other structures, and accelerate the filling of ditches and streams [33,34]. Furthermore, due to hyper-eutrophication, which is also caused by pesticide runoff, the formation of anoxic areas promotes algae blooms by shifting the main primary producers from photoautotrophic biofilms and vascular macrophytes to phytoplankton [31,35,36]. As a side effect, such nutrient loading (both internal from the sediment and external from runoff) contributes to harmful algal blooms (HABs), increases greenhouse gas emissions, and reduces the efficiency of fish reproduction. Several studies on the health effects of HAB aerosols also highlight physiological effects in humans, including respiratory symptoms, neurological effects, gastrointestinal problems, and systemic effects [37–39]. Algal toxins are also associated with cancer, heart disease, neurological disorders, and reproductive problems [40,41].

The effects of pesticide runoff on non-target organisms, such as microalgae, are often neglected. Microalgae play a pivotal role in aquatic food webs, the production of oxygen, and the absorption of carbon dioxide [42–44]. Any interference with their growth can negatively impact the stability of ecosystems, biodiversity, and pose a threat to other living organisms. We aim to analyze how pesticide exposure affects microalgae growth. Pesticides enter the aquatic environment via washout from agricultural areas and wind erosion of treated soils. We pre-cultivated the strains two weeks before inoculating them with a serial dilution of the respective pesticides. Optical density (OD) and in vivo fluorescence (ChlF) measurements were used to monitor growth for eight consecutive days, and pulse-amplitude-modulated (PAM) fluorescence measurements were obtained on the first and last day to evaluate the overall photosynthetic performance before and after exposure. We presumed that higher concentrations of flonicamid and azoxystrobin reduce microalgae growth to some extent, although the chemicals do not entirely stop growth. On the contrary, we expected serious negative effects from the herbicide at low doses [45,46], because a few studies have already proven the toxicity on certain algae species. As mentioned earlier, (S)-metolachlor was banned in the European Union very recently, but it is still used in other countries. The European Food Safety Authority (EFSA) identified critical concerns regarding the herbicidal active substance (S)-metolachlor, particularly its potential impact on mammalian health and groundwater quality. Risk assessment revealed that (S)-metolachlor and its herbicidally active metabolites were detected in groundwater at concentrations exceeding the parametric drinking water limit of 0.1 µg/L, raising concerns about their potential genotoxic and carcinogenic properties. On this basis, the European Commission decided not to renew the approval of (S)-metolachlor under Regulation (EC) No 1107/2009, leading to the withdrawal of authorizations for plant protection products containing this active substance within the European Union. Amidst the ban on (S)-metolachlor, major manufacturers such as Syngenta Global AG and UPL Limited continue to produce it primarily for export to target markets, including the United States, Argentina, Russia, and other non-EU countries [25,47]. According to market research, the global (S)-metolachlor market in 2024 was estimated at USD 1.2 billion worldwide (including the United States, Canada, and Mexico, as well as European markets such as Germany, the United Kingdom, and France, and Asia such as Korea, Japan, China, and India and the Middle East), and forecasted to grow at an expected compound annual growth rate (CAGR) of 5.2% between 2026 and 2033, expected to reach USD 1.8 billion by 2033 [48,49]. One purpose of this study was to demonstrate that using pesticides requires more attention to

non-target organisms. After all, their impact on microalgae is directly related not only to the reduction of biodiversity, but also to human health.

2. Materials and Methods

2.1. Culture and Media Preparation

Two pre-cultures obtained from the Culture Collection of Algae of the University of Göttingen (SAG, Göttingen, Germany) were tested in the experiment: *Chlorella vulgaris* SAG 211-11b (Chlorophyta) and *Synechococcus leopoliensis* SAG 1402-1 (Cyanoprokaryota). Both species are freshwater organisms and are known for their rapid and robust growth in culture. Their reliability and simplicity allow for reproducible tests, and their photosynthetic ability makes them ideal for testing compounds that affect cellular respiration and metabolic pathways, as well as the effects of oxidative stress [50–53]. BG-11 medium was used to grow and maintain the organisms [54]. The initial microalgae cultures we used for inoculation were pre-cultivated for two weeks to ensure vital specimens. The assays were grown at 21 °C under low light intensity (30 µmol photons m^{-2} s^{-1} photosynthetically active radiation, provided by warm-white fluorescent tubes). The flasks were placed on a shaker (75 rpm) to promote algae growth (Figure 2).

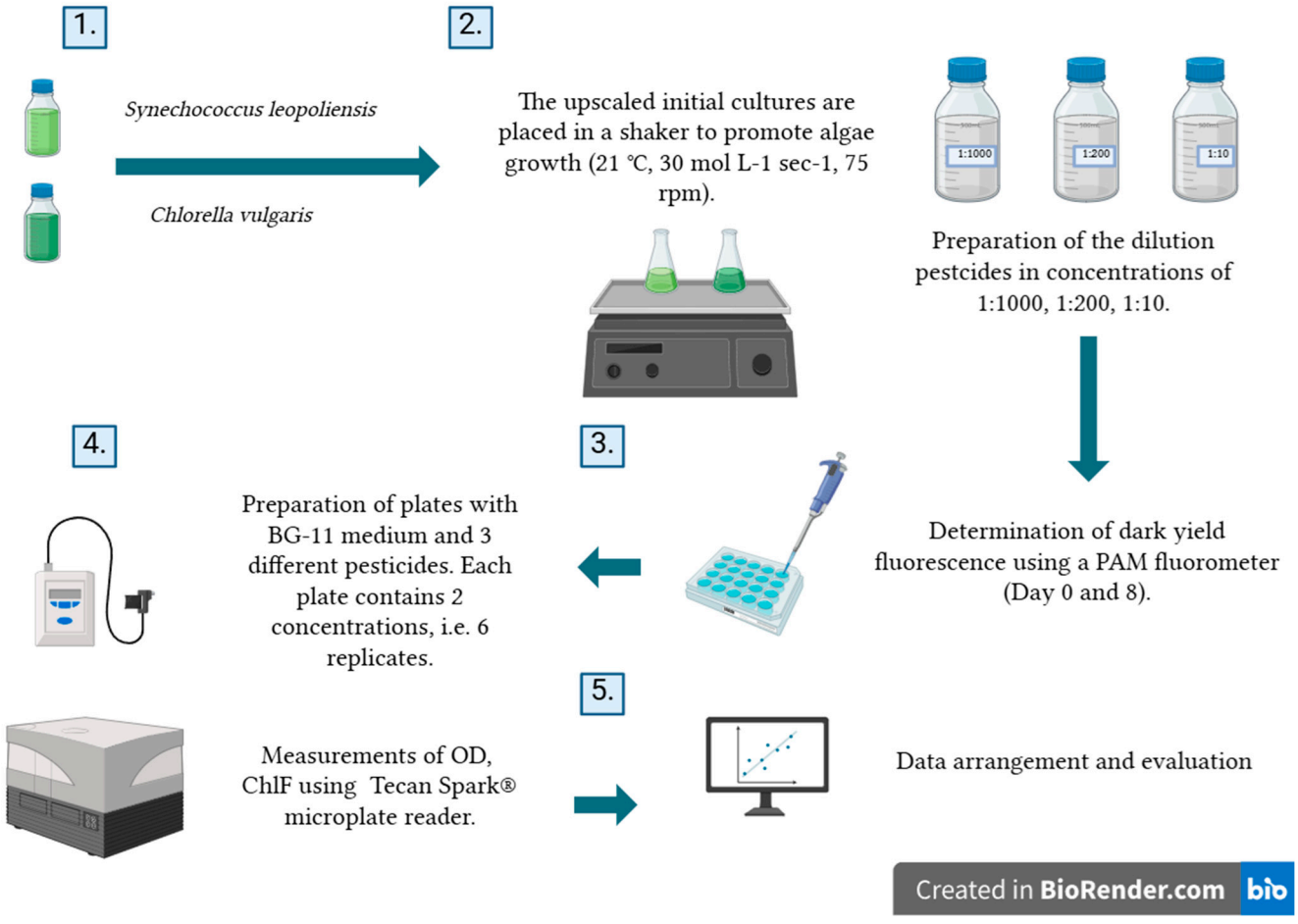

Figure 2. Project workflow. Created with BioRender (Science Suite Inc., Toronto, CA, USA, 2025).

2.2. A 12-Well Plate Assay

For the serial dilutions, we used transparent, flat-bottomed 12-well plates with lids (CytoOne multiwell plate; HH, DE). Each plate contained two different concentrations with six replicates. Percentages of 25%, 10%, 5%, 1%, and 0.1% (v/v) of the ready-to-use solution, commonly used in the field for pest control, represent the dilution coefficients for sequential

dilution. We selected non-linear concentration–response profiles for each pesticide instead of sequential dilution, allowing the detection of both the threshold and sub-threshold effects. The standard field application rates for the tested pesticides vary depending on the product formulation and intended use. The concentration of the ready-to-use solution is for Teppeki 2800–7000 mg/L^{-1}, for Ortiva 2500 mg/L^{-1}, and for Basar 960,000 mg/L^{-1}. Purchasable stock solutions contain 50–80% (*v*/*v*) of the active ingredient flonicamid in Teppeki, 20–25% (*v*/*v*) azoxystrobin in Ortiva, and 85% (*w*/*w*) of (S)-metolachlor in Basar (see also Table 1). The % (*v*/*v*) form allows us to directly compare the three types of pesticides on a relative basis (% of commonly applied concentrations based on the supplier's Safety Data Sheets). Optical density (OD) was measured using a Tecan Spark® microplate reader (Tecan Group Ltd., Männedorf, Switzerland; ZH, CH) at a wavelength of λ = 750 nm. Furthermore, in vivo *chlorophyll a* (ChlF) content was determined using the Spark® microplate reader device (top-reading fluorescence, excitation wavelength of λ = 410 nm and an emission wavelength of λ = 670 nm). Before each measurement, the samples were thoroughly mixed by repeated up-and-down pipetting. To minimize transmission contamination, mixing was carried out sequentially from the lowest to the highest pesticide concentration. Before and after the measurements, the samples were sealed with parafilm to avoid contamination and evaporation (Figure 2).

The toxicity of pesticides on microalgae was assessed via ChlF. In addition, half maximum effective concentration (EC50) values were obtained on day 3 and day 8 with the online tool Quest Graph™ EC50 Calculator for 3 d and 8 d, respectively (AAT Bioquest, Inc., Pleasanton, CA, USA, https://www.aatbio.com/tools/ec50-calculator (accessed on 31 July 2025)). For modelling the EC50 values, we first calculated the growth rate $\mu = (\ln(ChlF_{TX}) - \ln(ChlF_{To}))/X$ with T_0 = initial ChlF, and T_X = values after the respective period X. μ of controls were set to 0% response, μ = 0 was set to 100% response.

2.3. Pulse-Amplitude-Modulated (PAM) Dark Fluorescence Yield

PAM fluorescence of Photosystem II (PSII) was used to measure the efficiency of photosynthesis on the initial (T_0) and final (T_8) days. Maximum dark fluorescence yields of PSII (F_v/F_m) were measured with a PAM-2500/US device, specifically developed for low-concentration microalgal suspensions (Walz company, Effeltrich, Germany). We calculated the maximum quantum efficiency (F_v/F_m) after 10 min of dark acclimation of the cultures. F_v/F_m is defined as $F_v/F_m = (F_m - F_o)/F_m$ [55] and provides basic information of the photosynthetic performance; reduced F_v/F_m values indicate stress of the organism. F_m represents the maximum dark fluorescence after a strong flash of light (all PSII reduced), F_0 is the minimum fluorescence in the dark (all PSII oxidized). Absolute F_v/F_m values are listed in Supplementary File S2. Maximum F_v/F_m values for vascular plant species are about 0.83 [56,57] and indicate vital, non-stressed plants. For cyanobacteria, F_v/F_m is between 0.4 and 0.6 [58], because phycobiliproteins interfere with the fluorescence signal [59] (Figure 2).

2.4. Statistics

For contour plots of ChlF measurements, relative values were calculated to standardize the data for different concentrations and conditions. The values shown in the graphs refer to the relative maximum observed value of the respective treatments to ensure an optical comparison between the treatments. ChlF data for monitoring growth were analyzed with a general linear mixed model (GLMM). ChlF percentage values were taken as the dependent variable, measurement days and doses were set as fixed factors, and the replicates as fixed random factors. Statistical analysis was performed with the jamovi package V2.3.28.0 [60], jmw and GAML3 suite installed.

For comparing initial and final F_v/F_m values, we first calculated differences between the initial and the final F_v/F_m of concentrations offered for a specific strain and pesticide, followed by a Kruskal–Wallis test. If the main test indicated significant differences, Dunn–Bonferroni tests were performed. The graphs were created using RStudio 2023.12.0.369 [61–63].

3. Results

3.1. Visual Growth

One of the most striking differences between the pesticide treatments was that Basar $\geq$ 5% of the ready-to-use solution caused sudden death after inoculation for both strains (Figure 3). After one day of exposure, a similar effect was observed with Ortiva and *Synechococcus leopoliensis*. In contrast, *Chlorella vulgaris* did not show immediate cell death, but a noticeable reduction in color indicated growth inhibition in Ortiva. After exposure in Teppeki, visible growth inhibition of both *Chlorella* and *Synechococcus* was observed with the naked eye at doses $\geq$ 10%.

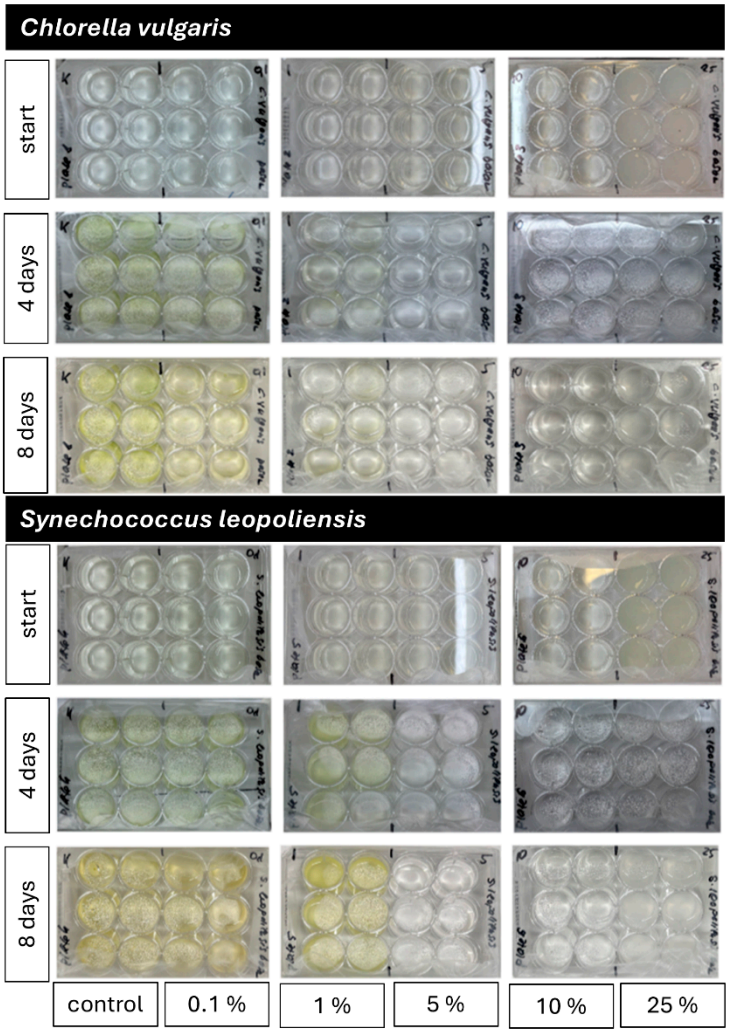

Figure 3. Development of *Chlorella vulgaris* (**top**) and *Synechococcus leopoliensis* (**bottom**) exposed to the herbicide. The plates are allocated in ascending order, with the concentration of the solution increasing from the control on the left to the right and ending with the highest content of the tested 25% (*v*/*v*) solution. For each concentration, 6 replicates were prepared. Please note the turbidity was already visible in the highest concentration at the start of the measurements.

3.2. Biomass Development

In vivo *chlorophyll a* fluorescence (Ch1F) and optical density (OD) were used as a proxy for algal biomass. Results with Teppeki exposure indicated a strong relationship between OD and ChlF (Figure 4A as an example for *Synechococcus leopoliensis*), whereas the herbicide treatment showed a considerable discrepancy between the two parameters (Figure 4B; *Chlorella vulgaris*). Furthermore, Ortiva treatments resulted in comparatively large differences (Supplementary File S1). The differences are caused by the chemicals, which showed increased turbidity at higher concentrations on the first day (see Discussion).

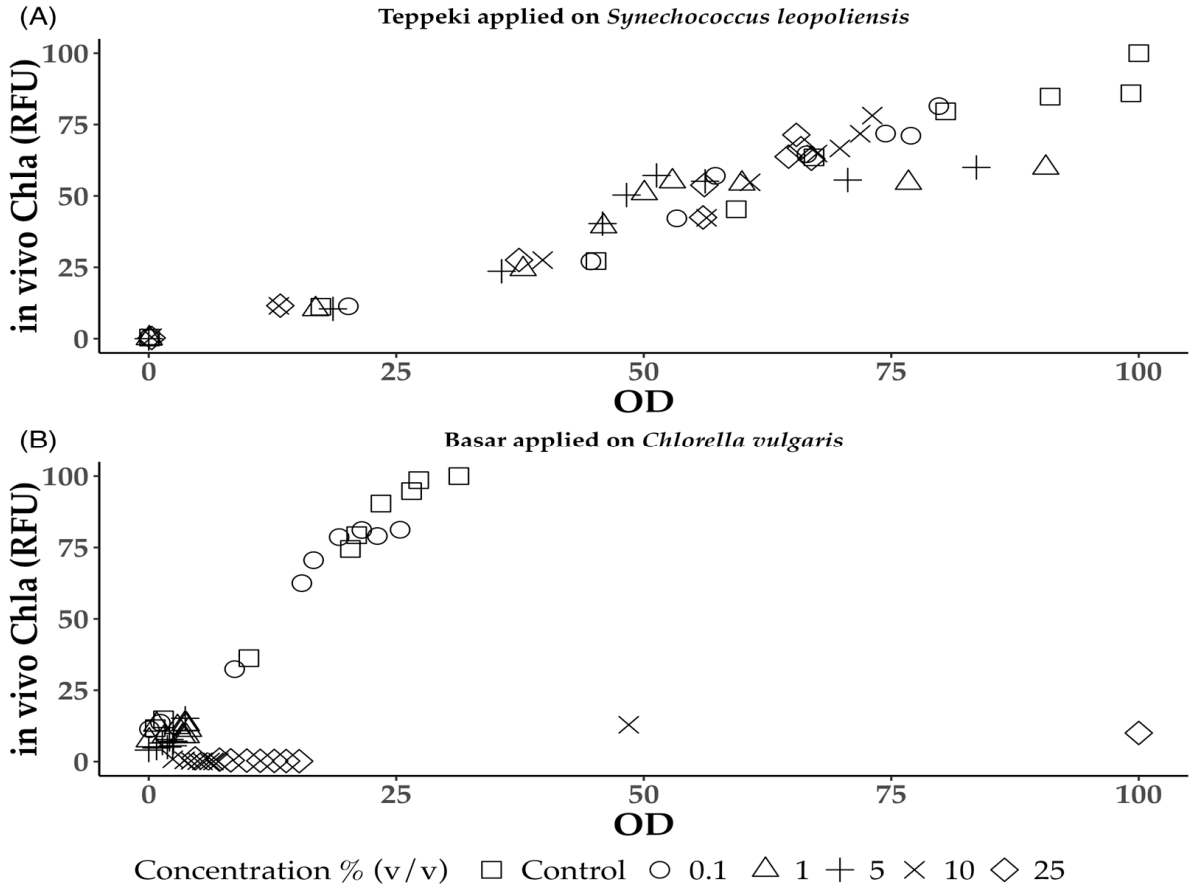

Figure 4. The scatter diagrams of (**A**) *Synechococcus leopoliensis* and (**B**) *Chlorella vulgaris* highlight the potential interference of pesticide exposure on the relationship between OD measurements and ChlF fluorescence.

During Teppeki insecticide treatment, biomass development of *Chlorella vulgaris* and *Synechococcus leopoliensis* consistently increased across all six tested concentrations throughout the experiment (Figure 5). At the same time, growth of *Chlorella vulgaris* remained when treated with Ortiva fungicide, though lower values were observed at doses $\geq$ 10% (v/v). For *Synechococcus leopoliensis*, no growth regarding the fungicide was noted at concentrations $\geq$ 10% (v/v); at around 5% a pronounced reduction in growth compared to lower doses was observed (Figure 5).

The Basar herbicide treatment yielded markedly different results: mainly the control and concentrations to around 1% (v/v) exhibited growth for *Synechococcus leopoliensis*; for *Chlorella vulgaris*, no growth was observed even at the lowest dose of 0.1% provided (Figure 5).

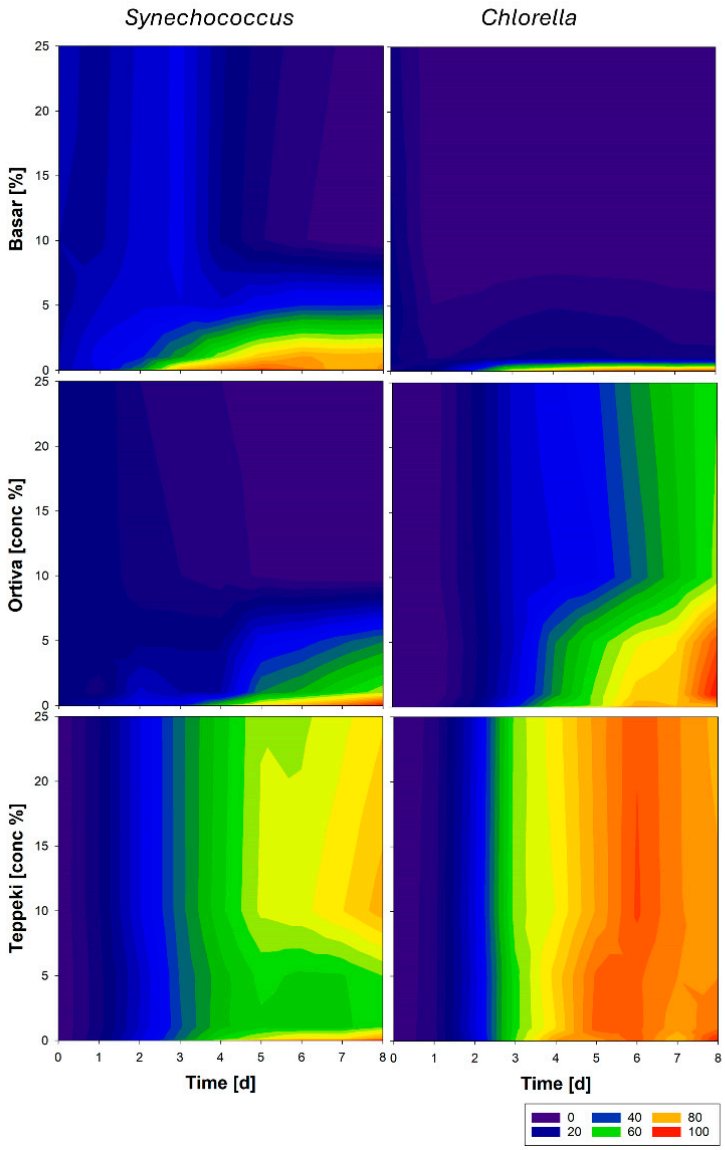

Figure 5. Changes in Chlorophyll-*a* in vivo fluorescence of *Synechococcus leopoliensis* (left column) and *Chlorella vulgaris* during exposure with the herbicide (**top**), the fungicide (**mid**), and the insecticide (**bottom**). Contour plots are shown on a color scale from 0 to 100% with 0% the minimum value of the treatment and 100% the maximum value, ensuring uniformity across different concentrations and conditions. Values represent the mean of 6 replicates.

3.3. In Vivo Fluorescence

GLMMs revealed significant differences between pesticide concentrations for Ortiva and Basar along with the effects of exposure time for each strain. Among the tested compounds, Basar exhibited the strongest inhibitory effect (p value < 0.001), completely suppressing growth in both *Chlorella vulgaris* and *Synechococcus leopoliensis* at concentrations $\geq$5 mgL^{-1}. In *Chlorella vulgaris*, growth inhibition was evident as early as day 1, with no subsequent recovery across the experimental period. In contrast, *Synechococcus leopoliensis* exhibited a delayed response, with growth suppression becoming apparent by day 4 (Supplementary File S2).

Conversely, Ortiva showed a selective and time-specific toxicity (p value < 0.001) that impaired the growth of *Synechococcus leopoliensis* only at high concentrations $\geq$ 10% from day 4 onwards, while algal growth at lower doses increased over time (Supplementary File S2).

Teppeki did not show significant differences between controls and the serial dilutions for *Chlorella*, but for *Synechococcus*, significant differences were calculated (Supplementary File S2).

The marked reduction in algal growth in both *Chlorella vulgaris* and *Synechococcus leopoliensis* due to Basar treatment is also reflected in the half maximum effective concentration (EC50) values. In *Chlorella vulgaris*, EC50 values were about 0.82 mgL^{-1} and 0.86 mgL^{-1} after 3 and 8 days, respectively. Similarly, *Synechococcus leopoliensis* exhibited suppressed growth, with values of 1.04 mgL^{-1} on day 3 and 1.21 mgL^{-1} on day 8. For Ortiva-treated *Synechococcus leopoliensis*, growth remained in the lag phase after 3 days, making calculation impossible. By day 8, however, EC50 was at 0.11 mLL^{-1}, indicating a delayed but partial recovery. These findings underscore the strong inhibitory effect of the herbicide on both algal strains and highlight the differing temporal responses under exposure.

3.4. Overall Photosynthetic Performance

Photosynthetic activity of *Chlorella vulgaris* remained unaffected by Teppeki treatment across all tested concentrations (Kruskal–Wallis test, $p = 0.258$), with mean F_v/F_m values ranging from 0.443 to 0.528 (Figure 6). In contrast, Ortiva exposure led to significant alterations in F_v/F_m values across concentrations ($p = 0.001$). Dunn–Bonferroni post hoc analysis identified significant differences between 0.1% (mean $F_v/F_m = 0.519$) and 25% (mean $F_v/F_m = 0.387$, $p_{adj} = 0.002$) and between 1% (mean $F_v/F_m = 0.484$) and 25% ($p_{adj} = 0.016$), suggesting that higher concentrations of Ortiva markedly reduced photosynthetic performance in *Chlorella vulgaris*. Similarly, treatment with Basar resulted in significant concentration-dependent effects on F_v/F_m values ($p < 0.001$). Post hoc comparisons showed significant differences between 0.1% (mean $F_v/F_m = 0.475$) and 10% (mean $F_v/F_m = 0.065$, $p_{adj} = 0.015$), 0.1% and 25% ($p_{adj} = 0.010$), and 0.1% and 5% (mean $F_v/F_m = 0.069$) concentrations ($p_{adj} = 0.005$).

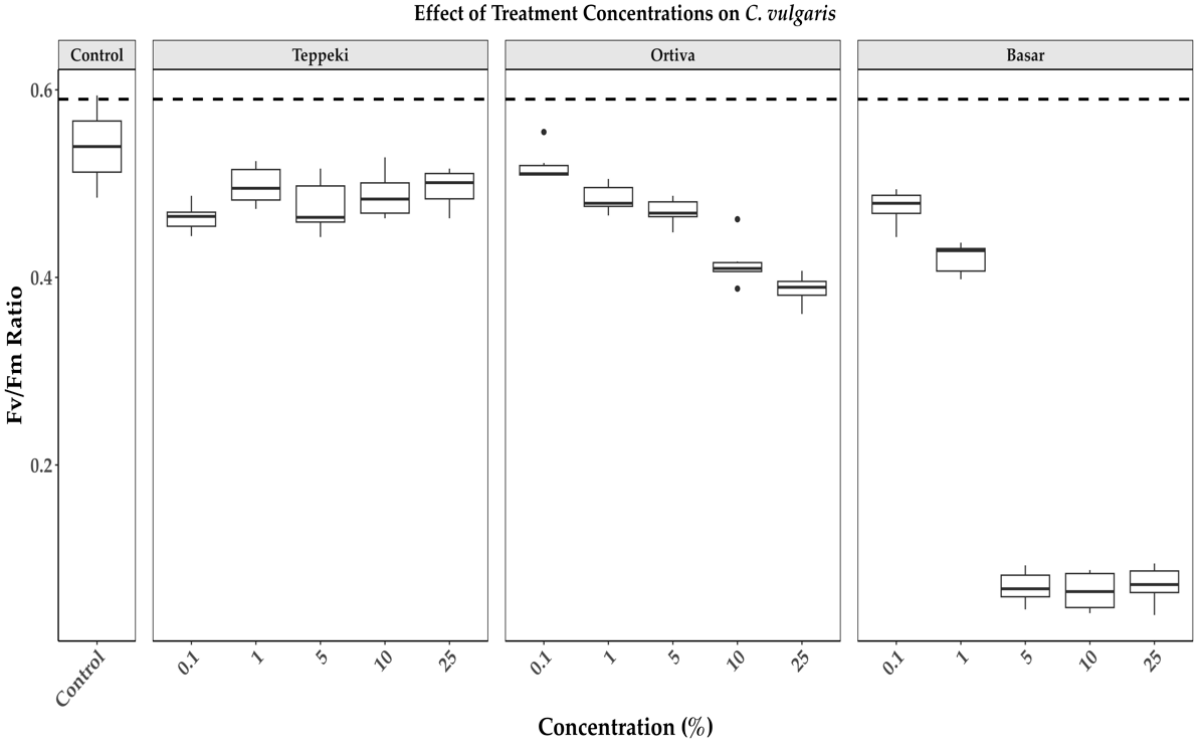

Figure 6. Median box plots showing F_v/F_m levels of *Chlorella vulgaris* after treatment with the insecticide, fungicide, and herbicide. The plots depict the difference between the initial (T_0—provided as a dashed line) and final (T_8) measurements (n = 6 for each box).

Significant differences in F_v/F_m values across Teppeki concentrations were observed in *Synechococcus leopoliensis* (Kruskal–Wallis, $p < 0.001$), with the mean F_v/F_m values ranging from 0.289 to 0.552 (Figure 7). Dunn–Bonferroni post hoc analysis revealed that the 25% concentration (mean $F_v/F_m = 0.328$) significantly differed from both 1% (mean $F_v/F_m = 0.532$, $p_{adj} < 0.001$) and 5% (mean $F_v/F_m = 0.528$, $p_{adj} = 0.001$). Similarly, Ortiva exposure resulted in significant differences in F_v/F_m values across concentrations in *Synechococcus leopoliensis* (Kruskal–Wallis, $p < 0.001$), with mean F_v/F_m values ranging from 0.046 to 0.488. Post hoc comparisons revealed significant effects of the 25% concentration (mean $F_v/F_m = 0.115$) relative to 0.1% (mean $F_v/F_m = 0.438$, $p_{adj} = 0.001$), 1% (mean $F_v/F_m = 0.425$, $p_{adj} = 0.004$), and 10% (mean $F_v/F_m = 0.209$, $p_{adj} = 0.017$). Additionally, the 10% treatment differed significantly from 1% ($p_{adj} = 0.046$). Basar exposure also resulted in a significant concentration-dependent effect on F_v/F_m values in *Synechococcus leopoliensis* (Kruskal–Wallis, $p < 0.001$), with mean F_v/F_m values ranging from 0.038 to 0.617. Significant differences were observed between 0.1% (mean $F_v/F_m = 0.569$) and 10% (mean $F_v/F_m = 0.076$) concentrations ($p_{adj} = 0.028$), 0.1% and 25% (mean $F_v/F_m = 0.051$) concentrations ($p_{adj} = 0.001$), and 0.1% and 5% (mean $F_v/F_m = 0.071$) concentrations ($p_{adj} = 0.035$).

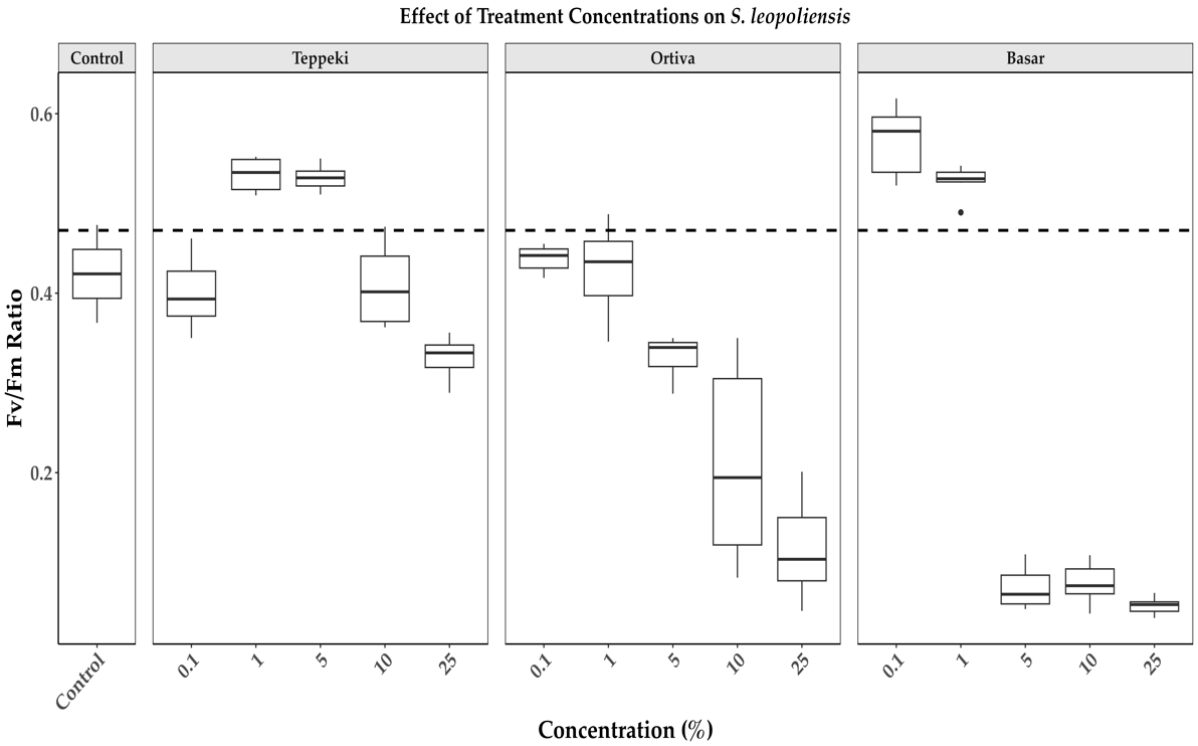

Figure 7. Median box plot showing the F_v/F_m levels of *Synechococcus leopoliensis* after treatment with the insecticide, fungicide, and Basar. The plots depict the difference between the initial (T_0—provided as a dashed line) and final (T_8) measurements (n = 6 for each box).

4. Discussion

We applied two methods for algal biomass estimation to increase the reliability of the results. Immediately after adding Basar to the cultures, some turbidity was observed at elevated doses. The turbidity was obviously caused by the Basar formulation itself, not by algal growth, as evidenced by its characteristic milky texture. Consequently, the elevated optical density (OD) readings at high concentrations at the beginning of the experiment reflect the emulsion of Basar, not increased biomass. Such distortion effects must be considered to avoid misinterpretation of toxicity. For our study, OD as a proxy

for algal biomass was not appropriate in the first days. In contrast, ChlF proved a good estimator for biomass and is thus more appropriate for evaluating pesticide effects.

We observed that the insecticide caused a slight reduction in the growth of *Synechococcus leopoliensis* at concentrations $\geq$ 25%, whereas no clear inhibition pattern was detected for *Chlorella vulgaris*. In the case of the fungicide, previous studies have shown that azoxystrobin inhibits the growth of various organisms, including microalgae such as *Chlorella pyrenoidosa* and *Microcystis aeruginosa*, fungi belonging to Chytridiomycota or *Magnaporthe oryzae*, soil microbiota such as actinobacteria and organotrophic bacteria, and freshwater fish such as *Danio rerio* [64–67]. We assumed that a similar effect can be observed at high doses, which was confirmed. Both OD and ChlF measurements correlated well with what we have visually observed. In agreement, the F_v/F_m, indicating the photosynthetic performance, showed reduced values at higher doses.

Treated with the herbicide, *Synechococcus leopoliensis* showed reduced growth at concentrations of 5% (*v*/*v*); at higher doses, no growth was observed. We assume that the threshold value of inhibition for this herbicide is located between 5% (*v*/*v*) and 1% (*v*/*v*), which is also indicated by the EC50 values. Also, for *Chlorella vulgaris*, our results revealed that an increased concentration of the herbicide had a statistically significant dose-dependent inhibitory effect on algal biomass development, with the most noticeable decrease occurring already at the lowest dose provided.

Both biomass development (ChlF, GLMM), and F_v/F_m (Kruskal–Wallis test) showed significant negative effects of higher doses of the insecticide on cyanobacterial growth, potentially threatening aquatic ecosystems where *Synechococcus leopoliensis* makes a significant contribution to primary production. The observed impact highlights the sensitivity of this species to chemicals, underscoring the need for strict control over the entry of pesticides into the environment. Future research should focus on clarifying the long-term environmental consequences of chronic exposure to Teppeki on cyanobacteria biodiversity and ecosystem functioning. Interestingly, the cyanobacterial strain was more affected than the eukaryote *Chlorella vulgaris*. The reduced values can be partially explained by the photophysiological sensitivity of the species or by suboptimal light penetration affecting the efficiency of photosynthesis.

F_v/F_m clearly pointed out that the overall photosynthetic performance significantly decreased after only one day of applying the herbicide. As previous studies have already proven, certain pesticides inhibit the growth of *Chlorella vulgaris* at specific concentrations, such as the herbicide florasulam with concentrations of 2 mgL^{-1}, 2.84 mgL^{-1}, and 6 mgL^{-1} [68] or fludioxonyl fungicide with an EC50 of 1.87 mgL^{-1}, both of which are still commercially available [69]. We found EC50 between 0.82 and 0.86 mgL^{-1} for *Chlorella vulgaris* and 1.04 and 1.21 mgL^{-1} for *Synechococcus leopoliensis* against Basar. Although *Chlorella vulgaris* has advanced detoxification systems that allow it to cope with various environmental factors, such as glutathione-mediated detoxification by modulating glutathione pathways [70] as well as detoxification of heavy metals with metallothionenes, phenolic compounds, phytochelatins, etc., for detoxification of heavy metals [71], the species is quite sensitive to this herbicide. *Chlorella vulgaris* has a rigid cell wall, including polysaccharides and chitin-like and cellulose-like components that make green microalgae robust; it is quite susceptible to this toxin [72]. For the case of *Synechococcus leopoliensis*, the differences are due to specific metabolic properties and enzymatic systems. This species contains phycobiliproteids, such as phycocyanin, which play a crucial role in light absorption and can influence their sensitivity to certain chemicals [73]. A study conducted by Deng et al., 2012 [74] showed that *Synechococcus* sp. PCC 7942 is more sensitive to herbicides such as irgarol than to diuron (algicide and herbicide), both of which are PSII inhibitors. A study by Noaman et al., 2004 [75] showed that *Synechococcus leopoliensis* produces antimicrobial

compounds active against Gram-positive bacteria, such as *Staphylococcus aureus*. However, the production of these compounds is influenced by environmental factors (temperature, pH, and nutrient availability). *Synechococcus leopoliensis* appeared to be more sensitive to both physical and biochemical stressors. Despite a relatively thin layer of peptidoglycans (~10 nm), characteristic of Gram-negative bacteria, this structural characteristic does not necessarily provide increased resistance to external influences [76].

Also, other water organisms not related to vascular plants are heavily influenced by herbicides: Mai et al., 2013 [77], examined the effects of (S)-metolachlor, irgarol, and diuron on the sensitivity of gametes and embryos of Pacific oysters. The results showed that all three substances significantly reduced fertilization effectiveness and increased malformations when treated with these pesticides, with Lowest Observed Effective Concentration (LOEC) values reaching 0.1 µg L^{-1} for c, 1 µg L^{-1} for irgarol, and 4 µg L^{-1} for diuron. The similar biological activity of irgarol and (S)-metolachlor is also due to their more similar chemical properties than that of diuron, since both irgarol and (S)-metolachlor are S-triazines, whereas diuron is a urea compound [78].

Pulse-amplitude-modulated (PAM) measurements showed that the insecticide had no significant effect on the efficiency of photosynthesis in *Chlorella vulgaris* (p = 0.2575), while the fungicide and the herbicide caused a decrease in F_v/F_m. In the case of the herbicide, a significant difference between 0.1% and 25% (v/v) can be observed (p_{adj} = 0.002). Similarly, photosynthetic performance was reduced between 1% and 25% in the fungicide (p_{adj} = 0.0164). For *Synechococcus leopoliensis*, even more negative effects were observable at higher concentrations of all three pesticides. Exposure at the highest concentration (25%) of each of them caused a noticeable decrease, indicating a significant response to stress.

In summary, this study highlights the sensitivity of both *Synechococcus leopoliensis* and *Chlorella vulgaris* to commonly used pesticides with *Synechococcus leopoliensis* showing a higher vulnerability, particularly to herbicidal and fungicidal treatments. The observed physiological disruptions—reflected in both growth and overall photosynthetic performance—underscore the potential ecological risks associated with the application of pesticides like Teppeki, Ortiva, and Basar in the environment. The findings emphasize the importance of assessing both lethal and sublethal effects of pesticides on non-target organisms, especially primary producers that form the foundation of aquatic food webs. Understanding species-specific responses and the mechanisms underlying these effects is crucial for accurate environmental risk assessment.

5. Conclusions

Although OD is commonly assumed and used as a reliable indicator of algal biomass, special attention must be paid to emulsions, e.g., Basar, which cause turbidity due to their residues (milky substance), thus impacting the measurements.

In agreement with our assumption, we observed that the herbicide exhibited the most pronounced sublethal effect among the three pesticide types tested. Even at a very low dose of one per thousand relative to the recommended field application rate, this corresponded to 3.84 mg/L of (S)-metolachlor in the ready-to-use solution. The fungicide showed selective and time-specific (day 4) toxic inhibition at the tested concentrations $\geq$ 10% with *Synechococcus leopoliensis* used as the target organism. Our results indicate that, under the tested experimental conditions, Teppeki did not induce statistically measurable impacts on the physiological or growth-related parameters of *Chlorella vulgaris*, but for *Synechococcus leopoliensis*, observable trends point to possible sublethal effects that require further investigation with greater replication or over prolonged exposure periods. Additionally, *Synechococcus leopoliensis* appeared more sensitive to the fungicide, showing a rapid decline

in F_v/F_m from 5% (*v*/*v*) of the ready-to-use solution onwards, while *Chlorella vulgaris* exhibited more resilience.

This study highlights the urgent need to assess pesticide impact on non-target organisms, such as algae or bacteria, to ensure improved ecological and environmental management. Furthermore, long-term studies are needed to evaluate the severe influence of pesticide exposure, particularly in a realistic setting where multiple stressors co-exist. Such insights are extremely valuable for formulating sustainable agricultural practices and developing strategies that can minimize harm to biodiversity and other ecosystem functions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w17182716/s1. Supplementary File S1: Stock concentrations, measurement values, correlation indices (OD and in vivo fluorescence) and statistical test results (Shapiro–Wilk, Kruskal–Wallis); Supplementary File S2: Results of the generalized linear mixed model (GLMM) analysis.

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Abbreviations

The following abbreviations are used in this manuscript:

EC50	Half maximum effective concentration
OD	Optical Density
ChlF	In vivo Chlorophyll-a Fluorescence
PAM	Pulse-Amplitude-Modulated
PSII	Photosystem 2

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