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Pesticide Mixtures Cause Short-Term, Reversible Effects on the Function of Autotrophic Periphyton Assemblages

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Abstract: In a laboratory experiment we investigated the effects of pesticide mixtures on the structure and function of freshwater biofilms, with focus on their photoautotrophic component. We identified 6 herbicides and 1 fungicide commonly found in Swedish streams at relatively high concentrations and created 3 ternary mixtures that were tested in concentration series ranging from observed environmental concentrations to up to 100 times higher. Biofilms were exposed to these pesticide mixtures for 8 d and then allowed to recover for another 12 d. Our results show a rapid and consistent inhibition of photosynthesis after just 24-h exposure to the highest test concentration of pesticides, as well as in some treatments with lower concentrations (i.e., 10 times the environmental level), on exposure. Interestingly, the observed effects were reversible because biofilm photosynthesis recovered rapidly and completely in clean media in all but one treatment. In contrast to the functional response, no effects were observed on the algal assemblage structure, as assessed by diagnostic pigments. We conclude that the pesticide mixtures induce a rapid but reversible inhibition of photosynthesis, without short-term effects on biofilm structure. *Environ Toxicol Chem* 2020;39:1367–1374.

Keywords: Pesticides; Mixture toxicology; Algae; Biofilm; Pigments; Photosystem II

INTRODUCTION

The current agricultural use of pesticide equals some 5.8 million tonnes per year globally, of which 7% are herbicides (Food and Agriculture Organization 2017), and is expected to increase with global warming (Kattwinkel et al. 2011) and population development (Godfray et al. 2010). In conventional agriculture, an investment in pest control generally translates into some 4-fold crop return in the United States (Pimentel 2005). A major drawback when using pesticides, however, is their frequent transport (through soil leakage and runoff, as well as spray drift) from the sites of application to surface waters, where they pose a threat to nontarget organisms, affecting both ecosystem structure (e.g., changes in community composition [Liess and Schulz 1999; Hasenbein et al. 2017]) and function (e.g., effects on photosynthesis, algal growth, and leaf litter decomposition [Molander and Blanck 1992; Schmitt-Jansen and Altenburger 2007; Rasmussen et al. 2012]). Pesticides in surface waters are commonly found in mixtures of at least 2 to 5 compounds (Schreiner et al. 2016; Bighiu et al. 2020). The widespread and extensive use of pesticides along with their potential combined effects on organisms and ecosystems call for a better understanding of their mixture effects on biota, including primary producers such as biofilms.

Biofilms cover all submerged surfaces and provide habitat for consortia of bacteria, fungi, algae, and protozoans that fulfill important ecosystem functions such as primary production and biogeochemical cycling (Battin et al. 2016). Biofilm organisms respond rapidly to environmental changes and are widely used for the assessment of water quality and ecological integrity (Sabater et al. 2007). Biofilms are also hot spots for the sorption and active uptake of pesticides and thereby control their fate and bioavailability in the aquatic environment (Katagi 2006; Rooney et al. 2020). For example, Lundqvist et al. (2012) found that biofilms can both increase and decrease the bioavailability of insecticides in freshwater, depending on their chemical characteristics and water dissolved organic matter (DOM) concentrations. Different organism groups in biofilms will be impacted by pesticides with specific modes of action, such as those targeting fungi (fungicides), autotrophs (photosynthesis-inhibiting herbicides), and invertebrates (insecticides, fungicides). Currently used herbicides have some 20 known modes of action (Duke 2012) and a wide range of physical–chemical properties

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and often occur in mixtures (Bundschuh et al. 2014; Kim Tiam et al. 2014), further complicating their fate and risk assessment.

In the present laboratory study, we investigated the effects on and recovery of biofilm structural and functional response variables of common mixtures of agricultural pesticides. More specifically, we tested 3 pesticide mixtures of 3 compounds each at concentrations that were observed in monitoring programs and at successively higher mixture concentrations. We measured photosynthetic activity as a functional endpoint, whereas structural changes in algal assemblages were scored by taxonomic and pigment analyses. We hypothesized that photosynthetic activity of exposed biofilms would decrease because all tested mixtures contained at least one photosystem II (PS II) inhibitor. We also hypothesized that shifts in biofilm community structure would occur, with more tolerant taxa groups occurring at the higher pesticide concentrations.

MATERIALS AND METHODS

Experimental preparation

Biofilms were removed with a brush from cobbles collected at 0.5 to 1 m depth in the littoral zone of Lake Erken in Sweden (59°50′15.6″N, 18°38′06.1″E) and transported (as suspension in lake water) to the laboratory in cooling boxes. In the laboratory, this suspension was used as an inoculum for biofilm colonization of unglazed ceramic granite tiles (3 × 3 cm). Biofilms were grown for 9 wk in plastic tanks containing 15 L of L16V medium (Lindström 1991) at 11.8 ± 0.2 °C and a 16:8-h light:dark cycle (Fluora fluorescent light bulbs) and a light intensity of 924 ± 144 lux, with continuous aeration through aquaria pumps. After 9 wk, well-established biofilms (Supplemental Data, Figure S1) were transferred to the experimental units and exposed to pesticide mixtures.

Pesticides, 6 herbicides and 1 fungicide, that repeatedly occur at relatively high concentrations in Swedish agricultural streams that are regularly monitored (Boström et al. 2016) were selected for this experiment. Moreover, these pesticides often co-occur in the monitored streams in various binary (e.g., up to 66%) and ternary (up to 33% of 129 water samples in 2015) combinations (Supplemental Data, Table S1). Note that this co-occurrence is valid for the selected combinations of 3 compounds and not for all 7. Hence, 3 ternary mixtures were prepared by our accredited pesticide laboratory. Briefly, the pesticides (Dr. Ehrenstorfer) were prepared in L16V medium with acetone (pesticide grade) as carrier medium (Table 1). Controls consisted of L16V medium and acetone but without pesticides. The final acetone concentration in all treatments was 0.01%. Experimental concentrations for each mixture covered 5 different mixture concentrations (MCs), where MC1 corresponded to the maximum observed sum of toxic units (ΣTU) of the pesticides during one season in situ (i.e., weekly average concentrations in time-integrated samples from national monitoring; see Bundschuh et al. [2014] and Boye et al. [2019] for details), MC5 was 5 times higher than that, and so on (i.e., MC1, MC5, MC10, MC50, MC100). The toxic units were based on published median effect concentration data for growth inhibition of green algae (predominantly *Pseudokierchneriella subcapitata*; Supplemental Data, Table S2; Lewis et al. 2006) and calculated according to Equations 1 and 2, where TU_{obs} corresponds to the observed environmental concentration of pesticides (C) and P is the proportion of each pesticide in a mixture, in the present study arbitrarily set to 50, 25, and 25%. The concentrations were normalized based on the highest observed ΣTU to have equal toxic potential among the 3 different mixtures.

$$TU_{obs} = \frac{C}{EC50} \quad (1)$$

$$\Sigma TU = \frac{\Sigma TU_{obs} \times P}{100} \quad (2)$$

The pesticide concentrations in the stocks used for preparing the concentration series were analytically confirmed by liquid chromatography-positive electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) according to our accredited method. Briefly, the stocks (in acetone) were diluted with MilliQ water (pH 5), cleaned up using an online SPE system (Strata-X, C18, 2.1 × 20 mm) and injected into an Agilent 1260 HPLC coupled to an Agilent 6470 Triple Quadrupole LC/MS. The analytical column used was an Agilent Zorbax C18-EC, 100 mm × 3.5 μm × 3.0 mm. The results, together with quality assurance/control data are listed in Supplemental Data, Table S3, and indicate that all pesticides but metamitron were within 20% of the nominal concentration, which is the acceptable error of our accredited laboratory. Likely the lower values for the metamitron stock are underestimates due to some degradation of the compound because the study was carried out.

Experimental design

Eight biofilm-covered tiles were placed in each of 80 experimental units (i.e., [3 treatments + 1 control] × 5 exposure

TABLE 1: Pesticide properties, their nominal concentration in mixture concentration (MC1; μg L^{-1}), and percentage in each mixture[a]

Pesticide	Mixture I	Mixture II	Mixture III	MOA (target site)	Log K_{OW}
Prochloraz	50% [0.223]			Membrane function	3.5
Metribuzin	25% [0.142]			Photosystem II	1.65
Metamitron	25% [2.835]	25% [2.835]		Photosystem II	0.85
Terbuthylazine		25% [0.324]		Photosystem II	3.4
Diflufenican		50% [0.018]	50% [0.018]	Carotenoid synthesis	4.2
Metazachlor			25% [0.547]	Cell division	2.49
Isoproturon			25% [1.458]	Photosystem II	2.5

[a]All are herbicides, except for prochloraz (fungicide). Analytical confirmation of the nominal concentrations is in Table S3.
MOA = mode of action.

concentrations × 4 replicates), consisting of 1-L glass crystallization bowls (i.d. = 140 mm) that contained 0.5 L L16V medium and that were covered with plastic Petri dishes to minimize evaporation and aerated continuously using glass Pasteur pipettes and aquarium pumps. Experimental units were incubated under identical conditions as those described for the biofilm colonization phase. Before the start of the experiment (day 0), biofilms from 4 randomly selected tiles were sampled prior to the addition of pesticide mixtures and used to quantify pre-exposure biofilm algal composition and photosynthetic activity (see sections *Periphyton taxonomic composition* and *Quantification of biofilm photosynthetic efficiency*). Experimental units were then randomly allocated to each treatment and controls.

On day 0 a pulse of pesticide mixtures was added using an automated pipette, followed by an 8-d exposure phase. During this exposure phase, one tile per replicate of treatments and controls was removed after 1, 2, 4, and 8 d. Biofilms were removed from tiles using a rubber cell scraper, homogenized, and divided into 3 subsamples. One subsample was used for the quantification of photosynthetic efficiency of the photoautotrophs in the biofilm immediately after sampling, the second subsample was preserved in Lugol's solution for later taxonomic analysis, and the third subsample was frozen for future analyses of pigments and nutrients (see section *Biofilm pigments*). On day 8, the medium in all treatments was replaced with clean L16V medium, and biofilms were allowed to recover for another 12 d (i.e., until day 20). During this recovery phase, tiles were collected on days 12, 16, and 20 and handled as described. The various analyses at each point in time are summarized in Figure 1. Nutrient water concentrations were analyzed in L16V medium samples taken from controls on days 0, 8, and 20. In addition, biofilm samples from controls and from the lowest (MC1) and highest (MC100) pesticide mixture concentrations were also analyzed for NH_4-N, PO_4-P, total P, total N, NO_2-N + NO_3-N, and Si according to the standard laboratory methods: ISO 15923-1:2013 (NH_4-N); Bran Luebbe G-175-96 Rev.15, Multitest MT 18 (PO_4-P); SS-EN ISO 6878:2005 mod., Bran Luebbe, Method No G-175-96 for AAIII (Total-P); SS EN 12260:2004 (Total-N); ISO 15923-1:2013 (NO_2-N + NO_3-N); SS-EN ISO 17294-2:2005 (Si).

Quantification of biofilm photosynthetic efficiency

Photosynthetic efficiency of biofilm photoautotrophs was determined using a Hansatech FMS 2 Pulse-Amplitude Modulated fluorometer (Schreiber et al. 1995). The biofilms were removed from the tiles as described and suspended in 300 µL tap water in plastic multiwell plates with a black bottom. The probe of the fluorometer was placed right above the wells, and chlorophyll *a* fluorescence yield was measured in both light-adapted and dark-adapted (20 min) biofilms, with a gain and pulse set to 50 and 60, respectively. By doing so we measured both the PS II operating efficiency (Y_{eff}, the fraction of absorbed light that is used for PS II photochemistry) and the maximum quantum yield (Y_{max}). These chlorophyll fluorescence parameters were calculated according to Genty et al. (1989) following Equations 3 and 4.

$$Y_{eff} = \frac{Fm' - Fs}{Fm'} \quad (3)$$

$$Y_{max} = \frac{Fv}{Fm} \quad (4)$$

In Equations 3 and 4, Fm and Fm′ are the maximum fluorescence for dark- and light-adapted biofilm, respectively; Fs is the steady-state fluorescence yield; and Fv is the variable fluorescence. Quality assurance/control was performed by measuring blanks consisting of L16V medium only.

Periphyton taxonomic composition

Taxonomic analysis of biofilm algal assemblages was done by accredited laboratory staff on Lugol's preserved samples from days 0 and 8 (preexposure and end of the exposure phase) from the highest mixture concentration treatments and from controls using an inverted microscope (Nikon) with differential interference contrast and ×100 to ×400 magnification. Taxa were identified to the genus level and/or allocated to trait-based groups (e.g., single or colonies, filamentous or coccoidal).

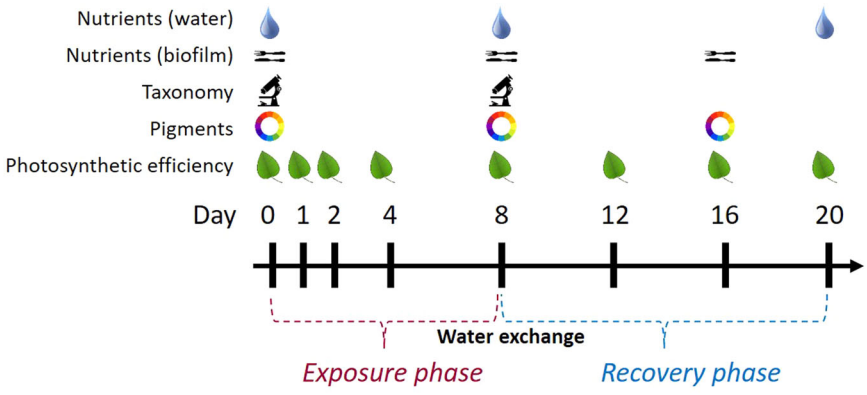

FIGURE 1: Schematic overview of the experimental design and analyses in biofilm (all) and water (nutrients) for each sampling day. $n = 4$ for photosynthetic efficiency, $n = 3$ for pigments and nutrients in biofilms, $n = 1$ for taxonomy and nutrients in water (per time point). See text for details.

Biofilm pigments

For pigment analyses, freeze-preserved biofilms from the controls and the treatments with the lowest and highest pesticide concentrations (i.e., MC1 and MC100) were freeze-dried and extracted according to Hagerthey et al. (2006) using methanol:acetone:*N*,*N*-dimethylformamide:water, 30:30:30:10 (MAD) medium. Briefly, 12 mg biofilm were frozen overnight in 2 mL MAD medium and then sonicated using a Vibra-cell® sonication probe, operating at 80% in 5-s pulses (see Torstensson et al. 2018). Extracts were analyzed for pigments using HPLC with an absorbance diode array–based detector (Spectraphysics UV6000LP), as detailed in Wright and Jeffrey (1997). A 150 × 3 mm Phenomenex Kinetex® 2.6-µm C18 100 A column was used for separation, and 12 pigments were identified by their retention time and absorbance spectra (400–700 nm) and compared with those for pigment calibration standards (DHI Water and Environment). Fucoxanthin, neoxanthin, and echinenone were used as chemotaxonomic markers for diatoms, green algae, and cyanobacteria, respectively.

Data analysis

Repeated measures analyses of variances (ANOVAs) were used for testing the effect of both pesticide mixture concentrations and exposure time on photosynthetic efficiency of biofilms (Y_{eff} and Y_{max}). Normality of residuals was evaluated using quantile plots. Recovery of biofilm photosynthetic efficiency was analyzed with Dunnett's test with control, for each day separately. Correlation analysis was done for PS II parameters and chlorophyll a concentration. All tests were run using the JMP PRO Ver 13 statistical software, and alpha was set to 0.05.

RESULTS

The ANOVAs showed significant effects of pesticide mixture concentration for all mixtures (Table 2) and consisted of decreasing photosynthetic efficiency with increasing mixture concentration. Temporal effects on both photosynthesis parameters were significant for all mixtures during the exposure phase but not during the recovery phase. The interaction term between mixture concentration and time was significant only for Y_{eff} during the exposure phase for mixtures II and III (Table 2); thus, photosynthesis inhibition occurred first at the highest exposure and then successively at the lower concentrations.

Photosynthetic efficiency showed a rapid inhibition of PS II maximum efficiency (Y_{max}) and PS II operating efficiency (Y_{eff}) by up to 18 and 13%, respectively, already after 1 d of exposure for all the mixtures and mixture concentrations, indicating a decline in the photosynthetic activity of the biofilms (Figure 2). Although this initial decline was concentration-dependent and apparent in all treatments, it was significant only for the highest mixture concentration tested (i.e., MC100). On day 2, treatments with pesticide concentrations of MC1, MC5, and MC10 resulted in higher photosynthetic efficiency than in controls (i.e., stimulation of photosynthesis), whereas treatments with higher mixture concentrations again showed inhibition and very large variability. In particular, mixture I at MC5 induced the strongest stimulation of photosynthetic efficiency relative to controls (i.e., 11% for Y_{max} and 13% for Y_{eff}). By the end of the exposure phase (day 8), significant inhibition of photosynthesis was also observed for treatments MC10 and MC50. The strongest Y_{max} inhibition of $22 \pm 6\%$ occurred at the highest mixture concentration (i.e., 100) in mixture II after 4 d of exposure, whereas the strongest inhibition of Y_{eff} of $26 \pm 7\%$ was found in treatments with the highest mixture concentration for mixture III after 8 d of exposure (Figure 2).

Four days after the medium exchange (i.e., on day 12), most treatments except mixture II again showed PS II values that were similar to or higher than those in controls, indicating recovery over 4 d in a clean medium (Figure 2). At pesticide concentrations observed in our monitoring program (i.e., MC1 based on weekly average values; Supplemental Data, Figure S2), the recovery of Y_{eff} was a maximum of 1.2% per day. By the end of the recovery

TABLE 2: Output of the repeated measures analysis of variance for the effects of time and treatment (mixture concentration) on photosynthetic parameters[a]

		Y_{eff}				Y_{max}			
		Exposure		Recovery		Exposure		Recovery	
Mix	Factors	*p*	*F* ratio	*p*	*F* ratio	*p*	*F* ratio	*p*	*F* ratio
I	Time	***	14.85	ns	0.70	**	8.98	ns	3.61
	MC	***	18.09	ns	1.12	****	24.36	ns	0.69
	Time × MC	ns		ns		ns		ns	
II	Time	**	7.12	ns	1.33	***	13.21	ns	1.27
	MC	***	21.71	***	16.79	****	32.17	**	8.77
	Time × MC	*	6.46	ns		ns		ns	
III	Time	*	5.26	ns	1.65	***	13.06	ns	0.07
	MC	****	57.69	***	20.65	****	70.51	**	13.97
	Time × MC	*	4.36	ns		ns		ns	

[a]Photosystem II operating efficiency (Y_{eff}, left) and maximum photosynthetic yield (Y_{max}, right), during the exposure phase (days 0–8) and the recovery phase (days 8–20).
*Indicate significance levels, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.
MC = mixture concentration; df = 1 for both Time and MC (treated as continuous variables); ns = not significant.

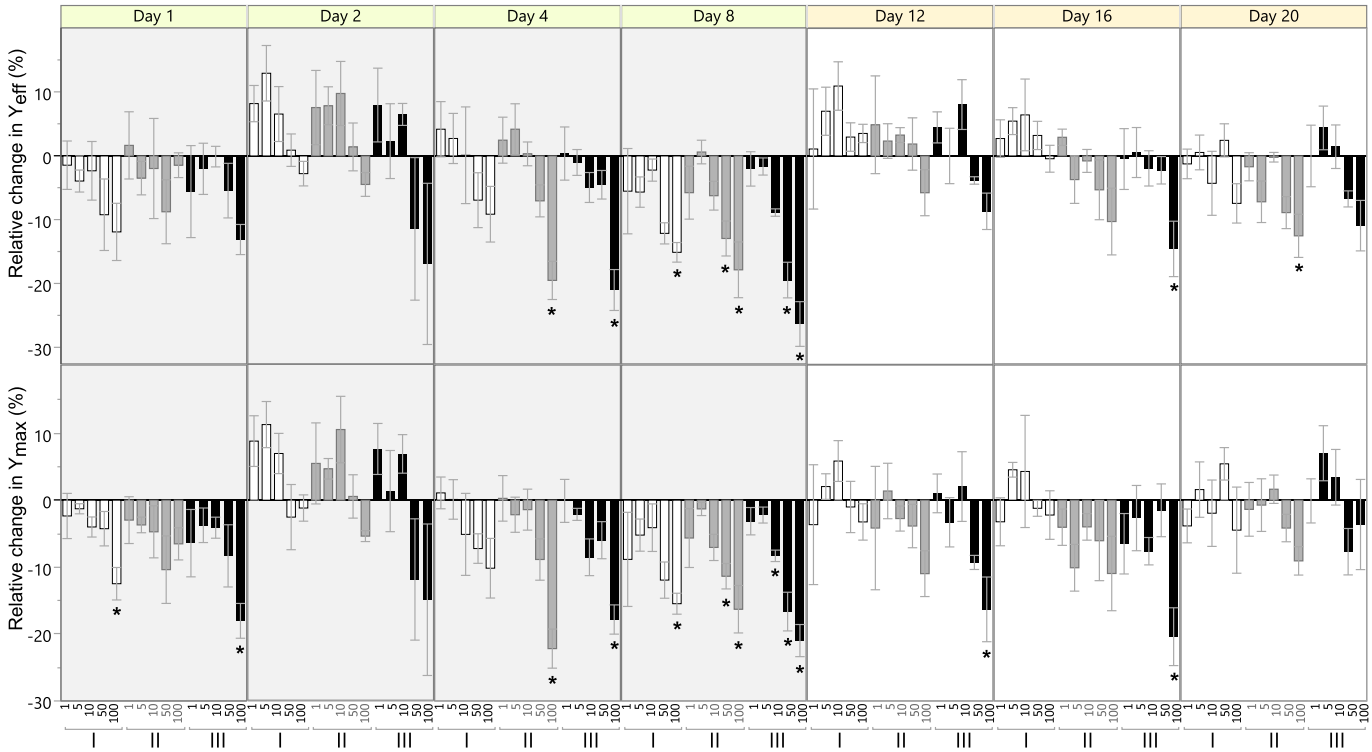

FIGURE 2: Temporal changes in photosynthetic efficiency, relative to control, in different pesticide treatments (means ± standard errors) for the exposure phase (shaded panels) and the recovery phase. The 3 mixtures are denoted by Roman numerals and mixture concentrations by Arabic numerals. Asterisks indicate treatments that differ significantly from control (see text for details). Y_{eff} = photosystem II operating efficiency; Y_{max} = maximum photosynthetic yield.

period, biofilm photosynthetic efficiency in mixtures I and III had fully recovered and was again similar to that in controls. However, for mixture II, Y_{eff} for biofilms previously exposed to MC100 still was significantly lower than that of the controls ($p = 0.0133$).

Nutrient water concentrations (measured in controls) decreased markedly after the first 8 d of biofilm growth, that is, from 1650 to 242.5 µg total N L^{-1} and from 307 to 15.5 µg total P L^{-1} (data not shown). Anionic forms of nitrogen and phosphorus nutrients were generally below the detection limit after 8-d exposure and after 12-d recovery. Nutrient concentrations in biofilms ranged between 0.33 and 0.93 µg mg^{-1} for NH_4-N, 0 and 0.15 µg mg^{-1} for $NO_2 + NO_3$-N, 1.23 and 4.25 µg mg^{-1} for PO_4-P, and 2.28 and 10.96 µg mg^{-1} for total P. During both the exposure and the recovery phases, all of the biofilm nutrient concentrations were generally higher in the pesticide-exposed biofilms than in the control biofilms (i.e., up to 33% higher), though this difference was not statistically significant.

Taxonomic analysis of biofilms identified 17 algal taxa to the genus level and showed that our experimental biofilms were largely predominated by green algae (10 taxa, 90% of total biovolume), followed by diatoms (2 taxa, 8%) and cyanobacteria (5 taxa, 1%; Supplemental Data, Figure S3). After the first 8 d of incubation, green algae had increased in biovolume by a factor of 3.5 in controls, whereas diatoms increased 2.6 times and cyanobacteria 1.2 times, suggesting rapid growth. The biovolume in the mixture treatments increased, on average, by a factor of 2 for green algae, 0.6 for diatoms, and 3.3 for cyanobacteria. The most predominant taxa, present in all samples, comprised the green algae *Scenedesmus*, *Spirogyra*, *Oedogonium*, *Pediastrum*, and *Cosmarium* and the diatom *Epithemia*. Microscopic analysis also identified several trait-based groups, most notably that the majority of diatoms (88%) were single-celled, whereas most green algae and cyanobacteria were filamentous (75 and 69%, respectively; data not shown).

Pigment analysis revealed the highest concentrations of fucoxanthin, neoxanthin, violaxanthin, lutein, and beta-carotene in the controls, although the data showed high variability for many of the samples and no differences between controls and pesticide-exposed biofilms (Figure 3). In contrast, chlorophyll a and zeaxanthin concentrations were highest in mixture II (MC100 and 1, respectively), and echinenone was highest in mixture I (MC1; Supplemental Data, Table S4). The total pigment concentrations, indicative of biofilm biomass, did not differ among treatments ($p > 0.05$). No correlation ($p > 0.05$) was found between chlorophyll a concentrations determined by HPLC and chlorophyll a fluorescence. Biofilm pigment concentrations in treatments with mixture II increased the least after transfer to clean media (i.e., average 7.6% increase), whereas for mixtures I and III the average increase was 22.4 and 26.3%, respectively.

DISCUSSION

Exposure to pesticide mixtures affected the function of the biofilms but not their structure, as illustrated by the observed

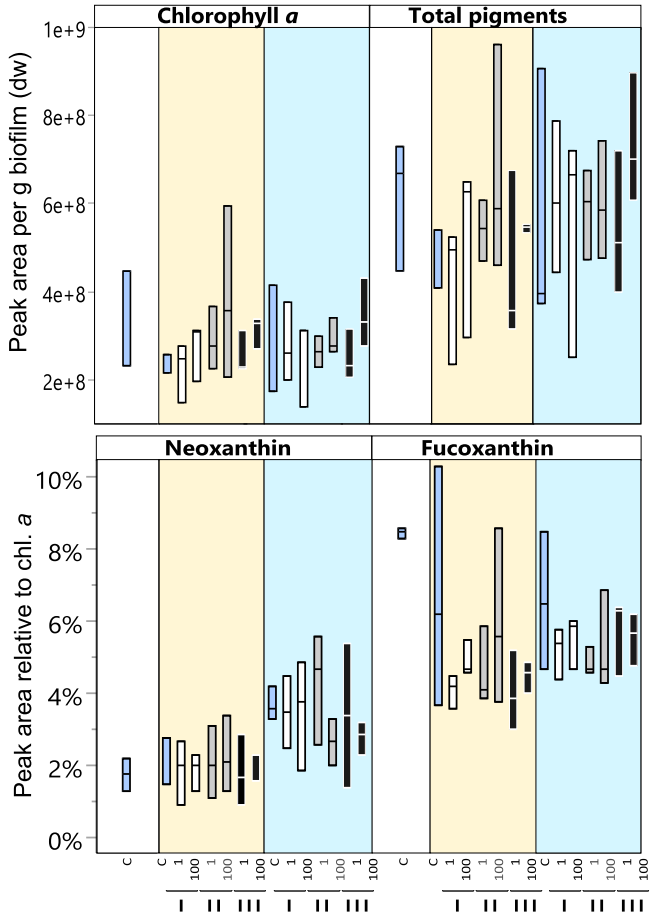

FIGURE 3: Pigment composition of biofilms before exposure to pesticide mixtures (white panels), after 8 d of exposure (orange panels), and after 8 d of recovery (blue panels). The 3 mixtures are denoted by Roman numerals and mixture concentrations by Arabic numerals. C = control; chl = chlorophyll; dw = dry weight.

decrease in photosynthetic efficiency and lack of effects on diagnostic pigment concentrations. However, these effects were observed generally at the highest concentrations tested and to a smaller extent at levels close to environmental concentrations (i.e., MC10, mixture III, day 8). Effects at lower mixture concentrations occurred later in the experiment, likely due to the pesticides' toxicokinetics, with more time needed for reaching high enough pesticide bioconcentration to trigger a response (Ashauer and Escher 2010). Functional recovery occurred rapidly in almost all of the biofilms, likely due to the short generation times of algae (DeLorenzo et al. 2001).

All of the pesticide mixtures negatively affected photosynthetic efficiency, despite apparent differences in their modes of action. Mixture III was the most diverse in terms of the modes of action of its constituent pesticides, namely one inhibiting PS II (isoproturon), another one inhibiting the synthesis of biocarotenoids (diflufenican), and a third one acting on cell division (metazachlor). In contrast, mixtures I and II were dominated by PS II inhibitors that bind to the exchangeable quinone in the reaction center, thereby blocking electron transfer (Rutherford and Krieger-Liszkay 2001). Hence, we expected a stronger inhibition of photosynthesis of these mixtures based on their mode of toxic action. Such a lack of pronounced effects may be partly due to the fact that we used mature, dense biofilms that limit the diffusion of contaminants, as demonstrated by Ivorra et al. (2002) for metal toxicity.

The unexpected slight increase in photosynthetic efficiency observed after 48 h of exposure to pesticide concentrations ≥MC10 (Figure 2) could indicate hormesis, with biofilm compensating for the toxicant-induced disturbance. This phenomenon of an increase in chlorophyll a in response to stress is also known as the "greening effect" in algae, a mechanism of shade adaptation (Sabater et al. 2016). Our findings concur with those of Tlili et al. (2008) and Feckler et al. (2018), who also showed increased chlorophyll a fluorescence in river biofilms exposed to diuron and diflufenican, respectively.

Almost all of the biofilms significantly and rapidly increased their photosynthetic efficiency after the exposure phase and the transfer to pesticide-free water. The fast recovery of the photosynthetic efficiency suggests that the tested pesticide mixtures caused a down-regulation of PS II rather than damage to the photosynthetic apparatus (Baker 2008). Despite this reversible effect of pesticides on photosynthesis, the replacement of sensitive species can still occur over longer timescales (Gustavson et al. 2003). In the present study, some green algae (e.g., *Aphanochaete*, *Closterium*) and cyanobacteria (*Lyngbya*, *Pseudoanabaena*) were only found in control biofilms, which suggests that they might be sensitive to pesticides. However, no overall significant differences in algal groups (as shown by diagnostic pigments) between treatments and controls were detected. Nonetheless, biofilms containing the highest percentage of diatom accessory pigments (fucoxanthin) were associated with control treatments, whereas the percentage of cyanobacteria markers (zeaxanthin and echinenone) was generally higher in the pesticide mixtures than in the controls, especially in the recovery phase.

The lack of significant effects on photosynthetic efficiency in biofilms exposed to environmental levels of pesticides suggests that the investigated compounds might not pose a risk for this biofilm function in the field. Indeed, our lowest tested concentrations are consequently below the predicted environmental concentrations obtained from step 4 in the European Food Safety Authority's tiered approach for pesticide risk assessment (Supplemental Data, Figure S2). However, it is important to keep in mind that our choice of mixture concentrations is based on weekly average measurements from our national monitoring program, while high pesticide peaks can occur for much shorter time periods (Holvoet et al. 2007), resulting in higher short-term exposure. In the present study, we observed significant effects on photosynthetic efficiency after 24-h exposure to MC100. In addition, pesticide contamination of surface waters in Sweden is lower than in other countries in Europe (Kattwinkel et al. 2011; Bighiu et al. 2020), where, for instance, diflufenican concentrations similar to those found in our treatment MC5 (Carabias Martínez et al. 2000), terbuthylazine corresponding to our MC10 level, and metribuzin even up to our MC100 level occur in southern European rivers (Konstantinou et al. 2006). It is, however, challenging to extrapolate our results to field conditions because of the

multitude of additional stressors encountered in the field, such as nutrient limitation, turbidity, shading, and grazing. Nonetheless, the strength of our controlled laboratory study is the use of natural, multispecies, rather than monospecific, biofilms, which carry more environmental relevance and allow for the purification of pesticide effects on photosynthesis. The diversity in algal species ensured a functional redundancy of the biofilms, as reflected in the rapid recovery of photosynthetic efficiency after the pesticide pulse. However, in the field, biofilms are usually exposed to repeated pulses of pesticides, leading to adaptation in the long term (i.e., pollution-induced community tolerance [Blanck et al. 1988]). In addition, the continuous supply of nutrients in surface waters will likely aid biofilm functional recovery. We observed a strong decline in nutrient concentrations in the test media during the first 8 d of the experiment, which did not reflect nutrient limitation because the algal biovolume increased up to 3-fold during this period (Supplemental Data, Figure S3). Hence, nutrients were incorporated in the biofilms, as confirmed by our analyses (e.g., up to 10.96 $\mu g\ mg^{-1}$ total P).

Because biofilms have a high potential for pesticide bioaccumulation (Lundqvist et al. 2012; Rooney et al. 2020), they likely are hot spots for pesticide exposure and effects. Our documented effects on biofilm function were almost entirely reversible within a time frame of 12 d, illustrating that algal biofilms adapt rapidly to changing conditions. However, the lack of effects on biofilm structure shows that taxonomic composition, as measured through diagnostic pigments, responds more slowly or not at all to the short-term herbicide exposure. Although herbicides have been pointed out as the most problematic group of pesticides in Swedish streams (Bundschuh et al. 2014; Gustavsson et al. 2017), their effects may be short-term because they primarily act on the functional responses of benthic algae. We conclude that the investigated pesticides may pose a risk for biofilms, especially in streams with larger pesticide concentrations than our tested level MC50.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4722.

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Data Availability Statement—All data can be made available on request to the corresponding author (maria.bighiu@slu.se).

Author Contribution Statement—W. Goedkoop: study design with input from S. Gottschalk, manuscript revision; S. Gottschalk: laboratory experiment, manuscript revision; Å. Arrhenius: pigment analysis, manuscript revision; M. Bighiu: data analysis, writing–together with W. Goedkoop, manuscript revision.

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