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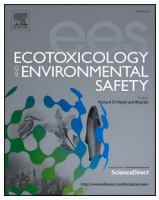

# Pesticide mixture toxicity to algae in agricultural streams – Field observations and laboratory studies with *in situ* samples and reconstituted water

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ABSTRACT

Long-term pesticide water concentrations were investigated in four agricultural streams and their mixture toxicity on algae was assessed, based on realistic (i.e. observed) concentrations in laboratory tests using (i) natural weekly water samples and (ii) reconstituted pesticide-spiked water samples representing mixtures with predicted high mixture. This approach both covered the full complexity of natural water samples and the controlled approach of reconstituted water samples. Long-term monitoring data (time-integrated, weekly samples) revealed more than 11 pesticides (range 11.0 ± 0.25–24.0 ± 0.44) in 75% or more of the almost 1600 samples collected between 2002 and 2018. $\sum TU_{algae}$ exceeded 0.1 for 29 observations (or 1.8%). Despite the multitude of pesticides in a sample, $\sum TU_{algae}$ was frequently set by one or a few dominating pesticides that contribute to more than 90% of the mixture's toxicity. Algal growth inhibition tests with *in situ* stream water showed a high frequency of inhibition, despite the low $\sum TU$ for most of these samples (range 0.000014–0.3858). These "false positive" results were attributed to confounding effects of turbidity, the complexation of nutrients, and toxic effects of metals and/or other unknown contaminants. Algal inhibition tests with spiked reconstituted water showed significant inhibitory effects in the range of 1–10x the $\sum TU_{algae}$ observed in worst-case field samples. Although these tests disregard the chemical complexity of natural water, they show that inhibitory effects of pesticides on algae may occur at the $\sum TU_{algae}$ observed in monitoring. Furthermore, considering that the $\sum TU_{algae}$ of stream water are based on weekly average concentrations and likely underestimate short-term peak concentrations of pesticides, these results strongly suggest that inhibitory effects on algae may occur in the agricultural streams of southern Sweden. We conjecture, however, that the rapid recovery of algae contributes to ameliorate these short-term effects and that pesticide contamination should be seen as one of many stressors in the streams that drain agricultural landscapes.

## 1. Introduction

Pesticides enter aquatic ecosystems through runoff, tile-drainage or spray-drift (Liess et al., 1999; Brown and van Beinum, 2009). Moreover, single occasions (in the range of hours–days) with peak water concentrations of pesticides may have long-lasting effects on benthic invertebrates (Liess and Schulz, 1999; Heckman and Friberg, 2005), primary producers (e.g. Nyström et al., 1999; DeLorenzo et al., 1999), as well as on heterotrophic microbes (e.g. DeLorenzo et al., 2001; Widenfalk et al., 2004,) and the decomposition processes they perform (Zubrod et al., 2011; Rasmussen et al., 2012; Gardeström et al., 2015). Hence, pesticides interfere with the functioning of aquatic ecosystems (Schäfer et al., 2012) and will probably do more so under warmer and wetter climate scenarios (Kattwinkel et al., 2011). These documented effects of pesticides on non-target aquatic microbes, flora, and fauna are not surprising, as these compounds are designed to affect physiological mechanisms that are general across habitats.

Although contamination of surface waters with multiple chemical pesticides is well documented by ongoing monitoring (e.g. Kreuger, 1998; Gilliom, 2007; Rasmussen et al., 2011; Ansara-Ross et al., 2012), little is known about their concerted effects on non-target aquatic organisms and communities (e.g. Kortenkamp et al., 2009; Bighiu et al., 2020a). Despite the widespread awareness that the occurrence of multiple pesticides in water samples is the rule, ecological risk assessment

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and maximum residue limits, however, are solely based on results from single-compound standardized toxicity tests and the application of safety factors. The current approach in ecological risk assessment therefore disregards combined effects of multiple pesticides and likely underestimates true toxicity (Junghans et al., 2003; Lydy et al., 2004; Gustavsson et al., 2017). Assessment of pesticide combination effects is usually based on models of concentration addition (CA, assuming a similar mode of action for the mixture components) or independent action (IA, assuming a dissimilar mode of action). The predictive power of these models to estimate mixture toxicity has been documented in several studies (Faust et al., 2001; Backhaus et al., 2004; Tang and Escher, 2014).

In order to protect Europe's surface waters from chemical pollution, the European commission has agreed on science-based environmental quality standards (EQS) for priority substances, of which twenty are pesticides (EU, 2013). EQS sets the highest estimated surface water concentration of a single compound at which no adverse ecosystem effects are expected. Priority substances should not exceed their EQS in order to achieve good chemical status according to the European Water Framework Directive (EU, 2000). In addition to this, national water quality standards may supplement directive 2013/39/EU (EU, 2013) for the evaluation of national environmental goals (e.g. KEMI, 2008). In Sweden, pesticide monitoring has been operative since 2002 in four agricultural catchments generating consistent, long-term results on pesticide concentrations in surface waters (Boye et al., 2019). Monitoring data demonstrate that EQS are exceeded regularly for a number of pesticides (Nanos et al., 2012) and that summed toxic units (∑TU) regularly exceed the uniform principles of the European Union (EU, 2011), especially so for algae (Bundschuh et al., 2014). Gustavsson et al. (2017) also identified algae as the most exposed organism group in Swedish agricultural streams, but also concluded that the high detection limits for insecticides inflated appropriate risk assessment, resulting in a systematic underestimation of their toxicity.

In this study, we present long-term data of pesticide water concentrations and summed toxic units (∑TU) for pesticides for four streams in model catchments predominated by agricultural land use. Furthermore, the pesticides' mixture toxicity on algae, based on realistic (i.e. observed in long-term monitoring) concentrations was assessed in a two-step procedure. Firstly, the inhibition of algal growth in natural water samples was studied using slightly modified, standardized laboratory procedures and the results were related to observations of the mixture toxicity of these water samples. Secondly, mixtures with observed high ∑TU from long-term monitoring data of Swedish streams were selected and the effects of these mixtures on algal growth were tested in controlled experiments with pesticide-spiked, reconstituted water. This approach thus covered the full complexity of natural water samples and a controlled design with reconstituted water samples. We hypothesized that algal growth would be inhibited by the concentrations of pesticides found in the investigated streams and that observed mixture effects would exceed those predicted by models of concentration addition.

## 2. Materials and methods

Long-term (2002–2018) data from the Swedish national monitoring program for pesticides were compiled for four agricultural streams in southern Sweden, referred to as O18, E21, N34 and M42, with catchments (size 8–16 $km^2$) predominated by clay soils. A detailed description of the sites' locations, characteristics, long-term pesticide use, and the design of the pesticide monitoring program (including analytical methods used) is given by Boye et al. (2019). These streams have between 85% and 93% agricultural land use in their catchments, are all well buffered (alkalinity $>$ 0.883 mmol/L for N34 and $>$ 4 mmol/L for the other streams), have a circumneutral or slightly alkaline pH (range 7.1–8.0), and are nutrient-rich (mean annual total-P range 0.048–0.344 mg/L, mean annual total-N range 3.66–9.40 mg/L). Inorganic water chemistry data have been extracted from a data base for the 21 agricultural catchments in Sweden that are monitored for water chemistry and nutrient run-off (Kyllmar et al., 2014), of which the four streams in our study form a subset.

From all four streams water subsamples for pesticide analysis were collected every 90th minute using Teflon tubing and an automatic sampler (ISCO™, initially 3700FR, but 6712FR from 2008 in M42, from 2011 in O18, and from 2013 in E21 and N34) and immediately transferred to refrigerated containers (4 °C), one in high-density polyethylene (for glyphosate analysis) and one in glass (for analyses of other compounds). Composite water samples were collected on a weekly basis during May and October/November (with some samples missing during periods of severe drought) and analyzed in our accredited laboratory. Hence results of our pesticide analyses represent weekly average concentrations. In brief, composite water samples were transported to the laboratory by overnight freight in coolers and immediately extracted or stored in a freezer. Pesticide analysis was done using gas chromatography mass spectrometry (GC–MS and GC-MS/MS, Agilent Technologies Inc.) and liquid chromatography (tandem) mass spectrometry (LC–MS, Waters Corp., and LC–MS/MS, Agilent Technologies Inc.). A detailed description of the sample pre-treatment and analytical methods (OMK 49, 50, 51, 53, 57, 58, 59) used over the years is given by Boye et al. (2019). Jansson and Kreuger (2010) provide a detailed description of the combined liquid chromatography and tandem mass spectrometry method (OMK57/OMK58) that has been used for the majority of substances since 2009. The ambition of the long-term monitoring in these four catchments has been to analyze both all pesticides permitted for use in Sweden and those listed as priority substances by the EU in Annex II of Directive 2008/105/EC (EU, 2008), i.e. including those that have never been permitted for use in Sweden.

### 2.1. Algal growth inhibition with in situ water samples

During two consecutive years, 61 water samples collected for pesticide analysis during the growing season (i.e. from May through October/November, see above) were split into two, one of which was allocated to pesticide analysis while the other was frozen in glass flasks and later used for algal growth inhibition experiments. This approach provided us with a data set where pesticide concentrations could be combined with the results of algal growth inhibition tests.

Inhibition tests were performed in 15 test runs, each with a separate control, following the methods outlined in the standardized algal growth inhibition OECD-test (OECD, 2006). Before the start of each test, freeze-preserved stream water was slowly thawed to the experimental temperature (21–22 °C). Growth medium, prepared from stock solutions of nutrients (N and P) and trace elements according to the freshwater alga growth inhibition test (OECD, 2006), was used as control water. Stream water samples were supplemented with the same concentrations of nutrient and trace element stock solutions (OECD, 2006) as the controls to exclude effects on algal growth due to nutrient limitation. Test vessels consisted of 250 ml glass flasks and 3–5 replicates were run for both treatments and controls. At the start of each test run, nutrient stock solutions and an inoculum of the green alga *Pseudokirchneriella subcapitata* (in exponential growth phase) was added to each test vessel to achieve an initial density of approximately 40,000 cells/ml. After mixing, 100 ml aliquots of this algal suspension were transferred to each experimental unit. Test vessels were then randomly placed on a shaking table at gentle agitation in a climate room with a constant light of 110 μE $m^{-2}$ $s^{-1}$ and a temperature of 21–22 °C. Extra water-filled vessels were placed along the edges of the shaking table to avoid any edge effects and all vessels were gently agitated. pH was checked before and after every test run and ranged 7.5–9.0.

After 0 and 72 h, 5-ml subsamples were collected from each test vessel for quantification of algal cells. In the first four test runs, cells were counted using a light microscope (M75, Carl Zeiss Germany) at 100x magnification. For these test runs, algal samples were preserved with Lugol's solution prior to counting. In the next eleven test runs,

fluorescence was measured with a digital filter fluorometer (Turner® Quantech, Barnstead International) and cell numbers were back calculated from fluorescence using the previously established relationship for *Pseudokirchneriella subcapitata* in our laboratory:

$$y = 4855\ x + 25597 \quad (1)$$

where $x$ is the fluorescence and $y$ is the number of cells/ml. Algal growth rates and growth inhibition was calculated according to the OECD-guideline (OECD, 2006):

$$\mu_{i\text{-}j} = (\ln X_j - \ln X_i) / (t_j - t_i) \quad (2)$$

and

$$I_y = 100*(Y_c - Y_T) / Y_c \quad (3)$$

where $\mu_{i\text{-}j}$ is the average specific growth rate from time $i$ to $j$, $Xi$ is the biomass at time $i$ and $Xj$ is the biomass at time $j$. $I_y$ is the relative inhibition of the yield (as %), $Y_c$ is the mean value for yield in the control and $Y_T$ is the yield for the treatment replicate.

### 2.2. Calculation of predicted mixture toxicity

Identification of stream water samples with estimated high mixture toxicity was done using data on weekly average concentrations from long-term pesticide monitoring in the four streams (i.e. O18, E21, N34, M42). Pesticide mixture toxicity was calculated for each water sample as the summed toxic units (ΣTU), based on the acute effect concentration of each pesticide for aquatic primary producers:

$$\Sigma TU_{algae} = \Sigma(Ci/EC_{50}i) \quad (4)$$

where $TU_{algae}$ is the toxic unit for the pesticide $i$, $Ci$ is the concentration of the pesticide $i$ (i.e. the detected concentration or the LOD if only traces where found) and $EC_{50}$ is the concentration when 50% of the organisms exposed to pesticide $i$ were affected (OECD, 2006). $EC_{50}$-values were obtained from the established pesticide properties database (PPDB, http://sitem.herts.ac.uk/aeru/ppdb/). Effect concentrations were generally $EC_{50}$-values from 72-hours or 96-hours green algal toxicity tests, i.e. primarily data for *Pseudokirchneriella subcapitata* (previously *Selenastrum capricornutum*) and secondarily for *Scenedesmus subspicatus,* resulting in data for 45 and 20 pesticides, respectively. If no data for these species were available, as was the case for seven pesticides, data for any other green algal species was used. For four pesticides, i.e. benazolin, endosulfan-alpha, endosulfan-beta and flupyrsulfuronmethyl-sodium, $EC_{50}$-values for blue-green algae (cyanobacteria) were used, whereas for propiconazole $EC_{50}$-values for the diatom *Navicula seminuluma* were included in our calculations of toxic units. For chlorpyrifos, deltamethrin, flamprop and permethrin, the algal species was not specified in the reference (see Appendix A).

### 2.3. Algal growth inhibition tests with reconstituted spiked water

Among the long-term monitoring data, eight samples with a $\Sigma TU_{algae}$ exceeding 0.2 were identified and used for algal growth inhibition tests with reconstituted water (Table 1). Only pesticides that contributed with more than 0.1% of the total $\Sigma TU_{algae}$ were included in the test mixtures. The eight mixtures contained the following dominant pesticides: diflufenican (mixture 1), cyanazine (2, 5) and metribuzin (2, 3, 6, 8), metazachlor (4), and prochloraz (7) (Table 1). Diflufenican is a carboxamide herbicide that inhibits carotenoid biosynthesis, cyanazine and

**Table 1**
Concentrations of pesticide (Conc.) and their absolute and relative toxic units for algae (TU) for mixtures 1–8, identified as the most toxic mixtures (highest $\sum TU_{algae}$) in stream water samples (O18, E21, N34, M42). All pesticides are herbicides except those marked[1]), which is an insecticide, and those marked[2]), which are fungicides. Pesticides that contributed less than 0.1% of the total $\sum TU_{algae}$ were not included in the mixtures that we used in our tests.

| Mixture | Conc. (μg/L) | TU | TU (%) | Mixture | Conc. (μg/L) | TU | TU (%) |
|---|---|---|---|---|---|---|---|
| *Mix 1:* | | | | *Mix 5:* | | | |
| Diflufenican | 0.32 | 7.1E-1 | 98.2 | Cyanazine | 4.4 | 2.2E-1 | 90.6 |
| Isoproturon | 0.80 | 1.1E-2 | 1.5 | Metribuzin | 0.13 | 1.9E-2 | 7.6 |
| Metazachlor | 0.035[a] | 1.3E-3 | 0.2 | Iodosulfuronmethyl-sodium | 0.10 | 1.4E-3 | 0.6 |
| *Total* | *1.2* | *0.72* | | Metazachlor | 0.027[a] | 1.0E-3 | 0.4 |
| | | | | Tribenuron methyl | 0.061 | 7.6E-4 | 0.3 |
| *Mix 2:* | | | | Aclonifen | 0.017[a] | 5.9E-4 | 0.2 |
| Metribuzin | 0.84 | 1.2E-1 | 51.3 | Imidacloprid[1]) | 3.0 | 3.0E-4 | 0.1 |
| Cyanazine | 2.2 | 1.1E-1 | 47.0 | *Total* | *7.7* | *0.24* | |
| Metsulfuron-methyl | 0.050 | 1.1E-3 | 0.5 | | | | |
| Metazachlor | 0.028[a] | 1.0E-4 | 0.4 | *Mix 6:* | | | |
| Iodosulfuronmethyl-sodium | 0.045[a] | 6.4E-4 | 0.3 | Metribuzin | 4.0 | 5.7E-1 | 98.9 |
| Tribenuron methyl | 0.023[a] | 2.9E-4 | 0.1 | Fenpropimorph[2]) | 0.53 | 3.1E-3 | 0.6 |
| *Total* | *3.2* | *0.23* | | Thifensulfuron-methyl | 0.040 | 2.5E-3 | 0.4 |
| | | | | *Total* | *4.6* | *0.58* | |
| *Mix 3:* | | | | | | | |
| Metribuzin | 2.6 | 3.7E-1 | 99.2 | *Mix 7:* | | | |
| Cyanazine | 0.030[a] | 1.5E-3 | 0.4 | Prochloraz[2]) | 2.9 | 5.3E-1 | 93.9 |
| Metazachlor | 0.028[a] | 1.0E-3 | 0.3 | Picoxystrobin[2]) | 0.70 | 1.2E-2 | 2.2 |
| *Total* | *2.7* | *0.37* | | Diflufenican | 0.006[a] | 1.2E-2 | 2.2 |
| | | | | Metribuzin | 0.052 | 7.4E-3 | 1.3 |
| *Mix 4:* | | | | Terbuthylazine | 0.011[a] | 6.9E-4 | 0.1 |
| Metazachlor | 10 | 3.7E-1 | 96.0 | Aclonifen | 0.017[a] | 5.9E-4 | 0.1 |
| Metribuzin | 0.085 | 1.2E-2 | 3.2 | *Total* | *3.7* | *0.56* | |
| Cyanazine | 0.030[a] | 1.5E-3 | 0.4 | | | | |
| Isoproturon | 0.060 | 8.3E-4 | 0.2 | *Mix 8:* | | | |
| Metsulfuron-methyl | 0.020[a] | 4.4E-4 | 0.1 | Metribuzin | 2.0 | 2.9E-1 | 93.1 |
| *Total* | *10* | *0.38* | | Cyanazine | 0.27 | 1.4E-2 | 4.4 |
| | | | | Metazachlor | 0.060 | 2.2E-3 | 0.7 |
| | | | | Thifensulfuron-methyl | 0.025 | 1.6E-3 | 0.5 |
| | | | | Prosulfocarb | 0.21 | 1.3E-3 | 0.4 |
| | | | | Picoxystrobin[2]) | 0.050 | 8.9E-4 | 0.3 |
| | | | | Iodosulfuronmethyl-sodium | 0.035 | 5.0E-4 | 0.2 |
| | | | | *Total* | *2.7* | *0.31* | |

[a] indicates concentrations that were below the LOQ. The value given is the mean of the LOD and LOQ for the specific pesticide.

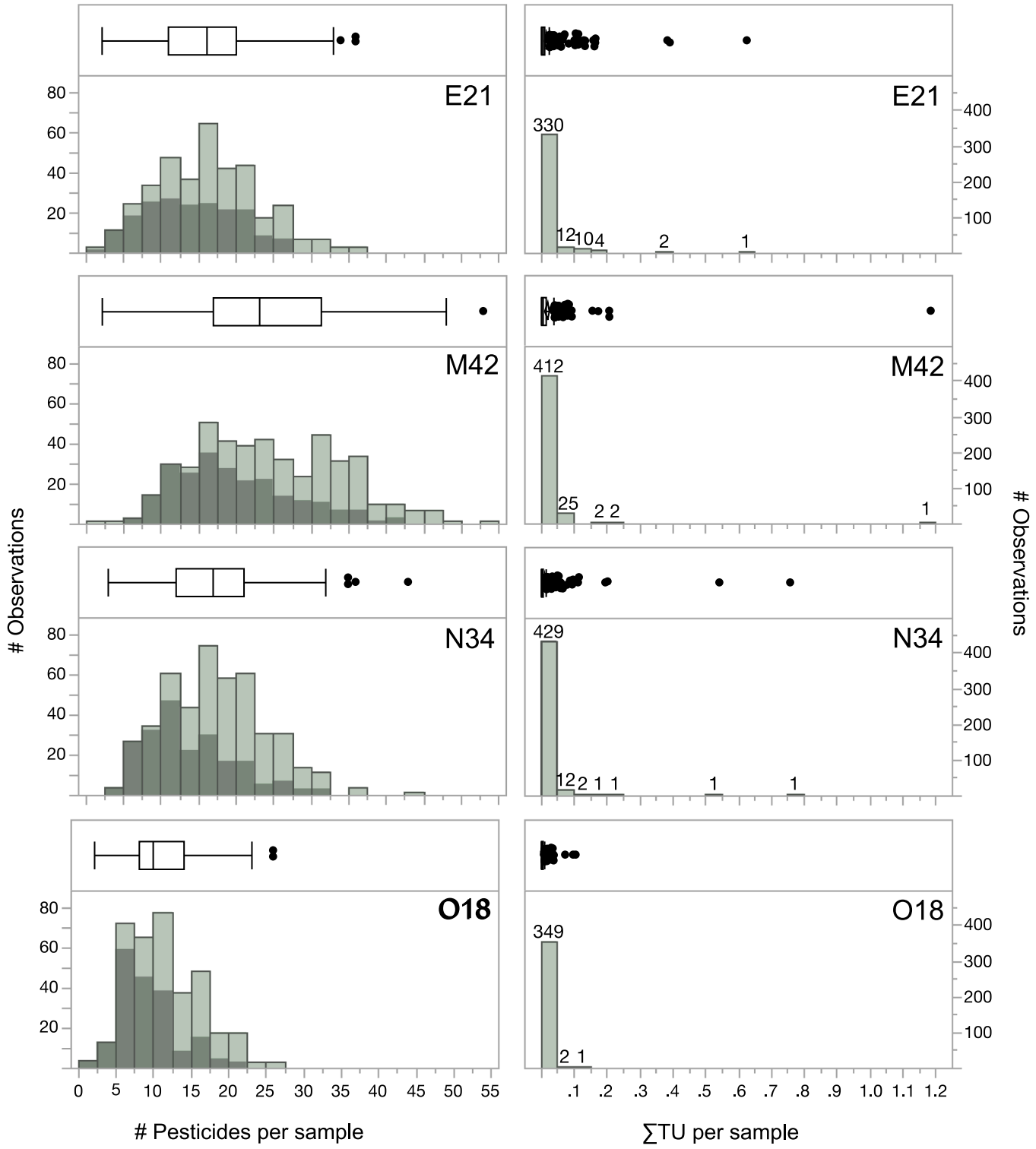


**Fig. 1.** Frequency plots for the number of pesticides per sample (left panels) and the $\sum TU_{algae}$ per sample (right panels) for water samples collected during 2002–2018 from four monitoring streams E21 (n = 358), M42 (n = 442), N34 (n = 446), O18 (n = 352) (see text). The shaded area in the left panels represent the samples from 2002 to 2008, while the non-shaded area represents samples from 2009 to 2018.

metribuzin are triazine herbicides that inhibit photosystem II, metazachlor is a chloroacetamide herbicide that inhibits the synthesis of long-chain fatty acids, while prochloraz is an imidazole fungicide that primarily inhibits ergosterol biosynthesis (Paranjape et al., 2015).

Pesticide stock solutions (in 100% acetone, pesticide grade) were prepared to obtain the appropriate pesticide concentrations and a final acetone concentration of 0.01% in each treatment. Algal growth medium was spiked with the selected pesticide mixtures at concentrations of 0 (controls), 0.5, 1, 10, 50 and 100 times the concentration of each pesticide to obtain the $\Sigma TU_{algae}$ of the different mixtures. Controls received only acetone (no pesticides added), while acetone controls (no acetone added) were run to test for possible effects of acetone on algal growth. Treatments and controls were run in four replicates and followed the outline of the OECD-guideline (OECD, 2006). At the start of the tests, an algal inoculum representing an initial density of approximately 40,000 cells/ml (OECD, 2006) and 200 ml of spiked growth medium was added to each of the test vessels. The vessels were then randomly placed on a shaking table in a climate room using the same set-up and experimental conditions as described for tests with *in situ* water samples above. After 0 and 72 h, 8-ml subsamples were collected from each test vessel and algal cells were counted. Algal concentrations were verified by cell counts using light microscopy (as above).

### 2.4. Data analysis

$EC_{50}$-values were calculated for each mixture using the established response curves. Predictions of effect concentrations for the mixtures by concentration addition (CA) were calculated according to Faust et al. (2001):

$$ECx_{mix} = \sum_{i=1}^{n} \left( \frac{P_i}{ECx_i} \right)^{-1} \quad (5)$$

where $ECx_{mix}$ is the predicted toxic effect of the mixture, $p_i$ is the fraction of component $i$ in the mixture and $ECx_i$ is the individual effect concentrations when applied singly. As concentration addition is the more conservative model, several studies have recommended this method both for scenarios with similar and dissimilar modes of action in order to achieve a worst-case scenario (Belden et al., 2007a, 2007b; Cedergreen et al., 2008).

Effects on algal growth inhibition in tests with *in situ* stream water samples were evaluated with two-sided *t*-tests. Effects on algal growth inhibition tests with reconstituted spiked water were tested with one-way ANOVA, while Tukey HSD-tests were used for pairwise comparisons. If needed, the data were log-transformed to meet the criteria of normality and homogeneity of variance. All analyses were performed in JMP10® (SAS Institute Inc.).

## 3. Results

### 3.1. Pesticide concentrations and $\sum TU_{algae}$ in stream water

More than 11 pesticides were detected in 75% or more of the 1598 water samples, with 90-percentiles for the different sites ranging 8–30 pesticides (Fig. 1, left panels). The average number of pesticides in a single sample ranged from 11.0 ± 0.25 in O18 (SE used throughout) to 24.0 ± 0.44 in M42, while the maximum was 53 pesticides in a single sample. The number of analyzed pesticides gradually increased over time, i.e. from 76 to 84 during 2002–2008 and from 110 to 131 during 2009–2018, due to improved analytical methods and more targeted compounds. This also contributed to a higher number of compounds detected in more recent time. These results stress the complexity of exposure by pesticides in agricultural streams. $\sum TU_{algae}$ during 2002–2018 exceeded 0.1 in 29 water samples, while 420 had a $\Sigma TU_{algae}$ exceeding 0.01 (Fig. 1, right panels). Site O18 generally showed slightly lower $\sum TU_{algae}$ than the other streams. The herbicides metazachlor, metribuzin, diflufenikan, cyanazine, and prochloraz were most frequently contributing to the observed high $\sum TU_{algae}$. Eight samples with $\sum TU_{algae}$ ranging 0.23–0.72, i.e. the worst-case mixtures, were selected from long-term monitoring data for algal growth inhibition tests with reconstituted spiked water (see below).

The 61 time-integrated weekly *in situ* stream water samples from long-term monitoring that were used for algal growth inhibition tests contained detectable concentrations of between 6 and 24 pesticides (on average 13 ± 2). The most common pesticides in these samples were the herbicides bentazon (n = 60, $conc_{max}$ 0.41 µg/L), glyphosate (n = 53, $conc_{max}$ 1.6 µg/L), isoproturon (n = 48, $conc_{max}$ 2 µg/L), metazachlor (n = 45, $conc_{max}$ 10 µg/L) and fluroxypyr (n = 44, $conc_{max}$ 2 µg/L) (Appendix A). Samples with the highest $\Sigma TU_{algae}$ were predominated by metribuzin or metazachlor that contributed most to the highest predicted toxicity (96–99%). The latter was not surprising, as metribuzin and metazachlor are common herbicides with a relatively low $EC_{50}$ (20 and 16 µg/L, respectively). Appendix A provides detailed information on the level of detection, level of quantification, maximum observed concentrations, frequency of occurrence, as well as Water Quality Standards and $EC_{50algae}$ for all pesticides detected in water samples collected during 2002–2018.

### 3.2. Algal growth inhibition tests

Algal growth inhibition tests performed with *in situ* water samples showed significant inhibitions in 30 of the 61 analyzed samples compared to controls (Fig. 2). Samples collected from M42 showed by far the highest frequency of significant inhibitions, i.e. on 13 of 16 dates. Each of the streams also showed a single occasion when algal growth in stream water was stimulated compared to controls. In general the $\sum TU_{algae}$ for these *in situ* samples were quite low: 41 samples had a $\sum TU_{algae}$ less than 0.02, whereas only 5 samples had $\sum TU_{algae}$ that exceeded 0.05. For example, five of the samples for site M42 showed a significant inhibition of algal growth, but the range in $\sum TU_{algae}$ for these samples was 0.0027–0.0365. There was also no difference in $\sum TU_{algae}$ between samples that showed a significant inhibition and those that showed a significant stimulation (*t*-test, $p > 0.05$). These results suggest that other factors than the concerted action of pesticides was driving the

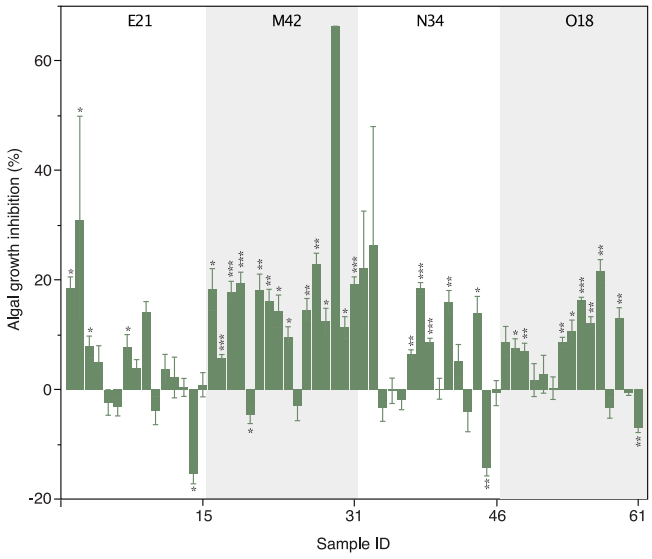


**Fig. 2.** Growth inhibition (mean ± 1 SE) of *Pseudokirchneriella subcapitata* relative to controls for 61 *in situ* water samples collected during a two-year period from the four streams. Asterisks adjacent to the bars indicate the significance levels (*t*-tests), ***$p < 0.001$, **$0.01 < p < 0.001$, and *$0.01 < p < 0.05$. Note that negative inhibition indicates a stimulation of algal growth.

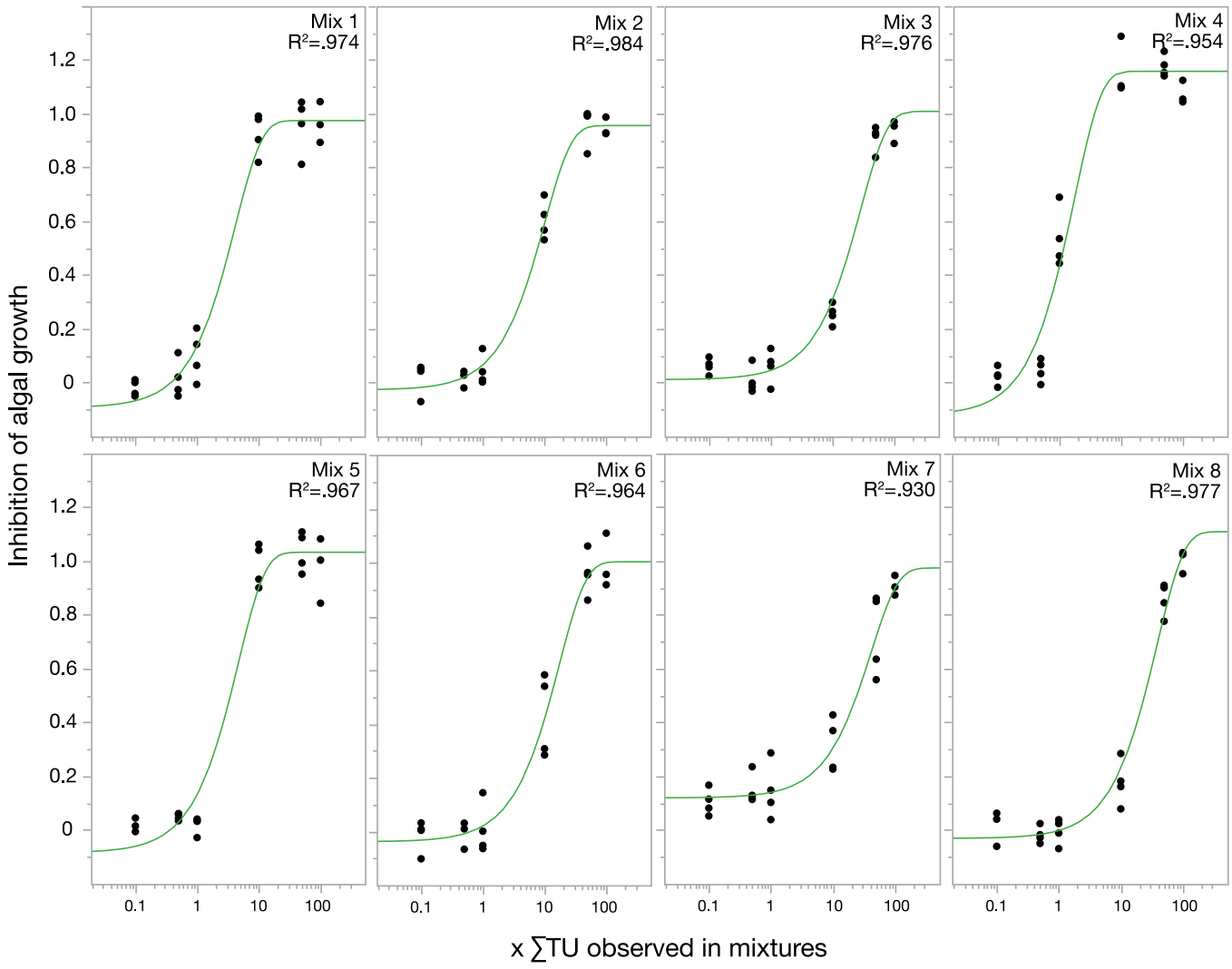


**Fig. 3.** Growth inhibition (GI) response curves of *Pseudokirchneriella subcapitata* in reconstituted water samples for selected pesticide mixtures (Mix1–8) at $\sum TU_{algae}$ of 0.1, 0.5, 1.0, 10, 50 and 100 times the $\sum TU_{algae}$ of selected pesticides (see Table 1 for details). $GI_{mix1} = 0.9772–1.0671*e^{(-0.2434* x\sum TU)}$, $GI_{mix2} = 0.9589 – 0.9832* e^{(-1005* x\sum TU)}$, $GI_{mix3} = 1.0114 – 0.9973* e^{(-0.0360 * x\sum TU)}$, $GI_{mix4} = 1.1605 – 1.2782* e^{(-0.5758* x\sum TU)}$, $GI_{mix5} = 1.0357 – 1.1170* e^{(-0.2196 * x\sum TU)}$, $GI_{mix6} = 1.0051 – 1.0408* e^{(-0.0592 * x\sum TU)}$, $GI_{mix7} = 0.9773 – 0.8549* e^{(-0.0248* x\sum TU)}$, $GI_{mix8} = 1.1122–1.1404* e^{(-0.0266* x\sum TU)}$.

**Table 2**
$EC_{50}$ values of the dominant pesticide(s) according to the literature ($EC50_{dom}$), calculated for the mixtures in our test ($EC50_{mix}$), estimated according to the concentration addition model ($EC50_{CA}$), and their ratios. The abbreviations *S. subspicatus* and *P. subspicata* refer to the closely related green algae *Scenedesmus subspicatus* and *Pseudokirchneriella subcapitata*, respectively.

| Mix | $Pesticide_{dom}$ | Algal species, end point, duration | $EC50_{dom}$ (μg/L) | $EC50_{mix}$ (μg/L) | $EC50_{CA}$ (μg/L) | $EC50_{Mix}$/ $EC50_{CA}$ | $EC50_{mix}$/ $EC50_{dom}$ |
|---|---|---|---|---|---|---|---|
| 1 | Diflufenican | *S. subspicatus*, growth 72 h | 0.45[b] | 3.45 | 1.60 | 2.2 | 7.7 |
| 2 | Cyanazine | *P. subspicata*, biomass 96 h | 20[b] | 23.5 | 13.8 | 1.7 | 1.2 |
| | Metribuzin | *S. subspicatus*, biomass 96 h | 7[b] | | | | 3.3 |
| 3 | Metribuzin | *S. subspicatus*, biomass 96 h | 7[b] | 30.6 | 7.11 | 4.3 | 4.4 |
| 4 | Metazachlor | *P. subspicata*, growth 72 h | 27[b] | 9.85 | 26.5 | 0.37 | 0.36 |
| 5 | Cyanazine | *P. subspicata*, biomass 96 h | 20[b] | 18.4 | 31.9 | 0.58 | 0.92 |
| 6 | Metribuzin | *S. subspicatus*, biomass 96 h | 7[b] | 53.1 | 7.92 | 6.7 | 7.6 |
| 7 | Prochloraz | *S. subspicatus*, biomass 72 h | 5.5[a] | 50.5 | 2.95 | 17 | 9.2 |
| 8 | Metribuzin | *S. subspicatus*, biomass 96 h | 7[b] | 56.0 | 8.71 | 6.4 | 8.0 |

a) Andersson and Kreuger (2011)
b) KEMI (2008)

algal response in these tests.

Controlled algal growth inhibition tests with reconstituted spiked water were run to specifically link the algal growth response to the concerted effects of pesticides in eight mixture samples that were identified as high-risk scenarios (Table 1). These samples each contained 10–25 pesticides, although one or a few compounds commonly explained 99% of the mixture's toxicity. The eight selected mixtures were reconstituted with the predominant 38 pesticides, with a total concentration of 1.16–10.20 μg/L and a $\Sigma TU_{algae}$ of 0.23–0.72. In seven of these eight mixtures, a single pesticide was responsible for more than 90% of the $\Sigma TU_{algae}$, i.e. the herbicides diflufenican (in mixture 1), metribuzin (3, 6 and 8), metazachlor in (4) and cyanazine (5), and the fungicide prochloraz (mixture 7). In mixture 2, instead two herbicides (cyanazine and metribuzin) stood for around 50% each of the $\Sigma TU_{algae}$. The other pesticides in the mixtures were mainly other herbicides that together contributed less than 8% to $\Sigma TU_{algae}$.

Algal growth inhibition tests with these reconstituted mixtures showed significant inhibition in the range of 1–10x the $\sum TU_{algae}$ observed in monitoring data (Fig. 3). Algal growth in mixture 4 was inhibited by 54% already at 1x the observed $\sum TU_{algae}$, while the other mixtures all showed significant growth inhibition at ten times the observed $\sum TU_{algae}$ in stream samples. No differences were found between controls and solvent controls ($p > 0.05$), showing that the final acetone concentration of 0.01% did not contribute to algal growth inhibition in any of the experiments. For all mixtures except 2 and 5, calculated $EC_{50}$-values were between 3 and almost 10-fold higher than literature values for the dominant pesticide (Table 2). The $EC_{50}$-value for mixture 2 was instead close to that set by the dominant pesticide, while that for mixture 5 was only 36% of the literature $EC_{50}$-value. The calculated $EC_{50}$-values for most of the mixtures were 2–17 times higher than those estimated by the model of concentration addition. However, mixtures 4 and 5 where these instead were 2–3 times lower.

## 4. Discussion

Our long-term monitoring data show that multiple pesticides are commonly found in water samples from agricultural streams, but that $\sum TU_{algae}$ exceeds 0.1, i.e. the uniform principles for algae of the European Union (EU, 2011), in only 29 of 1600 samples (or less than 2%), which the vast majority occurring in E21 (Fig. 1). These water samples represent weekly average, time-proportional samples, a design that does not capture the peak concentrations may occur during shorter time intervals, i.e. hours to days (Liess et al., 1999; Xing et al., 2013). For example, a comparison between flow-proportional samples and in time-integrated samples for one of the streams showed that concentrations of pesticides (e.g. MCPA, metamitron, quinmerac, metazachlor, isoproturon and glyphosate) can be $>10$ times higher in flow-proportional samples, in which also more compounds were detected (Boye et al., 2019). Hence, our toxicity estimates ($\sum TU_{algae}$) based on weekly average concentrations should likely be seen as underestimates of true exposure and toxicity. Our long-term data also showed that the number of pesticides in a single water sample increased gradually over time, but despite the multitude of pesticides in a sample $\sum TU_{algae}$ was frequently set by one or a few compounds that contributed to more than 90% of the mixture's toxicity. Also in the eight samples with the highest $\sum TU_{algae}$ 95% of the mixture toxicity in our high-risk water samples was made up by one or a few pesticides (Table 1).

Approaches with natural water samples and reconstituted mixtures using artificial water provide sound assessments of toxicological effects of environmentally relevant mixtures (Tang and Escher, 2014), but also have their inherent strengths and limitations. Our algal inhibition tests with *in situ* stream water were run as an add-on to ongoing chemical monitoring to study if effects occurred in stream water at ambient field concentrations. These tests showed a high frequency of algal growth inhibition (Fig. 2), despite the additions of nutrients to avoid limitation. However, the $\sum TU$ for these samples were generally low (range 0.000014–0.3858, 10- and 90-percentiles 0.0013–0.0608) and at the low end of the range where effects can be expected. Hence, there was no relationship between $\sum TU_{algae}$ and the algal growth response. Likely, observed inhibitions of algal growth for *in situ* water samples depended on other factors than pesticide concentrations, such as complexation of nutrients and/or effects of dissolved metals or other contaminants (see also Tang and Escher, 2014). We can also not exclude that additional pesticides not captured by our analyses contributed to the observed algal growth inhibition in the natural samples. The latter is unlikely, however, as the analyses included all pesticides used in the catchment and reported by farmers. Lastly, other micropollutants in the water samples may have confounded our results. Although our tests with *in situ* water samples provide insight in the algal response at realistic, field-level conditions, we cannot correct for confounding factors that affected test results.

Our algal inhibition tests with spiked reconstituted water were performed with a standardized medium and controlled pesticide additions, thus creating a causal link between added pesticides and observed effects. These tests showed significant inhibitory effects (ANOVA, $p < 0.05$) in the range of 1–10x the $\sum TU_{algae}$ (Tukey HSD, $p < 0.05$) observed in all the 8 worst-case field samples extracted from the monitoring results. In mixture 4 significant growth inhibition already occurred at $\sum TU_{algae}$ that were equal to those found in the stream water sample. The toxicity in this mixture was mainly due to high concentrations of the herbicide metazachlor, which has an $EC_{50}$ of 16.2 μg/L in 72-h inhibition tests with *Pseudokirchneriella* (PPDB). Although these tests disregard the chemical complexity of natural water (Junghans et al., 2003; Lydy et al., 2004), they show that inhibitory effects of pesticides on algae may occur at the observed *in situ* $\sum TU_{algae}$. Considering that our monitoring data and $\sum TU_{algae}$ represent weekly averages and underestimate peak concentrations of pesticides by more than 10 times (see above), these results strongly suggest that inhibitory effects on aquatic algae may occur in Swedish streams in agricultural landscapes. Brock et al. (2000) suggested, based on a meta-analyses of results from freshwater mesocosm studies, that effects of herbicide contamination can occur when $\sum TU_{algae}$ exceeds 0.1. In accordance with this, our long-term data show that toxicity on algae may occur in at least 1.8% of the water samples. If our weekly average concentrations underestimate short-term exposures by 10–100 times (see above), then negative effects on algae should be more common, as 420 of our 1600 water samples (or 26%) had a $\sum TU_{algae}$ exceeding 0.01.

Comparisons of the calculated $EC_{50}$-values for pesticide mixtures were higher than those estimated by the CA-model for six of the eight mixtures (Table 2), suggesting that the model in most cases provided conservative estimates of mixture toxicity (Tang and Escher, 2014). However, CA underestimated toxicity in mixtures 4 and 5 by 2.6 and 1.7 times, respectively. Theoretically, the CA model predicts the toxicity of chemicals with a similar mode of action, while independent action (IA) is meant to predict toxicity of dissimilarly acting chemicals. However, mixtures acting strictly similar or dissimilar should be rare in nature. Indeed, our eight tested mixtures all include pesticides with dissimilar modes of action, and all except mixture 1 and 6 also include similarly acting pesticides. As CA predicts higher toxicity than IA, and as mixture toxicities higher than predicted by CA are rare, CA is suggested to be a better, more conservative approach in risk assessment, regardless mechanisms of action of the mixture components (Junghans et al., 2006; Backhaus and Faust, 2012; Tang and Escher, 2014). Our results support this conclusion.

### 4.1. Ecosystem implications and conclusion

Toxic effects of pesticides on algae, i.e. both phytoplankton or the algae that inhabit the biofilms on submersed surfaces, may affect primary production and indirectly higher trophic levels and important ecosystem processes (Lamberti and Steinman, 1997; Fleeger et al., 2003). However, long-term exposure of pesticides can induce tolerance

in algae (Molander and Blanck, 1992; Berard et al., 2002), which can ameliorate effects on primary production, but can itself be considered an ecological effect. However, considering the short generation times and subsequent rapid recovery of algal communities in their nutrient-rich habitats (Debenest et al., 2009; Weber et al., 2012; Brain et al., 2012), these effects are likely short in their duration. For example, Bighiu et al. (2020b) showed that recovery of algal photosynthesis from herbicide exposure was rapid and complete within a time frame of 12 days, suggesting rapid recovery of their ecological function. The inherent capacity of algal/microbial biofilms to degrade and mineralize pesticides (e.g. Bighui and Goedkoop, 2021) further contributes to the resilience of these assemblages.

Our long-term monitoring results show that multiple pesticides commonly occur in water samples, but that their mixture toxicity (∑TU) is frequently low and large driven by only a few compounds. The fact that we detected negative effects on algal growth in standardized tests in the range of 1–10x the $\sum TU_{algae}$ in pesticide mixtures representing worst-case scenarios suggest that such negative effects may occur. Our finding of a high frequency of algal growth inhibition *in situ* water samples, however, shows that other factors than pesticide contamination also contribute to algal toxicity under field conditions. We speculate that high-turbidity (and their effect on under-water light climate), as well as complexation of nutrients and toxicity of metals and/or other unknown compounds contribute with equal or larger shares than pesticide contamination to the multiple stress scenarios that algal communities in agricultural streams experience. Hence, the joint effects of agricultural land use in general, rather than specific and hard-to-unravel single stressors should likely form the basis for ecological assessments.

## CRediT authorship contribution statement

Jenny Rydh Stenström has carried out the research (i.e. laboratory bioassays, data compilation, statistics) and produced a first draft of the manuscript. Jenny Kreuger is responsible for the pesticide monitoring program and the data obtained. She has contributed with data and provided valuable comments to earlier versions of the ms. Willem Goedkoop is the corresponding author for the paper and the PI of the FORMAS-project that provided the funding. He has reworked the original draft of the manuscript and fine-tuned the graphical presentations.

## Declaration of Competing Interest

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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## Appendix A

See Table A1.

**Table A1**
Level of detection (LOD, range as μg/L), level of quantification (LOQ, range as μg/L), maximum observed concentrations (as μg/L), number of observations (n), Water Quality Standards (WQS) and $EC_{50}$ values (as μg/L) of analyzed pesticides detected in 1598 weekly water samples collected from four monitoring streams in southern Sweden during 2002–2018. Pesticides lacking a LOQ were not detected above that limit during the investigation period

| Pesticide | Group* | LOD | LOQ | $Conc_{max}$ | n | WQS | $EC_{50algae**}$ |
|---|---|---|---|---|---|---|---|
| Amidosulfuron | H | 0.001–0.05 | 0.002–0.06 | 2.3 | 223 | 0.2[b] | 47000 |
| AMPA | M | 0.005–5 | 0–2 | 5.5 | 651 | 500[b] | 200000 |
| Atrazine | H | 0.001–0.02 | 0.002–0.03 | 0.097 | 511 | 0.6[c] | 59 |
| Azoxystrobin | F | 0.001–0.06 | 0.002–0.2 | 1.0 | 625 | 0.9[b] | 360 |
| BAM | M | 0.002–0.03 | 0.01–0.05 | 0.47 | 483 | 400[a] | 10000 |
| Benazolin | H | 0.003–0.025 | 0.01–0.05 | 0.034 | 4 | 30[a] | 16000 |
| Bentazon | H | 0.003–0.02 | 0.01–0.03 | 25 | 1452 | 30[b] | 10100 |
| Beta-cyfluthrin | I | 0.0002–0.03 | 0.006–0.05 | 0.011 | 1 | 0.0001[b] | > 10 |
| Bitertanol | F | 0.003–0.1 | 0.04–0.1 | 1.6 | 14 | 0.3[b] | 1380 |
| Carbofuran | I | 0.001–0.1 | 0.002–0.05 | 0.03 | 2 | 0.3[b] | 6500 |
| Carfentrazonic acid | M | 0.002–0.08 | 0.01–0.25 | 0.86 | 32 | 0.8[b] | 787 |
| Chloridazon | H | 0.0015–0.1 | 0.002–0.1 | 4.4 | 510 | 10[b] | 3000 |
| Clopyralid | H | 0.003–0.2 | 0.01–0.2 | 2.2 | 585 | 50[b] | 35000 |
| Cyanazine | H | 0.0015–0.2 | 0.01–0.1 | 4.4 | 82 | 1[b] | 200 |
| Cyprodinil | F | 0.001–0.02 | 0.01–0.05 | 2.0 | 176 | 0.2[b] | 2600 |
| 2,4-D | H | 0.002–0.03 | 0.02–0.05 | 0.67 | 41 | 30[a] | 24200 |
| Desethylatrazine | M | 0.0010.07 | 0.002–0.07 | 0.036 | 334 | 0.6[c] | 100 |
| Desethylterbytylazine | M | 0.001–0.03 | 0.002–0.05 | 0.078 | 419 | 0.02[d] | 140 |
| Dichlorprop | H | 0.002–0.02 | 0.01–0.04 | 4.9 | 55 | 10[b] | 1100000 |
| Diflufenican | H | 0.001–0.01 | 0.004–0.08 | 0.19 | 338 | 0.005[b] | 10 |
| Esfenvalerate | I | 0.0001–0.02 | 0.003–0.1 | 0.045 | 7 | 0.0001[b] | 6.5 |
| Ethofumesate | H | 0.003–0.03 | 0.01–0.05 | 1.6 | 123 | 30[b] | 3900 |
| Fenitrothion | I | 0.001–0.08 | 0.02–0.08 | 0.3 | 2 | 0.009[b] | 1300 |
| Fenmedifam | H | 0.001–0.6 | 0.002–0.5 | 1.1 | 55 | 2[b] | 86 |
| Fenpropimorph | F | 0.001–0.1 | 0.01–0.05 | 0.53 | 25 | 0.2[b] | 327 |
| Flamprop | H | 0.003–0.02 | 0.02–0.04 | 0.17 | 5 | 20[a] | 6800 |
| Fluazinam | F | 0.001–0.005 | 0.004–0.01 | 0.94 | 20 | 0.4[b] | 160 |
| Fluroxypyr | H | 0.003–0.04 | 0.01–0.08 | 2.0 | 449 | 100[b] | 49800 |
| Flurtamone | H | 0.001–0.1 | 0.002–0.2 | 1.2 | 226 | 0.1[b] | 20 |
| Glyphosate | H | 0.005–0.5 | 0.02–1 | 57 | 1205 | 100[b] | 4400 |
| Imidacloprid | I | 0.001–2 | 0.002–0.8 | 5.0 | 347 | 0.06[a] | > 10000 |
| Iodosulfuron-methyl-sodium | H | 0.001–0.04 | 0.02–0.05 | 0.2 | 27 | 0.08[a] | 70 |

(*continued on next page*)

**Table A1** (*continued*)

| Pesticide | Group* | LOD | LOQ | $Conc_{max}$ | n | WQS | $EC_{50algae**}$ |
|---|---|---|---|---|---|---|---|
| Isoproturon | H | 0.001–0.07 | 0.002–0.01 | 8.1 | 781 | 0.3[b] | 13 |
| Lindane (Gamma-HCH) | I | 0.0001–0.02 | 0.001–0.02 | 0.031 | 22 | 0.02[c] | 2500 |
| MCPA | H | 0.002–0.01 | 0.01–0.05 | 28 | 776 | 1[b] | 79800 |
| Mecoprop | H | 0.002–0.01 | 0.01–0.03 | 6.6 | 503 | 20[b] | 237000 |
| Metalaxyl | F | 0.001–0.05 | 0.002–0.1 | 2.0 | 563 | 60[b] | 36000 |
| Metamitron | H | 0.003–0.05 | 0.01–0.4 | 17 | 251 | 10[b] | 140 |
| Metazachlor | H | 0.001–0.03 | 0.002–0.06 | 12 | 858 | 0.2[b] | 16.2 |
| Metribuzin | H | 0.0015–0.04 | 0.01–0.1 | 4.0 | 335 | 0.08[b] | 20 |
| Metsulfuron-methyl | H | 0.001–0.04 | 0.002–0.05 | 0.42 | 81 | 0.02[b] | 875 |
| Pirimicarb | I | 0.001–0.02 | 0.002–0.05 | 0.47 | 282 | 0.09[b] | 140000 |
| Propiconazole | F | 0.003–0.04 | 0.001–0.2 | 1.2 | 237 | 7[b] | 93 |
| Propyzamide | H | 0.001–0.05 | 0.002–0.06 | 1.8 | 185 | 10[b] | 2800 |
| Prosulfocarb | H | 0.002–0.05 | 0.001–0.05 | 5.0 | 81 | 0.9[b] | 49 |
| Quinmerac | H | 0.001–0.06 | 0.002–0.06 | 6.8 | 844 | 100[b] | 48500 |
| Rimsulfuron | H | 0.001–0.03 | 0.002–0.12 | 0.3 | 24 | 0.01[b] | 1200 |
| Sulfosulfuron | H | 0.001–0.05 | 0.002–0.07 | 0.2 | 90 | 0.05[b] | 221 |
| Terbuthylazine | H | 0.001–0.03 | 0.002–0.03 | 0.08 | 195 | 0.02[b] | 12 |
| Terbutryn | H | 0.002–0.04 | 0.006–0.05 | 0.11 | 8 | 0.002[a] | 2.4 |
| Thifensulfuron-methyl | H | 0.001–0.04 | 0.002–0.04 | 1.6 | 38 | 0.05[b] | 800 |
| Tribenuron methyl | H | 0.001–0.04 | 0.002–0.05 | 0.24 | 115 | 0.1[b] | 110 |
| Triflusulfuron-methyl | H | 0.001–0.05 | 0.002–0.06 | 0.099 | 57 | 0.03[b] | 46 |

a) Andersson and Kreuger (2011) b) KEMI (2008).
* H=herbicide I=insecticide F=fungicide M=metabolite.
** Data from PPDB (see text).
AMPA=aminomethylphosphonic acid (metabolite of glyphosate).
BAM=2,6-dichlorobensamid (metabolite of dichlobenil).
MCPA=2-methyl-4-chlorophenoxyacetic acid.

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