

Environmental Chemistry

Combining Polar Organic Chemical Integrative Samplers (POCIS) with Toxicity Testing on Microalgae to Evaluate the Impact of Herbicide Mixtures in Surface Waters

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Abstract: Pesticide risk assessment within the European Union Water Framework Directive is largely deficient in the assessment of the actual exposure and chemical mixture effects. Pesticide contamination, in particular herbicidal loading, has been shown to exert pressure on surface waters. Such pollution can have direct impact on autotrophic species, as well as indirect impacts on freshwater communities through primary production degradation. The present study proposes a screening method combining polar organic chemical integrative samplers (POCIS) with mode of action-specific toxicity testing on microalgae exposed to POCIS extracts as a standard approach to effectively address the problem of herbicide mixture effects detection. This methodology has been tested using Luxembourgish rivers as a case study and has proven to be a fast and reliable information source that is complementary to chemical analysis, allowing assessment of missing target analytes. Pesticide pressure in the 24 analyzed streams was mainly exerted by flufenacet, terbuthylazine, nicosulfuron, and foramsulfuron, with occasional impacts by the nonagricultural biocide diuron. Algae tests were more sensitive to endpoints affecting photosystem II and reproduction than to growth and could be best predicted with the concentration addition model. In addition, analysis revealed that herbicide mixture toxicity is correlated with macrophyte disappearance in the field, relating mainly to emissions from maize cultures. Combining passive sampler extracts with standard toxicity tests offers promising perspectives for ecological risk assessment. The full implementation of the proposed approach, however, requires adaptation of the legislation to scientific progress. *Environ Toxicol Chem* 2022;41:2667–2678. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

The body of evidence suggesting that pesticide risk assessment as implemented within the European Union (EU) Water Framework Directive (WFD) is largely insufficient to protect surface waters has been constantly growing over recent years (Carvalho et al., 2014; Weisner et al., 2022). The main critiques concern inadequate sampling schemes, missing the main exposure periods (Bundschuh et al., 2014; la Cecilia et al., 2021; Norman et al., 2020), insufficient broadness in target

analytes (Moschet et al., 2014), as well as missing concepts to address the impacts of pesticide mixtures and repeated seasonal exposures (Weisner et al., 2021). These deficits also explain the scarcity of convincing field data showing relationships between deterioration of biological quality elements and pesticide exposure. Although the impact of insecticides on macroinvertebrates has been documented in several field studies (Liess & Von der Ohe, 2005; Liess et al., 2021), primary producer impairment proves difficult to relate to herbicide exposure. On the one hand, effects from pulsed exposures on periphyton seem to be rapidly reversible (Bighiu et al., 2020); on the other hand, macrophytes are more responsive to factors that are independent of anthropogenic pressures (Bucior et al., 2021; Demars et al., 2012). Algae tests involving photosystem II (PSII) inhibition and growth are an alternative to evaluate pesticide mixture impact in grab samples (De Baat et al., 2018; Glauch & Escher, 2020) as well as in passive sampler extracts (Vermeirssen et al., 2010).

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The aim of the present study was to establish a relationship between macrophyte index impairment and herbicide exposure on a gradient of small size, agriculturally used catchments. Passive sampler extracts were also used to verify if the algal toxicity in the samples in different modes of action could be entirely explained by the chemically identified contributors.

MATERIALS AND METHODS

Sites of study and monitoring plan

Herbicide exposure was monitored in 24 river stretches in Luxembourg (Supporting Information, Figure S1.1.) using polar organic chemical integrative samplers (POCIS), following calibration and evaluation methods established in earlier projects (Gallé, Frelat et al., 2020; Gallé et al., 2019). Sites with a watershed size between 10 and 50 km² were preferred to ensure continuous water flow throughout the monitoring season. The main criterion for site selection was to establish a gradient of Macrophyte Biological Index for Rivers (Indice Biologique Macrophytique en Rivière [IBMR]) respecting official WFD quality classes (Hauray et al., 2006). In addition, catchments were evaluated for arable and maize surface as well as eutrophication and morphological quality of the segments, referring to the structural mapping of the river networks in Luxembourg (Zumbroich & Meier, 2014). Water flow data, data on physical–chemical parameters, and macrophyte quality assessment data were provided by the Luxembourgish Water Administration. Catchment properties were extracted using a geographic information system after topographically defining catchment boundaries upstream of the sampling point. Topographic and land-use data were provided by the cadastral service. Information on crops on the plot level came from the Agricultural Administration Luxembourg. Twelve rivers were monitored constantly from June to August 2013 and the remaining 12 from mid-May to the end of September 2014 (sites and catchment property distribution in Supporting Information, S1.1 and S1.2). In the 24 rivers POCIS were exposed in triplicate for a period of 2 weeks, allowing for time-integrative monitoring expressed as an average exposure. The sampling period focused on herbicide applications in maize and their subsequent emission to surface waters. The field data were analyzed to identify the main temporal and spatial contamination patterns to define the toxic mixture of interest to be tested in mixture assays. An additional objective was to highlight relationships between herbicide impact and macrophyte biodiversity. To achieve this objective, the maximum and average daily sum of toxic units (Backhaus et al., 2013) were calculated for the exposure periods, based on ecotoxicological data for water plants (median effect concentration [EC50] at 7 days for water plants, Pesticide Properties DataBase [PPDB]; Supporting Information, Table S2), following Equation 1:

$$\text{Sum of toxic unit}_{\text{exposure period}} = \left(\sum_{i=1}^n \frac{EnvC_i}{EC50_i} \right) \quad (1)$$

In Equation 1, n is the number of herbicides detected, $EnvC_i$ is the time-weighted average (TWA) for 14-day exposure of

herbicide i in the sample, and $EC50_i$ is the corresponding literature EC50 value for water plants.

Values of sum of toxic units were correlated with the IBMR as well as catchment properties and selected water quality measurements.

Selection of monitored herbicides

The economic service of the Luxembourgish Ministry of Agriculture establishes an annual survey of pesticide use in Luxembourgish agriculture based on purchase accountancy of selected farms. The survey follows approximately 500 farms that are distributed over the whole country. From this representative statistical population, average doses of specific pesticides per culture can be established considering the type of pesticide purchased and its application to specific crops in Luxembourg. Supporting Information, Table S2, shows the results of such calculations for the most prominent pesticides used in maize for the year 2014. The seasonally used herbicides terbuthylazine, s-metolachlor, flufenacet, and mesotrione are the most dominant and are often used in combination. Glyphosate is not used in crops but rather after harvest to prepare the next culture. Glyphosate cannot be monitored with OASIS-HLB-based passive samplers and was therefore not considered in the present study. Note also that glyphosate has quite high EC50 values for aquatic plants (12 mg/L, PPDB; Supporting Information, Table S2) and is therefore not expected to have a high impact in aquatic ecosystems. The impact of the herbicides depends not only on their initial dose but also on half-lives and sorption affinity in soils, both of which largely determine their mobilization potential and hence the probability to be transported in surface runoff. In addition, there are substantial differences in EC50 values for algae and macrophytes. Supporting Information, Figure S2, shows that in general impacts on algae and macrophytes are aligned but that there is a three orders of magnitude higher impact for some compounds in macrophytes (nicosulfuron, foramsulfuron, mesotrione, pethoxamide, and 2-methyl-4-chlorophenoxyacetic acid [MCPA]). Lower doses of these compounds might therefore be compensated by higher impact. Figure 1 attempts a summarizing view in a three-dimensional plot combining dose, half-life in soils, and the macrophyte EC50. It is easily discernible that terbuthylazine and flufenacet are expected to have the highest impact.

Passive samplers and analytics

The passive samplers used were POCIS, each disk contained 200 mg of OASIS-HLB adsorbent between two polyethersulfone membrane sheets. Theory and modeling for POCIS have been described already (Gallé, Frelat et al., 2020). Three POCIS disks were exposed in the small EST-Lab canisters. After exposure of 2 weeks, the OASIS-HLB powder was recovered and filled into 5-ml solid-phase extraction columns and covered with a frit. The recovered powder was weighted to compensate for losses on transfer. Columns were dried under vacuum and subsequently extracted twice with a

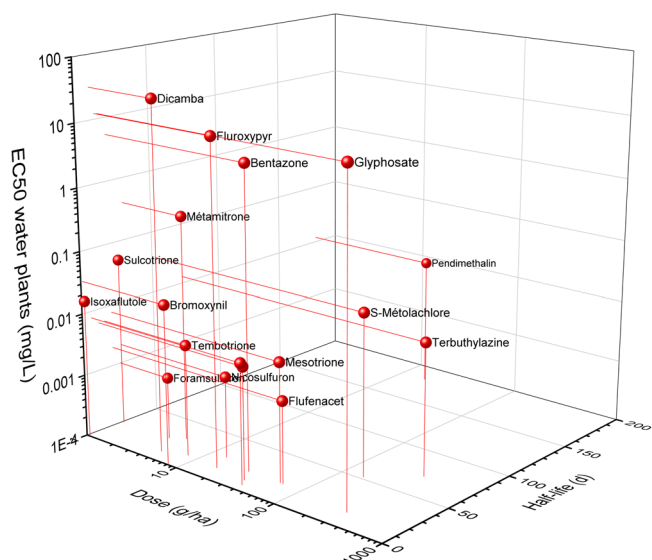


FIGURE 1: The combination of average dose in maize culture, half-life of the compound in soil, and water plant EC₅₀ can be used to identify the drivers of macrophyte impact. EC₅₀ = median effect concentration.

50/50 solvent solution of dichlormethane and acetonitrile. Solvent was reduced to dryness after internal standard addition and taken up in 50/50 acetonitrile/water for analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Internal standards were available for three fourths of the compounds and served to compensate for matrix effects and handling losses. Quantification was calibrated on internal standards. Sampling rates for POCIS were derived from low-flow field and complementary laboratory calibrations and expressed as TWAs for 14 days (see Supporting Information, S3, and Gallé et al. [2019] for the principle of field calibration and POCIS sampling rates). Analytical and internal standards for quantification had been purchased from LGC Standards.

Test organism and culture conditions

Synchronous cultures of unicellular freshwater chlorophytes *Scenedesmus vacuolatus* were used as the test organism. *Scenedesmus vacuolatus* were grown photoautotrophically in a sterile inorganic medium at pH 6.4 (modified from Grimme & Boardman, 1972). The strain (SAG 211-15) was bought from the experimental phycology and culture collection of algae at the University of Goettingen. Algae were cultured under synchronous conditions as described by Faust et al. (2001) and Altenburger et al. (1990). Cultures were grown in sterile glass vessels (Supporting Information, Figure S4) under the following parameters: a bubbling regime of compressed air and 1.5% CO₂, at 28 °C ± 2 °C, and under illumination of saturated white light at an intensity of 400 μmol photons s⁻¹ m⁻² in a 14:10-h light:dark cycle. Illumination parameters were achieved using 4 × Osram L36W/827 Lumilux Interna and 4 × Osram L36W/865 Lumilux Daylight fluorescent lamps. An inoculum culture in fresh medium was performed daily to allow exponential growth in the control culture throughout the incubation period without

risk of nutrient depletion (maximum cell density 10⁶ cells/ml measured on a Multisizer 3 Coulter cell counter; Beckman).

Laboratory experimental setup

The toxicity assay selected to test herbicide effects was the synchronous algae test (adapted from Neuwoehner et al., 2008, 2010). Three different endpoints (cell growth, cell reproduction, and photosynthetic efficiency) were tested to cover the different toxic modes of action of the target compounds: photosynthetic quantum yield, reproduction, and biovolume. Laboratory toxicity tests were conducted on both field extracts from passive samplers and spiked artificial samples, adapting the method presented in Vermeirssen et al. (2009). Spiked artificial samples included single-herbicide and mixture tests. Single-herbicide tests included three endpoints (photosynthetic efficiency, growth, and reproduction) and allowed the identification of the effect levels and toxic modes of action of 10 selected representative herbicides: terbutylazine, bentazone, isoproturon (endpoint photosynthesis inhibition), mesotrione (endpoint inhibition of the synthesis of the carotenoids, effects on photosynthesis), MCPA, foramsulfuron, metholachlor (endpoint growth inhibition), metazachlor, flufenacet, andnicosulfuron (endpoint cell division/reproduction inhibition). Herbicide standards were diluted in ultrapure acetonitrile solvent (Sigma-Aldrich). Two replicates were done per water and solvent control sample. The performance of *S. vacuolatus* was assessed in three different scenarios: (1) 12 single dilutions of herbicides, (2) 12 dilutions of one artificial spiked mixture of herbicides at known concentrations prepared based on measured herbicides in a selected POCIS extract, and (3) 12 dilutions of one selected POCIS extract. The concentration range of single dilutions of herbicide mixture in the test tubes included concentrations detected in natural samples from the present study. The effects in the assay treated with the whole field extracts were compared with the effects measured in assays contaminated with spiked “artificial” mixtures of herbicides. The extract toxicity tests were performed after the end of the experimental campaigns and after the analysis of the field samples, to select the most representative mixture. The samples were stored at –20 °C in the unextracted POCIS to minimize degradation of the compounds; the extraction was performed shortly before the exposure.

Growth and reproduction inhibition algal assays

Growth inhibition and reproduction inhibition of the unicellular green alga *S. vacuolatus* cultures during the exponential growth phase were measured in a 24-h bioassay in terms of biovolume and cell division rate inhibition in laboratory conditions. The algae were exposed to single herbicides and compound mixtures as well as whole field extracts. Sterile test tubes were prepared with autoclaved 7.2 ml of modified GB medium (Grimme & Boardman, 1972), 20 μl NaHCO₃ 0.6 M, and stirring bars. Herbicide standards were diluted in ultrapure acetonitrile solvent (Sigma-Aldrich). A volume of 8 μl of each

test substance was placed in the test tubes to obtain the 12 final test concentrations: 10,000, 5000, 2500, 1000, 500, 250, 200, 100, 50, 25, 10, and $5 \mu\text{g L}^{-1}$. In three controls $8 \mu\text{l}$ of Milli-Q water was added, and in three solvent controls $8 \mu\text{l}$ of ultrapure solvent (acetonitrile) was added. Finally, each tube was inoculated with 0.8 ml of the algal suspension ($\sim 7.5 \times 10^5$ cells/ml cell density) directly prior to the start of the test. The final volume was 8 ml per test tube. Two replicates were done for each single-herbicide dilution, artificial mixture of herbicides, or whole field extract sample. Magnetic stirring of the test tubes was maintained for 30 s , stirring at 240 rpm with a pause of 4 min during the test. The tests were performed in a constant-temperature water bath at $28 \pm 2^\circ\text{C}$ and under a laminar flow hood to ensure sterility. Aliquots of $200 \mu\text{l}$ of each test tube were resuspended in 10 ml Isoton solution and analyzed on a Multisizer 3 Beckman Coulter after 18-h exposure for biovolume determinations and after 24 h for cell division rate determinations. The growth rate of controls was the quality control of the results. If it was equal to or higher than the specific growth rate of the microalgae culture in exponential phase, the results were accepted. Effects on growth and reproduction were quantified according to the inhibition percentage measured at the end of the test sequence.

Photosynthesis inhibition algal assays

Photosynthesis inhibition was studied in acute exposure (test duration 2000 s). Microalgae cultures were used when they reached the exponential growth stage. The test was performed on black, 96-well, flat-bottomed microplates; each test well contained $400 \mu\text{l}$ of culture medium (initial density of $\sim 1 \times 10^6$ cells ml^{-1}) plus $4 \mu\text{l}$ of single-herbicide dilutions or mixture dilutions and Milli-Q water or solvent in the control groups. Herbicide standards were diluted in acetonitrile ultrapure solvent (Sigma-Aldrich). Two replicates were done per water and solvent control sample. The effects of 12 concentrations of single herbicides (Supporting Information, Table S11.1), of the selected spiked mixture, and of the selected POCIS extract were measured with a MAXI Imaging-pulse amplitude modulated (PAM) instrument (Heinz Walz) equipped with light-emitting diode lights with a wavelength of 470 nm . The central part of the 96-well microplate was used for the tests to ensure uniform lighting (Supporting Information, Figure S5). The gain was adjusted in such a way that in the absence of actinic illumination the fluorescence signal was in the range of a minimum $150\text{--}200$ units. After 5 min of dark adaptation, minimum fluorescence yield (F_0) was measured on a measuring light with low intensity ($3 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ photosynthetic active radiation [PAR], frequency 5 Hz , modulated pulses of $100 \mu\text{s}$). The saturation pulses had an intensity of $7700 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ PAR lasting for 600 ms and were repeated every 100 s during the test sequence of 2000 s . Maximal PSII quantum yield (Max YII) was determined under saturation pulse after dark adaptation, and it was calculated as $\text{Max YII} = F_v/F_m$, where $F_v (=F_m - F_0)$ is the unquenched variable fluorescence. The effective PSII quantum yield (YII) was calculated by the formula $\text{YII} = (F'_m - F)/F'_m$, where F is the level of

fluorescence immediately before the saturation pulse (3 s average) and F'_m is the maximal level of fluorescence reached in the following saturation pulses. Four saturation pulses were then applied each 100 s , maintaining the sample in the dark phase to check the homogeneity of the replicates. The test substances were added during a short break after pulse 4, in the absence of actinic illumination; after approximately 200 s , the microplate was replaced in the apparatus, and the measurement continued until the end of the sequence (2000 s), maintaining measuring light at low intensity ($3 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ PAR, frequency 5 Hz , modulated pulses of $100 \mu\text{s}$). Phytotoxicity was quantified according to the inhibition of PSII quantum yield (YII) measured at the end of the test sequence.

Mixture algal assays

One POCIS extract was selected for the mixture tests: river Girsterbach, sampling period June 13–26, 2013. One aliquot of the extract was used for the chemical analysis to obtain the concentrations of herbicides in the field (Supporting Information, Table S11.2) and to calculate the remaining absolute mass of herbicides in the extract. Considering the total absolute mass of measured herbicides remaining in the extract, eight test dilutions from the extract were prepared covering the range from 5 to $500 \mu\text{g L}^{-1}$ of total herbicide concentrations ($500, 250, 200, 100, 50, 25, 10,$ and $5 \mu\text{g L}^{-1}$). Eight spiked test mixture dilutions were prepared with the most representative herbicides measured in the extract (terbuthylazine, bentazone, metholachlor, metazachlor, mesotrione, and nicosulfuron) covering the same concentration range from 5 to $500 \mu\text{g L}^{-1}$ (total herbicide concentration) and respecting the same proportion of the single herbicides in the extract (Supporting Information, Table S11.2). Herbicide standards were diluted in acetonitrile ultrapure solvent (Sigma-Aldrich). The mixture tests were performed using the same procedure as single-herbicide tests.

Data modeling

Herbicide concentrations and growth, reproduction, and PSII quantum yield inhibition values of microalgae cultures, obtained from tests, were the input data for the calculation of concentration–response curves using Matlab software (Ver R2017a).

The inhibition was calculated according to the following equation:

$$\text{Inhibition} = \left(\frac{X_{\text{control}} - X_{\text{sample}}}{X_{\text{control}}} \right) \quad (2)$$

In Equation 2, X indicates the endpoints: biovolume of cells (cubic millimeters) for growth tests, density of cells (per milliliter) for reproduction tests, and PSII quantum yield for photosynthesis inhibition tests. All data were log-transformed prior to data analysis to increase normality of data and homogeneity of variances. The results were fitted using a simple sigmoidal dose–response curve implemented in Matlab (Equation 3).

$$y = \frac{1}{1 + \exp\left(-\left(\frac{x-a}{b}\right)\right)} \quad (3)$$

The EC50, which is the concentration causing a 50% response, is calculated using a simple power function with 10 as the base and coefficient a as the exponent. Concentration–response graphs were also plotted in Matlab. Values of EC50 and expected growth inhibition from single and artificial metal mixtures of contaminants were calculated using this software, interpolating or extrapolating the missing information. The additivity hypothesis has been tested for compounds of identical modes of action and in fully mixed situations. Mixture models were based on the concentration addition (Berenbaum, 1985) and independent action (Hewlett & Plackett, 1959) hypotheses. In the case of concentration addition the equation applied was

$$EC_{X_{\text{Mix}}} = \left(\sum_{i=1}^n \frac{p_i}{EC_{X_i}} \right)^{-1} \quad (4)$$

In Equation 4, n is the number of mixture components, p_i is the relative fraction of chemical i in the mixture, and x is a common effect level, which is provoked by an exposure to a single substance (EC_{X_i}) or a mixture concentration ($EC_{X_{\text{Mix}}}$).

In the case of independent action the equation applied was

$$E(c_{\text{Mix}}) = 1 - \prod_{i=1}^n [1 - E(c_i)] \quad (5)$$

The individual effects of mixture constituents $E(c_i)$ can be calculated from concentration response functions F_i determined for single substances: $E(c_i) = F_i(c_i)$. Again, the individual concentrations c_i can be expressed as relative proportions p_i of the total concentration c_{mix} , and under the condition that total effect $E(c_{\text{mix}})$ equals $x\%$, c_{mix} is defined as $EC_{X_{\text{mix}}}$. Thus, by substitution we can transform Equation 5 into

$$x\% = 1 - \prod_{i=1}^n \{F_i[p_i(EC_{X_{\text{mix}}})]\} \quad (6)$$

which implicitly provides a prediction of effect concentrations of a mixture under the hypothesis of independent action.

RESULTS

Field results

The present study consisted of two field campaigns in 2013 and 2014, each comprising 12 sites and spanning an array of seasonally confirmed IBMR values. The outcomes of the two campaigns were very different in terms of herbicide occurrence, impact, and correlation with catchment parameters. The reasons for these differences will be discussed at the end of this paragraph. Pesticide mobilization by surface runoff is governed by relatively intense precipitation events shortly after pesticide application. Supporting Information, Figure S6, shows the comparison between the two years: 2013 featured a strong precipitation event on June 20, which largely drove the herbicide

mobilization and impact during the first campaign. Figure 2 shows the occurrence of different herbicides in one specific representative site that is illustrated by 14-day TWAs in a step-type graph (Gallé, Frelat, et al., 2020). All four parent compounds featured showed the same behavior. While metolachlor and terbuthylazine (probably stemming from the same combined product) exceeded the micrograms per liter concentration two to four times, pethoxamide and nicosulfuron only reached 200–300 ng/L. However, because these are 14-day averages, if this average was only caused by one (daily peak), the event mean concentration on that day would be approximately 10 times higher because of the time proportionality of passive sampling (Gallé, Frelat, et al., 2020). Note also the rather constant base flow–driven occurrence of the transformation products metolachlor ethane sulfonic acid (ESA) and metazachlor ESA, which increased slightly later in the season because of the slow metabolization of the parent compounds (further site profiles in Supporting Information, S7).

Figure 3A shows the combined impact of all herbicides on macrophytes as the sum of toxic units over the season for the formerly discussed site (Girsterbach) as well as the sum of toxic units per compound for all sites and over the entire season to evaluate the overall impact. The sum of toxic units followed the evolution of the pesticide exposure in Figure 2. The more interesting feature of the figure is the dominant herbicides that caused the impact on water plants. This is shown by the fractional impact in Figure 3B. At the peak of the sum of toxic units in the period featuring the large precipitation event terbuthylazine and nicosulfuron dominated over metolachlor. This was due to the large differences in EC50 values between the different compounds. Later in the season, and at lower sum of toxic unit levels, terbuthylazine was largely dominant because of its longer-lasting presence (higher soil half-life value). Note that during lower-impact periods those compounds with very low EC50s like foramsulfuron and nicosulfuron were more dominant because small concentrations, even close to quantification limit, can lead to higher calculated impact (further sites in Supporting Information, S8). The sum of toxic units per compound over all sites were dominated by flufenacet, terbuthylazine, nicosulfuron, and foramsulfuron (Figure 3C).

Table 1 displays the Pearson correlations that were tested between measured concentrations, sum of toxic units, IBMR, and catchment property features like arable or agricultural surface fractions. The parameter values for all sites can be found in Supporting Information, S9, and distributions of land-use properties and measured parameters for both years in Supporting Information, S10. There are differences in the mid-term representativeness of the measured parameters. Atrazine-desethyl, for instance, is the transformation product of atrazine, a compound that was prohibited in 2005 but was still emitted to surface water from groundwater contribution at the time of monitoring in 2013–2014. It therefore represents long-term agricultural impact. The same holds for nitrate, an indicator of intensive agriculture, which because of its storage in soils and groundwater is also indicative of long-term impact. The transformation products of metolachlor and metazachlor are somewhere in between because they are also stored in groundwater

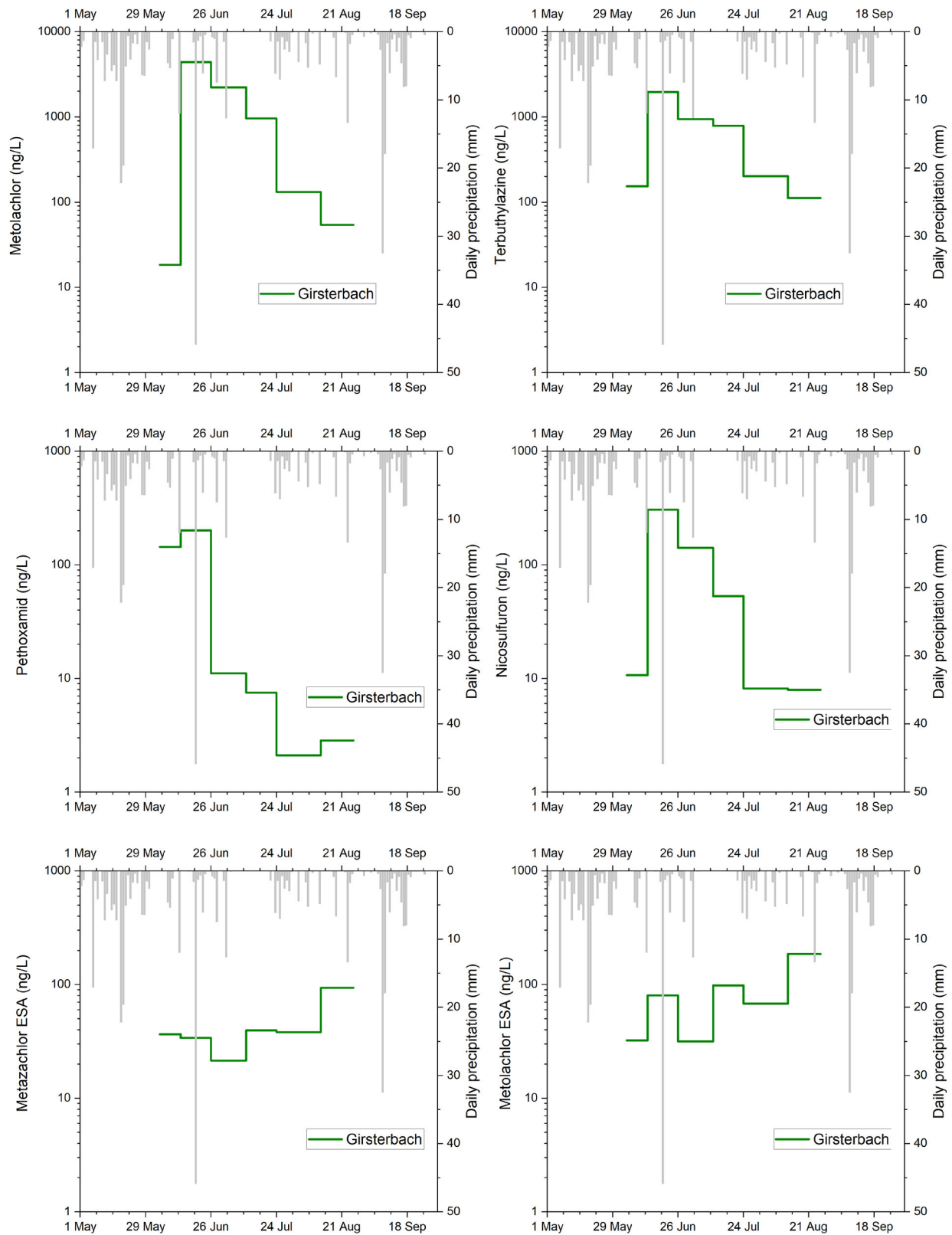


FIGURE 2: Seasonal time-weighted average profiles for relevant compounds at one monitoring site (Girsterbach). Top bars are daily precipitation data. ESA = ethane sulfonic acid.

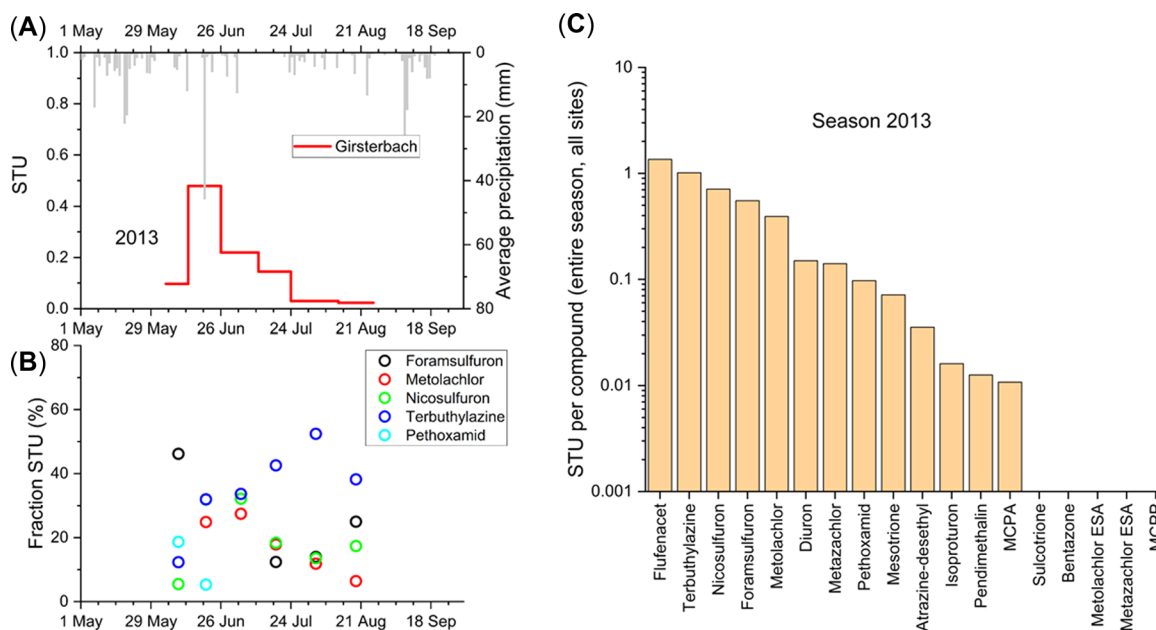


FIGURE 3: Sum of toxic units (STU) profiles over the season for one site (Girsterbach) (A) with the main fractional contributing compounds (B) and the overall contribution of the different compounds for all sites (C). Compounds with STU <0.001 are shown for the record. MCPA = 2-methyl-4-chlorophenoxyacetic acid; ESA = ethane sulfonic acid; MCPP = methylchlorophenoxypropionic acid.

and document a use in earlier years than the current season. On the other side, the sum of toxic units is clearly linked to the current seasonal use of herbicides and is dependent on the weather conditions driving their mobilization. The correlation matrix shown in Table 1 gives a quite coherent picture in terms of the origin of the pressure on macrophytes, although correlations were only moderate and not significant at the 5% level with an n of 12 ($\alpha = 0.05$, $R > 0.574$). Atrazine-desethyl was correlated to metolachlor ESA, nitrate, and the maize fraction of land use. Maize was also the strongest driver on IBMR impact ($R = -0.52$) and was also correlated to the seasonal sum of toxic unit ($R = 0.52$). The correlation with IBMR was stronger than that of sum of toxic unit (-0.37). This indicates that long-term impact on macrophytes might outweigh short-time, event-driven

impact. The 2014 season did not show any notable correlations between impact and catchment properties (Supporting Information, S9.2 and S9.3), which could be partly due to the lack of intense precipitation during the season but is probably mainly a consequence of the much narrower distribution of maize surface fraction (Supporting Information, S10). These findings underline that a broad array of catchment properties and a consequent monitoring are necessary to establish sound causal relationships. Note that catchment selection could only be made based on information on crop localization from former years. The true crop distribution for the current campaign shown in our study was only available 1 year after the campaign was performed. This is a difficulty in smaller catchments where shifts can be important because the number of farmed plots is limited,

TABLE 1: Correlation matrix showing Pearson factors for different measured parameters and catchment properties

	Atrazine-desethyl ^a	Metazachlor ESA ^a	Metolachlor ESA ^a	NO ₃	Arable surface ^b	Agri surface ^b	Maize surface ^b	IBMR	STU average	STU maximum
Atrazine-desethyl	1.00	-0.47	0.62	0.48	-0.22	-0.16	0.40	-0.42	0.60	0.44
Metazachlor ESA	-0.47	1.00	0.01	-0.56	0.03	-0.46	-0.38	0.34	-0.42	-0.35
Metolachlor ESA	0.62	0.01	1.00	0.31	0.06	-0.29	0.22	-0.33	0.58	0.55
NO ₃	0.48	-0.56	0.31	1.00	0.19	0.14	-0.15	-0.14	0.16	0.06
Arable surface	-0.22	0.03	0.06	0.19	1.00	0.54	0.30	0.04	-0.14	-0.19
Agri surface	-0.16	-0.46	-0.29	0.14	0.54	1.00	0.44	-0.40	0.08	0.06
Maize surface	0.40	-0.38	0.22	-0.15	0.30	0.44	1.00	-0.52	0.52	0.37
IBMR	-0.42	0.34	-0.33	-0.14	0.04	-0.40	-0.52	1.00	-0.37	-0.31
STU average	0.60	-0.42	0.58	0.16	-0.14	0.08	0.52	-0.37	1.00	0.96
STU maximum	0.44	-0.35	0.55	0.06	-0.19	0.06	0.37	-0.31	0.96	1.00

^aWater quality parameters: pesticide transformation products average of time-weighted averages at low-flow or of grab samples for NO₃ (Supporting Information, Table S9.2).

^bCatchment properties: fractions of land use in percentage used (Supporting Information, Table S9.2). Agricultural surface = arable land + green land.

Numbers in bold are only to guide the reader to the correlations discussed in the text. For $n = 12$, significance of $\alpha = 0.05$ requires an R of 0.574 and $\alpha = 0.1$ an R of 0.5 (Berthouex & Brown, 2002).

ESA = ethane sulfonic acid; IBMR = Macrophyte Biological Index for Rivers (Indice Biologique Macrophytique en Rivière); STU = sum of toxic units.

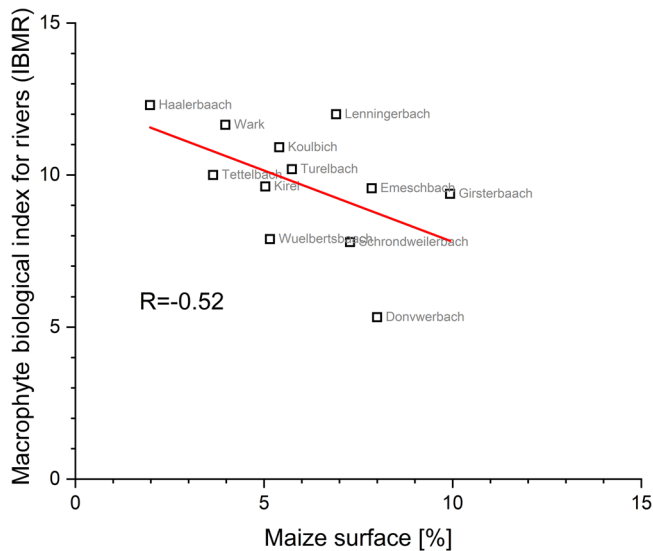


FIGURE 4: Relationship between fractional maize surface in the catchment and IBMR for the 2013 sites. The linear regression is significant at $\alpha=0.1$. IBMR = Macrophyte Biological Index for Rivers (Indice Biologique Macrophytique en Rivière).

meaning that some culture might not be present at all in a given year (Figure 4).

Exposure assays to single herbicide

Several key herbicides were selected for single-exposure assays, based on both POCIS field campaigns and literature toxicity data: terbuthylazine, bentazone, isoproturon, metolachlor, metazachlor, MCPA, flufenacet, mesotrione, foramsulfuron, and nicosulfuron. Mesotrione (inhibitor of *p*-hydroxyphenylpyruvate dioxygenase, an essential enzyme in the biosynthesis of carotenoids) and MCPA (growth regulator, affects the synthesis of auxins) did not show any effect for the three endpoints measured at a concentration of 10 mg/L. Except for bentazone (lower EC₅₀ in photosynthesis inhibition test), all herbicides tested showed lower EC₅₀ values in reproduction tests (see Supporting Information, Figures S12–S14), the most sensitive test for detecting herbicidal contamination.

In biovolume tests, in addition to mesotrione and MCPA, nicosulfuron and isoproturon did not show any measurable adverse effect (Supporting Information, S13).

Only three herbicides showed effects in the photosynthesis tests (Supporting Information, S12). Terbuthylazine and isoproturon had a steep concentration–effect curve, whereas bentazone showed a flatter impact.

The most toxic compound was terbuthylazine, with measured effects at low concentrations (EC₅₀ = 0.03, 0.04, and 0.08 mg/L, respectively, in reproduction, photosynthesis inhibition, and biovolume tests). Flufenacet was very effective in reproduction tests (EC₅₀ = 0.02 mg/L), probably because of its mode of action. In fact, flufenacet is a long-chain fatty acid inhibitor, and it affects cell membranes of meristematic tissues, interfering with both membrane selectivity and permeability and preventing cell division. Metolachlor showed low EC₅₀ values (0.50 mg/L) in reproduction tests.

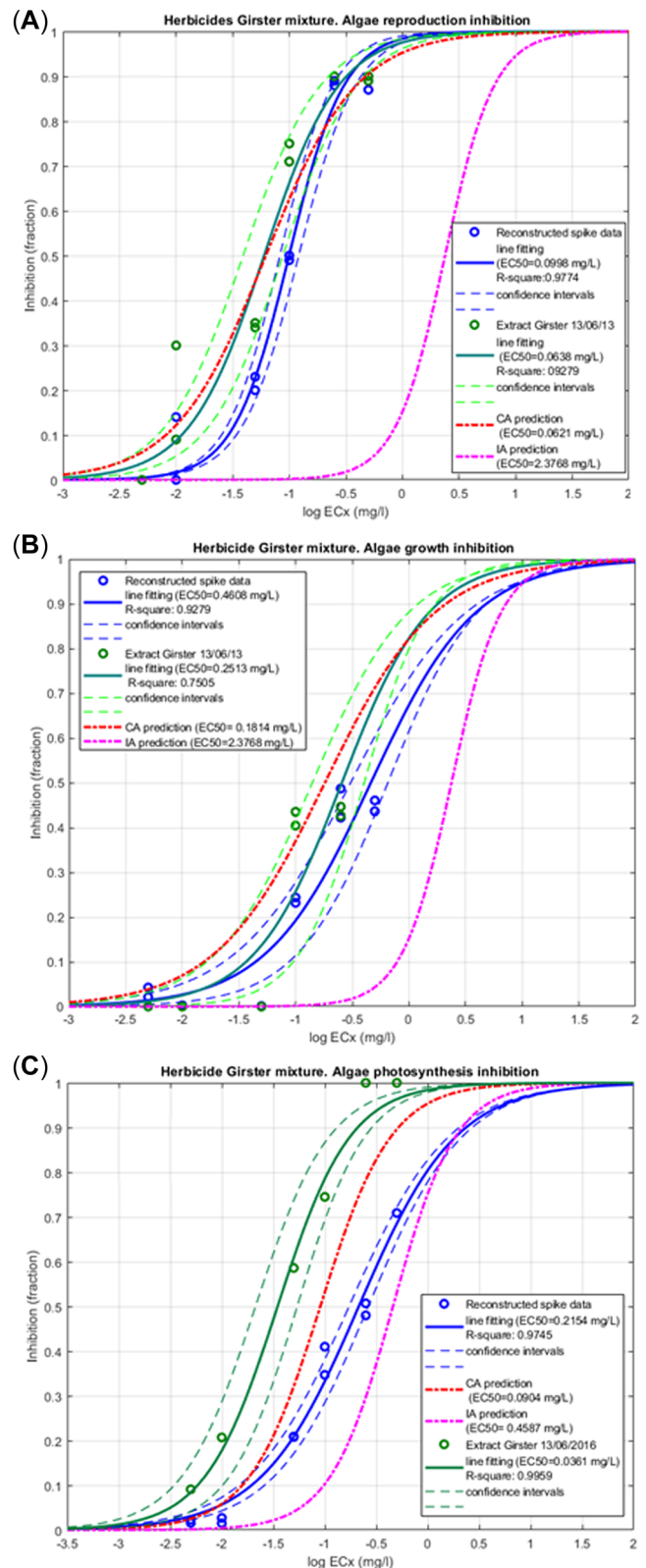


FIGURE 5: Reproduction (A), growth (B), and photosynthesis (C) inhibition test results of the effective herbicide mixtures. The equations of the concentration–response curves, r^2 , and median effect concentrations are indicated in the graphs (fitting parameters and uncertainty values available in Supporting Information, S16 table). EC_x = *x*% effect concentration; CA = concentration addition; IA = independent action.

Exposure assays to herbicide mixtures

Following the analysis of the field data, the field extract selected for its broad mixture of herbicides was the extract from the Girsterbach sampling point (sampling period June 13–26, 2016). The concentration range used to obtain the dose–response curve was 5–500 $\mu\text{g L}^{-1}$, as indicated in *Materials and Methods*. Unfortunately, it was not possible to expose the algae to higher concentrations, to obtain 100% inhibition values, because of the small mass in the extract; it was possible to obtain dose–response curves with high r^2 coefficients. The concentration addition and independent action predictions were calculated considering all herbicides analyzed in the single-herbicide tests. As expected, for all endpoints, the effects of the extract were slightly higher with respect to the reconstructed spike (Figure 5), indicating the presence of other herbicides or other toxic compounds not included in the spike. On the other hand, the main effects could be associated with the selected herbicides, which were in this way identified as key compounds (Figure 5). The largest gap between extract EC50 and reconstructed spike EC50 turned out to be in the PSII algae test, hinting at missing compounds with that mode of action. The concentration addition prediction was, as expected, the most appropriate model to reproduce the effect of the mixture, whereas the independent action model showed a tendency to underestimate the effects, especially in reproduction and growth inhibition assays (Figure 5).

DISCUSSION

Field results

Herbicides are used on a large scale in Luxembourgish agricultural and domestic applications and are frequently detected at a level $>1 \mu\text{g/L}$ in surface waters during seasonal flood events (Gallé, Frelat, et al., 2020). Although herbicides have minor ecotoxicological effects on nontarget species (Hasenbein et al., 2017) such as macroinvertebrates and fish, two important WFD biological indicator groups, their occurrence may lead to an insufficient ecological quality estimated using macrophytes. This often causes the failure of good ecological status in Luxembourg and in other EU countries. Macrophytes are known to be highly sensitive to herbicides (Vonk & Kraak, 2020), yet the current method to determine whether the calculated index is poor is solely based on eutrophication. The present study is, to our knowledge, the first to show a relationship between a macrophyte index deterioration and herbicide exposure in the field. Maize cultures correlated more strongly than sum of toxic unit with IBMR and can therefore be identified as a main driver of herbicide emissions and, thus, of macrophyte impact. The 2013 correlation matrix gave a coherent output of interrelationships between catchment properties, herbicide concentrations, as well as calculated (sum of toxic units) and measured (IBMR) impacts. Macrophyte indices are known to be easily confounded by other geochemical parameters (Kaijser et al., 2021). In addition, impact is strongly dependent on weather conditions and agricultural practice in the catchments, which makes the finding of only moderate correlations plausible. The second season showed no clear correlations at all, which was probably related to the much

too narrow fraction of maize culture surface within the land-use distribution. This, however, could also be seen as an indirect confirmation of the first season's result because the distribution of arable surface fraction in the second year was very large but did not correlate with any exposure or impact. These results make a case for the recognition that continuous monitoring is key to establish cause–effect relationships in field investigations (Moschet et al., 2014).

Exposure assays to single herbicides

Cell growth, cell reproduction, and photosynthesis efficacy are considered to be the most important parameters in ecotoxicological bioassays with small phototrophic species (see Escher et al., 2008). The main technique in ecotoxicological bioassays which allows one to observe these effect parameters is synchronous algae assays (Neuwoehner et al., 2008, 2010). The tests proposed in the present study demonstrate several useful attributes. The results are repeatable and robust, the short generation time of the algae guarantees fast results, and, after a reasonable investment in the needed laboratory equipment and instrument maintenance, costs are low. In addition, the tested species was very sensitive to the target compounds (i.e., herbicides), and the combination of different endpoints was demonstrated to cover the different herbicide modes of action.

Many studies have evaluated the impact of different chemical classes of herbicides using different doses, organisms, and bioassays. Our single-herbicide EC50s are in agreement with previous studies in freshwater green microalgae from reference sources such as the US Environmental Protection Agency (2020) and other literature sources (Supporting Information, S15). The variability of EC50 values between our tests and literature values can be explained by specific toxicity mechanisms, differences in resistance of the specific algae strain, and boundary conditions.

Exposure assays to herbicide mixtures

Organisms are rarely exposed to single pollutants in their environment, and the action of a single compound in a mixture of different compounds may affect the organism in a totally different way than the same concentration on its own (Faust et al., 2001). It has been observed that mixtures of chemicals might have significant toxicity effects even if single compounds are detected in small concentrations only (Carafa et al., 2011). Mixtures can lead to antagonistic effects where two or more agents in combination have an overall effect that is less than the sum of their individual effects, but they can also induce synergism where the effect caused by the exposure of two or more chemicals at a time results in health effects that are greater than the sum of the effects of the individual chemicals (Altenburger et al., 2009).

Identifying the toxicants in complex mixtures remains an open challenge, but it is possible to assess indirectly the toxicity by linking exposure information to biological effects (Blackwell et al., 2019; Brack et al., 2019). Extracts from POCIS in combination with tests on microbial communities have already been proposed (Cernoch et al., 2011; Hamers et al., 2018; Pesce

et al., 2011), but the drawbacks of this method are the difficulties in the replicability and the wide range of biofilm responses that differ according to origin of the biofilms tested, revealing spatial variations in the sensitivity of natural communities in the studied stream. For this reason, it is probably preferable for large-scale application to use highly replicable standard methods (De Baat et al., 2020). Booij et al. (2013) proposed a PAM assay in marine microalga *Dunaliella tertiolecta* exposed to passive sampling extracts. In particular, algal bioassays seem the most sensitive and appropriate method to detect herbicide mixture effects (Neale et al., 2020). In our study, we were able to successfully identify the target compounds (i.e., herbicides) responsible for the main effects in algae exposed to the POCIS extracts. Nevertheless, other studies showed that it is not always possible to identify key compounds. For example, Claessens et al. (2015) exposed the marine diatom *Phaeodactylum tricomutum* to contaminant mixtures that were previously collected in the field through passive sampling, but they could not correlate effects with the mixtures of compounds analyzed in the extracts.

The effects of the herbicide mixtures in a laboratory were investigated in many studies (Faust et al., 2019; Knauert et al., 2008). Several theoretical models have been made and applied to explain the behavior of the contaminants in the mixture. These models are based on the modes of action of the chemicals. They describe functional or anatomical change at the cellular level resulting from exposure to the specific substance (Altenburger et al., 1996).

In our study the combined effect of the herbicide mixture was shown to be predictable by using the concentration addition concept, whereas independent action underestimated the mixture toxicity. Altenburger et al. (2004) recommends using concentration addition in conservative risk assessment of chemical mixtures both for chemicals with a similar mode of action and for those with a different mode of action. The independent action model is preferable in multispecies-level and ecosystem-level analyses, according to Cedergreen et al. (2008).

Because several herbicide modes of action were investigated, the differences between effects measured on spiked mixtures and whole extracts can be attributed to herbicides that were not analyzed with LC-MS-MS. The EC₅₀s of the extracts were always lower relative to the EC₅₀s of the reconstructed spikes, but this is not always the case for the EC₅₀s predicted by the concentration addition model. That is, for the reproduction endpoint concentration addition dose–response curves match the extract curve quite well, whereas for the growth endpoint the EC₅₀ values of extracts are slightly higher than the concentration addition predictions. Note that photosynthetic inhibition assay EC₅₀ values of the extract are approximately one third of the predicted EC₅₀ from the concentration addition model. The greatest difference in dose–response curves of the exposure to the reconstructed spike was observed in photosynthesis inhibition assays. These results might indicate that some of the herbicides affecting directly photosynthetic mechanisms were not included in the reconstructed spikes. The most probable candidate is a triazine used as a biocide: terbutryn is regularly detected in Luxembourgish surface waters during base flow as well as in flood waves (Gallé et al., 2019; Gallé, Bayerle, et al.,

2020). The compound could have been emitted during the high-flow events from facade emissions or accidental product spilling during low flow. The other biocide, diuron, also a PSII active compound, was too low in concentration to have this effect.

The approach used in the present study can allow an initial fast screening of water bodies simply using tests on POCIS extracts, and then, when required, more information on key compounds can be gathered by coupling reconstructed spike tests or simply by comparing concentration addition model predictions. Finally, as for field results, laboratory results also showed a certain degree of uncertainty (see Supporting Information, Table S16 and Figure 5), especially for growth and reproduction tests, whereas the photosynthetic inhibition test seems the more robust. To establish sound causal relationships between measured toxic effects in aquatic plants and key herbicide compounds, it is recommended to collect and compare marketing, monitoring, and laboratory toxicity data.

CONCLUSIONS

The present study proposes a method combining passive sampling with toxicity testing to evaluate toxic pressure on primary producers in surface waters. The suggested methodology is applicable at all scales (EU, river basin, and site-specific levels) for the identification of mixtures presenting significant risks. The method has proven to be very sensitive considering the small absolute mass that can be extracted from the passive sampler POCIS. The repeatability of the tests is confirmed by the similar results obtained in independent test replicates; the assays' duration time is short, and they have low maintenance costs. The main effects detected in the algae exposed to POCIS extracts could be associated with the selected target herbicides, measured in the extracts, and used in the spiked artificial mixtures. The complementarity of the results in our three different mixture assays demonstrated that short-time microalgae ecotoxicological assays should cover different endpoints, to better discriminate between effects of compounds with different modes of action and consequently serve as an effective screening method. The concentration addition model is the one that most closely predicts effects of the tested field extract, but a previous selection of the possible target substances, based on relevance, is anyway an essential prerequisite to obtain a good predictive model.

Finally, based on our field data, it is possible to establish causal relationships between biodiversity loss for macrophytes and herbicide impact drivers. Considering the many confounding factors in the field, this is only possible with a large array of catchment properties (in our case, maize surface fractions), favorable weather conditions (large precipitation events), and a continuous monitoring of the sites.

Risk assessments of aquatic pollutant mixtures require cross-cutting initiatives, including new EU chemical legislation. Combining passive sampler extracts with algal toxicity tests offers promising perspectives for ecological risk assessment in surface waters. Nevertheless, more evidence is needed on questions regarding mixture behavior and their direct and indirect effects on the environment.

Supporting information—The Supporting Information is available on the Wiley Online Library at <https://doi.org/10.1002/etc.5461>.

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Conflict of Interest—There are no conflicts of interest.

Author Contributions Statement—**Roberta Carafa**: Conceptualization; Funding acquisition; Supervision; Investigation; Formal analysis; Visualization; Writing—original draft; Writing—review & editing. **Tom Gallé**: Conceptualization; Formal analysis; Supervision; Validation; Visualization; Writing—original draft; Writing—review & editing. **Sandrine Massarin, Viola Huck**: Investigation. **Michael Bayerle, Denis Pittois**: Investigation; Validation. **Christian Braun**: Software.

Data Availability Statement—All original data that supported the study and which are not documented in the Supporting Information are available on demand from the authors (tom.galle@list.lu; roberta.carafa@aecom.com).

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