APPLICATION OF AN INDUCED FLUOROMETRY-BASED METHOD IN ALGAL GROWTH INHIBITION TESTS

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Abstract

Aquatic ecosystems are strongly exposed to various micropollutants from agricultural origin. The harmful effect can be expressed directly on aquatic organisms and indirectly through the food chain. The use of ecotoxicity assays mainly in aquatic environments, and corresponding water quality assessment are undoubtedly important. Project Aquafluosense was designed to develop instrument prototypes of a fluorescence-based setup for *in situ* measurement of algal biomass and for application of flurescence in ecotoxicity assays. Fluorescence-based determination of algal density was validated by conventional methods and signals obtained by the fluorometer correlated well with the conventional methods for algal density determination. The applicability of the fluorometer developed was demonstrated in ecotoxicity assays using the herbicide active ingredient isoxaflutole in neat and formulated forms.

Introduction

The composition and quantity of the algal biomass are essential indicators of quality and ecological status of natural water bodies and aquatic ecosystems. Micropollutants of agricultural origin, including pesticide active ingredients, co-formulants, mycotoxins and fertilizers, can enter the surface water system by leaching and runoff, and can directly and indirectly trigger adverse effect on aquatic organisms and human health via contamination of the drinking water base [1].

Algal biomass, as an indicator of water quality and ecotoxicological effects, can be measured by several techniques. The most commonly applied methods include the determination of algal cell numbers by microscopy and optical density (OD) by spectrohotometer, measurement of dry mass and the main photosynthetic pigment content of algal cells [2]. The primary source of endogenous fluorescence in algae is the induced fluorescence signal by chlorophylls responsible for photosynthesis [3]. Thus, efficiency of photosynthesis can also be characterized by fluorescence induction kinetics describing changes in the photosynthetic process and the physiological state of algal cultures [4]. Monoculture of various microalgal species (e.g. *Raphidochelis subcapitata*) are often used as test organisms in ecotoxicity assays to determine side effects of agricultural pollutants.

Isoxaflutole (5-cyclopropyl isoxazol-4-yl-2-mesyl-4-trifluoromethylphenyl ketone) is an active ingredient in several commercially available herbicidal formulations for preemergence control of weeds. The mode of action of isoxaflutole is to inhibit the enzyme 4-hydroxyphenylpyruvate dioxygenase. As a pigment inhibitor, it blocks the biosynthesis of carotenoid pigments, which protect chlorophyll from decomposition by sunlight. Thus, chlorophyll pigments are photo-oxidized, chloroplasts break down, and as a result the entire plant necrotizes and eventually dies

[5,6]. Isoxaflutole was first registered for use on corn in 1999, and it was also registered in 2020 for use on genetically modified (GM), herbicide-resistant soybean. The US Environmental Prottection Agency is presently considering an application to register isoxaflutole use on herbicide-resistant GM cotton [5,7]. With introduction of GM crops in agricultural practices, the application rate and the amount of the corresponding active ingredient increases, as the word-wide application of glyphosate on glyphosate-resistant GM crops showed [8]. Due to its increasing use, isoxaflutole has been detected, as emerging pollutants, in surface waters together with its metabolites of diketonitrile and benzoic acid [9], and it has been classified as a persistent water pollutant substance [5].

The aim of this study was to determine the ecotoxic effect of isoxaflutole herbicide active ingredient by the conventionally applied guideline for growth inhibition and by using the newly developed instrument for investigating the biomass and the efficacy of the PSII photocemical system of microalgae. The instruments were developed in the frame of project Aquafluosense (NVKP_16-1-2016-0049). The project strategy applied induced fluorescence as a signal to be detected, and utilize it for a range of purposes in water quality analysis [10,11]. One of the purposes is ecotoxicity assessment of plant protection products (PPPs). PPPs contain, in addition to their active ingredients, various additives, co-formulants, which are considered to be inert from the point of view of the main biological effect of the PPP. Nonetheless, numerous studies have confirmed higher toxicity of PPP formulations than of the active ingredients themselves, suggesting additive or synergistic effects between the active ingredients and the additives in different formulations [12]. In the present work we report the use of our novel fluorometer prototype detecting algal density in water for the assessment of algal toxicity of agrochemicals.

Experimental

The model green alga species, *R. subcapitata*, Korshikov (NIVA-CHL1) was obtained from public collections. Monocultures of test species were maintained and diluted in Z8 medium [13]. Ecotoxic effects of isoxaflutole (Figure 1) and its formulated PPPs (Merlin Flexx and Merlin 750 WG) were determined by the OECD 201 guideline [14] and by the newly developed fluorescence-based instrument. The active ingredient content for Merlin Flexx and Merlin 750 WG are 20.3% and 75%, respectively. Beside the active ingredient, both PPPs contain co-formulant agents at different concentrations (Table 1).

Table 1. Co-formulant agents in isoxaflutole-based plant protection products (Merlin Flexx and Merlin 75 WG) investigated in this study.

Plant protection product			
Merlin Flexx		**Merlin WG**	
co-formulant	**concentration (%)**	**co-formulant**	**concentration (%)**
cyprosulfamide	20.3	kaolin	5 – 15
benzisothiazol-3(2H)-one	0.005 – 0.05	other ingredients (non-hazardous) to 100%	10 – 20
alkyl polyglycoside	1 - 5		

The response of the guideline applied is the reduction of growth in a series of algal cultures exposed to different concentrations of test substances. Growth and growth inhibition are quantified from measurements of the algal biomass with determination of optical density and chlorophyll-a-content by spectrophotometer (UV/VIS Camspec single beam M330, Camspec,

Crawley, United Kingdom). For the quantification of chlorophyll content the Felföldy-formula was applied [15].

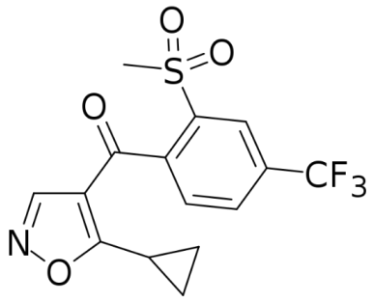

Figure 1. Chemical structure of the herbicide active ingredient, isoxaflutole.

For the determination of adverse effect of test substances on PSII photochemical system and vitality index, the newly developed, fluorescence-based instrument, FluoroMeter Module (FMM) was applied. The FMM [16] was also equipped with an instrument capable of accommodating standard size 96-well microplates (Figure 1b). The FMM instrument allows the recording of conventional Kautsky induction kinetic curves, which were simultaneously detected at two maxima of Chl-a fluorescence. The instrument was equipped with laser diode (10 mW) as excitation source providing excitation wavelength of 635 nm. Detection was performed at 690 nm ($\Delta\lambda = 10$) and 735 nm ($\Delta\lambda = 10$). For the instrument, a 96-well microplate holder was developed that allows parallel determination of samples. The photochemical efficiency of the algae photosynthetic system PSII (Fv/Fp, where Fv refers to the variable fluorescence, while Fp refers to the peak of the fluorescence) and changes in the fluorescence decrease ratio (Rfd= Fq/Fs, where Fs is the observed steady-state fluorescence and Fq is the fluorescence quenching capacity calculated as Fp-Fs) characterized the photosynthetic activity and vitality index, respectively [17].
Validation of fluorescence-based method was performed by comparison with other methods and correlation coefficients (R^2 value) were determined. A three-fold dilution series of six concentrations of green *R. subcapitata* were used for quantitative measurements. OD, chlorophyll content and cell concentration with Bürker chamber were measured for each concentration, together with the determination of the fluorescence signal intensities using the FMM. ODs were measured at 750 nm with a CAM-Spec.M33 UV–visible spectrophotometer. Chlorophyll was extracted and measured following the method of Wetzel et al. [18]. Samples were placed in a Bürker chamber and cell numbers were counted using a Nikon Labophot 2 microscope at 20x and 40x magnifications.

Results and discussion

Ecotoxicity assays were performed to determine the effects of isoxaflutole and its formulated products on the growth of *R. subcapitata* by measurement of OD and chlorophyll-a content in the algal growth medium. The same trend in toxicity was determined for both parameters that are recommended by the OECD 201 guideline. Isoxaflutole was determined as the most toxic component (EC_{50}OD = 0.034 mg/L). Co-formulants in Merlin Flexx (cyprosulfamide and 1,2-benzisothiazol-3(2H)-one) decreased the toxic effect of the active ingredient by nearly over 200-fold (EC_{50}OD = 33.7 mg/L), as the isoxaflutole-equivalent EC_{50} value was 6.84 mg/L. An EC_{50}OD value of 0.20 mg/L was determined for 1,2-benzisothiazol-3(2H)-one, while cyprosulfamide, used as a herbicide safener for isoxaflutole, did not exert algal toxicity below its OECD limit test value (100 mg/L). For Merlin 750 WG an EC_{50}OD of 0.64 mg/L was

determined indicating a somewhat weaker (one order of magnitude) decreasing effect on the toxicity of isoxaflutole by the additives (e.g. kaolin) present.
Determination of two photochemical parameters (Fv/Fp - photochemical efficiency of PSII photochemical system and Rfd – vitality index) were determined by the newly developed fluorescence-based instrument. Both parameters characterize the functioning of the PSII photochemical system in green plants. The Fv/Fp parameter was not found to be good in ecotoxicity studies. However, EC_{50} values of the Rfd parameter EC_{50} were determined to be 0.015 mg/L, 27 mg/L and 0.6 mg/L for isoxaflutole, Merlin Flexx and Merlin 750 WG.
For validation, results showed strong correlation among different methods applied for determination of algal biomass. The correlation of results obtained with FMM correlated with OD determination, Bürker-chamber cell counting and Chlorophyll-a-content by ethanol at the R^2 values of 0.98, 0.98 and 0.99, respectively.

Conclusion

Project Aquafluosense provided a fluorescence-based instrument appropriate for ecotoxicological assays on algae test species. Fluorescence-based determination of algal density and application of Rfd (vitality index) as endpoint in ecotoxicity assay was validated by conventional methods (Bürker chamber cell counting, optical density measurement, chlorophyll extraction with ethanol), and signals obtained by the fluorometer correlated well with the conventional methods for algal density. Isoxaflutole herbicide active ingredient and its formulated PPPs (Merlin Flexx and Merlin 75 WG) exerted harmful effect on algal growth. The most toxic effect was determined for neat isoxaflutole (EC_{50}OD=0.034 mg/L). Co-formulants in formulated products increased the effect of the active ingredient, however there occurred a significant difference between the products. The difference in amount and type of co-formulants resulted in difference in ecotoxic effect.

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