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Phytochemical Biomarkers in Eight Aquatic Plant Species Exposed to Metsulfuron-Methyl

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Herbicides are frequently detected in surface waters associated with agriculture (Gilliom et al. 1999). One of the most potent herbicides is the sulfonylurea, metsulfuron-methyl, which has been shown to have a 5% Hazard Concentration (HC_5) for a macrophyte community of < 10 ng L^{-1} (Cedergreen et al. 2004a). This is below the detection limit for most chemical analyses, which at best is around 10 ng L^{-1} (Køppen and Spliid 1998). Concentrations close to the HC_5 are expected to induce physiological and thereby phytochemical changes (biomarkers) in plants. Exposures to low metsulfuron-methyl concentrations may therefore be detected by the use of biomarkers before morphological changes are noted. Previous papers have discussed biochemical changes in plants after exposure to sulfonylurea. One of the biomarkers used to detect the effect of acetolactate synthase (ALS) inhibitors is 2-aminobutyric acid, which is a sensitive biomarker in potatoes under field conditions (Lober et al. 2002). Lydon and Duke found that sulfonylureas increase the level of cinnamate derived phenolic compounds (Lydon and Duke 1989). Other sulfonylurea biomarkers as anthocyanins increased in concentration in soybean seedlings four days after herbicide application together with an eightfold increase in extractable phenylalanine ammonia lyase (PAL) activity (Suttle and Schreiner 1982). In aquatic plants and algae levels of glutathione S-transferase (GST) have proved to increase significantly in response to both herbicides and other toxic compounds, but not consistently in all species (Pflugmacher et al. 2000a and 2000b). To get a more consistent response across species, it has been suggested that a larger battery of biomarkers would be necessary (Who and Peterson 1994).

High Performance Planar Chromatography (HPPC) is a method that relatively quickly and inexpensively can test for the presence and abundance of a large range of phytochemical compounds.

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MATERIALS AND METHODS

In this study, we tested eight aquatic plant species grown under similar conditions and exposed to a range of metsulfuron-methyl concentrations in six HPPC-systems. This was done in order to test the variability of a large number of different biomarkers.

The aquatic plant species used were collected in streams and ponds of Northern Sealand: *Lemna trisulca, Spirodela polyrrhiza, Elodea canadensis, Myriophyllum spicatum, Ceratophyllum dermersum, Ceratophyllum submersum* and *Sparganium emersum. Lemna minor* from an aseptic culture was also used.

The plants were grown for two weeks in a flow-through set-up at a day/night water temperature of 18°C/15°C, a photoperiod of 16 hours, irradiance of 140-220 µmol m^{-2} s^{-1} (PAR) and concentrations of metsulfuron-methyl of: 0, 0.01, 0.10, 1.0, 10, 100 and 1000 µg/L. For details see Cedergreen et al. (2004b). After a period of 14 days, all the plants were harvested, freeze-dried and weighed. The plant material was used for phytochemical screening.

All chemicals and solvents used were of analytical grade and were obtained from Merck, VWR-International (Albertslund, Denmark). Standards and reagents were obtained from SIGMA-ALDRICH (Copenhagen, Denmark) and ACROS, Bie & Berntsen A/S (Rødovre, Denmark).

The plant extracts were prepared using crushed freeze-dried plant material. Fifty mg plant material per ml 75% ethanol was extracted for 2 hours in an ice-cooled ultrasonic bath (Bransonic®, Danbury, USA). The extracts were centrifuged in a Micro Centrifuge (Capsule, Japan) at 6400 rpm for 10 minutes and immediately used for analysis. Extracts of the plants were placed on the Thin Layer Chromatography (TLC) plates 1.5 cm from the bottom of the plate using disposable Blaubrand® (IntraEnd, Wertheim, Germany) micropipettes in amounts of 5µl (plate A) and 10µl (plate B), both with ± 0.5% accuracy. The TLC plates were eluted in CAMAG flat bottom TLC chambers (Muttenz, Switzerland) containing 50ml solvent. One-dimensional TLC was performed until the solvent front was 0.5 cm from the top of the TLC-plate. The TLC-plates were then air-dried for one hour before derivatization.

Several High Performance Planar Chromatography (HPPC) systems were used. Each HPPC-system is composed of a TLC plate (20 cm x 10 cm), a chromatographic solvent, and a derivatization reagent. Two combinations of TLC plates and solvents were used while the derivatization reagents varied between the six test systems. For TLC plate A made of cellulose on TLC aluminum sheets (No. 1.05552 Merck) solvent 1 was used. Solvent 1 consisted of n-butanol and 50% formic acid (in water) in a ratio of 2:1. For TLC plate B made of silica gel 60 F_{254} on High Performance Thin Layer Chromatography (HPTLC) aluminum sheets (No. 1.05548 Merck), solvent 2 was used. Solvent 2 consisted of n-butanol, acetic acid and water in a ratio of 4:1:5 (using upper phase, 5 minutes shaking). The derivatization reagents were modified from Krebs et al. (1969).

HPPC-System A: TLC plate B, solvent 2 and the derivatization reagent freshly prepared from one part 2% vanilin in ethanol (96%) mixed with nine parts sulphuric acid (50%) in ethanol (96%). After derivatization the TLC plate was heated at 120°C for 2-3 min on a CAMAG TLC Plate Heater III (Muttenz, Switzerland). Higher alcohols, phenolic compounds, steroids, etheric oils, terpenoids and phenyl propanoids are detected as spots in various colors (Krebs et al. 1969).

HPPC-System B: TLC plate B, solvent 2 and the derivatization reagent consisting of 1% diphenyl boric acid 2-aminoethylester in 5% polyethylenglycol [in ethanol (96%)]. Phenolic compounds, such as α- and β-pyrones (hydroxyflavonols) (Krebs et al. 1969), flavanoids, aloins, anthrones and anthranols (Wagner et al. 1984) are detected as yellow, blue or green fluorescence spots on a dark blue plate.

HPPC-System F: TLC plate B, solvent 2 and a derivatization reagent consisting of 10% molybdatophophor acid in ethanol (96%). The TLC plate was heated to 120°C until visualisation of the compounds. The plate is placed in ammonia vapour for 5 min. Lipids, sterols, gallus acids, lipid acids, triglycerids, substituted phenolic compounds, indol-derivates, prostaglandins, etheric oils and alkaloids are detected as blue spots on a white plate (Krebs et al. 1969).

HPPC-System I: TLC plate B, solvent 2 and a derivatization reagent consisting of anisaldehyde reagent (0.50ml *p*-anisaldehyde mixed with 10 ml acetic acid added to 85ml ethanol (absolute), followed by 5ml sulphuric acid). The TLC plate was heated to 100°C for 5 min. Sugars, steroids, terpenes, etheric oils and saponins are detected as spots in various colors (Krebs et al. 1969).

HPPC-System R: TLC plate B, solvent 2 and a derivatization reagent consisting of 10% silver nitrate in ethanol (96%). After derivatization the TLC plate was heated to 105°C for 7 min. Carbohydrates and reducing sugars are detected as brown spots on a white plate (Krebs et al. 1969).

HPPC-System C: TLC plate A, solvent 1 and a derivatization reagent consisting of 0.5 % ninhydrin and 3% acetic acid in ethanol (96%). After treatment the plates was heated 110°C for 2 min. Spots were stabilized by a reagent prepared from 1.0 ml saturated aqueous copper (II) nitrate solution with 0.2 ml 10% nitric acid and 100 ml ethanol (absolute). Amino acids, amines and amino sugar compounds are detected as red, violet and yellow spots on a light red plate (Krebs et al. 1969).

For the detection of biomarkers, the plates were photographed using CAMAG VideoStore 2 Version 3.00 software using CAMAG Ultra Violet (UV) Lamp (254 nm, 366 nm and white light), a CAMAG Reprostar 3 with a HV-C20A Hitachi camera fitted (Canon TV zoom lens 11.5-69 mm). CAMAG VideoScan Programme Version 1.01 software was used for computer analysis. R_f-values were calculated as $R_f = D_1 D_2^{-1}$, where D_1 is the spot centre from the start point and D_2 is the distance of the solvent front from the start point (Gänshirt 1969). The

intensity of the compounds was evaluated from the computer- calculated area. Single compound tests have shown that the computer-calculated area relates to the concentration of a compound in a linear manner (correlation coefficients > 0.98) within the used area-range (obeying Lambert-Beers law for dissolved substances).

With respect to the interpretation of the results, the same biomarkers can be detected in several of the HPPC-Systems A, B, F, I and R. The same R_f-value will then be registered. In this case the different chemical reagents in the HPPC systems will detect the biomarkers, which have different chemical reactive groups. However, it may also be that two different biomarkers are detected at the same R_f-value. Since the HPPC screening system methods were not optimal for all the compounds detected in the different plant species, the compounds may be hidden behind each other and will therefore not be detectable with all the reagents used in this study. For the HPPC-system with the derivatization reagent C (ninhydrin), free amino acids are primarily detected. Similarity studies, comparing the R_f-values and the color of pure standards of amino acids with the detected biomarkers indicate which amino acid is detected as a biomarker.

Significant increases or decreases in phytochemical compounds were evaluated statistically using Pearsons correlation coefficients between the estimated compound concentration and log metsulfuron-methyl dose with control set to 0.001 $\mu g\ L^{-1}$ ($p < 0.05$) (Sokal and Rohlf 1995).

RESULTS AND DISCUSSION

Around 60 to 100 phytochemical compounds were detected in each species investigated. Most concentrations of the compounds in the plants were unaffected by metsulfuron-methyl compared with the control. Only compounds where a change was observed visually and computer calculated to be ± 15% of control are reported in this paper as biomarkers. Eigthy-nine different biomarkers were changed significantly in response to metsulfuron-methyl detected in the HPPC systems. However, the consistency in the changes of compounds across species was very small (Table 1 and 2). The HPPC system that detected the most biomarkers was system C followed by, B, R, F, I, and A.

Fifty-three biomarkers including the amino acids (Table 1 and 2) showed a significant increase or decrease in the eight tested aquatic plant species. In table 1 only biomarkers with correlation coefficient >0.50 are included. Significant correlation coefficients are marked with bold ($p < 0.05$). Subscribt numbers mark the metsulfuron-methyl exposure concentration in µg/L where the biomarker was first detected. If no subscript number is marked, the biomarker was detected from the lowest concentration 0.01 µg/L. For *L. trisulca* no biomarkers with a correlation coefficient of >0.50 were detected. The R_f-value represents the migration distance of a biomarker on the (Thin Layer Chromatography) TLC-plates.

Some of the biomarkers had similar R_f-values in different test systems. It is therefore likely that the same compounds were detected. The derivatization

reagents in the different HPPC systems detect either different reactive groups presented in the same biomarker or different compounds with the same migration on the TLC plate. However, the biomarkers have to be isolated and identified to verify this.

Considering that metsulfuron-methyl was expected to affect the same biosynthetic pathway in all plants, a closer consistency in the detected biomarkers across species was expected. However, this aspect of different reaction patterns has also been detected for several other terrestrial plant species exposed to the same herbicide (Ravn, unpublished data). The variance in toxicity between species expressed as the EC_{50} on specific leaf area (SLA) was relatively small (between 0.10 ± 0.09 µg/L in *Lemna minor* and 2.21 ± 1.83 µg/L in *Ceratophyllum submersum*) (Cedergreen et al. 2004b). *Sparganium emersum* was the only species that did not exhibit a significant growth or morphological response towards the metsulfuron-methyl treatment (Cedergreen et al. 2004b), but the changes in biomarkers were not different from the other species (Tables 1 and 2). Hence, despite the lack of response in growth during the experimental period, *S. emersum* was physiologically affected by the herbicide. Thus, the missing growth response was not due to the plant not taking up the herbicide, but was either caused by efficient detoxification of the herbicide or by a delayed growth response not detected within the experimental time.

The aquatic plant species tested in this study come from taxonomically different groups and sulfonylurea metabolism has been shown to vary between even more closely related crop species as barley, rice and wheat (Cobb 1992). Hence, the large variation in biomarkers between species could be related to species specific differences in detoxifying metsulfuron-methyl. Looking at a broad spectrum of plant metabolites it is likely that some phytochemicals behave in a consistent way across species in response to a metsulfuron-methyl exposure. On the other hand, metsulfuron-methyl is a selective herbicide and plants can inactivate the compound or overcome stresses in different ways (Cobb 1992). Our results do not exclude that consistent patterns across species do exist, but this was not detected in this study.

The amino acid biomarkers are presented in Table 2. The biomarker matching the standards for leucine and isoleucine decreased significantly in three species (Table 2). As metsulfuron-methyl inhibits the synthesis of leucine and isoleucine together with valine (Cobb 1992), this was expected. Theoretically a decrease in leucine and isoleucine in the other plant species was also expected, but not seen in this study. The reason could be either undetectable concentrations of the extracted amino acids or interference between the amino acids and other phytochemical compounds. Subsequent extraction studies have proved that a 10% ethanolic extraction solvent is much better for extraction of the amino acids than 75% ethanolic solvent as used in this study (Ravn, unpublished data). Other studies have shown a general increase in the pool of non-branched chain amino acids when plants were exposed to ALS-inhibitors (Royuela et al. 1991).

Table 1. Pearsons correlation coefficients on the estimated concentration of the biomarkers as a function of log dose metsulfuron-methyl for eight aquatic plant species.

Rf-value	Colour of spot	Rea-gent	*L. minor*	*S. polyr-rhiza*	*E. canad-ensis*	*M. spicat-um*	*C. subm-ersum*	*C. dem-ersum*	*S. emer-sum*
0.07	Green	A						**0.97**	
0.07	Green	I						**0.92**	
0.08-0.14	Brown	R		0.57	**0.88**	**0.93**	**0.92**		
0.17-0.19	Green	A					**0.85**		
0.18	Brown	R				0.57			
0.25-0.30	Blue	F	**-0.96**		**0.84**				0.74
0.28	Blue	A	**-0.96**						
0.30-0.38	Brown	R			0.79	**-0.84**			
0.32	Yellow	B	**-0.92**						
0.35	Yellow	B	-0.77						
0.35	Violet	I	**-0.93**						
0.36	Green	F	**-0.92**						
0.37	Blue	B							**-0.89**
0.40	Blue	F	**-0.86**					**-0.89**	
0.41	Blue	A			0.75				
0.44	Green	I	**-0.84**						
0.48-0.51	Yellow	B				**-0.98**			0.72
0.49-0.53	Brown	R	**-0.91**						**-0.94**
0.53-0.55	Blue	B	**-0.81**	-0.60		**-0.81**		0.67	**-0.83**
0.54	Blue	F	**-0.94**			**-0.84**			
0.54	Green	I	**-0.94**						
0.60-0.64	Blue	F	**-0.94**			-0.82[1]			
0.60-0.65	Brown	R	**-0.93**			**-0.84**			**-0.84**
0.60-0.66	Green	I	**-0.98**				**0.86**		
0.66-0.76	Yellow	B	**-0.84**			**-0.91**			-0.73
0.70	Brown	R							**-0.94**
0.71	Blue	F		**-0.82**					
0.80-0.82	Blue	F	-0.79	**-0.90**					
0.82-0.83	Yellow	B	**-0.87**			**-0.87**			
0.82-0.84	Green	I	**-0.93**						**-0.88**
0.91	Blue	A	**0.89**						
0.91	Blue	B							0.80
0.95	Blue	A	0.80						
0.96	Green	I							-0.66
0.99	Brown	R							-0.72

Table 2. Pearsons correlation coefficient on the estimated concentration of amino acids as a function of log dose metsulfuron-methyl for eight aquatic plant species.

Rf-value*	Colour	Suggested amino acids	*L. minor*	*L. trisulca*	*S. polyrrhi-za*	*E. canaden-sis*	*M. spicatum*	*C. submer-sum*	*C. demer-sum*	*S. emer-sum*
0.12	Violet	Histidine	**-0.84**							
0.17	Red	Arginine	**0.94**[1]			-0.94[10]				
0.20-0.23	Red	Glutamine	**-0.85**		-0.68[1]				-0.55	
0.25-0.26	Red	Glycine	**0.94**[1]				0.72			
0.27-0.28	Red	Aspartic acid Trans-4-hydroxyproline Glutamic acid	**-0.84**		-0.74[1]					
0.36-0.40	Red	Threonine								0.60
0.45	Red	Alanine			**0.92**					-0.94
0.46-0.49	Yellow	Proline						**-0.83**		
0.51-0.52	Red	-	0.76[10]							
0.56-0.63	Red	Methionine			**0.93**		-0.78			
0.73-0.77	Red	Leucine/ Isoleucine		**-0.89**		**-0.97**	**-0.91**			

*The Rf-value represents the migration distance of the amino acids on the (Thin Layer Chromatography) TLC-plates. Only biomarkers with correlation coefficient >0.50 are included. Significant correlation coefficients are marked with bold. Subscript numbers mark the metsulfuron-methyl exposure concentration in µg/L where an amino acid was first detected. If no subscript number is marked the biomarker was detected from the lowest concentration 0.01 µg/L. The identity of the amino acid was suggested from comparisons with standards.

Though the concentration of some of the detected amino acids in this study did increase significantly with increasing metsulfuron-methyl exposure, just as many were decreasing (Table 2). In the study on the effect on metsulfuron-methyl exposure on growth and morphology made on the same plants, the most consistent trait across species was the change in specific leaf area, which decreased with increasing metsulfuron-methyl exposure (Cedergreen et al. 2004b). This decrease may be related to starch accumulation in the leaves of the plants (Royuela et al. 1991). For the HPPC-system R (Table 1) detecting carbohydrates and reducing sugars, the compound with a R_f-value of 0.08-0.14 increased in half of the plant species, confirming the proposed increase in starch and sugars.

This study showed that there is a large inter-specific difference in the phytochemical composition and phytochemical response among aquatic plant species exposed to metsulfuron-methyl growing under the same environmental conditions. The biomarkers of a plant species exposed to metsulfuron-methyl have to be evaluated individually or together with plant species, which have the same biochemical response to the herbicide if the presented method should be used to detect metsulfuron-methyl exposure. However, a suitable model-plant could be *Lemna minor*, which showed highly significant changes in a large number of compounds and biomarkers in response to the metsulfuron-methyl exposure (Table 1 and 2). To do this, the pattern of biomarkers should be mapped and the sensitivity of herbicide exposure to the plant species under varying growth conditions should be estimated. *Lemna minor* is a sensitive test plant as biomarkers were detected at concentrations as low as 0.01 and 0.10 µg/L.

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