Advancements in effect-based water quality assessment

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CHAPTER 4

NATIONWIDE SCREENING OF SURFACE WATER TOXICITY TO ALGAE

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ABSTRACT

According to the European Water Framework Directive (WFD), chemical water quality is assessed by monitoring 45 priority substances. However, observed toxic effects can often not be attributed to these priority substances, and therefore there is an urgent need for an effect-based monitoring strategy that employs bioassays to identify environmental risk. Algal photosynthesis is a sensitive process that can be applied to identify the presence of hazardous herbicides in surface water. Therefore, the aim of this study was to employ an algal photosynthesis bioassay to assess surface water toxicity to algae and to identify the compounds causing the observed effects. To this end, *Raphidocelis subcapitata* was exposed to surface water samples and after 4.5 h photosynthetic efficiency was determined using PAM fluorometry. In this rapid high throughput bioassay, algal photosynthesis was affected by surface water from only one of 39 locations. Single compounds toxicity confirmation elucidated that the observed effect could be solely attributed to the herbicide linuron, which occurred at 110 times the EQS concentration and which is not included in the WFD priority substances list. In conclusion, applying the algal photosynthesis bioassay enables more efficient and effective assessment of toxicity to primary producers because it: (i) identifies the presence of herbicides that would be overlooked by routine chemical WFD monitoring, and (ii) avoids redundant chemical analyses by focusing only on (non-)target screening in samples with demonstrated effects.
INTRODUCTION

According to the European Union (EU) Water Framework Directive (WFD), chemical water quality is determined by monitoring surface waters for the presence of 45 (groups of) priority substances. However, the use of many of these compounds is restricted or banned, and concentrations of priority substances in European waters are, therefore, decreasing. Simultaneously, industries have switched to a plethora of thousands of alternative compounds, which potentially enter aquatic environments and can severely impact water quality. Hence, many substances on the priority list are not representative of present day contamination. Consequently, a large portion of toxic effects observed in surface waters cannot be attributed to compounds measured by water authorities, and toxic risks to freshwater ecosystems are thus caused by myriads of (un)known, unregulated and unmonitored compounds that are present in the environment. Understanding of these risks requires a paradigm shift, that allows for new monitoring methods that do not depend on chemical target analysis of priority compounds, but contrastingly consider adverse biological effects first. Therefore, there is a need for an effect-based monitoring strategy that employs bioassays to identify environmental risk. Bioassay responses to surface water samples are caused by mixtures of all bioavailable (un)known compounds and their metabolites, thereby overcoming the limitations posed by chemical analysis of a limited number of target compounds. The indication of surface water toxicity by bioassays in turn allows for identification of locations with environmental risks, although the compounds responsible for the observed toxicity are initially unknown. However, these can subsequently be elucidated with targeted or non-target chemical analysis, which will only be necessary for locations with indicated environmental risk.

The success of this approach will rely largely on the ease of use, endpoint specificity and scale of the selected bioassays. In vitro or small scale in vivo assays with specific drivers of adverse effects allow for focused identification and subsequent confirmation of toxic compounds. Adequate selection of bioassays employed in water quality monitoring can thus greatly aid in narrowing down the identification of compound(s) that cause environmental risks. Microalgal photosynthesis is an example of a sensitive and well-studied bioassay endpoint that can be applied to identify hazardous effects of herbicides in surface waters. In these bioassays photosynthesis is often quantified using pulse amplitude modulation (PAM) fluorometry, a rapid measurement technique suitable for quick screening purposes. Algal photosynthesis is preferably quantified in light adapted cells as effective photosystem II (PSII) efficiency (ΦPSII). This end point responds most sensitively to herbicide activity, as the most commonly applied herbicides either directly target PSII, or indirectly affect ΦPSII.

Herbicides are the most frequently detected pesticide group in North American and European surface waters, and are hence expected to have a significant effect on aquatic ecosystem functioning. Moreover, a wide variety of herbicides often exceed environmental quality standards (EQS) in European surface waters. Herbicides can be phytotoxic to non-target aquatic organisms such as algae, and effects on primary producers can cascade up
the food web altering community structure. Algae respond quickly to environmental changes, thus making identification of locations where algae are affected of great ecological importance, while simultaneously functioning as an early warning system for herbicide induced ecosystem changes. Triggered by the need to identify these herbicide induced risks to algae in surface waters, the aim of the present study was to employ an algal photosynthesis bioassay that allows for screening of surface water toxicity to algae and subsequent identification of the causing compound(s) on a nationwide scale. To this end, the microalga *Raphidocelis subcapitata* was exposed to surface water samples in 96-well plates. After 4.5 h, previously shown to be a sufficient exposure time for stable effect determination, effective ΦPSII was determined using PAM fluorometry connected to an autosampler, resulting in a rapid high-throughput bioassay. Inhibitory effects on ΦPSII of surface water samples from 39 locations were assessed, and chemical analysis at the location with observed toxicity was performed to elucidate responsible compounds. For accreditation of compound contribution to the observed toxic effect, subsequent toxicity tests with individual suspected compounds were carried out.

**MATERIALS AND METHODS**

**Sample collection**

Water grab samples were collected at 39 locations within the Netherlands during May, June and July 2016 (Figure 4.1). Locations were provided by the Dutch water boards and only partly originated from their regulatory monitoring networks, resulting in a scattered availability of chemical and ecological quality scores for the sampling sites. The time of sampling was chosen because late spring and early summer are relevant periods for agricultural pesticide application in The Netherlands. Water was collected in 1 L polypropylene (PP) bottles and filtered through pre-combusted (100 °C, to avoid sorption of contaminants to carbon residues on the filters) 1.2 μm glass fiber filters (GF/C Whatman) in the laboratory to eliminate autochthonous microalgae and stored overnight in the dark at 4 °C until bioassay analysis.

**Test species and culturing conditions**

The freshwater green microalga *Raphidocelis subcapitata* CCAP 278/4 (form. *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*) was used as test species in all bioassays. This species was selected as it is recommended as a sensitive species in regular OECD test guidelines for algal toxicity testing and it has been shown to respond sensitively to herbicides relative to several other green microalgal species. Batch cultures of *R. subcapitata* were maintained in 300 mL Erlenmeyer flasks in 200 mL algal growth medium (BG-11 medium, Sigma-Aldrich) under constant aeration with compressed air at 20 °C with 16:8 h light-dark regime (~100 μmol m⁻² s⁻¹, Sylvania BriteGro F58W/2084-T8). Algae inoculum was prepared 2–4 days prior to bioassay analysis to ensure exponential growth at the start of the test.
PAM bioassay and surface water toxicity

Water samples were tested for herbicide activity in a short-term algal bioassay using PAM fluorometry adapted from Sjollema et al. (2014).71 *R. subcapitata* were exposed to water samples in black PP 96-well plates (Greiner Bio-One) with a final volume of 280 μL and at a cell density of $1 \times 10^7$ cells/mL, and ΦPSII was determined after 4.5 h of incubation. Per location, algae were exposed to surface water in six separate wells, hence surface water effects were determined six replicate times. Cell densities were determined using a CASY Counter TT (Roche INNOVATIS) at the start of the experiment. To expose algae to undiluted surface water at the proposed cell density, algae were taken from the inoculum and centrifuged at 2500g. Subsequently, the cell pellet was washed twice with Dutch standard water (DSW, deionized water with 200 mg/L CaCl$_2$·2H$_2$O, 180 mg/L MgSO$_4$·H$_2$O, 100 mg/L NaHCO$_3$ and 20 mg/L KHCO$_3$; hardness is 210 mg as CaCO$_3$/L and pH 8.2 ± 0.2), centrifuged again and resuspended in surface water. To investigate the potential confounding effects of nutrient concentration differences between samples and to avoid nutrient deficiency during analyses, all samples and control treatments were tested with and without added nutrients. Nutrients were added as 50× concentrated BG-11 medium. Plates were incubated under continuous light (~50 μmol m$^{-2}$ s$^{-1}$, Sylvania BriteGro F58W/2084-T8) for 4 h and 20 min. The final 10 min of incubation, as well as PAM measurements, were performed under actinic LED light (659 nm, ~45 μmol m$^{-2}$ s$^{-1}$). ΦPSII of algal suspensions was determined using a WATER-PAM (Fiber version, Heinz Walz GmbH).
Minimum and maximum fluorescence (F and F’_m, respectively) were determined and ΦPSII was calculated as \([F’_m - F]/F’_m\). Test validity required the minimum ΦPSII of unexposed control algae to be at least 0.250 and ΦPSII at a fixed concentration of atrazine (207 μg/L) to be 50 ± 10% of the negative control. Water samples were considered toxic if they caused ΦPSII inhibition >10% relative to the negative control, which was previously set as trigger value for this test.

Outliers (>1.5 interquartile range) were excluded from the dataset. All data were checked for normality using a Shapiro-Wilk test. Differences between treatments with and without nutrients were tested with t-test or Mann-Whitney U test. To compare ΦPSII of the water sample with the corresponding control, inhibition of ΦPSII by water samples was tested using ANOVA with TukeyHSD post-hoc test when normally distributed, and Kruskal-Wallis with Nemenyi post-hoc test when not normally distributed. Statistical analyses for the herbicide screening were performed using RStudio statistical software (RStudio Team) with \(\alpha = 0.05\).

**Chemical analysis**

Water samples that caused a response in the PAM bioassay were subjected to chemical screening for 151 commonly used pesticides (Table S1) at the laboratory of the water board of Fryslân using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), as well as gas chromatography coupled to mass spectrometry (GC–MS). Liquid chromatography (LC) was performed with untreated water samples on an Accela 1250 HPLC system (Thermo Fisher Scientific) equipped with a CTC autosampler. Chromatographic separation was performed on a Hypersil GOLD™ HPLC column (50 × 2.1 mm, 3 μm particle size, Thermo Fisher Scientific) preceded by a Hypersil GOLD aQ™ SPE column (20 × 2.1 mm, 12 μm particle size, Thermo Fisher Scientific) using a methanol/water system (Tables S2–4). Detection of compounds was performed on a TSQ Vantage triple quadrupole mass spectrometer (MS) equipped with an electrospray ionization source (Thermo Fisher Scientific). Target compounds were analysed in positive and negative modes. Identification of target compounds was based on selected reaction monitoring (SRM), and compound concentrations in the original samples were calculated based on multiple external standard calibrations. For gas chromatography (GC), compounds were extracted from water samples by liquid-liquid extraction using dichloromethane. Extracts were evaporated to 1 mL using a Kuderna-Danish concentrator under a constant flow of N₂ gas. Extracts were then analysed in duplicate by GC on a Trace GC system (Thermo Fisher Scientific) fitted with a DB-5MS fused silica column (30 m × 0.25 mm, 0.25 μm film thickness, J&W Scientific). Detection of compounds was performed on a dual stage quadrupole (DSQ) MS (Thermo Fisher Scientific) set to selected ion recording (SIR) mode. Identification of target compounds was based on retention time and three compound specific masses, one quantification mass and two masses for peak ratios, for confirmation of a compound. Identified compound concentrations in water samples were quantified with a five-point calibration curve, applying a correction for internal standard recovery for each compound of interest.
Single compound toxicity

Based on chemical analysis of the water samples, compounds suspected to have caused the observed toxicity were identified and subjected to toxicity testing. To this purpose, toxicity of the herbicides linuron and dimethenamid and the herbicide metabolite desethylterbuthylazine to algal photosynthetic efficiency was determined using the PAM bioassay. All tested compounds were obtained from Sigma-Aldrich and stock solutions were made in acetone, protected from light and stored at 4 °C (CAS numbers and stock concentrations in Table S5). Working stocks were prepared by evaporation of the appropriate volume of concentrated acetone stock solutions in a fume hood, followed by dissolution in DSW under continuous stirring to obtain solvent free working stocks. Toxicity experiments were performed in DSW according to the abovementioned method described for the surface water toxicity experiment. There were six test concentrations per compound, within the ranges listed in Table 4.1. A positive atrazine control and a negative control in DSW were included as well. There were eight replicate measurements per test concentration. It was decided to only measure the actual concentrations of those compounds contributing significantly to the observed toxicity of the field samples, which was the case for linuron.

To determine the actual linuron concentrations in the test medium, water samples were taken at the end of the 4.5 h exposure period. Per test concentration, the medium from all replicates was pooled into a single sample and centrifuged at 1.4 × 10^4 rpm to dispose of suspended algae. Subsequently 1 mL of supernatant was combined with 0.5 mL methanol and stored at −20 °C awaiting analysis. Linuron concentrations in the supernatant were measured at the laboratory of the University of Amsterdam using a HPLC system (Prominence UFLC-XR, Shimadzu) coupled to a tandem mass spectrometer (QTRAP 4000, Applied Biosystems). Chromatographic details of linuron analysis are provided in the Supporting Information. Of each sample 10 μL was injected. Blanks and controls showed no signal of linuron. Only the highest test concentration (1.5 mg/L) and spiking stock solution (5 mg/L) required 10-fold dilution with 50% methanol/DSW. The concentration of the stock solution was 101% of the nominal value, and the actual test concentrations were 82–90% of the nominal values (Table 4.1).

The log-logistic dose-response model described by Haanstra et al. (1985)126 was used to determine the 50% reduction (EC_{50}) in ΦPSII by the single compounds, and was calculated as $y = c / (1 + e^{b(log(x)−log(a)))}$, where $y$ is the ΦPSII, $x$ is the concentration of the toxicant (μg/L), $a$ is the EC_{50} value (μg/L), $b$ is the slope of the curve and $c$ is the ΦPSII of the control. EC_{50} values were calculated using SPSS statistical software (IBM SPSS Statistics 24).

RESULTS

Surface water toxicity

Water samples from 39 locations throughout The Netherlands were successfully screened for algal toxicity applying the short-term bioassay using PAM fluorometry. For these locations, the ΦPSII of unexposed control algae was always well above 0.25, and the ΦPSII of the positive control with atrazine was 50 ± 10% of the negative control, thereby meeting the validity criteria.
Table 4.1. Test concentrations in the algal toxicity tests with *R. subcapitata* and corresponding 4.5 h acute EC<sub>x</sub> concentrations and quality standards and surface water concentrations at a location with indicated surface water toxicity to algae.

<table>
<thead>
<tr>
<th>compound</th>
<th>toxicity test conc. range (µg/L)</th>
<th>surface water conc. (µg/L)</th>
<th>quality standard (µg/L)</th>
<th>4.5 h effect concentrations ΦPSII R. subcapitata (µg/L, 95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MAC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>linuron&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 – 1351</td>
<td>32</td>
<td>0.29</td>
<td>0.17</td>
</tr>
<tr>
<td>dimethenamid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.3 – 1000</td>
<td>3.7</td>
<td>1.6</td>
<td>0.13</td>
</tr>
<tr>
<td>desethylterbutylazine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 – 3000</td>
<td>0.12</td>
<td>38</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> actual concentration. <sup>b</sup> nominal concentration. <sup>c</sup> maximum acceptable concentration environmental quality standard (EQS) from the Dutch National Institute for Public Health and the Environment (RIVM). <sup>d</sup> annual average EQS from RIVM. <sup>e</sup> maximum acceptable risk concentration from RIVM in 2016.
Addition of nutrients did not result in differences in ΦPSII of algae exposed to water samples \( (p = 0.31) \) nor controls \( (p = 0.57) \) (Figure S1). Therefore, only tests with added nutrients were selected for subsequent analyses to ensure minimum variation due to confounding factors present in the water samples. After 4.5 h of exposure to the field samples, the ΦPSII of 38 locations remained within 10% deviation from the control, set as the trigger value for this test (Figure 4.2). The response of the algae to surface water from only one location, Sexbierum, strongly exceeded the bioassay trigger value, with a ΦPSII of 56% of the corresponding control (Figure 4.2). Subsequently, water samples from this location were subjected to chemical analyses and water samples were repeatedly collected from the same location during the three following weeks, to monitor the development of the observed effect over time.

**Pesticide screening of surface water samples**

The Sexbierum sample was chemically analysed for the presence of 151 commonly used pesticides. Two herbicides were present in concentrations exceeding the corresponding environmental quality standard (EQS) as stated by the Dutch National Institute for Public Health and the Environment (RIVM). The PSII inhibitor linuron exceeded the annual average EQS (AA-EQS) 188 times and the maximum acceptable concentration (MAC-EQS) 110 times. The long-chain fatty acid inhibitor dimethenamid exceeded the AA-EQS 28 times and the MAC-EQS 2.3 times (Table 4.1). For herbicide metabolites, no EQS existed at the time of sampling, but desethylterbuthylazine exceeded by 50 times the indicated maximum acceptable risk (MAR) concentration as stated by the RIVM and was hence included in subsequent toxicity determination. However, it was present below the EQS concentrations that in the meantime became available (Table 4.1). Desethylterbuthylazine is a metabolite of the PSII inhibitor

![Figure 4.2. Average \( (n = 6) \) ΦPSII (% of control) of *R. subcapitata* after 4.5 h of exposure to surface water samples from 39 field sites. Error bars represent standard deviation. Dashed lines represent the 10% deviation from the control which was set as trigger value for this test.](image)
terbuthylazine (present at 0.03 μg/L in the initial sample), used extensively in The Netherlands as the replacement product for the EU-wide banned herbicide atrazine, which is used as a positive control in the present study.

Additional water samples were taken at the Sexbierum location three times in the three weeks following the toxicity identification (7, 10 and 23 days after initial sampling) and were subjected to the PAM bioassay as well as chemical analyses (Figure 4.3 and Table S6). The concentrations of the three compounds that exceeded the quality standards in the initial sample all decreased over time (Figure 4.3). Desethylterbuthylazine was present above the MAR concentration in all but the last sample, in which it was not detected. Dimethenamid exceeded the EQS in the first two samples and was present below the EQS in the last two samples. The linuron concentration exceeded the EQS in all samples, however the exceedance decreased notably over time. Corresponding to the decreasing compound concentrations and quality standard exceedances, the negative effect of the water samples in the PAM bioassay also decreased over time. Nonetheless, the bioassay response to the last water sample still exceeded the trigger value, hence still indicating surface water toxicity to algae at the Sexbierum location three weeks after the initial toxicity identification.

Figure 4.3. Average (n = 6) $\Phi_{PSII}$ (% of control ± st. dev; black squares) of *R. subcapitata* after 4.5 h of exposure to water samples taken over a period of three weeks from the Sexbierum location with corresponding concentrations of the herbicides linuron (green dots) and dimethenamid (red inverted pyramids) and the herbicide metabolite desethylterbuthylazine (desethyl-TBA; blue pyramids) in μg/L.
Single compound toxicity

To investigate the contribution of the three compounds present at concentrations above the quality standards to the toxicity observed in the surface water samples, they were subjected to single compound toxicity tests. Dimethenamid caused no toxicity at the highest test concentration (1000 μg/L), far exceeding the concentration measured in the field (3.7 μg/L) (Table 4.1). Hence, contribution of dimethenamid to the observed toxicity was negligible. For desethylterbuthylazine a clear dose-response relationship was obtained (Figure 4.4a), from which the EC$_{50}$ was calculated to be 445 μg/L (95% C.I. 305.0–585.6). However, as the desethylterbuthylazine concentration in the initial surface water sample was only 0.12 μg/L, the contribution of desethylterbuthylazine to the toxicity observed in surface water samples was negligible. Indeed, when the EC$_x$ for this concentration was calculated using the log-logistic dose-response model this resulted in <0.1% inhibition of photosynthetic efficiency and thus in no exceedance of the trigger value of the test.

Also for linuron a clear dose-response relationship was obtained (Figure 4.4b), from which the EC$_{50}$ based on actual concentrations in the medium, was calculated to be 32.3 μg/L (95% C.I. 21.6–43.0). Hence, as the linuron concentration in the initial surface water sample was 32 μg/L and the corresponding ΦPSII 56%, the field concentration of linuron fully explained the toxicity observed in the surface water sample. Moreover, the toxicity observed in the samples taken in the weeks subsequent to the initial sample was also fully attributable to the linuron field concentrations. The contribution of linuron to ΦPSII inhibition in the samples is illustrated by plotting the bioassay response observed at the respective field concentrations in the dose-response graph in Figure 4.4b.

DISCUSSION

The present study aimed to employ an algal photosynthesis bioassay to identify surface water toxicity to algae, and subsequently identify the causing compound(s), on a nationwide scale. Surface water from 39 field locations was tested, and toxicity was observed at only one location. Chemical screening for 151 commonly applied pesticides identified three suspect compounds with herbicide activity that were present in the water sample above their respective quality standards, hence identifying risk of herbicides at this location. The toxicity of these compounds was elucidated in single compound toxicity tests, and their respective contribution to the observed toxicity in the sample was determined. One of the tested herbicides, dimethenamid, did not cause toxicity in the tested concentration range. The lack of a response in the PAM bioassay to dimethenamid, despite its presence above the EQS concentration, may be attributable to its mode of action as a long-chain fatty acid inhibitor. As this herbicide does not directly target PSII, it is expected that a response on PSII efficiency is limited, especially within the short exposure time of 4.5 h applied in the present study. Dimethenamid is, however, expected to cause a long-term effect on microalgal health. Such an effect may be elucidated in a longer exposure growth test. The herbicide metabolite desethylterbuthylazine did cause toxicity in the PAM bioassay, however the effect of the field concentration was negligible, which implies
that desethylterbuthylazine also did not contribute to the toxicity observed in the field sample. Contrastingly, the field concentration of the herbicide linuron, which was present at 110 times its MAC-EQS concentration, was shown to be solely and fully responsible for the toxicity observed in the field sample. Moreover, toxicity observed in samples at the same location taken in the weeks following the first identification of herbicide risk could also be fully explained by the linuron concentration. The success of attributing toxic effects to detected compounds in bioassays with field samples largely depends on the endpoint specificity of the applied bioassay.\textsuperscript{31,44,48} Adequate selection of bioassays employed in water quality monitoring can thus greatly aid in narrowing down the identification of compound(s) that cause environmental risks. Bioassays targeting PSII inhibition by herbicides are more often successful in explaining a majority of effects, most likely due to the specific mode of action of most herbicides and the sensitivity of PSII inhibition as an endpoint.\textsuperscript{19} Nonetheless, the full attribution of field sample toxicity to only one responsible compound as presented in the current study is very rare. Quite likely, a concentration that exceeds the EQS so dramatically as linuron did in the present study is caused by a spill or

Figure 4.4. Acute (4.5 h) effect of desethylterbuthylazine (a) and linuron (b) on photosynthetic efficiency of \textit{R. subcapitata}. Open circles represent average (\(n = 8\)) \(\Phi_{\text{PSII}}\) (% of control \(\pm\) st. dev), the line represents the fitted log-logistic dose-response model, the red square the calculated EC\textsubscript{50} value with 95\% C.I., and the green diamonds represent \(\Phi_{\text{PSII}}\) (% of control \(\pm\) st. dev) from field samples with corresponding compound concentrations.
incident. Although the sampled water lies alongside potato fields, regular application of linuron could not have resulted in such elevated concentrations in the surface water. The continued concordance of the decreasing PSII inhibition with decreasing linuron concentrations over the following 3-week period demonstrates the consistency of the PAM bioassay and chemical analysis. Considering the reported aerobic soil half-life of 22 days for linuron,\textsuperscript{128} the decreasing field concentrations may indicate a single contamination event that could have impacted PSII functioning in exposed biota for another several weeks in this case. Yet, occasional traditional grab sampling will only rarely capture such instances of elevated herbicide levels. Alternatively, passive sampling strategies can concentrate water samples over longer periods of continued field exposure and incorporate compound fluctuations over time, thereby lowering detection limits and leading to more frequent detection of ecotoxicological risks (Jones \textit{et al.} 2015).\textsuperscript{49} The combination of passive sampling and effect monitoring has recently been successfully applied,\textsuperscript{14,48} also specifically with PSII inhibition bioassays,\textsuperscript{112,129} and is hence recommended for future application of the present PAM bioassay.

The presence of a herbicide at 110 times the MAC-EQS concentration will evidently cause a trigger value exceedance of the PAM bioassay (>10% reduced ΦPSII), as was demonstrated in the present study. This raises the question at which herbicide concentration the trigger value of the here applied PAM bioassay is exceeded. To answer this question, the log-logistic dose-response model by Haanstra \textit{et al.} (1985)\textsuperscript{126} can be applied to calculate at which compound concentration the effect level will exceed the trigger value of the test. This way, the trigger value concentration (>10% reduced ΦPSII) for linuron was calculated to be 1.2 μg/L (EC10 in Table 1), which is approximately 4 times the MAC-EQS and 7 times the AA-EQS concentration. The value of 1.2 μg/L lies very close to the previously defined chronic no observed effect concentration for linuron of 0.5 μg/L which was used for EQS determination,\textsuperscript{130,131} meaning that the present PAM bioassay would identify toxicity at similar levels of linuron as previously described to have no chronic effect on primary producers. For desethylterbuthylazine the EC\textsubscript{10} was calculated to be 14 μg/L, which lies between the MAC-EQS and AA-EQS for this compound (Table 4.1). These findings indicate that the here applied PAM bioassay is sensitive comparable to previously applied chronic primary producer bioassays used to study the harmful effects of PSII inhibiting herbicides like linuron, while it is notably easier to perform and requires a much shorter exposure time.

To support the wide application of PAM bioassays in surface water toxicity screening, investigations into the current limitations and future challenges of this approach are necessary. Presently, the detection limit of the PAM assay was 4 times the EQS. The applicability of this assay as a tool in routine monitoring of herbicide risk to primary producers would require an increase in sensitivity of the assay to a detection limit at or below the quality standards of herbicides. Only this way, the absence of toxicity would indeed ensure the absence of herbicide risk. Moreover, toxic effects of non PSII inhibiting herbicides may be overlooked, and the sensitivity of the test to a wider variety of herbicides should be investigated. Nonetheless, it is concluded that PSII inhibition bioassays applying PAM fluorometry are a rapid, high throughput and sensitive tool in screening surface water toxicity to algae.
Previous research by Sjollema et al. (2014)\textsuperscript{71} did not identify hazardous field concentrations of herbicides at several coastal and estuarine locations in The Netherlands, and in their study only concentrated samples induced PSII inhibition. This lack of effect can likely be explained by the distance and dilution from the pollution source to the studied marine locations. The present study revealed that surface waters in closer proximity to agricultural lands can be at risk from herbicides, although this was only identified at one of 39 study sites, indicating no extensive toxicity to algae at the selected locations at the time of the screening. Contrastingly, studies in Southern European countries have recorded herbicide concentrations in surface waters often exceeding quality standards,\textsuperscript{132–135} even at levels that would result in a potential affected fraction of approximately half the algal species in sensitivity distributions.\textsuperscript{136,137} Hence, there seems to be an influence of country or area specific dilution and prevailing agricultural practice on the likeliness of herbicide risk in surface waters.

Our test setup enabled a countrywide screening of surface water toxicity to algae and resulted in identification of herbicide risk and confirmation of the responsible compound at a location with observed herbicide toxicity. This compound, linuron, is not present on the priority substances list of the EU-WFD. Thus, applying the algal photosynthesis bioassay may avoid redundant chemical analyses at locations without toxicity to algae, while simultaneously identifying the presence of hazardous compounds that would be overlooked by routine chemical WFD monitoring.

**PERSPECTIVES**

Following from the observations presented in this study, it is relevant to consider the future development of bioanalytical tools and their employment in environmental risk assessment. Compared to traditional chemical target analysis, the use of bioanalytical tools offers several advantages when applied as a screening tool in regular monitoring strategies. Chemical analyses allow for direct comparison of surface water concentrations to priority substance-based legal guidelines, however, there are restraints to this approach that limit the reliability of the ensuing risk assessment. Priority substances lists are not representative of present day contamination, and chemical analyses overlook bioavailability and mixture toxicity.\textsuperscript{37–39,41,43} Hence, low concentrations or absence of priority substances do not guarantee the absence of ecotoxicological risks. In contrast, bioanalytical tools incorporate bioavailability and mixture toxicity of all substances, including metabolites and unknown compounds.\textsuperscript{35,45} There is still a lack of a generally accepted classification for bioanalytical tools that can be applied in regular monitoring frameworks, however in recent years valuable research efforts have been made to develop classifications, in the form of effect-based trigger values, that allow for standardised environmental risk assessment.\textsuperscript{21,30,138} Hence, although effect monitoring is still limited by the low substance specificity, which complicates identification of compounds causing adverse effects, its advantages far outweigh its disadvantages. Effect monitoring can be achieved with a limited number of assays, allowing cost-effective risk assessment, where the absence of observed effects reliably guarantees the absence of ecotoxicological risks. This in turn permits water authorities
to invest money where it matters most: identification of compounds causing adverse effects at locations with indicated ecotoxicological risks, as was demonstrated in the present study. Hence, the surface water toxicity screening and subsequent herbicide risk assessment advocated here can offer significant advantages to water authorities. Such tools, that permit the analysis of tens to hundreds of samples on a single day, can readily be integrated in a bioassay battery employed in effect-based monitoring programs. The simplicity of this approach makes upscaling to region- or countrywide screening of herbicide risk in surface waters feasible. This not only applies to small countries like The Netherlands, but also makes it decidedly fit for application in large nations in which monitoring programs require an even higher throughput capacity. Hence, it is evident that there is an important place for fluorescence based algal bioassays specifically, and effect-based tools in general in future water quality monitoring.

**SUPPORTING INFORMATION**

The supporting information to this chapter is available online at:
https://doi.org/10.1016/j.scitotenv.2018.07.214