Lifting the veil: Impact of contaminants on coastal phytoplankton
Sjollema, S.B.

Citation for published version (APA):
Sjollema, S. B. (2014). Lifting the veil: Impact of contaminants on coastal phytoplankton

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Chapter 3

Identification of photosynthesis inhibitors of pelagic marine algae using 96 well plate micro-fractionation for enhanced throughput in Effect-Directed Analysis


*Environmental Science and Technology* 48(14), 8003-8011
Abstract

Because of large-scale production and use of an increasing diversity of chemicals in modern society, estuarine and coastal waters may be contaminated with numerous substances. Some of these compounds have the potential to affect microalgae at the base of the pelagic food chain. Therefore, we identified the main chemical stressors that negatively affect the effective photosystem II efficiency ($\phi$PSII) in marine microalgae of the Dutch estuarine and coastal waters. An enhanced effect-directed analysis (EDA) was carried out by combining reversed-phase ultra performance liquid chromatography fractionation of extracts from passive samplers, followed by effect assessment using the pulse amplitude modulation fluorometry assay and chemical analysis of biologically active fractions using high-resolution mass spectrometry. This study focuses on a novel microfractionation technique using 96-well plates to enhance throughput in EDA, structure elucidation, and the analytical and effect confirmation of the compounds that are identified. Although there are numerous unknown compounds present in estuarine and coastal waters, our EDA study shows that atrazine, diuron, irgarol, isoproturon, terbutryn, and terbutylazine are the main contributors to the observed effect on the $\phi$PSII of marine microalgae.
Chapter 3 – Identification of phytotoxic contaminants

Introduction

In recent years, sources, types, and levels of chemicals in the estuarine and coastal waters have increased as a consequence of anthropogenic activities worldwide. It has been proposed that some of these chemicals can potentially affect microalgae at the base of the pelagic food chain (Hylland and Vethaak, 2011). A variety of contaminants were demonstrated or designed to have a negative effect on photosynthesis (Vermeirssen et al., 2010) and thus these contaminants may have a direct impact on plankton communities. On a European level, the Water Framework Directive (WFD) requires a good ecological and chemical status of all water bodies, including transitional and coastal waters up to one nautical mile from shore, by 2015. In Europe, the chemical status of freshwaters, estuarine, and coastal waters is predominantly assessed by means of chemical monitoring and compliance with environmental quality standards (EQSs) for individual compounds. However, the European WFD currently identifies 45 chemical substances of concern only (Directive 2013/39/EU). In addition, the OSPAR commission for the protection of the marine environment of the North-East Atlantic, which aims to reduce discharges, emissions and losses of hazardous substances, agreed to a list of only 40 substances or groups of substances for priority action in marine waters (OSPAR, 2011). Routine monitoring of only the limited set of compounds on, for example, the WFD and OSPAR lists is not expected to be sufficient to investigate, which pollutants are responsible for the toxic pressure on marine microalgae. Effect-directed analysis (EDA) may help to provide evidence on the main chemical stressors beyond priority pollutants to improve the ecological status of a coastal ecosystem (Weiss et al., 2011). Recent reviews, for example Richardson et al. (2011), have provided insight into the multitude and variety of newly detected contaminants from domestic, commercial, and industrial use, for example, artificial sweeteners, perfluorinated compounds, pharmaceuticals, hormones, drinking water disinfection byproducts, sunscreen/UV filters, flame-retardants, siloxanes, musks, algal toxins, pesticides, and transformation products. Therefore, identification of intermediates and degradation products becomes important, as well as the evaluation of resulting toxicity or biological activity of the transformation products. Herbicides used in for example, agriculture and antifouling agents, are known to affect photosynthesis and enter the marine environment through urban and agricultural runoff, antifouling applications, ballast water discharge and industrial discharge (Sjollema et al.,
The use of certain herbicides has been restricted by some countries and in Europe a large amount of data has been gathered as part of the Biocidal Products Directive (Directive 98/8/EC). Although some herbicides have been banned for several years, they are still present in surface waters (Quednow and Püttmann, 2007). It is evident that a ban on use does not necessarily lead to immediate disappearance of banned substances from the environment. The high number and the wide range of chemicals that can affect microalgae pose a big challenge for analytical methods and risk assessment studies. In the present EDA study, we aim to identify and subsequently confirm the compounds in extracts from passive samplers deployed in the Dutch estuarine and coastal waters causing toxic effects on pelagic marine microalgae by using an enhanced microfractionation procedure. EDA combines analytical chemistry with bioassay analysis to isolate and ultimately identify the compounds in a complex sample that are responsible for the observed effects. For example, an EDA review discussed key pollutants in European river basins in which priority pollutants were identified but also other compounds that were neither regulated nor considered as environmental pollutants before (Brack et al., 2007).

Examination of surface water can be difficult because of the extremely low concentrations of the compounds, which are usually below any toxic or inhibitory level (Weller 2012). To overcome this concentration problem, we use passive samplers to concentrate large volumes of water, enabling trace level detection, and integrative sampling. Passive sampling can result in a more concentrated extract and time-weighted average concentration compared to spot water sampling for which large volumes are needed to concentrate compounds and represent one time point only (Booij et al., 2013). Although concentrations of individual compounds can pose a low actual risk, mixtures of these compounds can result in potential effect levels in bioassays (Sjollema et al., 2014b). By using ultra performance liquid chromatography (UPLC), extracts from passive samplers were fractionated in 96 well plates. Microfraction techniques reduce the complexity of extracts compared to lower resolution fractionation in typical EDA studies, thereby facilitating the identification process of responsive compounds and have successfully been applied in recent studies for bioactivity guided screening purposes (Kool et al., 2011; Pieke et al., 2013). Another very important aspect of the implementation of 96-well plate microfractionation is the enhanced EDA throughput, by avoiding time-consuming evaporation steps of the excess solvent of conventional fractions.
Another novelty of our approach is the use of a keeper during the evaporation process to increase the recoveries of the compounds of interest. After semi-automated parallel evaporation of the solvent from all 96 wells, the well plates were directly used for microalgae toxicity assessment using the Pulse Amplitude Modulation (PAM) fluorometry assay. The PAM assay is a sensitive and rapid high-throughput assay used for direct inhibition measurements of photosynthesis in algal species (Sjollema et al., 2014a). Acute effects on photosynthesis in the PAM assay are indicative for chronic effects, for example, algae growth (Magnusson et al., 2008). High-resolution time-of-flight mass spectrometry was used for compound identification and confirmation, which benefits from the high mass resolution and accuracy as demonstrated by recent literature (Weiss et al., 2011; Hernández et al., 2012; Zedda et al., 2012). In general, a stepwise approach using high-resolution mass spectrometry is applied to handle the large amount of data produced in nontarget analysis to identify compounds (Weiss et al., 2011). For a successful nontarget analysis, several steps are used, including (i) determination of the elemental composition from accurate mass, (ii) a search for the molecular formula in libraries or databases, and (iii) a ranking of the proposed structure (Zedda et al., 2012).

The objective of our study was to improve throughput in EDA by using microfractionation directly in 96-well plates and to assist the identification process. Our second aim was to identify the main photosynthesis inhibitors of pelagic microalgae in the Dutch estuarine and coastal waters.

**Materials and methods**

**Sampling locations**

Passive samplers were deployed at three different locations in the Dutch estuarine and coastal waters: Hansweert (estuary), Fort IJmuiden (inshore coastal waters), and Texel (coastal waters) (see Supporting Information Fig. S1).
Chemicals, passive sampling, extraction, and PAM assay

The chemicals used in this study are described in the Supporting Information. The passive sampling, extraction methods, and the PAM assay have been described in detail elsewhere (Booij et al., 2013; Sjollema et al., 2014a,b). In short, polar organic integrated samplers (POCIS) and silicone rubber sheets were deployed at the 3 sampling locations for 6 weeks in the autumn of 2011 (see Supporting Information 2.3). Sepra ZT (pyrrolidone modified styrene-divinylbenzene polymer, 30 μm, 85 Å, Phenomenex) used as sorbent in POCIS was extracted with methanol (see Supporting Information 2.1 for information on chemicals used). Silicone rubber sheets (9 × 5.5 cm) were Soxhlet extracted with acetone/hexane (1:3, v/v). After evaporation, the extract was dissolved in Milli-Q water resulting in a silicone rubber sheet extract containing 8.4 sheets/mL and a POCIS extract equivalent to 1.3 g sorbent/mL. The extracts were tested with the PAM assay. The PAM assay was performed with the marine microalgae *Dunaliella tertiolecta* (Butcher, CCAP 19/27) and effective photosystem II efficiency (ΦPSII) was measured after 4.5 h exposure. Dose-response curves were made for individual compounds and extracts from field samples by log-logistic curve fitting as described by Sjollema et al. (2014b) and in the Supporting Information.

Fractionation by ultra performance liquid chromatography

Fractionation was performed using an UPLC-system (Agilent Technologies, Middelburg, The Netherlands) at 35 °C, with a Waters (Etten-Leur, The Netherlands) Acquity BEH C18 column and guard column (2.1 × 100 mm and 2.1 × 5 mm, 1.7 μm) column. A flow rate of 0.5 mL/min was used. A gradient started at 100% A (acetonitrile/water, 10%/90%, v/v), increased to 100% B (acetonitrile) in 40 min, and kept at this composition for 10 min. Twenty microliters of the passive sample extract was injected and fractionated for the PAM assay and another 20 μL of the same field extract was fractionated for chemical analysis. Fractions, covering a time window of 20 s, were collected in two black 96-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) with a total runtime of 50 min. Detailed parameters of the microfractionation are described in the Supporting Information. In the second well plates in which the relatively nonpolar compounds elute, no active fractions were determined, therefore the second plate is not discussed further in
this paper. Fifty microliters of glycerol (Sigma-Aldrich, Zwijndrecht, The Netherlands) diluted with Milli-Q water (35 mg/mL) was added to each fraction of the 96-well plates for the PAM assay. Glycerol was added as a keeper to prevent compounds from evaporating and to increase the recovery. For the PAM assay the organic solvent in the well plates was evaporated in a CentriVap concentrator (Labconco, Kansis City, USA) at 40 °C in 6 h. After evaporation, 60 μL f/2 medium (Sigma- Aldrich, Zwijndrecht, The Netherlands) was added to each well, the well plates were stored in the refrigerator during the night and the PAM assay was performed on the next day according to the protocol described by Sjollema et al. (2014b). Ten microliters of the internal standard isoproturon-d6 (1 μg/mL) was added to each fraction of the 96-well plates for chemical analysis. The well plates were sealed with a plate heat-sealer (Waters, Etten-Leur, The Netherlands) and stored at 4 °C until chemical analysis. A mixture of herbicides (see Supporting Information section 2.2.) was fractionated to determine the relation between retention time and log Kow of compounds in the well plates.

Chemical analysis with liquid chromatography coupled to high-resolution mass spectrometry

Fractions were considered active if the percentage inhibition in the PAM assay was >10% relative to the control. Active fractions and the nonactive fractions eluting before the active fraction (considered as the reference) were analyzed with an HPLC Agilent 1290 Infinity system coupled to a micrOTOF II mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (ESI). Detailed technical parameters of the LC-ToF-MS analysis are described in the Supporting Information S2.4. An outline of the method used in the effect-directed analysis is shown in the Supporting Information (Fig. S3).

Identification and Confirmation Strategy

The workflow of the identification and confirmation strategy is described in Fig. 3.1, including aspects of each step. With the software program DataAnalysis (version 4.0 SP4; Bruker Daltonics, Bremen, Germany) an
internal mass calibration was performed for each chromatogram based on high precision calibration (HPC). The MetaboliteDetect software (version 2.0; Bruker Daltonics, Bremen, Germany) was used to subtract the chromatograms of the active and non-active fractions to discriminate the peaks of interest and to generate a difference chromatogram. The eXpose algorithm was used, accounting for tolerances in retention time and mass position, as well as intensity ratios of mass peaks in the sample, for example, active fraction and reference, for example, nonactive fraction. The tolerance for a shift in mass was set at 0.01 m/z and the tolerance for a shift in retention time was set at 0.1 min. A peak detected in this time window with a specific m/z in the active fraction needed to exceed 5 times the abundance of a peak detected in the nonactive fraction to be accepted in the difference chromatogram. The result is a data set with abundant masses with S/N > 5 compared to the nonactive fraction that are exclusively detected in the sample. The difference chromatogram was loaded in DataAnalysis software, checked for the number of peaks, and an average

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**Fig. 3.1.** Description of the identification and confirmation strategy of the active compounds present in the fractions, the advantages/limitations and the software used for each step.

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**LC-ToF-MS**
- Analysis of active and non active fractions
- Electrospray positive mode
- Software: Hystar / Compass 1.3

**Internal calibration of each chromatogram**
- High precision calibration
- Software: DataAnalysis

**Subtraction of active fraction and non-active fraction chromatogram**
- Masses exclusively detected in the active fraction
- No true reference
- Software: MetaboliteDetect

**Calculation of possible molecular formulas**
- Several molecular formulas fitting a single m/z value
- Software: SmartFormulas

**List of identified and confirmed compounds**

**Biological confirmation**
- Effect in the PAM assay
- Availability of standards

**Chemical confirmation**
- Retention time
- Mass spectra full scan MS
- Availability of standards

**Query molecular formulas in databases**
- Set of databases available
- Software: DataAnalysis / CompoundCrawler
spectrum of each peak with $S/N > 3$ was taken. The m/z of the most abundant mass of each peak spectrum in the difference chromatogram was extracted from the original sample file. The peak in the original sample file was checked for peak shape, isotopes of the molecular ion, adducts, and accurate mass. We considered that all peaks were protonated, whereas adducts were not observed. On the basis of the accurate mass, the possible molecular formula of the peaks of interest was calculated using the SmartFormula tool within the DataAnalysis software. Parameter settings were $P 0−9$, $S 0−10$, $F 0−16$, $Cl 0−16$, $Br 0−4$, $Si 0−8$, based on the rules described by Kind and Fiehn (2007). The parameters $C$, $H$, $N$, and $O$ were automatically included by the software. The tolerance was set at 5 ppm as a maximum deviation between the calculated elemental composition and the measured exact mass. The mSigma value is a value for the agreement between the theoretical and measured isotopic pattern of the mass peak of interest and combines the standard deviation of the masses and intensities for all isotopic peaks. The maximum acceptable mSigma value was set at 50, lower numbers indicating a better fit. The relative intensity was set at 20%. The accurate mass was automatically checked for ring structures and double bonds, location of monoisotopic peaks and the number of carbon atoms as calculated by the software program. A library search of the molecular formula was performed by using the CompoundCrawler tool in the DataAnalysis software, which connects to several databases including ChemSpider, NIST, and METLIN. The analytical chemical confirmation of the tentatively identified compounds, for which pure standards were available, was conducted by evaluating the chromatographic and mass spectrometric behavior of the standard on the LC-ToF-MS. Fragmentation (MS/MS) behavior could not be performed as the used instrument was a ToF and not a QToF. For a satisfactory match, we accepted a mass window of 5 ppm and a retention time window of 0.3 min between the peaks observed in the chromatogram of the injection standard and the extracts. Quantification was accomplished using a 7 point calibration curve ranging from 1 to 350 ng/mL.
Correlation between measured concentration in passive sampler extracts and PAM activity

To correlate the measured concentrations in the passive sampler extracts with the PAM assay activities, we used the concentration addition model based on toxic units (TU). See the Supporting Information for a detailed description of the calculation of the TU. For fractions in the 96-well plates, a different approach was followed. To investigate the correlation between the concentration of the compounds in the active wells and the PAM activity in the well, the % inhibition in the PAM assay was calculated based on the chemically determined concentrations of the individual compounds in the active fractions. The criteria used in this approach were that the % inhibition determined in the PAM assay should be in the linear range (30–70%) and the concentration should be quantified with chemical analysis.

Results and Discussion

Microfractionation in 96-Well Plates

The complexity of the extracts was reduced by using high-resolution fractionation directly into 96-well plates. Fractions covering a time window of 20 s were collected, implicating that with the UPLC setup used the chromatographic peaks eluted in 2 to 3 wells. Ideally, this would lead to an improved correlation of the bioactivity as determined in the bioassay and the identity of the compounds established by high-resolution mass spectrometry (Pieke et al., 2013). Historically, most EDA studies deal with a lower number of fractions, which often complicates the identification of the compounds responsible for the observed effects. To overcome this problem, in many EDA studies active fractions are fractionated further and the next series of fractions is tested again for activity in the bioassay. Multiple fractionation steps are therefore time-consuming and may cause a loss of compounds or lower recoveries in the second fractionation step. The enhanced single-step microfractionation approach used in our study resulted in far more and less complicated fractions than commonly encountered in EDA. Another advantage of microfractionation is that the 96 well plates can be used directly for the PAM assay after evaporation of the organic solvent.
Organic solvents used for fractionation are rarely compatible with bioassays and therefore care should be taken to remove the organic solvent, that is, by evaporation, before the bioassay will be carried out. Evaporation of the organic solvent can cause losses of compounds, a phenomenon that is somewhat overlooked in many EDA studies. By adding glycerol as a keeper, the losses of compounds during evaporation can be reduced. A keeper is a nonvolatile solvent, which is added to a sample to prevent volatile compounds from evaporation during the evaporation step of organic solvents. Fig. 3.2 shows the recovery of 27 herbicides after evaporation in the CentriVAP with and without addition of glycerol. By adding glycerol prior to the evaporation procedure, recoveries increased considerably for many of the test compounds, for example, the recovery of monolinuron increased from 17 ± 1 % to 64 ± 3 %. At the concentration used, glycerol did not affect the PAM bioassay (data not shown).

![Fig. 3.2. Recoveries of triazine and urea herbicides and standard deviation (n=3) after evaporation with the CentriVap with and without glycerol keeper.](image)
Fractions and PAM assay results as starting point for identification

In this study, two passive samplers types (silicone rubber sheets and POCIS) were used. These passive samplers have different mechanisms of adsorption of molecules and can be used to extract compounds with different polarities and chemical structures (Booij et al., 2013). The PAM percentage inhibition results of the fractionated passive sampler extracts from the different locations are shown in Fig. 3.3. Fractions with a percentage inhibition of >10 % were considered as active fraction containing compounds that affect the ΦPSII and plotted on the x-axis. Fractions with a percentage inhibition of <10 % were considered nonactive. At all three locations, a response in the PAM assay was observed, indicating that compounds affecting ΦPSII were present in the passive sampler extracts. In general, there was a significant overlap in active
fractions between the POCIS and silicone rubber sheets for all three locations. This is an indication that both passive samplers are able to sample the same type of compounds that are responsible for the effect on the $\Phi_{PSII}$ in the PAM assay. In general, the silicone rubber sheets resulted in more and different active fractions compared to the POCIS exposed at the same location during the same period, indicating that more $\Phi_{PSII}$ inhibitors were extracted with silicone rubber sheets.

**Identification of chemical stressors**

The extracts of passive samplers contained 3 active fractions in the POCIS extract from Texel to 11 active fractions in the silicone rubber sheets extracts from Hansweert (Tab. 3.1). To identify which compounds are present in the active fractions, the data handling strategy is presented in Fig. 3.1 and consists of 7 steps to be performed to obtain a list of identified and confirmed biological active compounds in the sample of interest. The software gives direct access to online databases including ChemSpider (containing over 30 million structures) and METLIN, which enables very easy searching for data supporting the identity, occurrence and relevance of the mass under investigation. In Tab. 3.1, the results of several steps in the procedure are given, represented by the number of peaks remaining after a specific step. The first step

<table>
<thead>
<tr>
<th>Sample</th>
<th>Passive sampler</th>
<th>Step 1**</th>
<th>Step 3**</th>
<th>Step 4**</th>
<th>Step 5**</th>
<th>Step 6**</th>
<th>Step 7**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hansweert (estuary)</td>
<td>SR-sheets*</td>
<td>7</td>
<td>19</td>
<td>170</td>
<td>14</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>early autumn</td>
<td>POCIS</td>
<td>7</td>
<td>7</td>
<td>44</td>
<td>7</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Hansweert (estuary)</td>
<td>SR-sheets*</td>
<td>11</td>
<td>28</td>
<td>544</td>
<td>27</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>late autumn</td>
<td>POCIS</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Fort Ijmuiden (inshore)</td>
<td>SR-sheets*</td>
<td>6</td>
<td>7</td>
<td>40</td>
<td>9</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>late autumn</td>
<td>POCIS</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Texel (coastal)</td>
<td>SR-sheets*</td>
<td>10</td>
<td>24</td>
<td>133</td>
<td>23</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>late autumn</td>
<td>POCIS</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* SR-sheets = silicone rubber sheets

**Steps = 1: Number of active fractions; 3: Number of peaks after subtraction active and non-active fractions; 4: Number of possible molecular formulas; 5: Number of tentatively identified compounds; 6: Number of compounds confirmed by retention time; 7: Number of compounds confirmed in the PAM assay.
in the identification process is the analysis of fractions on the LC-ToF-MS. The second step is the internal calibration of each chromatogram to improve the calibration and to increase the mass accuracy. The third step is the comparison of chromatograms between the active fractions and adjacent nonactive fractions to extract a difference chromatogram, which includes peaks and masses exclusively detected in active fraction. Peaks in this difference chromatogram were evaluated for identification. We considered the nonactive fraction before the active fraction as a reference, since a real reference is not available in this study. This is a limitation in EDA studies in general and, as an alternative, a solvent procedure blank or a nonactive fraction adjacent to the active fraction is used as a reference (Weiss et al., 2011, Bandow et al., 2009). In the case of consecutive fractions, we assumed that the same compound is responsible for the effect in the PAM assay in both fractions, since we know that compounds can be distributed over two fractions, because of typical width of chromatographic peaks. Therefore, we used the same nonactive fraction for consecutive active fractions. Ideally, a reference contains the same compounds as the sample excluding the chemical stressors responsible for the effects in bioassays. Practically, this condition is very difficult to obtain or create: even an unexposed silicone rubber sheet or POCIS extract is not a good reference, since they are not exposed to estuarine and coastal waters. Finally, the most abundant m/z values of the peaks in the difference chromatogram were considered as the potential toxic compounds. This resulted in only 2 peaks detected in the fractions of the POCIS extract from Texel to 28 peaks in the fractions of the silicone rubber sheets from Hansweert (Tab. 3.1). In the fourth step, m/z ratios of the peaks observed in the difference chromatograms were extracted from the original chromatogram of the active fraction. m/z values observed in the chromatograms were all below 500 Da. The accurate mass was used to calculate the possible molecular formulas. A large difference in the number of potential molecular formulas was found between the fractions. Ranging from 3 possible molecular formulas in the POCIS extract from Fort IJmuiden to 544 possible molecular formulas in the silicone rubber sheet extract from Hansweert (Tab. 3.1). For some molecular formulas, more than 1000 isomers were found in the ChemSpider database. Sorting the number of references associated with each compound in descending order raises the most useful candidates to the top of the list for further evaluation (Little et al., 2012). The number of references in ChemSpider contain citations and relevant publications related to the
compounds and was considered as an indicator for the commercial importance or for the presence in the environment, and thus as an indicator for the probability of the detection in estuarine and coastal waters. To filter the amount of data, only compounds that indicated more than 50 references in ChemSpider were considered as tentatively identified compounds. Several of these formulas could not be found in databases like ChemSpider, because of unrealistic and unnatural structures. Each tentatively identified compound was checked whether the compound’s log Kow matched with the retention window of the microfractionation and the chemical analysis by LC-ToF-MS. For simplicity, we derived that the log Kow in the active fractions lies between 2.1 and 4.1 (±0.5) log units, based on a tested triazine and urea mixture (Finizio et al., 1997). This log Kow range is correlated to a retention time window from 5 to 12 min on the LC-system. The log Kow values were calculated with EPI Suite (KOWWIN v.1.67, U.S. EPA) and are hence theoretical values. After these data analysis steps the number of tentatively identified compounds resulted in 2 compounds in the POCIS extract from Texel to 27 compounds in the silicone rubber sheet extract from Hansweert (Tab. 3.1). There was some overlap in tentatively identified compounds between extracts, which reduced the total number of candidate compounds to 54 tentatively identified compounds.

**Confirmation of the identity of chemical stressors**

For the confirmation of the identity of the candidate chemical stressors, the behavior of the compound in the analytical system with respect to the retention time and the mass spectrum as well as the effect that the compound exhibits in the PAM assay were investigated (steps six and seven, Fig. 3.1). The availability of the compound as an analytical standard is crucial for confirmation. In our case, 19 of the 54 tentatively identified compounds were commercially available and purchased for confirmation. These 19 tentatively identified compounds are shown in Tab. S1 (see Supporting Information) with the monoisotopic mass, the molecular structure, the CAS number, the estimated log Kow, the molecular formula, the usage and the corresponding active fraction. Atrazine, isoproturon, diuron, terbutylazine, terbutryn, and igerol were confirmed by retention time and accurate mass spectra in the analytical system and showed an effect on the ΦPSII in the PAM assay. The retention times for standards of adamantanecarboxylic acid, coumatetralyl, domosedan, epler-
enone, 2-pyridyl-benzimidazole, xanthone, dodecalactam, cyclo-dodecanone oxime, N-octylpyrrolidone, and todralazine did not match with the retention time determined in the chromatograms of the passive sampler extracts. For 2-methoxy-4-(2-methyl-2-propanyl)phenol a m/z of 203.1041 was observed in the chromatogram of the standard solution instead of 181.1220 as observed in the spectra of passive sampler. The retention time for N,N-diethyl-3-methyl-benzamide and tris(2- chloroethyl)phosphate (TCEP) was confirmed; however, these compounds did not show any effect on the ΦPSII in the PAM assay up to levels of 1 mg/L.

Passive samplers and PAM assay for confirmation of the identity of chemical stressors

Silicone rubber sheets and POCIS were used to extract a broad range of chemical stressors from estuarine and coastal waters that affect the ΦPSII of marine microalgae (Booij et al., 2013). However, our EDA study showed that similar chemical stressors were found with the POCIS and silicone rubber sheets. The six confirmed compounds listed in Tab. 3.2 are all herbicides and were found in both passive samplers, which confirmed that POCIS and silicone rubber sheets extracts contained the same compounds that are responsible for the ΦPSII in the PAM assay. The limit of detection (LOD) for these six compounds in the fractions were determined on the LC-ToF-MS and compared to the EC_{10} levels in the PAM assay. The EC_{10} level in the PAM assay was considered as the no observed effect concentration (NOEC), which was below the analytical LODs of diuron and terbutryn. These LODs were relatively high because of the small volumes of sample available from the 96 well plates, indicating that the PAM is a very sensitive assay compared to chemical analysis with LC-ToF-MS.

Explaining toxicity and effects of chemical stressors

Herbicides are known to inhibit the photosynthesis by interrupting the electron transport system. Although atrazine is banned in the European Union since 2004, detectable concentrations of this compound are still present in estuarine and coastal waters. The five other herbicides are still allowed as active ingredients in several products in The Netherlands (College voor de toelating
van gewasbeschermingsmiddelen en biociden, CTGB). Atrazine, diuron, isoproturon, irgarol, and terbutryn are listed as priority substances in the WFD (Directive 2013/39/EU). Terbutylazine is not included in the Dutch monitoring program, nor listed as a priority substance by the WFD. Because of the relatively high toxicity (EC$_{10}$ in the PAM assay of 0.4 $\mu$g/L), this compound should be further investigated in terms of toxic pressure on the marine ecosystem. In addition to herbicides, there are hundreds of compounds present in marine and coastal waters, but they do not seem to affect the $\phi$PSII of *D. tertiolecta* at measurable levels.

Mixture effects are rarely taken into account in water quality regulations. Fluctuating numbers and concentrations of individual contaminants make experimental testing in the PAM assay of every potential mixture unfeasible. Hence, different modeling approaches for prediction of mixture toxicity can be applied. We assumed the identified herbicides to have the same mode of action, which allows the addition of toxic units that is based on the concentration addition model for the prediction of the mixture toxicity. The concept assumes addition of concentrations of different components of a mixture standardized to their effect concentration at a defined effect level. The validity of this concentration addition model was shown for the algal toxicity of photosynthesis inhibitors by Faust *et al.* (2000). Tab. S2 (see Supporting Information) shows the dose response curve parameters determined in the PAM assay for the confirmed herbicides and passive samplers extracts in which a is the concentration factor for the passive samplers extracts (CF EC$_{50}$ extract) at which 50% inhibition was determined. Overall, the CF EC$_{50}$ extract were lower.

### Tab. 3.2. Overview of toxic units at EC$_{50}$ levels of the PAM assay of the POCIS and silicone rubber sheet extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Passive sampler</th>
<th>irgarol</th>
<th>diuron</th>
<th>terbutylazine</th>
<th>terbutryn</th>
<th>atrazine</th>
<th>isoproturon</th>
<th>ΣTU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hansweert (estuary)</td>
<td>SR-sheets</td>
<td>1.1</td>
<td>0.9</td>
<td>1.7</td>
<td>1.0</td>
<td>0.0</td>
<td>0.3</td>
<td>5.8</td>
</tr>
<tr>
<td>early autumn</td>
<td>POCIS</td>
<td>0.6</td>
<td>1.4</td>
<td>1.1</td>
<td>&lt;LOD</td>
<td>0.0</td>
<td>0.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Hansweert (estuary)</td>
<td>SR-sheets</td>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.5</td>
<td>0.0</td>
<td>0.1</td>
<td>2.6</td>
</tr>
<tr>
<td>late autumn</td>
<td>POCIS</td>
<td>0.4</td>
<td>0.8</td>
<td>0.4</td>
<td>1.3</td>
<td>0.0</td>
<td>0.1</td>
<td>3.0</td>
</tr>
<tr>
<td>Fort Ijmuiden (inshore)</td>
<td>SR-sheets</td>
<td>2.0</td>
<td>0.3</td>
<td>0.1</td>
<td>&lt;LOD</td>
<td>0.0</td>
<td>&lt;LOD</td>
<td>2.4</td>
</tr>
<tr>
<td>late autumn</td>
<td>POCIS</td>
<td>0.9</td>
<td>0.7</td>
<td>0.1</td>
<td>&lt;LOD</td>
<td>0.0</td>
<td>&lt;LOD</td>
<td>2.7</td>
</tr>
<tr>
<td>Texel (coastal)</td>
<td>SR-sheets</td>
<td>1.4</td>
<td>&lt;LOD</td>
<td>0.9</td>
<td>2.0</td>
<td>0.0</td>
<td>&lt;LOD</td>
<td>4.4</td>
</tr>
<tr>
<td>late autumn</td>
<td>POCIS</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>0.2</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*SR-sheets = silicone rubber sheets, <LOD below limit of detection, toxic units cannot be calculated.*
in silicone rubber sheets compared to POCIS, indicating that silicone rubbers sheets were able to sample higher concentration and/or a higher number of chemical stressors. Silicone rubber sheets exposed at Hansweert showed a CF EC$_{50}$ extract of 0.12, the lowest CF EC$_{50}$ extract compared to other passive sampler extracts and indicating that this site was most polluted. The parameters in Supporting Information Tab. S2 were used to calculate the toxic units. Tab. 3.2 shows the calculated toxic units in the extracts of passive samplers. The sum of the toxic units in the passive sampler extracts is above 1, except for POCIS Texel, meaning an overestimation of the mixture toxicity and probably an under estimation of the unresolved toxicity (Faust et al., 2000). Overestimation and thus overprotection with respect to the precautionary principle is not very problematic (Brack et al., 2008). It has been reported that the concentration-addition model tends to overestimate the combined effects of mixtures including dissimilarly acting compounds (Faust et al., 2000; Grote et al., 2005). Concentrations in the extract from POCIS Texel were very low or below LOD. Since EC$_{10}$ levels observed in the PAM assay (see Tab. S3 Supporting Information) for diuron and terbutryn are below the LOD the sum of toxic units is probably an underestimation of the mixture toxicity for this POCIS extract. Isoproturon and diuron eluted in the same fraction similar to irgarol and terbutryn, for these compounds the toxicity cannot be explained for the single compound, therefore the % inhibition calculated was based on the effect addition approach. Tab. 3.3 shows an example in which the % inhibition determined in the PAM assay was compared to the calculated % inhibition in the active fraction. For atrazine the calculated % inhibition is below the determined inhibition in the PAM assay, meaning that there might be other compounds in this active fraction that could not be identified.

<table>
<thead>
<tr>
<th>Active fraction</th>
<th>Compound(s)</th>
<th>Early autumn PAM inhibition (%)</th>
<th>Late autumn PAM inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>measured</td>
<td>calculated</td>
<td>measured</td>
</tr>
<tr>
<td>D5</td>
<td>atrazine</td>
<td>18</td>
<td>4.0</td>
</tr>
<tr>
<td>G5</td>
<td>isoproturon/diuron</td>
<td>61</td>
<td>78</td>
</tr>
<tr>
<td>H5</td>
<td>isoproturon/diuron</td>
<td>30</td>
<td>43</td>
</tr>
<tr>
<td>B6</td>
<td>terbutylazine</td>
<td>67</td>
<td>80</td>
</tr>
<tr>
<td>F7</td>
<td>irgarol/terbutryn</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>G7</td>
<td>irgarol/terbutryn</td>
<td>64</td>
<td>31</td>
</tr>
</tbody>
</table>

*Tab. 3.3. Measured and calculated percentage inhibition in the PAM assay in fractionated extracts from silicone rubber sheets at Hansweert.*
In our study, it was not possible to identify all bioactive compounds and explain toxicity in all active fractions. Activity in the fractions A5, A6, E6, and E7 could not be identified and explained. However, the percentage inhibition in these fractions is relatively low (<25.6%) compared to other active fractions. Therefore, we considered atrazine, diuron, irgarol, isoproturon, terbutryn, and terbutylazine as the main contributors to the toxic pressure on microalgae in the Dutch estuarine and coastal waters because of the effect that could be explained. Our study focused only on one marine microalgae species whereas in the estuarine and coastal waters many different species are present. For better interpretation of our results and to support our conclusions, effects on other species and community effects should be further investigated. Although the implementation of high resolution mass spectrometry equipment has improved the identification success of several EDA studies, confirmation of the identify of compounds is still somewhat lagging behind (Weiss et al., 2011, Regueiro et al., 2013) and the compounds confirmed usually only represent a small percentage of the total effect that can be explained (Grote et al., 2005). On the other hand, there are many online databases now, and many software options although these have not been used extensively in EDA studies yet. The advantage of our current identification strategy is the possibility of online compound database searching which was not an option in some of our previous EDA studies (Weiss et al., 2011).

In general, every specific step in EDA from the extraction until the confirmation puts constraints on the results to be obtained. These choices undoubtedly lead to the exclusion of valuable information. To retain the maximum of information, any EDA study will benefit from measures taken to avoid losses of compounds during the EDA procedure. Current EDA studies are usually labor intensive because of the exhaustive extraction and fractionation techniques, and multiple evaporation steps leading to a decrease in recovery of compounds. The relatively low throughput has been one of the major bottlenecks of EDA until now and in order to become more broadly accepted higher throughput is a prerequisite. One approach to speed up the EDA process is microfractionation into 96-well plates using an UPLC system instead of conventional fractionation into large fractions containing several milliliters using normal bore columns. In addition, the use of a keeper in the 96-well plates
showed to be an efficient approach to reduce the loss of compounds during the handling of fractions.

Passive samplers used in this study extract an average time-weighed concentration, which is not representative for the water concentration, since the ratio of compounds in passive sampler extracts is different compared to field water samples. Concentrations of herbicides vary over time in estuarine and coastal waters and are relatively low compared to effects in the PAM assay. Therefore, further research is recommended to investigate the effect of these compounds in the estuarine and coastal waters to determine the toxic pressure of these herbicides under environmental conditions.

Acknowledgments

This study was part of a PhD project funded by Deltares, Delft, The Netherlands.

Supporting information is available: Fig. S1-3, Tab. S1-3. This information is available online (doi: 10.1021/es405428t)