Lifting the veil: Impact of contaminants on coastal phytoplankton
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Chapter 2

Laboratory algal bioassays using PAM fluorometry: effects of test conditions on the determination of herbicide and field sample toxicity


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Abstract

Pulse Amplitude Modulation (PAM) fluorometry, based on chlorophyll a fluorescence, is a frequently used technique in algal bioassays to assess toxicity of single compounds or complex field samples. It is known that several test conditions can influence the test results and since a standardized test protocol is currently lacking, it is difficult to link the results of different studies. Therefore, the aim of this study was to gain insight in the effects of test conditions of laboratory algal bioassays using PAM fluorometry on the outcome of toxicity tests. To this purpose we described the results from several pilot studies on test development in which we provide information on the effects of the main test factors during the pre-test phase, the test preparation, the exposure period and the actual measurement. The experiments were focused on individual herbicides and complex field samples and included the effects of culturing conditions, cell density, solvent concentration, exposure time and the presence of actinic light. Several of these test conditions demonstrated to influence the outcome of the toxicity test and the presented information provides important background information for the interpretation of toxicity results and describes which test conditions should be taken into account when using an algal bioassay with PAM fluorometry. Finally the application of PAM fluorometry in algal toxicity testing is discussed.
Chapter 2 - PAM fluorometry bioassay

**Introduction**

Over the past decades, toxic effects of contaminants have been investigated on numerous aquatic organisms at various trophic levels. Algae, primary producers in the aquatic food chain, play a key role in aquatic ecosystems due to their position at the base of the food chain and toxic effects of contaminants at this level could result in unfavorable effects on higher trophic levels (Hylland and Vethaak, 2011). To determine the toxicity of the contaminants, a large range of different toxicity tests with different endpoints are being used. For microalgae, the growth inhibition test has been used in many studies (Walsh, 1972; Casotti et al., 2005; Vallotton et al., 2008) and is described in several guidelines (ISO 8692:2012, ISO 10253:2006, OECD 201). Alternatively, the toxicity of a compound to microalgae can be determined by Pulse Amplitude Modulation (PAM) fluorometry, which is based on chlorophyll a fluorescence and was first described in 1986 (Schreiber, 1986). A wide range of photochemical processes linked to photosynthesis can be determined with this technique, providing information on the overall ‘health’ of the microalgae (Ralph et al., 2007) as photosynthesis is the main function of photosynthesizing organisms. PAM fluorometry is a non-destructive, non-invasive and rapid technique which can be used in a quick bioassay, since it provides direct information on the photosynthetic activity of the algae in contrast to the standardized growth inhibition test which requires at least 72 hours. Over the years, PAM fluorometry has been used to study the toxic effect of a range of compounds on microalgae. Due to the direct impact on algal photosynthesis a large number of studies using this technique are performed on herbicides as reviewed by Juneau et al. (2007), but it has also been used to test the toxicity of other compounds like metals (Peña-Vázquez et al., 2010), Polycyclic Aromatic Hydrocarbons (PAHs) (Wiegman et al., 2003) and field samples with a mixture of unknown compounds (Bengtson Nash et al., 2006; Escher et al., 2006; Muller et al., 2008). Based on these studies we argue that PAM fluorometry is an excellent tool that can be used for the rapid screening of large numbers of samples under laboratory conditions, testing phytotoxic effects towards microalgae. However, several studies described that different test conditions can influence the toxicity results obtained using PAM fluorometry (Schreiber et al., 2007, Conrad et al., 1993, Bengtson Nash and Quayle 2007). And since no standardized guidelines (in which these conditions are described) are available (Ralph et al., 2007) it is difficult to link the test results from
different studies using different experimental conditions. Therefore, the aim of this study was to gain insight in the effects of test conditions of laboratory algal bioassays using PAM fluorometry on the outcome of toxicity tests. To this purpose we present here the results from several pilot studies on test development in which we provide information on the effects of the main test factors during the pre-test phase, the test preparation, the exposure period and the actual measurement. These factors include culturing conditions, cell density, presence of a solvent control, exposure time and the effect of actinic light. All these factors can potentially affect the outcome of the test results, resulting in an under or overestimation of the toxic effect. Due to their specific mode of action on algal photosynthesis, herbicides are most likely causing toxic effects on microalgae. Consequently the effects of the above mentioned test conditions were determined for this type of compounds. To be able to determine the effects of field samples, containing a complex mixture of unknown compounds, experiments with field extracts were included. Additionally, the correlation between PAM fluorometry and the traditional $^{14}$C incorporation test was determined for a range of field samples and the application of PAM fluorometry in algal toxicity testing is discussed.

Materials and methods

Test species, chemicals and PAM fluorometry

Two different microalgal test species were selected for the different tests described in this study. The fresh water diatom *Pseudokirchinnella subcapitata* (Korshikov, UTEX 1648), formerly known as *Selenastrum capricornutum* (Printz), was cultured in Woods Hole Medium (Stein, 1973) in a chemostat at 20ºC and permanent cool white light with an intensity of 100 µmol m$^{-2}$ s$^{-1}$ (32W, Sylvania circline, Havells Sylvania, Raunheim, Germany). The algae in the chemostat were at steady state with a growth rate ($\mu$) of 0.6 d$^{-1}$. The marine flagellate *Dunaliella tertiolecta* (Butcher, CCAP 19/27) was cultured in batches under a light-dark regime of 16:18 h at 16 ºC with a light intensity of 50 µmol m$^{-2}$s$^{-1}$ (F58W/BriteGro2084, Havells Sylvania, Raunheim, Germany) on artificial seawater. Artificial sea salt (Aqua Bio Solutions, Wormerveer, The Netherlands) was dissolved in MilliQ water to obtain a salinity of 33 ‰ and enriched with commercially available f/2 medium (Guillard, 1975) (Sigma
All experiments were performed with exponentially growing cells and test were performed at a cell density of $1\times 10^6$ cells/mL unless indicated otherwise. Cell densities of *D. tertiolecta* were determined manually with a Bürker counting chamber and *P. subcapitata* cell densities with a Coulter counter (Coulter® Multisizer II).

Diuron (CAS: 330-54-4, analytical standard) and isoproturon (CAS: 34123-59-6, analytical standard) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands) and Irgarol®1051 (CAS: 28159-98-0, >97 %) from Ciba Specialty Chemicals Inc. (Basle, Switzerland). All stock solutions were made in methanol (ULC/MS grade, Biosolve, Valkenswaard, The Netherlands). The PSI inhibitor atrazine (CAS: 1912-24-9, analytical standard, Riedel-de Haën, Selzee, Germany) stock was made in ethanol (99.7 %, Merck, Amsterdam, The Netherlands). All presented concentrations are nominal concentrations. For several tests a concentration of 94 µg/L of atrazine was used. The concentration of this model toxicant was based on previous experiments as a clear photosynthetic inhibition can be observed at this concentration. In addition, extracts of organic micro pollutants present in natural waters were obtained by using two different concentrations methods: 1) XAD and 2) passive sampling. XAD samples were collected in the river Meuse (Eijsden) and Rhine (Lobith) and extracted with XAD-4 and XAD-8 according to the protocol described in Durand *et al.* (2009). Silicone rubber sheets (AlteSil™ translucent material, Deltares, Utrecht, The Netherlands) were used as passive samplers and deployed in estuarine water in Hansweert (Western Scheldt, the Netherlands) for six weeks starting in March, July and September and were soxhlet extracted according to Booij *et al.* (2013). Based on monitoring data, a large range of compounds, including non PSII inhibitors, are expected to be present in the XAD and passive sampler extracts of these locations (Waterbase, accessed Dec. 2012).

Chlorophyll *a* fluorescence was measured with a Pulse Amplitude Modulation (PAM) fluorometer (WATER-PAM, Heinz Walz GmbH, Effeltrich, Germany) connected to a robot for quick measurements. In the presence of actinic light, the actual fluorescence (F) and maximal fluorescence (F’*m*) were determined and the effective PSII efficiency (ΦPSII) was calculated as \( \frac{[F'_{m}-F]}{F'} \) (Genty *et al.*, 1989). After 10 minutes of dark adaptation, minimum fluorescence (F₀) and maximal fluorescence (Fₘ) were determined and the
maximal PSII efficiency ($F_v/F_m$) was calculated as \( \frac{F_m - F_0}{F_m} \) (Kitajima and Butler 1975). The fluorescence parameters $F$ and $F_0$, measured before the saturation pulse, represent the actual fluorescence intensity and fluorescence intensity with all PSII reaction centers open, while $F_m$ and $F'_m$ represent the maximal fluorescence intensity with all PSII reaction centers closed for dark and light adapted cells respectively (Kooten and Snel, 1990). The settings of the PAM measurement are given in the supporting information (Tab. S1). \( \Phi \) PSII inhibition was expressed as percentage of the corresponding control (% control).

**Pilot experiments**

In a series of pilot studies on test development, the effect of the main test factors relevant during the test were investigated. An overview of the test setup including the selected treatment, test concentrations, number of replicates, toxic endpoint, test species and test material of all experiments is given in Tab. 2.1.

**Culturing conditions**

To study the effect of the culturing conditions prior to a test on photosynthetic parameters and toxicity measured with PAM fluorometry, three modified culturing conditions (compared to the standard conditions described above) were tested: 1. double light intensity; 2. double light intensity and double nutrient (nitrate/phosphate) concentrations; 3. elevated temperature (25°C). These test factors are all ecological relevant for microalgae, and the selected test range of these parameters was based on commonly used laboratory conditions. The photosynthetic parameters $F$, $F'_m$ and $\Phi$PSII were determined after 4.5 h. Additionally, algae cultured under the different conditions were exposed to a concentration range of atrazine and median effect concentrations (EC50) values based on $\Phi$PSII after 4.5 h of exposure were determined.
Cell density

Three cell densities were tested to investigate the effect of cell densities on the response observed in the PAM bioassay. *P. subcapitata* suspension with a cell density of 1e6 cells/mL and an increased and decreased cell density of 1e7 and 1e5 cells/mL respectively were exposed to 94 µg/L atrazine. The ΦPSII was determined after 4.5 h of exposure.

Solvent concentration

A carrier solvent, sometimes needed to dissolve a test compound, should not affect the test results. To determine the effect of the commonly used carrier solvent methanol (ULC/MS grade, Biosolve, Valkenswaard, The
Netherlands) on ΦPSII, *D. tertiolecta* was exposed to five test concentrations (Tab. 2.1) and ΦPSII after 4.5 h of exposure was determined.

**Exposure time**

Depending on the mode of action of a compound, differences in exposure time might result in different responses determined by the PAM bioassay. Therefore *P. subcapitata* was exposed to the PSII inhibitor atrazine as well as a field sample extract (XAD) with a mixture of unknown compounds with different modes of action. Concentration ranges were tested for atrazine as well as the field sample extract and EC₅₀ values, based on ΦPSII, were determined after 0.5, 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5 h.

**Actinic light**

Algae in the test are exposed to actinic light, used to drive photosynthesis, during exposure as well as during the actual PAM measurement. The effect of actinic light intensities on algal sensitivity to atrazine was determined for both situations separately. To determine the effect of light intensity during the exposure period, *P. subcapitata* was exposed to a concentration range of atrazine (Tab. 2.1) for 4.5 h with an actinic light intensity of 50, 100 or 150 µmol m⁻² s⁻¹ after which ΦPSII was measured at 100 µmol m⁻² s⁻¹. To determine the effect of light intensity during the actual PAM measurement, algae were exposed to the same atrazine concentration for 4.5 h with an actinic light intensity of 100 µmol m⁻² s⁻¹ after which the ΦPSII was measured under different actinic light intensities (50, 100 and 150 µmol m⁻² s⁻¹). For all treatments, the EC₅₀ values based on ΦPSII after 4.5 h of exposure was determined. In addition, the response of dark adapted cells was compared to the response of cells exposed to actinic light. To this purpose, *D. tertiolecta* was exposed to 10µg/L atrazine, diuron, isoproturon and Irgarol®1051 as well as to three 50x concentrated passive sampler field extracts. After 4.5 h of exposure maximum PSII efficiency (Fᵥ/Fₘ) was determined using dark adapted cells and compared to ΦPSII determined in the presence of actinic light. These two parameters are the most commonly used fluorescence parameters used in chlorophyll a based bioassays in ecotoxicology (Ralph *et al.*, 2007).
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PAM fluorometry versus 14C incorporation

Concentration ranges of 11 XAD extracted field samples from different locations in The Netherlands were tested and EC50 values were calculated for ΦPSII and 14C incorporation of P. subcapitata. ΦPSII was determined after 4.5 h of exposure as described in this study with only a deviated test volume of 20 mL. The 14C incorporation test were performed in 50mL vials with sodium bicarbonate-14C (Amsersham, 50-60mCi mmol⁻¹) according to Tubbing et al. (1993).

Statistical analysis

A 50% reduction in ΦPSII compared to the control (EC50) was calculated according the log-logistic dose-response model described by Haanstra et al. (1985). SPSS (IBM SPSS Statistics 20) was used to obtain the dose response curve and was calculated as \( y = \frac{c}{1 + e^{b \cdot (\log(x) - \log(a))}} \), where \( y \) is ΦPSII, \( x \) is the concentration of the toxicant (µg/L), \( a \) is the EC50 value (µg/L), \( b \) is the slope of the curve and \( c \) is ΦPSII of the control. Likelihood ratio tests were used to compare EC50 values (\( \chi^2 > 3.84, p<0.05 \)). Significant differences between treatments were tested with a t-test or one-way ANOVA. Data was tested for normality and equality of variances and a Bonferoni test (\( p=0.05 \)) was performed to determine significant differences between treatments. All statistical analyses were performed with SPSS (IBM SPSS Statistics 20). Correlation between EC50 obtained by ΦPSII and 14C incorporation, both determined after 4.5 h of exposure, was determined by linear regression (Microsoft Excel 2010).
Results and discussion

Effect of test factors on the toxic response

Culturing conditions

Although an increased light intensity significantly (p<0.05) reduced the actual fluorescence (F) compared to the standard culturing conditions, no significant difference in F was observed for the other culturing conditions and none of the culturing conditions affected the maximal fluorescence (F’\textsubscript{m}) (Fig. 2.1). When the fluorescence parameters are combined to calculate Φ\textsubscript{PSII}, no significant (p>0.05) effects of changed culturing conditions were observed in any of the treatments, making it a reliable toxic endpoint (Fig. 2.1). Moreover, EC\textsubscript{50} values based on Φ\textsubscript{PSII} after 4.5 h of exposure to atrazine were not significantly different (p>0.05) compared to the standard conditions (Fig. 2.1). However, although light and temperature did not affect the test results in this study, an effect can be observed when more extreme light and temperature conditions are tested (Bengtson Nash and Quayle 2007; Chalifour and Juneau 2011).

Cell density

The Φ\textsubscript{PSII} inhibition after exposure to atrazine was significantly decreased (p<0.05) for a cell density of 1\textsuperscript{e}\textsuperscript{7} cells/mL compared to the inhibition of a cell density of 1\textsuperscript{e}\textsuperscript{6} cells/mL. No significant difference (p>0.05) was observed for a lower cell density of 1\textsuperscript{e}\textsuperscript{5} cells/mL compared to 1\textsuperscript{e}\textsuperscript{6} cells/mL. These results show that cell density can affect the outcome of the toxicity test which can be caused by for example a changed bioavailability of the compound. Since PAM fluorometry is an optical method, the intensity of the measured signal is of great importance and the cell density should be high enough to be detected by the PAM fluorometer, although high densities should be avoided as they can affect the toxicity results as demonstrated in this study. Because fluorescence signals are different for different algal species, additional testing is required when other microalgal species are used in PAM bioassays.
Solvent concentration

Concentrations of the carrier solvent methanol of 0.5, 1.0, 1.5, 2.0 and 2.5 % resulted in an inhibition of $\Phi$PSII compared to the control of 1, 4, 10, 10 and 15% respectively. Although El Jay (1996) demonstrated that methanol concentration > 0.1 % affected growth of C. vulgaris and P. subcapitata after 4 days, no difference in chlorophyll content of these algae was observed after one day at the highest test concentration of 1 %. Due to the short exposure time needed for the PAM bioassay as described in this study, a lower solvent
concentration of 0.01 % as described for several standardized algal growth inhibition tests (ISO 8692:2012, ISO 10253:2006, OECD 201) is not necessary, making it easier to test effects of compounds in concentrations close to the water solubility.

**Exposure time**

The EC$_{50}$ value after exposure to atrazine does not significantly (p<0.05) increase or decrease after 1.5 h (Fig. 2.2). Schreiber et al. (2007) obtained a stable value for the toxicity of the PSII inhibitor diuron even after 10 minutes although a different test species was used. While a longer exposure time might be needed to obtain a stable EC$_{50}$ value for other compounds like Triclosan (Franz et al., 2008), we demonstrated that for a complex field extract with a mixture of unknown compounds a significantly (p<0.05) stable EC$_{50}$ value was obtained after 4.5 h (Fig. 2.2). Since the field extract will most likely also consist of non-PSII inhibitors, these results are corresponding with Fai et al. (2007) where non-PSII inhibitors needed a longer exposure time to show an effect. Compared to the standardized toxicity test on algal growth inhibition of several days, PAM fluorometry provides a more rapid alternative to test the toxicity of a range of individual compounds as well as for complex field samples.

![Fig. 2.2. Determination of the EC$_{50}$ value of ΦPSII of atrazine (solid line) and a concentrated field extract (dotted line) after 0.5, 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5 hours. Error bars represent standard error of the EC$_{50}$ values. N=2.](image-url)
Actinic light

The EC_{50} value of ΦPSII after 4.5 h of exposure to atrazine was not significantly (p>0.05) affected by the intensity of actinic light used during the exposure time or the actual PAM measurement. Nevertheless, several studies demonstrated that light conditions can influence the test results (Guasch and Sabater, 1998; Bengtson Nash and Quayle 2007; Schreiber et al., 2007). Additionally, the present study showed that, except for atrazine, a significantly (p<0.05) lower toxicity was observed in total absence of light (F_v/F_m) compared to measurements in the presence of actinic light (ΦPSII) (Fig. 2.3A). Dark measurements are often performed in PAM studies because high fluorescence signals are obtained, but here we demonstrate that in PAM bioassays toxic effects of chemicals can be underestimated if only F_v/F_m is determined. The significantly (p<0.05) higher toxic response for ΦPSII in all field extracts (Fig. 2.3B), sampled in different periods of the year, confirms that ΦPSII is also a good endpoint to determine toxicity of complex mixtures. Our observations corroborates the study of Ralph et al. (2007) who reviewed several studies comparing different toxic endpoints and concluded that ΦPSII is generally the most sensitive. Considering the facts that PAM measurements are based on optical signals and that photosynthetic processes in algae are primarily driven by the presence of light it is evident that light conditions plays a crucial role in the setup of PAM bioassay. However, as ΦPSII and F_v/F_m provide complementary information, they can be determined together and the selection of a suitable endpoint will depend on the required information. For the purpose of the present study, focusing on herbicides and herbicide containing mixtures, ΦPSII is more sensitive indicator for toxicity compared to F_v/F_m (Ralph et al., 2007). The results presented here demonstrated that although the tested light intensities did not affect the test results, the total absence of light can give an underestimation on the toxic effect. Nevertheless the light intensity should be taken into account as demonstrated by other studies.
Fig. 2.3. Inhibition of PSII (% control) after exposure to four toxicants (a) and three field extracts (b) for effective (ΦPSII, white bars) and maximum (Fv/Fm, black bars) PSII efficiency. Toxicity was determined for 10 µg/L atrazine, diuron, isoproturon and Irgarol® 1051 (N=6) and three concentrated field extracts sampled in March, July and September (N=2) after 4.5 h of exposure. * = significant difference between ΦPSII and Fv/Fm (p<0.05). Error bars represent standard deviation.
Application of PAM fluorometry in algal toxicity testing

Little preparation and analysis time combined with a short exposure time makes a PAM bioassay a quick high throughput technique, measuring the effect on algal photosynthesis with small volumes and large sample sizes for commercially available compounds as well as field extracts. Next to the logistical benefits of such a rapid bioassay, the advantage over a long-term test like the standardized 72-96 h growth inhibitions tests is that also changes in for example pH and contaminant concentration, due to degradation and/or adhesion to the test material, are less likely to occur. However, the question arises if such a short term test is representative for ecological relevant processes like population growth rate. Versteeg et al. (1990) demonstrated that photosynthetic activity test based on 14CO2 fixation (35 minutes) and O2 generation (30 minutes) was less sensitive than a 4-day population growth test for diethyl phthalate, pentachlorophenol, carbaryl, copper, cadmium, atrazine and simazine with ratio’s (based on EC50 values) ranging between 1.4 and >161. This illustrates that toxic effects on algal populations after long term exposure to contaminants cannot always be predicted based on a short term toxicity test. However, with PAM bioassays a good correlation between 14C and ΦPSII was observed for periphyton exposed to the PSII inhibitors atrazine (R2=0.82), prometryn (R2=0.72) and isoproturon (R2=0.79) (Schmitt-Jansen and Altenburger, 2008). In addition, this study demonstrates a high correlation (R2=0.88) between 14C incorporation and PAM fluorometry for field samples with a complex mixture of compounds (Fig. 2.4). Furthermore, a consistent

![Fig. 2.4. EC50 values of 11 XAD extracted field samples based on ΦPSII determined by PAM fluorometry after 4.5 hours of exposure plotted against EC50 values of the same samples based on inhibition of 14C incorporation after 4.5 hours of exposure. The solid line represents linear regression (R²=0.88).](image-url)
relationship between inhibition of growth rate and \( \Phi_{\text{PSII}} \), both determined after 3 days, of the PSII inhibiting herbicides atrazine, diuron and hexazinone has been demonstrated for benthic microalgae (Magnusson et al., 2008). For marine phytoplankton it was demonstrated that effect concentrations after 3 days of exposure to the PSII inhibitor Irgarol®1051, were very similar for \( \Phi_{\text{PSII}} \) and growth inhibition for several algal species (Buma et al., 2009). These results suggest that, at least for these PSII inhibitors and algal species, \( \Phi_{\text{PSII}} \) provides a good prediction on the effect of algal growth underpinning the ecological relevance of this algal bioassay. The ecological relevance of this technique can be further increased by application in more field relevant experiments like recovery experiments (Jones and Kerswell 2003; Buma et al., 2009) and toxicity tests at a community level (Schmitt-Jansen and Altenburger 2007; Magnusson et al., 2012). Recently, the PAM bioassay as described in this study, with D. tertiolecta as a test species, has been incorporated in an Effect Directed Analysis (EDA) to identify phytotoxic compounds in the water.

Concluding, when performing laboratory bioassays using PAM fluorometry it is important to optimize the selected test conditions during the entire test setup since they might influence the outcome of the toxicity test. The information presented in this study provides important background information for the optimization of the bioassay and for interpretation of toxicity results. We have described which test conditions should be taken into account when using an algal bioassay with PAM fluorometry, however the exact settings have to be selected based on specific aims of the study.

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Supporting information available: Tab. S1. This information is available online (doi:10.1002/etc.2537).