Photoenhanced toxicity of azaarenes to marine phytoplankton.
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The role of UV adaptation of a marine diatom in photoenhanced toxicity of acridine

Abstract

Cultures of the marine diatom *Phaeodactylum tricornutum* were grown under laboratory light with a different fraction of ultraviolet radiation (UV) to study the potential role of photoadaptation in determining the sensitivity to photoenhanced toxicity of acridine. In short-term experiments a higher acridine concentration was needed to inhibit the photosynthetic electron flux, monitored with chlorophyll *a* fluorescence, in algae exposed to fluorescent light (low UV) than to mercury light (high UV), consistent with the expected role of UV. The two types of light in long-term exposures led to changes in the pigment composition and PS I to PS II stoichiometry to optimise the utilization of fluorescent and mercury light. Despite the adaptation of the photosynthetic apparatus to a small fraction of UV, long-term exposure to mercury light did show a constant sensitivity of the photosynthetic efficiency of *P. tricornutum* to the phototoxic acridine. It is concluded that the prime receptor of photoenhanced toxicity may be unrelated to the photosynthetic machinery.

Introduction

Several aromatic hydrocarbons, among which azaarenes (heterocyclic PAHs containing one in-ring nitrogen atom) absorb radiation in the ultraviolet range. This may cause photoenhanced toxicity by two photochemical mechanisms: photosensitization (formation of reactive species) and photomodification (formation of oxygenated products) (Larson and Berenbaum, 1988; Leifer, 1988; Veith et al., 1995; Arfsten et al., 1996; Huang et al., 1997a). Both photochemical reactions were found to contribute to adverse effects on growth and photosynthesis of algae (Wiegman et al., 1999; Marwood et al., 2001; Wiegman et al., 2001) and macrophytes (Huang et al., 1993; Huang et al., 1997b).

Photoenhanced toxicity of anthracene, a three-ringed PAH, has been shown to increase with decreasing content of carotenoid pigments in the green alga Selenastrum capricornutum, suggesting that carotenoid pigments can protect algae from the adverse effects of reactive species such as singlet oxygen formed in photosensitization reactions (Gala and Giesy, 1993). Carotenoids have natural photoprotective functions in the photosynthetic apparatus, extensively reviewed by (Demmig-Adams, 1990; Young, 1991; Niyogi, 1999): on the one hand carotenoids dissipate excess excitation energy as heat, on the other hand these pigments act as active quenchers of triplet-state chlorophyll, preventing the formation of oxygen radicals, and singlet oxygen ($^1$O$_2$).

Different studies reported that algae, transferred from low to high intensities of light and from cool-white fluorescent light to light with UV radiation, adapted to these light conditions by increasing their amount of carotenoids (Goss et al., 1999; Masojidek et al., 1999; Underwood et al., 1999). This type of UV protection would suggest that UV adapted algae are more protected against the adverse effects of PAH photosensitization than algae in the absence of UV radiation.

The present study investigated, therefore, the interference of UV adaptation and photoenhanced toxicity of acridine, a three-ringed azaarene, to the photosynthesis of Phaeodactyllum tricornutum, using the Pulse Amplitude-Modulated (PAM) fluorometry. Two sets of experiments were carried out, in which the short-term effects of acridine on P. tricornutum, cultured under cool-white fluorescent light, were determined in the presence of fluorescent
light and mercury light (UV) and, secondly, in which the long-term effects of acridine on *P. tricornutum* were monitored in the presence of mercury light.

Pulse amplitude-modulated (PAM) fluorometry, measuring chlorophyll *a* fluorescence, is a rapid and sensitive technique to determine the effects of environmental stressors on the photosynthetic electron transport of photosystem II (PS II) in macrophytes and algae in vivo (Samson and Popovic, 1988; Huang *et al.*, 1997b; Snel *et al.*, 1998; Marwood *et al.*, 1999). PAM fluorometry provides fluorescence parameters correlating with biomass (chlorophyll *a* content), electron transport efficiency of photosystem II (PS II) and the relative energy dissipation or the quenching of chlorophyll *a* fluorescence (Genty *et al.*, 1989; Bilger *et al.*, 1995; Schreiber *et al.*, 1996). Light absorbed by chlorophyll *a* is distributed between photochemical quenching (*q*P), the dissipation of energy as fluorescence, and, the dissipation of energy as heat, non-photochemical quenching, expressed as *q*N or NPQ (Masojidek *et al.*, 1999). With these fluorescence parameters it is attempted to analyse the mechanisms associated with UV protection and photoenhanced toxicity of acridine to the photosynthesis of *P. tricornutum*.

**Materials and methods**

* Culturing of Phaeodactylum tricornutum*

The marine diatom *P. tricornutum* (Bohlin) was cultured in batches artificial seawater medium (Admiraal and Werner, 1983) under cool-white fluorescent light (fluence rates for wavelengths < 400 nm are 0.1 µmol m⁻² s⁻¹ for wavelength > 400 nm 90 µmol m⁻² s⁻¹) at 20 °C. The light-dark regime was 16:8 h. Tris-buffer (3.7 mM, Merck) was added to the artificial seawater medium to maintain the pH at 8.

*Experimental set-up*

Photoenhanced toxicity of acridine (97% purity; Aldrich) to *P. tricornutum* was assessed in an incubator equipped with a mercury light source (fluence rates for wavelengths < 400 nm are 5 µmol m⁻² s⁻¹ and for > 400 nm 80 µmol m⁻² s⁻¹, Philips HPI-T 400 W), under the same conditions as the
culture. Algal cells in growing phase were added to fresh medium containing nutrient concentrations as described in Admiraal and Werner (1983).

Acridine was added to the medium using the carrier solvent dimethyl sulphoxide (DMSO, > 99.5%, Merck). Separate stock solutions were made for each acridine treatment by dissolving the highest used acridine concentration in DMSO, followed by a dilution series in DMSO. An equal volume of each stock solution was added to the corresponding experimental treatment, resulting in equivalent DMSO concentrations in all treatments. Besides controls (algae without acridine and without DMSO), DMSO controls (algae with DMSO but without acridine) were incorporated to check for effects of the carrier solvent on *P. tricornutum*. The acridine concentration series (including all controls) was tested in duplicate. Water samples (1–2 ml) were taken to determine the cell numbers, chlorophyll *a* fluorescence and acridine concentrations in the medium. Cell numbers and volumes of *P. tricornutum* (in triplicate) were determined with a Coulter counter (Coulter Multisizer) with a counting tube aperture of 70 μm. At the end of the experiment, the remaining algal suspension was filtered on GFF filters (glass microfibre filters, Whatman) for the determination of algal pigment content. GFF filters were frozen immediately and stored at -18 °C until spectrophotometric analysis.

**Short-term effects of acridine**

35 ml of algal suspension, originating from the batch culture under fluorescent light, were added to 50 ml glass jars and exposed to the following average actual acridine concentrations; 0.5, 1.2, 1.3, 4.8, and 10.6 μM (0.1% v/v DMSO). At the start of the experiment (t₀), the algal cell and acridine concentration in the medium was determined.

Due to photoinhibition by mercury light (data not shown), the fluorescence of chlorophyll *a* was diminished in all treatments, and consequently the electron transport of photosystem II (i.e. quantum yield of PS II) was inhibited (see also Demmig-Adams, 1990; West *et al.*, 1999; Marwood *et al.*, 2000). In contrast, the fluorescence of chlorophyll *a* under fluorescent light was measurable. Therefore, after 3.5 h exposure to mercury light, all treatments were placed under cool-white fluorescent light for 3 more hours. After 3 h exposure to fluorescent light, the chlorophyll *a* fluorescence of the
control treatments exposed (3.5 h) to mercury light recovered. This recovery enabled us to measure chlorophyll \(a\) fluorescence in all treatments, either exposed to mercury (3.5 h) and fluorescent light (3 h) or to fluorescent light only (6.5 h).

**Long-term effects of acridine**

Long-term effects (up to 72 h) of acridine on *P. tricornutum* were measured under mercury light (80 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)). Culturing of algae is routinely performed under fluorescent light (standard culturing light in toxicity tests; NEN 6506; EPA/600/4-91/002), whereas experiments regarding photoenhanced toxicity are performed under mercury light containing UV radiation. Here, algae were pre-cultured under fluorescent light and under mercury light and acclimatized for 24 h to mercury light to determine the long-term effects of acridine (as in previous experiments; Wiegman *et al.*, 2001). 180 ml glass jars were filled with 130 ml of algal suspension of *P. tricornutum* and each jar was aerated with a glass Pasteur pipette to ensure a water circulation and to keep algae in suspension. After 24 h acclimatization, a concentration series of acridine (0.04% v/v DMSO) was added. The average actual acridine concentrations tested were 0.5, 1.0, 1.1, 4.2, and 8.5 \(\mu\)M. Water samples were taken daily to determine the cell numbers, chlorophyll \(a\) fluorescence and acridine concentrations in the medium.

**Chlorophyll \(a\) fluorescence**

Chlorophyll \(a\) fluorescence was measured with a Pulse Amplitude-Modulated fluorometer (MINI-PAM; Walz, GmbH, Effeltrich, Germany). The detection limit of the fluorometer was approximately \(10^6\) cells of *P. tricornutum* per ml. The settings (measuring light and gain) of the fluorometer were optimised before proceeding with the measurements. The chlorophyll \(a\) fluorescence nomenclature of van Kooten and Snel (1990) was used.

Aliquots of 1 ml algal suspension were collected in a plastic vessel (Kartell, path length of 1 cm) and placed in a holder that was fixed with respect to the fibre-optics of the fluorometer to keep the distance between the sample and the fibre-optics constant during all measurements. In each sample, the chlorophyll \(a\) fluorescence was determined for light-adapted (cool-white
light: 80 μmol m⁻² s⁻¹) and dark-adapted (30 min) algae.

\( F_0 \) was measured in dark-adapted algae with low-intensity modulated light (0.15 μmol m⁻² s⁻¹), and represents the minimum chlorophyll \( a \) fluorescence. \( F_m \) was measured with a 0.6 s lasting pulse of saturating white light (10⁴ μmol m⁻² s⁻¹) and is the maximum chlorophyll \( a \) fluorescence. Maximum quantum yield of PS II photochemistry \( (F_s/F_m) \) was calculated according to Genty et al. (1989) and is a measure for the maximal efficiency of electron transport:

\[
F_s/F_m = (F_m - F_0)/F_m
\]  

(5.1)

In the presence of actinic light (80 μmol m⁻² s⁻¹), the parameters \( F_s, F'_m \) and effective quantum yield of PS II photochemistry \( (\Delta F/F'_m) \) were measured (light-adapted algae). \( F_s \) is the steady state and \( F'_m \) is the maximal chlorophyll \( a \) fluorescence under actinic light and both are determined by applying a minimum of 6 pulses of saturating light every 20 s. The effective quantum yield was calculated as the average of the last three successive pulses:

\[
\Delta F/F'_m = (F'_m - F_s)/F'_m
\]  

(5.2)

Directly after measuring \( F_s \) and \( F'_m \), actinic light was switched off and \( F'_0 \) was measured. With \( F'_0 \) the quenching coefficients \( qP \) (photochemical quenching) and \( qN \) (non-photochemical quenching) were calculated (Hofstraat et al., 1994):

\[
qP = (F'_m - F_s)/(F'_m - F'_0)
\]  

(5.3)

\[
qN = 1 - (F'_m - F'_0)/(F_m - F_0)
\]  

(5.4)

With the Stern-Volmer formula, the non-photochemical quenching of chlorophyll \( a \) fluorescence (NPQ), associated with the heat dissipation of excited energy in the antenna system (Demmig-Adams, 1990), was also calculated:

\[
NPQ = (F_m - F'_m)/F'_m
\]  

(5.5)

In addition, the relative electron flux (the amount of PS II units capable of
electron transport) can be estimated using the quotient of $F_0$ and effective quantum yield of PS II ($\Delta F/F'_m$), providing that the optic pathway for light is equal for all treatments (Geel, 1997).

Analysis of acridine by HPLC

After centrifugation (3000 rpm, 10 min), 1 ml of the supernatant was taken for analysis by high performance liquid chromatography (HPLC), using UV detection (Applied Biosystems model 785A). A $150 \times 4.6$ mm Lichrosorb 5 $\mu$m RP-18 analytical column was used with a $4 \times 4$ mm Lichrosorb 5 $\mu$m RP-18 guard column. The column temperature was kept at room temperature ($20 ^\circ C$). The flow of the mobile phase, an isocratic mixture of 65% acetonitrile (J.T. Baker Analyzed HPLC Reagent, > 99.9%) and 35% water (J.T. Baker Analyzed HPLC Reagent), was 1 ml/min. Of each sample, 20 $\mu$l were injected automatically. Acridine concentrations were calibrated with standards of acridine in methanol (J.T. Baker Analyzed HPLC Reagent, > 99.8%).

Algal pigments determination

The chlorophyll $a$ concentration, phaeopigment concentration and ratio of carotenoids (430 nm/665 nm) were determined in 90% acetone (Acros Organics Pro Analyse, > 99.5%), according to Lorenzen (1967) using an UV-visible spectrophotometer (UV-1601, Shimadzu).

For *P. tricornutum* cultured under fluorescent light and cultured under mercury light (duplicate), the pigment concentrations were determined using HPLC analysis. An ISCO 77 system equipped with a $250 \times 3.2$ mm 5 $\mu$m RP-18 Hypersil ODS-2 column and connected to an ISCO detector and a Diode Array Detector (DAD 440, Kontron Instruments) was used for the separations and identification of individual pigments. The pigment absorption was recorded at 665 nm (chlorophyll $a$) and at 436 nm (carotenoids) in the ISCO detector, and at 452 nm (chlorophyll $c$) in the Diode Array Detector. Of each sample, 100 $\mu$l (70% v/v methanol) was injected. The solvent program is described in Kraay *et al.* (1992), with 90% v/v acetonitrile and 10% v/v water as solvent A and 100% ethyl acetate as solvent B. All solvents were HPLC grade (J.T. Baker Analyzed HPLC Reagent, > 99.8%). For both the cultures, the chlorophyll $a$ absorption (at 665 nm) per
algal cell was equal ($p = 0.75, n = 4$) and, the separate pigment concentrations were calculated relative to total chlorophyll $a$ (chlorophyll $a$, chlorophyll $a$ allomer and chlorophyll $a$ epimer) absorption.

**Low temperature fluorescence (77K)**

Low temperature fluorescence was determined for *P. tricornutum* cultured under fluorescent light as well as under mercury light. From each culture, vials containing 10 ml algal suspensions were kept in dark for 1 h. From each vial, three samples were collected with glass Pasteur pipettes (2 mm inner diameter) and frozen in liquid nitrogen. From each sample immersed in liquid nitrogen, the fluorescence emission was measured in an Aminco-Bowman Series 2 Luminescence spectrometer equipped with a Dewar flask mounted between the light beam and the detector. The fluorescence emission was recorded from 650 to 750 nm with an excitation wavelength of 440 nm. A bandwidth of 4 nm was used for both excitation and emission. PS II related fluorescence was taken at an emission of 685 nm and PS I related fluorescence at 720 nm (Murakami, 1997).

**Statistical analyses**

The acridine concentrations that reduced the chlorophyll $a$ fluorescence (or the chlorophyll $a$ concentration or the growth rate) of *P. tricornutum* to 50% of the (DMSO) controls (EC50 values) were obtained by fitting the following equation (Haanstra et al., 1985) through the dose-response plots with Kaleidagraph:

$$Y = c/(1 + \exp^{b(X - a)})$$  \hspace{1cm} (5.6)

where $Y$ is effect on fluorescence parameter, $X$ is $10\log$ acridine concentration ($\mu$M), $a$ is $10\log$ EC50, $b$ is slope of the logistic curve and $c$ is average of the (DMSO) controls. The corresponding 95% confidence intervals were calculated according to the method described by Miller and Miller (1984).

The difference between the treatments was tested with one-way ANOVA or the non-parametric test Kruskal-Wallis in SPSS 9.0 (SPSS Inc.) at a significance level of $p < 0.05$ for treatment numbers ($n$) > 2; for treatment numbers of $n = 2$, a two-tailed student’s $t$ test was used (Microsoft Excel 2000). To
test for differences in toxicity of acridine between the two cultures, the toxicity data were fit simultaneously to logistic models that differed in their slope parameters but had the same EC50 parameter, using SPSS 9.0 (SPSS Inc.) for windows (van Gestel and Hensbergen, 1997).

**Results and discussion**

*Short-term effects of acridine*

Cultures of the marine diatom *Phaeodactylum tricornutum* were exposed to a concentration series of acridine in the presence of UV radiation (mercury light) or in the absence of UV (cool-white fluorescent light) to determine the short-term photoenhanced toxicity of acridine. Clear dose-response curves were observed for the effect of acridine on the chlorophyll $a$ fluorescence parameters $F_0$, $F_m$, $F_s$, $F'_m$, the maximum quantum yield ($F_m/F_m$) and the effective quantum yield ($\Delta F/F'_m$) of PS II of algae exposed to mercury light (Figure 5.1).

The dose-response curves of $F_s$, $F'_m$ and the effective quantum yield (all light-adapted state parameters) of *P. tricornutum* exposed to fluorescent light were similar to those obtained under mercury light. In contrast, a difference in the dose-response curves for the dark-adapted state parameters $F_0$, $F_m$ and the maximum quantum yield ($F_m/F_m$) was observed between algae exposed to acridine under fluorescent and mercury light. In the presence of fluorescent light, a higher acridine concentration was needed to affect the capacity of PS II than in the presence of mercury light, which is consistent with the expected role of a higher UV fraction in mercury light (Figure 5.1). Thus, short-term exposure to mercury light (3.5 h) enhanced the toxicity of acridine, as previously demonstrated for anthracene (Huang *et al.*, 1997b; Marwood *et al.*, 1999).

In Figure 5.2 the chlorophyll $a$ content, the ratio of total carotenoids to chlorophyll $a$ (430/665 nm absorbance) and the fluorescence parameter NPQ (non-photochemical quenching) are plotted against the different acridine concentrations. For phaeopigment concentration, no differences were observed between acridine treatments and light sources (data not presented). A significant increase of the carotenoids to chlorophyll $a$ content
was observed after treatment with acridine under mercury light \( (p < 0.05) \), i.e. the treatments in which photoenhanced toxicity took place (Figure 5.2).

![Graph](image)

**Figure 5.1.** Short-term effects: Fluorescence parameters (relative fluorescence units) of *Phaeodactylum tricornutum* exposed to mercury light or to fluorescent light plotted against the acridine concentration in the medium \((\mu M)\). Error bars represent the standard error of the mean of two replicates \((n = 4\) for the controls).

Since carotenoids play a key role in excess excitation energy dissipation and can act as active quenchers of oxygen radicals (Demmig-Adams, 1990; Young, 1991; Niyogi, 1999), our findings may indicate that photoenhanced toxicity of acridine mimic some of the physiological effects of UV as found by Gala and Giesy (1993). Under both light sources, the relative energy dissipated as heat, expressed as NPQ, was rising rapidly with increasing acridine concentration. However, in the treatment with the highest acridine concentration exposed to mercury light, NPQ decreased. This could indicate
that due to photosensitization of acridine permanent damage in the photosystem II of *P. tricornutum* occurred (Wiegman et al., 2001).

![Diagram of chlorophyll a concentrations, ratio of 430 nm/665 nm absorbance and non-photochemical quenching (NPQ) of *Phaeodactylum tricornutum* exposed to mercury light or to fluorescent light plotted against the acridine concentration in the medium (μM). Error bars represent the standard error of the mean of two replicates (n = 4 for the controls).](image)

**Figure 5.2.** Short-term effects: Chlorophyll a concentrations, ratio of 430 nm/665 nm absorbance and non-photochemical quenching (NPQ) of *Phaeodactylum tricornutum* exposed to mercury light or to fluorescent light plotted against the acridine concentration in the medium (μM). Error bars represent the standard error of the mean of two replicates (n = 4 for the controls).

Under fluorescent light, effective (ΔF/F'_m) and maximum (F_v/F_m) quantum yields were lower than under mercury light (Figure 5.1). In addition, the non-photochemical quenching (NPQ) of control treatments was higher under mercury light than under fluorescent light (Figure 5.2). These results show that a 3 h recovery period of *P. tricornutum* from photoinhibition by mercury light was not sufficient and the diatoms may still have been suffering from direct UV-stress besides from the toxic effects of acridine.
(Demmig-Adams, 1990; Casper-Lindley and Bjorkman, 1998; West et al., 1999; Marwood et al., 2001). It is concluded that on relatively short-time scales, as used in the present study, UV-stress interferes with the assessment of photoenhanced toxicity of algae cultured under fluorescent light.

To study this transient effect of UV on photosynthesis of *P. tricornutum* and photoenhanced toxicity of acridine, a second, long-term experiment was performed. To this purpose, *P. tricornutum* was cultured under fluorescent and under mercury light and the physiological adaptations to these light sources of both cultures were determined prior to a second toxicity test in which the different UV adapted cultures were exposed to acridine under mercury light.

**Physiological adaptation to UV**

*P. tricornutum* contains the photosynthetic pigments chlorophyll *a*, chlorophyll *c*, the photo-protective carotenoids; fucoxanthin, \( \beta \)-carotene and the xanthophylls violaxanthin, zeaxanthin, diadinoxanthin, and diatoxanthin (Young, 1991; Caron et al., 2001). *P. tricornutum* cultured under mercury light had higher chlorophyll *c* content than cultured under fluorescent light \((p < 0.05, \text{ Figure 5.3})\). Fucoxanthin and chlorophyll *a* contents were equal for both cultures, but the xanthophylls \((\Sigma XC)\) and \( \beta \)-carotene contents were higher under fluorescent light than under mercury light \((p < 0.05 \text{ for the xanthophylls, Figure 5.3})\). The ratio of xanthophylls to carotenoids, the ratio of carotenoids to chlorophyll *a*, and, accordingly, the spectrophotometrically determined carotenoids to chlorophyll ratio (430 nm/665 nm) were higher for *P. tricornutum* cultured under fluorescent light than for *P. tricornutum* cultured under mercury light (Figure 5.3). In addition to their photoprotective role, carotenoids act as accessory light-harvesting pigments (Demmig-Adams, 1990). Both the carotenoids and chlorophyll *c* absorb light in a wavelength region that is not accessible for chlorophyll *a*, extending the light-absorbing range for photosynthesis (Young, 1991; Caron et al., 2001). The difference in pigment composition of algae in the absence and presence of (high intensities of) UV, as observed in Underwood et al. (1999), Estevez et al. (2001) and Zudaire and Roy (2001), are associated to either the photoprotective or the light-harvesting role of pigments. In our experiments, both under fluorescent and under mercury light, *P. tricornutum* received moderate light intensities. Therefore, it is more likely that *P. tricornutum* opti-
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mised the composition of the light-harvesting pigments (chlorophyll c and carotenoids) to the used light sources rather than that of radical scavengers (xanthophylls and β-carotene).

Figure 5.3. Pigment composition of Phaeodactylum tricornutum, cultured under fluorescent light or under mercury light. Pigment content expressed as percentage of total chlorophyll a, including chlorophyll a allomer and epimer, (left panel) and ratios of pigments and 430 nm/665 nm absorbance (right panel). Error bars represent the standard error of the mean of two samples (**p < 0.05, *p < 0.1).

The fluorescence emission of PS II and PS I of both cultures was measured with cold (77 K) fluorescence (Figure 5.4). For P. tricornutum cultured under fluorescent light the PS II and I fluorescence emissions were equal, whereas for P. tricornutum cultured under mercury light, the fluorescence emission of PS II was higher than that of PS I (Figure 5.4). The ratio of fluorescence emission of PS II and I reflects the amount of antennae associated to each photosystem (Murakami, 1997). To optimise the electron flow between the PS II and I, both antenna size and photosystem stoichiometry in
algae can be modulated (MacIntyre et al., 2000; Chitnis, 2001). Under high light intensities and light without UV radiation excitation of PS I is preferred, whereas under low light intensities and light with UV radiation the excitation of PS II is favoured (MacIntyre et al., 2000). The observed decrease in the amount of PS I antenna pigment of \textit{P. tricornutum} cultured under mercury light compared to fluorescent light is, therefore, a direct consequence of the presence of UV radiation in mercury light.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fluo.png}
\caption{77K cold fluorescence emission (relative fluorescence units) of photosystem II and I of \textit{Phaeodactylum tricornutum}, cultured under fluorescent light or under mercury light.}
\end{figure}

Figure 5.5 shows the light-response curves for \textit{P. tricornutum}, cultured under fluorescent and mercury light measured with modulated fluorometry. These curves, recorded with relative short illuminations periods of fluorescent light compared to the traditional PI curves, are indicative for the rapid photoadaptation of algae to fast and large changes in light intensities (Henley, 1993; White and Critchley, 1999). Although the pigment composition (Figure 5.3) and the amount of antenna pigment of PS I (Figure 5.4) changed under mercury light, the amount of antenna pigments of PS II
and, accordingly, the photosynthetic capacity of PS II of *P. tricornutum* remained unaltered.

![Graph showing light saturation curves for *Phaeodactylum tricornutum* cultured under fluorescent light or under mercury light. Error bars represent the standard error of the mean of two replicates.]

**Figure 5.5.** Light "saturation" curves for *Phaeodactylum tricornutum*, cultured under fluorescent light or under mercury light. Error bars represent the standard error of the mean of two replicates.

The physiology of *P. tricornutum* cultured under mercury light clearly differed from that of the algae cultured under fluorescent light. Because of these UV adaptations a different response of the photosynthetic electron transport of PS II (chlorophyll *a* fluorescence) to acridine between the two cultures was expected.

**Long-term effects of acridine**

The long-term experiment started with *P. tricornutum* cells pre-cultured under fluorescent light or mercury light and was completed under mercury light. The growth rates of the control treatments (calculated over the 72 h exposure period to acridine) and the 72 h EC50 values of acridine for
growth were not different for algae pre-cultured under fluorescent light and mercury light ($p > 0.5$; Table 5.1 and 5.2). So physiological conditions due to different light sources did not persist through the 24 h acclimatization period under mercury light. However, the levels of acridine that affected growth were much lower than the EC50 values for chlorophyll $a$ fluorescence of algae exposed to mercury light in the short-term experiment. Toxicity of aromatic compounds will increase with increasing exposure time (Marwood et al., 2001). Therefore, the observed inhibition of growth by acridine may have proceeded via accumulated damage to the photosynthetic apparatus, resulting from lowered photosynthetic performance to reduced synthesis of new biomass, as has been observed for macrophytes exposed to anthracene and creosote (Huang et al., 1997b; Marwood et al., 2001).

**Table 5.1.** Long-term effects: Cell number and growth rates ($\pm$ standard error) for cultures of *Phaeodactylum tricornutum* under mercury light, pre-cultured under fluorescent or mercury light

<table>
<thead>
<tr>
<th>Precultured</th>
<th>Cell number ($10^6$ cells/ml)</th>
<th>72 h Growth rate (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_0$ (SE)</td>
<td>$t_{72}$ (SE)</td>
</tr>
<tr>
<td>Fluorescent light</td>
<td>1.24 (0.04)</td>
<td>12.9 (0.17)</td>
</tr>
<tr>
<td>Mercury light</td>
<td>1.84 (0.03)</td>
<td>19.9 (1.72)</td>
</tr>
<tr>
<td>$p$ ($n = 4$)</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

**Table 5.2.** 72 h effect concentrations (95% confidence limits) of acridine on growth rate and regression coefficient ($r^2$) for the dose-response curve

<table>
<thead>
<tr>
<th>72 h EC50 value</th>
<th>$\mu$M (95% CL)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescent light</td>
<td>1.55 (1.37-1.75)</td>
<td>0.99</td>
</tr>
<tr>
<td>Mercury light</td>
<td>1.53 (1.27-1.83)</td>
<td>0.98</td>
</tr>
<tr>
<td>$p$ ($n = 14$)</td>
<td>&gt; 0.05</td>
<td>-</td>
</tr>
</tbody>
</table>

Acridine was observed to have an effect on the fluorescence parameters after addition of only very high concentrations to cultures. Yet, clear dose-response curves were obtained after 24-, 48- and 72-h, and EC50 values for acridine on the fluorescence parameters of *P. tricornutum* pre-cultured under fluorescent or under mercury light were calculated. In Table 5.3 the EC50 values for $F_0$, $F_s/F_m$, $\Delta F/F_m$ and $F_0 \times \Delta F/F_m$ are reported; the EC50 values for $F_m$, $F_s$ and $F'_m$ equalled those for $F_0$ and were, therefore, omitted.
Table 5.3. Long-term effects: Effect concentrations of acridine (EC50, 95% confidence limit) in μM on *Phaeodactylum tricornutum* pre-cultured under fluorescent or mercury light, based on four fluorescence parameters. Regression coefficients ($r^2$) for the dose-response curves

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fluorescent light</th>
<th>Mercury light</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h EC50</td>
<td>$r^2$</td>
</tr>
<tr>
<td>$F_0$</td>
<td>9.22 (4.64–18.3)</td>
<td>0.77</td>
</tr>
<tr>
<td>$F/F_m$</td>
<td>4.73 (4.19–5.34)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98</td>
</tr>
<tr>
<td>$\Delta F/F'_m$</td>
<td>3.87 (3.43–4.38)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.98</td>
</tr>
<tr>
<td>$F_0 \times \Delta F/F'_m$</td>
<td>2.63 (1.90–3.63)</td>
<td>0.92</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fluorescent light</th>
<th>Mercury light</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h EC50</td>
<td>$r^2$</td>
</tr>
<tr>
<td>$F_0$</td>
<td>1.37 (0.96–1.95)</td>
<td>0.95</td>
</tr>
<tr>
<td>$F/F_m$</td>
<td>3.51 (3.08–4.00)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98</td>
</tr>
<tr>
<td>$\Delta F/F'_m$</td>
<td>3.30 (2.68–4.07)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92</td>
</tr>
<tr>
<td>$F_0 \times \Delta F/F'_m$</td>
<td>0.92 (0.65–1.29)</td>
<td>0.95</td>
</tr>
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</table>

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<thead>
<tr>
<th>Parameters</th>
<th>Fluorescent light</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>72 h EC50</td>
<td>$r^2$</td>
</tr>
<tr>
<td>$F_0$</td>
<td>0.56 (0.51–0.63)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.98</td>
</tr>
<tr>
<td>$F/F_m$</td>
<td>2.92 (2.52–3.37)</td>
<td>0.91</td>
</tr>
<tr>
<td>$\Delta F/F'_m$</td>
<td>2.51 (1.65–3.80)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98</td>
</tr>
<tr>
<td>$F_0 \times \Delta F/F'_m$</td>
<td>1.01 (0.51–2.03)</td>
<td>0.95</td>
</tr>
</tbody>
</table>

<sup>a</sup>significant difference between EC50 values ($p < 0.05$), <sup>b</sup>significant difference between slopes of dose-response curves ($p < 0.05$).

A gradual decrease in EC50 values with time is observed when the EC50 was based on the parameters $F/F_m$ and $\Delta F/F'_m$. For $F/F_m$ the 24 h EC50 values were 3.80 and 4.73, the 48 h EC50 values were 2.79 and 3.51 and 72 h EC50 values were 3.17 and 2.92 μM acridine for *P. tricornutum* pre-cultured under mercury or fluorescent light, respectively. Using the biomass-related parameter $F_0$, a stronger decrease in EC50 values with time is observed: the 24 h, 48 h and the 72 h EC50 values were 23.4 and 9.22, 1.45 and 1.37, and 1.17 and 0.56 μM acridine, respectively. Low, long term EC50 values measured with cell numbers converged with time to the EC50 values determined with $F_0$. In a previous study the progressing damage to cells of *P. tricornutum* was described effectively by the amount of UV absorbed by acridine, indicating that acridine is a reactive sensitizer (Wiegman *et al.*, in press). However, photosynthetic efficiency ($F/F_m$ and $\Delta F/F'_m$) remained relatively high, even after 72 h incubation with acridine.
under mercury light (EC50 values > 1.28 μM). Hence, we argue that the photosynthetic machinery during long-term exposure to acridine is not very sensitive to the phototoxic action. This is not opposing the earlier observations on the capacity of microalgae to mitigate phototoxic stress through their accessory pigments (Gala and Giesy, 1993). Yet, cellular damage unrelated to damage to the photosynthetic apparatus seems to determine growth inhibition of microalgae. Also, Rohacek and Bartak (1999) and Marwood et al. (2001) indicated the naturally well developed capacity in microalgae to manage the redox climate of their chloroplasts and to resist the action of reactive species such as singlet oxygen formed by photosensitization of PAHs and mixtures of PAHs.

Membrane damage due to oxidation of lipids by singlet oxygen has frequently been proposed as a mechanism for photoenhanced toxicity (McCloskey and Oris, 1991; Arfsten et al., 1996), suggesting that the membrane function may be the primary target for photoenhanced toxicity, instead of the functioning of the (remaining) PS II complexes (Rohacek and Bartak, 1999). For these reasons, we caution for the bias that may arise from the application of PAM fluorometry to measure photoenhanced toxicity or oxidative stress in microalgae.