Photobiological transformation of azaarenes in the water column

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UITGEBREIDE SAMENVATTING

DOEL VAN HET ONDERZOEK

In het huidige ecotoxicologisch onderzoek naar microverontreinigingen wordt de invloed van licht, met name die van ultraviolette (UV) straling, nog grotendeels genegeerd. Voor slechts weinig polycyclische aromatische koolwaterstoffen (PAK) is de relatie tussen de acuut toxische effecten en de versterking hiervan door UV straling op aquatisch organismen onderzocht. Ook de toxiciteit van afbraakproducten, die gevormd worden door biologische processen, is nauwelijks bestudeerd.
Polycyclische aromatische koolwaterstoffen komen door natuurlijke processen en menselijke activiteiten in het milieu. Het merendeel van de PAK zijn heterocyclische aromatische verbindingen, waaronder azaarenen. Azaarenen zijn beter oplosbaar in water dan hun moederverbindingen, de PAK, waardoor de beschikbaarheid voor aquatische organismen groter kan zijn.
Tot nu toe is risico-beoordeling van aromatische koolwaterstoffen alleen gebaseerd op toxiciteit van PAK, daarmee voorbijgaand aan de toxiciteit van heterocyclische aromaten en afbraakproducten. Daarom zijn in dit twee-jarig project, wat aan de Universiteit van Amsterdam is verricht en onder BEON's speerpunt microverontreinigingen valt, de onderstaande onderzoeksvragen beantwoord. Verwacht wordt dat dit BEON-project tot een beter begrip van transport en effecten van heterocyclische aromatische koolwaterstoffen en hun afbraakproducten in het milieu zal leiden.

PROBLEEMSTELLINGEN

- In welke mate worden azaarenen omgezet onder invloed van licht? Wordt deze omzetting van azaarenen door fytoplankton versneld?
- Welke producten worden door foto-biologische omzetting van azaarenen gevormd en zijn deze producten minder of juist meer toxisch of genotoxisch dan de oorspronkelijke azaarenen?
- Bestaat er een relatie tussen de nadelige effecten, de afbraak door micro-organismen en/of licht en de molecuulstructuur van azaarenen? Is het mogelijk om de biologische activiteit van deze stoffen met behulp van QSARs te voorspellen?

RESULTATEN EN CONCLUSIES

Voor acht verschillende azaarenen (heterocyclische PAK, waarin één koolstofatoom vervangen is door een stikstofatoom) is de fotochemische afbraaksnelheid bepaald onder twee soorten lichtbronnen, UV-B (300 nm) en UV-A (350 nm). De UV-B lamp brak de acht azaarenen (oplopend van twee-ring tot vijf-ring structuren) sneller af dan de UV-A lamp. Met een fotochemisch model (naar Zepp en Cline) werden de halfwaardetijden van deze acht stoffen geschat in oppervlaktewateren, onder natuurlijk licht. Dit model schatte voor de meeste stoffen een snelle afbraak en een sterke seizoensgebonden variatie in levensduur. Om deze theoretische halfwaardetijden te valideren, is de halfwaardetijd van acridine experimenteel bepaald. Deze kwam overeen met de halfwaardetijd die aan de hand van de kinetiek parameters van de UV-B lamp was bepaald.
De afbraak van de meeste azaarenen door UV-A leidde tot een toename in toxiciteit. In fotosynthese remmingsexperimenten met mariene diatomeeën (*Phaeodactylum tricornutum*) nam met name de toxiciteit van de twee-ringen met twee orde groottes toe. De fotolyse van de azaarenen door UV-B en UV-A straling, resulteerde in de meeste gevallen in hogere genotoxiciteit (Mutatox-assay). De resultaten laten zien dat UV-A een grotere impact op de nadelige effecten van azaarenen heeft dan UV-B (er trad zowel een toename in toxiciteit als genotoxiciteit op) door de vorming van (geno)toxischer stoffen.

De toxiciteit van de azaarenen op mariene algen nam toe met toenemend aantal ringen en daarmee toenemende lipofiliteit. De op lipofiliteit gebaseerde moleculaire descriptoren, gebruikt in QSAR analyse, verklaarde dan ook grotendeels de toxiciteit van azaarenen. Dit verschijnsel is bekend als narcotische toxiciteit. In groei-experimenten met een mariene flagellaat (*Dunaliella tertiolecta*) was met name de toxiciteit van acridine, een drie-ring structuur, veel hoger dan verwacht op basis van de narcotische werking. Dit verschil in toxiciteit tussen isomeren (fototoxiciteit) werd niet gevonden in de fotosynthese remmings-experimenten met diatomeeën (*Phaeodactylum*).

Omdat acridine vooral straling in de UV regio absorbeert en daarnaast reeds door enkele auteurs is aangetoond dat licht de toxiciteit van acridine op aquatische organismen verhoogt, is de mogelijke afbraak van acridine door algen vergeleken met afbraak door licht. De twee soorten mariene algen (diatomee en flagellaat) konden lage concentraties acridine omzetten en de omzetting werd versterkt door licht. De belangrijkste metaboliet was 9(10*H*)-acridon, een product dat ook gevormd werd onder invloed van zonlicht en UV-A straling. In dezelfde periode werd een natuurlijke mariene fytoplanktongemeenschap buiten (onder natuurlijk licht) blootgesteld aan lage concentraties acridine. Dat leidde tot het verrassende resultaat dat fototoxiciteit in de open lucht op natuurlijk fytoplankton één tot twee orde groottes hoger is dan fototoxiciteit op monocultures van algen onder kunstmatig licht in laboratoria. Effectconcentraties van azaarenen die gebaseerd zijn op laboratorium experimenten kunnen in situ effecten op fytoplankton onderschatten. Daarom is het essentieel in risico-beoordeling van heterocyclische aromatische koolwaterstoffen dat fototoxiciteit en biologische effecten van afbraakproducten worden geintegreerd.
Op grond van de in dit rapport gepresenteerde resultaten zijn er indicatieve maximaal toelaatbare risico niveau's (MTR's) voor azaarenen opgesteld. Hoewel gemeten azaareen-concentraties in het veld schaars zijn, laten deze zien dat de geschatte MTR's worden overschreden.

AANBEVELINGEN

- De waterkwaliteitsnormen voor lichtgevoelige aromatische verbindingen (heterocyclische en homocyclische PAK), gebaseerd op laboratorium experimenten (met kunstmatig licht), zijn niet geschikt voor in situ condities. Biologische effecten in het veld kunnen onderschat worden. Het is dan ook essentieel om fototoxiciteit in berekeningen van risico-parameters te integreren.

- Voor een schatting van biologische activiteit van aromatische verbindingen in het aquatische milieu is monitoring van de meeste PAK in het water niet toereikend, daar afbraakproducten een niet te verwaarlozen effect kunnen veroorzaken. Monitoring van genotoxische en specifiek toxische effecten, waaronder effecten van afbraakproducten, kunnen een aanvulling leveren op (heterocyclische)PAK-monitoring.

DANKWOORD
Graag willen wij de medewerkers van het Programma Bureau BEON danken voor hun logistieke hulp bij het tot stand komen van dit rapport. Daarnaast heeft prof. dr. R.W.P.M. Laane, als voorzitter van de begeleidingsgroep 'Microverontreinigingen' van BEON, waardevol commentaar geleverd op zowel de voortgangsrapporten als het definitieve rapport.

CONTENTS

ABSTRACT

In ecotoxicological test schemes the influence of light on ecotoxicolgical effects, especially UV, is hardly included. The relationship between acute toxic and phototoxic effects of azaarenes to aquatic organisms is sparsely investigated, while the toxicity of degradation products is virtually unknown. In the present study these problems were investigated, using different marine algae as test-organisms.

In the first stage of the project, acute toxicity of eight azaarenes, ranging from two-ringed to five-ringed structures, was determined in growth experiments with the green algae *Dunaliella tertiolecta*. The toxicity of azaarenes to *Dunaliella* increased with increasing number of aromatic rings and consequently lipophilicity. This pattern is also observed for toxicity to freshwater algae, midges and daphnids. Furthermore, toxicity of isomer structures of three-ringed and four-ringed azaarenes differed, probably due to photo-enhanced toxicity. So, for some of the azaarenes a higher toxicity can be found in situ, than expected based on their solubilities (chapter 2).
The second stage of the project focused on the photokinetics of azaarenes and the effects of photolytic products, formed under influence of short wavelength radiation, on algae (toxicity) and bacteria (genotoxicity). For acute toxicity of azaarenes and mixtures with phototransformed azaarenes the marine diatom *Phaeodactylum tricornutum* was chosen, with inhibition of photosynthetic activity as endpoint. For genotoxicity, the sensitive Mutatox test was used.
In chapter 3 photolysis rates of the eight azaarenes were quantified. Azaarenes were shown to degrade rapidly in the presence of short-wavelength light, with half-life periods of 11 hours in the UV-B region (300 nm) and 3.5 days in the UV-A region (350 nm).
Acute toxicity of azaarenes increased also with increasing number of rings, and was in the same order as for *Dunaliella*, although different algae and endpoints were used. Here, no differences between toxicity of isomers were found. Photolysis of azaarenes by UV-B irradiance led to detoxification. By UV-A irradiance, toxicity was generated upon lysis. For the two-ringed structures toxicity increased one to two orders of magnitude compared to parent compounds (chapter 4).
All azaarenes tested caused genotoxic effects. Photolysis of azaarenes results in higher genotoxic activities, despite occasional detoxification. In addition, light in the 350 nm range (UV-A) was equally or more active in formation of genotoxicity than 300 nm (UV-B) radiance. It is concluded that photolysis by UV-A or UV-B enhances toxic and/or genotoxic effects of azaarenes. Furthermore, UV-A has a higher impact on the adverse effects of azaarene mixtures than UV-B, by the formation of both genotoxic and toxic products (chapter 5).
In the last section degradation of acridine by marine algae and in situ, acute toxicity on phytoplankton were investigated. Bacteria and fungi are known to metabolize PAHs, especially the low ringed PAHs. This study showed that the marine algae *Dunaliella* and *Phaeodactylum* can metabolize low concentrations of acridine. In addition, co-action of metabolism and light enhanced lysis rates.
In outdoor experiments the phototoxicity of acridine for natural marine phytoplankton was even one to two orders of magnitude higher than (photo)toxicity to single species of microalgae, in laboratory studies under low light conditions. It is concluded that effect concentrations of azaarenes based on laboratory studies underestimate in situ effects on natural phytoplankton.

This study demonstrates that incorporation of phototoxicity and biological effects of degradation products are essential in risk assessment of azaarenes. In addition, monitoring of genotoxic and specific toxic effects, including the effects of these products, have to complete PAH/azaarene-monitoring.

1 GENERAL INTRODUCTION

In this report the results are presented of a study carried out at the University of Amsterdam (UvA), in co-operation with the National Institute for Coastal and Marine Management (RIKZ) and the National Institute for Public Health and Environment (RIVM), in the framework of BEON in the period 1996-1997. This research focused on the ecotoxicological effects of nitrogen containing heterocyclic polyaromatic compounds, but it is claimed to be relevant for the development of policies on photo-unstable aromatic compounds in general.

In nitrogen containing heterocyclic polyaromatic hydrocarbons (NPAHs) one or more carbon atoms of the aromatic ring have been replaced by nitrogen. Nitrogen containing heteroaromatic compounds exist in biological systems as mycotoxins, defense toxins of plants and sponges, electron carriers, alkaloids and nucleotides (Kaiser *et al.*, 1996; Kuhn and Suflita, 1989; Schmitter *et al.*, 1982; Tomkins and Ho, 1982). Apart from their natural origin, NPAHs also enter the environment as spills or waste materials generated by the mining industry, coal tar- and oil shale processing operations, wood preserving facilities and chemical manufacturing plants (Kaiser *et al.*, 1996).
Several field surveys indicated that NPAHs can be present in detectable concentrations in fresh- and marine waters (Van Genderen *et al.*, 1994), sediments (Fernández *et al.*, 1992; Swartz *et al.*, 1995; Kozin *et al.*, 1997) and in groundwater contaminated with coal tar (Pereira *et al.*, 1983).

As a consequence of their chemical structure, NPAHs are more soluble in water than non-substituted PAHs (Pearlman *et al.*, 1984), which often implies a higher bioavailabillity to organisms in aquatic surroundings. Although two-thirds of the four million known organic compounds are heterocyclic chemicals (Adrian and Suflita, 1994), most research has focused mainly on homocyclic compounds. Besides the lack of direct toxicity data, hazard assessment for this group of compounds is further hampered by the dynamic behavior of NPAHs in the environment. There is evidence for a strong photolysis of NPAHs in the water column (Mill *et al.*, 1981), possibly connected to the algal photosynthesis, resulting in the formation of new toxic and genotoxic products (Schoeny *et al.*, 1988). This influence of light, especially of UV, is until now hardly included in ecotoxicological test-protocols. At present, the relationship between the acute toxic, genotoxic and phototoxic effects of this family of compounds to aquatic organisms is unclear (Stahl, 1991; Vasseur, 1995).

In the absence of a proper insight in the mechanisms leading to the adverse biological effects of NPAHs, a hazard assessment based on residual concentrations of parent compounds is hardly satisfactory. Attention must therefore be focused on the flux of NPAHs in the water column and on research on the formation and effects of transformation products.
The present study aims to answer the following questions:

- To what extent do NPAHs degrade under influence of daylight? Is phytoplankton enhancing the degradation of NPAHs?
- Are phototransformation products of NPAHs more toxic or genotoxic than the parent compounds?
- Is there a relationship between the adverse biological effects, degradation by microorganisms (especially microalgae) and/or by light, and the molecular structure? Can biological activity of NPAHs be predicted by quantitative structure activity relationships (QSARs)?

Answering these questions will result in a better insight in poorly investigated contaminants, as mentioned in the "BEON speerpunt microverontreinigingen", especially NPAHs. It aims at finding a new approach for reactive compounds in general, which can be formed not only within organisms but also in the aquatic environment.

2 TOXICITY OF AZAARENES TO THE GREEN ALGA *DUNALIELLA TERTIOLECTA*

2.1 INTRODUCTION

Azaarenes (PAHs containing one nitrogen atom in place of a carbon atom) are better soluble in water and are therefore expected to be more toxic than their homocyclic analogues. However, acute toxic effects of azaarenes have been studied in only a few aquatic organisms (Bleeker *et al.*, 1996, Cooney and Gehrs, 1984; Johnson *et al.*, 1990; Kraak *et al.*, 1997a,b; Van Vlaardingen *et al.*, 1996). For several species acridine and quinoline were the only compounds tested. Even less is known about toxic effects of azaarenes to marine algae. Therefore, the acute toxicity of the two-ringed structures quinoline and isoquinoline, the three-ringed structures acridine and phenanthridine, the four-ringed structures benz[*a*]acridine and benz[*c*]acridine and the five-ringed structures dibenz[*a,i*]acridine and dibenz[*c,h*]acridine to the green marine alga *Dunaliella tertiolecta* was determined. Structures of the compounds are given in figure 2.1.

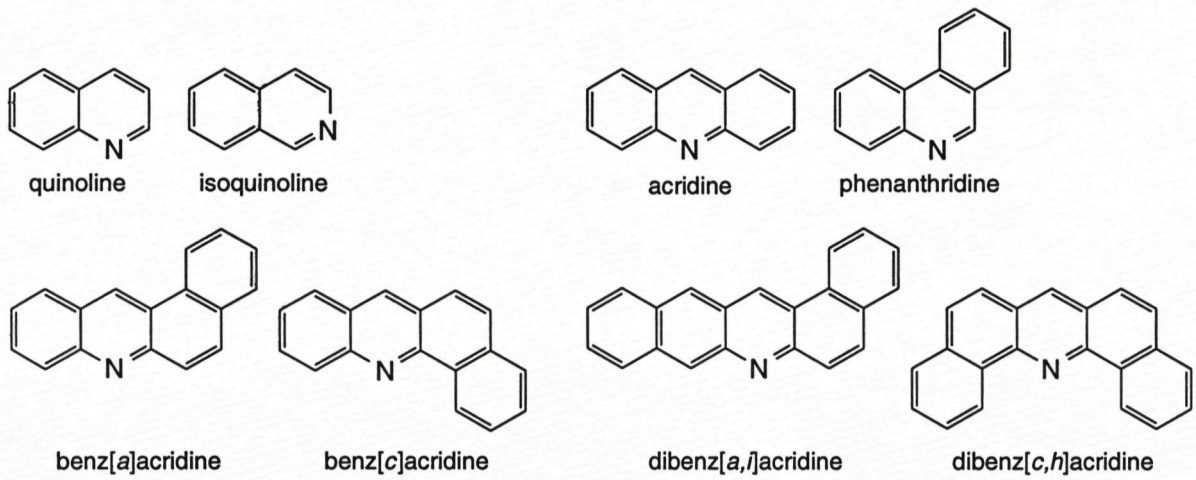


Figure 2.1. Structural formulas of the azaarenes used in this study.

2.2 MATERIALS AND METHODS

Experimental set-up for growth tests

The green alga *Dunaliella tertiolecta* (obtained from L. Peperzak, RIKZ) was cultured in batch at 20°C in an artificial seawater medium (Admiraal and Werner, 1983) from which silicate was omitted (to avoid precipitation in the medium).

Toxicity of the eight different azaarenes was assessed in a 72 hour growth test. The algae were placed in 500 ml borosilicate serum bottles closed with a screw cap with Teflon inlay to minimize sorption of azaarenes. Bottles contained 250 ml of medium, leaving an equal volume of air to allow equilibration of dissolved gasses. Algae were kept in suspension by incubating the bottles in water at 20°C on a rolling device (25 rpm). During the experiments cultures were illuminated with three mercury lamps (Philips HPI-T 400 W) each with an intensity of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$. The light dark regime was 16:8 h. The initial algal concentration was 10^4 cells per ml. To avoid a lag phase in algal growth, the experiment started with a day of acclimatization, before azaarene addition. Cell numbers and volumes (samples of 5 ml) were measured daily using a Coulter counter© (Coulter Multisizer) with a counting tube aperture of 70 µm.

Preparation of azaarene solutions

Azaarenes were added to the medium one day after algae were added, using the carrier solvent dimethylsulphoxide (DMSO, Merck, >99.5%). Separate stock solutions were made for each

treatment, by dissolving the highest used azaarene concentration in DMSO, followed by an appropriate dilution in DMSO. To each experimental treatment 50 µl of the corresponding stock solution was added. In this way DMSO concentrations were equal in all azaarene treatments (0.033% v/v). Besides controls without azaarenes, azaarene controls in sterile medium without algae (of lowest azaarene concentration) and DMSO controls were incorporated. For each compound, the series of concentrations (including DMSO controls and controls) was tested in duplicate.

The nominal concentrations of the azaarenes tested were: quinoline 3, 9, 27, 81, 242 mg/L, isoquinoline 1, 3, 9, 27, 81 mg/L, acridine 0.04, 0.16, 0.63, 2.5, 10 mg/L, phenanthridine 0.16, 0.63, 2.5, 10, 40 mg/L, benz[*a*]acridine 0.004, 0.016, 0.063, 0.25, 1 mg/L, benz[*c*]acridine 0.001, 0.004, 0.016, 0.063, 0.25 mg/L, dibenz[*a,i*]acridine 0.001, 0.002, 0.004, 0.007, 0.015, 0.029 mg/L and dibenz[*c,h*]acridine 0.0001, 0.0002, 0.0004, 0.0007, 0.014 and 0.029 mg/L.
The purity of the compounds used, was: quinoline (Aldrich) 99% purity, isoquinoline (Aldrich) 97%, acridine (Aldrich) 97% en phenanthridine (Aldrich) >99%, benz[*a*]acridine (CRM 157) >99.5%, benz[*c*]acridine (CRM 158) >99.5%, dibenz[*a,i*]acridine (CRM 152) >99.5% and dibenz[*c,h*]acridine (CRM 156) >99.5%. The last four chemicals are reference materials, obtained from the 'Community Bureau of Reference (BCR)' of the Committee of the European Community, where they are synthesized and purity is controlled in the framework of an European Community project (chemicals are registered with a CRM number).

Analysis of azaarenes by HPLC
Water samples (2 ml) were taken directly after addition of azaarenes (t_0) and after 72 h, in order to determine actual concentrations in the test medium. After centrifugation (3000 rpm, 10 min.) 1 ml of the supernatant was taken for analysis by High-Performance Liquid Chromatography (HPLC), using fluorescence detection (Kratos Spectroflow 980) for the (di)benzacridines and UV detection (Applied Biosystems model 785A) for the other compounds. A 150·4.6 mm Lichrosorb 5 µm RP-18 analytical column was used with a 4·4 mm Lichrosorb 5 µm RP-18 guard column. The column temperature was kept at room temperature (20°C). The flow of the mobile phase, a mixture (isocratic) of 65% (v/v) acetonitrile (J.T. Baker Analyzed HPLC Reagent, min. 99.9%) and 35% water (J.T. Baker Analyzed HPLC Reagent), was 1 ml/min. Of each sample 20 µl was automatically injected. Quinoline was detected at a wavelength of 225 nm, isoquinoline at 215 nm, acridine at 249 nm and phenanthridine at 247 nm. Benz[*a*]acridine and benz[*c*]acridine, eluted with an isocratic mixture of 80% acetonitrile and 20% water, were detected at excitation wavelengths of 276 nm and 275 nm respectively and emission wavelengths of >354 nm. Azaarene concentrations were calibrated with standards of corresponding azaarenes in methanol (J.T. Baker Analyzed HPLC Reagent, min. 99.8%).

From the azaarene concentrations measured at t=0 and t=72 h, an average exposure concentration was calculated assuming exponential decrease with time. Recovery was defined as the quotient of the actual concentration at 72 hour and the actual start concentration.
For each concentration, the measured algal concentration and biovolumes were plotted logaritmically against exposure time. The growth rate µ (day^{-1}) was calculated with linear regression. Growth rate values were plotted against corresponding average actual concentrations in the water. Negative growth rate values were set to zero in curve fitting calculations. EC_{50} values (including 95% confidence limits) were obtained by fitting the following equation (Haanstra *et al.*, 1985) through the dose-response plots with Kaleidagraph™ (Synergy Software 1997):

$$Y = \frac{c}{1 + e^{b(X-a)}}$$

in which Y = effect on growth rate, X = ^{10}log concentration (mg/L), a = log EC_{50}, b = slope of the logistic curve and c = average growth rate of controls.

2.3 Results and discussion

Figure 2.2 shows growth rates of *Dunaliella tertiolecta*, exposed to the different azaarenes. Clear dose-response relationships were observed for the effects of all compounds, except for the two five-ringed structures. The water solubilities of the five-ringed structures dibenz[*a*,*i*]acridine and dibenz[*c*,*h*]acridine were found to be too low to cause effects on the growth of the algae (the maximal achieved concentrations were resp. 0.029 mg/L and 0.0014 mg/L). For the other compounds tested EC_{50} and 95% confidence limits were calculated (table 2.1).

Toxicity increased with increasing number of rings. Both three-ringed molecules, acridine and phenanthridine, were significantly more toxic than the two-ringed structures quinoline and isoquinoline ($p<0.05$). Benz[*a*]acridine and benz[*c*]acridine, both four-ringed molecules were more toxic than the three-ringed structures, although the toxicity of acridine did not differ significantly from the toxicity of benz[*a*]acridine due to high confidence limits (table 2.1).

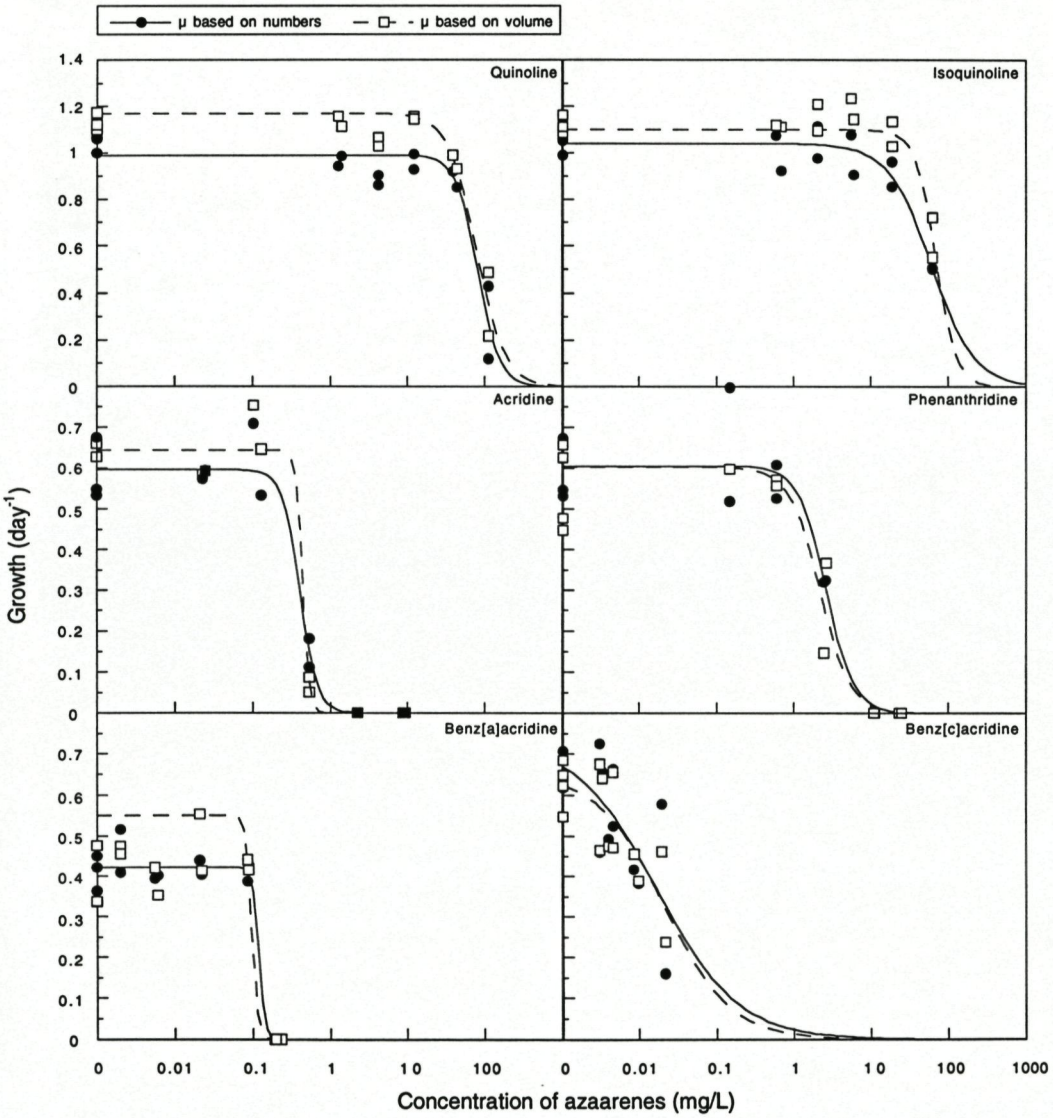


Figure 2.2. Growth rates of the marine alga *Dunaliella tertiolecta* during 72 h exposure to different concentrations of quinoline, isoquinoline, acridine, phenanthridine, benz[*a*]acridine and benz[*c*]acridine. The lines represent the curve-fit after Haanstra *et al.* (1985).

Toxicity increase with an increasing number of aromatic rings, appears to be a general pattern for the acute toxicity of N-heterocycles. Acridine was more toxic to the algae *Scenedesmus acuminatus* than quinoline and isoquinoline (Van Vlaardingen *et al.*, 1996). For some aquatic invertebrates the same pattern was observed. Bleeker *et al.* (1998) exposed first instar larvae of *Chironomus riparius* during 96 h to a series of azaarenes and also observed that toxicity increased with an increasing number of rings. In other studies acridine was found to be more toxic than quinoline for *Chironomus tentans* (Cushman and McKamey, 1981; Millemann *et al.*, 1984), *Daphnia magna* (Millemann *et al.*, 1984), *Gammarus minus* and *Physa gyrina* (Millemann *et al.*, 1984). Benz[*a*]acridine was more toxic than acridine for *Daphnia pulex* (Southworth *et al.*, 1978).

Table 2.1. EC_{50} values (72 h; mg/L) and the corresponding 95% confidence limits for the effects of the eight azaarenes on growth, based on cell number and volume, of the green algae *Dunaliella*, recovery (± standard deviations, n=10) and log K_{ow} of the used azaarenes.

Compound	EC_{50} based on	[1]EC_{50} (mg/L)	95% confidence limits	Recovery (%)	[2]Log K_{ow}
Quinoline	number	73.9	57.1-95.6	100	2.03
	volume	82.2	71.9-94.1		
Isoquinoline	number	60.0	46.4-77.5	100	2.08
	volume	70.0	60.0-81.6		
Acridine	number	0.38	0.24-0.58	82.6± 9.9	3.40
	volume	0.42	**		
Phenanthridine	number	2.63	1.96-3.53	99.8 ± 9.6	3.43
	volume	1.99	1.31-3.04		
Benz[*a*]acridine	number	0.12	0.05-0.29	78.1 ± 11.7	4.49
	volume	0.11	0.01-1.94		
Benz[*c*]acridine	number	0.02	0.01-0.04	40.7 ± 30.2[3]	4.49
	volume	0.02	0.01-0.04		
Dibenz[*a,i*]acridine	-	n.e.	-	-	-
Dibenz[*c,h*]acridine	-	n.e.	-	-	5.67

n.e. is no effect. [1]Actual concentrations. [2]Log K_{ow} values were taken from the ClogP model provided by the Environmental Science Center of Syracuse Research Corporation. [3]n=7. **95% Confidence interval could not be calculated.

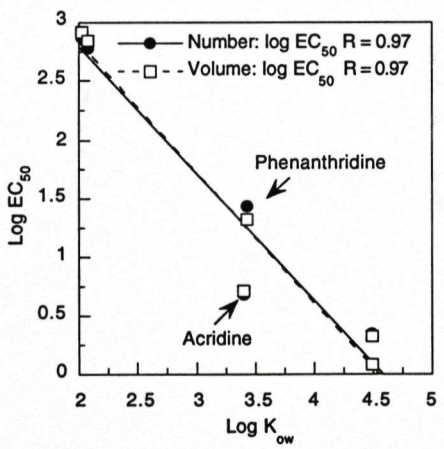


Figure 2.3. Relationship between the 72 h log EC_{50} (mg/L) of six azaarenes, based on cell number and volume, for *Dunaliella* and the log K_{ow}.

Because toxicity increased with increasing number of rings, a high correlation (r=0.97) between toxicity to *Dunaliella* (log EC_{50}) and lipophilicity (log K_{ow}) of the azaarenes (fig. 2.3) can be

expected. However, this graph clearly points out an exceptionally high toxicity of acridine, compared to its isomer phenanthridine. Differences in toxicity between isomers were also described by several other authors. Van Vlaardingen *et al.* (1996) found that acridine was far more toxic to the green alga *Scenedesmus acuminatus* than the isomers phenanthridine, benzo[*h*]quinoline and benzo[*f*]quinoline. Furthermore, Bleeker *et al.* (1998) found that acridine was far more toxic to *Chironomus riparius* than the isomers and they also observed a significantly higher toxicity for benz[*c*]acridine than its isomer benz[*a*]acridine.

Toxicity differences between isomers can be explained by different electronic interactions. One assumption is that the position of the N atom within the molecular structure plays a crucial role; if the N atom is positioned more centrally and the region of electron density is centralized, the formation of toxic metabolites is enhanced and thus causing a greater toxicity (Leslie, 1996). Another possibility is photo-enhanced toxicity of azaarenes under the influence of UV light (Morgan and Warshawsky, 1977; Bowling *et al.*, 1983; Newsted and Giesy, 1987; Mekenyan *et al.*, 1994a,b; Dijkman *et al.*, 1997; Van Vlaardingen *et al.*, 1996), in contrast with narcotic toxicity, due to association with membranes as a consequence of their hydrophobic nature (Southworth *et al.*, 1978; Catallo *et al.*, 1994). Indeed, photo-enhanced toxicity of acridine under mercury light, the light source in our experiments, has been reported by several authors (Morgan and Warshawsky, 1977; Van Vlaardingen *et al.*, 1996).

To gain better insight in the toxicity of acridine and differences between toxicity of isomers, quantitative structure activity relationships (QSARs) can be used as a tool to predict toxicity on both a molecular and a macroscopic physicochemical level (De Voogt, 1990). Several authors have developed a QSAR model for predicting the photo-induced toxicity of different polycyclic aromatic hydrocarbons, in which the molecular descriptor HOMO-LUMO gap is correlated with photo-enhanced toxicity of the compounds (Mekenyan *et al.*, 1994a; Veith *et al.*, 1995; Ankley *et al.*, 1997). Mekenyan *et al.* (1994a) demonstrated that PAHs and azaarenes exhibiting photo-induced toxicity, fall within a HOMO-LUMO gap window of 7.2 ± 0.4 eV and that linearity of the molecules affects the HOMO-LUMO gap. Only benz[*a*]acridine, benz[*c*]acridine and acridine fall into this highly phototoxic region (Bleeker *et al.*, 1998). This may explain why acridine was more toxic than phenanthridine for *Dunaliella* (this study) and also more toxic than all other isomers for *Scenedesmus acuminatus* (Van Vlaardingen *et al.*, 1996).
However, for the freshwater molluscs *Dreissena polymorpha* a different order of toxicity for the four isomers was observed (Kraak *et al.*, 1997b): phenanthridine was significantly more toxic than acridine. But since mussels are protected from UV light by valves, it is assumed that here acridine displayed no photo-enhanced toxicity.

CONCLUSIONS

- Acute toxicity of azaarenes to the green alga *Dunaliella tertiolecta* increases with increasing number of aromatic rings and consequently lipophilicity of this compounds.
- Toxicity of isomer structures can differ due to photo-enhanced toxicity.

3 DIRECT PHOTOLYSIS OF AZAARENES IN WATER, IRRADIATED WITH SHORT-WAVED LIGHT AND UV RADIATION

3.1 INTRODUCTION

Many chemicals present in aqueous media can undergo photochemical transformation under influence of sunlight via direct or indirect photoreaction. Direct photoreaction refers to those reactions in which a chemical absorbs sunlight and undergoes a chemical reaction. Indirect (or sensitized) photoreaction refers to those reactions in which another material absorbs sunlight and initiates a chemical reaction that transforms the chemical. In the environment, rate of direct photoreactions depends on solar irradiance, the chemical's molar absorptivity and its reaction quantum yield (Leifer, 1988). In order to asses the role of direct photochemical reactions in the aquatic and marine environment for azaarenes, kinetics of photolysis in water were determined in laboratory studies and extrapolated to field situations.

3.2 MATERIALS AND METHODS

Preparation of azaarene solutions

In this study, the same eight azaarenes were used as in chapter 2. In addition, fluoranthene (Fluka, >97%) a homocyclic PAH, was used as a photochemical standard (fig. 3.1). Its quantum yields at wavelengths 313 and 366 nm were reported by Zepp and Schlotzhauer (1979).

For all compounds, an analytical stock solution was made in acetonitrile (Rathburn, HPLC grade, >99%), instead of DMSO, used in toxicity experiments, because it can be mixed with water and has a refractive index very close to water. Furthermore acetonitrile does not absorb radiation at wavelengths greater than 290 nm (Leifer, 1988) whereas DMSO acts photosensitizing. However, this hampers the comparison of the two sets of experiments and therefore one compound, acridine dissolved in water with DMSO as carrier (0.033% v/v), was irradiated with the 350 nm lamp.

These stock solutions were kept in the dark at a temperature of 5° C in crimp-cap (sealed with teflon) sealed bottles (10 ml or 20 ml), to minimize evaporation of azaarenes and/or solvent. In order to measure UV-spectra and photoreaction kinetic parameters from these stocks, dilutions were made in milliQ-water (Millipore) (concentrations in table 3.1), with a specific resistance of 18.2 M .

UV-vis absorption spectra and photoreaction of azaarenes

UV-vis absorption spectra of each compound in aqueous solution were measured with a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer (with PECSS software) using quartz vessels (100 and 80 ml) with Teflon-lined screw caps and a path length of 1 cm. Each spectrum was corrected for absorption of the solvent in the range of the wavelengths used in the photolytical experiments. The scanning velocity was 30 nm·min.$^{-1}$ and absorbance was measured at 2 nm intervals.

Because azaarenes are chemicals that may ionize or protonate, when dissolved in water, spectra for the most alkaline azaarenes were recorded with and without a 5 mM phosphate buffer (pH 7), in order to determine whether azaarenes in the solutions used without buffers added (table 3.1) are all in the same molecular form. Since no differences were observed between these spectra, no buffers were added in the experiments. Furthermore, to avoid bimolecular reactions than direct photoreactions, azaarene concentrations in photochemical experiments were always 10^{-5} M or lower (table 3.1).

From each UV spectrum, the molar absorptivity (ε), at a fixed wavelength, was calculated for each compound with the Beer-Lambert law. With the molar absorptivity, the overlap between the UV absorption spectrum of the chemicals and the irradiance of the emission of the lamps used, i.e. the sum of the products of intensity of the light source and the molar absorptivity of a chemical over the relevant wavelength range (fig. 3.2), was calculated: $(I \cdot \varepsilon)_\lambda$.

Table 3.1. Concentrations of azaarenes (mol/L) and ratios between milliQ-water and carrier solvent acetonitrile, used for recording UV spectra and for photoreaction experiments.

	UV spectra		Photoreaction	
Compound	conc. (mol/L)	mQ/solvent	conc. (mol/L)	mQ/solvent
Quinoline	$5.8 \cdot 10^{-5}$	1000/1	$1 \cdot 10^{-5}$	1000/1
Isoquinoline	$5.6 \cdot 10^{-5}$	1000/1	$1 \cdot 10^{-5}$	1000/1
Acridine	$9.8 \cdot 10^{-6}$	1000/1	$1 \cdot 10^{-5}$	1000/1
Phenanthridine	$2.0 \cdot 10^{-5}$	1000/1	$1 \cdot 10^{-5}$	1000/1
Benz[*a*]acridine	$8.5 \cdot 10^{-7}$	90/10	$1 \cdot 10^{-6}$	90/10
Benz[*c*]acridine	$9.9 \cdot 10^{-7}$	90/10	$1 \cdot 10^{-6}$	90/10
Dibenz[*a,i*]acridine	$9.5 \cdot 10^{-7}$	90/10	$1 \cdot 10^{-6}$	90/10
Dibenz[*c,h*]acridine	$9.5 \cdot 10^{-7}$	80/20	$1 \cdot 10^{-7}$	90/10
Fluoranthene	$1.0 \cdot 10^{-7}$	90/10	$5 \cdot 10^{-7}$	90/10

Fluoranthene is a homocyclic PAH and is used as a photochemical standard (Zepp and Schlotzhauer, 1979).

Freshly prepared aqueous solutions (100 ml in closed quartz vessels) were irradiated in a Rayonet RPR-208 merry-go-round-reactor (MGRR) containing eight UV-lamps. Two types of lamps were used, one with a irradiation optimum around 300 nm (Rayonet, 21W) and a second one having its optimum around 350 nm (Rayonet, 24W). The wavelength distribution of both lamps as given by the manufacturer are shown in figure 3.1 (The Southern New England Ultraviolet Co., Hamden, CT, USA). The lamps were cooled with air, which kept the working temperature at 27° C.

Analysis of azaarenes by HPLC

The photochemical experiments were conducted at the laboratory of RIVM (Bilthoven).

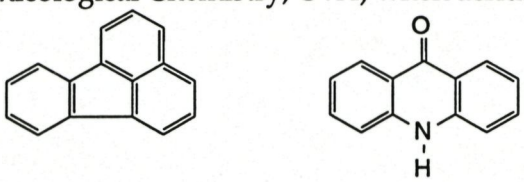
To measure the relative azaarene concentrations after different irradiation times, solutions were analyzed by High-Performance Liquid Chromatography (HPLC, Hewlett Packard model 1050) using fluorescence detection (Kratos Spectroflow 980), UV detection (Applied Biosystems model 785A) and if necessary diode-array detection. A 100·3 mm Lichrosorb 7 mm RP18 analytical column was used with a R2 reversed phase guard column (Chrompack). 2% (v/v) triethylamine (TEA, Aldrich, 99+%) was added to the milliQ-water in the mobile phase of the HPLC and pH was set to 6.5 with diluted acetic acid (Merck, 99%). The flow of the mobile phase, an isocratic mixture of acetonitrile (J.T. Baker Analyzed HPLC Reagent, min. 99.9%) and triethylamine/acetic acid/milliQ-water was 0.4 ml/min. Of each sample 5 µl was automatically analyzed (in duplicate) and the average of two analysis was used in the calculations.

Benz[*a*]acridine, benz[*c*]acridine, dibenz[*a,i*]acridine, dibenz[*c,h*]acridine and fluoranthene were eluted with a mixture of 90% acetonitrile and 10% TEA buffered milliQ-water. Quinoline, acridine and phenanthridine were eluted with 80% acetonitrile and 20% TEA buffered milliQ-water, isoquinoline with 60% acetonitrile and 40% TEA/milliQ-water.

Quinoline was detected at a wavelength of 225 nm and isoquinoline at 215 nm and. Acridine was detected at an excitation wavelength of 248 nm and an emission wavelength of 416 nm, phenanthridine at excitation wavelength of 247 nm and an emission wavelength of 359 nm. Benz[*a*]acridine and benz[*c*]acridine were detected at excitation wavelengths of 276 nm and 275 nm and emission wavelengths of 383 and 385 nm respectively, dibenz[*a,i*]acridine and dibenz[*c,h*]acridine were detected at excitation wavelengths of 299 nm and 289 nm and emission wavelengths of 492 and 395 nm respectively.

A solution of 9[10*H*]-acridone (fig. 3.1) in water (0.548 mg/L) was used to demonstrate the formation of acridone (a metabolite of acridine that was analysed by MS-GC at the dept.

Environmental and Toxicological Chemistry, UvA) when acridine was irradiated with light.



fluoranthene 9(10*H*)-acridone

Figure 3.1. Structural formulas of fluoranthene and 9[10*H*]-acridone.

Photoreaction kinetics

Concentrations of azaarenes, as determined with HPLC, were plotted as ln C_0/C_t against time, in which C_0 is the start concentration and C_t the concentration of azaarene at a given time t. The slope of the linear regression line through the obtained time-concentrations plot equals the pseudo first-order photoreaction rate constant k_{exp}. From k_{exp} the associated half-life of the compounds was calculated with the equation: $t_{1/2} = \ln 2/k_{exp}$. All azaarene photoreactions accorded to a first-order model, except one, that was better described with a zero-order model. In this case the azaarene concentration was plotted as C_0/C_t against time and the half-life was calculated with: $t_{1/2} = -0.5 \cdot C_0/k_{exp}$.

Leifer (1988) stated that for direct photoreaction of a compound x the following equation applies:

$$k_{exp} = \Sigma(I \cdot \varepsilon)_\lambda \cdot 2.303 \cdot j^{-1} \cdot \phi_x \qquad (1)$$

In which j is a conversion factor (1 Einstein/mol) and ϕ_x is the quantum yield of the photoreaction of compound x. This means that the rate constant of direct photoreaction is proportional to the quantum yield and the overlap in absorption spectrum of the compound and emission spectrum of the UV lamp used.

Absolute measurements of light intensity I were not available, and therefore no absolute value for $(I \cdot \varepsilon)\lambda$ was calculated. The quantum yields of azaarenes were calculated relatively to the quantum yield of fluoranthene. For all compounds it is assumed that the quantum yield is independent of the wavelength (Leifer, 1988).

Using equation 1, the following equation was derived from the ratio of the experimentally determined rate constant of compound x ($k_{exp,x}$) and a reference compound ($k_{exp,ref}$), determined under the same conditions:

$$\phi_x = \phi_{ref} \cdot \frac{k_{exp,x} \cdot \Sigma(I \cdot \varepsilon)_{\lambda,ref}}{k_{exp,ref} \cdot \Sigma(I \cdot \varepsilon)_{\lambda,x}} \qquad (2)$$

In which ϕ_x is the quantum yield of compound x and ϕ_{ref} is the quantum yield of the reference compound.

3.3 Results and discussion

Table 3.2. Summation of the product of light intensity I of the light source and the molar absorptivity ε of the compound over the relevant wavelength range for each lamp.

Compound	$(I \cdot \varepsilon)\lambda$, 300 nm	$(I \cdot \varepsilon)\lambda$, 350 nm
Quinoline	11910	6035
Isoquinoline	9381	1868
Acridine	10067	139322
Phenanthridine	21060	16219
Benz[*a*]acridine	76425	145929
Benz[*c*]acridine	94670	195884
Dibenz[*a,i*]acridine	186528	86537
Dibenz[*c,h*]acridine	157789	119981
Fluoranthene	28913	110597

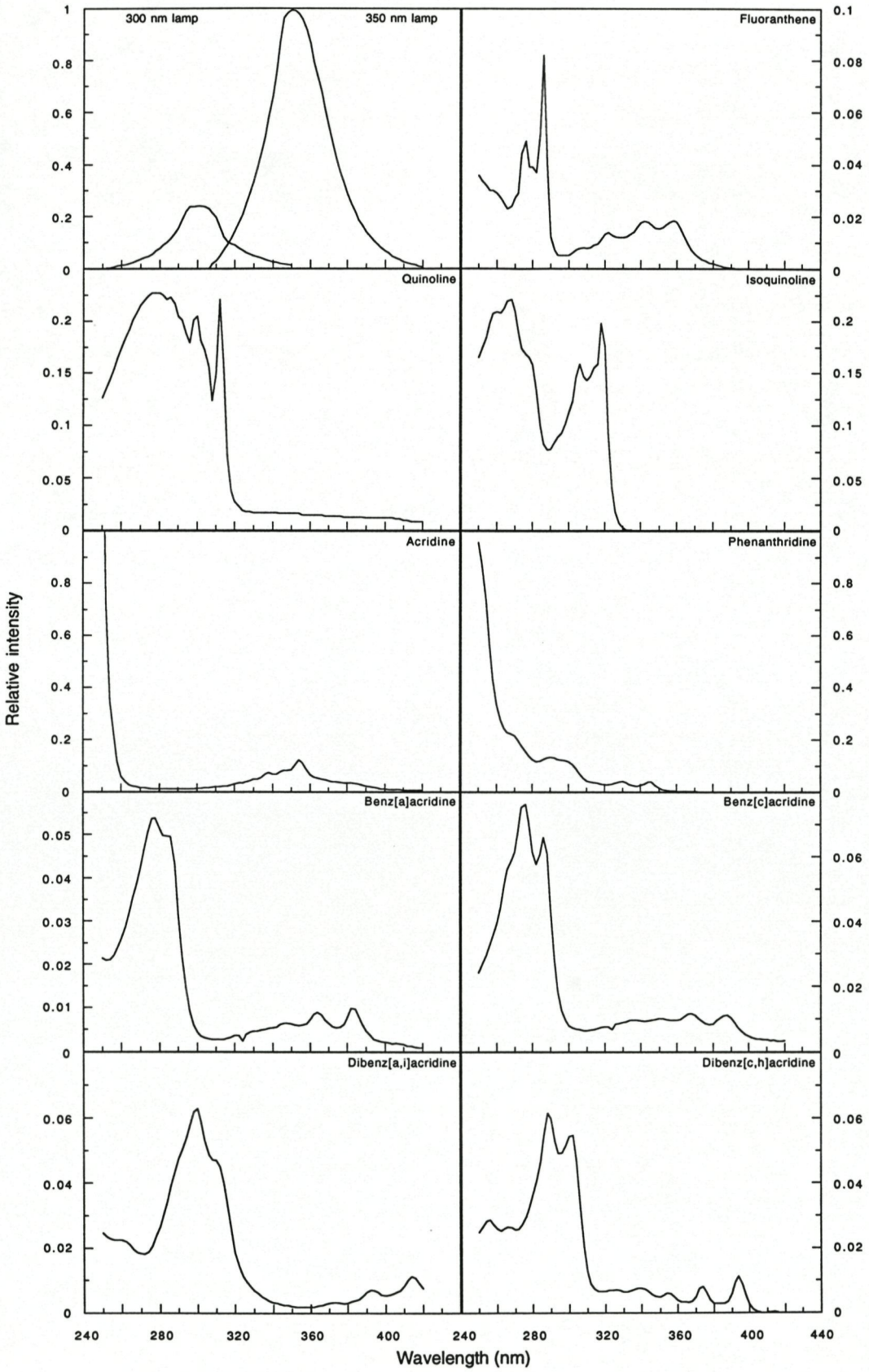


Figure 3.2. Spectral distribution of both lamps, sorption spectra of fluoranthene and eight azaarenes.

Table 3.3. Photoreaction rate constants (k_{exp}), half-lives and quantum yields (ϕ) of azaarenes in milliQ-water irradiated with Rayonet 300 or 350 nm lamps. k_{exp} are derived from a pseudo first-order model, except for dibenz[*a,i*]acridine (from a zero-order model). Correlation coefficients (r^2) of the regression lines are between 0.99 and 1.0, except for quinoline (r^2=0.97). In the final columns quantum yields are calculated relatively to the ϕ of quinoline from Mill *et al.* (1981) instead of fluoranthene.

	Irradiated with 300 nm lamps						Irradiated with 350 nm lamps						300 nm	
Compounds	n	k_{exp} (1/min)	$^1t_{1/2}$	$^2t_{1/2}$	ϕ (10^{-3})	-log ϕ	n	k_{exp} (1/min)	$^1t_{1/2}$	$^2t_{1/2}$	ϕ (10^{-5})	-log ϕ	ϕ (10^{-3})	-log ϕ
Quinoline	12	0.0104 ± 0.0004	67	0.046	2.12	2.674	15	0.00014 ± 0.000004	5148	3.575	0.33	5.479	0.320	3.495
Isoquinoline	10	0.0076 ± 0.00009	91	0.063	1.96	2.708	15	0.00075 ± 0.000008	927	0.644	5.95	4.225	0.297	3.527
Acridine	6	0.0058 ± 0.0003	120	0.083	1.40	2.854	16	0.0019 ± 0.00006	370	0.257	0.20	5.699	0.211	3.675
Acridine (DMSO)	-	-	-	-	-	-	14	0.00178 ± 0.00009	408	0.283	0.18	5.741	-	-
Phenanthridine	10	0.0098 ± 0.0003	71	0.049	1.12	2.950	14	0.0038 ± 0.0003	184	0.128	3.46	4.461	0.171	3.768
Benz[*a*]acridine	15	0.0010 ± 0.00004	663	0.460	0.03	4.479	15	0.00069 ± 0.00002	1008	0.700	0.07	6.154	0.005	5.319
Benz[*c*]acridine	11	0.0017 ± 0.00005	398	0.276	0.05	4.351	16	0.0013 ± 0.00002	520	0.361	0.10	5.995	0.007	5.182
Dibenz[*a,i*]acridine	10	0.0901 ± 0.0036	8	0.006	1.17	2.931	16	°-24.330 ± 1.18	17	0.012	nc	nc	0.177	3.752
Dibenz[*c,h*]acridine	14	0.0316 ± 0.0017	22	0.015	0.49	3.313	16	0.01206 ± 0.00063	57	0.040	1.51	4.821	0.073	4.134
Fluoranthene	15	0.0014 ± 0.00002	485	0.337	*0.12	3.921	15	0.0015 ± 0.00004	466	0.324	*0.20	5.699	0.018	4.751

n=number of observations. nc=not calculated. ^{1}min., ^{2}days. ° mol·L^{-1}·min^{-1}. * ϕ of fluoranthene from Zepp and Schlotzhauer, 1979.

Figure 3.2 shows the emission spectra of the two UV lamps, used in the experiments, and the light absorption spectra of fluoranthene and the eight azaarenes irradiated. Table 3.2 presents the overlap between each UV lamp and the light absorption of azaarenes (the available amount of energy for each molecule). In general this overlap was highest for the two four-ringed and two five-ringed structures and lowest for the two two-ringed compounds. The overlap in spectra of acridine, benz[*a*]acridine and benz[*c*]acridine was higher for the 350 nm lamp than for the 300 nm lamp (see also fig. 3.2). These results might indicate that by irradiation the four and five-ringed structures may degrade faster than quinoline and isoquinoline, and also that acridine, benz[*a*]acridine and benz[*c*]acridine may degrade quickest in light in the region of 350 nm.

Table 3.3 shows the experimentally determined photochemical rate constants (k_{exp}), the associated half-lives and quantum yields (ϕ) of azaarenes and fluoranthene. For dibenz[*a,i*]acridine irradiated with 350 nm, the k_{exp} was calculated with a zero-order model and ϕ was not calculated. The change of concentration of quinoline with time can be represented by a zero-order aswell a first order model, the latter with less accuracy but including all the measurements ($r^2 = 0.97$).
All photochemical rate constants of the tested azaarenes are highest at irradiation in the 300 nm wavelength range, although the overlap between spectra of acridine, benz[*a*]acridine and benz[*c*]acridine and the 300 nm lamp were lower. Because these k_{exp} are proportional to overlap spectrum (table 3.2) and efficiency of photochemical reactions (ϕ), the quantum yields must also be higher at the irradiance emission of 300 nm, as shown in table 3.3. The net result is lower half-lives of the eight azaarenes at lower wavelengths.
The five-ringed structures dibenz[*a,i*]acridine and dibenz[*c,h*]acridine were in both photoreaction tests the most photochemically unstable structures. Benz[*a*]acridine and benz[*c*]acridine on the contrary were the most stable compounds under both light regimes. Quinoline degraded slowly under the 350 nm lamp, because this compound absorbed hardly any irradiance in this range of wavelength. In the experiments with 300 nm lamps, the half-life of quinoline was comparable to the ones of isoquinoline, phenanthridine and acridine.

Although it was assumed that quantum yields are independent of the wavelengths (Leifer, 1988), large differences were observed between ϕ of azaarenes at 300 and 350 nm. Zepp and Schlotzhauer (1979) discussed the anomalities in the quantum yield of fluoroanthene at 313 nm and 366 nm. Although they could not give a full explanation, they (and others) have indicated that normally the quantum yield could not change drastically over such a short wavelength range. However, the difference between a quantum yield of fluoranthene at both wavelengths, as given in their paper, amounts to about 2 orders of magnitude. Hence, fluoranthene is not appropriate for normalization of a quantum yield data at 350 nm.
The quantum yield of quinoline in water under 313 nm has been measured before and does not seem to change with wavelength, if buffered water with a similar pH of around 7 was used (Mill *et al.*, 1981). Normalization therefore may also be done on the basis of quinoline or may be used as a comparison (see final columns, table 3.3). The quantum yield of quinoline based on actinometry is around 7 times higher than of normalization on fluoranthene at 313 nm.

The photochemical reaction of acridine with DMSO as solvent (0.033% v/v) proceeded slower than the reaction with acetonitrile (0.1% v/v), the difference in half-life is significant ($p<0.05$) and amounted to 10%. In both the photoreactions the formation of a strong fluorescencing metabolite could be observed, which was identified as 9[10*H*]-acridone. The amount of the photoproduct acridone was three times higher if DMSO was used (fig. 3.3), because it could have act sensitizing (Leifer 1988).

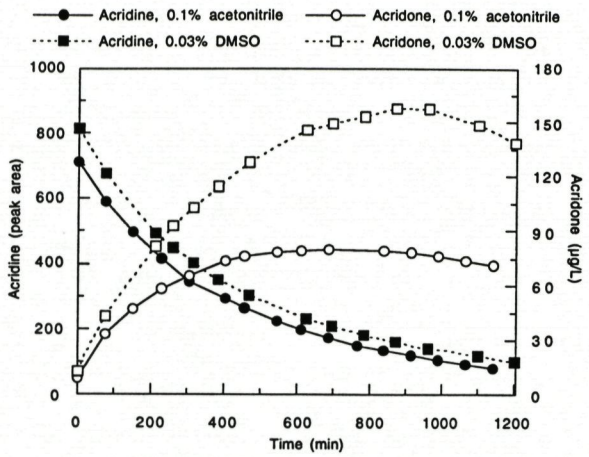


Figure 3.3. Decrease of acridine ($C_0 = 10^{-5}$ M) with solvent acetonitrile (0.1% v/v) and solvent DMSO (0.033% v/v) and formation of the photoproduct 9[10*H*]-acridone, plotted against time (min). Solutions were irradiated with Rayonet 350 nm lamps.

In the natural environment, the source of radiation is sunlight, which is polychromatic light and lies in the visible and ultraviolet regions. Sunlight has a broad spectral distribution in the range of 290 to 800 nm. Solar radiation entering aquatic media still contains a large amount of energy and when the chemicals in water absorb light, a diverse number of chemical reactions occurs (Leifer, 1988). Whether or not a PAH reacts depends on the overlap spectrum of the compound (ε_λ) and solar light (W_λ). Rough estimates of this type for selected azaarenes suggest the following order in outdoor transformation: acridine>quinoline>benz[*c*]acridine>dibenz[*a,i*]-acridine>phenanthridine>benz[*a*]acridine>dibenz[*c,h*]acridine>isoquinoline. This order differs from that of quantum yields or half-lives observed in table 3.3.
To generate half-lives of azaarenes irradiated with sunlight, quantum yields and absorption spectra of the eight azaarenes used in this chapter were entered in the program GCSOLAR (Zepp and Cline, 1988). With this program half-lives of chemicals in aquatic surroundings (at depts between 0.001 and 5.0 cm) exposed to sunlight were calculated, in which variables as latitudes, moment of the year, thickness of the ozone layer and thickness of the water layer were taken into consideration. The calculated half-lives of eight azaarenes in near surface waters (at depts between 0.001 and 5.0 cm) on 50° and 60° northern latitude (the Netherlands is located in between), integrated over the whole day, are given in tables 3.4 and 3.5.

Table 3.4. Calculated half-lives (days) of azaarenes in near surface water at 50° northern latitude and 5° eastern longitude, in four seasons using quantum yields determined after irradiation with a 300 nm and a 350 nm lamp.

Season	Spring		Summer		Autumn		Winter	
Compound	300 nm	350 nm	300 nm	350 nm	300 nm	350 nm	300 nm	350 nm
Quinoline	1.68	421	1.17	321	3.90	910	9.91	1920
Isoquinoline	1.0	78.5	0.71	52.8	2.4	192	6.3	611
Acridine	0.154	47	0.119	37	0.337	100	0.721	201
Phenanthridine	0.92	27	0.68	20	2.1	59	4.7	130
Benz[*a*]acridine	7.2	99	5.5	78	16	208	34	413
Benz[*c*]acridine	3.5	47	2.7	37	7.7	99	17	199
Dibenz[*a,i*]acridine	0.17	-	0.11	-	0.39	-	1.1	-
Dibenz[*c,h*]acridine	0.43	6.3	0.32	4.9	0.96	13	2.2	28

These calculated half-lives only have a relative meaning, because the quantum yields presented in table 3.3 were not derived from the equation in which the absolute intensity of light is needed.

Because of the large differences between the quantum yields of the eight azaarenes at 300 and 350 nm (table 3.3), the corresponding half-lives in tables 3.4 and 3.5 differed a lot.
Although in this study large differences were found between the half-lives derived from tests with 300 nm and 350 nm lamps and consequently between half-lives calculated for the natural environment, Leifer (1988) found a good agreement between experimental and theoretical (GCSOLAR) calculated half-lives.

Table 3.5. Calculated half-lives (days) of azaarenes in near surface water at 60° northern latitude and 5° eastern longitude, in four seasons using quantum yields determined after irradiation with a 300 nm and a 350 nm lamp.

Season	Spring		Summer		Autumn		Winter	
Compound	300 nm	350 nm	300 nm	350 nm	300 nm	350 nm	300 nm	350 nm
Quinoline	2.18	506	1.33	345	8.11	1650	33.5	5350
Isoquinoline	1.3	108	0.80	61	5.1	463	24	3090
Acridine	0.184	55	0.128	39	0.618	174	2.14	543
Phenanthridine	1.1	32	0.75	22	4.0	110	15	399
Benz[*a*]acridine	8.6	116	5.9	82	29	358	103	1090
Benz[*c*]acridine	4.2	55	2.9	39	14	172	51	530
Dibenz[*a,i*]acridine	0.22	-	0.13	-	0.85	-	3.9	-
Dibenz[*c,h*]acridine	0.53	7.4	0.35	5.2	1.9	24	6.9	76

During the photoreaction experiments it was found that acridine degrades quickly (fig. 3.3). To validate the half-lives of acridine in table 3.4 and 3.5, 100 ml quartz vessels with acridine (10^{-5} M) and fluoranthene ($5{\cdot}10^{-7}$ M), as standard, in milliQ-water with resp. 1% and 11% (v/v) acetonitrile were exposed to direct sunlight in the summer and the decrease in concentration of these two compounds is presented in figure 3.4. In this experiment a metabolite, with comparable HPLC retention time as 9(10*H*)-acridone, was formed.
Under influence of both UV-A and sunlight 9(10*H*)-acridone, a metabolite of acridine was formed.

The influence of light on the fate of PAHs in water is to a large extent determined by the presence or absence of reactive species. In the presence of oxygen, PAH-oxygen complexes will be formed (also called charge transfer or CT complexes), which can be excited by photons. Upon excitation, the CT complex can dissociate along several pathways (Onodera *et al.* 1985). For PAHs the excited complex may give rise to excited triplet state PAH and $^1O_2$ or ground state oxygen (^3O_g). Triplet state PAH can excite ground state oxygen (22 kcal/mol required) provided the triplet-singlet conversion of the PAH leads to an energy gain (usually more than 38 kcal/mol). Excited oxygen can react with the arene or will deactivate to the ground state. $^1O_2$ will generally react with PAH through a $^4s + ^2s$ mechanism. This leads to 1,4-adducts also called endoperoxides and involves covalent bonding. For 1,2,3,4-tetramethylnaphthalene in the presence of oxygen this type of product has been shown to result after irradiation with light of 366 nm (Yamaguchi *et al.*, 1985).
Endo compounds can dissociate into the parent PAH and $^1O_2$. This does occur only at relatively short wavelengths, however, and not at irradiation wavelengths close to 400 nm. At such wavelengths, cleavage of the O-O bond occurs, leading to aromatic epoxides or to hydroxyarenes containing bridged epoxides (Schmidt *et al.*, 1984). When the favoured oxygen-bridge formation involves the nitrogen atom, the actual product will probably be thermodynamically unstable and dissociate into a Zwitterion type of compound containing a positively charged nitrogen atom and a negatively charged peroxide ion coupled to the para oriented carbon atom. However, this intermediate can be stabilised by the presence of polar solvents such as water. In case of acridine, the product transforms into 9(10*H*)-acridone.

Excited oxygen will attack those carbon atoms in the benzene ring opposite (i.e. para oriented) to those having the highest electron density of the HOMO. Compared to parent PAHs, the nitrogen atom in azaarenes exhibits a much higher electron density than the corresponding carbon atom. Although this may suggest that the peroxide bridge will be attached to the nitrogen atom, the location of the bridge formation depends more on the difference in eigen values of opposite atoms. Hence, other opposite carbon atom pairs present in the molecule may have a more appropriate (higher) electron density difference (Schmidt *et al.*, 1984). For the azaarenes investigated in this study, with the exception of acridine, it is expected that all possible opposite pairs will have electron density differences that do not vary significantly and the peroxide bridge can be formed between all opposite positions.

The calculated half-life of acridine was 0.96 day, when acridine was exposed to 50 hours of sunlight at 52° northern latitude (fig. 3.4). Because the first two days were sunny and the other three days were cloudy (this explains why the acridine decrease is not following first-order kinetics perfectly in fig. 3.4B) the half-life of the first 12 hours was also calculated: 0.5 day. This was not in good agreement with the theoretically derived half-lives of acridine of 0.119 and 0.128 days, but lied closer to the theoretically derived half-lives of 0.34 and 0.63 day in the autumn, all derived from the 300 nm lamp (table 3.4 and 3.5).
The half-life of acridine differed completely from the ones derived with the 350 nm lamp, resp. 37 and 39 days. The theoretical half-lives of acridine calculated with the parameters of the 350 nm lamp give, especially in spring and summer (more UV light and higher intensity), an overestimation of the life span of acridine in the environment. This half-life also indicates that best comparison with sunlight was irradiation under 300 nm. It is likely that the same holds for the other azaarenes, although experimental results are not yet available. The results and implications for other azaarenes suggest that normalization on fluoranthene should be chosen to be based on 300 nm.

Thus, theoretically and practically derived half-lives of azaarenes in aquatic environments suggest that hazard of these compounds will be greatest in winter, in which azaarenes are more persistent than in the other seasons. Still, most of the tested azaarenes will degrade rapidly in the aquatic environment.

CONCLUSIONS

- All tested azaarenes degrade rapidly (half-lives are between 0.006 and 3.575 days) in the presence of short wavelength light, but QSAR analysis has still to be applied to demonstrate relationships between half-lives of compounds and molecular structures.
- Using the model of Zepp and Cline it is calculated that, in near surface waters, azaarenes degrade under influence of daylight and are especially labile in summer (with half-lives less than 6 days).

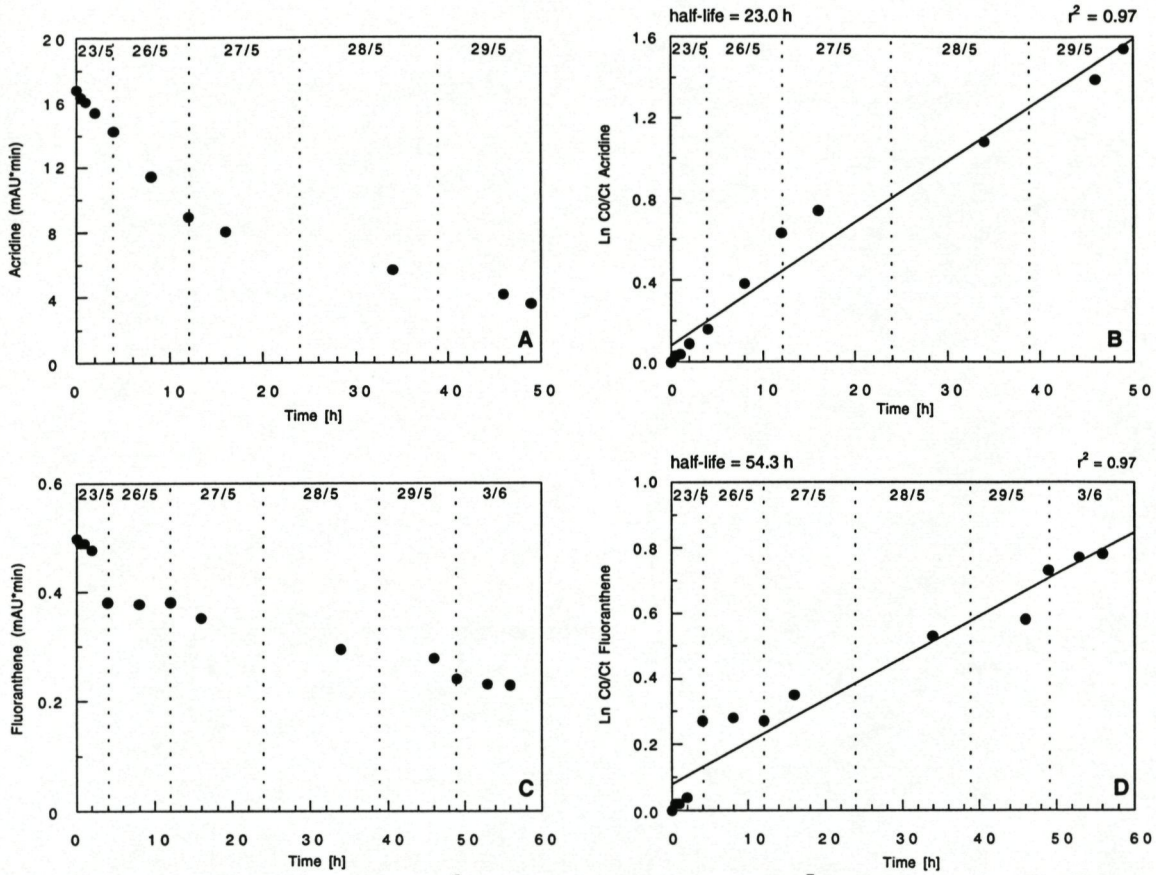


Figure 3.4. Decrease of acridine ($1 \cdot 10^{-5}$ M) and fluoranthene ($5 \cdot 10^{-7}$ M) dissolved in milliQ-water in quartz vessels, by sunlight irradiance and their half-lives calculated over resp. 50 and 60 h. Vessels were placed in sunlight during the day.

4 TOXICITY OF AZAARENES AND THEIR PHOTOTRANSFORMATION PRODUCTS TO THE DIATOM *PHAEODACTYLUM TRICORNUTUM*

4.1 INTRODUCTION

Absorption of sunlight (UV light) by azaarenes results in photochemical transformation of these compounds by two different modes: photomodification (photo-oxidation and/or photolysis) and photosensitization reactions (Leifer, 1988; Veith *et al.*, 1995; Huang *et al.*, 1997). Photomodification reactions structurally alter azaarenes to a variety of products, mainly oxygenation products (mostly with increased water solubility). In photosensitization reactions, azaarenes in excited state funnel energy to molecular oxygen primarily forming singlet oxygen, or other radicals (Veith *et al.*, 1995; Krylov *et al.*, 1997). Radicals have a very short life span, but are extremely reactive, when formed within an organism, because of their capability of oxygenating and oxidizing many different biomolecules (Halliwell and Cutteridge, 1985).

The relatively short life span of most of the azaarenes tested (chapter 3) suggests that the hazard of these compounds in the aquatic and marine environment is low, especially in summertime when the degradation rates are high and thus bioavailibility of parent compounds is low. However, taking the extreme reactivity of radicals into consideration, degradation would imply an increase rather than a decrease in toxicity.

Still, little is known about the effects of parent PAHs together with photoproducts formed by sunlight irradiance in aquatic and marine environments. Therefore the acute toxicity of azaarenes and photochemical transformation products to the diatom *Phaeodactylum tricornutum* was determined. The inhibition of ^{14}C photosynthetic activity was chosen as effect parameter.

4.2 MATERIALS AND METHODS

Preparations of azaarene test solutions

Aqueous azaarene solutions were irradiated, based on the half-lives derived from photochemical reactions described in chapter 2 to generate samples for ^{14}C photosynthesis tests. With these samples inhibition of the photosynthetic activity of the marine alga *Phaeodactylum tricornutum* was determined.

Acetonitrile at 10% (v/v) is known to be toxic to algae, therefore ratios of acetonitrile and milliQ-water (and consequently azaarene concentrations) used in these experiments were lower than in photochemical reaction experiments (table 4.1).

Table 4.1. Initial concentrations of azaarenes (µM), used for ^{14}C photosynthesis test, before irradiation (t_0) and ratios between milliQ-water and solvent acetonitrile. Percentages of azaarene present after $2 \cdot t_{1/2}$ irradiation with the 300 nm and 350 nm lamp. Azaarene solutions (µM) in 0.07% acetonitrile, for determining EC50 values, referred to as parent solutions (P).

	Initial solutions (t_0)		Irradiated solutions ($2 \cdot t_{1/2}$)		Solutions (P)
Compound	C_0 (µM)	mQ/solvent	300 nm (%t_0)	350 nm (%t_0)	(µM)
Quinoline	10	1000/1	25	11	2023
Isoquinoline	10	1000/1	24	28	1863
Acridine	10	1000/1	69	20	55.8
Phenanthridine	10	1000/1	46	70	108
Benz[*a*]acridine	0.5	99/1	36	43	4.36
Benz[*c*]acridine	0.1	99/1	18	32	2.62
Dibenz[*a,i*]acridine	0.1	99/1	29	16	0.95
Dibenz[*c,h*]acridine	0.01	99/1	28	42	0.024

Some of the initial azaarene concentrations (t_0, table 4.1) were below the effect concentrations on growth of *Dunaliella tertiolecta* (chapter 2). To achieve full scale dose-response curves and compare azaarene toxicity to *Phaeodactylum* with toxicity to *Dunaliella*, new solutions with higher azaarene concentrations were made in milliQ-water with 0.07% (v/v) acetonitrile and these are referred to as parent solutions (table 4.1).
The eight solutions, which were irradiated in the same way as described in chapter 3, were sampled at times: t=0 (untreated sample) and on circa $t=2 \cdot t_{1/2}$. At $t=2 \cdot t_{1/2}$ at least 75% of the initial azaarene concentration had disappeared and it was expected that a considerable amount of photoreaction products was formed.
At both sampling times 1 ml samples from each azaarene solution were collected for HPLC analysis and 20 ml samples were taken for ^{14}C photosynthesis experiments. Photosynthesis samples were immediately frozen in liquid nitrogen and stored at -70°C, HPLC samples were stored at 5°C.

Experimental set-up
The diatom *Phaeodactylum tricornutum* (obtained from AquaSense BV) was cultured in the laboratory in a continuous culture in artificial seawater (Admiraal and Werner, 1983) at 20°C with a dilution rate of 24 ml/h. The culture was illuminated with circular fluorescent tubes (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) and a light dark regime of 16:8 h. Algal density of the continuous culture was $\pm 2 \cdot 10^6$ cells/ml.
Azaarene solutions were diluted to a series of 66.6%, 20%, 6.6%, 2%, 0.66%, 0.2% and 0% solution in 20 ml scintillation vials in duplicate. Macro salts (NaCl, $MgCl_2$, $CaCl_2$, Na_2SO_4, K_2SO_4, $NaHCO_3$) were added in order to keep the salinity of all solutions equal to the salinity of the algal medium.
Algae were added to the scintillation vials, such that the starting concentration of algae used in ^{14}C photosynthesis experiments was $2 \cdot 10^5$ cells/ml.
Scintillation vials with algae and azaarene solution were placed on a swirl-table, equipped with cool-white fluorescent tubes of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Osram, L13W/20) for 1 hour at 20°C (pre-incubation). After one hour 100 ml $NaH^{14}CO_3$ (Amersham; 50-60 $mCi \cdot mmol^{-1}$) with an activity of 0.5 µCi was added to every vial. The biological activity in the scintillation vials, incubated with ^{14}C, was stopped after 1 hour by adding 0.6 ml formaldehyde (Merck, 37%). Besides the controls, algae without toxicant and algae with 1.7% (v/v) acetonitrile, two vials were used for determination of the incorporation of abiotic ^{14}C. Non-incorporated ^{14}C was removed during the night, after adding 100 µl 6N HCL (Merck) to the vials, in a fume hood. Activity was measured, after adding 7 ml scintillation liquid (Instagel; Hewlett Packard), using a Liquid Scintillation Analyzer (Packard Tricarb, Model 1600 TR). At the same time two vials of 100 µl $NaH^{14}CO_3$ with 1 ml Carbosorb® (Packard) were measured for the activity of the used ^{14}C source.
The photosynthetic activity is calculated from the activity of the samples (corrected for count efficiency) minus the activity of abiotic ^{14}C incorporation (corrected for counting efficiency) and is expressed as disintegrations per minute (dpm).
Photosynthetic activity in treatments was expressed as the percentage of the mean of the two corresponding controls. The photosynthetic activity values were plotted against the corresponding actual concentrations in the water. EC_{50} values (including 95% confidence limits) were obtained by fitting the equation from Haanstra *et al.* (1985) through the dose-response plots with Kaleidagraph™ (chapter 2), in which Y = Inhibition (%), X = ^{10}log concentration (µmol/L), c = photosynthetic activity of controls (100%).

Analysis of azaarenes by HPLC
Samples from parent azaarene solutions, from azaarene solutions before irradiance (t_0), and from azaarene solutions after $2 \cdot t_{1/2}$ irradiance with 300 nm and 350 nm lamps, all in milliQ-water, for HPLC analysis, were measured in the same manner as described in materials and

methods of chapter 2. Of the irradiated solutions only the concentrations of parent compounds could be quantified, photoreaction products were, although observed in the chromatograms in some cases, not identified.
To compare toxicity of the mixtures and toxicity of the parent azaarenes, toxicity of the mixtures was expressed in molarity of the remaining (parent) azaarenes.

4.3 Results and discussion

Figure 4.1 shows the inhibition of photosynthetic activity of the marine diatom *Phaeodactylum tricornutum* at different parent azaarene concentrations. Clear dose-response relationships were observed for quinoline, isoquinoline, acridine, phenanthridine and benz[*a*]acridine. The tested concentrations of benz[*c*]acridine, dibenz[*a,i*]acridine and dibenz[*c,h*]acridine had no effect and therefore it is concluded that the EC_{50} for these compounds exceeded the highest obtained concentration (table 4.2).

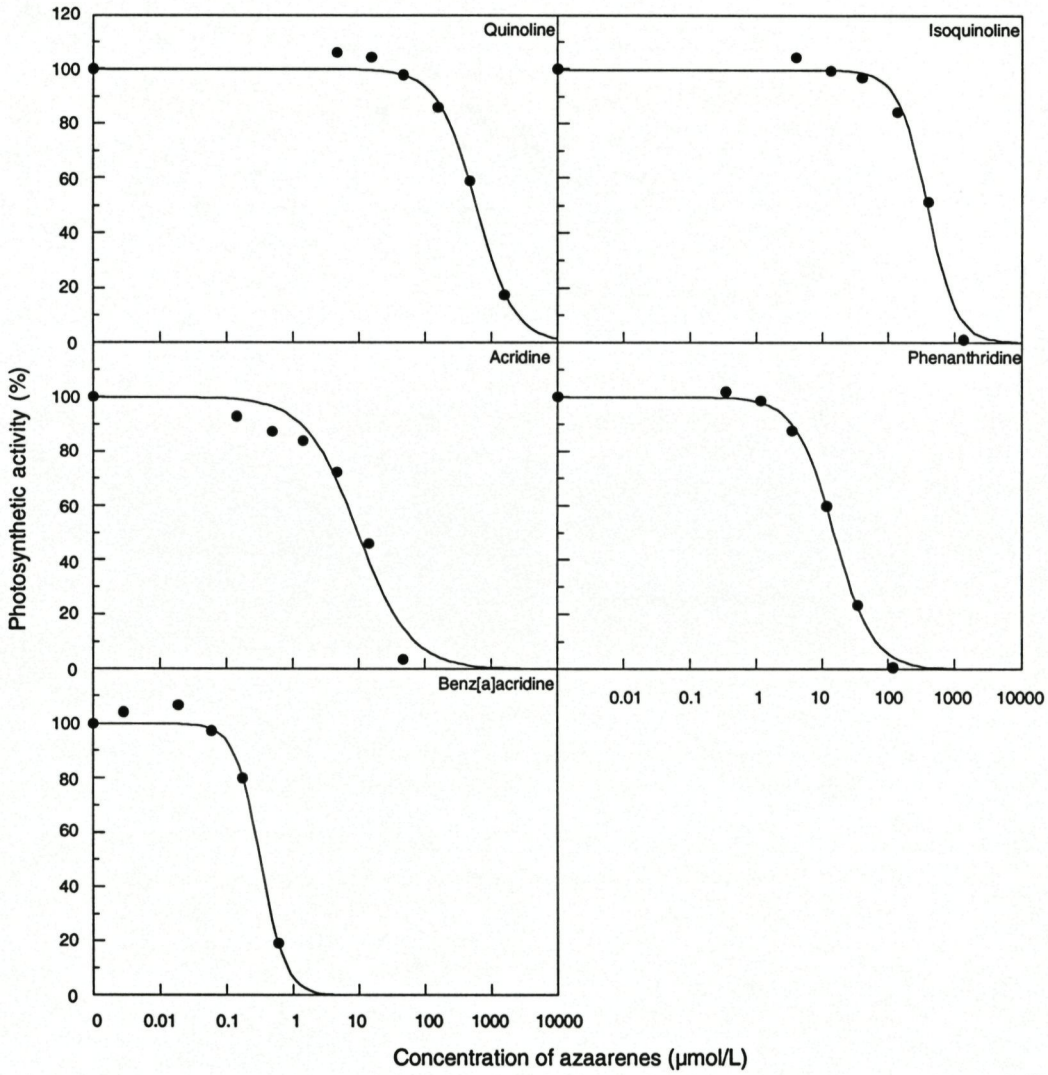


Figure 4.1. Photosynthetic activity (%) of the marine diatom *Phaeodactylum tricornutum* at different concentrations of the parent compounds: quinoline, isoquinoline, acridine, phenanthridine and benz[*a*]acridine, plotted as percentage of the corresponding controls. The lines represent the curve-fit after Haanstra *et al.* (1985).

The same pattern was observed as for the effect on the growth of the green alga *Dunaliella tertiolecta* (chapter 2): toxicity increased with increasing number of rings. Benz[*a*]acridine was significantly ($p<0.05$) more toxic than the isomers acridine and phenanthridine, while these compounds were significantly more toxic than the isomers quinoline and isoquinoline.
The effect concentrations of quinoline, isoquinoline, phenanthridine and benz[*a*]acridine (table 4.2) in short term experiments were also in good agreement with the observed effect concentrations on the long term growth of *Dunaliella*, 572 µM, 464 µM, 15.2 µM and 0.47 µM, respectively (chapter 2, table 2.1). However, the effect concentration of acridine for *Phaeodactylum* in this experiment equaled the one of phenanthridine, although for *Dunaliella* the effect concentration of acridine differed significantly from the phenanthridine concentration ($p<0.05$).
This difference may be partly explained by the duration and set-up of experiments. *Dunaliella* was exposed to acridine for 72 hours, with growth as effect parameter and *Phaeodactylum* was incubated with acridine for only 2 hours, with photosynthetic activity as effect parameter. But the most likely explanation is the use of different light sources (and intensities) and thus a different mode of toxicity. For the growth of *Dunaliella* mercury lamps were used with a small portion of the emission spectrum in UV light (10% of the emitted light is in the region of <410 nm) and with a higher intensity. Thus, toxicity was a combination of the narcotic effect and photo-enhanced toxicity of acridine. In this experimental set-up acridine only caused a narcotic effect to *Phaeodactylum*.

Table 4.2. EC_{50} values (µM) and the corresponding 95% confidence limits for the effects of azaarenes and for the effects of azaarenes at t_0, at $2 \cdot t_{1/2}$ after irradiation with 300 nm lamps and at $2 \cdot t_{1/2}$ after irradiation with 350 nm lamps on the photosynthetic activity of *Phaeodactylum tricornutum*.

	EC_{50} values (95% confidence limits) in µM			
Compound	Parent	t_0	[a]$2 \cdot t_{1/2}$ 300 nm	[a]$2 \cdot t_{1/2}$ 350 nm
Quinoline	554 (479-641)	n.e.	n.e.	1.8 (1.24-2.6)
Isoquinoline	385 (308-481)	n.e.	n.e.	4.3 (0.88-20.8)
Acridine	12.6 (10.1-15.8)	10.8 (6.83-17.2)	10.8 (6.9-16.8)	2.23 (1.36-3.65)
Phenanthridine	14.8 (13.1-16.7)	11.1 (7.21-16.9)	8.13 (4.54-14.6)	5.85 (2.24-15.2)
Benz[*a*]acridine	0.29 (0.23-0.36)	0.09 (0.02-0.54)	n.e.	n.e.
Benz[*c*]acridine	[b]>0.38	n.e.	n.e.	n.e.
Dibenz[*a,i*]acridine	>0.95	n.e.	n.e.	n.e.
Dibenz[*c,h*]acridine	>0.02	n.e.	n.e.	n.e.

[a]Effect concentrations are based on present azaarene concentration of parent compounds, [b]benz[*c*]acridine concentration is based on HPLC analysis, dibenz[*a,i*]acridine and dibenz[*c,h*]acridine were not quantified. n.e is no effect.

Table 4.2 shows the effect concentrations of azaarenes at t_0, $2 \cdot t_{1/2}$ at 300 nm irradiation and $2 \cdot t_{1/2}$ at 350 nm irradiation. In the t_0 treatments quinoline and isoquinoline had no effect, because highest used concentrations were below the effect concentrations of the parent group. Acridine, phenanthridine and benz[*a*]acridine showed a slight effect on the photosynthetic activity at the highest concentrations tested (which explains the high confidence limits of the EC_{50} values of these compounds). Again, the tested concentrations of benz[*c*]acridine, and both dibenzacridines had no effect (table 4.2). The maximal azaarene concentrations used in this test were 10^{-5} M, to avoid other reactions than photoreactions (chapter 3).
After 2 times half-life irradiation with the 300 nm lamp the EC_{50} of the parent compounds acridine and phenanthridine equaled the EC_{50} of the mixtures. So, toxicity of the mixtures was probably caused by the parent compounds only. In contrast, after irradiation with the 350 nm lamp, toxicity of the acridine and phenanthridine mixtures increased, although the difference was only significant for acridine. Consequently, toxicity of these mixtures was caused by the parent compounds and the formed photoproducts jointly (fig. 4.2).

Toxicity of the quinoline and isoquinoline, after irradiation with the 350 nm lamp, differed two orders of magnitude from that of the parent compounds and toxicity of these mixtures were also caused by the parent compounds and the formed photoproducts jointly. Furthermore, toxicity of quinoline and isoquinoline did not differ anymore from toxicity of acridine and phenanthridine. Irradiation caused differences in toxicity between two and three-ringed structures to disappear.
The observed differences can be explained by the presence of different photochemical products in the 300 nm and 350 nm treatment. Because the overlaps of absolute spectra of azaarenes with the 350 nm lamps differed from that of the spectra with the 300 nm lamps (fig. 3.2, chapter 3), other photoreaction, with the formation of other photoproducts, could have occurred.

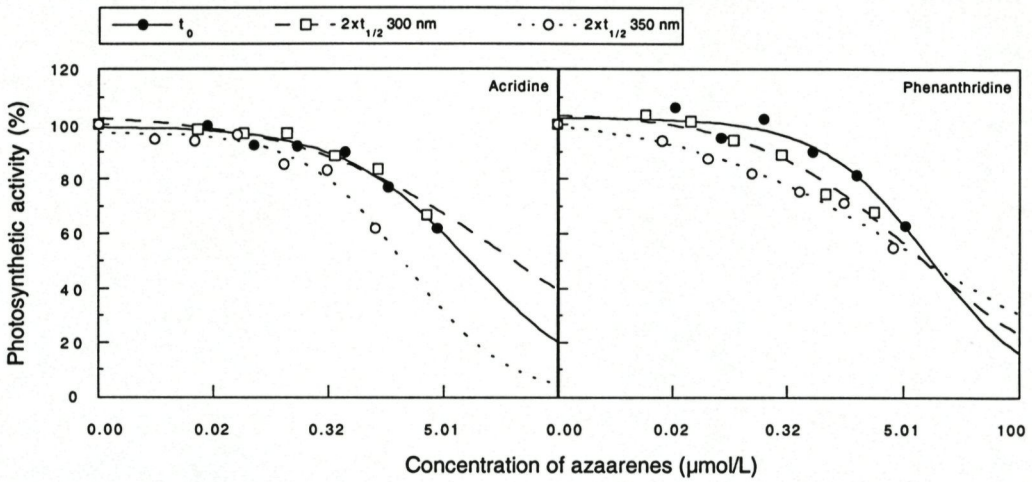


Figure 4.2. Photosynthetic activity (%) of *Phaeodactylum tricornutum* at different acridine and phenanthridine concentrations of the treatments t_0, $2 \cdot t_{1/2}$ 300 nm and $2 \cdot t_{1/2}$ 350 nm, plotted as percentage of the corresponding controls. The lines represent the curve-fit after Haanstra *et al.* (1985).

Krylov *et al.* (1997) exposed the duckweed *Lemna gibba* to 16 different PAHs and the photochemical mixtures (with less than 10% of the parent PAHs present), derived with simulated solar radiation (SSR, 100 $\mu E \cdot m^{-2} \cdot s^{-1}$). Of the 16 photomodified PAHs 13 displayed higher toxicity to *Lemna gibba* than parent PAHs. In their experimental set-up *Lemna gibba* took up the contaminants and in the tissue of the plant the PAHs and photoproducts were still exposed to light. So, two pathways of toxicity played a role: photomodification and photosensitization.
Photomodification is responsible for the formation of more polar compounds via oxidation, photosensitization for the formation of oxygen radicals and these radicals on their turn are capable of oxygenating and oxidizing many different biomolecules, with catastrophic consequences for the organisms (Larson and Berebaum, 1988; Huang *et al.*, 1993). By using a QSAR model it was determined that both pathways contributed to phototoxicity for *Lemna* and it explained why almost all the photochemical mixtures were more toxic than parent PAHs.
In the present experiment the toxicity of only four of the eight azaarene mixtures increased. One of the reason might be that toxicity of parent azaarenes and photodegradation products jointly was not enhanced by phototoxicity during the ^{14}C incorporation experiments (only a cool-white fluorescence light source was used). Furthermore, because photosensitization could not occur (radicals have short life spans), here only photomodification processes played an important role in the toxicity of the phototransformation products.
In the aquatic and marine environment azaarenes can be even more hazardous for organisms, because the broad spectrum (especially in the UV region, compared to the used lamps) and high intensity (1500 $\mu E \cdot m^{-2} \cdot s^{-1}$) of sunlight, inducing phototransformation of azaarenes, can form more toxic photoproducts with an overall higher toxicity.

CONCLUSIONS

- Short term toxicity tests using photosynthesis as an endpoint indicate similar toxicity of azaarenes as long term growth tests.
- Under the low irradiance levels used (and the low fraction of short wavelengths present) phototoxicity of some isomers is not detectable; the toxicity consistent with lipophilicity predominates.
- Photolysis of azaarenes by UV-A (350 nm) does not lead to detoxification, on the contrary the photolytic products introduce new toxicity. For quinoline and isoquinoline the new compounds are two orders of magnitude more toxic than quinoline and isoquinoline, in tests with *Phaeodactylum tricornutum*.

5 GENOTOXICITY OF AZAARENES AND THEIR PHOTOTRANSFORMATION PRODUCTS

5.1 INTRODUCTION

Hazard assessment for azaarenes is mostly based on the direct toxicity, although azaarenes induce also mutagenic, teratogenic and carcinogenic effects, or combinations of these effects (Bleeker *et al.*, 1998).
Absorption of sunlight (UV light) by azaarenes may results in photochemical transformation of these compounds which structurally alter azaarenes to a variety of products, mainly oxygenation products. Besides a possible higher bioavailibility for aquatic and marine organisms, some of these products displayed increased toxicity (chapter 4) and can also display increased genotoxicity. In this chapter genotoxicity of azaarenes and of the products of photochemical transformation will be determined using the Mutatox™, a sensitive genotoxicity test (Ulitzur, 1982).

5.2 MATERIALS AND METHODS

Preparations of azaarenes
Based on the half-lives derived from photochemical reactions described in chapter 3 (table 3.3) azaarene solutions (chapter 4, table 4.1) were irradiated to generate samples, from which genotoxicity to the bacteria *Vibrio fischeri* were derived, using the Mutatox™ tests.
Solutions of eight azaarenes (carrier solvent acetonitrile), which were irradiated in the same manner as described in chapter 3 (§2), were sampled at times: t=0 (untreated sample) and $t=2 \cdot t_{1/2}$. At both sampling times, 1 ml of each azaarene solution was collected for HPLC analyse and 0.75 ml samples for Mutatox tests. Samples for Mutatox were immediately frozen in liquid nitrogen and stored at -70°C. HPLC samples were stored at 5°C.

Experimental set-up
The Mutatox test uses a special dark mutant of luminescent bacteria *Vibrio fischeri* (strain M169) to detect the presence of genotoxic agents. The strain exhibits increased light production when grown in the presence of sub-lethal concentrations of genotoxic agents, because genotoxicants can restore the luminescent ability (Ulitzur, 1986; Johnson, 1992). The tests are carried out as follows: 0.7 ml sample is added to 1 ml Reconstitution Solution and of this mixture 0.75 ml is used for the 1:1 (v/v) dilution series. A small amount of bacteria suspension (10^5 cells) is added to the dilution series of test samples in growth media following the suppliers protocol (Microbics Corp.). Incubation time is 45 min at 20°C. Light emitted from the bacteria is measured, from 8 hours of the start of incubation in a Microbics Model 500 Analyser, every hour till maximal responses have been reached (duration could sometimes exceed 20 hours after incubation). Agents are denoted genotoxic when light emission increased to at least 4 times the average control reading in at least two sample dilution cuvettes. Genotoxicity is expressed as LOEC the Lowest Observed Effect Concentration. CMR, the Concentration of Maximum Response, is the test concentration at which the light emission reaches highest values and is determined by quadratic regression through the data points determining the top of the response curve. These normal procedure was simplified at this stage of the research. Four classes were arbritary formed: no genotoxicity, genotoxicity, moderate and high genotoxicity.

Analysis of azaarenes by HPLC
Samples taken from azaarene solutions before irradiation, and from azaarene solutions after irradiating (with 300 nm or 350 nm lamps) were measured with HPLC, in the same manner as described in materials and methods of chapter 2. Of the irradiated solutions only the concentrations of parent compounds were quantified, photoreaction products were not

identified or quantified. The maximal azaarene concentrations used for the dilution series in the Mutatox tests are given in table 5.1.

Table 5.1. Maximal azaarene concentrations (μM) used in the Mutatox test. *Nominal concentrations.

	Qui	Isoq	Acr	Phe	B[*a*]a	B[*c*]a	Dib[*a*,*i*]a	Dib[*c*,*h*]a
t_0	3.698	3.411	2.932	3.676	0.027	*0.036	*0.044	*0.004
300 nm	1.023	0.956	2.275	1.691	*0.010	*0.007	*0.013	*0.001
350 nm	0.526	0.956	0.639	2.665	*0.012	*0.011	*0.007	*0.002

5.3 Results and discussion

After irradiation of azaarene solutions with the 300 nm or 350 nm lamp, between 60 and 90% of the initial concentrations of most azaarenes were disappeared. Of the four-ringed and five-ringed structures the initial and remaining concentrations could not be quantified, because concentrations were below detection limits of HPLC analysis (also table 4.1, chapter 4). Low azaarene concentrations were used in these experiments to avoid other reactions than photoreactions (chapter 3 and 4). Also, sorption processes can have a clear but not quantifiable influence on the available concentration (Klamer *et al.*, 1997).

Table 5.2 shows the results of the Mutatox tests with untreated (t_0) and $2 \cdot t_{1/2}$ irradiated solutions. All azaarenes, except benz[*a*]acridine, induced genotoxicity (t_0, table 5.2). Genotoxicity of quinoline, isoquinoline, acridine and phenanthridine was in the same order, while the benz[*c*]acridine and dibenzacridines were one to two orders more genotoxic.

After irradiation with 300 nm (UV-B), no genotoxicity was observed for the samples with isoquinoline and benz[*c*]acridine, but now the benz[*a*]acridine mixture became genotoxic. Genotoxicity of the acridine, phenanthridine and benz[*a*]acridine mixtures increased, probably due to formation of new and/or more genotoxic products.

After irradiation with 350 nm (UV-A), the quinoline mixture was no longer genotoxic, but of the other azaarene mixtures genotoxicity increased (again no genotoxicity was found for benz[*a*]acridine).

Table 5.2. Genotoxicity of the untreated azaarenes (t_0 solutions) and of the $2 \cdot t_{1/2}$ radiated solutions with respectively 300 nm and 350 nm. Degree of genotoxicity is specified into: - no genotoxicity, + genotoxicity, +/+ + and ++ moderate genotoxicity, +++ high genotoxicity. LOEC's of the same azaarenes (μM) and the metabolites 9(10*H*)-acridone and 6(5*H*)-phenanthridinone, obtained from the studies Bleeker *et al.* (1996) and Bleeker *et al.* (submitted).

Compounds	t_0	$2 \cdot t_{1/2}$ 300 nm	$2 \cdot t_{1/2}$ 350 nm	LOEC (μM)
Quinoline	+	+	-	88.03
Isoquinoline	+	-	++	>107
Acridine	+	++	++	1.88
Phenanthridine	+	++	+/++	1.23
Benz[*a*]acridine	-	++	-	0.01
Benz[*c*]acridine	++	-	+++	>0.19
Dibenz[*a*,*i*]acridine	++	++	+++	>0.10
Dibenz[*c*,*h*]acridine	++	++	+++	>0.03
9(10*H*)-acridone	n.i.	n.i.	n.i.	0.005
6(5*H*)-phenanthridinone	n.i.	n.i.	n.i.	4.41

n.i. is not incorporated in our experiments.

Bleeker *et al.* (1996) found in a similar Mutatox series (with acetone instead of acetonitrile as carrier solvent) that most of the tested azaarenes (table 5.2) gave a direct genotoxic response, with no differences between isomers of two-ringed or three-ringed structures. From the four- and five-ringed structures, only benz[*a*]acridine gave a direct (and strong) genotoxic response, while in this study this compound gave no genotoxic response at all.

Photolysis of acridine by light (artificial or sky) resulted into the formation of different products with 9(10*H*)-acridone as the most important compound (chapter 3). This metabolite was also present in the acridine solutions, after irradiation with the 300 nm, although not quantified yet, and in the ones radiated with 350 nm light. Bleeker *et al.* (submitted) tested the genotoxic responses of the metabolites 9(10*H*)-acridone and 6(5*H*)-phenanthridinone and found a much stronger response for the metabolite of acridine, compared to all the other azaarenes tested. For phenanthridinone a similar response as the parent compound phenanthridine was observed. QSAR analysis indicated that the mode of action for genotoxicity of acridone was probably different from that of all other azaarenes, including the metabolite phenanthridinone (Bleeker *et al.*, submitted).
The results of Bleeker *et al.* (submitted) could explain why genotoxicity of the acridine solutions increased after irradiation, but did not explain why the genotoxicity of the phenanthridine solutions increased. It is possible that stronger genotoxic compounds were formed instead or besides 6(5*H*)-phenanthridinone.
Nevertheless, the present results suggest that photolysis of azaarenes results in the formation of more genotoxic compounds than parent compounds.

5.4 GENOTOXIC AND TOXIC EFFECTS OF PHOTOMODIFIED AZAARENES

In chapter 4 the acute toxicity of the eight azaarenes and photomodified azaarenes were examined. To compare these results with results of Mutatox (this chapter), the increase of toxicity and genotoxicity of the eight azaarenes, due to irradiance, was expressed as the (geno)toxicity of photomodified azaarenes relatively to the (geno)toxicity of untreated azaarenes (table 5.3). Because different test organisms (resp. a diatom *Phaeodactylum tricornutum* and a stain of bacteria *Vibrio fischeri*) and different endpoints were used, no quantitative comparison could be made. Furthermore, differences were observed in the recoveries of parent compounds in modified azaarene solutions: for acridine 69% (300 nm), for phenanthridine 46% and 70% (resp. 300 nm and 350 nm) and for benz[*a*]acridine 43% (350 nm). As a result of this, concentrations of modified products could have been to low to induce (geno)toxic effects, although they were the same for the treatments in both experiments (toxicity and genotoxicity). With these problems taken into account, table 5.3 gives a summary of the results of this chapter and chapter 4.

Table 5.3. Increase of toxicity and genotoxicity of azaarenes by irradiance with 300 nm and 350 nm lamps, respectively. Increase of (geno)toxicity is classified in the following manner: +/- no increase of (geno)toxicity, + slightly increased (geno)toxicity, + + (+) high increase of (geno)toxicity. No (geno)toxicity is -.

	300 nm (UV-B)		350 nm (UV-A)	
Compounds	Toxicity	Genotoxicity	Toxicity	Genotoxicity
Quinoline	-	+/-	+++	-
Isoquinoline	-	-	+++	++
Acridine	+/-	++	++	++
Phenanthridine	+/-	++	+	+
Benz[*a*]acridine	-	+	-	-
Benz[*c*]acridine	-	-	-	+
Dibenz[*a,i*]acridine	-	+	-	+
Dibenz[*c,h*]acridine	-	+	-	+

Irradiance with the 300 nm lamp strongly increased the genotoxicity of the acridine and phenanthridine solutions, and slightly increased the genotoxicity of the benz[*a*]acridine and dibenzacridines solutions. Acute toxicity did not increase after irradiance with 300 nm, which was partly caused by a too short irradiance time in the case of acridine and phenanthridine. Also, UV-B radiation was very reactive with a high light intensity, shown by the short life-

spans of azaarenes under this light source (chapter 3, table 3.3). During this irradiance, newly formed '(geno)toxic' products could have been further degraded into small carbon fragments and other non-toxic products as demonstrated by Kochany and Maquire (1994) for quinoline.
Irradiance with the 350 nm lamp (a less reactive light source than UV-B) strongly increased the acute toxicity of the quinoline, isoquinoline and acridine solutions, and slightly increased the toxicity of the phenanthridine solutions. Genotoxicity of the quinoline solution did not increase after irradiance with 350 nm.

Kochany and Maquire (1994) investigated the degradation of quinoline by sunlight and 315 nm light, respectively and identified two quinoline metabolites, 2-hydroxyl-quinoline and 8-hydroxyl-quinoline. It was possible that here these products were also formed and induced toxicity, but did not induce genotoxicity. It is more likely that the bacteria, used in Mutatox, were capable to further degrade quinoline and metabolites. Studies have shown that bacteria and fungi easily metabolise quinoline (e.g., Pereira *et al.*, 1987; Sutherland *et al.*, 1994a; Sutton *et al.*, 1996). It is not yet clear if isoquinoline is degraded in the same way (Oudendijk, 1997), and this may explain the high genotoxicity of the isoquinoline solution (table 5.3).
The acute toxicity and genotoxicity of phenanthridine solution increased slightly and no (geno)toxicity was found for benz[*a*]acridine, after irradiance with UV-A (probably due to less phototransformation of parent compounds). The acridine solution was strongly (geno)toxic. Of the four- and five-ringed structures, only the genotoxicity of the benz[*c*]acridine and dibenzacridines solutions increased, after irradiance with UV-A.
Genotoxicity increased more often than toxicity, after irradiance with short-wavelength light. Also, lower concentrations of azaarenes and metabolites were needed to induce genotoxicity than to induce toxicity, what suggested that genotoxicity tests are more sensitive than toxicity tests.

Theoretically, three independent events can restore the luminescence in the repressed mutant, *Vibrio* (Ulitzur, 1986): 1. Blocking the formation of the repressor, i.e. altering its or the operator site's structure, 2. inactivating the repressor of the luminescence system, and 3. changing the physical configuration of the DNA. The first mode of action is expected from direct mutagens. The second event was found to be associated with the activity of different DNA-damaging agents and with the action of DNA synthesis inhibitors. DNA-intercalating agents act via the most potent and rapid way of restoring the luminescence by changing the physical configuration of the DNA (Ulitzur, 1986). Because QSAR analysis indicated that the genotoxicity of acridone was operating in a different mode of action, Bleeker *et al.* (submitted) suggested that acridone was the only DNA-intercalating agent, while the others interacted with the repressor, either blocking or inactivating it. In our study, other metabolites formed by photolysis may have act in a similar way as the metabolite acridone, binding to DNA and changing its physical configuration. This could explain why the phenanthridine solution was as genotoxic as the acridine solution and low (photomodified) azaarene concentrations could have induced genotoxicity.

From these results it can be concluded that sunlight, containing fractions of UV-A and UV-B, has the potential to transform azaarenes in more toxic and genotoxic products, with hazardous effects for aquatic life.

CONCLUSIONS

- Low azaarene concentrations (between 3.7-0.004 µmol/L for resp. two-ringed and five-ringed compounds) cause genotoxic effects on micro-organisms.
- Photolysis by UV-A (350 nm range) or UV-B (300 nm) enhances toxic and genotoxic effects of two-to five-ringed azaarenes, despite occasional detoxification.
- UV-A has a higher impact on the adverse effects of azaarenes than UV-B, by the formation of both genotoxic and toxic products.

6 BIOTRANSFORMATION OF ACRIDINE BY *PHAEODACTYLUM TRICORNUTUM*

6.1 INTRODUCTION

Many xenobiotic organic compounds that enter surface waters or soils are transformed by microorganisms. Transformation reactions generally results in oxygenated products which have enhanced water solubility. Products of quinoline and indole, transformed by bacteria were determined (Pereira *et al.*, 1987; Johansen *et al.*, 1997). Transformation of azaarenes by algae is sparsely investigated, most research has focused on metabolism of homocyclic PAHs by algae. Cerniglia *et al.* (1980) demonstrated that 18 different marine algal species can metabolize naphthalene. In this study, the potential transformation of acridine by the diatom *Phaeodactylum tricornutum* was investigated.

6.2 MATERIALS AND METHODS

Phaeodactylum tricornutum, a marine diatom, was cultured in the same manner as *Dunaliella tertiolecta* (chapter 2), but now silicate was added. Biotransformation of acridine by *Phaeodactylum tricornutum* was assessed in a 48 hour test in closed 80 or 100 ml quartz vessels (chapter 3) at 20°C.

Quartz vessels were exposed to UV light overnight, to make the vessels free of bacteria. Eight 100 ml vessels were filled with 50 ml algal suspension and to four of them a non-lethal concentration of acridine (0.1 mg/L) was added, using stock solutions in DMSO. Of the four vessels containing algal suspension and acridine, two were exposed to light and two were wrapped in aluminum foil, so light could not penetrate. Of the four other vessels, containing only algal suspension, also two were exposed to light and two were wrapped in aluminum foil. In addition, two 80 ml vessels containing 40 ml sterile seawater medium and 0.1 mg/L acridine, were also exposed to the light and dark treatment respectively. All vessels were placed in front of one mercury lamp (chapter 2), emitting an intensity of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ at a distance of 100 cm. The initial algal concentration in all treatments, measured with a Coulter counter©, was $6 \cdot 10^6$ cells/ml. After 48 hours, algal concentrations were measured in each vessel.

At times t=0, t=3, t=5, t=23, t=26, t=29 and t=48 h, water samples were taken, in order to determine the actual acridine concentrations. Acridine concentrations were measured using the HPLC as described in chapter 2.

To compare the rates of degradation of acridine between treatments, acridine concentrations were plotted as $\ln C_0/C_t$ against time, in which C_0 is the initial concentration of acridine and C_t the concentration at time t.

6.3 RESULTS AND DISCUSSION

The initial algal concentration was $6.01 \cdot 10^6$ cells/ml and the average end concentration of the vessels containing algae was $6.75 \cdot 10^6$ cells/ml. In all the vessels algal densities increased slightly, so the used light intensity and the acridine concentrations were not lethal to *Phaeodactylum*.

Figure 6.1 shows the decrease of acridine concentrations in the different treatments: acridine was degraded much faster in presence of both algae and light in comparison to acridine in presence of light or algae only. Still, the acridine decrease was much higher in the treatment with algae in the dark than the decrease of acridine by photolysis. *Phaeodactylum* seemed to be able to transform low concentrations of acridine. HPLC-chromatograms showed peaks at elution time of 9[10*H*]-acridone, but also other products with HPLC retention times near acridine were formed.

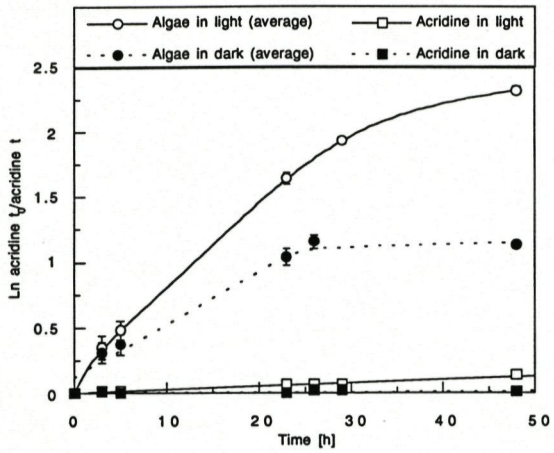


Figure 6.1. Decrease in acridine (C_0 = 0.08 mg/L) by the alga *Phaeodactylum tricornutum* and a mercury lamp, plotted against time (h.).

Bacteria and fungi are known to metabolize several azaarenes (Pereira *et al.*, 1988; Bollag and Kaiser, 1991; Sutherland *et al*, 1994a,b). Previous studies also demonstrated that marine and freshwater algae metabolize PAHs and azaarenes (Cerniglia *et al.*, 1980; Dijkman *et al.*, 1997; Van Vlaardingen *et al.*, 1996; Warshawsky *et al.*, 1995). The freshwater alga *Scenedesmus acuminatus* was capable to transform indole and *Selenastrum capricornutum* acridine (Dijkman *et al.*, 1997; Van Vlaardingen *et al.*, 1996).
Table 6.1 shows the decrease of low acridine concentrations by the marine diatom *Phaeodactylum*, the marine alga *Dunaliella* (Haradoni, 1998), both with the same experimental set-up, and by the freshwater alga *Selenastrum capricornutum* (Van den Berg, 1996), and by other processes involved.

Table 6.1. Decrease of acridine (%) by the marine diatom *Phaeodactylum tricornutum*, by the marine alga *Dunaliella tertiolecta*, by the freshwater alga *Selenastrum capricornutum* and by other degradation processen. Initial concentrations were 0.08, 0.13 and 0.16 mg/L, respectively.

	Decrease of acridine (%) by marine and freshwater algae		
Different treatments	*Phaeodactylum*[a]	*Dunaliella*[a]	*Selenastrum*[b]
Living cells in light	68.7±0.94	51.1±2.23	84±20.5
Living cells in dark	43.2	36.0±8.0	-
Dead cells	-	-	18.3±2.3
Bacteria	-	-	10
Sterile medium in light	12.8	8.9	1.83±0.06
Sterile medium in dark	0	-	-

[a]experiments performed with quartz vessels (48 h.), [b]with borosilicate vessels (24 h.).

In all experiments algae were not cultured axenic, so bacteria might have been partly responsible for the decrease in acridine concentration. In both set-ups the same light sources were used (mercury lamps). Because of substantial fractions of short-waved light in this light source, phototransformation of acridine played an important role (chapters 3). With these assumptions and absorption of acridine to cell walls (dead cells) taken in consideration, *Selenastrum* was capable of transforming minimally 54% of the acridine, after 24 hours test. *Phaeodactylum* transformed minimally 28% in the light and 15% acridine in the dark and *Dunaliella* minimally 14% in the light and 8% in the dark, after 48 hours.
The differences between *Selenastrum* on the one side and *Phaeodactylum* and *Dunaliella* on the other side, were partly caused by differences in phototransformation rates and initial concentrations. Van der Berg (1996) used a higher light intensity (more lamps), but the

borosilicate bottles were on a larger distance from the light sources, which were shielded with glass absorbing short-waved light. In the present experiment, algae were placed in quartz vessels, which hardly absorb radiation below 340 nm contrary to the borosilicate bottles (Leifer, 1988). Less short wavelength light reached the acridine in the experiments with *Selenastrum*, as shown by the difference of phototransformation percentages in sterile medium (1.8% for *Selenastrum* test and 6.4% for marine algae tests). Furthermore, several authors reported a range of azaarene concentration, in which transformation can take place (Dijkman *et al.*, 1997; Kraak *et al.*, 1997a; Van Vlaardingen *et al.*, 1996). Below this range transformation was not initiated and above it acridine hampered metabolism (toxic). As shown in figure 6.1, *Phaeodactylum* transformed more acridine during the first 20 hours than afterwards. Because acridine concentrations could have dropped below threshold concentrations, the decrease in acridine concentration by marine algae was less than by *Selenastrum*.

The small difference between decreases in acridine concentration by *Phaeodactylum* and *Dunaliella* were partly caused by differences in algal densities and light intensity. The initial algal concentration of *Dunaliella* was $0.81 \cdot 10^6$ cells/ml and the average end concentration of the vessels containing algae in the light was $1.60 \cdot 10^6$ and in the dark $1.07 \cdot 10^6$ cells/ml. In all the vessels algal densities increased, more in the light than in the dark treatment.
The decrease of acridine concentration in the treatment with living cells of *Dunaliella* in the light and in the sterile medium in the light were lower than in the corresponding treatments of the *Phaeodactylum* test, due to aging of the lamp used (pers. observations).

CONCLUSIONS

- The marine algae *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* are able to metabolize acridine.
- Co-action of photolysis and metabolism of acridine is a particularly effective transformation mechanism.

7 EFFECTS OF ACRIDINE ON A NATURAL MARINE PHYTOPLANKTON COMMUNITY MEASURED WITH FLOW CYTOMETRY

In cooperation with G. Vriezekolk (RIKZ, Middelburg)

7.1 INTRODUCTION

Recently, several authors have suggested photo-induced toxicity of acridine to different aquatic and marine organisms (Bleeker *et al.*, 1998; Dijkman *et al.*, 1997; Sinks *et al.*, 1997; Wernersson and Dave, 1997). In general, algae are still indicated as more tolerant to these adverse effects in comparison to other aquatic organisms (Gala and Giesy, 1990). Partly, this is caused by the comparison of different endpoints of toxicant effects to algae and to invertebrates, growth of algae and the more sensitive sublethal effects to invertebrates. Flow cytometry provides a fast and quantitative method to determine effects of a toxicant to individual algal cells within a population, in contrast to growth, a standard population-based endpoint (Gala and Giesy, 1990).

Still little is known about the effects of phototoxic compounds on natural algal communities under natural skylight. An accurate and sensitive characterization of such chronic photo-induced toxicity of azaarenes is to natural algal communities, in situ, essential in hazard assessments of azaarenes. Therefore, (photo)toxicity of acridine to a natural phytoplankton community under skylight was assessed in a 72 hour outdoor experiment, using flow cytometry.

7.2 MATERIALS AND METHODS

Effects of acridine to phytoplankton were assessed in a 72 hour experiment on the roof of the Biological Center in Amsterdam. For this outdoor experiment, seawater complete with phytoplankton and suspended matter, was collected from the Oosterschelde (at Neeltje Jans) on 9 and 22 September 1997.

On 9 September four full-glass aquaria were filled with 4 L seawater each, on 22 September eight aquaria were filled with 4 L seawater. Aquaria with seawater were enriched with nutrients (25% of the concentrations of vitamins, N, P, Si, trace metals from Admiraal and Werner, 1983) and acclimated for 24 hours before acridine was added.

Acridine was dissolved in DMSO and to each experimental treatment 0.4 ml solution of a dilution series was added. On September 9 only one acridine concentration was tested (0.1 mg/L) and on September 22, acridine concentrations 0.016 mg/L, 0.04 mg/L and 0.10 mg/L were tested in duplicate. Besides duplicate controls without toxicant, an acridine control in 0.2 µm filtered seawater and a quartz tube with fluoranthene ($5 \cdot 10^{-5}$ M) were included, the latter as a photochemical control.

Each day, water samples were taken for flow cytometric analysis of the phytoplankton community, and for HPLC analysis of acridine. Before samples were taken, seawater was stirred in order to suspend attached algae.

Because direct flow cytometric analysis was not possible, samples were fixed with 0.01% paraformaldehyde (Merck, >95%) and 0.1% glutaraldehyde (Merck, 25%) and were stored at 5°C in dark. The fixative was prepared by dissolving 2.5 mg paraformaldehyde in 125 ml water at 65°C. To this solution, 1 to 3 drops of 1N NaOH were added in order to get a clear solution. 100 ml of glutaraldehyde was added and pH was adjusted to 7. The end volume of the solution was adjusted to 250 ml with water. The solution was filtered with 0.2 µm polycarbonate filters (Nucleopore inc.) and stored at 5°C in dark. To every phytoplankton sample (50 ml) 0.5 ml was added and samples were kept at 5°C, till the analysis with flow cytometry was performed.

The parameters measured for each sample, were cell numbers/L, fluorescence/L, average fluorescence/cell and average scatter/cell. The used method can not separate cells from chains of cells and counts chains as only one cell. In combination with the fluorescence/L and average fluorescence/cell, flow cytometry shows the growth of individual cells or chains. The

fluorescence/L is a flow cytometric measure for the amount of pigments/L, the average scatter/cell is a measure for the size of cells.

7.3 Results and discussion

Figure 7.1 shows the decrease of the acridine concentration in the different treatments and the decrease of fluoranthene concentration in the presence of natural light. In the control of acridine and fluoranthene, the photodegradation rates were much lower than measured in previous experiments (without algae, in milliQ-water) performed in the summer (chapter 3, fig. 3.4). Also, the decrease of the acridine concentration was in the same order as the decrease of the fluoranthene concentration, which in previous experiments was much higher than that of the fluoranthene concentration (chapter 3). The exposure concentrations of acridine to phytoplankton did not alter drastically within 72 hours, and therefore it can be concluded that bio- and phototransformation of acridine played no important role during these experiments.

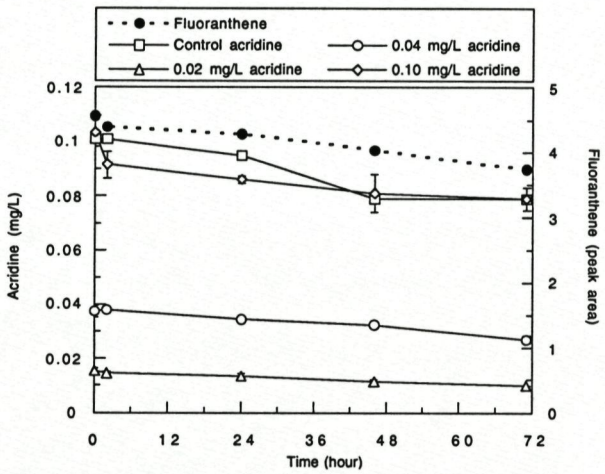


Figure 7.1. Decrease of acridine concentration (mg/L) in seawater and of fluoranthene concentration (HPLC peak area) in milliQ-water, plotted against time (h).

In the control treatments, microscopical observations showed an extreme dominance of one diatom species (*Nitzschia closterium*) and occasionally other algae, mainly small pennate diatoms, after 72 hours. The opportunistic species *Nitzschia closterium* (Van der Werff and Huls, 1974), was aggregated with other diatoms and particular matter. In the 0.02 mg/L and 0.04 mg/L treatment algae were still present, but here *Nitzschia* was not the dominant species: the phytoplankton community consisted of a considerable lower number of small diatom species, mainly pennate diatoms(*Chaetoceros danicus, Asterionella japonica, Navicula* spec., *Thalassiosira rotula* and others).

Figure 7.2 shows the toxicity of acridine to natural phytoplankton communities (collected on 22-9-1997), determined with different parameters of flow cytometry.
After 24 hours of exposure, the four parameters showed that in the 0.1 mg/L treatment the number of algal cells diminished drastically (below detection limits of flow cytometry). The same results were observed for the 0.1 mg/L treatments on 11-9 (only the results from 22-9 are presented). During the first 24 hours, the control treatments (without toxicant) did not differ from the 0.02 and 0.04 mg/L treatments. However, after 72 hours, 0.02 mg/L acridine inhibited cell multiplication (fig. 2) and 0.04 mg/L diminished the algal cells drastically. These observed inhibitory effects to phytoplankton, are directly related to acridine concentration, suggesting that NOEC are far below 0.02 mg/L acridine.

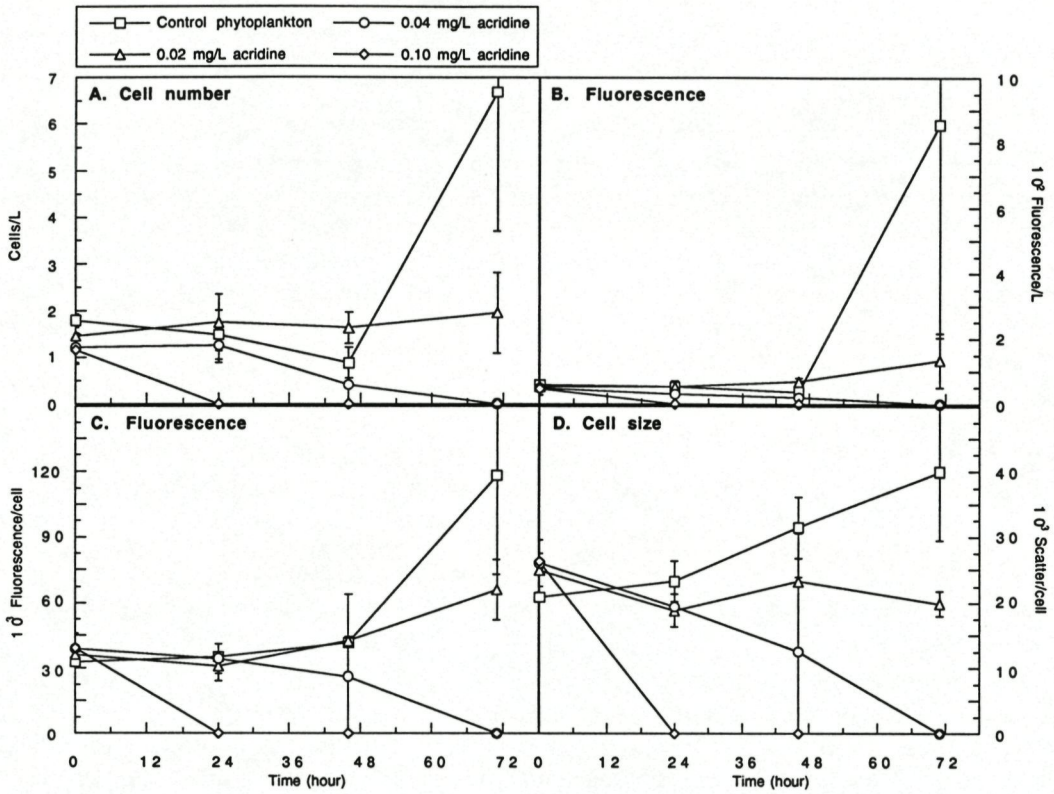


Figure 7.2. Effects of acridine (n=2, ± SD) on natural phytoplankton in seawater, determined with the flow cytometric measures cell·L^{-1}, fluorescence·L^{-1}, average fluorescence·$cell^{-1}$ and average scatter·$cell^{-1}$, during 72 hour exposure.

For the green algae *Selenastrum capricornutum* only with the flow cytometric measure sizes, small differences were observed in photo-induced toxicity test of the PAH anthracene. Cell viability (measured by the use of a stain) proved to be a more sensitive parameter (Gala and Giesy, 1994) than size. However, in our experiment, the flow cytometric measures in combination with microscopic observations proved to be a sensitive method to determine photo-induced toxicity of acridine to a marine phytoplankton community.

Table 7.1. An overview of the effect concentrations of acridine (mg/L), obtained from laboratory tests, to different freshwater (F) and marine (M) algal species.

Effects of acridine to:	F/M	h	EC_{50}	Based on:	References
Scenedesmus acuminatus	F	72	0.32-0.41	growth rate (μ)	Van Vlaardingen *et al.*, 1996
Scenedesmus acuminatus	F	72	0.26	chlorophyll-*a*	Van Vlaardingen *et al.*, 1996
Scenedesmus acuminatus	F	96	0.32-0.44	growth rate (μ)	Dijkman *et al.*, 1997
Selenastrum capricornutum	F	72	0.27-0.78	growth rate (μ)	Dijkman *et al.*, 1997
Selenastrum capricornutum	F	-	4.7 (EC_{25})	^{14}C incorporation	Giddings, 1979
Chlamydomonas eugametos	F	72	0.78-0.84	growth rate (μ)	Dijkman *et al.*, 1997
Dunaliella tertiolecta	M	72	0.38-0.42	growth rate (μ)	Chapter 2
Staurastrum chaetoceras	F	96	0.22	growth rate (μ)	Dijkman *et al.*, 1997
Staurastrum manfeldtii	F	96	0.35-0.46	growth rate (μ)	Dijkman *et al.*, 1997
Navicula salinarum	M	96	0.33-0.82	growth rate (μ)	Dijkman *et al.*, 1997
Nitzschia sigma	M	96	0.08-0.13	growth rate (μ)	Dijkman *et al.*, 1997
Phaeodactylum tricornutum	M	2	2.26	^{14}C incorporation	Chapter 4
Phytoplankton assemblage	M	72	0.01-0.02	Flow cytometry	Chapter 7

Sinks *et al.* (1997) and Wernersson and Dave (1997) reported photo-induced toxicity of acridine to a freshwater ciliate *Tetrahymena pyriformis* and the waterflea *Daphnia magna* respectively. In the presence of UV-A radiation toxicity of acridine increased with one order of magnitude to *Daphnia*. Also, an increase of UV-B intensity increased equally the toxicity to *Tetrahymena*. Both experiments showed the relevance of outdoor experiments with acridine, to validate phototoxicity under varying light and varying UV-A intensities.
Table 7.1 shows (photo-induced) effect concentrations of acridine to different algal species, determined under different laboratory conditions. All observed effect concentrations, except the effect concentration to *Nitzschia sigma*, exceeded the concentrations used in the present (outdoor) experiment (0.1 mg/L). Although *Nitzschia sigma* showed to be a sensitive species, photo-induced toxicity is an order of magnitude higher in outdoor experiments and therefore, it can be concluded that all laboratory derived effect concentrations give an underestimation of the effect under natural conditions.
In addition, these observations (in the experiments carried out in September) were marked by a moderate photodegradation of acridine and fluoranthene, in accordance with the season. Much higher phototoxicity of acridine can be expected in summertime, as indicated by higher photodegradation rates (chapter 3) and observed increases in phototoxicity with increasing UV-A intensity (Sinks *et al.*, 1997).

CONCLUSIONS

- Toxicity of acridine is one to two orders of magnitude higher in outdoor experiments, compared to laboratory derived toxicity data under artificial light.
- The hazard of phototoxic azaarenes and their photoproducts to marine phytoplankton is likely to be much higher in summer than indicated by these experiments in autumn.
- The toxic effect of acridine is selective to certain species in mixed algal assemblages, which can also be expected for other (phototoxic) azaarenes.

8 QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS (QSARS) FOR AZAARENE TOXICITY

Quantitative structure-activity relationships (QSARs), which correlate the physicochemical properties of molecules to biological responses, are useful for understanding the action mechanisms of groups of related chemicals and for predicting the environmental risk associated with those chemicals (De Voogt, 1990). With QSARs it can be established which physical or molecular properties of chemicals contribute to biological impacts, and they can help in elucidating chemical and biological mechanisms of toxicity. Based on established QSARs the potential hazard of untested compounds can be predicted.

To elicit a biological effect on receptors, a chemical has to cross membranes and interact with one or more receptors. Therefore, both hydrophobic and electronic forces play a role; so hydrophobicity-related and electronic molecular parameters determining the transport and partition processes involved, should be selected (Bleeker *et al.*, submitted). Therefore, in this study the following descriptors were chosen: molecular volume (Vol), surface area (SA), dipole moment (D), ionization potential (HOMO/IP) and electron affinity (LUMO/EA). In addition log K_{ow}, a parameter related to hydrophobicity was included, because good correlations have been found between acute toxicity of organic pollutants and their octanol/water partition coefficients (Könemann, 1981; Veith *et al.*, 1983) and between the descriptors Vol and SA and log K_{ow} (De Voogt *et al.*, 1988; De Voogt *et al.*, 1990).
QSARs for the photolysis of compounds as described in chapter 3 are currently under construction and will be reported elsewhere.

Table 8.1. Log K_{ow}, volume (Vol), surface area (SA), heat of formation (HF), dipole moment (D), electron affinity (EA), ionization potential (IP) of azaarene molecules and correlation coefficients (r) between the molecular descriptors and the corresponding effect concentrations on the algae *Dunaliella* (n=6) and *Phaeodactylum* (n=5).

Compound	log K_{ow}	Vol ($Å^3$)	SA ($Å^2$)	HF (kcal)	D	EA (eV)	IP (eV)	IP-EA gap
Quinoline	2.03	121.95	143.05	52.12	1.88	-0.4677	-9.1825	-8.7148
Isoquinoline	2.08	121.84	142.77	50.10	2.23	-0.5608	-9.0244	-8.4636
Acridine	3.40	166.25	187.50	77.05	1.84	-1.0413	-8.5754	-7.5341
Phenanthridine	3.48	166.03	186.25	67.93	2.07	-0.6031	-8.9708	-8.3677
Benz[*a*]acridine	4.49	210.19	230.41	92.19	1.91	-0.9924	-8.6242	-7.6318
Benz[*c*]acridine	4.49	210.35	231.06	91.30	1.44	-0.9813	-8.5448	-7.5635
r (*Dunaliella*)	0.96*	0.95*	0.95*	0.97*	0.63	0.92*	0.94*	0.93*
r (*Phaeodactylum*)	1.00*	0.99*	0.99*	0.98*	0.37	0.82*	0.84*	0.83*

*Significant positive correlation, $p<0.05$.

Although, from the series of eight azaarenes five and six EC_{50} values for *Phaeodactylum* and *Dunaliella* respectively, were observed, significant correlations between log K_{ow}, molecular volume, surface area or heat of formation of azaarenes and their toxicity to resp. *Dunaliella* and *Phaeodactylum* were found (r 0.95 and $p<0.05$, table 8.1). This indicates that for most of the tested azaarenes toxicity coincides with lipophilicity. Toxicity of the whole series of azaarenes is probably caused by narcotic effect, due to association with membranes.
The tested concentrations of the compound benz[*c*]acridine were too low to cause effect on *Phaeodactylum*, due to insolubility and low recovery. Based on the high correlation (r=1.0) between the EC_{50} of *Phaeodactylum* and the log K_{ow} of azaarenes (fig. 8.1), it is expected that the effect concentration of benz[*c*]acridine will equal the concentration of benz[*a*]acridine, 0.29 (0.23-0.36) µM.

Acridine and benz[*c*]acridine were far more toxic to *Dunaliella* in comparison to the toxicity of the isomers phenanthridine and benz[*a*]acridine. Most descriptors, however, were insufficiently discriminating between isomers, although correlation with toxicity of the whole series of azaarenes tested was high. Of all the descriptors, heat of formation discriminated most between the toxicity of isomers and provided a good explanation for observed effects for the whole series of compounds (fig. 8.1). Since heat of formation is related to the stability of molecular structure, it is also related to the photochemical stability of compounds. This suggests that photochemical reactions were contributing to the observed toxicity of isomers.

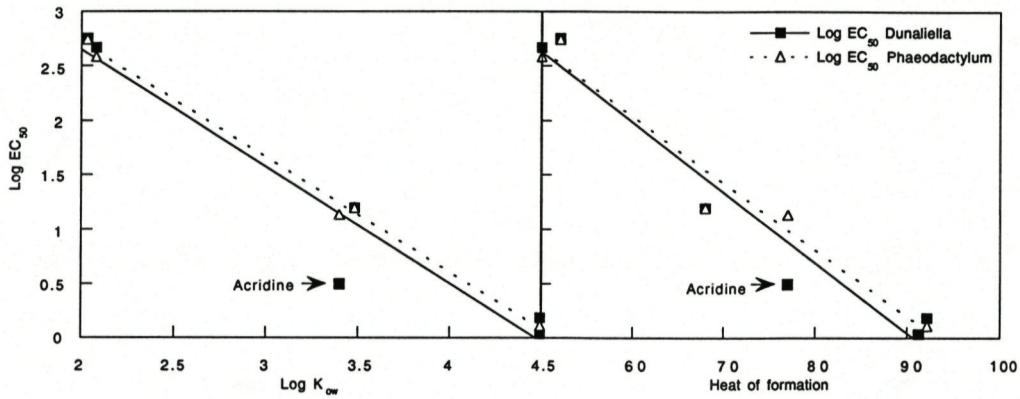


Figure 8.1. Effect concentrations (log base values) of azaarenes, plotted against the octanol/water partition coefficients (log Kow) and heat of formations of corresponding compounds. Curve fits represent the linear correlation.

Recently, several authors examined the relationship between photo-induced toxicity of PAHs and azaarenes with molecular descriptors and found that the difference in the energies of the HOMO (highest-occupied-molecular-orbital and LUMO (lowest-unoccupied-molecular-orbital) was an useful descriptor to evaluate the molecular stability, light absorbance and photo-induced toxicity of PAHs and azaarenes (Mekenyan *et al.*, 1994a,b; Veith *et al*, 1995; Sinks *et al.*, 1997). From this relationship, a "phototoxic window" or threshold value, defining phototoxic and non-phototoxic compounds, was determined. This window or HOMO-LUMO gap was found to be 7.2 ± 0.4 eV (Mekenyan *et al.*, 1994a) and only acridine, benz[*a*]acridine and benz[*c*]acridine fall in this phototoxic region. The high correlation between heat of formation and toxicity, and HOMO-LUMO values of acridine, benz[*a*]acridine and benz[*c*]acridine in this phototoxic region, are strong indicators for the photo-induced toxicity of these compounds.
Acridine was also far more toxic to *Dunaliella* in comparison to the toxicity for *Phaeodactylum*, probably due to photo-induced toxicity (chapter 2 and 4). Consequently, correlation coefficients between log K_{ow}, volume or surface area and toxicity to *Phaeodactylum* were much higher than between these parameters and toxicity to *Dunaliella*. In contrast, correlation coefficients of heat of formation, dipole moment, electron affinity or ionization potential and toxicity to *Phaeodactylum* were much lower than the correlation coefficients between log K_{ow}, molecular volume, surface area or heat of formation and toxicity. More important, correlation coefficients of these descriptors were also much lower than the correlation coefficients between the same descriptors and toxicity to *Dunaliella*.

Thus, toxicity consistent with lipophilicity of azaarenes is described best with the descriptors molecular volume, surface area and the parameter log K_{ow}, illustrated by the toxicity to *Phaeodactylum*. In contrast, the descriptors heat of formation, electron affinity and ionization potential, all based on electronic interactions, indicate that the increased toxicity of acridine and benz[*c*]acridine to *Dunaliella*, was caused by photo-enhanced toxicity.

CONCLUSIONS

- Lipophilicity-related descriptors explained most of the toxicity of azaarenes, in a range of two to four ringed structures.
- Descriptors related to electronic interactions (HF and HOMO-LUMO gap), were effective in describing phototoxic effects being superimposed on the narcotic effect. High phototoxicity was observed for especially acridine and benz[*c*]acridine.

9 RELEVANCE FOR ENVIRONMENTAL WATER QUALITY CRITERIA

A large fraction of NPAHs found in the environment are from anthropogenic origin, since almost no NPAHs have been found in older sediments (Wakeham, 1979). NPAHs are present in amounts up to 1-10% of those of the analog PAHs (Wild and Jones, 1995), but are more soluble than their homocyclic analogs. For example, the solubility of acridine (three-ringed NPAH) is almost two orders of magnitude higher than that of anthracene, its homocyclic analog (Pearlman *et al.*, 1984). Although this higher solubility would imply a higher bioavailabillity to organisms in water, most research has focused on homocyclic compounds (PAHs). Recent research has shown that especially aquatic organisms living in close contact with sediments (e.g. bivalves, oligochaetes and diatoms), bioaccumulate PAHs (Stronkhorst *et al.*, 1994; Volz, 1995; BEON report, 1996; Baumard *et al.*, 1998; Leppänen and Kukkonen, 1998). In contrast to NPAHs, the presence and effects of PAHs in the environment are extensively examined and it is known that the release of PAHs in air, waters and sediments exceeds maximal acceptable risk concentrations (MTRs) (Kalf *et al.*, 1995). Particularly for azaarenes, the presence in the environment is badly documented and water quality criteria (MTRs, VR), as for some PAHs (table 9.1), are lacking (NW4, 1997).

Table 9.1. Octanol-water partition coefficient (Kalf *et al.*, 1995) and quality criteria for PAHs in surface waters and sediments of The Netherlands (NW4, 1997).*For these compounds no toxicity data are available.

		Surface waters		Sediment (dry)
PAH	log K_{ow}	MTR (µg/L)	Target values (µg/L)	MTR (µg/mg)
Naphtalene	3.3	1.2	0.01	0.1*
Anthracene	4.5	0.08	0.0008	0.1
Phenanthrene	4.5	0.3	0.003	0.5*
Fluoranthene	5.2	0.5	0.005	3*
Benzo[*a*]anthracene	5.8	0.03	0.0003	0.4
Chrysene	5.7	0.9*	0.009	11*
Benzo[*k*]fluoranthene	6.0	0.2	0.002	2*
Benzo[*a*]pyrene	6.1	0.2	0.002	3
Benzo[*ghi*]perylene	6.6	0.5*	0.005	8*
Indenopyrene	6.4	0.4*	0.004	6*

In addition, existing quality criteria for PAHs are susceptible to criticism. For some PAHs, MTRs and NOECs are based on empirical results and for others toxicity data are generated, using QSAR analysis (Kalf *et al.*, 1995; BEON report, 1996; NW4, 1997). Criteria are mostly based on narcotic toxicity of single PAHs and other modes of action like mutagenicity and phototoxicity of PAHs in combination with biodegradation products are not considered (BEON report, 1996). For PAHs all these different modes of toxicity are evident (Zeiger *et al.*, 1992; Ankley *et al.*, 1997; Mekenyan *et al.*, 1994b; Huang *et al.*, 1997; Krylov *et al.*, 1997; Duxbury *et al.*, 1997) and toxic degradation products, formed by metabolism or photodegradation, have been identified (Kaiser *et al.*, 1996; Pereira *et al.*, 1987; McConkey *et al.*, 1997). Research on toxicity of mixtures of PAHs is very limited, although these compounds are always present in mixtures in the environment. For PAHs, future quality criteria should be based on toxicity of mixtures of PAHs and should incorporate heterocyclic compounds as well as biotransformation products.

If quality criteria for azaarenes are based on minimal narcotic toxicity, MTRs (minimal toxicity/50) can be calculated using the lowest EC_{10} values of azaarenes on the marine algae *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* (table 9.2). Minimal toxicity is routinely calculated for at least three groups of organisms (fish, daphnids and algae) and corresponding

MTR is determined with effect concentrations to the most sensitive organisms (BEON report, 1996). In this study, only marine algae were tested (table 9.3, [1]MTR), excluding other sensitive organisms and this could tend to lead to higher MTRs for azaarenes compared to PAHs (table 9.1).

Table 9.2. Effect concentrations of azaarenes. [1]Minimal effects on growth (72h) of *Dunaliella tertiolecta*. [2]Minimal effects on photosynthetic activity (2h) of *Phaeodactylum tricornutum*. [3]Effects of azaarenes, after irradiation with UV, expressed as concentrations of remaining parent compounds.

	narcotic toxicity		(geno)toxicity of mixtures	
Compound	$^1EC_{10}$ (mg/L)	$^2EC_{10}$ (mg/L).	$^3EC_{50}$ (µg/L)	^{3}Mutatox (µg/L)
Quinoline	30.3 (17.8-51.6)	14.9 (10.6-20.8)	0.23 (0.16-0.34)	0.130
Isoquinoline	12.7 (6.4-25.2)	16.5 (9.61-28.5)	0.56 (0.11-2.69)	0.120
Acridine	0.19 (0.08-0.45)	0.5 (0.32-0.88)	0.40 (0.24-0.65)	0.110
Phenanthridine	1.02 (0.34-3.04)	0.59 (0.45-0.77)	1.05 (0.40-2.73)	0.300
Benz[*a*]acridine	0.10 (0.002-5.32)	0.02 (0.01-0.03)	0.07 (0.02-0.08)	0.002
Benz[*c*]acridine	0.003 (0.001-0.02)	-	-	0.002

Table 9.3. Octanol-water partition coefficients of azaarenes and lowest observed effect concentrations (LOEC, µg/L) of 9(10*H*)acridone and 6(5*H*)phenanthridinone for genotoxic responses and the LOEC of acridine on natural phytoplankton in an outdoor experiment. Maximal acceptable risk concentrations (MTR, µg/L) based on narcosis[1] or including other modes of action[2].

Compound	log K_{ow}	LOEC (µg/L)	[1]MTR (µg/L)	[2]MTR (µg/L)
Quinoline	2.03	-	298	**2.6**
Isoquinoline	2.08	-	254	**2.4**
Acridine	3.4	16.0	3.80	**0.3**
9(10*H*)Acridone	2.84	0.98	-	**0.02**
Phenanthridine	3.48	-	11.8	6.0
6(5*H*)Phenanthridinone	2.70	10620	-	212
Benz[*a*]acridine	4.49	-	0.40	**0.04**
Benz[*c*]acridine	4.49	-	0.06	**0.04**

MTR values of 0.30 and 0.25 mg/L for respectively quinoline and isoquinoline in water, still could cause adverse biological effects. In combination with degradation products, these maximal acceptable risk concentrations are as well toxic as genotoxic (table 9.2 and table 9.3), which stresses the importance of including effects of biodegradation products and genotoxicity in risk assessment.
The MTRs of PAHs (table 9.1) and of most azaarenes (table 9.3) can be compared, when their lipophilic nature are considered. To visualise the influence of other modes of action, MTRs of azaarenes, incorporating these other modes, and MTR of PAHs, based on narcotic toxicity, are plotted against their corresponding log K_{ow} in figure 9.1. The line represents an estimation model for narcotic toxicity to *Daphnia* (Nendza and Hermens, 1995. Most MTRs of azaarenes, based on other modes of action (here, genotoxicity and toxicity of transformation products), are far below the range of MTRs of PAHs with narcotic action, except for anthracene (phototoxicity of this compound was incorporated in MTR).

Although literature on the presence of azaarenes in water is limited, a few studies have detected elevated concentrations of azaarenes (Wakeham, 1979; Furlong and Carpenter, 1982; Kozin *et al.*, 1997). In Pudget Sound sediments (Washington, USA) concentrations were for quinoline <6.6 µg/g OC, for isoquinoline <2.2 µg/g OC and for acridine <0.63 µg/g OC. In Ketelmeer sediments (The Netherlands) concentrations were 0.045 µg/g DW for benz[*a*]acridine, 0.095 µg/g DW for benz[*c*]acridine and for dibenz[*c,h*]acridine 0.0076 µg/g DW. Pereira and coauteurs found azaarenes in groundwater under a coal tar distillation site (Pereira *et al.*, 1983) and in aquifer contaminated with wood-treatment chemicals (Pereira *et al.*, 1987) up to the

following concentrations: quinoline 288 µg/L; isoquinoline 29 µg/L; acridine 106 µg/L (Pereira *et al.*, 1983); 9(10*H*)acridone 119 µg/L. In the Netherlands even the occurrence of azaarenes in surface waters have been reported, concentrations of quinoline, isoquinoline and acridine were in the range of 0.1-1 µg/L (Van Genderen *et al.*, 1994).

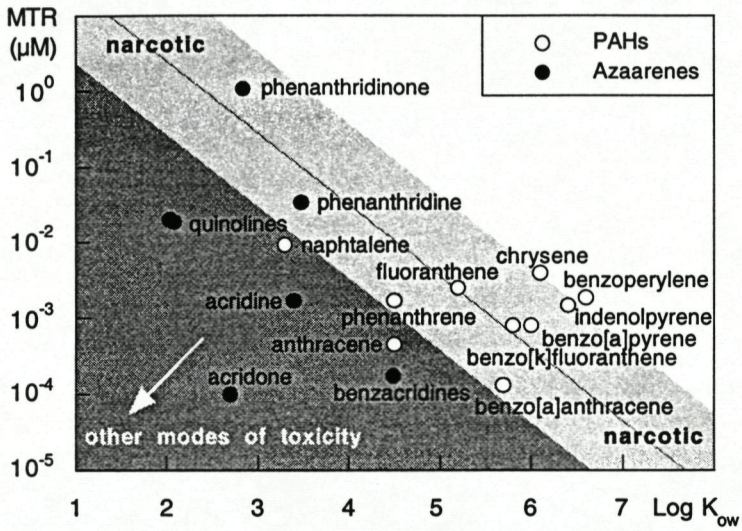


Figure 9.1. Schematic view of the relation between log K_{ow} and log MTRs, determined for the narcotic effects of PAHs and the ones of azaarenes, determined for toxic effects including other modes as phototoxicity and genotoxicity. The line represents an estimation model for narcotic toxicity to *Daphnia magna*; log NOEC=-0.95·log K_{ow}-2.0, after Nendza and Hermens (1995).

Especially near pollution sources, azaarene concentrations can reach toxic levels as shown for quinoline and acridine in groundwater and aquifer (Pereira *et al.*, 1983; Pereira *et al.*, 1987). Monitoring of PAHs poorly reflects the hazard of azaarenes and other heterocyclic aromatic compounds and therefore hazard assessment of aromatic compounds have to be completed with different biological end-points as genotoxic and specific toxic effects, including the effects of biotransformation products.

CONCLUSIONS

- Monitoring of azaarenes can not be used as a tool to estimate biological risk, because of the predominant effect of degradation products.
- Tentative environmental criteria (MTR) for some azaarenes are developed and indicate that the few measured concentrations in the field do exceed these MTR values.

10 CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Azaarenes degrade rapidly in the presence of short-wavelength light, with half-live periods of eleven hours (UV-B region) and four days (UV-A region) for most compounds. Extrapolations, according to the model of Zepp and Cline to surface conditions in the sea in midsummer indicate half-live periods of less than six days.

Photolysis of azaarenes by UV-A (350 nm) does not lead to detoxification, in contrast the photolytic products introduce new toxicity. For quinoline and isoquinoline the new compounds were two orders of magnitude more toxic in tests with *Phaeodactylum tricornutum.*

Photolysis of azaarenes by UV-B (300 nm) or UV-A causes enhanced genotoxic effects, due to formation of new or more genotoxic compounds.

Differences between toxicity of azaarene isomers to *Dunaliella tertiolecta,* a green alga, are greatly increased by light and this influence was quantified in QSAR analysis, using electronic descriptors (HF, HOMO-LUMO gap).

The marine algae *Phaeodactylum* and *Dunaliella* are able to metabolize acridine, especially in co-action with light conditions that enhance photolysis. HPLC diagrams showed the presence of different metabolites, formed by biotransformation and/or photodegradation.

Toxicity of acridine is one to two orders of magnitude higher in outdoor experiments to natural phytoplankton, compared to toxicity observed in mono-cultures under artificial light. This accords with a strong photobiological response. Hazardous effects of phototoxic azaarenes and their photoproducts to phytoplankton observed in autumn are therefore expected to be even stronger in summer.

The phototoxic effect of acridine selectively affects certain species in mixed algal assemblages, which can also be expected for other azaarenes.

Monitoring of azaarenes can not be used as a tool to estimate biological risk, because of the predominant effect of degradation products. Tentative environmental criteria (MTR) for some azaarenes are developed and indicate that the few measured concentrations in the field do exceed these MTR values.

POLICY RECOMMENDATIONS

- Water quality criteria, based on laboratory studies (artificial light) for light sensitive polyaromatic compounds (azaarenes, as well as parent PAHs) are not applicable to in situ conditions. Biological effect concentrations may be overestimated by several orders of magnitude. Therefore, incorporation of phototoxicity in calculations of risk parameters is essential.
- Monitoring of most PAHs in seawater is insufficient as a tool to estimate biological risk, because of the predominant effect of degradation products. Monitoring of genotoxic and specific toxic effects, including the effects of degradation products, has to complete PAH/azaarene-monitoring.

11 REFERENCES

Adrian, N.R. and J.M. Suflita, 1994. Anaerobic biodegradation of halogenated and nonhalogenated N-, S-, and O-heterocyclic compounds in aquifer slurries. Environ. Toxicol. Chem. 13 (10): 1551-1557.

Admiraal, W. and D. Werner, 1983. Utilization of limiting concentrations of orthophosphate and production of organic phosphates in cultures of marine diatoms. J. Plankt. Res. 5: 495-513.

Ankley, G.T., R.J. Erickson, B.R. Sheedy, P.A. Kosian, V.R. Mattson and J.S. Cox, 1997. Evaluation of models for predicting the phototoxic potency of polycyclic aromatic hydrocarbons. Aquat. Toxicol. 37: 37-50.

Baumard, P., H. Budzinski and P. Carrigues, 1998. Polycyclic aromatic hydrocarbons in sediments and mussels of the Western Mediterranean Sea. Environ. Toxicol. Chem. 17 (5): 765-776.

Bleeker, E.A.J., W. Admiraal, M.C. Buckert-de Jong, H.G. van der Geest, M.H.S. Kraak, P. van Vlaardingen, P. de Voogt and E. Wind, 1996. NPAHs in Dutch marine and freshwater sediments and toxic, mutagenic and teratogenic effects on aquatic invertebrates. ARISE, University of Amsterdam. Project-report.

Bleeker, E.A.J., H.G. van der Geest, M.H.S. Kraak, P. de Voogt and W. Admiraal, 1998. Comparative ecotoxicity of NPAHs to larvae of the midge *Chironomus riparius*. Aquatic Toxicol. 41: 51-62.

Bleeker, E.A.J., H.G. van der Geest, H.J.C. Klamer, P. de Voogt, E. Wind and M.H.S. Kraak. Toxic and genotoxic effects of azaarenes: isomers and metabolites. submitted.

Bollag, J.M. and J.P. Kaiser, 1991. The transformation of heterocyclic and aromatic compounds and their derivatives under anaerobic conditions. Crit. Rev. Environ. Cont. 21 (3,4): 297-329.

Bowling, J.W., G.J. Leversee, P.F. Landrum and J.P. Giesy, 1983. Acute mortality of anthracene-contaminated fish exposed to sunlight. Aquat. Toxicol. 3:79-90.

Catallo, W.J., Hoover, D.G., Vargas, D., 1994. Effects of an environmental toxicant on ultrastructure in *Paramecium caudatum* and the protective effect of dissolved calcium. Aquatic Toxicology 29: 291-303.

Cerniglia, C.E., D.T. Gibson, and C. van Baalen, 1980. Oxidation of naphthalene by cyanobacteria and microalgae. J. Gen. Microbiol. 116: 495-500.

Cooney, J.D. and C.W. Gehrs, 1984. Effects of temperature, feeding and acridine on development and mortality of eggs and nauplii of *Diaptomus clavipes* Schacht. Aquat. Toxicol. 3: 79-90.

Cushman, R.M., and M.I. McKamey, 1981. A *Chironomus tentans* bioassay for testing synthetic fuel products and effluents, with data on acridine and quinoline. Bull. Environ. Contam. Toxicol. 26: 601-605.

De Voogt, P., J.W.M. Wegener, J.C. Klamer, G.A. Van Zijl and H. Govers, 1988. Prediction of environmental fate and effects of heteroatomic polycyclic aromatics by QSARs: The position of n-octanol/water partition coefficients. Biomed. Environ. Sci. 1:194-209.

De Voogt, P., 1990. QSARs for the environmental behaviour of polynuclear (hetero)aromatic compounds. Ph.D. thesis, Free University, Amsterdam, 254 pp.

De Voogt, P., G.A. van Zijl, H. Govers and U.A.Th. Brinkman. 1990. Reversed-phase and thin-layer chromatography and structure-activity relationships of polycyclic (hetero)aromatic hydrocarbons. J. Planar. Chromatogr. 3: 24-33.

Dijkman, N.D., P.L.A. van Vlaardingen and W. Admiraal. 1997. Biological variation in sensitivity to N-heterocyclic PAHs; effects on seven species of micro-algae. Environ Pollut. 95 (1): 121-126.

Duxbury, C.L., D.G. Dixon and B.M. Greenberg, 1997. Effects of simulated solar radiation on the bioaccumulation of polycyclic aromatic hydrocarbons by the duckweed *Lemna Gibba*. Environ. Tox. Chem. 16 (8): 1739-1748.

Fernández, A., M. Griffol, A.M. Solanas, J.M. Bayona and J. Albalgés. 1992. Bioassay-directed chemical analysis of genotoxic components in coastal sediments. Environ. Sci. Technol. 26: 817-829.

Furlong, E.T. and R.T. Carpenter, 1982. Azaarenes in Puget Sound sediments. Geochim. Cosmochim. Acta 46: 1385-1396.

Gala, W.R. and J.P. Giesy, 1990. Flow cytometric techniques to assess toxicity to algae. In: *Aquatic Toxicology and Risk Assessment*, 13th Volume (W.G. Landis and W.H. van der Schalie, eds.). STP 1096. American Society for Testing and Materials, Philadelphia, PA, pp. 237-246.

Gala, W.R. and J.P. Giesy, 1994. Flow cytometric determination of the photo-induced toxicity of anthracene to the green alga *Selenastrum capricornutum*. Environ. Toxicol. Chem. 13 (5): 831-840.

Giddings, J.M., 1979. Acute toxicity to *Selenastrum capricornutum* of aromatic compounds from coal conversion. Bull. Environ. Contam. Toxicol. 23: 360-364.

Haanstra, L., P. Doelman and J.H. Oude Voshaar, 1985. The use of sigmoidal dose response curves in soil ecotoxicological research. Plant and Soil 84: 293-297.

Halliwell, B. and J.M.C. Cutteridge, 1985. Free radicals in biology and medicine. Clarendon, Oxford, U.K.

Haridoni, E., 1998. Degradation of acridine by marine algae and artificial light. M.Sc. Thesis. ARISE, University of Amsterdam

Huang, X.-D., D.G. Dixon and B.M. Greenberg, 1993. Impacts of ultraviolet radiation and photomodification on the toxicity of polycyclic aromatic hydrocarbons to the higher plant *Lemna gibba* L. G-3 (duckweed). Environ. Toxicol. Chem. 12: 1067-1077.

Huang, X.-D., S.N. Krylov, L. Ren, B.J. McConkey, D.G. Dixon and B.M. Greenberg, 1997. Mechanistic quantitative structure-activity relationship model for the photoinduced toxicity of polycyclic aromatic hydrocarbons: II. An empirical model for the toxicity of 16 polycyclic aromatic hydrocarbons to the duckweed *Lemna gibba*. Environ. Toxicol. Chem. 16 (11): 2296-2303.

Johansen, S.S., E. Arvin, H. Mosbæk and A.B. Hansen, 1997. Degradation pathway of quinolines in a biofilm system under denitrifying conditions. Environ. Toxicol. Chem. 16 (9): 1821-1828.

Johnson, D.W., M.V. Haley and W.G. Landis, 1990. The aquatic toxicity of the sensory irritant and riot control agent dibenz[*b,f*]-1,4-oxazepine (CR). In: *Aquatic Toxicology and Risk Assessment*, Thirteenth Volume (W.G. Landis and W.H. Van der Schalie, eds.). STP 1096, ASTM, Philadelphia, PA, pp. 199-221.

Johnson, D.W., 1992. An evaluation of a genotoxicity assay with liver S9 for activation and luminescent bacteria for detection. Environ. Toxicol. Chem. 11: 473-480.

Kaiser, J.P., Y. Feng and J.M. Bollag, 1996. Microbial metabolism of pyridine, quinoline, acridine and their derivatives under aerobic and anaerobic conditions. Microbiol. Rev. 60 (2): 483-498.

Kalf, D.F., G.H. Crommentuijn, R. Posthumus and E.J. van de Plassche, 1995. Integrated environmental quality objectives for polycyclic aromatic hydrocarbons (PAHs). RIVM report nr. 679101018, Bilthoven, The Netherlands

Klamer, H.J.C., L.A. Villerius, J. Roelsma, P.G.-J. de Maagd and A. Opperhuizen, 1997. Genotoxicity testing using the Mutatox™ assay: Evaluation of benzo[*a*]pyrene as a positive control. Environ. Toxicol. Chem. 16 (5): 857-861.

Kraak, M.H.S., C. Ainscough, A. Fernández, P. van Vlaardingen, P. de Voogt and W. Admiraal, 1997a. Short-term and chronic exposure of the zebra mussel (*Dreissena polymorpha*) to acridine: effects and metabolism. Aquat. Toxicol. 37: 9-20.

Kraak, M.H.S., P. Wijnands, H.A.J. Govers, W. Admiraal and P. de Voogt, 1997b. Structural-based differences in ecotoxicity of benzoquinoline isomers to the zebra mussel (*Dreissena polymorpha*). Environ. Toxicol. Chem. 16: 2158-2163.

Krylov, S.N., X.-D. Huang, L.F. Zeiler, D.G. Dixon and B.M. Greenberg, 1997. Mechanistic quantitative structure-activity relationship model for the photoinduced toxicity of polycyclic

aromatic hydrocarbons: I. Physical model based on chemical kinetics in a two-compartment system. Environ. Toxicol. Chem. 16 (11): 2283-2295.

Kuhn, E.P. and J.M. Suflita, 1989. Dehalogenation of pesticides by anaerobic microorganisms in soil and ground water: a review. In: *Reactions and Movements of Organic Chemicals in Soils* (B.L. Sawhnew and K. Brown, eds.). SSSA special publication no. 22. Soil Science Society of America and American Society of Agronomy, Madison, Wis.

Kochany, J. and R.J. Maquire, 1994. Photodegradation of quinoline in water. Chemosphere 28 (6): 1097-1110.

Könemann, H., 1981. Quantitative structure-activity relationships in fish toxicity studies. Part I: Relationship for 50 industrial pollutants. Toxicol. 19: 209-222.

Kozin, I.S., O.F.A. Larsen, P. de Voogt, C. Gooijer and N.H. Velthorst, 1997. Isomer-specific detection of azaarenes in environmental samples by Shpol'skii luminescence spectroscopy. Anal. Chim. Acta 354: 181-187.

Larson, R.A. and M.R. Berenbaum, 1988. Environmental phototoxicity. Environ. Sci. Technol. 22: 354-360.

Leslie, H. 1996. Azaarenes: Ecotoxicity, Biotransformation and Molecular structures. M.Sc. Thesis, University of Amsterdam.

Leifer, A., 1988. The kinetics of environmental aquatic photochemistry. ACS Professional Reference book. Salem. MA, USA.

Leppänen, M.T. and J.V.K. Kukkonen, 1998. Relative importance of ingested sediment and pore water as bioaccumulation routes for pyrene to oligochaete (*Lumbriculus variegatus*, Müller). Environ. Sci. Technol. 32: 1503-1508.

McConkey, B.J., C.L. Duxbury, D.G. Dixon and B.M. Greenberg, 1997. Toxicity of a PAH photooxidation product to the bacteria *Photobacterium phosphoreum* and the duckweed *Lemna gibba*: Effects of phenanthrene and its primary photoproduct, phenanthrenequinone. Environ. Toxicol. Chem. 16 (5): 892-899.

Mekenyan, O.G., G.T. Ankley, G.D. Veith and D.J. Call. 1994a. QSAR estimates of excited states and photoinduced acute toxicity of polycyclic aromatic hydrocarbons. SAR QSAR Environ. Res. 2: 237-247.

Mekenyan, O.G., G.T. Ankley, G.D. Veith and D.J. Call, 1994b. QSARs for photo-induced toxicity: I. Acute lethality of polycyclic aromatic hydrocarbons to *Daphnia magna*. Chemosphere 28: 567-582.

Mill, T., W.R. Mabey, B.Y. Lan and A. Baraze, 1981. Photolysis of polycyclic aromatic hydrocarbons in water. Chemosphere 10: 1281-1290.

Millemann, R.E., W.J. Birge, J.A. Black, R.M. Cushman, K.L. Daniels, P.J. Franco and J.M. Giddings, 1984. Comparative acute toxicity to aquatic organisms of components of coal-derived synthetics fuels. Trans. Am. Fish. Soc. 113 (1): 74-85.

Morgan, D.D. and D. Warshawsky, 1977. The photodynamic immobilization of *Artemia salina* nauplii by polycyclic aromatic hydrocarbons and its relationship to carcinogenic activity. Photochem. Photobiol. 25: 39-46.

Nendza, M. and J.L.M. Hermens, 1995. Properties of chemicals and estimation methodologies. In: *Risk assessment of chemicals: an introduction*, first edition (C.J. van Leeuwen and J.L.M. Hermens, eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 271-278.

Newsted, J.L. and J.P. Giesy, 1987. Predictive models for photo-induced acute toxicity of polycyclic aromatic hydrocarbons to *Daphnia magna*, Strauss (Cladocera, Crustacea). Environ. Toxicol. Chem. 6: 445-461.

NW4 (Vierde Nota waterhuishouding Regeringsvoornemen), 1997. Ministerie van Verkeer en Waterstaat, The Hague, The Netherlands.

Onodera, K., G. Furusawa, M. Kojima, M. Tsuchiya, S. Aihara, R. Akaba, H. Sakuragi and K. Tokumaru, 1985. Mechanistic considerations on photoreaction of organic compounds via excitation of contact charge transfer complexes with oxygen. Tetrahedron 41: 2215-2220.

Oudendijk, O.C., 1997. Metabolism of azaarenes. M.Sc. Thesis. ARISE, University of Amsterdam (in Dutch).

Pearlman, R.S., S.H. Yalkowsky and S. Banerjee. 1984. Water solubilities of polynuclear aromatic and heteroaromatic compounds. J. Phys. Chem. Ref. Data 13 (2): 555-562.

Pereira, W.E., C.E. Rostad, J.R. Garbarino and M.F. Hult, 1983. Groundwater contamination by organic bases derived from coal-tar wastes. Environ. Toxicol. Chem. 2: 293-294.

Pereira, W.E., C.E. Rostad, D.M. Updegraff and J.L. Bennett, 1987. Fate and movement of azaarenes and their anaerobic biotransformation products in an aquifer contaminated by wood-treatment chemicals. Environ. Toxicol. Chem. 6: 163-176.

Pereira, W.E., C.E. Rostad, I.J. Leiker, D.M. Updegraff and J.L. Bennett, 1988. Microbial hydroxylation of quinoline in contaminated groundwater: evidence for incorporation of the oxygen atom of the water. Appl. Environ. Microbiol. 54 (3): 827-829.

Polycyclic aromatic hydrocarbons (PAHs), 1996. BEON report nr. 96-13, The Hague, The Netherlands.

Schmidt, R., K. Schaffner, W. Trost, and H.-D. Brauer, 1984. Wavelength-dependent and dual photochemistry of the endoperoxides of anthracene and 9,10-dimethylanthracene. J. Phys. Chem. 88: 956-958.

Schmitter, J.M., H. Colin, J.L. Excoffier, P. Arpino and G. Guiochon, 1982. Identification of triaromatic nitrogen bases in crude oils. Anal. Chem. 54: 769-772.

Schoeny, R., T. Cody, D. Warshawsky and M. Radike, 1988. Metabolism of mutagenic polycyclic aromatic hydrocarbons by photosynthetic algal species. Mut. Res. 197: 289-302.

Sinks, G.D., T.W. Schultz and R.S. Hunter, 1997. UVB-induced toxicity of PAHs: Effects of substituents and heteroatom substitution. Bull. Environ.Toxicol. 59: 1-8.

Southworth, G.R., J.J. Beauchamp and P.K. Schmieder, 1978. Bioaccumulation potential and acute toxicity of synthetic fuel effluents in freshwater biota: Azaarenes. Environ. Sci. Technol. 12:1062-1066.

Stahl, R.G., 1991. The genetic toxicology of organic compounds in natural waters and waste waters. Ecotox. Environ. Saf. 22: 94-125.

Stronkhorst, J., P.C. Vos and R. Misdorp, 1994. Trace metals, PCBs and PAHs in benthic (epipelic) diatoms from intertidal sediments: a pilot study. Bull. Environ. Contam. Toxicol. 52 (6): 818-824.

Sutherland, J.B., J.P. Freeman, A.J. Williams and C.E. Cerniglia, 1994a. N-oxidation of quinoline and isoquinoline by *Cunninghamella elegans*. Exp. Myocol. 18: 271-274.

Sutherland, J.B., F.E. Evans, J.P. Freeman, A.J. Williams, J. Deck and C.E. Cerniglia, 1994b. Identification of metabolites produced from acridine by *Cunninghamella elegans*. Mycol. 86 (1): 117-120.

Sutton, S.D., S.L. Pfaller, J.R. Shann, D. Warshawsky, B.K. Kinkle and J.R. Vestal, 1996. Aerobic biodegradation of 4-methylquinoline by a soil bacterium. Appl. Environ. Microbiol. 62 (8): 2910-2914.

Swartz, R.C., D.W. Schults, R.J. Ozretich, J.O. Lamberson, F.A. Cole, T.H. DeWitt, M.S. Redmond and S.P. Ferraro, 1995. PAH: A model to predict the toxicity of field-collected marine sediment contaminated by polynuclear aromatic hydrocarbons. Environ. Toxicol. Chem. 14: 1977-1987.

Tomkins, B.A. and C.H. Ho, 1982. Determinations of polycyclic aromatic amines in natural and synthetic crudes. Anal. Chem. 54: 91-96.

Ulitzur, S., 1986. Bioluminescence test for genotoxic agents. Meth. Enzym. 133: 264-274.

Van den Berg, M.C. 1996. Metabolism of the NPAH acridine by the green alga *Selenastrum capricornutum* (Chlorophyta). M.Sc. Thesis. ARISE, University of Amsterdam (in Dutch).

Van Genderen, J., T.H.M. Noij and J.A. Van Leerdam, 1994. Inventory and toxicological evaluation of organic micropollutants. RIWA-report, Amsterdam, The Netherlands.

Van Vlaardingen, P.L.A., W.J. Steinhoff, P. de Voogt and W.A. Admiraal. 1996. Property-toxicity relationships of azaarenes to the green alga *Scenedesmus acuminatus*. Environ. Toxicol. Chem. 15: 2035-2042.

Van der Werff, A. and H. Huls, 1974. Diatomeeën flora van Nederland. Sprey, Abcoude, The Netherlands.

Vasseur, P., 1995. Indices for carcinogenicity in aquatic ecosystems: significance and development. In: *Evaluation and Monitoring Health of Large-Scale Ecosystems*, (D.J. Rapport, C.L. Gaudet and P. Calow, eds.). Springer-Verlag Berlin, Heidelberg. Vol. 28: 179-193.

Veith, G.D., D.J. Call and L.T. Brooke, 1983. Structure-activity relationships for fathead minnow, *Pimephales promelas*: a narcotic industrial chemicals. Can. J. Fish. Aquat. Sci. 40: 743-748.

Veith, G.D., O.G. Mekenyan, G.T. Ankley and D.J. Call, 1995. A QSAR analysis of substituent effects on the photo-induced acute toxicity of PAHs. Chemosphere 30 (11): 2129-2142.

Volz, J., 1995. Reservoirs and nature - a contradiction? Aqua 44 (1): 30-34.

Warshawsky, D., T. Cody, M. Radike, R. Reilman, B. Schumann, K. LaDow and J. Schneider, 1995. Biotransformation of benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons and heterocyclic analogs by several green algae and other algal species under gold and white light. Chemo-Biological Interactions 97: 131-148.

Wernersson, A.-S., and G. Dave, 1997. Phototoxicity identification by solid phase extraction and photo-induced toxicity to *Daphnia magna*. Arch. Environ. Contam. Toxicol. 32: 268-273.

Wakeham, S.G., 1979. Azaarenes in recent lake sediments. Environ. Sci. Technol. 13: 1118-1123.

Wild, S.R. and K.C. Jones, 1995. Polynuclear aromatic hydrocarbons in the United Kingdom environment: a preliminary source inventory and budget. Environ. Pollut. 88 (1): 91-108.

Yamaguchi, K., Y. Ikeda and T. Fueno, 1985. Charge-transfer interactions, exciplex formations and ionic dissociations in singlet oxygen reactions. Tetrahedron 41: 2099-2107.

Zeiger, E., B. Anderson, S. Haworth, T. Lawlor and K. Mortelmans, 1992. Salmonella Mutagenicity Tests: V. Results from the testing of 311 chemicals. Environ. Mol. Mutagen. 19 (21): 2-141.

Zepp, R.G. and P.F. Schlotzhauer. 1979. In: *Polynuclear Aromatic Hydrocarbons*, (P.W. Jones and P. Leber, eds.). Ann Arbor Science: Ann Arbor, MI, USA.

Reeds verschenen BEON rapporten:

BEON rapport nr.	1.	BEON Meerjarenplan 1988-1993.	1987
BEON rapport nr.	2.	BEON Jaarwerkplan 1988.	1988
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BEON rapport nr.	4.	BEON meerjaren Uitvoeringsprogramma 1988-1993.	1989
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BEON rapport nr.	6.	Findings of the BEON Workshop in preparation for the Third North Sea Conference.	1989
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BEON rapport nr.	9.	BEON Jaarwerkplan 1990.	1990
BEON rapport nr.	10.	BEON Voortgangsrapport 1988-1989.	1990
BEON rapport nr.	11.	Beleidspresentatie BEON 31 mei 1990 Den Haag.	1990
BEON rapport nr.	12.	Beleidspresentatie BEON 20 juni 1991 Den Haag.	1991
BEON rapport nr.	13.	Effects of Beamtrawl Fishery on the Bottom Fauna in the North Sea. II. The 1990 - studies.	1990
BEON rapport nr.	13 A.	BEON Jaarwerkplan 1991.	1991
BEON rapport nr.	14.	BEON Jaarwerkplan 1992.	1992
BEON rapport nr.	15.	Beleidspresentatie BEON 19 juni 1992 Den Haag.	1992
BEON rapport nr.	16.	Effect of Beamtrawl Fishery on the Bottom Fauna in the North Sea. III. The 1991 - studies.	1992
BEON rapport nr.	17.	Beleidspresentatie BEON 12 december 1991.	1992
BEON rapport nr.	18.	Trace Element Geochemistry at the Sediment Water Interface in the North Sea and the Western Wadden Sea.	1993
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BEON rapport nr.	93-3	BEON Jaarwerkplan 1993.	1993
BEON rapport nr.	93-4	BEON Beleidspresentatie "Zee en Wadvogels. "Voorkomen en invloeden daarop" d.d. 10 december 1993.	1993

1994

BEON rapport nr. 94-1 Effecten van verschuivingen van nutriëntenconcentraties op biota in de Nederlandse kustwateren. Philippart, C.J.M. & E.G. de Groodt, A.G. Brinkman, R.G. Jak, M.C.Th. Scholten (IBN 93 E 02).

BEON rapport nr. 94-2 Vervalt; zie 96-3

BEON rapport nr. 94-3 Jaarwerkplan 1994.

BEON rapport nr. 94-4 Jaarverslag 1993: Algenonderzoek in mesocosms en modelleringlering. Riegman, R. (NIOZ 93 E 01).

BEON rapport nr. 94-5 Impact of anthropogenic activities on the productivity of the western Wadden Sea ecosystem. Veer, H.W. van der. (NIOZ 93 E 02).

BEON rapport nr. 94-6.1 Bentic nutriënt generation in the ERSEM ecosystem model of the North Sea. Ruardij, P. and W. van Raaphorst. (NIOZ 93 E 03)

BEON rapport nr. 94-6.2 The EcoWasp model and it's environment. Smit, J.P.C., A.G. Brinkman, E.G.M. Embsen, P. Ruardij, and W. van Raaphorst. (NIOZ 93 E 03)

BEON rapport nr. 94-7 Risico-analyse Mariene Systemen (RAM*2 project) Eindrapport van de RAM-Auditgroep.

BEON rapport nr. 94-8 Comparison of models describing species composition of marine phytoplankton Michielsen, H & Berg, A. van den & Joordens, J., et al.(project MANS-FYFY, WL 93 E 01).

BEON rapport nr. 94-9 Verslag BEON Workshop Risico-analyse, d.d. 27 april 1994, Den Haag.

BEON rapport nr. 94-10 BEON Beleidspresentatie "Microverontreinigingen: effecten en trends", d.d. 21 juni 1994.

BEON rapport nr. 94-11 De epi- en endofauna van de Nederlandse, Duitse en Deense kustzone: een analyse van 20 jaar bijvangsgegevens. Buijs, J., J.A. Craeymeersch, P. van Leeuwen, A.D. Rijnsdorp. (BEONADD IV/V)

BEON rapport nr. 94-12 De inductie van cytochroom P450 1 A in platvis door blootstelling aan polyaromatische koolwaterstoffen in de Noordzee. INP-programma 1991- 1992. Boon, J.P., H.M. Sleiderink, M.L. Eggens, A.D. Vethaak (NIOZ 93 M 05)

BEON rapport nr. 94-13 Directe effecten van de visserij met de 12m en 4m boomkorren op het bodemleven in de Nederlandse sector van de Noordzee. Bergman, M.J.N. en J.W. van Santbrink. (NIOZ 93 V 07)

BEON rapport nr. 94-14 Scavenging seabirds at beamtrawlers in the southern North Sea, distribution, relative abundance, behaviour, prey selection, feeding efficiency, kleptoparastism and the possible effects of the establishment of protected areas'. Camphuysen, C.J. (BEONADD IV/V)

BEON rapport nr. 94-15 The relationship between food supply, reproductive parameters and population dynamics in Dutch Lesser Black-backed Gulls Larus fuscus: a pilot study. Spaans, A.L., M. BukaciÑska, D. BukaciÑska. (BEONADD IV/V)

BEON rapport nr. 94-16 Pilot study on the influence of feeding conditions at the North Sea on the breeding results of the Sandwich Tern Sterna sandvicensis. Brenninkmeijer, A. & E.W. M. Stienen. (BEONADD IV/V)

BEON rapport nr. 94-17 BEON-studie naar de effecten van de teruglopende nutriëntenbelasting van de Nederlandse kustzone. Boddeke, R. en P. Hagel. (RIVO 93 E 03)

1995

BEON rapport nr. 95-1 Effecten van de schelpdiervisserij op het bodemleven in de Voordelta. Van der Land, M.A. (RIVO 94 V 06).

BEON rapport nr. 95-2 Jaarwerkplan 1995.

BEON rapport nr. 95-3 Trends in het voorkomen van vissen en epibenthische evertebraten in de Noordzee: Een vergelijking van datasets. Van der Veer, H.W., J.A. Craeymeersch, J. Van der Meer, A.D. Rijnsdorp, J.IJ. Witte. (NIOZ 93 A 04)

BEON rapport nr. 95-4 De ontwikkeling van een in vitro assay voor de bepaling van de invloed van biotransformatie op de bioaccumulatie van lipofiele organohalogeen verbindingen in mariene toppredatoren.
I. Validatie van de assay met PCBS en de eerste resultaten met Toxafeen. Boon, J.P., van Schanke, A., Roex, E., de Boer, J., Wester, P. (NIOZ 94 M 01)

BEON rapport nr. 95-5 BEON beleidspresentatie "Ontwikkelingen in het beleid", d.d. 9 december 1994.

BEON rapport nr. 95-6 BEON beleidspresentatie "Modellering: de stand van zaken en het belang voor beleid en beheer", d.d. 31 maart 1995.

BEON rapport nr. 95-7 Wetenschappelijke discussie. De visserij-intensiviteit van de Nederlandse boomkorvisserij op de Noordzee mede in het licht van de milieu effecten en gesloten gebieden.

BEON rapport nr. 95-8 Antropogene eutrofiëring en natuurlijke variaties. Consequenties voor de produktiviteit van de Noordzee. INP-MOORING/PELAGIC FOOD WEB/STED/ STRAECOS. Van Raaphorst, W., F.C. van Duyl, H. Ridderinkhof, R. Riegman, P. Ruardy. (NIOZ 94 E 01)

BEON rapport nr. 95-9 Effecten van antropogene aktiviteiten op de produktiviteit van het ecosysteem in de Westelijke Waddenzee. Van der Veer, H.W., J.J. Beukema, G.C. Cadée, J. Hegeman, B. Mom, W. Van Raaphorst, J. IJ.. Witte (NIOZ 93 E 02)

BEON rapport nr. 95-11 Biomarkers of Toxic effects chemoreceprion: effects of contaminated dredge spoil on chemoreception acuity in whelks.Ten Hallers-Tjabbes, C. and C.V. Fisher. (NIOZ 93 M 05)

BEON rapport nr. 95-12 Habitatkarakteristieken van de Nederlandse kustzone. Wintermans, C. et al. (IBN 94 H 02)

BEON rapport nr. 95-13 BEON Tweejaarverslag 1993-1994. Onderzoek en beleid kiezen samerhet ruime sop; PB-BEON; augustus 1995.

BEON rapport nr. 95-14 Toxische algen tussen Noordwijk- en Terschelling-raai. Peperzak, L. et al. (RIKZ 94 E 05; RKZ-040).

BEON rapport nr. 95-15 Korte en lange termijn veranderingen in macrofauna veroorzaakt door verschillende vormen bodemvisserij. Bergman, M. et al. (NIOZ 94 V 01).

BEON rapport nr. 95-16 Intercallibratie en toepassing Noordzee-modellen (MANS-FYFY) fase 2. (WL 94 E 04).

1996

BEON rapport nr. 96-1 De ontwikkeling van een in-vitro assay voor de bpaling van de invloed van biotransformatie op de bioaccumulatie en de mutageniteit van lipofiele organohalogeenverbindingen in mariene toppredatoren. II. Toxafeen. Boon, J.P., H.M. Sleiderink, J. De Boer, P. Wester, H.J. Klamer, B. Govers.(NIOZ 95 M 03).

BEON rapport nr. 96-2 Spisula subtruncata als voedselbron voor Zeeëenden in Nederland. Leopold, M.F. (IBN 95 V 29).

BEON rapport nr. 96-3 BENTOX. Toxische effecten van verontreinigde sedimenten voor marien benthos. 1e fase: Verkennend onderzoek met 'natuurlijk' verontreinigde sedimenten , 2e fase: Benzo(a)pyreen en fluoranteen, 3e fase: BaP concentratiereeks. Kaag, N.H.B.M., J.P. Boon, K. Booij, C.V. Fischer, E.M. Foekema, M.T.J. Hillebrand, H. Hummel, H. Kralt, M.C. Th. Scholten, B.M.H. Timmermans, A.P.M.A. Vonck, M. de Vries, E. van Weerlee. (TNO 93 M 04,TNO 94 M 06, TNO 95 M 16).

BEON rapport nr. 96-4 Algenbegrazing: Een nadere analyse van de invloed van toxicanten op het ontstaan van eutrofiëringsproblemen. Jak, R.G., Michielsen, B.F. (TNO 95 E 07).

BEON rapport nr. 96-5 Habitatkartering en beschrijving Nederlandse kustwateren (IBN 95 H 36)

BEON rapport nr. 96-6 Onderzoek naar de invloed van fluctuaties in de lokale voedselbeschikbaarheid op de populatiedynamiek van de grote stern Sterna sandvicensis: tussentijdse resultaten. Stienen, E.W.M. & A. Brenninkmeijer. (IBN 95 H 24).

BEON rapport nr. 96-7 Resultaten BEON Workshop NW4.

BEON rapport nr. 96-8 Thema bijeenkomst Boomkorvisserij.

BEON rapport nr. 96-9 Jaarwerkplan 1996.

BEON rapport nr. 96-10 SCREMOTOX (WL 95 M 21).

BEON rapport nr. 96-11 Effecten van de schelpdiervisserij op het bodemleven in de Voordelta: De schelpdierbestanden in de Voordelta in 1995. Van der Land, M.A. (RIVO 95 V 30).

BEON rapport nr. 96-12 Verslag van de BEON workshop ter voorbereiding van de Nederlandse inbreng van de tussenconferentie van Noordzee- en Visserijministers (IMM 97).

BEON rapport nr. 96-13 BEON thema bijeenkomst polycyclische aromatische koolwaterstoffen (PAK's). 22 februari 1996 Den Haag.

BEON rapport nr. 96-14 Evaluatierapport BEON 1996. Tussentijdse evaluatie Tweede Meerjarenprogramma BEON 1993-1997. Rapport naar aanleiding van de BEON evaluatieworkshop d.d. 2 februari 1996, Den Haag.

BEON rapport nr. 96-15 Onderzoek naar mogelijkheden tot vermindering van discard produktie door technische aanpassing van boomkornetten (NIOZ 95 V 05). Fonds, M. & W. Blom

BEON rapport nr. 96-16 INP-Mooring 94-96: Antropogene eutrofiëring en natuurlijke variaties in de open Noordzee: metingen op een verankeringsstation in de Oestergronden (NIOZ 95 E 01)

1997

BEON rapport nr. 97-1 Fluctuaties in de lokale voedselbeschikbaar in relatie tot de populatiedynamiek van de Grote Stern Sterna sandvicensis: resultaten 1995-1996 (IBN 95 H 24). Stienen, E.W.M. en A. Brenninkmeijer.

BEON rapport nr. 97-2 Vervallen.

BEON rapport nr. 97-3 Jaarwerkplan 1997.

BEON rapport nr. 97-4 De betekenis van het zout- en silicaatgehalte in Nederlandse kustwateren voor het zeegrasareaal. Kamermans, P., M.A. Hemminga, D. de Jong, K.S. Dijkema. (NIOO 96 EH 07).

BEON rapport nr. 97-5 GiftigeAlgen en de Reductie van de Nutriëntenbelasting (BEON-GARdeN) Competitie tussen algen. Jaarverslag 1996. Riegman, R., K. Peeters, H. Los.(NIOZ 95 E 02).

BEON rapport nr. 97-6 In vitro biotransformatie van organohalogeenverbidingen in zeezoogdieren en vogels. Mogelijke gevolgen voor bioaccumulatie en genotoxiciteit. III: Gebromeerde vlamvertragers (Polybroom difenylethers & polybroom bifenylen). Boon, J.P., M.J. Greve, J.B. Bouma, M.K. de Boer, W.E. Lewis, H.J.C. Klamer, D. Pastor, P. Wester, J. de Boer (NIOZ 95 M 03).

BEON rapport nr. 97-7 The impact of marine eutrophication on phytoplankton, zooplankton and benthic suspension feeders. Stratification in mesocosms, a pilot experiment (Escaravage, V, Wetsteyn, L.P.M.J., T.C. Prins, A.J. Pouwer, A. de Kruyff, M. Vink-Lievaart, C.M. van der Voorn, J.C.H. Peeters &A.C. Smaal (RIKZ 96 E 01).

1998

BEON rapport nr. 98-1 In vitro biotransformatie van organohalogeenverbindingen in zeezoogdieren en vogels. Mogelijke gevolgen voor bioaccumulatie en genotoxiciteit. IV. Polychloor terfenylen (PCT's). Boon, J.P. D.E.C. Smith, W.E. Lewis, H.J.C. Klamer, D. Pastor, P. G. Wester, J. de Boer (NIOZ 95 M 03)

BEON rapport nr. 98-2 De verspreiding van (epi) benthische macrotauna op het NCP in relatie met de microverspreiding van de boomkorvisserij vanaf 1993. Bergman, M.J.N., J. Buys, J. Craeymeersch, G.J. Piet, A.D. Rijnsdorp, J.W. van Santbrink. (NIOZ 96 V 26)

BEON rapport nr. 98-3 Effects of changing food availability on population dynamics of the Sandwich Tern *Sterna sandvicensis*. Stienen, E.W.M., A. Brenninkmeijer (IBN 95 H 24)

BEON rapport nr. 98-4 BEON-GARdeN (Eindrapport). Giftige Algen en de Reductie van de Nutriëntenbelasting. Riegman, R., K. Peelers, H. Los. (NIOZ 95 E 02)

BEON rapport nr. 98-5 De betekenis van het zout- en silicaatgehalte in Nederlandse kustwateren voor het zeegrasareaal. Kamermans, Hemminga, P. M.A., D. de Jong. (NIOO 96 EH 07)

BEON rapport nr. 98-6 Spisula en zeeëenden in de strenge winter van 1995/96 in het Noordzee kustgebied. Leopold, M.F., M.A. van der Land, H. C. Welleman. (IBN 96 VH 19)

BEON rapport nr. 98-7 In vitro biotransformatie van organohalogeenverbindingen inzeezoogdieren en vogels. Mogelijke gevolgen voor bioaccumulatie en genotoxiciteit. VChloordanen. Boon, J.P., D.E.C. Smith, W.E. Lewis, H.J.C. Klamer, D.Pastor, P.G. Wester, J. de Boer (NIOZ 95 M 03)

BEON rapport nr. 98-8 Beleidspresentatie BEON. Kartering habitats/ecotopen zoute wateren en het belang voor beleid en beheer. 25 oktober 1996 Den Haag.

BEON rapport nr. 98-9 Beleidspresentatie BEON. Stand van zaken van het microverontreinigingen onderzoek en het belang voor beleid en beheer. 19 juni 1997 Den Haag.

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