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Published in:
Environmental Toxicology and Chemistry

DOI:
[10.1897/1551-5028\(1996\)015<2035:PTROAT>2.3.CO;2](https://doi.org/10.1897/1551-5028(1996)015<2035:PTROAT>2.3.CO;2)

[Link to publication](#)

Citation for published version (APA):

van Vlaardingen, P. L. A., Steinhoff, W. J., de Voogt, P., & Admiraal, W. (1996). Property-toxicity relationships of azaarenes to the green alga *Scenedesmus acuminatus*. *Environmental Toxicology and Chemistry*, 15, 2035-2042. [2.3.CO;2" class="link">https://doi.org/10.1897/1551-5028\(1996\)0152.3.CO;2](https://doi.org/10.1897/1551-5028(1996)0152.3.CO;2)

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PROPERTY–TOXICITY RELATIONSHIPS OF AZAARENES TO THE
GREEN ALGA *SCENEDESMUS ACUMINATUS*

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(Received 2 January 1996; Accepted 10 May 1996)

Abstract—The toxicity of three two-ring and five three-ring azaarenes to the green alga *Scenedesmus acuminatus* and its relationship with molecular structure was investigated. Indole, quinoline, isoquinoline, and carbazole did not inhibit the growth rate of *S. acuminatus* in 96-h batch culture assays up to 10 mg/L. Chlorophyll-*a* content appeared to be a more sensitive response parameter than growth for five of six compounds. This parameter showed a relationship with hydrophobicity-related molecular descriptors like molecular surface area. Four benzoquinoline isomers showed notably different effects on growth rate and chlorophyll-*a* content. Median effect concentration (EC50) values obtained for growth rate were acridine, 0.32 mg/L; benzo[*f*]quinoline, 1.55 mg/L; phenanthridine, 5.24 mg/L; and benzo[*h*]quinoline, 6.65 mg/L. Similar differences existed in their EC50 values for chlorophyll-*a* content. The differences in toxicity between these isomers correlates with two electronical molecular descriptors, viz. the ionization potential and the homo–lumo gap. Several modes of action are put forward and their relative importance discussed.

Keywords—*Scenedesmus acuminatus* Toxicity Azaarenes Growth rate Structure–activity relationships

INTRODUCTION

Nitrogen heterocyclic polyaromatic hydrocarbons (NPAHs) comprise a group of compounds that has a widespread occurrence. Many molecules with a biological function, like porphyrin ring systems, coenzymes, vitamins, nucleotides, alkaloids, etc., are nitrogen heterocycles. Azaarenes are polycyclic aromatic hydrocarbons (PAHs) in which one carbon atom is substituted by a nitrogen atom. This type of compound is present in natural resources like crude oils or tars as well as in synthetic oils [1]. Apart from their natural origin, NPAHs enter the environment as spills or effluents of several industrial activities like production and combustion of synthetic fuels; oil drilling, refining, and storage; coal tar distillation; and coal gasification. Nitrogen heterocycles are also associated with wood preservation and pesticide use. Nitrogen heterocyclic polyaromatic hydrocarbons have been detected in automobile exhaust [2], ambient air [3], and air particulate matter [4]. The presence of azaarenes in marine and freshwater lake sediments has also been shown [5,2], and azaarenes have been detected incidentally at low concentrations in Dutch rivers [6].

Azaarenes can be divided into two classes based on the aromatic ring containing the nitrogen atom, molecules containing either a five-membered (pyrrole) or a six-membered (pyridine) ring. The pyridine derivatives are basic compounds, whereas the pyrrole derivatives are neutral. The presence of the nitrogen atom introduces polarity in the molecule. Therefore, azaarenes have a higher water solubility compared with homocyclic analogues [7]. Consequently, the octanol/water partition coefficient, K_{ow} , values of the heterocyclic compounds are lower, and partitioning into sediment or organic matter will be less than for the corresponding homocyclic compounds [8].

Hence, dissolved aqueous concentrations remain higher, and the route of exposure for aquatic organisms to NPAHs via the aqueous phase will prevail. Organisms for which uptake of organic contaminants is determined solely by partitioning, like algae, will be exposed to lower internal NPAH than PAH concentrations in the case of equal aqueous concentrations. However, due to the presence of the heteroatom, the biological activity of the azaarenes may be different, and possibly higher, than that of parent aromatics.

Research on the aquatic toxicity of azaarenes is not being carried out extensively, and even a set of basic toxicological data is lacking. Two- and three-ring azaarenes are mostly classified as mutagenic, whereas some four- (and higher) ring compounds are probably carcinogenic. In addition, teratogenic effects of azaarenes on frog embryos have also been reported [9].

Their presence in the hydrosphere together with the expected reactivity, differing from parent PAHs, and the sparingly investigated aquatic toxicity prompted us to investigate the effects of this family of polycyclic aromatics on aquatic biota. We chose the common freshwater chlorophyte *Scenedesmus acuminatus* as the test organism because it is representative of the primary producers at the base of the food chain in aquatic ecosystems. The aim of our study was to assess and compare the degree of toxicity of two- and three-ring azaarenes, both pyrrole and pyridine derivatives, to *S. acuminatus*. In addition, the differences in isomer and congener toxicities will be explained with the aid of molecular descriptors.

MATERIALS AND METHODS

Test organism

The green alga *S. acuminatus* is a coenobium of four cells (eight cells after cell division). Hereafter, the coenobium form

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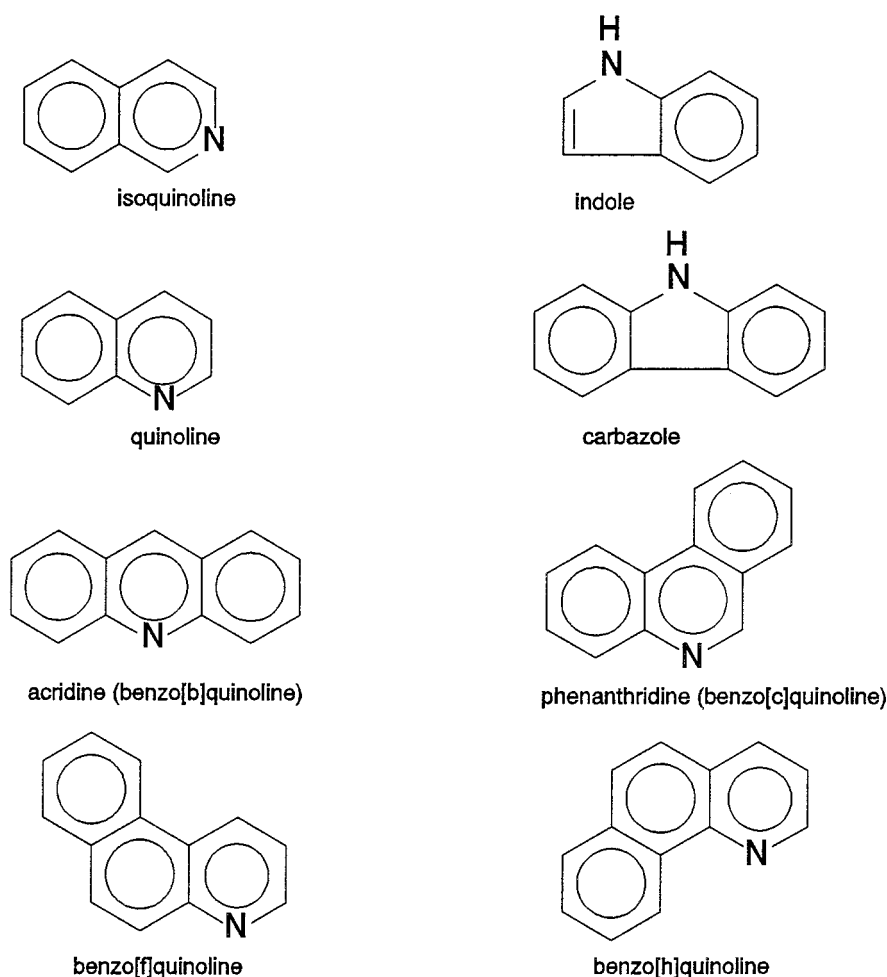


Fig. 1. Structural formulas of the azaarenes used in this study.

is referred to as "cell" unless stated otherwise. *Scenedesmus acuminatus* was kept in continuous culture under a 16:8-h light : dark regime with two cool white fluorescent tubes (Sylvania, FC40W/CW) as a light source. Woods Hole (WH) medium [10] was used with two modifications: silicium and Tris buffer were omitted. In batch cultures, HEPES-buffer, 2-[4-(2-hydroxyethyl)-1-piperazynil]-ethanesulfonic acid, was added instead of the described Tris buffer [10]. This medium was used throughout all experiments. In order to obtain algal cells in the exponential phase, a static (batch) preculture was inoculated 4 d before the start of a 96-h toxicity experiment.

Toxicants

Eight different NPAHs were tested (Fig. 1): indole (Janssen Chimica, Geel, Belgium, 99% purity), quinoline (Aldrich Chemical Co., Bornem, Belgium, 99%), isoquinoline (Aldrich, 97%), carbazole (Aldrich, 98%), acridine (benzo[b]quinoline, Aldrich, 97%), phenanthridine (benzo[c]quinoline, Aldrich, >99%), benzo[f]quinoline (Janssen Chimica, 99%), and benzo[h]quinoline (Janssen Chimica, 97%). The toxicants were applied to the test cultures as dimethylsulphoxide solutions (DMSO, Merck, Amsterdam, The Netherlands, $\geq 99.5\%$). From freshly prepared stock solutions with different toxicant concentrations, 100- μ l (0.033% v/v) portions were added to the test cultures. For all compounds except carbazole, the same concentrations were applied: 0.04, 0.16, 0.63, 2.5, and 10.0 mg/L. Carbazole has a lower water solubility (Table 1) and

was tested at 0.016, 0.06, 0.16, 0.4, and 1.0 mg/L. Two cultures with WH medium and algae but without DMSO or NPAH served as controls; two cultures with WH medium, algae, and DMSO (0.033% v/v) were incorporated to establish solvent effects. All concentrations, including the controls, were tested in duplicate. One bottle with WH medium without algae but with the highest toxicant concentration was used as a sterile control in order to determine changes in toxicant concentration due to sorption or chemical (photo-) degradation.

Toxicity experiments

Cultures were prepared in sterilized 500-ml glass bottles (borosilicate) containing 300 ml WH medium and closed with screw caps containing Teflon® inlays. An aliquot from the preculture was inoculated in each bottle, giving an initial cell density of approx. 1,000 cells/ml. After inoculation the cultures were allowed to acclimate for at least 2 h before the toxicant was added. The bottles were incubated horizontally on a rolling device at 25 rpm, partly submerged in water at 20°C. The light source consisted of three mercury lamps (Philips HPI-T, 400 W), giving a light intensity of 150 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ on the bottles. The light-regime was 16:8-h light : dark.

Each day a sample was taken aseptically from the cultures, starting directly after the addition of algae. Samples (5 ml) were counted in duplicate using a Coulter Counter (Coulter Multisizer; tube diameter, 70 μm) to determine the number and volume of algal cells. At $t = 0$ and $t = 96$ h, samples

Table 1. Physical and chemical data on azaarenes used in this study

Compound	Formula	Molecular weight	pK _a ^a	Log ^a K _{OW}	S _w ^b (mg/L)	Detector ^c	λ _{abs/exc} ^d (nm)
Indole	C ₈ H ₇ N	117.15	-2.4	2.27	1874	FLU	215
Quinoline	C ₉ H ₇ N	129.16	4.49	2.10	6329	UV	226
Isoquinoline	C ₉ H ₇ N	129.16	5.14	2.08 ^e	4521	UV	215
Carbazole	C ₁₂ H ₉ N	167.21	~-2	3.84	1.03	FLU	230
Acridine							
(benzo[<i>b</i>]quinoline)	C ₁₃ H ₉ N	179.22	5.60	3.45	46.6	UV	254
Phenanthridine							
(benzo[<i>c</i>]quinoline)	C ₁₃ H ₉ N	179.22	5.58	n.a. ^f	n.a.	FLU	254
Benzo[<i>f</i>]quinoline	C ₁₃ H ₉ N	179.22	5.15	3.40	78.7	FLU	254
Benzo[<i>h</i>]quinoline	C ₁₃ H ₉ N	179.22	4.25	3.60	n.a.	FLU	254

^a [26].^b Maximum water solubility at 20°C (unless noted otherwise), from Pearlman et al. [7].^c FLU = fluorescence detector; UV = absorbance detector.^d λ_{abs/exc} = used absorption (UV) or excitation (FLU) wavelength.^e [34].^f n.a. = not available.

were also taken for high-performance liquid chromatography (HPLC) analysis of azaarenes. At $t = 96$ h, the chlorophyll-*a* content of the cells was determined using the remaining culture. Samples from the cultures were filtered onto glass fiber filters (Whatman GF/C) in triplicate and were extracted with 80% methanol (Janssen Chimica) at 60°C for 30 min. The absorption was measured at 750 and 666 nm before and after acidification. The amount of chlorophyll-*a* was calculated using the extinction coefficient and acid ratio published by Marker et al. [11]. The extinction coefficients of chlorophyll-*a* in 80% and 100% methanol did not differ significantly on determination, giving values of (phaeophytin-corrected) absorbances of 0.206 ± 0.013 and 0.199 ± 0.022 , respectively (five replicates).

HPLC analysis of azaarenes

Concentrations of azaarenes were determined directly (i.e., no cleanup or preconcentration) in centrifuged water samples using reverse-phase liquid chromatography. A 20-μl sample was injected onto a 150- × 4.6-mm column packed with Li-chrosorb RP18 (Merck; particle diameter, 5 μm) with a 3- × 3-mm guard column containing the same packing material. Both columns were thermostated at 40°C. Either a fluorescence detector (Kratos Spectroflow 980) or an ultraviolet (UV) absorbance detector (Applied Biosystems 785) was used to detect the compounds. The detector used and excitation c.q. absorption wavelengths for each compound are given in Table 1. Emission wavelengths higher than 310 nm were detected using a cutoff filter. The mobile-phase composition was 80/20 or 85/15 methanol/water (J.T. Baker, Deventer, The Netherlands, HPLC grade). The mobile phase was vacuum-degassed directly before use. All (isocratic) elutions were performed at a flow rate of 1 ml/min. Concentrations were determined using the external standard method.

Capacity factors (k') for each benzoquinoline isomer were calculated from isocratic elutions with mobile-phase compositions of 100% methanol and 90, 85, 80, 70, and 60% methanol/water. The breakthrough of methanol was taken as the void volume. Log k_0 was calculated by linear extrapolation of the log k' values determined at the mentioned modifier concentrations to 100% water, as described by De Voogt et al. [12].

Dose-effect relationships

From the NPAH concentrations measured at $t = 0$ and $t = 96$ h, an average exposure concentration was calculated assuming exponential decrease with time. For each bottle, the relative growth rate μ (d⁻¹) of the algal population over 96 h and the amount of chlorophyll-*a* per cell (at $t = 96$ h only) were calculated. Growth rates were calculated on the basis of both cell number and cell volume. The parameters were plotted versus the ¹⁰log of the toxicant concentration and a logistic dose-response model [13] fitted through the data using a non-linear least-squares method. From this curve the toxicant concentrations giving 10% and 50% inhibition (EC10 and EC50, respectively) of either growth rate or chlorophyll-*a* content, relative to the controls, were calculated.

Structural descriptors

Correlation coefficients were calculated between the observed biological activity of the azaarenes and a selection of structural and physicochemical molecular descriptors. Based on the concept that to elicit a biological effect a compound has to pass membranes and interact with receptor(s) and that, therefore, both hydrophobic and electronic forces may play a role, hydrophobicity-related and electronic descriptors were selected. These included molecular volumes (vol) and surface areas (sa), molecular valence connectivity indices (χ^v), and, in addition, for the benzoquinoline isomers dipole moments (D) and energies of highest occupied and lowest unoccupied molecular orbitals. The latter two reflect the ionization potential (IP) and electron affinity (EA) of the molecule, respectively.

Molecular volumes and sa were calculated by the COORD/SAVOL program [14] using Cartesian coordinates and bond angles obtained from crystallographic data (assuming a planar molecule). Van der Waals radii used for hydrogen, aromatic carbon, and nitrogen were 1.01, 1.77, and 1.50 Å, respectively. Atom-atom distances used were C-H, 1.084; C-C, 1.395; and C-N, 1.338 Å. A solvent radius of zero was assumed.

Dipole moment, IP, and EA were calculated by the semiempirical quantum mechanical AM1 method after optimization of the molecular geometry by a molecular mechanics modeling method (MM1 option in MOPAC [15]). This program also generates heats of formation. Valence- χ indices for path orders 2, 3, 4, and 5 were calculated by the CFUNC program [16].

Table 2. The effect of eight azaarenes on growth rate and chlorophyll-*a* content of *S. acuminatus*^a

Compound	μ Cell numbers		μ Biomass volume		Chlorophyll- <i>a</i> /cell	
	EC10	EC50	EC10	EC50	EC10	EC50
Indole	>10		>10		2.43	9.42
Quinoline	>10		>10		>10	
Isoquinoline	>10		>10 ^b		1.25	8.77
Carbazole	>0.4		>0.4		>0.4	
Acridine	0.11	0.32	0.25	0.41	0.15	0.26
Phenanthridine	3.15	5.24 ^b	2.56	5.59 ^b	1.08	3.05
Benzo[<i>f</i>]quinoline	0.98	1.55	1.10	1.62	>0.6	>0.6
Benzo[<i>h</i>]quinoline	4.47	6.65	2.45	5.24	0.21	1.43

^a All concentrations are given in milligrams per liter. > means that no effect concentration could be determined.

^b μ determined over growth of first 72 h.

Correlation coefficients and related statistical parameters were calculated with the Statgraph Programme (Statistical Graphics Corp., Rockville, MD, USA).

RESULTS

Dose-effect relationships

In most cases, the algae showed exponential growth over the complete 96-h period, in control bottles as well as in bottles with toxicant; exceptions (μ determined over 72-h period) are mentioned in Table 2. The growth of algae in the solvent controls was similar to that in the control bottles.

Not all the tested azaarenes showed inhibitory effects within the tested range of concentrations (Table 2). Quinoline did not have any effect on the growth or chlorophyll-*a* content of the algae. Indole (Fig. 2), isoquinoline, and carbazole affected the chlorophyll-*a* content of the cells only at the highest concentration tested (10 mg/L and 1 mg/L, respectively). Figure 2 shows that the growth rate (based on cell number) of *S. acuminatus* under increasing indole concentrations is not negatively affected, and even a slight stimulation of growth at the highest concentration is noted. The growth rate of these cultures based on cell volume, however, is not affected (not shown). Thus, at 10 mg/L indole individual cell size was lower, partly explaining the observed decrease in chlorophyll-*a* concentration (Fig. 2).

The four benzoquinoline isomers had marked, but different, effects on the growth and chlorophyll-*a* content of the algae

(Table 2). Acridine (benzo[*b*]quinoline) is most toxic (Fig. 3), followed by benzo[*f*]quinoline. Both benzo[*h*]quinoline (Fig. 4) and phenanthridine (benzo[*c*]quinoline) are more than one order of magnitude less toxic to growth, although reduction of the chlorophyll-*a* content by benzo[*h*]quinoline occurred at lower concentrations. Figure 3 shows the effect of acridine on growth (based on cell number) and on chlorophyll-*a* content. Figure 4 demonstrates the effect of benzo[*h*]quinoline on the growth rate (cell volumes) and on chlorophyll-*a* content. In Figure 3 the growth rate seems to increase at the highest acridine concentration. This is caused by disintegration of the *Scenedesmus coenobia* into separate cells, thus yielding higher cell number counts. This disintegration was confirmed by microscopic observation. The dose-effect relationship expressing the effect on the chlorophyll-*a* content shows a slight stimulation which was not observed in any of the other experiments. Data points at higher concentrations are absent because the amount of biomass was too low to give absorbance measurements exceeding the detection limit.

The effect of acridine on the growth rate of *Scenedesmus* was assessed in another series of experiments (N.D. Dijkman, submitted manuscript), giving EC50 values of 0.32 and 0.44 mg/L based on cell number and biomass volume, respectively. These results are in agreement with the effect concentrations obtained in this study (0.32 and 0.41 mg/L, Table 2). The reproducibility of these results shows that the reliability of the test system in completely independent experiments is satisfactory.

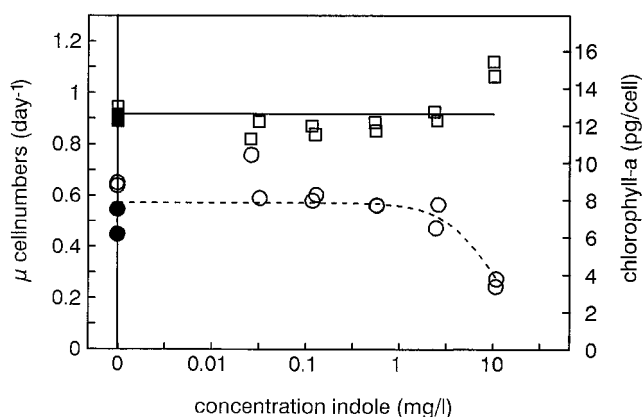


Fig. 2. Effect of indole on growth rate and chlorophyll-*a* content of *S. acuminatus*. Growth rates are based on measurement of cell number. \square = μ (96 h); \circ = chlorophyll-*a*. The DMSO controls are the black symbols.

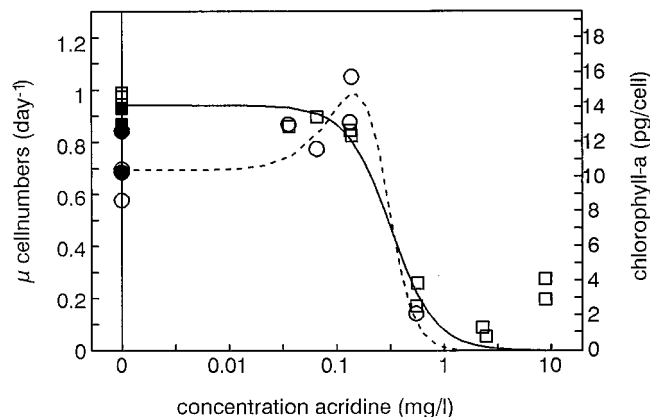


Fig. 3. Effect of acridine on growth rate and chlorophyll-*a* content of *S. acuminatus*. Growth rates are based on measurement of cell number. \square = μ (96 h); \circ = chlorophyll-*a*. The DMSO controls are the black symbols.

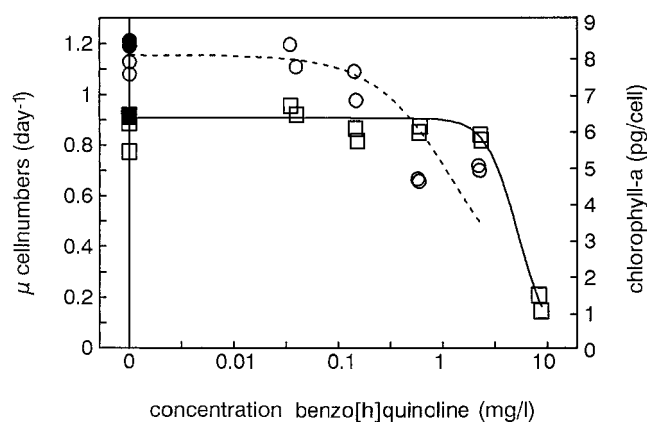


Fig. 4. Effect of benzo[h]quinoline on growth rate and chlorophyll-*a* content of *S. acuminatus*. Growth rates are based on measurement of cell volume. □ = μ (96 h); ○ = chlorophyll-*a*. The DMSO controls are the black symbols.

Toxicant concentrations

In general, toxicant concentrations decreased only slightly over the exposure period, leaving 85 to 100% of the initial concentration after 4 d. This decrease can be explained by sorption of the NPAHs to the glass surface of the bottle, stopper inlay, or the algal biomass and evaporation. The decrease in concentration in the sterile control bottles containing 10 mg/L of the tested azaarene in each experiment (1.0 mg/L for carbazole) was in the same range as the decrease observed in the bottles containing 10 mg/L with algae. This indicates that the major part of the decrease in concentration in the algal tests can be explained by evaporation or sorption to the glass and stopper of the bottle. In the indole experiment the decrease in concentration was much higher, being 100% at both 0.16-mg/L treatments and declining to 7% (average) at the 10 mg/L treatments. This was not observed with any of the other compounds. The strong decrease of the indole concentrations (notably at lower concentrations) might be attributed to transformation by the algal biomass.

Carbazole exhibited an effect on growth rates at the 1-mg/L treatment, but this effect was not reproduced on replication of the experiment. The maximum aqueous solubility of carbazole of 1.2 mg/L [7] was not reached in our experimental setup. High-performance liquid chromatography measurements showed that, on average, 0.48 ± 0.08 mg/L ($n = 5$) of the nominal concentration of 1.0 mg/L dissolved. The concentration in the 0.4-mg/L treatments could be accurately determined,

reached its nominal value, and showed no effects on the growth rate and chlorophyll-*a* content. We chose to present the effect concentrations of carbazole as being higher than 0.4 mg/L.

Structure-activity relationships

Table 3 presents the calculated molecular descriptors for the azaarenes. To investigate the benzoquinoline isomers more explicitly, quantum mechanical descriptors were included for these compounds. As can be expected for isomeric PAH compounds, differences in molecular size and hydrophobicity-related descriptors are generally small. Electronic descriptors derived from quantum mechanical calculations may show larger differences, as shown by D, EA, and IP-EA.

Correlation coefficients between the logarithm of the observed EC50 and several molecular descriptors are presented in Table 4. A distinction was made in growth rate EC50 (four values for benzoquinoline isomers available) and chlorophyll-*a* content EC50 (five values available: indole, isoquinoline, acridine, phenanthridine, and benzo[h]quinoline). A weakly significant correlation is observed for chlorophyll-*a* and *sa* values when logarithm-based EC50s are used. When EC50 values are used, *vol*, *sa*, and several χ^p values show high correlation coefficients ($r > 0.96$, not shown in Table 4) at a high level of significance ($p < 0.01$). Growth rate log EC50s for the benzoquinoline isomers correlate significantly with the electronic descriptors IP and IP-EA.

DISCUSSION

Toxicity

Azaarenes are susceptible to biodegradation in aqueous systems [17]. Also, bacterial photometabolism of (one-ring) heterocyclic aromatic compounds [18] and degradation of quinoline in particular [19] are well established. However, degradation by algae is hardly documented. The decrease of toxicant concentrations observed in the present experiment on indole strongly suggests transformation by algae. Similar observations were reported for the green alga *Selenastrum capricornutum* and the NPAH acridine (N.D. Dijkman, submitted manuscript). In the latter study, HPLC analyses showed the appearance of previously undetected peaks upon disappearance of acridine from the medium. No such metabolite peaks were detected in HPLC analyses in the present experiment on indole. Either complete mineralization occurred or the degradation products of indole could not be measured under the HPLC conditions used.

Indole, quinoline, isoquinoline, and carbazole appeared not

Table 3. Calculated molecular descriptors of benzoquinoline isomers and other azaarenes^a

Compound	Log K_0^b	Molecular <i>sa</i> \AA^2	Molecular <i>vol</i> \AA^3	Molecular connectivity indices				Quantum mechanical descriptors				Heat of formation
				$^2\chi^p$	$^3\chi^p$	$^4\chi^p$	$^5\chi^p$	IP (homo)	EA (lumo)	IP-EA	D	
Acridine	2.79	184.66	163.97	3.374	2.425	1.720	1.244	-8.299	-1.300	6.999	1.861	88.71
Phenanthridine	2.94	183.20	163.76	3.363	2.498	1.808	1.279	-8.804	-0.811	7.993	2.077	79.91
Benzo[f]quinoline	2.71	182.41	163.60	3.357	2.510	1.834	1.302	-8.588	-0.726	7.862	1.434	77.99
Benzo[h]quinoline	2.80	183.42	163.78	3.362	2.497	1.815	1.294	-8.720	-0.767	7.953	1.964	80.34
Indole	nc	132.32	106.71	2.065	1.462	0.995	0.542	nc	nc	nc	nc	nc
Carbazole	nc	179.32	157.58	3.216	2.414	1.799	1.238	nc	nc	nc	nc	nc
Quinoline	nc	141.23	120.56	2.196	1.512	1.016	0.664	nc	nc	nc	nc	nc
Isoquinoline	nc	141.64	121.13	2.223	1.550	1.027	0.664	nc	nc	nc	nc	nc

^a *sa* = surface area; *vol* = volume; IP = ionization potential; EA = electron affinity; D = dipole moment; nc = not calculated.

^b Extrapolated from HPLC log *k'* values determined in this study.

Table 4. Correlation coefficients between growth rate and chlorophyll-*a* content EC50 values for *S. acuminatus* and molecular descriptors of azaarenes^a

Toxicity parameter	Log k_0	vol	sa	D	$^2\chi^v$	$^3\chi^v$	$^4\chi^v$	$^5\chi^v$	Heat of formation	IP	EA	IP-EA
Log EC50 growth rate (four benzoquinolines)	0.440	-0.181	-0.571	0.378	-0.672	0.808	0.773	0.708	-0.769	-0.971**	0.835	0.930*
Log EC50 chlorophyll- <i>a</i> content (five azaarenes)	nc	-0.789	-0.807*	nc	-0.802	-0.766	-0.747	-0.775	nc	nc	nc	nc

^a See Table 3 for definitions of abbreviations.

* Significant at the 0.10 level.

** Significant at the 0.05 level.

to affect both growth rate parameters up to the highest concentrations tested (10 mg/L for indole, quinoline, and isoquinoline; 0.4 mg/L for carbazole). This is in agreement with effect concentrations of these compounds reported for other aquatic organisms (Table 5). Table 5 shows effect concentrations for isoquinoline in some test systems (*Photobacterium phosphoreum*, *Daphnia magna*, and chlorophyll-*a* content of *Scenedesmus*), while the isomer quinoline is moderately toxic to all five organisms mentioned. Further comparisons, however, are complicated due to absence of data or differences in test systems and end points.

The median lethal concentration (LC50) of 20.6 mg/L reported by Schultz et al. [20] for carbazole is questionable since this exceeds the maximum water solubility (1 mg/L, Table 1) by a factor of 20. In our study, concentrations equal to the maximum water solubility could not be reached, as HPLC measurements revealed. The presence of an undissolved portion, probably leading to monolayer/micel formation, makes exposure concentrations uncertain. However, since one of our experiments indicated effect at this "1-mg/L" treatment, car-

bazole toxicity to *Scenedesmus* seems to occur in the same range as found for *P. phosphoreum* (Table 5).

It was noted that the EC50 for chlorophyll-*a* content is lower than the EC50 for growth rate for five of six azaarenes (Table 2). Using the population-based parameter growth rate as an end point is nonspecific, i.e., it can indicate a toxic effect that might be caused by more than one mechanism at more than one site. It is likely that chlorophyll is one of the targets that reveals toxicity at lower levels than revealed by an inhibited population growth. Such an effect may also be found at a shorter timescale, e.g., within one generation. Clearly, it is important to study parameters other than growth rate in order to determine at what concentration the toxicity of nitrogen heterocyclic polyaromatic compounds is affecting cellular mechanisms.

Acridine was the most toxic of the azaarenes tested in the present study. Effects on both growth rate and chlorophyll-*a* content of *Scenedesmus* occur at lower concentrations than is found for other aquatic organisms (Table 5). This difference may be partly due to the light source used in the experiments.

Table 5. Effect concentrations of azaarenes on various aquatic organisms

Species	<i>Tetrahymena pyriformis</i>		<i>Daphnia magna</i>		<i>Daphnia pulex</i>	<i>Photobacterium phosphoreum</i>	<i>Rana pipiens</i>	<i>Scenedesmus acuminatus</i>		<i>Scenedesmus subspicatus</i>	<i>Selenastrum capricornutum</i>
	IGC50 60 h ^a	LC50 72 h ^b	LC50 48 h ^c	LC50 48 h ^d	LC50 24 h ^e	EC50 15 min	LC50 96 h ^b	EC50 96 h ⁱ	EC50 Chloro- phyll- <i>a</i> ⁱ	EC50 96 h ^j	EC25 ¹⁴ C fixation ^k
Indole	70.3	72.6						>10	9.4		
Quinoline	95.4	125.7	28.5	28.5			24.5	>10	>10	90	
Isoquinoline				4.1	39.9	2.8 ^f		>10	8.8		
Carbazole		20.6				0.9 ^g		>0.4	>0.4		
Acridine		7.3	2.3	2.3	2.9		1.1	0.3	0.3		~4.7
Phenanthridine								0.3 ^l			
Benzo[<i>f</i>]quinoline								5.2	3.1		
Benzo[<i>h</i>]quinoline								1.6	0.9		
								6.6	1.4		

^a IGC50 = 50% inhibition of growth rate, from Schultz and Moulton [35].

^b [20].

^c [36].

^d [37].

^e [25].

^f [38].

^g [39].

^h [24].

ⁱ This study.

^j 50% inhibition of biomass growth, from Kühn and Pattard [40].

^k [41].

^l N.D. Dijkman et al., submitted manuscript.

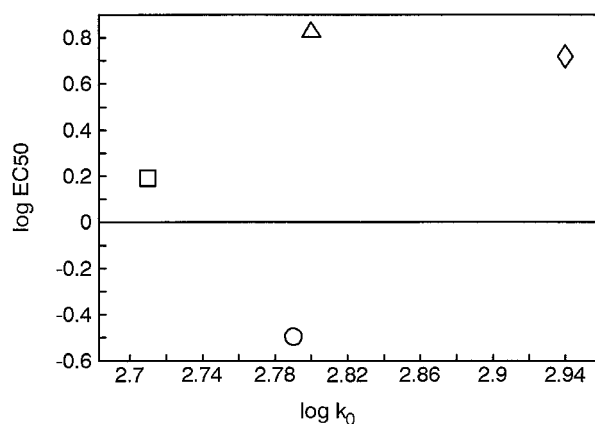


Fig. 5. Relationship between $\log k_0$ (hydrophobicity) and $\log EC_{50}$ for the four benzoquinoline isomers. The symbols represent EC_{50} values based on growth rates (cell number). ○ = acridine; □ = benzo[f]quinoline; △ = benzo[h]quinoline; ◇ = phenanthridine.

Light plays a key role in the extent to which PAHs exert their toxic effect, since many PAHs (e.g., acridine) show photoinduced toxicity [21]. Schoeny et al. [22] showed that the biological activity of benzo[a]pyrene to *Selenastrum capricornutum* depends on both its concentration and the light source used. Inhibition of growth was a function of the intensity of radiant energy below 410 nm. Morgan and Warshawsky [23] reported a photoinduced toxicity of acridine in mercury light of 366 nm. The mercury lamps used in the current study emit a relatively large percentage (11.4%) of their radiation in wavelengths below 400 nm, which is 10 times the proportion emitted by a fluorescent tube (Philips, No. 33) in this region but closer to skylight.

Structure–activity relationships

Several studies have established that toxicity increases with the number of aromatic rings in series of nitrogen heterocyclic compounds [20,24,25]. Linear relationships were observed between (log) toxicity and (log) K_{ow} [9]. Our results qualitatively support these findings for the pyridine derivatives, the benzoquinolines being more toxic than the two quinolines (Table 2). A higher toxicity of carbazole (three-ring, pyrrole derivative) compared to indole (two-ring, pyrrole derivative) is likely but not conclusive since we did not detect an EC_{50} for carbazole.

The isomers phenanthridine and benzo[h]quinoline are over one order of magnitude less toxic than acridine, which cannot be explained by their differences in hydrophobicity (note that acridine is most toxic and has the lowest K_{ow} , Table 1). Since a K_{ow} value for phenanthridine was lacking, we determined the relative hydrophobicity of the benzoquinoline isomers on our reverse-phase liquid chromatography system as described by De Voogt et al. [26]. No correlation between extrapolated $\log k_0$ values and toxicity is found, which is illustrated in Figure 5.

Subsistent in the application of most descriptors to isomers is that differences between values are small, causing increased uncertainty of quantitative predictions. Yet, if any predictive value can be assigned to a molecular descriptor, the order of these should correlate with the order of toxicity. Of the descriptors given in Table 3, IP and IP–EA were found to correlate significantly with the toxicity of the four benzoquinoline isomers. Although far from conclusive because of the relatively low number of compounds investigated, this may be

taken as a tentative indication that electronic interactions play a role in the (differences in) toxic action of these isomers. It is perhaps important to note here that IP–EA, referred to as the homo–lumo gap, has been shown to correlate with the phototoxicity of PAHs [27]. We are currently investigating the phototoxicity of the NPAHs from the present study.

Isomeric differences in molecular structure influence the strength of the toxic interaction, which may be the sum of effects caused by more than one specific mode of action. A better understanding of the toxicity of nitrogen heterocyclic polyaromatics to aquatic organisms is also needed for the application of quantitative structure–activity relationships in predicting their toxicity. Some of the mechanisms of toxic action are discussed briefly here.

As a consequence of their hydrophobic nature, azaarenes exhibit narcotic toxicity due to association with membranes [28]. This is reflected in the observed correlation between, e.g., *sa* and chlorophyll-*a* content EC_{50} s for two- and three-ring azaarenes (Table 4). Action on more specific sites, like binding to proteins [29], uncoupling of membrane-bound electron transport by proton scavenging (pyridinil–nitrogen), metabolic activation of azaarenes by monooxygenases known for parent PAHs [30] or dioxygenases (suggested for an *Oscillatoria* species [31]), intercalation between base pairs of nucleic acids as Lehninger [32] mentions for acridine, and phototoxicity [33], are other possibilities, several of which are obviously related with electronic interactions. Thus, the explanation for the differences in toxicity of the four isomers to *Scenedesmus* must be sought in a combination of physicochemical, photochemical, and/or structural properties that cause the strength of (several) modes of action to differ. The present limited data set does not allow for multiple-regression analysis, however. Moreover, the order and difference in toxicity of the isomers found for *Scenedesmus* might be species specific. The physiology may vary considerably between taxonomically closely related species of algae, thereby causing the importance of different metabolic targets and detoxification mechanisms to differ. We will therefore extend our studies to obtain a larger data set of related compounds.

Acknowledgement—We are grateful to Marion Buckert-de Jong for determination of the $\log k_0$ values and to Koen Roijackers for maintaining the continuous culture. We would like to thank Michiel Kraak and Diny Tubbing for their comments, which helped to improve the manuscript.

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