Chapter 5

Toxic and Genotoxic Effects of Azaarenes: Isomers and Metabolites

Abstract
Heterocyclic PAHs and their metabolites can show toxic, mutagenic, carcinogenic and teratogenic effects. In this study, a relation between toxic and mutagenic effects, and chemical structures of nitrogen substituted polycyclic hetero-aromatic hydrocarbons was sought using seven azaarenes and two azaarene metabolites. Survival of first instar larvae of the midge *Chironomus riparius* was determined to measure toxicity. Mortality increased with increasing number of aromatic rings of the compound, but metabolism of acridine and phenanthridine to 9(10H)-acridone and 6(5H)-phenanthridinone, respectively, strongly decreased toxicity. However, acridone was the most genotoxic of the azaarenes tested in the Mutatox™ test. Although mortality and genotoxic activity are well described by molecular descriptors relevant to bioaccumulation, in both cases exceptions are found on this general rule, even between isomers. Furthermore, it is shown that metabolism of azaarenes changed the impact on different biological endpoints, clearly putting severe limitations on environmental standards solely based on the narcotic effects of parent PAHs.
Chapter 5

Introduction

Hazard assessment for polycyclic aromatic hydrocarbons (PAHs) is mostly based on the narcotic effects of homocyclic compounds (e.g. Ministry of Transport, Public Works and Water Management, 1997). Substituted compounds and degradation products receive little attention. Furthermore, studies on other biological endpoints are scarce, although PAHs are known to induce mutagenic, teratogenic, and carcinogenic effects and direct toxicity (e.g. Davis et al., 1981; Santodonato and Howard, 1981; Walton et al., 1983).

The relatively low numbers of studies on heterocyclic substances are in contrast with the number of heteroaromatics: two-thirds of the 4 million known organic compounds are heterocyclic (Adrian and Suflita, 1994). Their availability to organisms may be higher than that of their homocyclic analogues, because their higher water solubility can imply higher water concentrations. Azaarenes are such a group of heterocycles, containing one nitrogen atom in place of a carbon atom. Apart from their natural origin (e.g. as alkaloids), azaarenes are formed and released into the environment by incomplete combustion of fossil fuels, in spills or effluents of several industrial activities, oil drilling, refining and storage (Kochany and Maguire, 1994) and coal tar distillation (Pereira et al., 1983). N-heterocycles are also associated with wood preservation (Pereira et al., 1983) and pesticide use (Kuhn and Suflita, 1989). They have been identified in freshwaters (Van Genderen et al., 1994) and in both freshwater and marine sediments (Blumer et al., 1977; Wakeham, 1979; Furlong and Carpenter, 1982; Bleeker et al., 1996).

Acute toxic effects of azaarenes have been studied in only a few aquatic macroinvertebrate species (Bleeker et al., 1998; Cooney and Gehrs, 1984; Johnson et al., 1990; Kraak et al., 1997b). For several species, acridine and quinoline are often the only compounds tested. Therefore, in previous experiments, the acute toxicity of a group of azaarenes was tested, which ranges from the two-ringed quinoline to two four-ringed benzacridines (reported in Bleeker et al., 1998) (Fig. 5.1).

Azaarenes have been shown to be degraded or metabolised by both microorganisms and higher organisms (e.g. Bean et al., 1985; Berry et al., 1987; Bleeker et al., 1996; Dijkman et al., 1997; Kraak et al. 1997a; Lemke and Kennedy, 1997), often leading to detoxification (e.g. Bleeker et al., 1996), but
metabolism can also increase (geno)toxicity (e.g. Möller, 1994). The present study aims at gaining more insight in the differences in the type and level of adverse biological effects of azaarenes and their degradation products, and relating molecular properties of the compounds with their effects. Therefore, two metabolites of the benzoquinolines acridine and phenanthridine, 9(10H)-acridone and 6(5H)-phenanthridinone, respectively, were tested (Fig. 5.1). These metabolites were chosen, because they were formed in laboratory experiments (Kraak et al., 1997a; Bleeker et al., 1996) and have also been found in the field (Pereira et al., 1987). Toxicity of azaarenes and their corresponding metabolites is compared with their genotoxicity.

![Structures of the compounds used.](image)

Figure 5.1. Structures of the compounds used.

Genotoxicity of various azaarenes has been determined using the Ames test (reviewed in Santodonato & Howard, 1981). Since then, however, several new and more sensitive genotoxicity tests have been developed, like the Mutatox™ test (Ulitzur, 1982). Therefore, genotoxicity was determined of the compounds previously (Bleeker et al., 1998) and presently tested for toxicity, using the Mutatox™ test.

**Materials and Methods**

**Acute toxicity**

The acute toxicity of quinoline, acridine, phenanthridine, benzo[f]quinoline, benzo[k]quinoline, benz[a]acridine and benz[c]acridine was reported previous-
In addition, the present study involves the metabolites 9(10H)-acridone and 6(5H)-phenanthridinone, which were tested in acute toxicity tests with chironomid larvae.

These experiments lasted for 96 h and were carried out according to Bleeker et al. (1998).

The compounds were added to the water once, at the start of the experiment, using stock solutions. Stock solutions were made using a generator column technique (Bleeker et al., 1998). This method resulted in a stock solution concentration of 0.56 μM for 9(10H)-acridone and of 0.41 μM for 6(5H)-phenanthridinone. The purity of the compounds was 99 % for 9(10H)-acridone (Aldrich) and 97 % for 6(5H)-phenanthridinone (Aldrich). 9(10H)-acridone and 6(5H)-phenanthridinone were tested at maximum water solubility only (0.56 and 0.41 μM respectively). Each compound (including controls) was tested in triplicate. Survival in experimental treatments was expressed as percentage of the mean of the two corresponding controls.

Water samples to determine actual azaarene concentrations in the water were taken and measured by HPLC according to Bleeker et al. (1998). Fluorescence detection (Kratos Spectroflow 980) was used at an excitation wavelength of 254 nm and emission wavelengths of > 354 nm. The flow of the mobile phase was 1 ml/min and the compounds were eluted with a mixture of 80 % acetonitrile and 20 % water. Average actual concentrations, start concentrations (at t = 0 h), and recoveries were calculated according to Bleeker et al. (1998).

**Genotoxicity**

The Mutatox™ test was used for testing genotoxicity. Azaarene stock solutions were made in acetone (Mallinckrodt, nanograde quality). Concentrations of these solutions differed for each compound: for quinoline it was 276 μM, for acridine 56.8 μM, for phenanthridine 19.4 μM, for 6(5H)-phenanthridinone 23.3 μM, for benz[f]quinoline 0.12 μM, for benz[h]quinoline 116 μM, for 9(10H)-acridone 0.59 μM, for benz[a]acridine 0.0855 μM and for benz[c]acridine 0.1868 μM. 10 μl of these stocks were added to 500 μl of the bacteria media used. The Mutatox™ tests were run in dilution series according to the supplier's protocol (Microbics Corp.). A nine-fold 1:1 (v:v) dilution series was made in Mutatox™ direct media. This series was incubated for 24 h at
27 ± 0.1 °C. Light levels were recorded at intervals of 1 h between 10 and 24 h. Lowest observed effect concentrations (LOEC) were defined as the lowest concentration at which the light levels are at least 4 times higher than those in the controls (Klamer et al., 1997).

**Molecular descriptors**

To elicit a biological effect, a chemical has to cross membranes and the cytosol, and interact with one or more receptors, and consequently both hydrophobic and electronic forces may play a role. Therefore, hydrophobicity-related and electronic molecular parameters were selected, which may describe the transport and partition process(es). Since photo-induced toxicity and/or transformation may also be involved when considering azaarenes (Bleeker et al., 1998), parameters related to the reactivity of the compound were also included.

Correlation coefficients were calculated between molecular descriptors and both the estimated log based LC$_{50}$ values for midges and LOEC values in the Mutatox™ test. The following molecular descriptors were used: molecular volumes (Vol) and surface areas (SA), dipole moments (D) and energies of the lowest unoccupied and highest occupied molecular orbits (LUMO and HOMO), which reflect the electron affinity (EA) and ionisation potential (IP), respectively. Log K$_{ow}$ was also incorporated, because in several studies, good correlations were found between acute toxicity of organic pollutants and octanol-water partition coefficients, K$_{ow}$ (Könemann, 1981; Veith et al., 1983; De Voogt et al., 1988).

Log K$_{ow}$ values are taken from the ClogP model provided by the Environmental Science Center of Syracuse Research Corporation (internet: http://esc.syrres.com/~ESC/kowexpdb.htm). Vol and SA were calculated by the SAVOL program using Cartesian co-ordinates obtained after optimisation of the molecular geometry by a molecular mechanics modelling method (MM1 option in MOPAC). Van der Waals radii used for hydrogen, aromatic carbon and nitrogen were 1.01, 1.77 and 1.50 (10$^{-10}$ m), respectively. Atom-atom distances used were: C-H, 1.084; C-C, 1.395; C-N, 1.338 (10$^{-10}$ m). A solvent radius of zero was assumed. D, IP and EA were calculated by the semi-empirical quantum mechanical AM1 method using the same optimised molecular conformations (MM1 option in MOPAC). This program also
generates heats of formation. Regression was calculated with the computer program KaleidaGraph® (Synergy Software, 1997). Because acridone and phenanthridinone can be present both in keto- and in enol-form, for both forms molecular descriptors were calculated.

Results and Discussion

Acute toxicity

The two metabolites, 9(10H)-acridone and 6(5H)-phenanthridinone, were not toxic to Chironomus riparius at maximum water solubility (4.8 and 3.5 μM, respectively) (Table 5.1), while for the parent compounds (acridine and phenanthridine, respectively) clear dose response curves were observed (Table 5.1; Bleeker et al., 1998). These resulted in LC50 values of 3.42 μM for phenanthridine and 0.40 μM for acridine. Clearly, metabolites of the two parent compounds show a strongly decreased toxicity (Table 5.1). To understand why oxidation of the parent compound decreases toxicity so much, more insight in the mechanism of toxicity is necessary.

Table 5.1. Toxicity and genotoxicity of azaarenes (effect concentrations in μM).

<table>
<thead>
<tr>
<th>Azaarene</th>
<th>Toxicity LC50 (95 % confidence intervals)</th>
<th>Genotoxicity LOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinoline</td>
<td>37.9119 (23.8464 - 60.2733)</td>
<td>88.0250</td>
</tr>
<tr>
<td>Benzo[f]quinoline</td>
<td>4.6379 (2.1287 - 10.1038)</td>
<td>&gt; 0.12</td>
</tr>
<tr>
<td>Phenanthridine</td>
<td>3.4209 (2.8858 - 4.0548)</td>
<td>1.2340</td>
</tr>
<tr>
<td>Benzo[h]quinoline</td>
<td>3.3808 (2.7832 - 4.1067)</td>
<td>2.5440</td>
</tr>
<tr>
<td>Acridine</td>
<td>0.3984 (0.3415 - 0.4646)</td>
<td>1.8930</td>
</tr>
<tr>
<td>Benz [a]acridine</td>
<td>0.0667 (0.0602 - 0.0737)</td>
<td>0.0101</td>
</tr>
<tr>
<td>Benz [c]acridine</td>
<td>0.0301 (0.0292 - 0.0314)</td>
<td>&gt; 0.19</td>
</tr>
<tr>
<td>9(10H)-acridone</td>
<td>&gt; 4.8</td>
<td>0.0050</td>
</tr>
<tr>
<td>6(5H)-phenanthridinone</td>
<td>&gt; 3.5</td>
<td>4.4080</td>
</tr>
</tbody>
</table>

Molecular descriptors could be a promising tool to gain this insight, since the narcotic effect of azaarenes was found to correlate well (r > 0.9) with their chemical structure (Bleeker et al., 1998). For the metabolites, however, no LC50 could be calculated, so correlations with acute toxicity could not be examined. Alternatively, theoretical LC50 values, above maximum water solubility, have
been calculated for the metabolites, based on the previously determined relationship between molecular descriptors and LC$_{50}$ values (Bleeker et al., 1998).

![Figure 5.2. Estimated LC$_{50}$ values for the metabolites, based on the relationship between log $K_{ow}$ and LC$_{50}$, without taking acridine into account, considering this to be the exception (as argued by Bleeker et al., 1998).](image)

Size parameters, such as molecular volume and surface area, showed high correlations with LC$_{50}$ values of parent azaarenes (Bleeker et al., 1998), but when such parameters are used to predict toxicity of the metabolites they appeared to be insufficient: LC$_{50}$ values calculated for the metabolites were in the range of concentrations tested, while the highest concentrations tested showed no effect at all. However, when log $K_{ow}$ is used in the calculations, the theoretical LC$_{50}$ values are in agreement with the experimental data (Fig. 5.2). Some care, however, should be taken in this respect. Both metabolites show tautomerism and because large differences can occur in molecular properties between the keto- and the enol-form, also their toxicity can differ strongly. It can be argued that in aquatic systems the best soluble tautomer-form will be the dominant one. Based on log $K_{ow}$ values, this would be the enol-form for acridone, and the keto-form for phenanthridinone. Further evidence for the dominance of these
tautomer forms may be found in the theoretical LC₅₀ values of the metabolites. These are only in agreement with experimental data when the enol-form of acridone and the keto-form of phenanthridinone are considered. In contrast, LC₅₀ calculations based on log K_{ow} values of the other tautomers, result in LC₅₀ values far below water solubility (Fig. 5.2), which have experimentally been proven to be wrong. These results and calculations indicate that, in agreement with previous studies (Könemann, 1981; Veith et al., 1983), octanol-water partition coefficients are quite reliable predictors for toxicity of organic pollutants, in this case suggesting that the low toxicity of the metabolites is caused by a decrease in bioconcentration factor.

Genotoxicity
While toxicity was tested on midges, the Mutatox™ test uses bacteria to assess genotoxicity. Since also the mode of action differs for mortality and genotoxicity, differences may be expected between the sensitivity of these different biological endpoints (shown in Table 5.1). Like for mortality, genotoxicity increases with the number of rings of the compound, but exceptions to this general rule occur more often. From the parent azaarenes, quinoline was the least, and benz[a]acridine the most genotoxic, with the three-ringed structures in between (benzo[h]quinoline < acridine < phenanthridine) (Table 5.1). However, benzo[f]quinoline and benz[c]acridine did not show any genotoxicity at the highest concentrations tested (0.12 and 0.19 µM, respectively) (Table 5.1), most likely due to the low concentrations at which these compounds were tested. The results imply that benz[c]acridine was at least 20 times less genotoxic than benz[a]acridine (Table 5.1). This is in contrast with results obtained by Wood et al. (1983), who found a more or less similar mutagenicity for both compounds using the Ames test, in which a different bacteria species (Salmonella typhimurium) is used. Hence, these differences in results may be explained by the difference in test organism, and therewith differences in test conditions and genetic endpoint. It stresses, however, the uncertainties involved when genotoxicity is determined using one test species only.

The difference in genotoxicity between the two benzacridines in our study cannot easily be explained by molecular descriptors, because only two four-ringed compounds were tested and benz[a]acridine shows higher values for all
descriptors calculated, except for the estimated log $K_{ow}$ (Table 5.2). However, these estimated log $K_{ow}$ values should be handled with care, because in benz[c]acridine the nitrogen atom is shielded by the benzene rings, and therefore this compound can be expected to be more hydrophobic than its isomer, resulting in a higher log $K_{ow}$ value for benz[c]acridine (cf. Nielsen et al. 1997). This also explains the lower LC$_{50}$ of benz[c]acridine compared to benz[a]-acridine (Table 5.1; Bleeker et al., 1998). The shielding effect in benz[c]acridine may also result in more mutagenic derivatives compared to benz[a]acridine. 7-methylbenz[c]acridine for example, was shown to be 3 fold more mutagenic than its isomer 12-methylbenz[a]acridine (as reviewed by Warshawsky, 1992). In our study, however, the reactive site of the molecule should be around the nitrogen atom, since benz[c]acridine is less genotoxic than benz[a]acridine.

Table 5.2. Molecular descriptors of azaarenes and their correlation with genotoxicity. Vol: molecular volume; SA: molecular surface area; $K_{ow}$: octanol-water partition coefficient; HF: heat of formation (kcal); D: dipole moment (D); IP: ionisation potential (eV); EA: electron affinity (eV); r: correlations with LOEC$_{genotoxicity}$.

<table>
<thead>
<tr>
<th></th>
<th>Vol</th>
<th>SA</th>
<th>log $K_{ow}$</th>
<th>HF</th>
<th>D</th>
<th>IP</th>
<th>EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinoline</td>
<td>121.95</td>
<td>143.05</td>
<td>2.03$^a$</td>
<td>52.1167</td>
<td>1.876</td>
<td>9.183</td>
<td>0.468</td>
</tr>
<tr>
<td>Benzo[f]quinoline</td>
<td>165.97</td>
<td>185.98</td>
<td>3.43$^a$</td>
<td>69.0352</td>
<td>1.959</td>
<td>8.877</td>
<td>0.594</td>
</tr>
<tr>
<td>Phenanthridine</td>
<td>166.03</td>
<td>186.25</td>
<td>3.48$^a$</td>
<td>67.9297</td>
<td>2.065</td>
<td>8.971</td>
<td>0.603</td>
</tr>
<tr>
<td>Benzo[n]quinoline</td>
<td>166.10</td>
<td>186.70</td>
<td>3.43$^a$</td>
<td>68.1384</td>
<td>1.529</td>
<td>8.736</td>
<td>0.559</td>
</tr>
<tr>
<td>Acridine</td>
<td>166.25</td>
<td>187.50</td>
<td>3.40$^a$</td>
<td>77.0543</td>
<td>1.840</td>
<td>8.575</td>
<td>1.041</td>
</tr>
<tr>
<td>Benz[a]acridine</td>
<td>210.19</td>
<td>230.41</td>
<td>4.49</td>
<td>92.1936</td>
<td>1.906</td>
<td>8.624</td>
<td>0.992</td>
</tr>
<tr>
<td>Benz[c]acridine</td>
<td>210.35</td>
<td>231.06</td>
<td>4.49</td>
<td>91.2963</td>
<td>1.441</td>
<td>8.545</td>
<td>0.981</td>
</tr>
<tr>
<td>Acridone$^b$</td>
<td>173.51</td>
<td>196.70</td>
<td>2.84</td>
<td>34.1309</td>
<td>2.079</td>
<td>8.377</td>
<td>1.011</td>
</tr>
<tr>
<td>Acridone$^c$</td>
<td>173.43</td>
<td>196.54</td>
<td>5.28</td>
<td>22.2254</td>
<td>4.227</td>
<td>8.475</td>
<td>0.471</td>
</tr>
<tr>
<td>Phenanthridinone$^b$</td>
<td>174.45</td>
<td>196.58</td>
<td>3.59</td>
<td>25.0385</td>
<td>1.473</td>
<td>8.761</td>
<td>0.583</td>
</tr>
<tr>
<td>Phenanthridinone$^c$</td>
<td>173.25</td>
<td>196.50</td>
<td>2.70</td>
<td>16.6438</td>
<td>3.249</td>
<td>8.723</td>
<td>0.636</td>
</tr>
</tbody>
</table>

| r$^d$ | 0.79 | 0.80 | 0.60 | 0.17 | 0.08 | 0.79 | 0.77 |
| r$^e$ | 0.96 | 0.95 | 0.96 | 0.62 | 0.11 | 0.66 | 0.70 |
| r$^f$ | 0.99 | 0.99 | 0.97 | 0.96 | 0.15 | 0.70 | 0.69 |

$^a$ these are literature values, others are calculated; $^b$ enol-form of the molecule; $^c$ keto-form of the molecule; $^d$ correlations without considering benzo[f]quinoline (BfQ) and benz[c]acridine (BcA) (acridone in enol-form and phenanthridinone in keto-form); $^e$ correlations without considering BfQ, BcA and acridone (phenanthridinone in keto-form); $^f$ correlations without considering BfQ, BcA and the metabolites.
The response of the Mutatox™ test to the metabolites was complex: genotoxicity of phenanthridinone is similar to that of the three-ringed parent structures, while acridone, isomer of phenanthridinone, is by far the most genotoxic of all compounds tested (Table 5.1). To gain more insight in the genotoxicity of the azaarenes and their metabolites, correlations with molecular descriptors were studied. For these correlations it was assumed that the metabolites were present as their most water soluble tautomer, i.e. acridone in enol-form and phenanthridinone in keto-form.

Since genotoxic compounds by definition interact with DNA, it can be expected that genotoxicity can be described by either reactivity or transport related molecular descriptors or both. If the role of molecular reactivity is the key factor for genotoxicity, then electronic descriptors (electron affinity, ionization potential and dipole moment) can considered to be the best descriptors for genotoxicity. This hypothesis, however, is in contrast with the low correlation coefficient observed between dipole moment and genotoxicity (Table 5.2, $r^d$). Even when homogeneity of the test compounds is improved by ignoring the metabolites (Table 5.2, $r^d$), correlation does not improve. Although electron affinity and ionization potential show reasonable correlations with genotoxicity, better results are obtained with size and transport related descriptors, especially when the metabolites are not considered (Table 5.2, $r^e$).

If the transport through the cell membrane and the cytosol is the limiting factor, size related parameters, such as volume and surface area, and also the more macroscopic octanol-water partition coefficient, should show good correlations with genotoxicity. When genotoxicity of the whole group of compounds is correlated with these parameters, results are poor (Table 5.2, $r^d$). However, when acridone is not taken into account, correlations strongly increase (Table 5.2, $r^d$), suggesting that for the other compounds transport through the cell is indeed a limiting factor, and emphasising the exceptional genotoxicity of acridone. We therefore conclude that the mode of genotoxic action for acridone is significantly different from that of the other compounds.

In the Mutatox™ test, restoring the luminescence of the repressed dark mutant can theoretically be achieved by three independent events (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, thus allowing unrepressed transcription of the luciferase operon. The first mode of action is expected from direct mutagens (Ulitzur, 1986). The second event was found to be associated with the activity of different DNA-damaging agents and with the action of DNA synthesis inhibitors, which seem to act through their ability to trigger the "SOS functions" that involve the inactivation of the luminescence system's repressor (Weiser, et al., 1981). 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). In this respect, azaarenes in general may induce genotoxicity by interacting with the repressor (either blocking or inactivating it), while acridone might be the only DNA-intercalating agent. This is suggested not only by the relative short time of response, but also by the fact that especially this molecule is very similar to the nucleotide bases of DNA, with a nitrogen atom at one side of the atom and an oxygen atom, capable of forming hydrogen bonds, at the opposite side. In addition, the structure of the keto-form of acridone is very similar to the nucleotide bases of DNA, suggesting that this is the most genotoxic tautomer of this compound. Further prove for this hypothesis can be found in the improvement of the correlation between genotoxicity and log K_{ow} when acridone is in the keto-form instead of being in the enol-form (r = 0.97 vs. r^2 = 0.60 in Table 5.2).

We conclude that both toxicity and genotoxicity of azaarenes are overall well described by transport related molecular descriptors (especially log K_{ow}). In both cases, however, exceptions can be found on this general rule, most likely due to a difference in mode of action. Multiple regression techniques might be a helpful tool in clarifying this difference. The present study showed that metabolism of azaarenes causes significant changes (both enhancing as well as decreasing) in the level of impact on different biological endpoints, even between isomers. This clearly puts severe limitations on environmental standards solely based on the narcotic effects of parent compounds. Therefore, we suggest that also other biological endpoints, especially genotoxicity, are incorporated in environmental hazard assessments for hetero-aromatic compounds.
References


