Toxicity of azaarenes: mechanisms and metabolism.

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Chapter 8
Concluding Remarks

Structural Diversity and Diversity of Effects

The present study showed that even within a limited series of related compounds, i.e. azaarenes, small differences in molecular structure (e.g. among isomers) lead to very different biological effects. Such differences in biological effects are enhanced by the capacity of aquatic biota to transform azaarenes (Chapters 2 and 3), which increases the number of compounds involved. Such biotransformation has shown to be compound or even isomer specific for different biological species. The prominent transformation products in this study were oxides, whereas in other studies hydroxides and epoxides are mentioned as major products (Chapter 3). Thus, different reactive groups are introduced in the basic structure of azaarenes. Subsequently, such products were shown to be genotoxic (Chapter 5). So far, these differences in biological effects between isomeric products like acridone and phenanthridone (Chapter 5) can not be predicted from previous observations on transformation of homocyclic PAHs, especially considering the direct genotoxicity of all azaarenes tested: all azaarenes induced genotoxicity without metabolic activation, in contrast with most homocyclic PAHs needing such activation.

The wide range of molecular structures of PAHs are likely to influence a wide variety of biological endpoints. In the present study, some of these effects have been identified, which resulted in compound and even isomer specific effects (Chapters 4 and 5). Even when acute toxicity was reduced by biotransformation, the metabolites showed life cycle effects comparable to their parent compounds. Furthermore, several sets of isomers showed significant differences in acute toxicity and genotoxicity. Yet, the endpoints tested in the present study are only few of the possibilities and, therefore, it seems likely that
the spectrum of different biological responses to azaarenes is considerably wider than could be demonstrated here.

In the present study, the genotoxic potential of azaarenes was identified using the Mutatox™ test. Although this Mutatox™ test generally discriminates well between carcinogens and non-carcinogens, *Salmonella* tests are generally more sensitive (Legault et al., 1994), indicating that genotoxic effects of azaarenes may occur at even lower concentrations (cf. Chapter 5). By using the Mutatox™ test, however, effects on the regulation of genes may be included. Such gene regulation is vital in developing organisms and its disturbance may result in long-term effects of azaarenes. In addition, the observed delay in emergence can be speculated to have great influence on subsequent generations (cf. Van Brummelen et al., 1996), especially considering hibernation of the species.

From the results in this thesis it is concluded that the many structurally different homocyclic and heterocyclic PAHs act upon a wide spectrum of biological receptors. Natural processes of biotic and abiotic transformation enhance the number of variables determining the specific effect on natural (aquatic) species. It seems unavoidable that several of these variables need further exploration. In the next paragraphs some of the approaches are indicated.

**Towards a Hierarchy of Toxic Effects**

An attempt was made to analyse the observed biological responses to azaarenes (Tables 5.1 and 6.2) and relate the different types of toxicity to the molecular structure of the compounds. Fig. 8.1 summarises some of the present observations showing a plot of the relationships between biological effects (two parameters) and a single physicochemical property (log \( K_{ow} \)), following the many studies that relate narcosis to log \( K_{ow} \) as a measure of lipophilicity (Könemann, 1981; Van Leeuwen et al., 1992; Verhaar et al., 1992). Comparing the present results for azaarenes with other studies, however, indicates that especially hydrophobic azaarenes are more toxic than can be predicted from other groups of narcotic compounds.
Figure 8.1. Acute toxicity (LC$_{50}$; black symbols) and genotoxicity (LOEC; open symbols) plotted versus log $K_{ow}$ values (results from Chapters 4-6). a-c: relationships based on QSARs for narcotic chemicals (taken from Van Leeuwen et al., 1992); a: based on 96 h LC$_{50}$ for the copepod Nitocra spinipes; b: based on 96 h LC$_{50}$ for the fish Pimephales promelas; c: based on 48 h LC$_{50}$ for the daphnid Daphnia magna; d: based on 96 h LC$_{50}$ for the midge Chironomus riparius (this study, black squares only); e: based on LOEC$_{genotoxicity}$ data (this study, open squares only). The diamonds in the figure are experimental LC$_{50}$ values (black diamonds) or LOEC$_{genotoxicity}$ values (open diamonds), which were not used in the relationships d and e (see text for details).

If lipophilicity related narcosis would predominate over other effects, it would be expected that simple linear relationships according to Königemann (1981) and Van Leeuwen et al. (1992) would provide reliable descriptions of effects observed in this thesis. The decreased effects of the metabolites, compared to their parent compounds, could indeed be explained by a change in log $K_{ow}$, although in this case only by recognising tautomerism, a special kind of isomerism exhibited by the metabolites. For other compounds, however, such a simple relationship evidently does not provide powerful explanations for the observations (Fig. 8.1). For narcotic effects (Chapter 4; acute toxicity) and genotoxicity (as tested with the Mutatox™ test; Chapter 5) several compounds are outlying (Fig. 8.1, diamonds vs. squares). The tautomeric forms of the
metabolites even enhance this observation, indicating that lipophilicity based toxicity is an oversimplification of azaarene toxicity. This is most likely also true for homocyclic PAHs.

A more complete explanation, therefore, has to be found. The difference in acute toxicity of acridine from a generalised log K\textsubscript{ow} model was explained by the photosensitivity of this compound, indicated by the HOMO-LUMO gap, lying in the phototoxic region as defined by Mekenyan et al. (1994) for PAHs. This suggests that this HOMO-LUMO gap should be incorporated into a predictive model for acute toxicity. Neither of the explanations given so far, however, could explain the toxicities of either benz[g]isoquinoline-5,10-dione or its isomer benzo[g]quinoline-5,10-dione, let alone the differences between the two. Although their structure is clearly different from the other azaarenes, due to the two double-bonded oxygen atoms, this cannot explain the differences in (geno)toxicity between these two isomers. It can, therefore, be argued that, apart from log K\textsubscript{ow} and the HOMO-LUMO gap, other molecular or physicochemical properties supersede narcosis. It is likely that for genotoxicity such parameters should also exist, most likely related to the way they interfere with DNA and gene regulation.

It is concluded that it seems essential for a systematic analysis of effects of (N)PAHs to construct calculation routines for toxic action that provide effective explanations for overall effects. So far, this seems to be effective for mixed narcotic-phototoxic effects, but for genotoxicity such a chemical cue could not be detected.

**Computing Toxicity**

Since narcosis is generally assumed to be related with membrane interactions, these interactions were studied more closely by using a computational membrane-model (Chapter 7), to calculate interaction energies for membrane-azaarene interactions. The high correlation coefficients between the two indicated that the membrane model showed good possibilities in describing this type of toxicity. Furthermore, once the target site is identified, a similar approach could be used for predicting other biological endpoints, showing the high potential of a mechanism-based approach in predicting (eco)toxicity.
To fully understand the toxicological impact a compound can exhibit, however, it appears to be essential to relate the different types of toxicity to the compound's structure, which would result in a multi-endpoint based QSAR. This would obviously imply a multiple regression approach, in contrast to the often used two-dimensional regression. Such an approach has been used in other studies (e.g. Geladi and Tosato, 1990), although in most cases different biological species are compared for a similar type of toxicity. Such multi-endpoint QSARs could also be useful in determining the role of biotransformation in toxicity, on the one hand incorporating effects of the parent compound and on the other the effects of its (stable) biotransformation product(s).

**Risk Assessment**

In the Netherlands risk assessment of PAHs is based on homocyclic compounds only, in particular a group of 10 representative PAHs (Slooff et al., 1989). Results presented in this thesis, however, showed the (eco)toxicological relevance of azaarenes, which is only one (small) group of heterocyclic compounds: at concentrations comparable to those in the field (0.1-1 μg/l; Van Genderen et al., 1994), toxicity of azaarenes has already been shown. Biotransformation not only enlarges the group of compounds involved, but it may also alter the toxic activity, either in intensity or in biological endpoint that is influenced. So, for a vast number of structures, some of them showing a much higher toxicity than the selected homocyclic PAHs, there is no protection provided by the current environmental standards. Quality criteria for PAHs, therefore, should incorporate heterocyclic compounds and biotransformation products.

It has been argued that the most important toxicant exposure route for aquatic life is via the water (cf. De Voogt et al., 1991). It can, therefore, be argued that quality criteria for PAHs should be related to water solubilities. The water solubility of two-ringed structures is generally so high (Pearlman et al., 1984) that concentrations found in the aquatic environment (0.1-1 μg/l for most two-ringed structures; Van Genderen et al., 1994) are likely to be too low to induce toxicity. For larger PAHs (structures with four or more rings) the opposite holds: these structures are generally strongly hydrophobic, resulting
in low bioavailabilities (water solubilities are low, resulting in disappearance of benzacridines from the test solution within 96 h; Chapter 4). It is argued, therefore, that three-ringed toxicants appear to be the most relevant in aquatic environments. Furthermore, this study has shown that primary biotransformation products of such three-ringed structures are quite soluble in water themselves, which enhances the toxicity routes involved. Such biotransformation processes may be incorporated in risk assessment models by estimating the reactive site(s) in a compound, especially for oxygenation and hydroxynation, which have been shown extensively as the primary biotransformation steps (e.g. Chapters 2 and 3; Kochany and Maguire, 1994).

Structure-activity relationships have been widely recognised as a useful tool in risk assessment. Such relationships, however, often focus on one particular biological endpoint, while neglecting others. Yet, one compound can influence several endpoints, depending on exposure dose and duration, indicating that if risk assessment is solely based on one-endpoint QSARs, the chance of missing other endpoints cannot be neglected. Multi-endpoint approaches, therefore, appear to be a necessity, incorporating different types of effects, including those of biotransformation products. For a proper estimation of such effects, some way of relating metabolite induced effects to those induced by the parent compound should be defined. One such an approach could include a comparison of the distribution of toxic effects for a number of species induced by either the parent compound or the metabolite. For instance, when one biological endpoint is stronger influenced by a compound, this would result in a skewed distribution of effect concentrations over the different biological endpoints.

Another point of concern is the inappropriate inclusion of time-dependency. Although this time-dependency has been recognised and has lead to the calculation of acute-to-chronic-ratios (Kenaga, 1982; Mayer, 1990; Länge et al., 1998; Roex et al., 1999), caution is necessary in using such ratios. Kenaga (1982) found that acute-to-chronic-ratios (ACRs) for PAHs were always below 10, whereas in our study we found values as high as 50. This suggests that the standard safety factor of 10 for extrapolating chronic effects from acute effects, which is used in risk assessment, should be adjusted. Such adjustment could include a summation of the different kinds of effects related to the molecular structure of the toxicant.
In conclusion, it is argued that the role of heterocyclic compounds and metabolism should be incorporated in order to obtain reliable structure-activity relationships, thus enabling a reliable risk assessment for polycyclic aromatic compounds.

References


