Toxicity of azaarenes: mechanisms and metabolism.
Bleeker, E.A.J.

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In the present study a systematic analysis of the relationships between molecular structure and several effects in aquatic biota was performed for azaarenes, a group of nitrogen heterocyclic polyaromatic hydrocarbons. Special attention was given to biotransformation, because this process enlarges the group of compounds involved, thereby altering the intensity or the mode of toxic action. Differences in toxicity between isomers were analysed in order to find molecular key factors determining biological effects. Also different biological endpoints, such as acute narcosis, genotoxicity and life-cycle effects, were studied comparatively and relationships were sought between the different adverse effects and molecular properties. Through a synthetic membrane model, a first step was taken towards a fully computational analysis of azaarene toxicity.

The first part (Chapters 2 and 3) of the thesis focuses on the metabolism of two azaarenes (the isomers acridine and phenanthridine) by aquatic organisms.

Chapter 2 describes the identification of these two major metabolites using different analytical methods, including HPLC, GC and hyphenated LC- or GC-MS. This study illustrates the typical problems with, as well as the potency of, chromatographic methods when elucidating metabolic routes of organic contaminants. The zebra mussel (Dreissena polymorpha), a green alga (Selenastrum capricornutum) and periphyton or bacteria were found to transform acridine into 9(10H)-acridinone (acridone). Its isomer 6(5H)-phenanthridinone (phenanthridone) was found as a biotransformation product of phenanthridine in experiments with midge (Chironomus riparius) larvae, although the actual producer of this metabolite, bacteria or midges, could not yet be identified. These findings indicate that closely related compounds like isomers may undergo species specific biotransformation, i.e. some species are capable of transforming one compound (e.g. acridine), while they cannot transform another (e.g. phenanthridine). It was
concluded that, unlike homocyclic PAHs, benzoquinolines are transformed to keto-metabolites.

Having assessed the strong species and compound specificity of biotransformation of azaarenes by aquatic invertebrates and algae, the metabolism of phenanthridine was further studied in separate experiments with midge larvae and carp (Chapter 3). In both experiments, again phenanthridone was observed as a major metabolite. In the midge experiment this metabolite appeared to be principally formed by bacteria growing on the food. In both experiments, after an initial increase, phenanthridone concentrations in the water decreased, indicating that the metabolite itself was further degraded to non-observed compounds. This metabolite transformation was due to bacteria and midges acting together in the midge experiment, and to carp in the fish experiment. Internal concentrations of phenanthridine and phenanthridone were below detection limits in the midge larvae, whereas high concentrations of both compounds were observed in carp organs, suggesting a major role of bile and liver. Only part of the phenanthridine loss could be accounted for by its transformation into phenanthridone. Hence, apart from phenanthridone formation, other metabolic pathways may be involved. The results described in Chapters 2 and 3 demonstrate that biotransformation capacity is strongly species specific, suggesting that the toxicity of organic compounds strongly depends on the biological species present in the environment.

To assess the toxicity of azaarenes, acute toxicity was determined to first instar *Chironomus riparius* larvae (Chapter 4). Lethal effects generally increased with increasing number of aromatic rings of the compound, resulting in strong relationships between the $LC_{50}$ values and size, shape and topology-related molecular properties. On-ring substituted azaarenes (i.e. ketones), however, did not correspond with these relationships: they either showed a toxicity higher than expected (benzo[9]quinoline-5,10-dione, and benz[g]isoquinoline-5,10-dione), indicating a new specific action, or no toxicity at all (acridone and phenanthridone). Four benzoquinoline isomers showed obvious differences in toxicity: acridine was significantly more toxic than the other benzoquinoline
isomers tested, due to photoactivation of acridine, as predicted by the HOMO-LUMO gap of this compound.

Chapter 5 describes the determination of the genotoxic potential of azaarenes using the bacterial Mutatox™ test. Genotoxic activity was partly described by molecular descriptors relevant to bioaccumulation. Acridone, however, showed an extremely high genotoxicity, especially when compared with its isomer phenanthridone. In this case the tautomeric properties of the metabolites is suggested as a causal factor. The relatively high genotoxicity of the isomers benzo[g]quinoline-5,10-dione and benz[g]isoquinoline-5,10-dione (results in Chapter 6) may be due to a different mechanism. The comparison between narcosis and genotoxicity showed that, although narcosis is reduced by biotransformation, genotoxicity may be enhanced, indicating that metabolism may influence the mode of toxic action, i.e. the biological endpoint that is influenced.

In Chapter 6 the adverse effects of azaarenes on larval development of the midge Chironomus riparius are examined. To this purpose six closely related three-ringed isomers and metabolites were selected: acridine and phenanthridine, acridone and phenanthridone, and benzo[g]quinoline-5,10-dione and benz[g]isoquinoline-5,10-dione. Midges were exposed to concentrations below acute LC$_{10}$ values from egg to adult stage. Effects on development were examined by comparing the average day of emergence of life-time exposed midges with that of controls. Fluctuating asymmetry (FA) in the pecten epipharyngis was examined as a measure of developmental integrity.

Delayed emergence was found at concentrations as low as 2% of the acute LC$_{50}$. Hence, emergence appears to be a useful and sensitive parameter to quantify life cycle effects beyond current expectations as to toxicity in short and long-term experiments. Yet, no differences in FA were found between exposed and control larvae, although all six azaarenes have been proven to be genotoxic and benz[g]isoquinoline-5,10-dione was known to induce teratogenic effect in crickets. The differences in the genotoxic, teratogenic and FA-inducing properties of these compounds indicate that different mechanisms are involved in expressing these adverse effects. This study also illustrates that the choice of
the morphological parameter strongly influences the results of developmental disturbance analyses, and thus the risk qualification of a potentially hazardous compound.

Since the commonly used molecular and physicochemical descriptors (e.g. hydrophobicity) were unable to distinguish between the toxic potential of isomers, in Chapter 7 a more mechanism based approach was used in describing narcosis, i.e. membrane disturbance. Interactions between a computationally constructed model membrane and the previously selected azaarenes were investigated. The membrane model was constructed from 16 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine molecules forming a bilayer. Membrane-azaarene interaction energies were then calculated from solvation energies to evaluate the importance of three different regions in the membrane: the polar headgroup, between the alkyl chains, and between the two layers. A QSAR approach was used to compare the resulting energies with the narcotic and genotoxic data from Chapters 4, 5 and 6. Significant differences between the compounds were only found for interactions situated in the headgroup and between the layers of the membrane. Of these two regions, the headgroup appears to be the most important for narcosis, resulting in significant relationships between interaction energies and narcosis. As expected, genotoxic activity was not related with membrane interaction energies. Based on the preliminary results presented in Chapter 7, the approach used is a promising tool in predicting narcosis.

From the present study it is concluded that the many structurally different heterocyclic PAHs act upon a wide spectrum of biological receptors. Abiotic and biotic transformation enhance the number of variables determining the specific effects on aquatic species. The number of outlying observations in relationships between toxicity and log $K_{ow}$ indicate that lipophilicity based toxicity is an oversimplification of azaarene toxicity. It seems, therefore, essential to construct more complete, multivariate relationships to provide effective explanations for overall effects. In addition, multi-endpoint QSARs could be useful in determining the influence of biotransformation in the
different types of toxic effects, both by predicting the metabolic pathway and the change of biological effect.

The present results show that the present environmental standards for PAHs fail to protect against the toxicity of a vast number of polyaromatic structures. Especially for the aquatic environment inclusion of polar, substituted PAHs in risk assessment appears to be necessary. For a proper estimation of toxic effects a multi-endpoint QSAR approach seems necessary in which biotransformation products and their toxicity should be incorporated.