Toxicity of azaarenes: mechanisms and metabolism.

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Chapter 3

Comparative Metabolism of Phenanthridine by Carp (Cyprinus carpio) and Midge Larvae (Chironomus riparius)

Abstract

Abiotic transformation of azaarenes in the environment has been analysed extensively, but metabolism is less well described. Large differences in the metabolism of azaarenes, however, have been noted in different aquatic species. Therefore, metabolism of phenanthridine was studied in separate experiments with midge larvae and carp. In both experiments, 6(5H)-phenanthridinone (phenanthridone) was found as a major metabolite, although in the midge experiment this metabolite was principally formed by bacteria growing on the food. Phenanthridone itself was further degraded to non-observed compounds in both experiments, due to bacteria and midges acting together in the midge experiment, and by carp in the fish experiment. Internal concentrations of phenanthridine and phenanthridone were non-detectable in the midge larvae, but concentrations of both compounds in carp organs suggest a major role of bile and liver. Since phenanthridone did not account for all phenanthridine loss, it was suggested that, apart from phenanthridone degradation, also other metabolic pathways may play a role. From this study it is clear that metabolism is an important factor in determining the effects of azaarenes and should, therefore, not be neglected in risk assessment.
Introduction

The major part of research on polycyclic aromatic hydrocarbons (PAHs) has been focused on homocyclic compounds. However, two thirds of the aromatic compounds known are heterocyclic (Kuhn and Suflita, 1989), having in-ring substitutions of oxygen, sulphur, and/or nitrogen. Azaarenes are such a group of heterocycles, containing one nitrogen atom. This makes them better soluble in water than their homocyclic analogues (Pearlman et al., 1984), and therefore, perhaps also more bioavailable. Although partly of natural origin (e.g. as alkaloids; Kaiser et al., 1996), the majority of the azaarenes found in the environment is from anthropogenic sources, originating from, for instance, combustion of fossil fuels (Santodonato and Howard, 1981), or wood preserving industry (Adams and Giam, 1984). Their presence has been shown in air (Santodonato and Howard, 1981), in groundwater (Pereira et al., 1987), and in both marine and freshwater environments (Blumer et al., 1977; Van Genderen et al., 1994; Kozin et al., 1997).

Our recent studies have demonstrated that metabolism of azaarenes may alter their toxicity. Whereas the biotransformation of acridine into acridone (De Voogt et al., 1999) led to a significant reduction of its toxicity to zebra mussels (Kraak et al., 1997) and midge larvae (Bleeker et al., 1999b), the genotoxicity appeared significantly enhanced (Bleeker et al., 1999b). These significant changes in toxicity and in biological endpoint that is affected, stress the importance of biotransformation for the environmental fate and (eco)toxicity of azaarenes.

Abiotic transformations of azaarenes in the aquatic environment have been reviewed (Kochany and Maguire, 1994), but information on azaaerene biotransformation is less well documented (De Voogt et al., 1999). A diversity of organisms can transform azaarenes, ranging from bacteria (Bollag and Kaiser, 1991) and fungi (Sutherland et al., 1994ab) to algae (Van Vlaardingen et al., 1996; Dijkman et al., 1997), mussels (Kraak et al., 1997), and fish (Bean et al., 1985). Strong species specificity, however, occurs between compounds that can or cannot be biotransformed. Zebra mussels have been found to transform acridine (Kraak et al., 1997), but such metabolism has not been found for midge larvae (Bleeker et al., 1999a). Indications were found that midge larvae were capable of transforming phenanthridine, one of the isomers of acridine, into
6(5H)-phenanthridinone (Bleeker et al., 1996; De Voogt et al., 1999). Furthermore, Dijkman et al. (1997) showed that only one out of the seven algal species tested was capable of acridine metabolism.

Although in previous studies (Bleeker et al., 1996; De Voogt et al., 1999) indications were found that midge larvae are able to transform phenanthridine, no conclusive evidence could be presented. The present study, therefore, aims to identify the separate contributions of bacterial and larval metabolic activity to the biotransformation process. In addition, carp (Cyprinus carpio) were exposed to phenanthridine in order to compare invertebrate with vertebrate metabolism. Since it is assumed that metabolism of phenanthridine into phenanthridone is mediated through Cytochrome P450 coenzymes (De Voogt et al., 1999), it is expected that fish are better capable of transforming phenanthridine into phenanthridone, due to their higher levels of Cytochrome P450, as well as the higher inducible activity of these coenzymes (Livingstone, 1998).

**Materials and Methods**

**Midge experiment**

Laboratory reared midge larvae (Chironomus riparius) (Postma, 1995) were exposed to phenanthridine in three different concentrations (0.3, 0.8 and 3.0 μM). All concentrations, including a control, were tested in triplicate. The experimental set-up consisted of 180 ml glass jars containing 100 ml of Dutch Standard Water (DSW), a moderate buffering artificial salt solution (demineralised water with 200 mg/L CaCl₂·H₂O, 180 mg/L MgSO₄·H₂O, 100 mg/L NaHCO₃, and 20 mg/L KHCO₃; pH ≈ 8.2), comparable with Dutch surface waters (Nederlands Normalisatie Instituut, 1980).

To avoid co-solvents, phenanthridine was added to the water using a generator column derived stock solution according to Bleeker et al. (1998). The stock solution concentrations in the present experiments varied between 44.4 and 69.0 μM, so dilutions were made to obtain the desired exposure concentrations. Actual azaarene concentrations were determined by analysing water samples (2.5 ml) taken at 1, 2, 4, 8, 16, 24, 48, 72, 96, 120, and 168 h, both in the first and in the second week of the experiment. Water samples were
centrifuged (3000 rpm) and 1 ml of the supernatant was injected in HPLC and eluted with an isocratic mixture of 80 % acetonitrile (HPLC grade, Rathburn) and 20 % water (HPLC grade, J.T. Baker). Both fluorescence detection (excitation $\lambda = 254$ nm and emission $\lambda > 354$ nm) and UV detection ($\lambda = 254$ nm) were used for detecting phenanthridine and phenanthridone. All HPLC measurements were performed according to Bleeker et al. (1998).

Three times a week 1 ml of uncontaminated food suspension was added from a stock, composed of 1 g of a fishfood mixture (Tetraphy:Trouvit, 1:20 w/w) in 20 ml DSW, supplying food ad libitum. The jars were constantly aerated, covered with plastic foil and kept under a 22 ± 1 °C and 16:8 h light:dark regime. The experiment lasted for 14 days and after 7 days water and toxicant were renewed. Food was not renewed, because it was used as sediment in which the larvae made protective tubes. Therefore, renewal of the food would have caused too much stress to the larvae. Three different treatments were used for each exposure concentration: DSW with toxicant (to check for loss of toxicant due to abiotic factors), DSW with toxicant and food (to check for loss of toxicant due to binding to the food and due to metabolism by bacteria growing on the food), and DSW with toxicant, food and midge larvae (to analyse the metabolic capacity of the midge larvae). In the latter treatment 50 first instar larvae ($< 24$ h old) were used per jar. At the end of the experiment midge larvae were collected and stored at -70 °C until further analysis.

**Fish experiment**

The carp (Cypinus carpio) were obtained from the Fish Culture and Fisheries Group of the Wageningen Agricultural University (Wageningen, The Netherlands). They were exposed to a higher concentration series (0.4, 3.2, and 13.8 $\mu$M) than the midge larvae, since in general fish are less sensitive to toxicants due to their size. All concentrations, including a control, were tested in duplicate. The set-up consisted of 4.5 L glass aquaria filled with 3 L of DSW and containing three fish (2.5 - 3 cm, ca. 4 weeks old). The aquaria were covered with glass plates to minimise evaporation and aerated constantly. Light and temperature were identical to the midge experiments. Total exposure time was 14 days and water was renewed after 7 days. Fish were fed daily with uncontaminated fourth instar chironomid larvae, five per fish during the first week and seven per fish during the second week, to compensate for the growth
of the fish. The midge larvae were consumed very rapidly (within minutes), so it was reasonable to assume that they would not have influenced the toxicant concentrations in the water and remained uncontaminated until consumption. Water samples were taken at 1, 2, 4, 8, 16, 24, 48, 72, 96, 120 and 168 h in both weeks of the experiment and measured using HPLC (see above). At the end of the experiment fish were killed in ice water, weighed and dissected to enable separate internal concentration measurements of different parts of the fish. Bile, kidney, liver, gills, and the remainder of each fish were stored separately at -70 °C until further analysis.

**Analysis of phenanthridine and phenanthridone**

For determining phenanthridine and phenanthridone concentrations, midge larvae and fish organs were extracted with ethyl acetate (glass distilled grade) using Soxhlet extraction. To achieve detectable azaarene concentrations, the triplicate treatments from the midge experiment and the duplicate treatments from the fish experiment were pooled. 400 μL hexachlorobenzene was added as internal standard. The extract (ca. 30 ml) was concentrated under a nitrogen flow and cleaned using an adsorption chromatographic column. This column consisted of 2.2 g of SiO₂·H₂O and 1 g of NaSO₄. Elution/fractionation was executed with 5 ml of hexane and 15 ml of ethyl acetate. The last 10 ml (analyte fraction) was concentrated to about 400 μL. 1 μL of each sample was analysed using GC-LRMS (Hewlett Packard 5890GC with Hewlett Packard 5970 MSD HRGC/LRMS system with on-column injection). A 30 m x 0.32 mm (film 0.25 μm) DB-5 (J&W) capillary column was used with helium as carrier gas. Pre-pressure was 5 psi and the transfer line was maintained at 250 °C. The temperature gradient of the GC furnace went from 80 °C to 250 °C at a rate of 20 °C per min.

**Results**

Survival in the midge experiment (ca. 30 %) was less than could be expected from the LC₅₀ for phenanthridine (Bleeker et al., 1998), most likely due to damage during transfer of the larvae into the test jars. This low survival, however, was not dose-dependent and since surviving larvae grew normally, it is assumed that the low survival did not influence the metabolism. In the fish
experiment all fish exposed to the highest phenanthridine concentration (13.8 μM), started to float within one hour after start of the experiment and died within 24 h. All other fish survived.

Figure 3.1. Phenanthridine and phenanthridone concentrations in test water during midge experiments at three different exposure levels, plotted versus time, with midge larvae absent (black symbols) and present (open symbols). The upper panel of each graph (square symbols, straight line) shows the phenanthridine concentrations (% of the initial concentration) and the lower panel of each graph (round symbols, dotted line) shows the phenanthridone concentrations (also as % of the initial phenanthridine concentration). In the upper right corner of each graph the average initial exposure concentration is given. The arrow on the time scale indicates the time of water renewal. Error bars represent the standard deviations.
Figure 3.2. Phenanthridine and phenanthridone concentrations in test water during the fish experiments at three different exposure levels, plotted versus time. The upper panel of each graph (black square symbols, straight line) shows the phenanthridine concentrations (% of the initial concentration) and the lower panel of each graph (open round symbols, dotted line) shows the phenanthridone concentrations (also as % of the initial phenanthridine concentration). In the upper right corner of each graph the average initial exposure concentration is given. The arrow on the time scale indicates the time of water renewal. Error bars represent the standard deviations. At the highest exposure concentration all fish died after 24 h.
Abiotic loss of phenanthridine in all cases was less than 10 % over a period of 7 days as was observed in the control experiments. Fig. 3.1 shows the decrease of phenanthridine and the increase of phenanthridinone over time during the midge experiment, for the treatments with and without midge larvae. The values shown are corrected for the abiotic loss of phenanthridine, hence all phenanthridine disappearance is due to metabolic activity. For all three exposure concentrations it is clear that midge larvae did not enhance the degradation of phenanthridine, in addition to the bacteria growing on the food. Furthermore, the transformation proceeded faster in the second week, compared to the first week, due to growth and/or adaptation of the bacteria.

At the lowest phenanthridine exposure no detectable concentrations of phenanthridone were found, but from the other two phenanthridine exposures it is clear that a larger proportion of phenanthridine shows up as phenanthridone in the second week, compared to the first week.

The highest phenanthridone concentration found in the experiments was about 40 % of the initial molarity of phenanthridine, although all phenanthridine disappeared, suggesting that either phenanthridone was further degraded in non-detected compounds or phenanthridine was transformed into other non-detected products, apart from phenanthridone. The further degradation of phenanthridone is indicated by the decreasing phenanthridone concentrations at the end of the experiments and seemingly enhanced by midge larvae.

Fig. 3.2 shows the results for the fish experiment, indicating clearly that carp are capable of transforming phenanthridine into phenanthridone. Even at the highest phenanthridine exposure detectable amounts of phenanthridone were found, although the fish died within 24 h. Again only a portion of the disappearing phenanthridine has been detected as phenanthridone, so also in the fish experiments other (non-identified) products are likely to be formed, either from phenanthridine or from phenanthridone.

Neither phenanthridine nor its transformation product phenanthridone was found in phenanthridine-exposed midges. Table 3.1 shows the internal concentrations observed in the fish. Recoveries, based on the internal standard hexachlorobenzene were 76 ± 14 % (average ± s.d.), and azaarene concentrations were corrected accordingly. Although all concentrations were low
Metabolism of Phenanthridine

(often below detection limits; see Table 3.1), some observations can be made. In general, phenanthridone concentrations are always higher than those of phenanthridine, except for fish exposed to the highest phenanthridine concentration (13.8 μM), in which phenanthridine concentrations always exceed those of phenanthridone. At low exposure concentrations, phenanthridone levels are high in bile and liver, compared to other tissues. At the highest exposure concentration, both the parent compound phenanthridine and its degradation product phenanthridone appear to show up in all tissues. In this latter case, especially kidneys show extreme phenanthridine levels.

Table 3.1. Internal concentrations of phenanthridine (phe) and phenanthridone (pho) in different organs of the fish. If concentrations were below detection limit, this is indicated by < followed by the detection limit. For the ‘rest’ fraction, detection limits were 0.01 and 0.06 pmol/mg for phe and pho, respectively.

<table>
<thead>
<tr>
<th>Initial exposure concentration (μM phenanthridine)</th>
<th>0.4</th>
<th>3.2</th>
<th>13.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>phe (pmol/organ)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bile</td>
<td>&lt;1.5</td>
<td>15.3</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>kidney</td>
<td>&lt;1.5</td>
<td>&lt;6.8</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>liver</td>
<td>&lt;1.0</td>
<td>8.4</td>
<td>3.2</td>
</tr>
<tr>
<td>gills</td>
<td>&lt;0.1</td>
<td>&lt;0.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>rest</td>
<td>0.4</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>pho (pmol/organ)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>kidney</td>
<td>&lt;6.8</td>
<td>&lt;6.8</td>
<td>&lt;6.8</td>
</tr>
<tr>
<td>liver</td>
<td>3.2</td>
<td>19.3</td>
<td>19.3</td>
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<tr>
<td>gills</td>
<td>5.4</td>
<td>88.3</td>
<td>88.3</td>
</tr>
<tr>
<td>rest</td>
<td>0.2</td>
<td>3.3</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Although the low concentrations caused some difficulties, an attempt was made to come up with a molar based azaarene balance in fish and water. A rough estimation showed that most of the phenanthridine loss in water (> 90 %) could not be accounted for, except for the highest exposure concentration, where only about 15 % of the dissolved phenanthridine could not be accounted for (detection limits in the water are 0.06 and 0.05 μM for phenanthridine and phenanthridone, respectively).

Discussion

In the present study, biotransformation of phenanthridine into phenanthridone has been found, which is in agreement with previous studies (Bleeker et al., 1996; Bleeker et al., 1999a; De Voogt et al., 1999) and confirms the impor-
tant role of ketones in the metabolic pathway of azaarenes (De Voogt et al., 1999). From the present study, however, it is clear that midge larvae are not responsible for the observed biotransformation. Hence, the bacteria growing on the midge food are principally responsible. Microbial oxidation of azaarenes has been demonstrated extensively (reviewed by Kaiser et al., 1996), but to our knowledge microbial oxidation of phenanthridine has not been reported previously. Yet, phenanthridone has been shown to be a minor metabolite in mammalian studies (Benson et al., 1983; LaVoie et al., 1985). The microbial transformation of phenanthridine may be similar to that of acridine, which involves an anaerobic mechanism in which acridine is transformed to 9-hydroxyacridine (Knezovich et al., 1990). Such a mechanism has also been found for the anaerobic transformation of quinoline, a two-ring azaarene (Pereira et al., 1988). In our study, however, it is more likely that an aerobic mechanism is involved, since the constant aeration provides high oxygen levels throughout the experiment. Therefore, the oxygen of 6(5H)-phenanthridinone is most likely originating from dissolved oxygen, in contrast with the anaerobic mechanism described above, in which the oxygen is taken from water molecules.

The maximum yield of phenanthridone in the experiments accounted for only about 40 % of the disappeared phenanthridine. Abiotic loss, e.g. binding to the food particles, could play a role, but other processes may be more important. Phenanthridone is clearly further degraded into yet unknown compounds, indicated by the decrease of phenanthridone concentrations near the end of the experiment. The low phenanthridone yield may also be caused by phenanthridine transformation via other metabolic pathways. For acridine and quinoline indications have been found that, apart from the heterocyclic part, the homocyclic part of the condensed ring structure can also undergo the initial oxidative attack (Knezovich et al., 1990), which, of course, results in other initial transformation products. In several mutagenicity and carcinogenicity studies with benzoquinolines (e.g. Adams et al., 1983; McMurtrey and Knight, 1984; Kandaswami et al., 1987), for instance, metabolic activation often leads to dihydrodiols, diol epoxides and other derivatives in which the homocyclic parts are attacked.
The further metabolism of phenanthridone proceeded faster when midge larvae were present together with the bacteria that grow on the food, suggesting that, although midge larvae do not contribute to the phenanthridine metabolism, they are able to transform phenanthridone. Analogous to mammalian studies (e.g. LaVoie et al., 1985), it may be expected that transformation products of phenanthridone include dihydrodiols and diolepoxides. In the present study, however, such compounds were not detected, since both the invertebrate and the fish experiments were focussed on detecting phenanthridone.

From the experiment with carp it is clear that these fish are able to transform phenanthridine. Even under severe stress, as at the highest, extremely toxic exposure concentration, detectable amounts of phenanthridone were found within 24 h of exposure. Similar to observations in the midge experiments, the phenanthridone concentrations do not explain all phenanthridine disappearance: in this case only about 15 % of the initial molar phenanthridine concentration has been found as phenanthridone. Although in these experiments abiotic factors can not be ruled out, they are likely to be similar or even less important than in the midge experiments, since glass and water surfaces are relatively small due to the larger water volume used in comparison to the midge experiments. Furthermore, at the 3.2 µM exposure, after an initial increase in phenanthridone concentration a decrease was observed, especially during the first week (Fig. 3.2), indicating that also in the fish experiments phenanthridone is further degraded. This is supported by the large percentage of phenanthridine loss that cannot be accounted for in a tentative molar balance. Similar to the midge experiments, however, other processes may play a role resulting in different (non-detected) biotransformation products, either from phenanthridine or phenanthridone. In experiments with rainbow trouts exposed to quinoline, 8- and 5-hydroxyquinolines were identified as the major transformation products (Bean et al., 1985). Hence such products may also be formed in our fish experiment, especially when considering that in mutagenicity studies with benzoquinolines hydroxylated compounds were found as major metabolites (e.g. Adams et al., 1983).

In the midge larvae neither phenanthridine nor phenanthridone were detected. Hydrophobic compounds, among which all sorts of aromatic
compounds, will accumulate mostly in hydrophobic parts (e.g. lipids) of an animal (Van Wezel et al., 1996). Since the lipid content of *Chironomus riparius* is low (1.6 ± 0.4 %; Legierse, 1998), this may be an explanation for not detecting azaarenes in midge larvae. For chlortion, an organophosphorous pesticide, Legierse (1998) reported a BCF value for *Chironomus riparius* that was about 2 to 3 times less than that of the water flea *Daphnia magna* although the lipid content of *D. magna* is about one third of that of *C. riparius*. In addition, the elimination rate constant for *C. riparius* was about twice that for *D. magna* (Legierse, 1998), indicating that *C. riparius* can efficiently eliminate aromatic compounds. Midge larvae, therefore, like the fish, may eliminate at least a substantial portion of the accumulated phenanthridine and phenanthridone, probably facilitated by the reduced azaarene concentrations near the end of the experiment.

Internal concentrations in fish suggest a highly efficient metabolism of phenanthridine in which bile and liver play a major role, indicated by highest phenanthridine and phenanthridone concentrations in these organs. This supports the assumption that P450 mixed function oxygenase systems are involved (De Voogt et al., 1999), since most MFO activity in fish is localised in the liver (Lindstrom-Seppa et al., 1981). At the highest exposure concentration, however, toxicity of phenanthridine was too high, which strongly decreased the efficiency of the metabolism. This was not only shown by the 100 % mortality, but also by high concentrations of both phenanthridine and phenanthridone in all organs (Table 3.1). These observations suggest that for these young carp both the LC₅₀ and the EC₅₀ for phenanthridine are between 3.2 and 13.8 µM phenanthridine, which is similar to that of midge larvae (96 h LC₅₀ = 3.4 µM; Bleeker et al., 1998).

Comparing fish metabolism of azaarenes with that of midge larvae, the major difference is that fish are able to transform phenanthridine, while midge larvae are not. This further supports the assumption that cytochrome P450 is involved in the initial oxidation of phenanthridine to phenanthridone, since the cytochrome P450 system in fish is more efficient than that in midge larvae (Livingstone, 1998). In the case of phenanthridone transformation, it is hard to compare efficiencies of both species, since in the midge experiments the role of bacteria cannot completely be excluded.
In uptake studies with fish bioconcentration factors (BCFs) are often calculated, because of their importance in risk assessment models. In the available models, however, the compound tested has to fulfil certain conditions. It should either be non-toxic (Banerjee et al., 1984), and/or not be transformed during the experiment (Banerjee et al., 1984; Sijm and Van der Linde, 1995). For phenanthridine, however, neither of these requirements is met, and consequently no BCFs could be calculated. This stresses the importance of including (bio)transformation in risk assessment and BCF modelling.

Furthermore, in previous studies (Bleeker et al., 1999b) it was shown that metabolites may affect different biological endpoints, compared to their parent compounds, indicating that metabolism is an important factor in (eco-) toxicology. We, therefore, argue that, conform the risk assessment for pesticides and insecticides, metabolism cannot be ignored in the risk assessment for PAHs.

Acknowledgement
We would like to thank Pieter Slot for his assistance in analysis of internal azaarene concentrations.

References


