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Formation and identification of azaarene transformation products from aquatic invertebrate and algal metabolism

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Abstract

The metabolism of two azaarenes, viz. acridine and phenanthridine, by aquatic organisms was studied in short-term and chronic laboratory tests. The identity of metabolites observed in the test waters was investigated with different analytical methods, including HPLC, GC and hyphenated LC– or GC–MS. The Zebra mussel (*Dreissena polymorpha*), one green alga species (*Selenastrum capricornutum*) and periphyton or bacteria transformed acridine into 9[*10H*]-acridinone. Phenanthridine was transformed into 5[*6H*]-phenanthridinone by midge (*Chironomus riparius*) larvae. The findings indicate that closely related isomers may undergo species-specific biotransformation. It was concluded that keto-metabolites are major products in the aquatic fate of benzoquinolines, which may be overlooked in the risk assessment of parent compounds. This study illustrates the typical problems with, as well as the potency of, chromatographic methods in the elucidation of metabolic routes of organic contaminants. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Azaarenes; Acridine; Phenanthridine

1. Introduction

Nitrogen heterocyclic aromatic hydrocarbons or azaarenes appear both in natural and anthropogenic sources, such as fossil fuels, tars and synthetic oils. As a result of combustion processes and accidental spills, azaarenes have been released into the environment. They have been detected in automobile exhaust [1], ambient air [2] and air particulate matter [3]. The presence of azaarenes in marine and lake sediments has also been shown [4,5] and they were detected in low concentrations in Dutch river sediments [6].

Metabolism of polyhomocyclic aromatic hydro-

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carbons (PAH) by invertebrates is well documented in the literature [7,8]. PAH may either be activated to reactive intermediates or detoxified as a result of metabolism. Which of these processes may occur depends on physicochemical properties of the PAH as well as on characteristics of the organisms concerned and environmental, nutritional and physiological factors [9]. In the presence of UV light, toxicity of PAH can be enhanced up to several orders of magnitude [10], probably as a result of activated oxygen species.

Abiotic transformations of azaarenes in the aquatic environment have been reviewed [11], but much less is known about their biotransformation. In mammalian liver microsomes, several metabolites of acridine and phenanthridine have been identified or their identity proposed [12–14]. Mutagenicity of azaphenanthrenes using the Salmonella assay has been shown to result in formation of dihydrodiols and N-oxides [15]. Fungi have been shown to transform acridine into mono or dihydroxylated [16], and quinoline and isoquinoline into N-oxidated metabolites [17]. Quinoline transformation has also been reported for bacteria [18,19] and fish [20], major products being hydroxylated metabolites.

Obviously, the information on azaarene biotransformation is still fragmentary and especially experimental data on invertebrates and algae are lacking. Therefore, the purpose of the present work was to assess the ability of invertebrates and algae to transform azaarenes and to elucidate the identity of unknown metabolites observed in acridine and phenanthridine toxicity tests with mussel (*Dreissena polymorpha*) [21], algae (*Scenedesmus acuminatus* and *Selenastrum capricornutum*) [22] and midge larvae (*Chironomus riparius*) [23]. The present work focuses on the practical problems and solutions encountered in the elucidation of metabolite identities.

This work fits into a continuing effort to elucidate azaarene environmental fate and toxicity mechanisms involving determination of azaarene concentrations in sediments [6], assessment of azaarene toxicity and genotoxicity towards invertebrates [23,24] and explaining differences in azaarene toxicity by structure–activity relationships [25,26].

2. Experimental

2.1. Chemicals

Phenanthridine (99%) and acridine (97%), 9[*10H*]-acridinone (99%) and 6[*5H*]-phenanthridinone (98%) were obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands. Acridine was further purified by adsorption chromatography on aluminium oxide. All solvents used were of HPLC grade, and all other chemicals were of analytical grade quality.

2.2. Toxicity experiments

The experiments with mussels, algae and midge larvae have been described in detail elsewhere [21–23]. The experiments with midge consisted of short term (96 h), semichronic (12 day) or chronic (4–5 weeks) toxicity tests. Experiments with algae lasted for 7 days. Experiments with mussels consisted of short-term (48 h) and chronic (i.e. 10 weeks) tests. In the chronic midge test water and toxicant were renewed every 7 days, and in the chronic mussel experiments every 48 h. During these experiments midge larvae were fed with commercial food (a suspension of Tetraphyl and Trouvit in water), and mussels were fed with alga *S. acuminatus*.

In all tests, organisms were exposed to dissolved concentrations of the azaarene, which were added to the medium either directly, by way of organic carrier (DMSO), or by using a generator column technique. Actual concentrations of the azaarene were monitored by RP-HPLC at regular intervals.

2.3. HPLC monitoring

Azaarene and metabolite concentrations were monitored directly by RP-HPLC in centrifuged water samples from exposure experiments with algae, mussels or chironomid larvae. A sample volume of 20 μl was injected onto a 150×4.6 mm Lichrosorb RP18 5 μm (Merck) column (using a 3×3 mm guard column containing the same stationary phase), operated at 40°C. Both fluorescence (Kratos Spectroflow

980) and UV (Applied Biosystems 785) detection were applied. For fluorescence excitation and UV absorption, a wavelength of 254 nm was used. For registration of signals at fluorescence emission wavelengths over 310 nm, a cutoff filter was applied. The mobile phase composition was maintained at 80:20 (v/v) acetonitrile–water (for phenanthridine) or 80:20 MeOH–phosphates buffer (for acridine). Samples were eluted at a flow-rate of 1.0 ml min^{-1} using a Gynkotec 480 pump.

2.4. GC–MS

In this study GC–MS was used as the initial method for confirmation of metabolite identities. Sample extracts were redissolved in ethyl acetate and injected in a HP5890/KRATOS Concept-s MACH 3 HRGC/HRMS system (Kratos) with cold on-column injection. A 60 m×0.25 mm (film: 0.25 μm) DB-5 (J&W) capillary column was used. The transfer line was maintained at 250°C. The mass spectrometer was operated in the EI mode using selected ion monitoring at the following conditions: ionization energy 70 eV, ion source temperature 250°C, analyzer pressure 1×10^{-7} Torr, scan time 1.02 s.

The temperature of the GC oven was programmed from 70°C to 200°C at 30°C min^{-1} and from 200°C to 250°C at 5°C min^{-1}. The following ions, corresponding to parent and (possible) metabolites, were monitored: 179.07/180.08, 151.06/152.06 (for acridine and phenanthridine), 195.07/196.07, 167.07/168.08 (monohydroxybenzoquinolines and their tautomers), 211.06/212.07 (dihydroxybenzoquinolines, hydroxyacridones) and 213.08/214.08 (benzoquinoline dihydrodiols). No quantitative analysis was performed.

2.5. LC–MS

The LC–MS was used after initial attempts to identify an unknown metabolite by GC–MS failed (see Section 3) and the LC–MS instrument became available at that time. The LC system consisted of a HP 1090 gradient system for delivering the mobile phase (0.4 ml min^{-1}) equipped with a Rheodyne six-port switching valve. The analytical column was a 250 mm×4.6 mm I.D. stainless-steel column packed with Supelcosil LC-18-DB, 5 μm deactivated-base C-18 material. The PROSPEKT-automated cartridge exchange, solvent selection and valve switching unit, with its solvent delivery unit, was used for sample trace enrichment. The preconcentration was carried out on 10×2.0 mm I.D. cartridges packed with 15–25 μm PLRP-S (Polymer Laboratories, Church Stretton, UK) styrene–divinylbenzene copolymer. The wavelength of a HP 1050 ultraviolet diode-array detector was set as 210 nm with 10 nm bandwidth.

A Hewlett Packard 5989 A MS Engine, equipped with a dual EI/CI ion source and high-energy dynode, was connected to the LC column outlet via an HP 58990 particle beam (PB) interface. All data were acquired on the DOS-based HP Vectra 486/66 computer using MS ChemStation software. The MS ion source block temperature was set at 250°C and that of the quadrupole analyser at 100°C. The scan range was from m/z 65 to 350 amu at a scan rate of 0.6 scans s^{-1}. The desolvation chamber temperature of the PB interface was set at 70°C and the helium nebuliser pressure at 35 psi.

After background subtraction the mass spectrum of each compound was compared with those in the (NIST and Wiley) libraries.

The PLRP-S precolumn was flushed at 5 ml min^{-1} with 5 ml of methanol and, next, 5 ml of HPLC-grade water prior to preconcentration. Subsequently, a 40 ml sample was preconcentrated at a flow of 4 ml min^{-1}. The analytes trapped on the precolumn were then desorbed in the forward flush mode with acetonitrile–water at a flow-rate of 0.4 ml min^{-1} and on-line transferred to the C-18 analytical column. The actual separation of the analytes was carried out using a 45-min linear gradient of acetonitrile–water mixture beginning with 10:90 (v/v) to 95:5 (v/v); this was held isocratically for another 10 min. The non-destructive UV DAD detector was positioned on-line in front of the PB-MS. All steps of analysis, including conditioning of the precolumn, preconcentration, separation and detection by both detectors, were performed in a fully automated way.

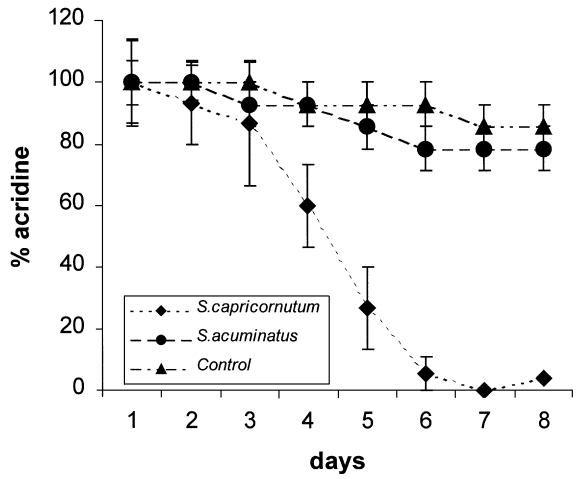

Fig. 1. Relative concentration of acridine vs. time plots from exposure experiments with algae. Triangles: control experiment. Circles, *S. acuminatus* experiment; diamonds, *S. capricornutum* experiment. Error bars represent standard deviation from three replicate experiments. Dotted lines are linear point-to-point extrapolations.

3. Results

3.1. Acridine experiments

Fig. 1 shows the concentration vs. time course for acridine from exposure experiments with two species of algae and a control experiment. As can be seen, *S. capricornutum* exhibits a significant decline of the aqueous acridine concentration, whereas in *S. acuminatus* the decline is negligible compared to the control. HPLC chromatograms of samples from the test solutions of the experiments with *S. capricornutum* invariably showed, besides the peak of acridine, an earlier eluting peak with unknown identity. In samples taken at the end of the experiment (Days 6 and 7), the peak area of the unknown peak was less than in samples taken earlier during the experiment.

Neither in short-term experiments with mussels nor in short-term or chronic (6 weeks) experiments with midge larvae metabolism of acridine was observed. In the chronic experiments with mussels, it was observed that disappearance of acridine from the medium started after 3 weeks, concurrent with a recovery of initially reduced filtration rates of the mussels. Fig. 2 shows a chromatogram obtained with the monitoring HPLC method of an aqueous sample from the chronic experiments with mussels exposed to acridine, taken just before the water and toxicant renewal. As can be seen, besides the acridine peak, an unknown peak is observed with a relatively high response in fluorescence mode and a shorter retention time, suggestive of a metabolite. In the UV mode the same peak was observed, but with a response much less than that of acridine. Within a 48 h-renewal period, the acridine concentration in the test water decreased significantly. All acridine had

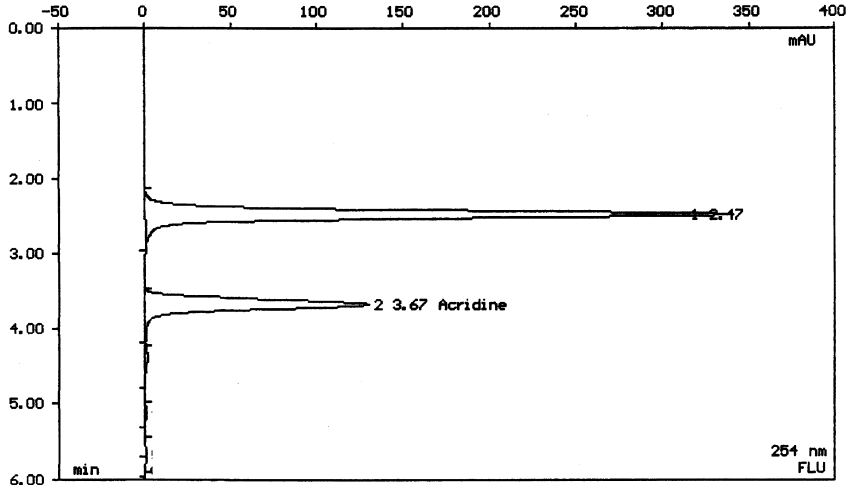

Fig. 2. Reversed phase LC–FLU chromatogram of a sample of test water taken from the chronic experiment with mussels exposed to acridine. Acridine (t_R=3.67) is preceded by a metabolite (t_R=2.47). For conditions, see Section 2.

disappeared after 48 h. Since the aquaria used in the chronic mussel experiments not only contained mussels but also periphyton and bacteria, which grow on glass walls and mussel shells, we also tested the metabolic capacity of these organisms in control treatments without mussels. Here, metabolism was also observed, but started only after Week 9. From one test solution at the end of the chronic experiment mussels were taken out of the aquarium, and water and toxicant were renewed once more. Again a significant decrease in acridine concentration was observed [21] although still some acridine was present after 48 h. Apparently bacteria or periphyton or both were able to degrade acridine.

To assess the identity of the unknown metabolite observed in the RP-HPLC chromatograms, initially it was decided to use the GC–MS technique. To that end aqueous samples taken from the algae and mussels experiments were first liquid–liquid extracted with dichloromethane (DCM). DCM was preferred to *n*-hexane since acridine is poorly soluble in the latter. DCM was also found to lead to higher recoveries for acridine than diethyl ether (100–110% vs. 50–55%, respectively). The DCM extracts had to be solvent exchanged because of its poor solvent effect performance in split/splitless injection. Initially, two solvents were selected for this purpose: cyclohexane and toluene, since it was expected that the metabolite would have a structure and, consequently, properties resembling those of acridine, which is soluble in these solvents. Thus, extracts were prescreened by GC/FID to evaluate the suitability of these solvents. In all extracts that were examined, whether redissolved in cyclohexane or toluene, invariably two peaks were observed in the chromatograms. These peaks appeared to correspond to acridine and DMSO. The latter had been used as the carrier solvent in the tests. To exclude possible discrimination effects of the injection technique, the redissolved samples were also injected by an on-column technique. Again, only peaks of acridine and DMSO were observed and no metabolite peak was present.

Referring to Section 1, it was expected that the metabolite could be a hydroxylated product of acridine. Direct gas chromatography of hydroxylated polyaromatic compounds usually results in poor chromatography. Therefore, the extracts were derivatized with acetic acid anhydride according to standardized textbook procedures into acetylated products, which can be chromatographed easily. However, extracts thus treated were found to contain no peaks at all.

These practical problems prompted us to focus further attempts regarding structure elucidation of the metabolite on LC–MS analysis. Initially an off-line procedure was used, consisting of a liquid–liquid extraction of 2.5 ml of aqueous test solution with DCM, followed by solvent exchange into methanol. The methanol extract was injected in the LC–MS. The resulting chromatogram showed two peaks. The mass spectra of these peaks corresponded to acridine and to DCM.

Since DCM would be present in all samples as it was used in the liquid–liquid extraction step, further attempts were made with an on-line procedure [27]. On-line LC–MS enabled us to refrain from elaborate solvent extractions of the test solutions. Instead, by using the preconcentration setup described in Section 2, the sample could be used in aqueous form and subsequently concentrated on-line to arrive at sufficiently high concentrations to allow for MS detection.

To this end 40 ml of the test solution from a mussel experiment was introduced into the system. The resulting total ion chromatogram (TIC) is shown in Fig. 3. The TIC shows two major and three minor distinct peaks. Through comparison with the available particle beam-MS spectral library peak 1 could be identified as acridine, peak 2 as 9[*10H*]-acridinone, also known as acridone, peak 3 as DMSO and peak 4 as 4,4′-(1-methylethylidene)bisphenol. Peak 5 could not be identified unequivocally due to its complicated mass spectrum.

The identity of the metabolite was confirmed by preparing solutions of acridone and injecting these into the LC system used for monitoring. Acridone is known to possess a relatively high fluorescence response factor [28] and to be poorly soluble in organic solvents like *n*-hexane, iso-octane, cyclohexane or toluene.

3.2. Phenanthridine experiments

Experiments with phenanthridine were carried out with midge larvae only. Short-term toxicity experi-

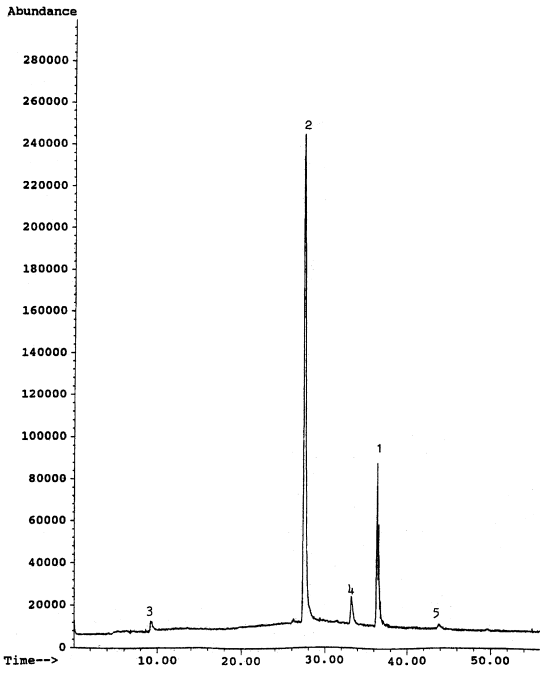

Fig. 3. Total ion chromatogram of on-line LC–PBMS analysis of test water from the chronic experiment with mussels exposed to acridine. For conditions, see Section 2.

ments were carried out at different toxicant concentrations. In these experiments the time course of the dissolved phenanthridine concentration invariably showed a steady decrease during the experiment. The relative disappearance of phenanthridine from the test medium in experiments with different nominal concentrations is shown in Fig. 4. Maximum disappearance is shown to occur at a nominal concentration of 0.16 mg l^{-1}.

Semichronic experiments showed that phenanthridine disappeared completely from the test medium after 11 days, whereas in the control treatment a decrease of no more than 10% in the concentration of phenantridine was observed (see Fig. 5).

HPLC chromatograms of the test solutions revealed one peak of unknown identity. A plot of the FLU response of metabolite vs. time is shown in Fig. 6. Apparently, the concentration of the unknown compound increases with decreasing phenanthridine concentration up to a certain maximum followed by a decline with time.

Considering the identity of the metabolite of

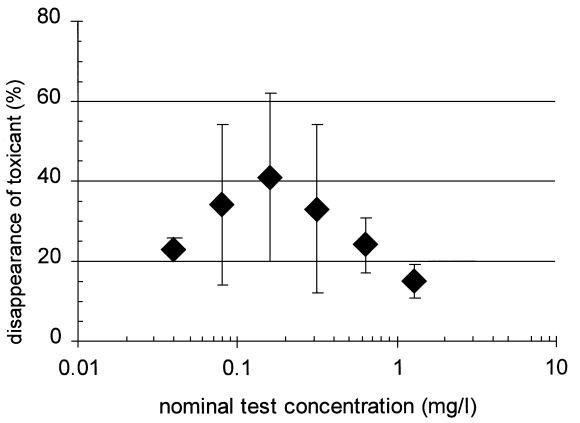

Fig. 4. Relative disappearance of phenanthridine in test waters taken at the end of short-term (96 h) toxicity tests with midge larvae. Each mark corresponds to a different exposure concentration. Error bars represent standard deviation from three replicate test tanks.

acridine found in the experiments described above, the possibility that its analogue, i.e. 6[*5H*]-phenanthridinone (phenanthridone) had been formed out of phenanthridine was investigated first. To that end stock solutions of phenanthridone were prepared and analysed using RP-HPLC. The resulting chromatograms showed a peak with a capacity factor equal to

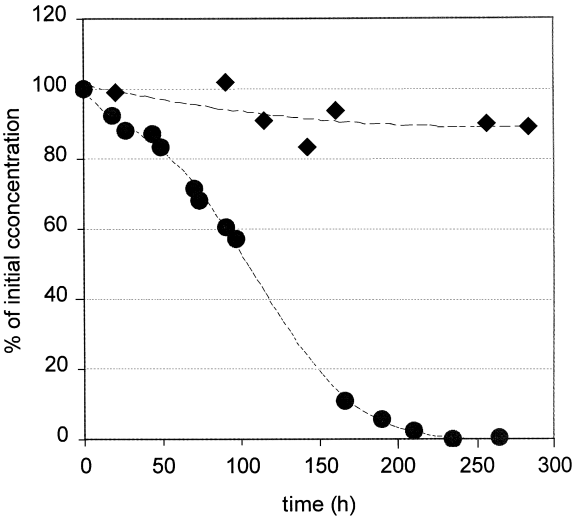

Fig. 5. Relative disappearance of phenanthridine in test waters during semichronic (12 day) exposure experiments with midge larvae. Diamonds, control experiment; circles, midge experiment; dotted lines represent polynomial fit.

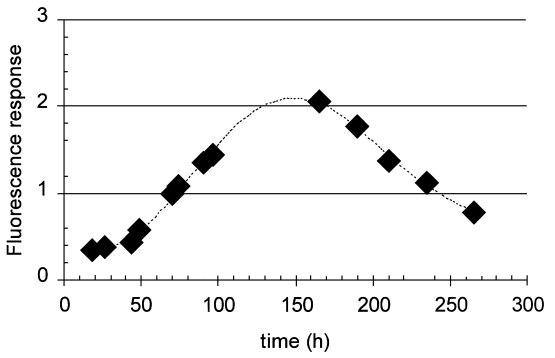

Fig. 6. Occurrence of metabolite, as reflected by its fluorescence response, in test water sampled during a semichronic experiment with midge larvae exposed to phenanthridine. Dotted line represents polynomial fit.

that of the unknown compound observed in chromatograms from test solutions.

Next, the test water from the semichronic experiment at $t=166$ h was extracted with *n*-hexane and concentrated to enable further analysis using HRGC–HRMS. Contrary to acridine, the gas chromatography of phenanthridine is less hampered by solubility problems and solute↔stationary phase interactions. Fig. 7 shows selected ion monitoring traces of the concentrated sample extract, monitoring ions 195.07 (M^+), 196.07 ($[M+1]^+$), 167.07 ($[M\text{-}CO]^+$) and 168.08 ($[M\text{-}CO+1]^+$). Retention times of the peaks as well as abundance ratios of selected ions matched those of a standard solution of phenanthridone. It was therefore concluded that the identity of the metabolite was phenanthridone.

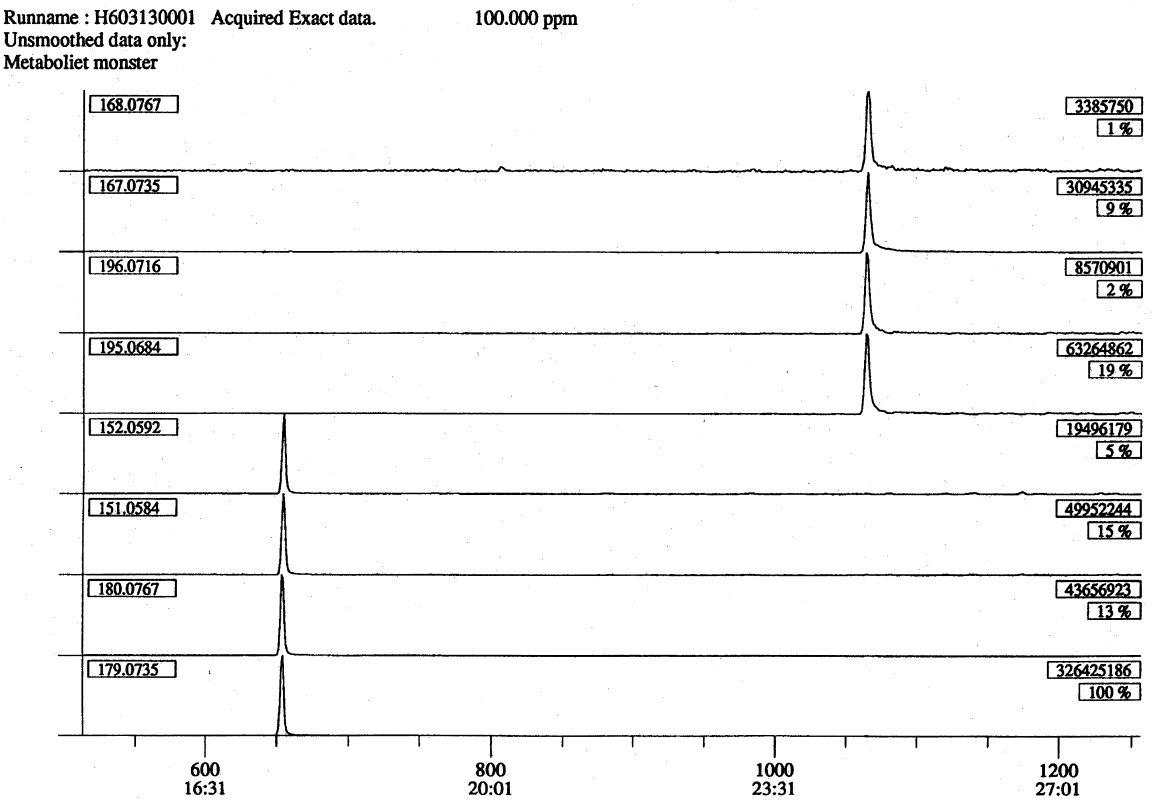

Fig. 7. High-resolution GC–MS of a test water sample extract from a semichronic experiment with midge larvae exposed to phenanthridine. For conditions, see Section 2.

4. Discussion

4.1. Acridine

The poor solubility of acridone in several organic solvents explains why the initial attempts to analyze the metabolite by GC failed. The metabolite was either extracted incompletely by the liquid–liquid extraction procedure with DCM, or did not dissolve in the solvent used for GC. Decomposition as a cause for its absence in GC runs can be ruled out since a standard solution in ethyl acetate does show a peak in GC–FID.

The initial quest for the identity of the metabolite was somewhat hindered by the fact that in RP-HPLC runs of some sterile control samples a peak eluted with a retention time similar to that of the metabolite. It was found that in some batches of commercial acridine, acridone can be present as a contaminant in significant concentrations (up to several %). Purifying acridine stock solutions through adsorption chromatography using alumina solved this problem.

Although a fairly well documented compound [28], acridone has not been mentioned in the literature as a major metabolite in transformation studies. This is somewhat surprising given the fact that oxidation at position 9 is not unlikely due to the relatively low electron density at this carbon atom compared to any other C atom in the acridine [28]. Acridone had already been suggested in 1904 as a possible product of metabolism of acridine by rabbits [29]. In studies with rat liver homogenates acridone has been observed as a minor metabolite [12,13]. One of the reasons for the relatively poor attention given to acridone in earlier studies may be that acridone is an intermediate in the overall metabolic breakdown of acridine. Although in the present study other acridine-related metabolites were neither observed in HPLC nor in LC–MS runs, we have indeed observed a decline in acridone concentrations after an initial increase in both the algal and in the chronic mussel experiments [26].

4.2. Phenanthridine

The identity of the metabolite appearing in the phenanthridine experiments was unambiguously confirmed by comparing retention times on HPLC of standard solutions of isomers, e.g. hydroxyphenanthridines and phenanthridine-N-oxide. The latter has been identified as a major metabolite in mammalian in vitro tests [14].

A possible explanation for the optimum observed in the relative % disappearance of phenanthridine from the medium (Fig. 4) at different nominal short-term test concentrations, may be that at lower concentrations substrate limitation has occurred. At higher concentrations the metabolic process may start to get inhibited by the toxicant itself.

In the semichronic experiment phenanthridine disappeared completely when midge larvae were present in the test vessel. The influence of food supply (which is essential in longer lasting tests) during the semichronic tests was also investigated. To this end a separate treatment with food only was performed. After 11 days 70% of the initial toxicant concentration had disappeared. This experiment showed that bacteria (growing on the midge food) were also able to transform phenanthridine, similar to the findings for acridine in chronic mussel experiments. HPLC runs of this experiment showed a metabolite peak having the same retention time as the metabolite observed in the extract of the midge experiment. It may be concluded that both bacteria and midge larvae are capable of transforming phenanthridine into phenanthridone.

The decrease observed in the metabolite peak area with time (Fig. 6) after the initial increase indicates that phenanthridone itself may be further degraded during the experiment by the larvae. In the separate food experiment described above, it was found that further transformation of phenanthridone did not occur. Hence the bacteria were not able to further degrade phenanthridone or did this only at a very low rate of transformation.

High-resolution MS was used in order to help in the identification of the actual metabolite. This technique enables one to distinguish fragments due to the loss of CH_2N (m/z: 28.016) (which is common in MS of benzoquinolines) from those due to loss of CO (m/z: 27.995) from the molecular ion (see Fig. 7). Since the latter loss was actually observed, the presence of an oxygen atom in the molecule was confirmed.

Phenanthridone, like acridone, has been found as a minor metabolite only in mammalian studies [14,30].

In the present study, none of the other metabolites mentioned in mammalian studies [14] were found in the test waters. Although in the present work only the test water has been analysed, and the detection limits of our methods for, for example, dihydrodiol derivatives may have been high (as no standards were available of these derivatives), we believe that one can conclude that the keto-derivatives are major metabolites in the aquatic invertebrate breakdown route of azaarenes.

5. Concluding remarks

This study has shown that lower aquatic organisms possess the capacity to transform azaarenes into keto-derivatives. Until now, to our knowledge, this type of metabolite has not been identified as a major product of biotransformation. Oxygen insertion is usually mediated through Cytochrome P450 coenzymes [31], which therefore are likely to be present in the organisms studied.

Risk assessment of azaarenes is usually based on the parent compounds. From the present study it appears that this may have to be extended to metabolites. The assessment is further complicated by the apparent outcome of our studies that closely related compounds like isomers exhibit species–specific biotransformation possibilities. Recent findings of our group [24] that the keto-metabolites may be more genotoxic than the parent azaarenes underline the importance of these statements.

This work is an example of a quest typical for metabolism studies, showing the experimental difficulties the analyst may encounter. It has shown that LC–MS and on-line preconcentration may be instrumental in the identification of transformation products from NPAH formed by aquatic biota.

Acknowledgements

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