

## TOXICITY OF NITROGEN-CONTAINING POLYCYCLIC AROMATIC HYDROCARBONS (NPAH) TO THE MIDGE *CHIRONOMUS RIPARIUS* (DIPTERA)

**Eric A.J. Bleeker, Marion C. Buckert-de Jong, Harm G. van der Geest  
and Michiel H.S. Kraak**

University of Amsterdam, ARISE, Section of Aquatic Ecotoxicology  
Kruislaan 320, 1098 SM Amsterdam, The Netherlands

**Keywords:** NPAH, toxicity, *Chironomus riparius*

### Summary

So far, most research on the toxicity of polycyclic aromatic hydrocarbons (PAHs) has been focused on the non-substituted PAHs. The substituted PAHs are most likely more reactive and potentially more toxic than parent PAHs and therefore the toxicity of heterocyclic PAHs is studied here. Acute toxic effects of quinoline, acridine, phenanthridine, benzo[*f*]quinoline and benz[*a*]acridine on *Chironomus riparius* larvae are determined. Growth was only reduced at concentrations very close to lethal doses. Toxicity increased with an increase of the number of aromatic rings of the compound, but also within the three benzoquinoline isomers significant differences in toxicity are observed. This indicates a specific action of some NPAHs thus complicating an assessment of environmental risk.

### INTRODUCTION

The research on polycyclic aromatic hydrocarbons (PAHS) has improved the knowledge on this group of compounds considerably. Some questions, however, are still unanswered. While there are many carcinogenicity data, only few data on toxicity to aquatic organisms have been generated. Furthermore, the relationship between the different kinds of effects of PAHs remains unclear (Groenendijk, 1993): are the observed effects caused by carcinogenicity, mutagenicity, teratogenicity, direct toxicity or combinations of these four? These uncertainties make it difficult to set environmental standards for this group of compounds.

Until now, research has been focused on non-substituted 'parent' PAHs. However, substituted PAHs, both external (H-atoms are substituted) and internal (C-atoms have been replaced), are most likely more reactive and potentially more toxic than parent PAHs. Likely, parent PAHs are only part of the total effective toxicity/mutagenicity in field samples. Especially the very diverse group of substituted PAHs would be responsible for observed effects (Opperhuizen *et al.*, 1993). Therefore, this study focuses on one of the family of substituted PAHs, the nitrogen-substituted PAHs (NPAHs). There are only few data on NPAHs, both on their presence in the field and on their effects (e.g. Santodonato & Howard, 1981, Southworth *et al.*, 1978). These data, however, are essential for the validation of standards for the large range of NPAHs.

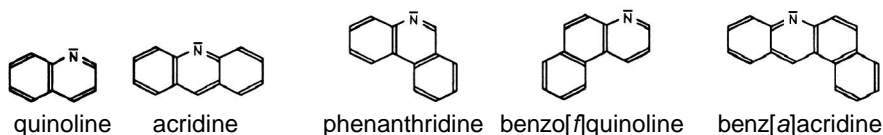
The few field surveys carried out so far, demonstrate that NPAHs can be present in detectable concentrations in waters and in sediments (Adams & Giam, 1984, Furlong & Carpenter, 1982, Van Genderen *et al.*, 1994). Data on the effects of NPAHs on aquatic organisms mostly concern studies on *Daphnia magna* and *D. pulex* (Johnson *et al.*, 1990, Parkhurst *et al.*, 1981, Southworth *et al.*, 1978). Because of the limited variety in test organisms, it is important to collect more effect concentrations of these compounds for other

aquatic organisms. In this study five NPAHs were tested in acute experiments on larvae of the midge *Chironomus riparius*.

## MATERIALS AND METHODS

Fifty first instar larvae were placed in a glass vessel containing 100 ml Dutch Standard Water (DSW) (Van de Guchte *et al.*, 1993). The larvae originated from a laboratory culture and were fed 1 ml of a solution of Tetraphyl<sup>®</sup> and Trouvit in DSW.

The toxicants tested were quinoline (1, 2, 4, 8, and 16 mg/L), acridine (0.04, 0.08, 0.16, 0.31, and 0.63 mg/L), phenanthridine (0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5 mg/L), benzo[*f*]quinoline (0.08, 0.16, 0.31, 0.63, and 1.25 mg/L) and benz[*a*]acridine (0.004, 0.008, 0.016, 0.031, and 0.063 mg/L). In figure 1 the structure formulas of these compounds are given. All concentrations of each compound, including two controls, were tested in triplicate. At the start of each experiment the length of 25 larvae was measured and after 96 h surviving larvae were counted and their length measured.



**Figure 1.** The structure formulas of the NPAHs tested.

After 1 h and after 96 h water samples were taken to determine the actual toxicant concentration in the water. 2.5 ml water was sampled and centrifuged (4000 rpm during 5 min.). 1 ml of the supernatant was then measured by High Pressure Liquid Chromatography (HPLC), using fluorescence detection (Kratos Spectroflow 980) for benz[*a*]acridine and UV detection (Applied Biosystems model 785) for the other compounds. A 150\*4.6 mm LiChrosorb 5  $\mu$ m RP-18 analytical column was used with a 4\*4 mm LiChrosphere 5  $\mu$ m RP-18 guard-column. Column temperature was set to 40 °C and 20  $\mu$ l per sample was automatically analysed. Quinoline was detected at a wavelength of 220 nm and was eluted with a mixture of 65 % methanol (J.T. Baker Analyzed HPLC Reagent, min. 99.8 %) and 35 % 13.4 mM phosphate buffer (Merck, 1.0998 Titrisol<sup>®</sup>, pH 7.00  $\pm$  0.02). Acridine, phenanthridine and benzo[*f*]quinoline were detected at a wavelength of 254 nm after elution with a mixture of 80 % methanol and 20 % phosphate buffer. Benz[*a*]acridine was detected at an excitation wavelength of 285 nm and an emission wavelength of >354 nm and eluted with a mixture of 80 % acetonitrile (J.T. Baker Analyzed HPLC Reagent, min. 99.9%) and 20 % water (J.T. Baker Analyzed HPLC Reagent).

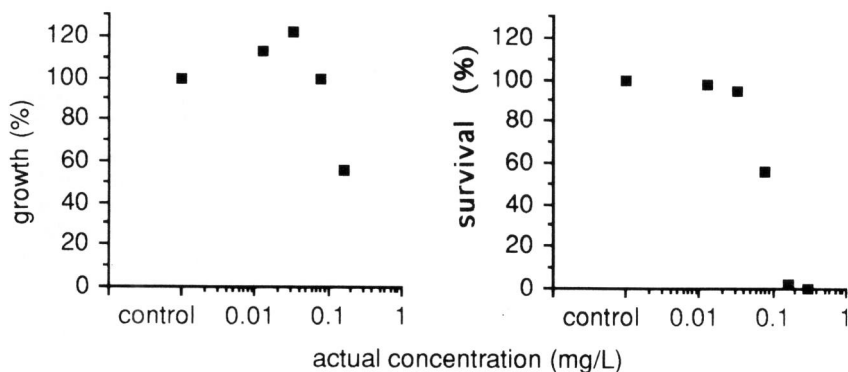
The LC50 was calculated using probit analysis (Finney, 1971), while the data on growth were analysed using the non-parametric statistic test of Kruskal-Wallis, according to Sokal & Rohlf (1981).

## RESULTS AND DISCUSSION

Figure 2 shows two typical dose response curves for the toxicity of NPAHs, in this case acridine, to the midge *Chironomus riparius*. A clear dose response curve was observed for survival, but when larvae survive they tend to grow normally. The lowest concentration that reduced growth, also caused about 98% mortality, so an EC50 for growth could not be calculated. The same pattern was found for all NPAHs tested.

The LC50 values are given in table 1. Acridine, phenanthridine and benzo[*f*]quinoline, all having three ringed molecules, are more toxic than the two ringed quinoline.

Benz[a]acridine, a four ring structure is much more toxic than each of the three ring compounds. Thus, toxicity increases with increasing number of rings. This is in agreement with results for *Chironomus tentans* (Cushman & McKamey, 1981) and for *Daphnia magna* (Parkhurst *et al.*, 1981), both exposed to quinoline and acridine and with results for the frog *Rana pipiens* exposed to pyridine, quinoline and acridine (Birge & Cassidy, 1983). This increase in toxicity coincides with an increase in lipophilicity. Another explanation can be that the smaller compounds degrade more easily than the larger ones. Rainbow trout (*Salmo gairdneri*) quickly took up and metabolised quinoline (Bean *et al.*, 1985) and micro-organisms can also degrade this compound (Bollag & Kaiser, 1991, Pereira *et al.*, 1988, Sutherland *et al.*, 1994b). Sutherland *et al.* (1994a) reported that also acridine was metabolised by mycelia of *Cunninghamella elegans*. In experiments with the mussel *Dreissena polymorpha* it was found that degradation of acridine took place, at least partly due to the activity of the mussels (Kraak *et al.*, in prep). Metabolisation of the other three-ring structures and of benz[a]acridine has not been reported. In our experiments with phenanthridine, however, an extra peak appeared in the HPLC-chromatograms. The area of this peak increased, while the peak of phenanthridine decreased, indicating that this peak was a metabolite of phenanthridine, so far not identified.



**Figure 2.** Growth and survival of *Chironomus riparius* larvae at different concentrations of acridine. Both survival and growth are plotted as percentage of the corresponding control.

The LC50-values in table 1 are based on both nominal and actual concentrations, because big differences were observed in the recovery of the different compounds. While all the quinoline remains in the water, the recovery for the other compounds is much lower. This is mostly due to the fact that the compounds bind to the glass wall of the aquarium, to the food and to the midges. Especially in the case of the relatively lipophilic four ring structure benz[a]acridine concentrations in the water rapidly dropped. In this case it is more realistic to look at the nominal LC50, because the actual concentration in the water is an underestimation of the total exposure to benz[a]acridine. The recovery percentage clearly reflects the increasing lipophilicity, and therewith this increasing importance of uptake via food, relative to uptake via water. However, further research on the uptake of these compounds via water or particles is needed.

From the LC50 values for acridine found for juvenile copepods (*Diaptomus clavipes*) (1.59 and 1.55 mg/L) (Cooney & Gehrs, 1984), the 24h LC50 (2.92 mg/L) and the 24h EC50 (1.71 mg/L) for *Daphnia pulex* (Southworth *et al.*, 1978), and the 48h LC50 (2.3 mg/L) and the chronic LOEC (0.8 mg/L) for *Daphnia magna* (Parkhurst *et al.*, 1981) it is clear that the effect concentrations for the species tested so far are in the same range. Also the 48h LC50

(1.96 mg/L) for *Chironomus tentans* (Cushman & McKamey, 1981), strongly related to our test species *C. riparius*, is in this range. This may indicate a similar mode of action at cellular level, e.g. a narcotic effect. The LC50 (0.07 mg/L) for our species, however, is much lower. An explanation for these differences between *C. riparius* and *C. tentans* can be that Cushman & McKamey (1981) used fourth instar larvae, while in these experiments the more sensitive first instar larvae have been used (Williams *et al.*, 1986). Another explanation can be that our experiments lasted for 96 h, while the experiments of Cushman & McKamey (1981) only lasted for 48 h. This can also explain the differences in LC50 for quinoline between Cushman & McKamey (1981) (57.2 mg/L) and this study (6.8 mg/L). For *Daphnia pulex* exposed to benz[*a*]acridine, a 24h LC50 of 0.449 mg/L was calculated (Southworth *et al.*, 1978). This is a much higher concentration than found in this study, even compared to the nominal LC50. The most likely explanation is, of course, the difference in test organism, but also the difference in exposure time can play a role.

	recovery (%)	LC50nominal (mg/L)	LC50actual (mg/L)
Qui	103.18 ± 4.99	5.0853 (4.4122 - 5.8612)	6.7550 (5.4640 - 8.3510)
Acr	39.82 ± 2.30	0.1535 (0.1343 - 0.1754)	0.0720 (0.0690 - 0.0750)
Phe	72.33 ± 1.53	0.7167 (0.6722 - 0.7640)	0.5131 (0.4810 - 0.5472)
BfQ	64.94 ± 1.19	1.0017 (0.8873 - 1.1308)	0.6060 (0.5480 - 0.6700)
BaA	7.87 ± 1.05	0.0902 (0.0769 - 0.1059)	0.0127 (0.0108 - 0.0149)

**Table 1.** Recovery and calculated effect concentrations of NPAHs. Qui = quinoline, Acr = acridine, Phe = phenanthridine, BfQ = benzo[*f*]quinoline, BaA = benz[*a*]acridine. Recovery : percentage of the added NPAH recovered in the water after 96 hours ± standard error. In the third and fourth columns LC50-values are given for the NPAHs tested, based on both nominal and actual concentrations (95% confidence limits are given between brackets).

Although toxicity clearly increased with the number of aromatic rings of the compound, significant differences in toxicity were also observed between the three isomers, acridine, phenanthridine and benzo[*f*]quinoline, indicating a more selective effect, in contrast with the hypothesis of a narcotic effect. This further complicates the setting of environmental standards for this group of compounds.

#### ACKNOWLEDGEMENTS

We like to thank the Institute for Inland Water Management and Waste Water Treatment (RIZA) and the National Institute for Coastal and Marine Management (RIKZ) for their financial support of this project and Prof. Dr. W. Admiraal for his comments.

## REFERENCES

- ADAMS, J., & C.-S. GIAM, 1984 — Polynuclear azaarenes in wood preservative waste water. *Environ. Sci. Technol.* **18**(5), 391-394.
- BEAN, R.M., D.D. DAUBLE, B.L. THOMAS, R.W. HANF & E.K. CHESS, 1985 — Uptake and biotransformation of quinoline by rainbow trout. *Aquat. Toxicol.* **7**, 221-239.
- BIRGE, W.J. & R.A. CASSIDY, 1983 — Structure-activity relationships in aquatic toxicology. *Fund. Appl. Toxicol.* **3**, 359-368.
- BOLLAG, J.-M. & J.-P. KAISER, 1991 — The transformation of heterocyclic aromatic compounds and their derivatives under anaerobic conditions. *Crit. Rev. Environ. Control* **21**(3,4), 297-329.
- COONEY, J.D. & C.W. GEHRS, 1984 — Effects of temperature, feeding and acridine on development and mortality of eggs and nauplii of *Diatomus clavipes* Schacht. *Aquat. Toxicol.* **5**, 197-209.
- CUSHMAN, R.M. & M.I. MCKAMEY, 1981. A *Chironomus tentans* bioassay for testing synthetic fuel products and effluents, with data on acridine and quinoline. *Bull. Environ. Contam. Toxicol.* **26**, 601-605.
- FINNEY, D.J., 1971 — *Probit analysis*. 3rd Edn., Cambridge Uni. Press, London.
- FURLONG, E.T., & R. CARPENTER, 1982 — Azaarenes in Puget Sound sediments. *Geochim. Cosmochim. Acta* **46**, 1385-1396.
- GROENENDIJK, D., 1993 — Stikstofhoudende polycyclische aromaten in het milieu met speciale aandacht voor de toxiciteit voor benthische organismen. M.Sc. thesis, University of Amsterdam, Amsterdam, The Netherlands, in Dutch.
- JOHNSON, D.W., M.V. HALEY & W.G. LANDIS, 1990 — The aquatic toxicity of the sensory irritant and riot control agent dibenz[*b,f*]-1,4-oxazepine (CR). In: *Aquatic Toxicology and Risk Assessment: Thirteenth Volume*, ASTM STP 1096 (W.G. Landis & W.H. van der Schalie eds.) ASTM, Philadelphia: 199-221.
- KRAAK, M.H.S., C. AINSCOUGH, A. FERNANDEZ, P.L.A. VAN VLAARDINGEN, P. DE VOOGT & W. ADMIRAAL. Short-term and chronic exposure of the zebra mussel (*Dreissena polymorpha*) to the NPAH acridine: effects and metabolisation. In prep.
- OPPERHUIZEN, A., G.J. DE MAAGD & W. SEINEN, 1993 — Polycyclische aromatische koolwaterstoffen: de probleemstoffen voor het Nederlands aquatische milieu in de jaren '90? Review RITOX RUU, Utrecht, The Netherlands, in Dutch.
- PARKHURST, B.R., A.S. BRADSHAW, J.L. FORTE & G.P. WRIGHT, 1981. The chronic toxicity to *Daphnia magna* of acridine, a representative azaarene present in synthetic fossil fuel products and waste waters. *Environ. Pollut. (A)* **24**, 21-30.
- PEREIRA, W.E., C.E. ROSTAD, T.J. LEIKER, D.M. UPDEGRAFF & J.L. BENNETT, 1988 — Microbial hydroxylation of quinoline in contaminated ground water: evidence for incorporation of the oxygen atom of water. *Appl. Environ. Microbiol.* **54**(3), 827-829.
- SOKAL, R.R., & F.J. ROHLF, 1981 — *Biometry*, 2nd ed. Freeman, NY.
- SOUTHWORTH, G.R., J.J. BEAUCHAMP & P.K. SCHMIEDER, 1978 Bioaccumulation potential and acute toxicity of synthetic fuel effluents in freshwater biota: azaarenes. *Environ. Sci. Technol.* **12**, 1062-1066.

- SUTHERLAND, J.B., F.E. EVANS, J.P. FREEMAN, A.J. WILLIAMS, J. DECK & C.E. CERNIGLIA, 1994a — Identification of metabolites produced from acridine by *Cunninghamella elegans*. *Mycologia* **86**(1), 117-120.
- SUTHERLAND, J.B., J.P. FREEMAN, A.J. WILLIAMS & C.E. CERNIGLIA, 1994b — N-oxidation of quinoline and isoquinoline by *Cunninghamella elegans*. *Exp. Mycol.* **18**, 271-274.
- VAN DE GUCHTE, C., E.M.M. GROOTELAAR & J.L. MAAS, 1993. "Chironomid, chronic toxicity test on larvae in spiked freshwater sediment" *OECD guideline for testing of chemicals: Draft Guideline*. Working document 93.155X, Institute for Inland Water Management and Waste Water Treatment (RIZA), Lelystad, The Netherlands.
- VAN GENDEREN, J., T.H.M. NOIJ & J.A. VAN LEERDAM, 1994 — Inventory and toxicological evaluation of organic micropollutants. RIWA-report, Amsterdam, The Netherlands.
- WILLIAMS, K.A., D.W.J. GREEN, D. PASCOE & D.E. GOWER, 1986 — The acute toxicity of cadmium to different larval stages of *Chironomus riparius* (Diptera: Chironomidae) and its ecological significance for pollution regulation. *Oecologia (Berlin)* **70**, 362-366