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STRUCTURAL-BASED DIFFERENCES IN ECOTOXICITY OF BENZOQUINOLINE ISOMERS TO THE ZEBRA MUSSEL (*DREISSENA POLYMORPHA*)

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Abstract—Effects of four benzoquinoline isomers on the filtration rate of the zebra mussel (*Dreissena polymorpha*) were analyzed, to study the effect of minor differences in chemical structure on adverse biological effects. Filtration rates were measured after 48 h of exposure to different concentrations of acridine, phenanthridine, benzo[*f*]quinoline, and benzo[*h*]quinoline in the water. The 50% effective concentration (EC50) values for filtration rate of the four isomers differed significantly. Effects increased in the order benzo[*f*], -[*h*], -[*b*], and -[*c*]quinoline, and the difference between the most toxic isomer and the least toxic isomer amounted to a factor of 30. Attempts were made to relate these differences in toxicity to the structure of the isomers. Size- or topology-related molecular descriptors provided insufficient resolution to distinguish between the benzoquinoline isomers, and none of the electronic descriptors separately provided a significant correlation with the observed effects. In an alternative approach, molecular shape, accessibility, and minimum agent–macromolecule distance were used to represent repulsive and attractive forces between the benzoquinoline isomers and biological membranes. This approach could tentatively explain the observed effects and is supported by a high correlation between the EC50 data and the reversed-phase C18-HPLC behavior of the benzoquinolines (k_o), which is likely to be governed by similar processes.

Keywords—Zebra mussel Filtration rate Polycyclic aromatic hydrocarbons Benzoquinoline molecular descriptor

INTRODUCTION

Benzoquinolines belong to the azaarenes, a family of nitrogen containing polycyclic aromatic hydrocarbons (NPAH), in which one C-atom of the aromatic ring structure has been replaced by an N atom. Four benzoquinoline isomers are commercially available: benzo[*b*]quinoline (acridine), benzo[*c*]quinoline (phenanthridine), benzo[*f*]quinoline, and benzo[*h*]quinoline (Fig. 1). Effects of acridine have been determined on several macroinvertebrate species, among which the midges *Chironomus riparius* (Bleeker et al., unpublished data) and *Chironomus tentans* [1,2], the amphipod *Gammarus minus* [2], the snail *Physa gyrina* [2], the mussel *Dreissena polymorpha* [3], the calanoid copepod *Diaptomus clavipes* [4], and two daphnid species, *Daphnia pulex* [5] and *Daphnia magna* [2,6–8]. However, the other benzoquinoline isomers have almost never been tested and therefore the present study examines if such closely related compounds differ in their adverse effects.

Field surveys indicated that benzoquinolines can be present in detectable concentrations in sediments [9–11], in city air [12], in groundwater contaminated with coal tar [13], and in river water [14]. Adams and Giam [15] measured a concentration of total azaarenes of 1,300 mg/kg in wood preservative wastewater, in which the benzoquinoline concentrations were 55 mg acridine/kg, 53 mg benzo[*h*]quinoline/kg, and 71 mg phenanthridine plus benzo[*f*]quinoline/kg. The four benzoquinolines are mutagenic [12]. In experiments on the growth rate of unicellular algae [16] benzoquinolines appeared to be the more toxic azaarenes. For the ciliate *Tetrahymena pyriformis*, acridine was one of the most toxic of 24 nitrogen heterocyclic compounds tested [17].

The zebra mussel (*D. polymorpha*) plays an important role

in various freshwater ecosystems. This mussel is able to reduce high phytoplankton abundances by its high filtration activity [18,19]. It is the main source of food for diving ducks and coots [20,21] that winter in large quantities in the Netherlands, and for benthivorous fish, such as roach (*Rutilus rutilus*) and carp (*Cyprinus carpio*) [22]. Zebra mussels are sedentary organisms that live attached to solid substrates above the sediment where they filter the water column. Consequently, the aqueous phase is the dominant uptake route for organic contaminants [23] and therefore, the zebra mussel is one of the benthic invertebrate species more likely to accumulate the relatively polar NPAHs. Filtration rate was chosen as an effect parameter, because it is a sensitive sublethal parameter [24] and it is an important parameter considering the ecological role *Dreissena* fulfills.

Attempts will be made to relate possible differences in toxicity to the structure of the isomers. The structure of the compounds can be quantified by parameters describing geometric molecular bulk properties (such as molecular surface area or volume), molecular shape parameters (such as kappa indices [25] or the globularity factor, cf [26]), electronic properties (e.g., charge distribution or dipole moment) and macroscopic physicochemical properties like partition coefficients (e.g., *n*-octanol/water partition coefficient or Henry's law constant), and chromatographic retention [27].

MATERIALS AND METHODS

Mussels and water were collected from Lake Markermeer (the Netherlands), a relatively clean location [28]. The water was sieved (25 μ m) and kept in a storage barrel from which it was pumped continuously over a sand filter. The mussels were sorted by length (1.6–2.2 cm) and distributed over the

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Fig. 1. Structures of the four benzoquinoline isomers tested in this study.

experimental treatments such that the average length did not differ between treatments. An experimental treatment consisted of 25 mussels placed in a glass aquarium (10 L), containing 3 L of filtered lake water. On the bottom of the aquaria stones from the collection side were placed to facilitate reattachment of the mussels, because the glass surface appeared to be too smooth. The water temperature was kept at 15°C, the hardness was 150 mg CaO/L and the pH was 7.9. The water was aerated and always saturated with oxygen. The aquaria were covered with glass plates, to reduce evaporation.

The following benzoquinoline concentrations were tested: acridine: 0 (control), 0.16, 0.31, 0.63, 1.25, and 2.50 mg/L; phenanthridine: 0, 0.08, 0.16, 0.31, 0.63, 1.25, 2.50, and 5.00 mg/L; benzo[f]quinoline: 0, 0.16, 0.31, 0.63, 1.25, 2.50, and 5.00 mg/L; benzo[h]quinoline: 0, 0.31, 0.63, 1.25, 2.50, and 5.00 mg/L. There were two replicates per treatment and a total number of 1,350 mussels were used. Benzoquinoline was added to the water in the aquaria the day after the mussels were collected, using generator column-derived stock solutions. Separate stock solutions were made for each benzoquinoline by dissolving 100 mg benzoquinoline in 50 ml methanol. Four g Chromosorb G AW (60–80 mesh; Chrompack, Middelburg, the Netherlands) was added, after which the methanol was evaporated, using a nitrogen gas flow in an incubator at 50°C. Next, the benzoquinoline-loaded chromosorb was placed in a 20-cm-long glass column. One liter of particle-free lake water was pumped into the bottom side of the column by a peristaltic pump at a flow rate of 1.7 ml/min. The benzoquinoline-containing water was left at the top end of the column, where it was directed into an erlenmeyer. Column and erlenmeyer were wrapped in aluminum foil to prevent photodegradation of the benzoquinoline. The purity of the compounds was 97% for acridine (Aldrich, Zwijndrecht, the Netherlands), phenanthridine (Aldrich) and benzo[h]quinoline (Janssen Chimica, Geel, Belgium) and 99% for benzo[f]quinoline (Acros, Geel, Belgium).

Test solutions were renewed after 48 h and filtration rate was measured. One hour after addition, just before renewal and 1 h after renewal, water samples (1 ml) were taken, which were analyzed for the corresponding benzoquinoline by HPLC. Concentrations of benzoquinoline were determined directly, without a clean-up or preconcentration step, using reversed-phase liquid chromatography (LC). The LC system and measuring conditions were as follows: a Gynkotek 480 pump was used in combination with a fluorescence detector (Kratos Spectroflow 980) and a UV-absorbance detector (Applied Biosystems 785). A 20-μl sample was injected onto a 150 × 4.6-mm column packed with Lichrosorb RP 18 (Merck, particle

diameter 5 μm), with a 30 × 3-mm guard column containing the same packing material. The columns were thermostated at 40°C. The mobile phase consisted of a mixture of methanol (J.T. Baker, Deventer, the Netherlands, HPLC grade, >99.8%) and a 15 mM phosphate buffer (pH 7.0) in water (J.T. Baker, HPLC grade) at an 80/20 or 85/15 ratio. The mobile phase was vacuum degassed directly before use. All elutions were performed isocratic at a flow rate of 1 ml/min. The four benzoquinolines were detected on the UV detector, set at a wavelength of 254 nm. The fluorescence detector was operated at an excitation wavelength of 254 nm; emission was detected at wavelengths >310 nm. The average actual concentrations of benzoquinoline to which the mussels were exposed during the experiments were determined from the measured benzoquinoline values, using integral calculus.

Filtration rate was measured after 48 h of exposure. To determine the filtration rate, the mussels were fed the green alga *Scenedesmus acuminatus* (20,000 cells/ml). The algal concentration decreased due to the filtration activity of the mussels. This decrease in algal concentration was quantified by taking triplicate water samples (5 ml) from each treatment at 0, 10, and 20 min after addition of the algae. The algal concentrations in the samples were measured using a Coulter counter. The filtration rate was calculated from the decrease in algal concentration, according to Coughlan's formula [29]:

$$m = \frac{M}{nt} \ln \frac{C_0}{C_t}$$

in which m = filtration rate in ml/mussel/h, M = volume of the test solution (3,000 ml), n = number of animals/aquarium (25), t = duration of the experiment in hours, C_0 = algal concentration at the beginning of the determination of the filtration rate, and C_t = algal concentration at time t .

The filtration rates in the experimental treatments were expressed as percentages of the corresponding control. The results were plotted in dose-response relationships, from which the EC50 values were calculated by curve fitting according to Haanstra et al. [30].

Correlation coefficients were calculated between the observed biological activity and a selection of structural and electronic molecular properties. It was assumed that to elicit a biological effect, a compound has to pass membranes and interact with receptor(s) and that, therefore, both size-related properties and electronic forces may play a role. Accordingly, the following descriptors were selected: (1) geometric bulk properties: molecular volume (VOL), surface area (SA), and valence-corrected molecular connectivity indices (c^v); and (2) electronic properties: dipole moments (D), and energies of lowest unoccupied and highest occupied molecular orbitals (HOMO and LUMO), which reflect the ionization potential (IP) and electron affinity (EA), respectively (see also Hickey and Passino-Reader [31]). In several studies, high correlations were found between toxicity of organic pollutants and hydrophobicity, often expressed as K_{ow} [32,33]. However, experimental K_{ow} values are not available for all benzoquinoline isomers and estimation methods for K_{ow} cannot resolve these isomers. Alternatively, the HPLC capacity ratio, k , can be used.

VOL and SA were calculated by the SAVOL program, using cartesian coordinates obtained after optimization of the molecular geometry by a molecular mechanics modeling method (MM1 option in MOPAC). Van der Waals radii used for hydrogen, aromatic carbon, and nitrogen were 1.01, 1.77, and

Table 1. EC50 values (48 h; mg/L) and the corresponding 95% confidence limits for the effects of the four benzoquinoline isomers on the filtration rate of the zebra mussel (*Dreissena polymorpha*)

Benzoquinoline isomer	EC50 filtration rate (mg/L)	95% confidence interval (mg/L)
Benzo[b]quinoline (acridine)	0.50	0.30–0.85
Benzo[c]quinoline (phenanthridine)	0.09	0.06–0.13
Benzo[f]quinoline	2.73	2.23–3.36
Benzo[h]quinoline	1.25	1.00–1.55

$1.50 \cdot 10^{-10}$ m, respectively. Atom–atom distances used were: C–H, 1.084; C–C, 1.395; C–N, $1.338 \cdot 10^{-10}$ m. A solvent radius of zero was assumed. D, IP, and EA were calculated by the semiempirical quantum mechanical AM1 method using the same optimized molecular conformations (MM1 option in MOPAC). This program also generates heats of formation. Valence-c connectivity indices for path orders 2, 3, and 4 were calculated by the CFUNC program. Log k_0 values for the four isomers were taken from Van Vlaardingen et al. [16]. Correlation coefficients and related statistical parameters were calculated with the Statgraph program (Statistical Graphics Corp., Rockville, MD, USA).

RESULTS

None of the 1,350 mussels died during the experiments and filtration rates in the controls of up to 140 ml/mussel/h were measured. These levels are similar to the higher values reported in the literature [34,35], as discussed by Kraak et al. [24]. In Figure 2 the filtration rate of *D. polymorpha*, expressed as a percentage of the corresponding control, is plotted against the average actual benzoquinoline concentration in the water. A clear dose–response relationship was observed for the effect of the four isomers. From these results, the EC50 values and their 95% confidence limits were calculated (Table 1). Adverse effects increased in the order benzo[f], -[h], -[b], and -[c]quinoline. All EC50 values differed significantly ($P < 0.05$) from each other and the difference between the most toxic isomer ([c]) and the least toxic isomer ([f]) amounted to a factor of 30.

Table 2 presents calculated molecular descriptors for the four benzoquinoline isomers. As could be expected for isomers, differences in size-related descriptors are generally small. Electronic descriptors derived from quantum mechanical calculations showed larger differences as can be seen for D, EA, and IP-AE. Correlation coefficients between the observed toxicity (log-based values) and the values for the corresponding molecular descriptors (Table 2) demonstrate that for none of the selected molecular descriptors a significant

Fig. 2. Filtration rates of zebra mussels (*Dreissena polymorpha*) exposed for 48 h to different concentrations of acridine, phenanthridine, benzo[f]quinoline, and benzo[h]quinoline in the water, expressed as percentages of the corresponding controls. The lines represent the curve-fits after Haanstra et al. [30].

correlation was observed. The only significant ($P < 0.05$) relationship was that between effects on filtration rate and log k_0 , the HPLC capacity factor extrapolated to pure water.

DISCUSSION

Comparative ecotoxicity of benzoquinoline isomers

Our results clearly demonstrated that the EC50 values for filtration rate of the four isomers differed significantly. Bleeker et al. (unpublished data) studied the effects of benzoquinolines on survival of the midge *C. riparius* and reported effect concentrations comparable to those observed in the present study. Van Vlaardingen et al. [16], testing the effects of the four isomers on growth rate of the alga *S. acuminatus*, observed a different order in decreasing isomer toxicity (unpublished data) (b, f, c, h) compared to Bleeker et al. (unpublished data) b, c/h, f) and compared to the present study (c, b, h, f). The high toxicity of acridine as observed by Bleeker et al. (unpublished data) and Van Vlaardingen et al. [16] was in both cases explained by photoinduced toxicity, to which acridine is susceptible, while the other isomers are not (Table 2 vs Mekenyan et al. [36]). In the present study, however, acridine was not the most toxic isomer. Photoinduced toxicity of acridine to the zebra mussel is indeed not expected because the mussel tissue is protected against UV light by the valves. The possibility to compare the present EC50 values with those for other test

Table 2. Log k_0 , molecular surface areas (SA), molecular volumes (VOL), second, third, and fourth order path molecular valence connectivity indices (2c_p , 3c_p , 4c_p), HOMO (IP), LUMO (EA), dipole moments (D), and heat of formation (HF) of the four benzoquinolines and the correlation coefficients (r) with the corresponding (log-based) 48 h EC50 values for the filtration rate of the zebra mussel (*Dreissena polymorpha*)

Compound	HPLC		Molecular VOL ($\text{\AA}^3$)	Molecular valence connectivity indices			Quantum mechanical descriptors			Heat of formation	
	cap. log k_0	Molecular SA ($\text{\AA}^2$)		2c_p	3c_p	4c_p	IP	EA	IP-EA	D	HF
Acridine	2.79	187.47	166.21	3.374	2.425	1.720	-8.573	-1.043	7.530	1.832	77.19
Phenanthridine	2.94	186.17	165.99	3.363	2.498	1.808	-8.976	-0.596	8.380	2.057	68.07
Benzo[f]quinoline	2.71	185.98	165.97	3.357	2.510	1.834	-8.884	-0.598	8.286	1.958	69.28
Benzo[h]quinoline	2.80	186.73	166.05	3.362	2.497	1.815	-8.746	-0.550	8.196	1.537	68.48
Correlation coefficient (r)	-0.96	-0.06	-0.09	-0.37	0.20	0.28	0.26	0.13	-0.05	-0.46	0.00

Fig. 3. Schematic two-dimensional representation of factors that play a role in the interactions between benzoquinoline isomers and lipid membrane alkyl chains. Distances a , b , and c represent the minimum distance between parallel alkyl chains, the minimum distance between the nitrogen atom and alkyl chain, and the distance between neighboring hydrogen atoms, respectively. Dashed lines represent linear alkyl chains. Dashed circles represent electron clouds around hydrogen atoms, using Van der Waals radii (1.01×10^{-10} m). Molecules are scaled with C-C atom distances of 1.40×10^{-10} m.

organisms is limited to acridine and can be found in Kraak et al. [3] and Bleeker et al. (unpublished data).

Molecular descriptors

Molecular and macroscopic descriptors have been used extensively to relate the toxicity of organic contaminants to their molecular structure. Such relationships have been particularly successful when a large range of effect concentrations was observed within a rather homologous series of compounds. For NPAHs De Voogt et al. [27] have observed such relationships in series of compounds ranging from two to five aromatic rings. When isomers are studied, however, many of the molecular descriptors lose much, if not all, of their discriminating power. For example, the uncertainty in calculated molecular volumes is acceptable (around 2%), but yet differences calculated between the four benzoquinoline isomers in this study amount to only 0.4%. Molecular volumes of the benzoquinoline isomers can therefore not be considered to differ significantly and consequently, it is concluded that molecular volume is insufficiently discriminating between isomers to explain the observed differences in toxicity.

Although connectivity indices provide accurate molecular measures without intrinsic statistical uncertainties, differences in molecular connectivities between the four isomers could not explain the high differences in effect on filtration rate, reflected in

the low correlation coefficients in Table 2. In conclusion, the size- or topology-related molecular descriptors selected in this study provided insufficient resolution to distinguish between the benzoquinoline isomers. From the electronic descriptors given in Table 2 it is apparent that the dipole moment and the electron affinity discriminate most between the isomers. None of these electronic descriptors, however, provided a significant correlation with the observed effects.

Molecular descriptors can explain differences in toxicity of (homologous) series of NPAHs, e.g., ranging from two to five aromatic rings, even if an integrated biological endpoint such as mortality is used (Bleeker et al., unpublished data). The results of this study, however, clearly demonstrated that molecular descriptors proved not very effective in explaining the large differences in effects of the four isomers on filtration rate. Therefore, an alternative approach is discussed below, considering the molecular interactions between the benzoquinolines and biological membranes in more detail.

Biological effects due to interaction with membrane lipids involve penetration into the membranes. Simplified, membrane lipids can be represented by relatively long alkyl chains, arranged in a longitudinal, parallel way, as depicted in Figure 3 by the horizontal dashed lines. It is obvious that stretched molecules, such as acridine, may penetrate more easily, as a result of the

Table 3. Distances *a*, *b*, and *c* in Figure 3 representing shape, minimal distance of N atom to lipid chain, and accessibility of N atom, respectively^a

Benzoquinoline isomer	<i>a</i>	<i>b</i>	<i>c</i>
Benzo[<i>b</i>]quinoline (acridine)	7.04	2.02	2.77
Benzo[<i>c</i>]quinoline (phenanthridine)	7.61	1.88	2.59
Benzo[<i>f</i>]quinoline	7.61	3.13	2.58
Benzo[<i>h</i>]quinoline	7.61	1.88	2.19

^a All distances in 10⁻¹⁰ m.

smaller repulsive forces, than bent molecules, such as the other benzoquinolines. This is reflected in the larger distances between consecutive alkyl chains that accommodate the isomers, indicated in Figure 3 by arrow *a*. Other orientations of the isomers, indicated by the tilted dashed lines in Figure 3 lead to even larger distances between consecutive parallel alkyl chains. Obviously, distance *a* is not the only variable involved in the interaction between the benzoquinolines and membranes, because acridine is not the most toxic isomer.

Most of the relevant attractive forces will deal with the nitrogen atom, and the closer the distance of the nitrogen atom to the lipid chains, the more possibilities it has to interact with lipid chain moieties. In Figure 3, this distance is depicted by arrows *b*. Phenanthridine and benzo(*h*)quinoline thus have the shorter N-to-lipid chain distances, whereas this distance is largest for benzo(*f*)quinoline. The accessibility of the nitrogen atom is also determined by shielding resulting from the electron clouds of the hydrogen atoms neighboring the nitrogen atom. The less shielded, the more easily interactions of this type may occur. This type of effect can be estimated by considering the "open space" left between two neighboring hydrogen atoms, as depicted by distance *c* in Figure 3. Accordingly, the nitrogen atom of benzo(*h*)quinoline has a relatively low accessibility, whereas for acridine accessibility of the nitrogen atom is highest.

Distances *a*, *b*, and *c* in Figure 3, were calculated from the distance matrices provided by the output of the geometry-optimized structures from the semiempirical quantum mechanical calculations (cf. methods section)(Table 3). Attempts were made to relate these values to the observed biological effects, but multiple regression was not possible with four dependent data using three variables. By combining variables, this problem could be bypassed to some extent, although it must be emphasized here that the result can only be taken as indicative. The combined variables related well ($r^2 = 0.90$) to the observed biological effects and it is concluded that this simple mechanistic approach can help to explain the observed differences in effect on mussel filtration rates. This membrane interaction approach is supported by the high correlation between the EC50 data and the reversed-phase C18-HPLC behavior of the benzoquinolines (k_0 ; Table 2), which is likely to be governed by the same processes as discussed above.

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