Toxicity of azaarenes: mechanisms and metabolism.

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Chapter 6

Effects of Exposure to Azaarenes on Emergence and Mouthpart Development in the Midge *Chironomus riparius* (Diptera: Chironomidae)

Abstract
Adverse effects of azaarenes on emergence and mouthpart development of the midge *Chironomus riparius* were analysed using six closely related three-ringed isomers and metabolites. Effects on growth rate were examined by comparing the average day of emergence of exposed midges with that of controls. Fluctuating asymmetry (FA) in the pecten epipharyngis was examined as a measure of developmental abnormality. Delayed emergence was found at concentrations as low as 2% of the acute LC$_{50}$, so emergence day appears to be a useful sensitive parameter to quantify life cycle effects. No differences in FA were found between exposed and control larvae, although in other studies all compounds have been proven to be genotoxic. The differences in the genotoxic and FA-inducing properties of these compounds indicate that different mechanisms are involved in expressing these adverse effects. This study also illustrates that the choice of the morphological parameter strongly influences the results of developmental disturbance analyses, and thus the risk qualification of a potentially hazardous compound.
Introduction

Azaarenes are derivatives of polycyclic aromatic hydrocarbons in which one carbon atom is substituted by a nitrogen atom. They can be of natural origin (e.g., alkaloids; Kaiser et al., 1996), but high environmental concentrations in the field result from human activities such as combustion of fossil fuels (Santodonato and Howard, 1981), wood preserving (Adams and Giam, 1984; Pereira et al., 1987), mining and chemical manufacturing (Kaiser et al., 1996), and coal-tar processing (Pereira et al., 1983). Azaarenes have been identified in air (Santodonato and Howard, 1981), marine and freshwater environments (both in sediments and water columns) (Blumer et al., 1977; Van Genderen et al., 1994; Kozin et al., 1997), and in groundwater (Pereira et al., 1987). The presence of the electronegative nitrogen atom makes azaarenes more water soluble than their homocyclic analogues (Pearlman et al., 1984), which may increase their significance (mobility, bioavailability, etc.) in aquatic environments.

Although their environmental distribution is documented to some extent, relatively little is known about the ecotoxicology of azaarenes compared to the relatively well-studied PAHs. These toxicity data are usually the basis for environmental quality criteria and risk or hazard assessment. To date, however, no environmental norms exist for heterocyclic PAHs in Dutch environmental law (Ministry of Transport, Public Works, and Water Management, 1994). In addition, research on aquatic species has often been restricted to acute toxicity testing, while chronic toxicity, genotoxicity and teratogenicity are often ignored, in contrast to mutagenic and carcinogenic risks of these compounds evaluated for humans (e.g., Warshawsky, 1992). Furthermore, compounds considered to be safe based on acute toxicity data may exhibit adverse chronic effects when other biological endpoints are examined. Bleeker et al. (1999), for instance, have shown that although 9(10H)-acridone is not toxic in a 96 h Chironomus riparius test, it is highly genotoxic when tested with the Mutatox™ test. Since 9(10H)-acridone is a metabolite of the acute toxic azaarene acridine (Kraak et al., 1997a), metabolism clearly enhances the diversity of biological effects of PAHs. Although progress is being made in the study of metabolism of heterocyclic aromatics (Kaiser et al., 1996; Warshawsky, 1992) and structure-effect relationships (De Voogt et al., 1988; Roghair et al., 1994; Bleeker et al., 1998), the
mechanisms involved in the adverse toxic effects are still poorly understood. This makes it difficult to predict the consequences of metabolism for the level and type of adverse biological effect. Furthermore, the large differences in effects observed between isomers of azaarenes (Bleeker et al., 1998; Bleeker et al., 1999; Kraak et al., 1997b) give rise to additional problems.

To clarify the role of metabolism and differences between isomers in expressing long-term effects, in the present study midge larvae were subjected to life cycle exposure to six three-ringed azaarenes, including isomers and metabolites. The chosen compounds have been shown to induce acute, chronic and genotoxic effects in freshwater invertebrates (Kraak et al., 1997a; Bleeker et al., 1998). One of the chosen compounds (benz[g]isoquinoline-5,10-dione) was highly teratogenic to crickets (*Acheta domesticus*), resulting in extra antenna, extra eyes, and even complete extra heads, while exposure to its isomer (benzo[g]quinoline-5,10-dione, also incorporated in this study) did not result in teratogenic effects at all (Walton et al., 1983), further emphasising the selectivity of isomers as well.

Literature on induction of morphological abnormalities by organic compounds in the field is scarce and in most cases related to co-occurring chemicals. For example, Van Urk et al. (1992) and Janssens de Bisthoven et al. (1998) showed that the frequency of mentum deformities in *Chironomus* increased with increasing sediment contamination (metals, PAHs and PCBs). Furthermore, malformations in fish from the Rhine (Slooff, 1982) and birds from Lake Michigan (Larson et al., 1996) were related to elevated levels of organic contaminants (PAHs, PCBs and insecticides).

Morphological abnormalities in chironomids are becoming common bioindicators of anthropogenic stress in aquatic systems and are being used more and more in ecological risk assessments and evaluations (e.g. Bird, 1994; Canfield et al., 1994). Chironomids from polluted sites are characterised by greater fluctuating asymmetry index values than those from unpolluted sites, not only for metals or organic pollution, but also for complex mixtures of various types of pollution (e.g. Vermeulen, 1995; Janssens de Bisthoven, 1995; Groenendijk et al., 1998). The ecological significance of deformities in mouth parts, such as the pecten epipharyngis, could be an impact on the organisms fitness in the field, reducing growth rate or reproduction, especially in areas
where food (organic matter) is limited. Van Urk and Kerkum (1986) and Janssens de Bisthoven et al. (1998) demonstrated that larvae with deformed mouthparts indeed showed a delay in development.

In this study developmental delay and morphological abnormalities (fluctuating asymmetry) were chosen as endpoints in an experimental set-up where both parameters could be studied in the same animals. Developmental delay was expected to be influenced at concentrations below acute (narcotic) toxicity. In addition, morphological abnormalities were expected to be induced primarily by the compounds characterised as genotoxic in other studies (Warshawsky, 1992; Bleeker et al., 1999).

### Materials and Methods

#### Experimental set-up

At the start of the experiment 3 half egg masses of *Chironomus riparius*, containing ca. 200 eggs each, were placed in each of four glass jars (180 ml) containing 100 ml 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). Two jars contained the azaarene being tested (Fig. 6.1; for exposure concentrations see the Azaarene concentrations in water section below) and the other two served as controls. On the fourth day, when the larvae had hatched, 0.5 ml of food suspension was added from a stock, composed of 1 g of a fishfood mixture (Tetraphyl®:Trouvit, 1:20 w/w) in 20 ml DSW. After one week, 100 second instar larvae from the original 2 jars were placed in 5 new jars (20 larvae/jar), which contained 100 ml DSW and 0.4 g shredded cellulose as an artificial substrate. Midge in these jars were fed the same food suspension three times a week, 0.5 ml per feeding in the first week, 1 ml in the second, and 1.5 ml for the rest of the experiment, providing food ad libidum during the whole experiment. Water was continuously aerated and renewed once a week, and plastic wrap was placed over the jars to prevent evaporation. The light: dark regime was 16:8 h, and temperature was held constant at 20 °C by placing the
jars in a waterbath. From the fourteenth day onwards, cages were placed on the jars to catch emerging adults.

![Structures of the azaarenes tested.](image)

**Figure 6.1.** Structures of the azaarenes tested.

**Azaarene concentrations in water**

The chosen compounds (Fig. 6.1) were the isomers acridine (ACR) (Aldrich, purity 97 %) and phenanthridine (PHE) (Aldrich, 99+ %), and their respective metabolites 9(10H)-acridone (ACO) (Aldrich, 99 %) (Kraak et al., 1997a) and 6(5H)-phenanthridinone (PHO) (Aldrich, 98 %) (Bleeker et al., 1999), and the isomers benzo[g]quinoline-5,10-dione (BQD) (synthesised at the Department of Organic and Inorganic Chemistry of the Vrije Universiteit Amsterdam, purity 99 %) and benz[g]isoquinoline-5,10-dione (BID) (Aldrich, 99 %). To reach concentrations near the maximum water solubility, toxicants were dissolved using a generator column technique (Bleeker et al., 1998), in which compounds are dissolved in organic solvent, to which chromosorb GAW (Chrompack) is added. The solvent is then evaporated under a nitrogen flow, followed by slowly pumping water over the chromosorb, enabling the toxicant to dissolve in the water. The organic solvent ethyl acetate (‘Baker Analysed® HPLC Reagent, 99.6 %, J.T. Baker) was used for ACO and PHO, while the other compounds were dissolved in methanol (‘Baker Analysed® HPLC Reagent, 99.8 %, J.T. Baker). For further details, see Bleeker et al. (1998). The generator column...
produced stock solutions with the following concentrations: 95.6 μM for ACR, 4.5 μM for ACO, 36.5 μM for PHE, 6.5 μM for PHO, 19.6 μM for BID, and 13.7 μM for BQD. Based on acute toxicity data (Bleeker et al., 1998; Bleeker et al., 1999), concentrations were chosen which induced no acute lethal effect (LC50). For ACO and PHO pure stock solutions were used, for ACR the nominal concentration chosen was 0.85 μM, for PHE 0.56 μM, for BID 0.19 μM, and for BQD 4.78 μM. To calculate the actual exposure concentration, water samples were taken at the start and at the end of the experiment (including egg exposure), and before and after water renewal. These samples were analysed with HPLC (according to Bleeker et al., 1998), and from these data the average actual exposure concentrations were calculated, assuming an exponential decrease with time between two renewals. All compounds were eluted with 80 % acetonitrile (HPLC Grade, Rathburn) and 20 % water (Analysed HPLC Reagent, J.T. Baker), except for BID (60 % acetonitrile and 40 % water). ACR, PHE, BID and BQD were measured using UV-detection (λ = 254 nm; BID: λ = 230 nm). ACO and PHO were measured using fluorescence detection (excitation: λ = 254 nm; emission: λ > 354 nm). Average actual azaarene concentrations in the water were 0.208 ± 0.017 μM for ACR, 2.441 ± 0.292 μM for ACO, 0.190 ± 0.004 μM for PHE, 3.451 ± 0.172 μM for PHO, 0.064 ± 0.003 μM for BID, and 3.185 ± 0.167 μM for BQD. In the PHE exposures small amounts of its metabolite PHO (0.038 ± 0.005) were also found.

Emergence

During the emergence period, midges were collected and sexed daily. From these data the average time to emergence was calculated separately for each sex, to see if sex-based differences appeared, apart from the previous observed protandry (e.g. Danks, 1978). Significance of the difference between the time to emergence for the exposed and control midges was determined using a student t-test. Survival was defined as the total number of emerging midges. Since fluctuations in day of emergence may occur in time, all data on exposed midges were compared to corresponding control midges.

Analysis of fluctuating asymmetry

After emergence was complete, fourth instar head capsules were collected for analysis of the mouth parts and placed overnight in a 10 % KOH (Merck)
solution to remove any remaining non-chitinous material in the capsules. The head capsules were then placed in glacial acid (99 %, Sigma) for 15 minutes to neutralise the KOH, and rinsed twice with ethanol (96 %). Any remaining tissue attached to head capsules after the clearing process, such as remains of the intestinal tract or parapodia, were removed using a small blade. Finally, the head capsules were rinsed once in Euparal grün (Chroma-Gesellschaft) and mounted, ventral side up, on a microscopic slide in Euparal grün.

Fluctuating asymmetry (FA) was determined in the pecten epipharyngis, a comb-like mouthpart structure situated in the anterior area of the larval head capsule. The difference in the number of teeth between left and right sides has been proven to be a good measure for environmental stress in Chironomidae (Janssens de Bishoven, 1995; Groenendijk et al., 1998; Clarke, 1993). The centre of the pecten epipharyngis was determined by an indentation on the anterior side of the pecten. The tooth or pair of teeth in the middle of this indentation served as the division point between right and left. FA scoring was carried out 'blind' (i.e. without knowing whether the organisms scored were control or exposed larvae) by two individuals independently. Head capsules regarded to be unscorable by one or both of the scorers were excluded from analysis.

In this study FA was defined as the sum of the squared signed differences between left and right, divided by the number of individuals (FA = [R-L]²/N). This FA index is recommended for small sample sizes if FA is independent of character size or character size variation is small relative to variation in FA, and there is neither directional asymmetry nor antisymmetry (Palmer and Strobeck, 1986). Before calculating FA indices, the assumptions on which these indices are based were tested.

As suggested by Palmer (1994) for meristic traits, a likelihood ratio (g-test; Sokal and Rohlf, 1995) comparing the counts in each asymmetry class and the counts in each class of deviations between replicates was used to yield an estimate of the statistical significance of the between-sides variation relative to counting error. To make this g-test possible (i.e. to avoid zero values), all counts were increased by 1. In addition, a two-way ANOVA was performed to confirm that the variation in R-L exceeded the variation in replicate counts (Palmer, 1994). Since both tests showed that counting error was relatively small, in
further analyses the average number of teeth of the replicate countings was used.

Considering size dependence, regression analyses between absolute size differences (1R-L1) and mean character size (\((R+L)/2\)) were performed. If a relationship was found, size variation was compared with FA to check whether size-scaling was necessary. Since in all cases size variation (S) was relatively small (\(S/\sqrt{FA} < 10\); Palmer and Strobeck, 1986), size-scaling was considered unnecessary.

To test whether one side contained significantly more teeth than the other, a one-sample t-test was used to determine the significance of a departure from the mean of (R-L) from an expected mean of zero (as is the case when both sides have the same number of teeth). Departures from normality were determined by calculating significance of skew and kurtosis, using a one-sample t-test (expected mean of zero). The significance of the difference in FA between control and exposed midges was tested using an F-test, which is considered to be the most powerful in this case (Lehman, 1959).

**Results**

For all compounds average measured azaarene concentrations in the water were below acute effect levels (Bleeker et al., 1998; Bleeker et al., 1999) except for acridine, for which the concentration was similar to the acute LC10. This generally resulted in high survival in both control (av. ± s.d.: 81.3 ± 7.0 %) and exposure treatments (93.8 ± 6.1 %), except for the BID and BQD experiments (control: 30.4 ± 17.7; exposure: 29.6 ± 15.7 %). This implies that emergence results for BID and BQD may be questionable. Sampling of the head capsules, however, did show most larvae (> 80 %) had reached the fourth instar stage, indicating that most of the mortality occurred in the fourth instar and/or pupal stage and, therefore, mortality did not interfere with FA analysis. In all experiments, in both controls and exposures, females emerged significantly (p < 0.05) later than males. All compounds tested caused a significant (p < 0.05; PHO p < 0.1) delay in emergence time for both males and females, except for PHE exposed female midges (p = 0.98) (Fig. 6.2). The delay in emergence in the BID and BQD trials relative to controls, was much higher (about five times) than that in the other exposures.
Table 6.1 shows that the difference between the number of teeth on the right and left sides was independent of the total number of teeth on the pecten epipharyngis (probability of the slope > 0.05), except for ACO and PHO exposed midges, but in these cases size variation was relatively small compared to FA. In general (except for BID exposed larvae), no directional asymmetry was observed. BID exposure induced a highly significant directional asymmetry (t-test, p = 0.003; Table 6.1, R-L): when differences occurred (35 individuals), in most cases (24 individuals) more teeth were observed on the left side than on the right side. Since the controls in both the BQD and the PHE experiments showed deviations from normality (as indicated by a significant skew and/or kurtosis, Table 6.1) these controls were not used for FA comparison. In these cases, FA indices of the exposed midges were compared with BID and PHO controls, respectively. These latter experiments were performed in the same period and the controls of neither BQD nor PHE showed a significant difference from those of BID and PHO, respectively (t-test, p > 0.05). With this in mind, FA indices showed no clear differences between control and exposed midges in either of the experiments.
Discussion

Emergence

The significant delays in time to emergence in 11 out of 12 trials occurred at very low exposure concentrations, which induced no acute effects (2-10 % of acute LC$_{50}$ in most cases; Bleeker et al., 1998; Bleeker et al., 1999). Kenaga (1982) found that acute to chronic ratios (acute LC$_{50}$ to chronic NOEC) for fused-ring aromatics, including PAHs, were always below 10, while in the present study effects are found at concentrations as low as 2 % of the acute LC$_{50}$, indicating that an acute to chronic ratio would be consistently larger than 10. This might be due to the fact that acute high doses of a toxicant provoke narcosis, while chronic low doses induce other, more specific effects (Musch, 1996). In risk assessment the standard safety factor of 10 is often used for extrapolation from acute to chronic effect concentrations (Van Leeuwen and Hermens, 1995), which clearly underestimates the chronic effects found for azaarenes in this study. In addition, the isopod Porcellio scaber exposed to different concentrations of benzo[a]pyrene showed no significant effects on growth in the first seven weeks, while after nine weeks growth was significantly (p < 0.05) reduced (Van Brummelen and Stuijfzand, 1993). Comparable results were found for the isopod Oniscus asellus exposed to five different PAHs (Van Brummelen et al., 1996). In these experiments not only growth was affected, but the number of brood pouches as well (Van Brummelen et al., 1996). These observations clearly demonstrate the importance of chronic toxicity tests for reliable hazard assessment, preferably including complete life cycles of test organisms. The present study indicates that time to emergence of insects is a very suitable and sensitive chronic sublethal parameter for quantifying the effects of PAHs, as has previously been observed for metals (Nebeker et al., 1984; Postma et al., 1995).

Considering the lack of acute toxicity of the metabolites ACO and PHO at maximal water solubility, it might have been concluded that metabolism of ACR and PHE neutralises toxicity (Bleeker et al., 1999). The present life cycle experiments, however, showed that these metabolites also delay emergence. This is in agreement with the acute to chronic ratio theory that chronic effects often occur below acute effect concentrations (Kenaga, 1982).
Table 6.1. Descriptive data of FA analysis (presented as suggested by Palmer, 1994). N: number of pecten epitharyngis scored; R-L/2: character size; Size dependence: determined by regression of IR-LI against (R+L)/2 and expressed here as the slope; R-L: right minus left differences; skewness and kurtosis describe the shape of R-L frequency distributions; $\sigma^2_m$: measurement error mean square; $\sigma_i^2$: non-directional asymmetry; df: approximate degrees of freedom for non-directional asymmetry after partitioning out measurement error; FA: fluctuating asymmetry index.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>(R+L)/2 Mean ± se</th>
<th>Size dependence (se, prob.)</th>
<th>(R-L) Mean ± se</th>
<th>IR-LI Mean ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR-c</td>
<td>29</td>
<td>6.12 ± 0.13</td>
<td>-0.01 (0.23, 0.96)</td>
<td>-0.17 ± 0.21</td>
<td>0.76 ± 0.16</td>
</tr>
<tr>
<td>ACR-e</td>
<td>34</td>
<td>6.04 ± 0.16</td>
<td>0.09 (0.13, 0.47)</td>
<td>0.18 ± 0.17</td>
<td>0.76 ± 0.11</td>
</tr>
<tr>
<td>ACO-c</td>
<td>21</td>
<td>6.73 ± 0.13</td>
<td>0.16 (0.21, 0.45)</td>
<td>0.07 ± 0.20</td>
<td>0.74 ± 0.12</td>
</tr>
<tr>
<td>ACO-e</td>
<td>34</td>
<td>6.66 ± 0.11</td>
<td>-0.30 (0.15, 0.04)</td>
<td>0.10 ± 0.15</td>
<td>0.63 ± 0.10</td>
</tr>
<tr>
<td>PHE-c</td>
<td>65</td>
<td>6.18 ± 0.10</td>
<td>0.08 (0.10, 0.47)</td>
<td>-0.09 ± 0.11</td>
<td>0.60 ± 0.08</td>
</tr>
<tr>
<td>PHE-e</td>
<td>43</td>
<td>5.92 ± 0.13</td>
<td>-0.12 (0.13, 0.35)</td>
<td>0.08 ± 0.18</td>
<td>0.94 ± 0.11</td>
</tr>
<tr>
<td>PHO-c</td>
<td>47</td>
<td>5.86 ± 0.15</td>
<td>0.02 (0.11, 0.85)</td>
<td>-0.06 ± 0.16</td>
<td>0.77 ± 0.11</td>
</tr>
<tr>
<td>PHO-e</td>
<td>68</td>
<td>5.91 ± 0.11</td>
<td>-0.22 (0.10, 0.02)</td>
<td>0.01 ± 0.14</td>
<td>0.82 ± 0.09</td>
</tr>
<tr>
<td>BID-c</td>
<td>48</td>
<td>6.10 ± 0.11</td>
<td>0.03 (0.15, 0.84)</td>
<td>0.19 ± 0.15</td>
<td>0.67 ± 0.11</td>
</tr>
<tr>
<td>BID-e</td>
<td>52</td>
<td>6.10 ± 0.14</td>
<td>-0.07 (0.13, 0.57)</td>
<td>-0.47 ± 0.16</td>
<td>0.94 ± 0.12</td>
</tr>
<tr>
<td>BQD-c</td>
<td>56</td>
<td>6.10 ± 0.11</td>
<td>-0.22 (0.14, 0.11)</td>
<td>0.07 ± 0.16</td>
<td>0.72 ± 0.11</td>
</tr>
<tr>
<td>BQD-e</td>
<td>45</td>
<td>6.36 ± 0.12</td>
<td>-0.11 (0.13, 0.41)</td>
<td>0.11 ± 0.15</td>
<td>0.78 ± 0.10</td>
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</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Skew ± se</th>
<th>Kurtosis ± se</th>
<th>$\sigma^2_m$</th>
<th>$\sigma_i^2$</th>
<th>df</th>
<th>FA Mean ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR-c</td>
<td>-0.32 ± 0.43</td>
<td>1.54 ± 0.85</td>
<td>0.060</td>
<td>0.62</td>
<td>25.46</td>
<td>1.29 ± 0.44</td>
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<tr>
<td>ACR-e</td>
<td>-0.03 ± 0.40</td>
<td>-0.55 ± 0.79</td>
<td>0.051</td>
<td>0.48</td>
<td>29.72</td>
<td>1.02 ± 0.22</td>
</tr>
<tr>
<td>ACO-c</td>
<td>0.37 ± 0.50</td>
<td>-0.92 ± 0.97</td>
<td>0.036</td>
<td>0.41</td>
<td>18.34</td>
<td>0.82 ± 0.20</td>
</tr>
<tr>
<td>ACO-e</td>
<td>0.46 ± 0.40</td>
<td>0.04 ± 0.79</td>
<td>0.029</td>
<td>0.35</td>
<td>30.39</td>
<td>0.71 ± 0.19</td>
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<tr>
<td>PHE-c</td>
<td>-0.98 ± 0.30</td>
<td>0.93 ± 0.59</td>
<td>0.075</td>
<td>0.37</td>
<td>52.50</td>
<td>—</td>
</tr>
<tr>
<td>PHE-e</td>
<td>-0.44 ± 0.36</td>
<td>-0.15 ± 0.71</td>
<td>0.064</td>
<td>0.67</td>
<td>38.22</td>
<td>1.38 ± 0.28</td>
</tr>
<tr>
<td>PHO-c</td>
<td>0.32 ± 0.35</td>
<td>0.24 ± 0.68</td>
<td>0.066</td>
<td>0.55</td>
<td>40.85</td>
<td>1.14 ± 0.26</td>
</tr>
<tr>
<td>PHO-e</td>
<td>0.25 ± 0.29</td>
<td>-0.13 ± 0.57</td>
<td>0.109</td>
<td>0.57</td>
<td>55.65</td>
<td>1.24 ± 0.21</td>
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<tr>
<td>BID-c</td>
<td>-0.17 ± 0.34</td>
<td>0.51 ± 0.67</td>
<td>0.036</td>
<td>0.50</td>
<td>43.77</td>
<td>1.05 ± 0.24</td>
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<td>BID-e</td>
<td>-0.32 ± 0.33</td>
<td>-0.30 ± 0.65</td>
<td>0.315</td>
<td>0.50</td>
<td>28.73</td>
<td>—</td>
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<tr>
<td>BQD-c</td>
<td>1.13 ± 0.32</td>
<td>2.22 ± 0.63</td>
<td>0.054</td>
<td>0.53</td>
<td>49.74</td>
<td>—</td>
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<tr>
<td>BQD-e</td>
<td>-0.13 ± 0.35</td>
<td>-0.38 ± 0.69</td>
<td>0.033</td>
<td>0.53</td>
<td>41.36</td>
<td>1.08 ± 0.20</td>
</tr>
</tbody>
</table>

\(^{a}\) c = control midges; e = exposed midges; \(^{b}\) computed using replicate measurements to partition out measurement error, all other values are computed after taking average of replicate measurements.
The more delayed emergence in BID and BQD trials, which also showed high mortality in late developmental stages, may be caused by a water renewal during (moulting to) pupal stage, a stage vulnerable to physical disturbances (Postma et al., 1995).

**Morphological abnormalities**

The use of fluctuating asymmetry as a measure for developmental stability and genetic stress are generally accepted (Parsons, 1990; McLachlan and Cant, 1995; Vermeulen, 1995; Rabitsch, 1997; Groenendijk et al., 1998), but because of the many assumptions involved which result in a complicated statistical analysis, data from different FA studies are nearly impossible to compare quantitatively (Palmer, 1994). Therefore, in this study we followed the primer of Palmer (1994) to provide data which could be compared quantitatively with other studies. However, since these data are scarce in the literature, in this paper comparisons are also made with less well documented FA values.

Control FA-values in the present experiments (average 1.10; excluding the non-normally distributed PHE and BQD controls), are highly comparable to values for organisms found in a clean river (average 0.92; Groenendijk et al., 1998). In azaarene exposed midges (excluding the directional asymmetric BID exposed midges) no significant differences were found with the corresponding controls. In contrast, in midges from a metal-contaminated river in Belgium FA values were considerably higher (1.40-1.91, average 1.70; Groenendijk et al., 1998). Furthermore, chironomids from sites contaminated with nitrates, phosphates, ammonia, methanol and suspended matter showed even higher FA-values (2.05 and 2.83 for the two sites examined; Clarke 1993).

The reason for the significant directional asymmetry in BID exposure is unclear, but it is most likely due to a type I error because preliminary results on the same compound (unpublished data) did not show this type of asymmetry after exposure to similar concentrations. Furthermore, this kind of asymmetry has never been reported in pecten epipharyngis of *Chironomus riparius* before (Janssens de Bisthoven, 1995).

The absence of fluctuating asymmetry in azaarene exposed midges was unexpected. Considering that the development of such abnormalities may imply damage at the DNA level, it was tempting to predict that most of the six compounds would elicit developmental disturbances, because all compounds
were proven to be genotoxic to a certain degree in the Mutatox™ test (Bleeker et al., 1999 and Table 6.2) and, if tested, in the Ames test as well (Warshawsky, 1992). However, this was not the case. Genotoxicity (Bleeker et al., 1999) and developmental disturbances (this study) proved to be unrelated. Because genotoxicity is related to DNA damage, this may indicate that developmental disturbances observed in the midge are not. This is in accordance with recent results from Vermeulen et al. (1997), who presented evidence that increased levels of deformation in C. riparius are the result of disturbance of epidermal growth during molting events, rather than being classical teratogenic effects. In contrast, Groenendijk et al. (1998) suggested that there could be some heredity of FA effects. In that study, however, direct DNA-damage was not studied, so their results might also be explained by hormonal disturbances in parent generations, rather than by DNA-damage. Further supporting evidence for the observations of Vermeulen et al. (1997) may be found in a comparison of results from the present study with results from Walton et al. (1983). These authors demonstrated strong teratogenicity of BID in crickets, in contrast to the lack of induction of morphological abnormalities in the midge C. riparius in the present study. Apparently, highly genotoxic or teratogenic compounds do not necessarily induce morphological abnormalities like FA. Thus, the choice of morphological parameter strongly affects the outcome of developmental disturbance analyses, and thus the risk qualification of potentially hazardous compounds.

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References


