Toxicity of HC Orange No. 1 to *Daphnia magna*, Zebrafish (*Brachydanio rerio*) embryos, and goldfish (*Carassius auratus*)

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Abstract

HC Orange No. 1 (HCO1; 2-nitro-4'-hydroxydiphenylamine) (CAS No. 54381-08-7) is used as a color additive in hair dyes and can be released into aquatic environments in wastewater. In this paper, the effects of HCO1 on aquatic organisms were studied using a battery of toxicological tests. These included measuring immobilization of *Daphnia magna*, inhibition of zebrafish embryo development, and acute lethality in zebrafish and goldfish, which are different species belonging to different trophic levels. HCO1 was toxic to all of the organisms studied. In our experiments, HCO1 remarkably restrained the mobility of *D. magna*, which may cause subsequent death. The EC50 value for restrained the mobility of *D. magna* at 48 h was 1.54 mg HCO1 l⁻¹. In addition, HCO1 showed toxicity in zebrafish and goldfish, where LC50 values at 96 h were 4.04 and 5.37 mg l⁻¹, respectively. The results also indicated that HCO1 remarkably retarded the development of zebrafish embryos, which may cause embryo abnormality and even lethality. The most sensitive toxicological endpoint in the development of the embryos was failure to hatch, which had an EC50 of 0.19 mg HCO1 l⁻¹. These results indicated that HCO1 is a potential teratogen to zebrafish embryos. In addition, as HCO1 concentrations increased, the outcomes of each of these toxicity tests changed in a concentration-dependent manner. Together, the results revealed that HCO1 appears to be toxic to multiple different species of aquatic organisms. The EC50 (LC50) values contain sufficient discriminatory power for risk assessment of HCO1 in aquatic environments. Based on the present results, more efficient risk assessment procedures for HCO1 will be designed in the future, integrating more flexible testing methods into the testing schemes that employ only the necessary tools for each case.

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Keywords: HCO1; Toxicology; Aquatic organisms; Battery of tests

1. Introduction

The use of hair dyes can be traced back to 4000 years BC, when the hair on Egyptian mummies was dyed with henna (Nohyne et al., 2004). Currently, synthetic organic compounds are used for coloration in commercial hair dyes. The majority of young women and an increasing proportion of men in industrialized countries regularly use hair dyes. For example, the natural hair color of the Chinese is black, but dying hair with other colors has also become fashionable. Unfortunately, the chemical components of these dyes can then be released into the aquatic environment, especially in some Asian countries where wastewater treatment is not always available.

Some hair dye products have been found to contain mutagenic and carcinogenic compounds (Ames et al., 1975; Flamm, 1985). Thus, it is not surprising that
potential carcinogenic effects of hair dye products have been observed in recent epidemiological studies (Rodstein et al., 1994; La Vecchia and Tavan, 1995; Cook et al., 1999; Gago-Dominguez et al., 2001).

HC Orange No. 1 (HCO1; 2-nitro-4'-hydroxydiphenylamine) (CAS No. 54381-08-7) is used as a colorant in semi-permanent hair dyes (Pang and Fiume, 1998). The product formulation data submitted to the US Food and Drug Administration (USFDA) in 1996 reported that HCO1 was used in a total of 95 hair dyes and colors (USFDA, 1996). The concentration reported to be used is approximately 0.15%, but information from manufacturers suggested that higher concentrations might be used in the future (Pang and Fiume, 1998). The effects of HCO1 on mammals have been investigated and there is no indication that semi-permanent hair dye formulations containing 0.15% HCO1 are carcinogenic in mice or rats (Burnet et al., 1976; Pang and Fiume, 1998).

With rapid development of the dyestuff industry in China, a relatively large amount of HCO1 has the potential to reach aquatic environments where it may have adverse effects. Furthermore, if HCO1 is not removed by sewage treatment due to inefficiency or a complete lack of sewage treatment in some rural locations, sewage could be a source of potential toxic materials. However, there are few published studies on the effects of HCO1, especially in aquatic life. Therefore, we conducted a study to determine the potential effects of HCO1 in aquatic organisms.

One approach to assess the potential harmful effects of chemicals in the aquatic environment is to use a set of global physical–chemical and biochemical parameters to predict the potential environmental fate and toxicity of the chemicals (Denis, 2003). However, chemical–physical properties alone cannot provide sufficient information on the potential harmful effects of chemicals in the aquatic environment (Vvryan et al., 1999). Acute and chronic toxicity, including subtle effects such as deformities and cancer, can also have long-term effects on populations. Thus, bioassays have been used to evaluate risk assessment of new registered chemicals as well as to investigate their effects (Repetto et al., 2001). Due to the diversity of aquatic organisms and differences in these possible endpoints, use of a single bioassay may not provide sufficient information for environmental protection. An alternative cost-effective approach is to apply a battery of simple tests (Isooma and Lilius, 1995; Bierkens et al., 1998; Repetto et al., 2001).

The aim of this study was to apply a battery of tests and endpoints to assess the potential effects of HCO1 on the aquatic environment. D. magna is a typical aquatic species in toxicity tests (USEPA, 1991). The fish acute toxicity test was deemed to be a suitable assay to minimize the number of fish used (Schulte and Nagel, 1994). The goldfish (Carassius auratus) (Vittozzi and De Angelis, 1991) was included in the battery of tests because it is abundant in the freshwater of China (Mashiro et al., 1999). Such a battery of test systems and indicators would be not only representative of a wide range of organisms but also simple and rapid, making it convenient for routine environmental monitoring. The endpoints studied include immobilization of the D. magna, inhibition of zebrafish embryo development, and acute lethality of adult zebrafish and goldfish.

2. Materials and methods

2.1. Toxicant exposure

HCO1 (purity > 99%) was purchased from Nanjing King-Pharm Co., Ltd., Nanjing. A stock solution of HCO1 was prepared in methanol and maintained in darkness at 0°C. Before each bioassay, stock solutions were warmed to room temperature and used to prepare the final test concentrations, which were sterilized by filtration through a 0.22 μm filter (Millipore, Beijing). The methanol concentration in the medium containing the solvent control was less than 0.1%. One control group and one solvent control group were designated for each exposure test, with the total mortality of test organisms near zero. Results of the preliminary studies indicated that HCO1 was stable enough that nominal concentrations changed less than 20% in a week. Reported concentrations are nominal. Before experiments, the test organisms were acclimatized in aquariums for two weeks under conditions similar to those under which the tests were performed.

2.2. Model systems

2.2.1. D. magna

Acute toxicity test conditions (48 h) conformed to the guidelines developed by the Organization for Economic Cooperation and Development (OECD, 1984). Specifically, eight groups (0.60, 1.00, 1.50, 2.50, 3.00, 3.50, 4.00, and 5.00 mg l⁻¹ of HCO1) of five neonates each were kept for 48 h in 20 ml of water contained in 50-ml glass beakers. Result of preliminary studies indicated that 6.00 mg HCO1 l⁻¹ restrains all D. magna from moving in response to stimulus within a few hours. Experiments were conducted in quadruplicate for each concentration. D. magna
(clone A) were maintained at 20.0 °C in aerated water at a pH of 7.3 ± 0.3 (95%CI). The water had a hardness of approximately 100 mg l⁻¹ CaCO₃ and a dissolved oxygen (DO) concentration greater than 6.0 mg l⁻¹. The D. magna were subjected to a 12 h light 12 h dark cycle and fed with green alga (Chlorella vulgaris). Adults were separated from neonates 24 h before the initiation of the test so that neonates less than 24 h old would be available for testing. The measured effect was death, which was defined as immobilization for 15 s after stimulation by a bright light.

2.2.2. Zebrafish embryos

The test design (72 h) conformed to the guidelines developed by the Organization for Economic Cooperation and Development (OECD, 1996). Adult zebrafish were maintained in groups of four females and eight males (Nagel, 1986). Fertilized eggs were used to investigate the acute toxicity of HCO₃⁻. The eggs were collected by a plastic box (12 x 24 cm) placed at the bottom of each tank before the light was turned on. The group mating occur during the first 30 min of the light period. The fertilized eggs were exposed to different concentrations (0.01, 0.05, 0.10, 0.20, 0.40, 0.80, 1.20, 1.60, 2.00, 4.00, and 8.00 mg l⁻¹) of HCO₃⁻ within 1 h of collecting and washing them and then incubated at 26.5 °C in embryo medium (Cheng et al., 2001). Twenty-four-well culture plates with 3 ml volume in each well were prepared with 2 ml test solution. In each plate, 20 wells contained a single concentration of the test solution and four wells were internal controls containing embryo medium. To exclude mutual influences, only one fertilized egg was placed in each well. The fertilized eggs collected for each well of a single plate were taken from the vessel with the same concentration of HCO₃⁻. In order to keep the concentration constant, the wells were covered with foil. The development of the eggs was not affected by this procedure (Schulte and Nagel, 1994).

Developmental stages of embryos are described as hours post-fertilization (hpf) and classified according to the morphological characteristics described in a study by Chen et al. (2004). Embryos were examined under an inverted stereomicroscope. Only those embryos that developed normally were selected for the subsequent experiments. Embryo viability was determined for each level of HCO₃⁻ at different developmental stages (4, 8, 12, 24, 48 and 72 hpf). All groups of eggs were observed and differences in the chosen parameters were documented (Table 1).

2.2.3. Adult zebrafish

Zebrafish were purchased from a local supplier and had an average body length and weight of 2.8 cm and 2.5 g, respectively. Before exposure, fish were acclimated to the water with a total mortality of one fish in 500. Fish were fed 0.2 g frozen brine shrimp (Artemia salina) per fish each day and were subjected to a 14 h light 10 h dark photoperiod. During the experiment, aerated water had a pH of 7.3 ± 0.3 (95%CI) and a temperature of 26.5 °C. The water hardness was approximately 100 mg CaCO₃ l⁻¹, and the dissolved oxygen (DO) concentration was greater than 6.0 mg l⁻¹. The test concentrations were selected based on data obtained from the preceding acute toxicity tests and preliminary test results. Exposure of fish to the higher concentration of HCO₃⁻ (10.00 mg l⁻¹) in the preliminary test caused a rapid loss of equilibrium, associated with a spiral swimming behaviour. Fish then became hypoactive and all died within a 4 h exposure period. Standard conditions included test containers containing 10 organisms each, with nine nominal concentrations of HCO₃⁻ (1.00, 2.00, 3.00, 4.00, 5.00, 5.40, 6.00, 7.00 and 8.00 mg l⁻¹) (each in triplicate). Fish were not fed during the 4 d acute toxicity tests. Mortality was recorded hourly during the first 4 h of exposure and daily thereafter.

2.2.4. Goldfish

The average length and weight of goldfish were approximately 9.8 cm and 30.8 g, respectively. During experiments, the aerated water temperature was 22.0 ± 2.0 °C, and lighting was 12 h light and 12 h dark. Prior to the experiments, the fish were acclimated with a total mortality of zero. In the preliminary test, 15.00 mg HCO₃⁻ l⁻¹ caused

### Table 1

The chosen toxicological endpoints at different stages of development of zebrafish embryos (hpf)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulated eggs</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Completion of gastrulation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Extension of the tail</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spontaneous movements within 20 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Development of the eye</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heartbeat</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Circulation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Development of the otolith</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Development of melanocytes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rate of malformed egg</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rate of hatched</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

“+” indicates that the morphological changes parameters are selected as toxicological endpoints at different stages of development.

![Fig. 1. Daphnia magna immobilization after exposure to different concentrations of HCO₃⁻ for 24 h and 48 h. Data were expressed as mean values ± SD from one representative experiment (n = 4). Significant difference from control value was at P < 0.05.](attachment:image)
all goldfish to die within a 4 h exposure period. For subsequent experiments, nominal concentrations of 4.00, 5.00, 6.00, 6.40, 7.00, 8.00, and 10.00 mg HCO₃⁻L⁻¹ were used. The goldfish were tested in triplicate in groups of eight goldfish in 50 l fishbowls containing 30 l of aerated water under a static test.

2.3. Calculations and statistical analysis

All the tests were considered valid if control mortality was ≤5%. All experiments were performed at least three times and at least in triplicate for each concentration. The EC₅₀ (50% effect concentration) values for zebrafish embryos and the respective 95% confidence limits were determined by probit analysis (version 4.0). LC₅₀ (EC₅₀) values for the other test species and the respective 95% confidence limits were determined using Trimmed Spearman-Karber method version 1.5 (USEPA, 1990).

3. Results

3.1. D. magna

The results of this study demonstrated that D. magna is moderately sensitive to HCO₃⁻, showing a dose-dependent curve of immobilization with an EC₅₀ of 4.47 mg l⁻¹ at 24 h and of 1.54 mg l⁻¹ at 48 h (Fig. 1).

3.2. Zebrafish embryo

HCO₃⁻ caused both deformities and lethality of zebrafish embryos. The developing embryo of zebrafish offers a sys-

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Fig. 2. Morphological images changes at development stages of zebrafish embryos exposed to HCO₃⁻. Bar is 50 µm. (a) Coagulated egg. (b) Gastrulation (8 h). (c) Completion of gastrulation (12 h). (d) Normally developed embryo (48 h) with apparent pigmentation. (e) Abnormally developed embryo (48 h). (f) Delayed hatch (72 h).
tem with natural interactions between cells. Fifty percent of the eggs coagulated after 4 h of exposure at a concentration of 4.93 mg HCO1 l−1. Coagulated eggs were milky white and appeared dark under the microscope (Fig. 2a) (OECD, 1996). After 8 h, the beginning of gastrulation was examined. In a process called epiboly the blastoderm spreads over the yolk cell. The blastoderm margin, clearly seen as a fold, should by this time have extended over the equatorial line of the egg (about 70%-epiboly stage; Fig. 2b) (OECD, 1996). Completion of gastrulation is documented after 12 h. At this time, the blastoderm has completely enveloped the yolk cell (100%-epiboly stage). The blastoderm margin has disappeared due to fusion at vegetal pole (Fig. 2c) (OECD, 1996). Embryogenesis in zebrafish is completed within the first 72 h and major development of most internal organs, including the cardiovascular system, gut, liver and kidney, occurs in the first 24–48 h. Spontaneous myotomal contractions start after approximately 20 h. After 24 h, side-to-side contractions involving the trunk and tail should be discernible approximately every 20 s. One endpoint in the development of the embryos that is sensitive to HCO1 is a lack of spontaneous movement at 24 hpf, which occurs with an EC50 value of 0.38 mg HCO1 l−1. After 48 h, deviation of pigmentation from normal development was evaluated. At 48 h, the most sensitive endpoint was a lack of melanocyte development, which occurs with an EC50 of 0.66 mg HCO1 l−1 (Fig. 2d) (OECD, 1996) (after HCO1 exposure). For HCO1, the most sensitive endpoint in the development of the embryos was failure to hatch (72 hpf), occurring with an EC50 of 0.19 mg HCO1 l−1. The concentration of HCO1 causing 50% of embryos to express abnormalities (72 hpf) was 0.43 mg l−1. The most common deformity was tail flexures (Fig. 2f) (after HCO1 exposure), which suggests that HCO1 is a potential teratogen. The magnitude and severity of the effects increased with time of exposure.

3.3. Adult zebrafish

In toxicity tests with adult zebrafish no lethality was observed for exposures of 96 h to HCO1 at concentrations of less than 2.00 mg l−1. However, concentrations greater than 7.00 mg l−1 caused rapid lethality. Exposure of fish to the higher concentration (8.00 mg l−1) of HCO1 resulted in a rapid loss of equilibrium, associated with a spiral swimming behaviour. Fish then became hypoactive and all died within a 24–48 h exposure period. LC50 values were 4.38 and 4.04 mg HCO1 l−1, for 48 and 96 h, respectively (Table 2).

3.4. Goldfish

Goldfish exposed to high concentrations of HCO1 (10.00 mg l−1) died almost immediately, and mortality was 100% within 48 h. LC50 values were 7.52, 6.08 and 5.37 mg HCO1 l−1 for 48, 72 and 96 h, respectively (Fig. 3).

Table 2
Toxic effect of HCO1 on the different models and bioindicators included in the proposed ecotoxicological battery

<table>
<thead>
<tr>
<th>Model system</th>
<th>Origin</th>
<th>Indicator</th>
<th>Exposure period (h)</th>
<th>EC50 (mg l−1) (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daphnia magna Clone A</td>
<td>Immobilization</td>
<td>24</td>
<td>4.47 (4.00–5.00)</td>
<td></td>
</tr>
<tr>
<td>Daphnia magna Clone A</td>
<td>Immobilization</td>
<td>48</td>
<td>1.54 (1.27–1.86)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>Coagulated egg</td>
<td>4</td>
<td>4.93 (4.80–5.09)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>Coagulated egg</td>
<td>8</td>
<td>4.01 (3.91–4.12)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>Gastrulation is not finished</td>
<td>12</td>
<td>3.86 (3.70–3.99)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>No spontaneous movement</td>
<td>24</td>
<td>0.38 (0.32–0.46)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>Coagulated egg</td>
<td>24</td>
<td>0.57 (0.40–0.82)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>No development of melanocytes</td>
<td>48</td>
<td>0.66 (0.55–0.80)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>No development of the otolith</td>
<td>48</td>
<td>0.70 (0.32–1.54)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>Coagulated egg</td>
<td>48</td>
<td>0.54 (0.40–0.72)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>All abnormality</td>
<td>72</td>
<td>0.43 (0.31–0.60)</td>
<td></td>
</tr>
<tr>
<td>Embryos Zebrafish</td>
<td>Embryo does not hatch</td>
<td>72</td>
<td>0.19 (0.11–0.35)</td>
<td></td>
</tr>
<tr>
<td>Zebrafish</td>
<td>Lethal</td>
<td>48</td>
<td>4.38 (3.94–4.87)</td>
<td></td>
</tr>
<tr>
<td>Zebrafish</td>
<td>Lethal</td>
<td>96</td>
<td>4.04 (3.61–4.52)</td>
<td></td>
</tr>
<tr>
<td>Goldfish</td>
<td>Lethal</td>
<td>48</td>
<td>7.52 (7.35–7.69)</td>
<td></td>
</tr>
<tr>
<td>Goldfish</td>
<td>Lethal</td>
<td>72</td>
<td>6.08 (5.06–6.55)</td>
<td></td>
</tr>
<tr>
<td>Goldfish</td>
<td>Lethal</td>
<td>96</td>
<td>5.37 (4.92–5.87)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Diagram of the concentration/response relationship for HCO1 in goldfish for 48 h, 72 h and 96 h. Data were expressed as mean values ± SD (n = 3). Significant difference from control value was at P < 0.05.
4. Discussion

There is no doubt that *D. magna* is an excellent test organism for screening the relative toxicity of chemicals. Invertebrates have been suggested to be sensitive species that can serve as surrogates for mice and rats in ecotoxicology studies. In support of this, LC$_{50}$ values for *D. magna* when exposed to some metals were found to correlate with the corresponding LD$_{50}$ values for the mouse and rat (Neuhausser et al., 1985). The predictive screening potentials of some aquatic invertebrate tests for acute oral toxicity in humans have been shown to be better than the rat LD$_{50}$ test for some chemicals (Calleja and Persoone, 1992). The major advantage of using invertebrate bioassays as a pre-screening method is to reduce the number of vertebrate animals required for toxicity testing. Since these methods are *in vivo* tests, biotransformation of chemicals is taken into account, making these tests preferable to *in vitro* methods that have been used to evaluate human acute toxicity (Ekwall et al., 1989, 1998). The association between the acute toxicity to *D. magna* and the corresponding oral LD$_{50}$ values in the rat is further evidence of a strong relationship between the two species (Guilhermino et al., 2000). The *D. magna* test seems to have a predictive capacity comparable to that of mammalian cytotoxicity tests. Since it is an *in vivo* test taking into account the biotransformation of toxicants and potential integrated effects that occur in the organism as a whole, the toxicity data produced by this test could be advantageous in many situations.

The toxic effects of HCO1 on the different models and bioindicators included in the proposed ecotoxicological battery are summarized in Table 2. *D. magna* was also relatively sensitive to the effects of HCO1 exposure. The toxicity of chemicals to *D. magna* can be predictive of effects on both invertebrates and mammals. All of the features discussed above are interconnected. *D. magna* is vulnerable to fish predation because of its large size. While *D. magna* is an aquatic invertebrate, zebrafish and goldfish aquatic vertebrates. The different trophic levels of these species result in the differences in their EC$_{50}$ values. This suggests that their tolerance to HCO1 is different. Their nutritional levels increase gradually, resulting in the increase in their EC$_{50}$ values, which suggests that their tolerance to HCO1 increases. The effect of HCO1 on fish is not very species-specific, with 96-h EC$_{50}$ values for lethality goldfish of 5.37 mg l$^{-1}$ becoming appreciably higher than the corresponding values for zebrafish. On the other hand, it is worth noting that EC$_{50}$ values for zebrafish embryos indicate the embryos cannot endure higher HCO1 concentrations

If the concentrations of HCO1 in *D. magna*, embryos and fish could be monitored, EC$_{50}$ (LC$_{50}$) values might be greater than those observed in this study because of bioaccumulation. The relationship between toxicity and body burden can be used to predict impact (Chapman, 1997). In the future, tests including the chronic toxicity test, the long term toxicity test in low dose exposure, and molecular toxicological tests will be used to assess the toxicology of HCO1 more accurately. Because of the potential for HCO1 to be released into aquatic environments, the discharge of sewage or effluent containing HCO1 should be monitored and controlled.

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