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Chronic toxicity of azo and anthracenedione dyes to embryo-larval fathead minnow

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ABSTRACT

The toxicity of selected azo and anthracenedione dyes was studied using chronic exposures of embryo-larval fathead minnows (*Pimephales promelas*). Newly fertilized fathead minnow embryos were exposed through the egg stage, past hatching, through the larval stage (until 14 days post-hatch), with dye solutions renewed daily. The anthracenedione dyes Acid Blue 80 (AB80) and Acid Blue 129 (AB129) caused no effects in larval fish at the highest measured concentrations tested of 7700 and 6700 μg/L, respectively. Both azo dyes Disperse Yellow 7 (DY7) and Sudan Red G (SRG) decreased survival of larval fish, with LC50s (based on measured concentrations of dyes in fish exposure water) of 25.4 μg/L for DY7 and 16.7 μg/L for SRG. Exposure to both azo dyes caused a delayed response, with larval fish succumbing 4–10 days after hatch. If the exposures were ended at the embryo stage or just after hatch, the potency of these two dyes would be greatly underestimated. Concentrations of dyes that we measured entering the Canadian environment were much lower than those that affected larval fish survival in the current tests. In a total of 162 samples of different municipal wastewater effluents from across Canada assessed for these dyes, all were below detection limits. The similarities of the structures and larval fish responses for the two azo and two anthracenedione dyes in this study support the use of read-across data for risk assessment of these classes of compounds.

1. Introduction

The presence of synthetic dyestuffs in aquatic ecosystems is a growing concern due to the possibility that they could impact environmental health (Vaidya and Datye, 1982); (Bafana et al., 2011). Synthetic dyes are extensively used in many industrial and consumer products, including paper production, leather tanning, food colouring, personal care products (e.g., hair colour, deodorant, etc.), as well as in textiles and paints (Vaidya and Datye, 1982). No method has been able to completely remove these pollutants from wastewater (Forgacs et al., 2004); (Bae and Freeman, 2007); and 10–15% of the dyes eventually enter aquatic ecosystems (Anliker, 1979), where very little is known about their ultimate fate and potential impacts.

In Canada, as part of the Government of Canada's Chemicals Management Plan (CMP), substances that are used in industry or imported in products are being assessed for potential human and environmental toxicity. Azo and anthracenedione dyes are among the groups of substances that have been identified as requiring further study (Hill et al., 2011), as there is currently little information regarding the aquatic toxicity of these compounds. Since the dyes may enter Canadian waterways from municipal wastewater effluents (MWWEs), our objectives were to study the potential effects of the dyes in fathead minnow (*Pimephales promelas*), and to compare levels of effect to environmental concentrations and concentrations in Canadian MWWEs.

Fathead minnow embryo-larval-adult and lifecycle exposures have been successfully used to study pulp mill effluents, oil sands sediments, estrogens, pharmaceuticals and personal care products (Colavecchia et al., 2004); (Parrott, 2005); (Parrott and Blunt, 2005); (Parrott and Bennie, 2009); (Parrott et al., 2013). Embryo-larval exposures are advantageous because the embryological and larval stages are sensitive to toxicant exposure, survival and growth of hatched larvae can also be assessed, and the duration of the exposures is substantially shorter than full lifecycle tests, making these methods more practical when testing numerous compounds. The fathead minnow embryo-larval assay was therefore used to examine lethal and sublethal effects of several anthracenedione and azo dyes: Acid Blue 80 (AB80) and Acid Blue 129 (AB129),

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Disperse Yellow 7 (DY7), and Sudan Red G (SRG; Dye structures are shown in Supplementary Information). In this study, we determined dose-response curves for fathead minnow survival over a 20-day exposure (5 days in the egg stage, through hatching, and 14 days in larval-juvenile stage). We also assessed growth (length, weight) and condition factor in larval fish. We compared the results to the concentrations of dyes in 162 municipal wastewater effluents across Canada.

2. Methods

2.1. Dye solutions

Disperse Yellow 7 (95% dye content), Sudan Red G (96% dye content), Acid Blue 80 (40% dye content) and Acid Blue 129 (25% dye content) were purchased from Sigma–Aldrich (Oakville, ON). Stock solutions were prepared (see Supplementary Information), and frozen in scintillation vials at −20 °C in aliquots. One vial of stock was removed from the freezer each day, thawed, and used to prepare new exposure solutions which were then placed in a 25 °C water bath to warm and equilibrate. Daily solution changeovers involved the transfer of eggs/larval fish and egg cups to a fresh beaker. Water samples (100 mL) were collected from each concentration at the beginning and end of the fish exposure solution changeover and stored in the dark at 4 °C pending chemical analysis for dye concentration.

2.2. Municipal wastewater effluents

Over the course of three years (2009–2012), samples collected on condition of anonymity from 162 municipal wastewater treatment plants across Canada were provided by S.A. Smyth (Environment Canada). Wastewater treatment plants featuring different methods of wastewater treatment were chosen (primary treatment, aerated lagoon treatment, lagoon treatment, secondary membrane treatment, and secondary activated sludge treatment), and samples were collected during winter, spring, summer, and autumn to account for possible seasonal variability. Twenty of the wastewater treatment plants are described in (Guerra et al., 2014a, 2014b). Samples were stored in amber glass solvent containers at 4 °C until they were extracted.

2.3. Fathead minnow embryo-larval tests

Fathead minnow early-life stage assays were performed in 2009–2010 in accordance with OECD TG 210 (OECD, 1992) guidelines but typically ended 14 days post-hatch (dph). Eggs were collected from an in-house fathead minnow (*P. promelas*) breeding culture, and were less than 24 h post-fertilization (0 days post-fertilization, dpf) at the start of the exposure. Replicates were started with eggs from at least 3 breeding groups to maximize genetic diversity and variability. There were 30 eggs per beaker, and 3–4 replicates of each dye exposure concentration. Temperature was maintained by housing all beakers within aquaria in a large water bath set to 25 °C. Temperature, pH, conductivity, dissolved oxygen and free ammonia were measured in fish exposure beakers several times during the test (see Supplementary Information). All water quality parameters were within acceptable limits over the test periods.

Static-renewal exposures began at 0 dpf and ended at 14 dph, for a total exposure of 20 days (with hatch at 4–5 dpf). Eggs and larvae were reared in aerated 1-L glass beakers (with 1 L test solution) in egg cups (glass cylinders with fine nylon mesh) to facilitate transfer between old and new solutions. Fish exposure solutions and beakers were renewed daily over the 20 day exposure (at hatch on day 5, and at first cull on day 14).

Nominal test concentrations for dye exposures were: 10, 100, 1000, and 10,000 μg/L for AB80 and AB129; 10, 18, 32, 58, and 100 μg/L for DY7, and 1, 10, 100, and 1000 μg/L for SRG. Control groups of both laboratory water and methanol controls were run alongside dye exposures. Methanol controls were necessary for DY7 and SRG, as they were less water-soluble and methanol was needed to prepare test solutions. Methanol controls were prepared to expose eggs and larvae to the same concentration of methanol (1–2 mL methanol/L) as the highest exposure concentration of the dye tested. Details on methods for preparation of dye solutions and methanol controls are shown in the Supplementary Information. If no MeOH controls were needed (as was the case for AB80 and AB129), the lab water control group replicates were doubled.

Embryos and larvae were inspected each day for mortalities, which were recorded and removed. Severely deformed and/or immobile larvae with necrosis, but still with heartbeats, were described, removed and euthanized via immersion in tricane methane sulfonate solution (250 mg/L) or clove oil solution (10 drops/L). Larvae were fed 20 μL/fish of a newly-hatched brine shrimp slurry daily (mean density of 6 nauplii/μL) 2 h prior to the solution changeover. The number of larvae in the beaker was randomly culled at day 14 of the test (8 dph) to a maximum of 15 larvae. The culled individuals were assessed for total length to 0.01 mm (at 6.3x magnification), and mass (to 0.01 mg). At day 20 of the test (14 dph) all remaining surviving larvae were euthanized and similarly assessed (length and weight measured, and condition factor calculated).

Survival was calculated at several timepoints during each test. Survival from 'egg to hatch' was the # larvae hatching on day 5/# eggs in replicate beaker at the start. Survival from 'egg to thin' was the # larvae alive on day 8 post-hatch (prior to the cull)/# eggs in that replicate beaker at the start. Survival from 'thin to end' was the # larvae alive on day 14 post-hatch/# larvae left after the cull on day 8 post-hatch (usually 15 larvae). Survival from 'start to end' was calculated as the product of 'egg to thin' and 'thin to end' survival rates.

2.4. Measurement of dyes in fish exposure solutions and municipal wastewater effluents

Municipal wastewater effluents (162) were shipped at 4 °C from sites across Canada, and stored in the dark at 4 °C until analysis. All MWWE samples and fish exposure solutions (100 mL) were filtered through a 3 cm bed of Celite 545 (Fisher Scientific) on a 0.7 μm GFF filter (VWR Scientific) using an Alihn funnel. For fish exposure solutions containing AB80, the filtrate was directly analysed by High Pressure Liquid Chromatography-Photodiode Array (HPLC-PDA). For all other solutions (fish exposures to DY7, SRG, AB129 and all MWWE samples), the filtrate was adjusted to pH 3 using 0.1 N HCl and loaded onto an ENVI-18 Solid Phase Extraction (SPE) cartridge (Supelco; Oakville, Canada) that had been previously conditioned by passing 5 mL methanol (MeOH) followed by 5 mL H_2O adjusted to pH 3 using 0.1 N HCl). Once the sample was loaded onto the SPE sorbent bed, the cartridge and sample container were both rinsed with water (adjusted to pH 3, using 0.1 N HCl) and eluted using MeOH (3 x 5 mL). The resulting eluate was evaporated to dryness under a stream of N_2, and the residue was reconstituted in 1 mL of MeOH for subsequent analysis by HPLC-PDA.

HPLC-PDA analyses for the determination of the extracted dyes were performed using an Agilent LC1100, equipped with a PDA detector using a Phenomenex Ultracarb C-18 column (ODS 30, 150 mm × 4.6 mm ID × 5 μm) at 30 °C into which was injected 10 μL sample aliquots. The detector was set to the λ_{MAX} values for the various dyes: 423 nm (DY7), 504 nm (SRG), 590 nm (AB80, AB129).