Published in final edited form as:

Environ Toxicol. 2018 May ; 33(5): 555–568. doi:10.1002/tox.22541.

Toxicity Implications for Early Life Stage Japanese Medaka (*Oryzias latipes*) Exposed to Oxyfluorfen

Doris K. Powe¹, Asok K. Dasmahapatra^{1,2}, Joseph L. Russell³, and Paul B. Tchounwou^{1,*}

¹Environmental Toxicology Research Laboratory, NIH RCMI-Center for Environmental Health, College of Science, Engineering and Technology, Jackson State University, Jackson, MS 39217, USA

²National Center for Natural Product Research, School of Pharmacy, University of Mississippi, University, MS 38677

³Department of Chemistry and Physics, Tougaloo College, Tougaloo, Mississippi, USA

Abstract

We investigated the potential toxic effects of Oxyfluorfen (OXY), an herbicide used in agriculture, on the embryo-larval development of Japanese medaka fish (*Oryzias latipes*). Embryos (1day post fertilization) and larvae (2 day post hatch) were exposed to OXY (0.5–8 mg/L) for 96 h and evaluated for mortality and hatching on embryos, and the mortality and growth on larvae during depuration. It was observed that the embryo-mortality was inconsistently altered by OXY; only the 2 mg/L group showed significant reduction on embryo survivability. However, larval-mortality was concentration-dependent and OXY exposure induced scoliosis-like phenotypic features in the surviving larvae; the calculated IC₅₀ was 5.238 mg/L. Our data further indicated that larval skeleton, both axial and appendicular, was the potential target site of OXY. Skeletal growth in larvae exposed to 2 mg/L was inhibited significantly until one week of depuration with regard to the linear lengths of neurocranium, Meckel's cartilage, caudal vertebrae (first 10) in the axial skeletons, and pectoral fin and urostyle in the appendicular skeletons. Moreover, the total protein content remained unaltered by OXY after 96 h exposure; while the RNA concentration was reduced significantly in larvae exposed to 2 mg/L. Expression analysis of several genes by quantitative real-time RT-PCR (RT-qPCR) showed significant upregulation of *zic5*, a zinc-finger type transcription regulator, at the transcription level. This study indicated that the scoliosis induced by OXY in Japanese medaka larvae was the result of stunted skeletal growth, probably due to deregulation of zinc-finger type transcription regulators, at the genomic level.

1 INTRODUCTION

Oxyfluorfen (OXY) is a diphenyl-ether herbicide globally used in agriculture to control grass and weeds.¹ The currently registered OXY is 98% pure and was evaluated for toxicity in laboratory animals.² In plants, diphenyl-ether herbicides inhibit photosynthesis by

*Corresponding author: Paul B. Tchounwou, Office of the Dean, College of Science, Engineering and Technology, Jackson State University, 1400 JR Lynch Street, Jackson, MS 39217, USA, Phone: 601-979-0777, Fax: 601-203-5142, paul.b.tchounwou@jsums.edu.

blocking chlorophyll synthesis through a mechanism that inhibits the activity of protoporphyrinogen oxidase, the enzyme that catalyzes oxidation of protoporphyrinogen IX into protoporphyrin IX.^{3–5} In humans, upon exposure, OXY is rapidly absorbed and excreted in feces and urine with trace amounts remaining in the body.¹ In animals, it showed low acute oral, dermal, and inhalation toxicity.² The primary toxic sites of OXY is the blood and liver. In rodents, OXY inhibits heme biosynthesis⁶ and induces variegate porphyria. This compound is also structurally related to a class of peroxisome proliferators that induces liver tumors through a nongenotoxic mode of action. Although in male mice OXY did not increase peroxisomes, it moderately increased the expression of *cyp2b10* and *cyp4a10* at the transcription level.⁷ Moreover, it did not produce reproductive or developmental toxicity in rodents and is considered a nonmutagenic or nongenotoxic compound.² A recent report indicated that OXY may induce birth defects such as transverse limb deficiency or craniosynostosis in babies of San Joaquin Valley of California, USA, where mothers were exposed to OXY through commercial agricultural pesticides.⁸ Even though it is not predicted to be carcinogenic in humans, USEPA has classified OXY as a possible human carcinogen.

As a persistent herbicide in the environment, OXY could be a serious threat to aquatic organisms including fish, with a negative impact on water quality. This herbicide has low solubility in water (0.116 mg/L), low vapor pressure (0.026 mPa at 25 °C), and biodegradation is very poor. Plants cannot metabolize OXY and it is only slowly assimilated by microorganisms.^{9–10} A recent survey in the lower Columbia River in Oregon and Washington states in the USA indicated that OXY could be accumulated in the liver tissue of fish (largescale suckers male) due to pollution.¹¹ Earlier reports indicated that OXY is able to induce hsp 70 protein in the liver and kidney of Egyptian Nile fish, *Oreochromis niloticus*¹² and to reduce acetylcholinesterase activity in the brain of *Oreochromis niloticus* and *Gambusia affinis*.¹³ With these brief reports the toxic potential of OXY on fish cannot be evaluated properly. The aim of this study is to evaluate the potential toxic effects of OXY in the development and growth of Japanese medaka fish (*Oryzias latipes*) embryos and larvae after 96 h waterborne exposure with different concentrations. Japanese medaka fish is a small laboratory fish and like zebrafish (*Danio rerio*), are extensively used in toxicological research. Our data indicate that the larvae were more sensitive to OXY than the embryos and this compound was able to affect larval morphology and growth by deregulating gene expressions at the transcription level.

2 MATERIALS METHODS

2.1 Chemicals

The herbicide oxyfluorfen (OXY), C₁₅H₁₁ClF₃NO₄, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl) benzene (also known as Goal) was purchased from Chem Service (West Chester, PA) in technical grade (purity 98%). A stock solution was prepared by dissolving 0.08 g oxyfluorfen (OXY) in 50 mL acetone and diluted to the desired concentration either in embryo rearing medium (ERM; 17 mM NaCl, 0.4 mM KCl, 0.6 mM MgSO₄, 0.36 mM CaCl₂, pH adjusted to 7.4 with NaHCO₃ and 0.0002% methylene blue to reduce fungal infection) or in balanced salt solution (BSS; 17 mM NaCl, 0.4 mM KCl, 0.3

mM MgSO₄, and 0.3 mM CaCl₂, pH adjusted to 7.4 by NaHCO₃) according to the nature of the experiments. The final acetone concentration was adjusted to 0.1% in all experiments.

2.2 Animal rearing, embryo collection, and exposure of embryos and larvae to OXY

The Institutional Animal Care and Use Committee (IACUC) of the Jackson State University approved all experimental protocol.

Methods on the maintenance of Japanese medaka fish adults (breeders), collection of fertilized eggs, identification of the different developmental stages¹⁴ and the culture conditions of Japanese medaka fish embryos have been described previously.^{15–16} In brief, the orange-red varieties of adult Japanese medaka fish (breeders) were maintained in BSS in Aquatic Habitats ZF0601 zebrafish standalone system (Aquatic Habitats, Apopka, FL, USA). The fish were fed twice daily with TetraMin flakes and brine shrimp nauplii (*Artemia*). Fertilized eggs, after collection, were gently separated and checked under a binocular microscope for screening. The embryos which were damaged during collections or remained unfertilized were removed from the rest of the embryos which were in stage 10 of development.¹⁴ Collected intact embryos were maintained in ERM at standard laboratory conditions (25 ± 1 °C with 16L: 8D light cycle). The embryos were selected for OXY exposure, when they reached Iwamatsu stage 20–22 (~30–40 hour post fertilization; hpf). In order to obtain larvae, the embryos were allowed to hatch in standard laboratory conditions in ERM and transferred to BSS once hatched. After 48h of hatching when they had overcome hatching stress, the larvae were used for experiments. The embryos (1 day post fertilization; dpf) were exposed to 0.5, 1.0, 2.0, 4.0 and 8.0 mg/L OXY for 96 h in loosely capped, Teflon-lined 60 mL vials (Chemware, Raleigh, NC) containing 20 embryos/20 mL media/vial. The control embryos were maintained in identical conditions in vehicle (0.1% acetone) and also in clean ERM (no vehicle or OXY). All the experimental embryos were maintained under a 16L: 8D light cycle in a precision High Performance Incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 26 ± 1 °C. Half of the media was removed and replaced daily. After the required period of treatment, the surviving embryos were transferred to a 48 well plate, maintained in clean ERM and allowed to hatch under standard laboratory conditions. The media (50% static renewal) was also changed every day. The mortality of the embryos was evaluated immediately after the removal of OXY (5 dpf) or on 12 dpf (one week depuration). The hatching efficiency was evaluated on 9 and 12 dpf. For larvae, the hatched embryos 2 day post hatch (dph) were used for OXY exposure. They were exposed to 1, 2, 4 and 8 mg/L OXY for 96 h in BSS and maintained in standard laboratory conditions (No food) in Teflon-lined 60 mL vials (20 larvae in 20 mL media/vial) (Chemware, Raleigh, NC). Half of the media was replaced once every day. After the required period of treatment (96 h), the surviving larvae were either homogenized for protein or RNA extraction or transferred to clean BSS in glass bowl (150–200 mL) and maintained for 1 and 3 weeks with food. In few occasions, the larvae were reared to 8 and 12 weeks of age in Aquatic Habitats ZF0601 zebrafish standalone system (Aquatic Habitats, Apopka, FL) for growth (length and weight) evaluation. The length and weight of the larvae were taken after 1 and 3 weeks of depuration, used for bone and cartilage staining¹⁵ and other required analyses.

2.3 Cartilage and bone staining and morphometric analysis

Both control and treated-larvae (2, 7, and 21 dph) were preserved in Gregg's fixative for 1–2 days, bleached in 10% hydrogen peroxide, and washed in several changes of deionized water over 2–4 days and used for cartilage/bone staining.¹⁵ Briefly, the fixed larvae were stained in 1% Alcian Blue (Sigma-Aldrich, St. Louis, MO, USA) and rehydrated with graded alcohols (95–30%) and finally washed in water. The samples were treated with trypsin (Sigma-Aldrich, St. Louis, MO, USA) either for 24 h at 4 °C, or longer depending upon the degradation of muscles from the cartilages and bones, followed by a wash in 5% KOH. The bones were stained in 0.25% Alizarin Red (Sigma-Aldrich), washed in 5% KOH and dehydrated in graded ethanol. The specimens were finally transferred to 5% KOH overnight and preserved in 1% methyl salicylate for storage. The photographs were taken in different anatomical positions (dorsal, ventral, lateral) using an Olympus BX 40 microscope and used for morphometric analysis (in the axial skeleton: total length of the neurocranium, Meckel's cartilage, cumulative lengths of the first 10 caudal vertebra, linear length of 15th caudal vertebra as individual vertebral length; in the appendicular skeleton: length of pectoral fin and the linear length of urostyle).

2.4 Extraction of RNA and protein, cDNA synthesis, priming strategy, and quantitative real-time polymerase chain reaction (RT-qPCR)

After the required period of treatment (96 h exposure to OXY), the larvae (10 larvae) were homogenized in 0.25 M sucrose and used for protein precipitation by 5% perchloric acid (Sigma-Aldrich, St. Louis, MO). The precipitated protein was freed from lipid by ethanol: chloroform: ether (2:1:1) mixture at 40 °C, washed with absolute ethanol, dried with ether wash and finally dissolved in 0.5M NaOH. Protein concentration was determined using BioRad DC protein assay technique (BioRad laboratories, Hercules, CA, USA). The data were expressed as mg protein/larvae. For extraction of RNA, 4–5 larvae were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer instruction. To remove the genomic DNA, the extracted RNA was treated with RNase-free RQ1 DNase (Promega, Madison, WI). The concentration of the purified RNA was determined in an Eppendorf Biophotometer (Hauppauge, NY) and the 260:280 nm ratios were within 1.8–2.0. The quality of the purified RNA was evaluated by 1% agarose gel electrophoresis. All purified RNA showed two distinct 18S and 28S ribosomal RNA bands. Purified RNA (1 µg) was used to synthesize cDNA using a first strand synthesis protocol (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis was made at 50 °C for 1h in a 20 µL reaction volume containing 4 µL 5X buffer, 1 µL 10 mM dNTP, 1 µL oligo dT and 200 units of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). One µL of the synthesized cDNA was used for quantification of the target gene mRNAs (Table 1) by quantitative real-time RT-PCR (RT-qPCR) with gene-specific primers (IDT, Coralville, IA, USA) as targets and β -actin primers as internal control (Table 1). The PCR reactions were conducted in a thermal cycler (DNA Engine Opticon 2; BioRad Laboratories, Hercules, CA, USA) with a 20 µL reaction volume containing 10 µL reaction buffer, 1 µL cDNA, 50 pM of each of the forward and reverse primers of the target or β -actin (internal control) genes. The volume was adjusted to 20 µL with required amount of nuclease-free water (Please see the supplementary MIQE Table). The amplification and fluorescence detections were performed in a real-time PCR thermal cycler (Opticon 2, BioRad Laboratories, Hercules, CA, USA).

The reaction conditions were: initial denaturation at 95 °C for 3 min, one cycle, followed by 40 cycles of denaturation at 94 °C, 30 sec, annealing at 65 °C, 1 min, and extension at 72 °C, 2 min, fluorescence data collection for one sec; to avoid the formation of primer dimers, a second set of fluorescence data was collected after incubating the samples at 76 °C for 1 sec. A final extension of one cycle at 72 °C for 10 min was made. The melting curve was constructed by plotting fluorescence data against temperature (65–95 °C with an interval of 0.2 °C). Samples with good melting curve were considered for analysis. The cycle threshold or C_t line was set manually using the Opticon monitor software (MJ Research, San Francisco, CA, USA). This threshold was applied to all wells for consistent analysis of individual samples and internal standards. For each sample, the threshold cycle for internal standard (β -actin) amplification (C_t , β -actin) was subtracted from the threshold cycle of the corresponding target gene amplification (C_t , target gene) to yield ΔC_t . The threshold cycle represents the cycle number at which the fluorescence signal was significantly above the base line (in our situation it was routinely between 0.05–0.1). For each treatment group, the mean ΔC_t of control samples was subtracted from each individual samples to yield individual ΔC_t . Fold induction relative to control were calculated with $2^{-\Delta C_t}$.

2.5 Statistics

The data were analyzed by one way ANOVA followed by Post-hoc Tukey's multiple comparison test or nonparametric "t"-test (GraphPad prism version 5; GraphPad Prism Inc., CA, USA). The results were expressed as Mean $\pm$ SEM with $p < 0.05$ considered as significant.

3 RESULTS

3.1 Effects of OXY on Japanese medaka fish embryos and larvae

3.1.1 Mortality and hatching efficiency of embryos exposed to OXY during embryonic development—Fertilized eggs of Japanese medaka fish after one day of development (Iwamatsu stage 17–18) in ERM were exposed to five different concentrations of OXY (0.5, 1, 2, 4, and 8 mg/L) for 96 h in ERM and then in clean ERM without OXY. The control eggs (no OXY) were exposed in both ERM and in vehicle (0.1% acetone) for 96 h and then in clean ERM. The media (50% static renewal) were changed daily. The viable embryos were maintained in clean ERM and allowed to hatch. The embryo mortality was evaluated after 96 h exposure (5 dpf) of OXY and on 12 dpf (after 7 day depuration). Hatching efficiencies (% hatch) were evaluated on 9 and 12 dpf. It was observed that the survivability of the control embryos either maintained in clean ERM or vehicle-treated (0.1% acetone) was almost identical. Moreover, except the 2 mg/L group (72% embryos survived), almost all the embryos, including 8 mg/L group, survived after 96 h of OXY treatment (Fig. 1A). The embryos surviving after 96 h of OXY treatment were further validated for mortality on 12 dpf (7 days maintenance in clean ERM for depuration) and was found that the survivability of the embryos/larvae on 12 dpf exposed to 2 mg/L OXY was significantly lower than the control embryos/larvae (Fig. 1B). The surviving embryos began hatching in 7 dpf (~168 hpf). It was observed that more than 60% of the controls (both ERM and vehicle-treated), and the embryos exposed to 0.5 mg/L OXY hatched successfully on 9 dpf (Fig. 1C). The hatching rates on 9 dpf were reduced although the difference was not

significant in the embryos exposed to 1, 2, 4, and 8 mg/L OXY when compared with the control (both in ERM and vehicle-treated) embryos (Fig. 1C). The hatching rates in all experimental groups (control, vehicle-treated, and OXY-treated embryos) were slightly increased on 12 dpf. However, the hatching rates in 2, 4 and 8 mg/L groups in 12 dpf embryos were observably lower than the control groups (both in ERM and vehicle-treated), even though the data failed to establish any significant difference with controls (Fig. 1D).

3.1.2 Larvae exposure to OXY—Japanese medaka larvae (2 dph) were exposed to OXY (0.25–8.0 mg/L) for 96 h (2 dph–6 dph) and evaluated for larval mortality. It was observed that mortality was increased with the increase of OXY concentration (Fig. 2A) and the calculated LC_{50} was 5.238 mg/L (Fig. 2B). Moreover, the larvae exposed to OXY showed scoliosis-like phenotypic features in a concentration-dependent manner (Figs. 2C–G). The scoliosis-like phenotypes were found to be maximum in larvae exposed to 8.0 mg/L and minimum in 2.0 mg/L OXY (Fig. 2G). As the mortality of the larvae increased with the increase in OXY concentration (less than 50% of the larvae survived at 6 dph with OXY concentration 4 mg/L and above), for the remainder of the study we restricted our experiments to only two concentrations of OXY (0.5 and 2.0 mg/L).

3.2 Effects of OXY on the growth of the larvae of Japanese medaka fish

The growth of Japanese medaka larvae (2 dph) exposed to 0.5 and 2 mg/L of OXY for 96 h (2 dph–6 dph) was evaluated as total body length (Figs. 3A–C) and weight (Figs. 3D–E) after 1, 3, 8 and 12 weeks of depuration. It was observed that after one week of depuration, the total body length of control larvae (maintained in BSS) was 7.33 mm ($n=15$) (Fig. 3A). There was no significant difference in total body length of the control larvae (ERM) with the larvae exposed either to vehicle (0.1% acetone) or to OXY (0.5 or 2 mg/L). After 3 weeks of depuration, the total body length of the larvae increased significantly in controls (both ERM and vehicle) and OXY (0.5 and 2 mg/L) groups, compared to the length at 1 week of depuration. In controls (ERM) the average increase after 3 weeks of depuration was approximately 28% (9.40 mm) of the total body length observed in 1 week (Fig. 3B). Moreover, the growth of the larvae with regards to total length remained at the same level in all other groups exposed either in vehicle or in OXY (Fig. 3B). After 8 weeks of depuration, the length of the control fish (ERM) was increased to 22.5 mm (approximately 200% increase compared to the larval length observed in 1 week of depuration). After 12 weeks of depuration, the total length of the control fish (ERM) was increased further to 25.0 mm which is approximately 250% of the total length of the larvae observed after one week of depuration. Moreover, the total length of the larvae exposed to vehicle (0.1% acetone) or OXY (0.5 and 2 mg/L) was unable to establish any significant difference in total length in comparison to controls (ERM) either in 8 or 12 week of depuration (Fig. 3C).

The weight of the larvae, as in total body length, also remained unaltered when compared with the larvae exposed to different concentrations of OXY (Figs. 3D–E). The weight of the control larvae (ERM) after 1 week of depuration was ~5 mg (Fig. 3D) and there were no significant differences in body weight of the larvae exposed either to vehicle or OXY (0.5 and 2 mg/L) with the control groups (ERM). However, in three weeks, the body weight of control groups (ERM) was approximately 30 mg/larvae which is approximately a 500%

increase in body weight over the larval weight at 1 week of depuration (Fig. 3D). The body weight of other groups (vehicle, and OXY 0.5 and 2 mg/L) was also increased significantly after 3 weeks of depuration, however, unable to establish any significant difference when compared with the corresponding controls (Fig. 3D). In 8 and 12 weeks of depuration, the body weight of the control larvae (both ERM and vehicle-treated) were enhanced further, and the body weight of the larvae exposed to OXY (both 0.5 and 2.0 mg/L) showed identical enhancement as in the control fish (Not statistically different from the control groups) (Fig. 3E).

3.3 Effects of OXY on cartilage and bone growth of Japanese medaka larvae

Both control and OXY-treated larvae were fixed in Greg's fixatives and used for morphometric analyses after 1 and 3 weeks of depuration considering some selected skeletal structures of the axial and appendicular skeletons. The lengths of neurocranium (Figs. 4A–D), Meckel's cartilage (Figs. 4A–D), the cumulative length of the first 10 caudal vertebrae (characterized by the presence of hemal arches) (Figs. 4E–G; 4J–K) and the individual length of the 15th caudal vertebra (Figs. 4E–I) were considered as axial skeletal features and the length of pectoral fin rays (Fig. 4E), and urostyle (Figs. 4H–I) were evaluated as appendicular skeletal features. It was observed that OXY exposures were able to retard the growth of neurocranium at 1 week of depuration only in larvae exposed to 2 mg/L, while in 3 weeks the neurocranial length of the larvae exposed to OXY (both 0.5 and 2.0 mg/L) was found to be identical with controls (Fig. 4.1). Consequently, the length of Meckel's cartilage of the larvae exposed to 2 mg/L was found to be shorter at 1 week of depuration (Fig. 4.2) not in week 3 (Fig. 4.3). The cumulative length of the first 10 caudal vertebrae was reduced significantly in larvae exposed to 2 mg/L OXY only after one week of depuration (Fig. 4.4); other OXY concentrations (0.5 mg/L) at 1 week and both 0.5 and 2.0 mg/L concentrations of OXY after 3 weeks were unable to establish significant difference in the cumulative length of the first 10 caudal vertebra when compared with corresponding controls (both ERM and vehicle-treated) (Fig. 4.5). However, the length of the 15th caudal vertebrae remained unaltered in both one and three weeks of depuration (Figs. 4.6 and 4.7). The length of appendicular skeletons (pectoral fin rays and urostyle) was also reduced in larvae exposed to 2 mg/L OXY at one week of depuration. However, in 3 weeks, the length of pectoral fin and urostyle appeared to be identical with controls (Figs. 4.8 and 4.9).

3.4 Effects of OXY exposure on total protein and RNA content of larvae after 1 and 3 weeks of depuration

Total protein and RNA content of the whole larvae were evaluated after 96 h of OXY exposure. It was observed that the total protein of Japanese medaka larvae was only ~3μg/larvae; the larvae exposed to 0.5 mg/L or 2.0 mg/L were unable to establish any significant difference in total protein between the larvae of controls (ERM and vehicle) and OXY (0.5 or 2.0 mg/L)-treated groups (Fig. 5A). However, the total RNA content was found to be reduced significantly in the larvae exposed to 2.0 mg/L but not in 0.5 mg/L when compared with the corresponding controls (Fig. 5B).

3.5 Effects of OXY exposure on mRNA expression of *emx2*, *en2*, *irx3a*, *otx2*, *wnt1*, *shh*, and *zic5*

We analyzed the mRNA expression of *emx2*, *en2*, *irx3a*, *otx2*, *wnt1*, *shh*, and *zic5* genes of Japanese medaka larvae exposed to 0.5 and 2 mg/L OXY for 96h by RT-qPCR (Fig. 6) after following the minimum information for publication of quantitative real-time experiments (MIQE) guidelines.¹⁷ (please see supplementary data file). The extracted RNA was reverse transcribed and the genes amplified by gene-specific primers (Table 1). *β-actin* (*actb*) was used as internal control. It was observed that compared to controls, the expression of *en2*, *otx2*, and *zic5* were found to be enhanced ~2-fold in both 0.5 (*en2*: 3.96±0.92; *otx2*: 2.51±0.20; *zic5*: 7.11±1.60 folds) and 2.0 mg/L OXY (*en2*: 2.75±0.62; *otx2*: 1.88±0.06; *zic5*: 5.76±0.77 folds), however, only *zic5* showed significant difference. The expression of other genes such as *emx2*, *irx3a*, *shh*, and *wnt1* was identical with controls (~1) both in 0.5 and 2 mg/L OXY (Fig. 6).

4 DISCUSSION

In continuation of our studies on the effects of environmental pollutants on Japanese medaka fish^{18–19}, a small laboratory fish, considered as complementary to zebrafish²⁰, we studied the potential toxic effects of OXY, an herbicide, widely used in agriculture. This compound is almost insoluble in water (0.116 mg/L at 25 °C); to improve the solubility of OXY in ERM or BSS, we used 0.1% acetone as a solvent and parallel control embryos/larvae were maintained in 0.1% acetone-containing media as vehicle control in addition to the normal control maintained in ERM. Our studies indicated that the herbicide was able to induce minimal toxic effects on Japanese medaka embryos upon waterborne exposure (Fig. 1). Both embryonic mortality and hatching efficiency remained unaltered (not significantly different) when compared with the corresponding controls (Figs. 1A–D). However, the survivability of 12 dpf embryo/larvae exposed to 2 mg/L OXY during embryogenesis was found to be significantly reduced when compared with the corresponding controls (Fig. 1B). As the OXY concentrations above 2 mg/L did not establish any concentration-dependent effects on mortality or hatching efficiency, we suggested that OXY at higher concentration (above 2 mg/L) was able to diffuse inconsistently through the chorion and unable to reach optimum level to produce potential toxic effects. We concluded that the chorion of the Japanese medaka embryos is the major barrier that interfere with the entry of OXY into the embryonic environment. We therefore continued our OXY studies using Japanese medaka larvae 2 dph instead of fertilized eggs.

Our data on Japanese medaka larvae documented that OXY caused an increase of larval mortality in a concentration-dependent manner (Fig. 2A). It was observed that approximately 7.9 % of the larvae survived after 96 h of OXY at a concentration of 8 mg/mL while more than 95% of the larvae survived in controls (both in ERM and vehicle) (Fig. 2A). Moreover, the larvae showed increasing scoliosis- like phenotypic features with increasing concentrations of OXY (2–8 mg/L) after 96 h of exposure (Figs. 2B–G). The calculated IC₅₀ value from a typical mortality curve was found to be 5.238 mg/L (Fig. 2B). This is in good agreement with the observation made¹³ in Egyptian Nile fish (*Oreochromis niloticus*) where the LC₅₀ after 96 h exposure to OXY was 3 mg/L. We therefore restricted our studies to only

two sub-lethal concentrations of OXY: 0.5 mg/L (~ 10% of IC₅₀ with no scoliosis) and 2.0 mg/L (~ 40% of IC₅₀ with ~10% of the larvae developed scoliosis).

OXY is rapidly absorbed and excreted unchanged in feces and urine with little remaining in the tissues of human.¹ Moreover, this compound is structurally related to a class of peroxisome proliferators that cause liver tumors through a nongenotoxic mode of action. Therefore, we aimed to investigate whether OXY absorbed during 96 h larval exposure was able to produce any measurable toxicity during later period of survival. In our studies, after 96 h of OXY exposure (0.5 mg/L and 2.0 mg/L), the larvae were maintained in clean BSS for a maximum period of 12 weeks and were evaluated for normal growth. We considered the total length and weight of the larvae as indices of growth (Fig. 3). It was observed that the linear length and weight of the larvae in both controls and OXY-treated larvae (0.5 and 2 mg/L) were gradually increased over time; however, the data (length and weight), were unable to establish any significant difference with the corresponding controls at any time points (1, 3, 8 and 12 weeks of depuration) under investigation, even though the values in OXY-treated larvae showed a tendency of reduction. As the growth of the larvae, especially the linear length, followed the normal trend¹⁴, we predict that the larvae, like human, rapidly excrete OXY during depuration and the potential toxic effects, if any, could be seen if OXY constantly exists in the surrounding environment.

The effect of OXY on the linear length of the larvae, as per our observation, was unable to draw any conclusive evidence, even though the larvae treated by OXY appeared to be shorter than controls. To verify the effects more accurately, we extended our studies to individual bones/cartilages in the axial and appendicular skeletons. Among axial skeletons, the linear length of the neurocranium, Meckel's cartilage, the cumulative lengths of the first 10 caudal vertebrae, and the individual length of the 15th caudal vertebra were considered for evaluation (Figs. 4A–J and Figs. 4.1–4.7). In the appendicular skeleton, the linear length of the pectoral fin and the length of the urostyle were measured (Figs. 4G–J, Figs. 4.8–4.9). Moreover, we predict that the larvae could overcome the toxic effects of OXY due to prolonged duration of depuration. Therefore, the morphometric analysis of the axial and appendicular skeletal structures were restricted only at 1 and 3 weeks of depuration. Our data showed that except the individual length of 15th caudal vertebra in the axial skeleton, the other parameters in both axial (neurocranium, Meckel's cartilage, cumulative length of first 10 caudal vertebrae) and appendicular (pectoral fin and urostyle) skeletons, showed significant reduction in length after one week of depuration when compared with the corresponding controls (Figs. 4.1–4.7). However, after 3 weeks of depuration, the linear length measured in both axial and appendicular skeletal components returned to normal as observed in corresponding controls (Figs. 4.1–4.7). Therefore, our data indicated that the larval skeleton is a potential target site of OXY and the skeletal growth was stunted by OXY for a limited period until the significant amount of OXY remained accumulated in the body. Although we were unable to determine the OXY concentration accumulated in the larvae after 96 h of exposure, our data evidenced that like human, the absorbed OXY was excreted and released from the body during depuration. Moreover, by three weeks, most of the accumulated OXY either completely metabolized or was excreted from the larval body and the growth of the larvae (with regard to linear lengths of axial and appendicular skeletal structures) which showed stunted during week 1, became normal in week 3. From these

limited observations it is very difficult to accurately predict any possible mechanism of action/sites of OXY on the growth (length and weight) of Japanese medaka larvae. Although in our studies 0.5 mg/L OXY was unable to produce any toxic effects on skeletal development (probably due to rapid excretion), continuous presence of OXY in the aquatic environment (even lower than 0.5 mg/L) might have the potential to induce toxic effects. To our knowledge there are no published reports on the effects of OXY on the skeletal growth of fish. Environmental chemicals including OXY, in general, probably disrupt key pathways associated with apoptosis and cell death¹ that can be reflected on growth retardation. Moreover, an available report on the OXY exposure of mothers in San Joaquin Valley of California through pesticides induced craniosynostosis and transverse limb deficiency in their babies⁸ which suggested that the neurocranium or other developing cartilages/bones in Japanese medaka larvae could be potential target sites of OXY effect.

We analyzed the total protein and RNA content of the larvae as additional supportive data to the growth (length and weight) of the larvae. Our data indicated that the total protein content of the larvae immediately after removal from OXY exposure remained unaltered when compared with controls (Fig. 5A), however, the total RNA content decreased significantly in 2.0 mg/L group (Fig. 5B). Further we analyzed the expression of several genes (*emx2*, *en2*, *irx3a*, *otx2*, *shh*, *wnt1* and *zic5*) at the transcription level in Japanese medaka larvae exposed to OXY (Figure 6). The expression of all these genes during embryogenesis (0–6 dpf) of Japanese medaka fish were analyzed previously and found to be developmentally regulated.²¹ Our data on larvae indicated that three genes (*en2*, *otx2*, and *zic5*) tended to be upregulated and others (*emx2*, *iro3*, *shh*, *wnt1*) remained unaltered when compared with the controls (Fig. 6). Among the upregulated genes, *zic5*, a zinc-finger-type transcription regulators,²² showed more than 5-fold enhancement in both 0.5 and 2 mg/L of OXY exposure (Fig. 6). In vertebrate embryos, zic proteins are critical regulators of ectodermal development.^{23–26} In Japanese medaka *zinc finger MYND-type containing 10* (*zymnd10*) mutant fish has the potential to develop scoliosis in the later stages of development²⁷ which suggests that *zymnd10* gene played a significant role in phenotypes with scoliosis. Although more studies are needed, we predict that the upregulation of *zic5* gene, a zinc finger type transcription regulator like *zymnd10*, might be the result of scoliosis-like phenotypic features observed in Japanese medaka larvae exposed to OXY (both 0.5 and 2.0 mg/L).

5 CONCLUSION

Taken together our data documented that OXY was unable to induce severe toxic effects on Japanese medaka embryos with regards to mortality and hatching. However, the larvae were highly sensitive to OXY which affected the mortality and induced scoliosis in a concentration-dependent manner. Our data further showed that the linear growth of neurocranium, Meckel's cartilage, and the proximal caudal vertebrae in the axial skeleton and the pectoral fin and urostyle in the appendicular skeleton were stunted after one week of depuration, while attenuated after three weeks. Moreover, transcription of several genes especially zinc-finger-type transcription regulator genes like *zic5* were upregulated by OXY exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work has been primarily supported by NIH/NIMHD Grant # G12MD0007581 through the RCM Center for Environmental Health at Jackson State University.

References

1. Narayanan KB, Ali M, Barclay BJ, Cheng Q, D'Abronzio L, Dornetshuber-Fleiss R, Ghosh PM, Guzman MJG, Lee T-J, Leung PS, Li L, Luanpitpong S, Ratovitski E, Rojanasakul Y, Romano MF, Romano S, Sinha RK, Yedjou MF, Al-Mulla F, Al-Temaimi R, Amedei A, Brown DG, Ryan EP, Colacci AM, Hamid RA, Mondello C, Raju J, Salem HK, Woodrick J, Scovassi AI, Singh N, Vaccari M, Roy R, Forte S, Memeo L, Kim SY, Bisson WH, Lowe L, Park HH. Disruptive environmental chemicals and cellular mechanisms that confer resistance to cell death. *Carcinogenesis*. 2015; 36:s89–s110. [PubMed: 26106145]
2. U.S. EPA. Oxyfluorfen: Human health risk assessment. Health effects division (HED) chapter for registration eligibility decision (RED) document. Registration case no. 2490. Chemical no. 111601. 2002
3. Matringe M, Camadro JM, Labbe P, Scalla R. Protoporphyrinogen oxidase inhibition by three per oxidizing herbicides: oxadiazon, LS 82–556 and M&B 39279. *FEBS Letters*. 1989a; 245:35–38. [PubMed: 2522396]
4. Matringe M, Camadro JM, Labbe P, Scalla R. Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. *Biochem J*. 1989b; 260:231–235. [PubMed: 2775186]
5. Camadro JM, Matringe M, Scalla R, Labbe P. Kinetic studies on protoporphyrinogen oxidase inhibition by diphenyl ether herbicides. *Biochem J*. 1991; 277:17–21. [PubMed: 1854333]
6. Rio B, Parent-Massin D, Lautraite S, Hoellinger H. Effects of diphenyl-ether herbicide, oxyfluorfen, on human BFU-E.CFU-E development and hemoglobin synthesis. *Human & Exp Toxicol*. 1997; 16:115–122.
7. Stagg NJ, LeBaron MJ, Eisenbrandt DL, Gollapudi BB, Klaunig JE. Assessment of possible carcinogenicity of oxyfluorfen to humans using mode of action analysis of rodent liver effects. *Toxicol Sci*. 2012; 128:334–345. [PubMed: 22539621]
8. Carmichael SL, Yang W, Roberts E, Kegley SE, Brown TJ, English PB, Lammer EJ, Shaw GM. Residential agricultural pesticide exposures and risk of selected birth defects among offspring in the San Joaquin valley of California. *Birth Def Res (part A)*. 2016; 106:27–35.
9. Sondhia S. Persistent and bioaccumulation of oxyfluorefen residues in onion. *Environ, Monit Assess*. 2010; 162:163–168.
10. Calderon MJ, Real M, Cabrera A, Koskinen WC, Cornejo J, Hermosin MC. Influence of olive oil mill waste amendment on fate of oxyfluorfen in southern Spain soils. *Clean Soil, Air, Water*. 2015; 43:1107–1113.
11. Nilsen E, Zaugg S, Alvarez D, Morace J, Waite I, Counilhan T, Hardiman J, Torres L, Patino R, Mesa M, Grove R. Contaminants of legacy and emerging concern in large suckers (*Catostomus macrocheilus*) and the foodweb in the lower Columbia River, Oregon and Washington, USA. *Sci Total Environ*. 2014; 484:344–352. [PubMed: 24462179]
12. Hassanein HMA. Toxicological effects of the herbicide oxyfluorfen on acetylcholinesterase in two fish species: *Oreochromis niloticus* and *Gambusia affinis*. *J Environ Sci Health*. 2002; A37:521–527.
13. Hassanein HMA, Banhaway MA, Soliman FM, Abdel-Rehim SA, Muller WE, Schroder HC. Induction of hsp70 by the herbicide oxyfluorfen (Goal) in the Egyptian Nile fish, *Oreochromis niloticus*. *Arch Environ Contam Toxicol*. 1999; 37:78–84. [PubMed: 10341045]
14. Iwamatsu T. Stages of normal development in the medaka *Oryzias latipes*. *Mech Dev*. 2004; 121:605–618. [PubMed: 15210170]

15. Hu Y, Willett KL, Khan IA, Scheffler BE, Dasmahapatra AK. Ethanol disrupts chondrification of the neurocranial cartilages in medaka embryos without affecting aldehyde dehydrogenase 1A2 (Aldh1A2) promoter methylation. *Comp Biochem Physiology C Toxicol Pharmacol*. 2009; 150:495–502.
16. Dasmahapatra AK, Khan IA. DNA methyltransferase expressions in Japanese rice fish (*Oryzias latipes*) embryogenesis is developmentally regulated and modulated by ethanol and 5-azacytidine. *Comp Biochem Physiol C Toxicol Pharmacol*. 2015; 176–177:1–9.
17. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009; 55:611–622. [PubMed: 19246619]
18. Spencer HB, Hussein WR, Tchounwou PB. Effects of tetrachloroethylene on the viability and development of embryos of Japanese medaka (*Oryzias latipes*). *Archives Environ Contam Toxicol*. 2002; 42:463–469.
19. Spencer HB, Hussein WR, Tchounwou PB. Growth inhibition in Japanese medaka (*Oryzias latipes*) fish exposed to tetrachloroethylene. *J Environ Biol*. 2006; 27:1–5. [PubMed: 16850867]
20. Wittbrodt J, Shima A, Scharf M. Medaka- a model organism from the far east. *Nature Rev Genetics*. 2002; 3:53–64. [PubMed: 11823791]
21. Wu M, Chaudhary A, Khan IA, Dasmahapatra AK. Ethanol teratogenesis in Japanese medaka: effects at the cellular level. *Comp Biochem Physiol Part B*. 2008; 149:191–201.
22. Aruga J, Kamiya A, Takahashi H, Fujimi TJ, Shimizu Y, Ohkawa K, Yazawa S, Umesono Y, Noguchi H, Shimizu T, Saitou N, Mikoshiba K, Sakaki Y, Agata K, Toyoda A. A wide-range phylogenetic analysis of zic proteins: implications for correlations between protein structure conservation and body plan complexity. *Genomics*. 2006; 87:783–792. [PubMed: 16574373]
23. Inoue T, Ogawa M, Mikoshiba K, Aruga J. Zic deficiency in the cortical marginal zone and meninges results in cortical lamination defects resembling those in type II lissencephaly. *J Neurosci*. 2008; 28:4712–4725. [PubMed: 18448648]
24. Warr N, Powles-Glover N, Chappell A, Robson J, Norris D, Arkell RM. Zic2-associated holoprosencephaly is caused by a transient defect in the organizer region during gastrulation. *Hum Mol Genet*. 2008; 17:2986–2996. [PubMed: 18617531]
25. Maurus D, Harris WA. Zic-associated holoprosencephaly: zebrafish zic 1 controls midline formation and forebrain patterning by regulating Nodal, Hedgehog, and retinoic acid signaling. *Genes Dev*. 2009; 23:1461–1473.
26. Sanek NA, Taylor AA, Nyholm MK, Grinblat Y. Zebrafish zic2a patterns the fore brain through modulation of Hedgehog-activated gene expression. *Development*. 2009; 136:3791–3800. [PubMed: 19855021]
27. Kobayashi D, Asano-Hoshino A, Nakakura T, Ansai S, Kinoshita M, Ogawa M, Hagiwara H, Yokoyama T. Loss of zinc finger MYND-type containing 10 (zmynd 10) affects cilia integrity and axonemal localization of dynein arms, resulting in ciliary dysmortality, polycystic kidney and scoliosis in medaka (*Oryzias latipes*). *Dev Biol*. 2017; 430:69–79. [PubMed: 28823919]

Figure 1. Effects of Oxyfluorfen (OXY) on the embryo-larval development of Japanese medaka Fertilized eggs after one day post fertilization (Iwamatsu stage 17–18) were exposed to OXY in embryo rearing media (ERM) for 96 h with 50% static renewal of the media every day. After treatment, the surviving embryos were maintained in clean ERM for depuration. The controls were maintained either in clean ERM or in vehicle (0.1% acetone) in identical conditions (26±1 °C; 16L: 8D light cycle). The embryos were evaluated for mortality at 96 h (Figure 1A) of exposure and also after 12 days (Figure 1B). Hatching efficiency was evaluated on 9 and 12 day post fertilization (dpf) (Figure 1C and 1D). The data are expressed as mean ±SEM of 4–6 observations. It was observed that OXY inconsistently established any significant toxic effects on Japanese medaka embryos with regard to mortality and hatching. The pound symbol (#) indicates that the data are significantly different from control groups (ERM); the asterisks (*) indicate that the data are significantly different from the vehicle (0.1% acetone) group.

Figure 2. OXY toxicity on Japanese medaka larvae

Two-day post hatch Japanese medaka larvae were exposed to OXY in balanced salt solution (BSS) for 96 h with 50% static renewal of the media. Controls were maintained either in ERM or 0.1% acetone (vehicle). The larval mortality was evaluated after 96 h of OXY exposure (6 dph). The data were expressed as mean $\pm$ SEM and $p < 0.05$ was considered significant. The bar head with pound symbol (#) and asterisks (*) indicated that the data were significantly different ($p < 0.05$) from controls (ERM) and vehicle (0.1% acetone),

respectively. It was observed that the OXY toxicity of Japanese medaka is a concentration-dependent phenomenon and the calculated EC_{50} is 5.238 mg/L (log 0.7192) (Figure 2B). Figures 2C–2F: representative figures of OXY-induced scoliosis in Japanese medaka larvae after OXY treatment for 96 h. 2C: control (0.1% acetone); 2D: larvae exposed to 2 mg/L OXY for 96 h. 2E: larvae exposed to 4 mg/L OXY; 2F: larvae exposed to 8 mg/L OXY for 96 h. All these data indicated that OXY induced scoliosis in Japanese medaka larvae in a concentration-dependent manner (Figure 2G).

Figure 3E

Figure 3. Effects of OXY on growth of Japanese medaka larvae

Two day post hatch larvae were exposed to 0.5 and 2.0 mg/L OXY for 96 h with 50% static renewal of the media daily. After treatment the surviving larvae were maintained in clean BSS for depuration of OXY. Growth of the larvae was evaluated as total body length (Figure 3A, 3B, 3C) and weight (3D, 3E) of the larvae after 1, 3, 8 and 12 weeks of OXY exposure. Data were expressed as Mean $\pm$ SEM of 4–6 observations. The larvae exposed to OXY failed to establish any significant difference ($p < 0.05$) from the corresponding control larvae at any time point.

Figure 4F

Figure 4G

Figure 4H

Figure 4I

Figure 4.2

Figure 4.3

Figure 4.4

Cumulative length of first 10 caudal vertebrae (1 week)

Figure 4.5

Cumulative length of first 10 Caudal vertebrae (3 weeks)

Figure 4.6

15th caudal vertebra (1 week)

Figure 4.7

15th caudal vertebra (3 weeks)

Figure 4. Morphometrical analysis of axial and appendicular skeletal structures of Japanese medaka larvae exposed to OXY

Two day post hatch larvae were exposed to 0.5 and 2.0 mg/L OXY for 96 h with 50% static renewal of the media then maintained in clean BSS for 1 and 3 weeks. The total length of neurocranium and Meckel's cartilage, cumulative length of first 10 caudal vertebrae and individual length on the 15th caudal vertebra were considered as axial skeletal structures. The length of the pectoral fin rays and the urostyle were considered as appendicular skeletal structure. Figure 4A–C: representative figures of the neurocranium (4A), splanchnocranium (4B, C; ventral side of the neurocranium) and Meckel's cartilage (4A, 4C) of Japanese medaka larvae 2 day post hatch. 4D: representative figure of the splanchnocranium of medaka larvae exposed to 2 mg/L OXY 96 h (6 day post hatch); 4E–G, 4J–K. The cumulative length of the first 10 caudal vertebrae at different periods of depuration (caudal vertebrae were identified by the presence of hemal arch; 4E: one week post hatch; 4F: three

week post hatch; 4G: 6 week post hatch; 4J: 8 weeks post hatch; 4K: 12 weeks post hatch). The fifteenth caudal vertebra was marked on figures 4E–I; The pectoral fin is shown in Figure 4E and the urostyle is shown in Figure 4H and Figure 4I. Axial and appendicular skeletal structures of Japanese medaka after three weeks of depuration used for morphometry (4E–F; ventral view; 4G: lateral view). Figure 4.1: morphometric data on total length of neurocranium (4.1), length of Meckel's cartilage (Figures 4.2 and 4.3), cumulative lengths of first 10 caudal vertebrae (Figures 4.4 and 4.5), individual length of 15th caudal vertebra (Figure 4.6 and 4.7), length of pectoral fin (Figure 4.8), and urostyle (Figure 4.9). Each bar represents the mean $\pm$ SEM of 10–15 individual length of axial and appendicular skeletal structures of Japanese medaka larvae of different age groups (1 and 3 weeks) exposed to OXY for 96 h after 2 dph. Bar head with pound symbol (#) or asterisks (*) indicate that the data are significantly different from the corresponding controls (ERM) or in vehicle, respectively.

Figure 5. Effects of OXY on the total protein and RNA content of Japanese medaka larvae
Each bar is the mean $\pm$ SEM of 4–6 observations. 5A= protein; 5B= RNA. Bar head with pound symbol (#) indicates that the data are significantly different from the corresponding controls (ERM) ($p<0.05$).

Figure 6. Gene expression analysis of Japanese medaka larvae exposed to OXY
 Total RNA was reverse transcribed to cDNA and amplified by gene specific primers (Table 1). Each bar is the mean $\pm$ SEM of 5–6 observations. Bar head with pound symbol indicates that the data are significantly different from the corresponding ERM controls ($p < 0.05$).

Table 1

The primers used for RT-qPCR for the expression analysis of Target genes.

mRNA	Sense (5'-3')	Antisense (5'-3')	Product (bp)	Target/Internal standard	Gene ID
<i>β-actin</i>	cctgaccctgaaagatccca	cgtcaggcagctcatagtc	542	Internal standard	ENSORLG000000013676
<i>emx2</i>	ccgagggaacccatcaggccc	gggggtgtgtgcggccagcgcgtg	198	Target	ENSORLG000000014157
<i>en2</i>	cgcgcagcggccatgctgtggcc	gtacagtcctctgtgccatcag	337	Target	NM_001104744.1
<i>Irx3a</i>	ccagaccacaaaatgccacc	cttgttaaatcctcatagccg	221	Target	ENSORLG000000013724
<i>otx2</i>	ggccctgagcttaactaccic	ggactcggggaagattgatttc	201	target	ENSORLG0000000 16191
<i>shh</i>	gatggaggagaaacacggagcc	cttcacaccgccagctctggac	241	target	ENSORLG000000010463
<i>wnt1</i>	gtggggagattctctcaaggac	cagcaccacatggaagatgcag	338	target	ENSORLG000000007641
<i>zic5</i>	cctctgaacatgggcaattcag	ctctgacgtgtgttcaccagc	283	target	XM_004073109.3