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Effects of formulations of the fungicide, pentachloronitrobenzene on early life stage development of the Japanese medaka (*Oryzias latipes*)

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Abstract

Quintozene is a fungicide containing the active ingredient, pentachloronitrobenzene (PCNB) that is used to control "snow mold" on golf courses in temperate regions of North America. In this study, quintozene and a formulation of quintozene widely used on golf courses, FFII^{TM} were tested for toxicity to early life stages of the Japanese medaka, *Oryzias latipes*. For medaka exposed in static non-renewal assays to quintozene for 17 d from the fertilized egg stage to yolk resorption at the fry stage, the LC_{50} for mortality was a nominal concentration of 707 μg l^{-1} and the effective concentration for 50% hatch (i.e. EC_{50}) was a nominal concentration of 71 μg l^{-1}. Eggs and fry showed developmental abnormalities, including ocular malformations and retarded development of the brain, notochord, organs and body segmentation, which were interpreted as teratogenic responses to exposure to PCNB. For medaka exposed to quintozene, the LOECs for abnormalities of the eye and all other developmental abnormalities were 750 and 100 μg l^{-1}, respectively. In medaka exposed to the FFII^{TM} formulation, similar patterns of mortalities, reduced hatching success and developmental abnormalities were observed, but at higher test concentrations that were consistent with the proportion of quintozene in the formulation. For medaka exposed to the formulation, the LOECs for abnormalities of the eye and all other developmental abnormalities were 10000 and 1000 μg l^{-1}, respectively. Overall, these data indicate that studies should be conducted to assess the risk of exposure of early life stages of fish to quintozene in watersheds impacted by golf courses.

Keywords: Quintozene; Pentachloronitrobenzene; Fungicide; Golf course; Japanese medaka; Teratogenicity; Embryotoxicity

1. Introduction

Quintozene is a fungicidal product that contains the active ingredient, pentachloronitrobenzene (PCNB). PCNB is moderately water soluble compound (i.e. 0.39 mg l^{-1}), with a log Kow value of 4.64 (Mackay et al., 1992). This substituted chlorobenzene compound (Fig. 1) is relatively persistent, with reported half-lives of 78.5 d in soil and 1042 d in water (Chemfinder database, 2005). These data indicate that PCNB should be persistent in both water and soil, and has potential for transport from the terrestrial environment into surface water. In acute toxicity tests with quintozene, the 96-h LC_{50} reported for rainbow trout was 656 μg l^{-1} and the 48-h LC_{50} reported for opossum (mysid) shrimp was 18 μg l^{-1} (PAN Pesticides DataBase, 2005).

Most agricultural products containing PCNB have been banned from use in North America (Verrin et al., 2004). However, PCNB is still used in a variety of formulations for the treatment of turfgrass, and as a seed treatment. Quintozene is registered in Canada and the USA as a fungicide for use on golf courses for control of snow mold that develops in temperate regions from infestations of golf course turf by *Typhula* fungus (McBeath, 2003). Quintozene has long been an industry standard for snow mold control throughout North America (Burpee and Lawton,

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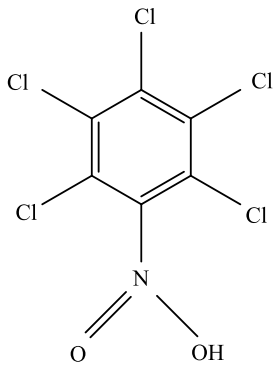

Fig. 1. Structure of pentachloronitrobenzene (PCNB).

1990; Vargas, 1994). Golf course superintendents attempt to make the last application of quintozene immediately before the permanent winter snow cover in the fall, and again in the early spring. Vincelli (2004) conducted computer simulations of runoff of several fungicides following applications on turfgrass and predicted that concentrations of PCNB would exceed lethal concentrations for aquatic organisms under some conditions. Johnson and Golob (2003) monitored leachate from a golf course and observed no detectable levels of PCNB, but detected the microbial degradation product, pentachloroaniline (PCA) at low ppb concentrations in all leachate samples. Hexachlorobenzene (HCB) has been identified as a contaminant in the quintozene technical product (Edwards et al., 1991).

Because of the potential for exposure of aquatic organisms to PCNB as a result of runoff from golf courses treated with quintozene, data are needed to assess the lethal and sublethal effects of this compound to aquatic organisms. However, few data are available on the toxicity of the active ingredient, PCNB, or the technical mixture, quintozene, and there are no data on the toxicity of the commercial formulations.

In this study, we assessed the lethal and sublethal effects of a technical mixture of PCNB (i.e. quintozene) and a commercial formulation of PCNB (ProTurf® FFII™) in early life stage toxicity tests with the Japanese medaka (*Oryzias latipes*). The early life stages of fish are especially sensitive to the toxic effects of organic compounds. The embryos and fry of the Japanese medaka have been widely used to assess the effects of organic compounds on early life stages of fish (Wisk and Cooper, 1990; Harris et al., 1994; Helmstetter et al., 1996; Hamm and Hinton, 2000; Metcalfe et al., 2000). This model species was used as an indicator of the potential for toxic effects among the early life stages of fish exposed to the test chemicals in the field.

Effects were monitored in static non-renewal assays over 17 d, beginning at the egg stage immediately after fertilization, and progressing through to hatch of the sac fry and development of the fry to complete yolk resorption (i.e. "swim-up"). Toxic endpoints included LC_{50}s, the EC_{50}s for reduced hatching success, and observations of developmental abnormalities.

2. Methods

2.1. Test chemicals

The fungicide quintozene was purchased from Sigma (Toronto, ON, Canada) as a technical mixture containing PCNB at a purity of 95.4%. Unfortunately, PCNB at a higher degree of purity are not currently available from commercial suppliers. A stock solution was prepared by dissolving the material in acetone, and various dilutions were prepared in acetone for toxicity testing. The FFII™ formulation marketed by ProTurf® was provided by a commercial golf course. This formulation is a mixture of turf fertilizer and fungicide reported on the label as containing 15.4% quintozene, 14% total nitrogen, 3% phosphate and 3% potash. A stock solution of the FFII™ formulation was prepared by placing 0.1 g of this material in 100 mL of acetone and agitating with a magnetic stir bar. Un-dissolved material was filtered out and water was removed from the solution by passing through a glass Buchner funnel containing 10 g of sodium sulfate. The filtrate was collected and diluted with acetone for toxicity testing.

The concentrations of PCNB, PCA and HCB in quintozene and the stock prepared from FFII™ were determined by gas chromatography with mass spectrometry in selected ion mode (GC–MS-SIM) using a Varian (Palo Alto, CA, USA) 3800 gas chromatograph coupled to a Varian Saturn 2200 ion trap mass selective detector. Electron impact ionization was used as the ionization source. The GC was equipped with a Varian 60 m × 0.25 mm DB-5 (0.25 μm) column. For analysis of PCNB, ions were monitored at *m*/*z* 265 (quantitation ion) and *m*/*z* 230 and 203 (qualifier ions). For analysis of HCB, ions were monitored at *m*/*z* 284 (quantitation ion) and *m*/*z* 249 and 214 (qualifier ions). For PCA, the quantitation ion was *m*/*z* 295 and the qualifier ions were *m*/*z* 265, 237 and 214. The analytes were quantified against a standard prepared in the laboratory from material purchased from Sigma (Toronto, ON, Canada).

2.2. Toxicity testing

The toxicities of quintozene and the FFII™ commercial formulation to early life stages of Japanese medaka were determined using a static non-renewal assay. All medaka originated from the brood culture of the golden strain originally purchased from Carolina Biological Supply (Burlington, North Carolina, USA) and held at Trent University for the past 10 years, with periodic augmentation with new medaka of the same strain to maintain genetic diversity. The broodstock was reared in dechlorinated city tap water with a pH ranging between 7.4 and 7.8, alkalinity between 1.2 and 1.4 meq l^{-1} and hardness between 80 and 100 mg l^{-1} as $CaCO_3$. Water temperature was held at 26 ± 1 °C and photoperiod was a standard 16 h light and 8 h dark. Fertilized eggs were collected from this brood-

stock to be utilized in the embryotoxicity assays. All eggs were maintained in embryo rearing medium, which was prepared by dissolving in 200 mL of distilled water: 10 g NaCl, 0.3 g KCl, 0.4 g $CaCl_2$ and 1.63 g $MgSO_4$. This solution was autoclaved and when cool, 20 mL of the solution was diluted to 1 L with distilled water and 2 drops of methylene blue were added to prevent fungal growth.

Different concentrations of the test compounds and a reagent blank control were placed in 2 mL glass vials and the acetone solvent was evaporated-off in a fume hood before adding 1 mL of embryo rearing medium. It is possible that there was some co-evaporation of the test compound, but removal of the acetone was necessary to ensure that there was no toxicity as a result of exposure to the carrier solvent. Newly fertilized eggs were collected from female medaka, separated and individually placed in exposure vials. Evaporation of the solvent in the vials was necessary to avoid solvent-induced mortalities to medaka. There were 10–20 embryos as replicates within each treatment, depending on the availability of embryos and survival past the first day post-fertilization. In tests with quintozene and the FFII^{TM} preparation, fish were tested at concentrations between 1 and 10 000 $\mu g\,l^{-1}$ and 10 and 50 000 $\mu g\,l^{-1}$, respectively.

The capped exposure vials containing individual medaka were incubated at 25 °C and the embryos were examined daily under a dissecting microscope to monitor mortality and development from Day 0 (i.e. fertilization) to Day 17 (i.e. normal point of total yolk resorption). The developmental endpoints monitored including hatching success, the presence of toxicopathic lesions (e.g. hemorrhage, cardiac edema), and developmental anomalies among the medaka.

The Lowest Observed Effect Concentrations (LOECs) for the various biological responses (i.e. mortality, no hatch, deformities) were determined as the lowest concentration where the response data differed statistically (Chi-square, $\alpha = 0.05$) from the control treatment. The No Observed Effect Concentration (NOEC) was the next test concentration below the LOEC. To evaluate concentration-dependent changes in toxicity, the concentrations (x-axis) were converted to a log scale and the toxic responses (y-axis) were calculated as a percentage relative to the 100% maximum response; generating a sigmoidal curve. The effective concentration for 50% no hatch (i.e. EC_{50}) was calculated as the concentration at which 50% of surviving medaka did not hatch over the duration of the test. The lethal concentration for 50% of the test population (i.e. LC_{50}) was calculated as the concentration at which the medaka showed 50% mortality over the duration of the test. The LC_{50} and EC_{50} values were calculated by plotting the log transformed nominal test concentration against the response data using GraphPad Prism® software (GraphPad Software, San Diego, CA). An equation for the sigmoidal concentration-response data was plotted by the software according to the equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{\log EC_{50} - x})$$

where Bottom = The lowest y-value

Top = The highest y-value.

3. Results

In the technical mixture of quintozene and in the FFII^{TM} formulation, analysis by GC–MS indicated that there were contaminants in the mixtures in addition to the PCNB active ingredient. For each gram of PCNB in the quintozene, there was 96 mg of HCB and 161 mg of PCA. In the FFII^{TM} formulation, there was no HCB detected, but for each gram of PCNB there was 16 mg of PCA. The amounts of PCNB detected analytically in the test solutions were generally consistent with the amounts reported on the labels. In the technical mixture of quintozene, PCNB comprised 91.5% by weight, and in the FFII^{TM} formulation, PCNB comprised 12.7% by weight.

Quintozene was toxic to early life stages of Japanese medaka at $\mu g\,l^{-1}$ (ppb) concentrations. From the data relating nominal concentrations to the biological responses (Table 1), the LC_{50} for mortality and the EC_{50} for no hatch were generated, along with 95% confidence intervals for these parameters. The LC_{50} and the EC_{50} for no hatch for quintozene were nominal concentrations of 707 (95% CI: 366–1366) and 71 (95% CI: 36–139) $\mu g\,l^{-1}$, respectively. In addition, the LOEC for no hatch was a nominal concentration of 10 $\mu g\,l^{-1}$ and the NOEC was a nominal concentration of 1 $\mu g\,l^{-1}$.

Striking alterations to development were observed among the embryos exposed to quintozene. Fig. 2 illustrates the normal development of medaka embryo from the control treatment at a point just before hatch. Among the embryos exposed to quintozene, the most obvious alterations to development were optical deformities, including "anisophthalmia", which is a condition where one eye is smaller than the other, or is completely absent (Fig. 3). In some cases, fusing of the eyes or total absence of the eyes (Fig. 4) was observed in medaka embryos. These conditions developed in medaka before hatch. Several other developmental abnormalities were observed in medaka exposed to quintozene, including lack of development of the brain, notochord, heart and body somites (Fig. 4). The data on the incidence of these developmental alterations showed a trend of increasing percent response with nominal test concentration (Table 1). However, the data did not yield EC_{50} values within reasonable confidence, limits. Chi-square analysis of the incidence data indicated that the LOECs for eye deformities and the other developmental deformities were 750 and 100 $\mu g\,l^{-1}$, respectively (Table 1).

The developmental abnormalities in medaka exposed to quintozene were interpreted as teratogenic effects resulting from arrested development of the embryo at approximately

Table 1
The percentage of mortalities, no hatch, abnormalities of the eye and other developmental abnormalities among Japanese medaka in the control treatments and in treatments with: (A) the quintozene technical mixture, (B) the FFII^{TM} formulation

Nominal conc. ($\mu g\,l^{-1}$)	PCNB conc. ($\mu g\,l^{-1}$)	Mortality (%)	No. hatch (%)	Eye deformities (%)	Other deformities (%)
(A) Quintozene					
7500	6863	100	100	60	100
5000	4575	100	100	30	70
2500	2288	88	100	50	40
1000	915	70*	100	30	60
750	686	40*	80	30*	40
250	229	10	60	10	30
100	92	12	47	0	35*
50	46	12	63	0	12
10	9	0	21*	0	0
1	1	0	0	0	0
Control	–	0	10	0	0
(B) FFII					
50 000	6350	100	100	20	80
25 000	3250	100	100	30	70
10 000	1270	63	100	21*	63
5000	635	60	70	0	10
1000	127	21*	63	7	32*
500	64	12	35	0	6
100	13	12	12*	0	0
50	6	0	0	0	0
10	1	0	0	0	0
Control	–	0	0	0	0

The LOEC values in which the incidence of the responses was statistically different from the control treatment (Chi-square analysis, $\alpha = 0.05$) are indicated by an asterisk (*). The concentrations of PCNB in the test solutions (rounded-off to the nearest whole number) were predicted from analytical data that showed 91.5% PCNB in the quintozene mixture and 12.7% PCNB in the FFII^{TM} formulation.

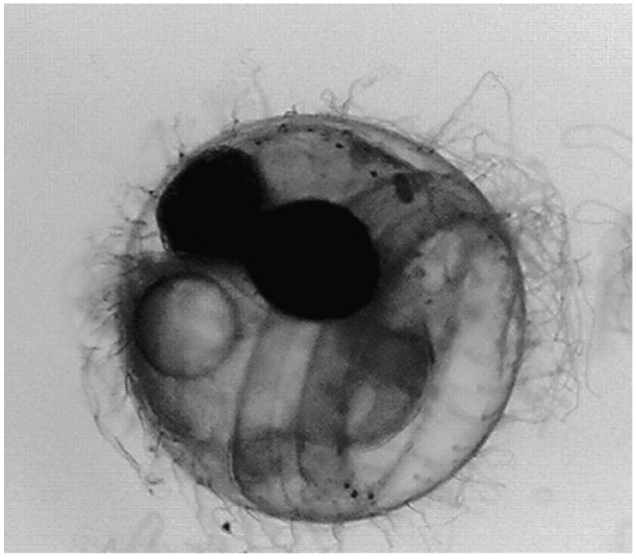

Fig. 2. Photomicrograph of a Japanese medaka embryo from the control treatment at 9 d of development, illustrating normal development to a point just before hatch.

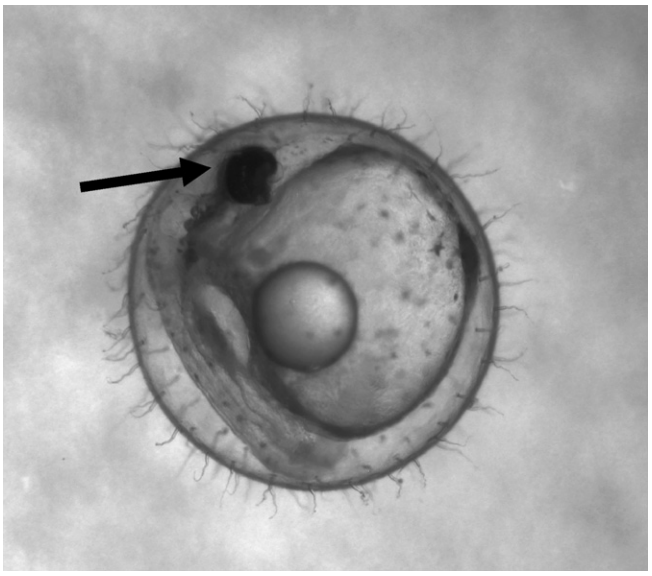

Fig. 3. Photomicrograph of a Japanese medaka embryo at 12 d of development which was exposed to 10 $\mu g\,l^{-1}$ quintozene, illustrating the eye deformity, anisophthalmia (arrow). This embryo did not hatch during the 17 d assay period.

stage 21. This stage of development normally takes place between 1 and 2 d after fertilization and coincides with the development of the neural crest and body somites (Iwamatsu, 1994). Medaka that did not develop normally typically did not hatch by the end of the 17 d test period. The LOEC and NOEC values for the altered developmental endpoints described above were nominal concentrations of 10 and 1 $\mu g\,l^{-1}$, respectively.

In treatments with the FFII^{TM} formulation, similar responses were observed for mortalities, hatching success and development abnormalities in medaka, but at higher nominal concentrations (Table 1). The LC_{50} and the EC_{50} for no hatch were calculated as nominal concentrations of the original FFII^{TM} formulation of 4060 (95% CI: 995–9548) $\mu g\,l^{-1}$ and 609 (95% CI: 228–1626) $\mu g\,l^{-1}$, respectively. In the treatments with FFII^{TM}, the LOEC for no hatch was a nominal concentration of 100 $\mu g\,l^{-1}$ and the NOEC was a nominal concentration of 50 $\mu g\,l^{-1}$.

The ratio of the LC_{50}s for quintozene and FFII^{TM} was 17%, and the ratio of the EC_{50}s for no hatch for quintozene and FFII^{TM} was 12%. These values are consistent with the relative proportions of PCNB in the quintozene and FFII^{TM} formulation (i.e. 91.5% and 12.7%, respectively). This can be observed in Table 1 where responses are similar at the estimated concentrations of PCNB in the quintozene and the FFII^{TM} formulation. When the lethal concentrations were calculated using the predicted concentrations of PCNB in the two exposure mixtures (Table 1), the LC_{50}s were estimated as 546 (95% CI: 298–944) $\mu g\,l^{-1}$ for the PCNB in quintozene and 282 (95% CI: 271–295) $\mu g\,l^{-1}$ for the PCNB in the FFII^{TM} formulation.

Teratogenic responses were also observed in the early life stages of medaka exposed to FFII^{TM}, including

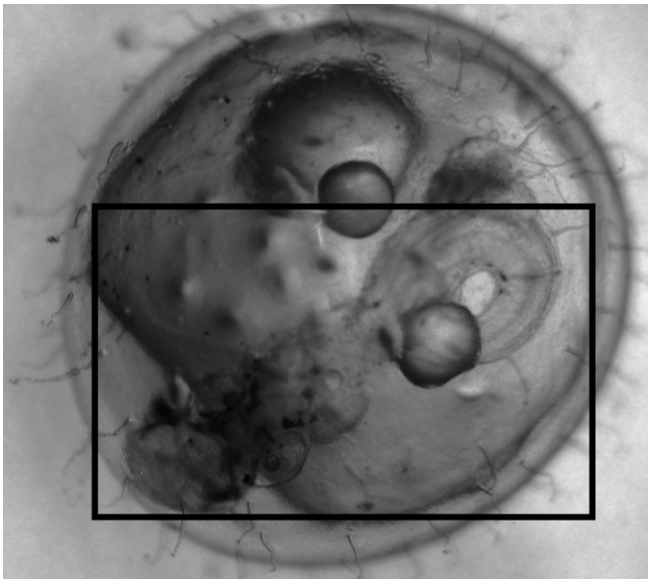

Fig. 4. Photomicrograph of a Japanese medaka embryo at 12 d of development (in box) which was exposed to 100 $\mu g\,l^{-1}$ quintozene, illustrating abnormalities in the development of the eyes, brain (arrow), notochord and body somites. Also note the lack of utilization of the yolk, relative to the embryo shown in Fig. 2. This embryo did not hatch during the 17 d assay period.

alterations to the development of the eye, brain, notochord, heart and body somites. These developmental abnormalities closely resembled the teratogenic effects observed in medaka from the quintozene treatments. The LOECs for the development of eye abnormalities and all other developmental abnormalities were 10 000 and 1000 $\mu g\,l^{-1}$, respectively (Table 1).

4. Discussion

The data presented in this study show that a technical mixture of PCNB (i.e. quintozene) and a commercial formulation of this compound (i.e. FFII^{TM}) were both toxic to early life stages of medaka at $\mu g\,l^{-1}$ (i.e. ppb) concentrations. The LC_{50} for quintozene determined for early life stages of medaka in this study (i.e. 707 ppb) is consistent with the reported 96-h LC_{50} of 656 ppb in juvenile rainbow trout (PAN Pesticides DataBase, 2005). However, while there were medaka mortalities at high ppb concentrations, sublethal effects on the early life stages of medaka were observed at nominal concentrations an order of magnitude lower. The toxic effects of the FFII^{TM} formulation were observed at concentrations that are consistent with the amounts of PCNB detected in the formulation. However, it is possible that some of the PCNB was lost during the preparation of the filtrate that was used for embryotoxicity testing. It would have been useful to measure the initial concentrations of PCNB in the solutions used to expose the medaka, but the volumes of exposure medium (i.e. 1 mL) were not sufficient for analysis. However, PCNB is a relatively hydrophobic compound with a log Kow value of 4.64, so we predict that this compound would rapidly partition from the exposure medium into the tissues of the developing embryo.

Quintozene was teratogenic to Japanese medaka; arresting development of the brain, notochord and body somites, causing ocular abnormalities, and reducing hatching success. To our knowledge, this is the first report of developmental abnormalities in early life stages of fish exposed to this substance. Although no adverse biological responses were observed in the control treatment that could be attributed to the exposure to the anti-fungal agent used in the rearing medium (i.e. methylene blue), it cannot be ruled out that there were interactions in the experimental treatments between methylene blue and the test substance.

Previously, a technical mixture of PCNB was shown to induce teratogenic effects (i.e. cleft palate) in developing mice exposed through maternal dosing (Courtney et al., 1976), but in the opinion of the authors of this study, teratogenicity was caused by the HCB present as a contaminant at a proportion of 11% in the technical mixture. In the present study with medaka, HCB was also present as a contaminant in the quintozene mixture, but was not detected at all in the FFII^{TM} formulation. More recently, Hayretdag and Kolankaya (1998) reported developmental abnormalities, including alterations to the formation of the lungs and kidneys in chick embryos exposed to PCNB, and they provided evidence that these responses were caused by alterations to the distribution of fibronectin in the developing embryo. Fibronectin is an adhesive glycoprotein that is involved in a range of cellular processes, including the migration and differentiation of cells in developing embryos (Yamada, 1991). Sadaghiani et al. (1994) demonstrated that the distribution of fibronectin was important for the normal development of the neural crest in fish embryos. Future work should investigate whether alterations to the distribution of fibronectin is the mechanism responsible for the teratogenic responses observed in early life stages of fish and other vertebrates.

It is possible that other components of the quintozene mixture other than PCNB were responsible for inducing these developmental effects in medaka. PCA was detected in the technical mixture and in the commercial formulation, but other unknown compounds may also have contributed to these effects. Future work should focus on determining whether purified PCNB is the ingredient in the technical mixture that is responsible for teratogenic effects in early life stages of fish. Work should also be done to determine whether the degradation products of PCNB, such as pentachloroaniline contribute to the biological effects.

Fish could be exposed to PCNB through the transport of this compound into aquatic ecosystems in runoff from golf courses. Since quintozene is typically applied to golf courses in the late fall and early spring to control snow mold, there is potential for PCNB to appear in spring runoff when the early life stages of fish are present in watersheds. PCNB and its metabolites were recently detected

in streams that drain golf courses in central Ontario, Canada (Metcalfe et al., in press). From an ecological perspective, alterations to fish development would reduce the survival of early life stages through to maturity. Studies are needed to determine whether PCNB and its degradation products are present in spring runoff within watersheds that drain golf courses, and whether best management practices can reduce exposure of fish to these compounds.

5. Conclusion

Quintozene and a formulation of quintozene (i.e. FFII^{TM}) that is widely used on golf courses caused mortalities and increased time to hatch in early life stages of Japanese medaka. Eggs and fry also showed developmental abnormalities, including ocular malformations and retarded development of the brain, notochord, organs and body segmentation. These data indicate that the active ingredient in the commercial formulation, pentachloronitrobenzene (PCNB) could have impacts on the development of early life stages of fish in watersheds downstream of golf courses. However, it cannot be ruled out that contaminants in the PCNB formulation may have caused these responses.

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References

Burpee, L., Lawton, M., 1990. Biological control of *Typhula* blight. Golf Course Manage. 58, 6–78.

Chemfinder database, <http://www.chemfinder.cambridgesoft.com>. Accessed, November 2005.

Courtney, K.D., Copeland, M.F., Robbins, A., 1976. The effects of pentachloronitrobenzene, hexachlorobenzene, and related compounds on fetal development. Toxicol. Appl. Pharmacol. 35, 239–256.

Edwards, I.R., Ferry, D.G., Temple, W.A., 1991. Fungicides and related compounds. In: Hayes, W.J., Laws, E.R. (Eds.), Handbook of Pesticide Toxicology. Academic Press, NY, pp. 6–8.

Hamm, J.T., Hinton, D.E., 2000. The role of development and duration of exposure to the embryotoxicity of diazinon. Aquat. Toxicol. 48, 403–418.

Harris, G.E., Kiparissis, Y., Metcalfe, C.D., 1994. Assessment of the toxic potential of PCB congener 81 (3,4,4′,5-tetrachlorobiphenyl) to fish in relation to other non-*ortho*-substituted PCB congeners. Environ. Toxicol. Chem. 13, 1405–1413.

Hayretdag, S., Kolankaya, D., 1998. The effects of pentachloronitrobenzene on the fibronectin distribution during lungs and kidney development in chick embryos. Toxicol. Lett. 95 (Suppl. 1), 210.

Helmstetter, M.F., Maccubbin, A.E., Alden III, R.W., 1996. The medaka embryo-larval assay: an *in vivo* assay for toxicity, teratogenicity and carcinogenicity. In: Ostrander, G.K. (Ed.), Techniques in Aquatic Toxicology. Lewis Publ., Boca Raton, FL, USA, pp. 93–124.

Iwamatsu, T., 1994. Stages of normal development in the medaka, *Oryzias latipes*. Zoological Sci. 11, 825–839.

Johnson, W.J., Golob, C.T., 2003. Analysis of PCNB in leachate from the Coeur d'Alene resort golf course floating green. Western Canada Turfgrass Association, Final Research Report (1997–2003), 74p.

Mackay, D., Shiu, W.Y., Ma, K.C., 1992. Illustrated Handbook of Physical–Chemical Properties and Environmental Fate for Organic Chemicals. Volume 1: Monoaromatic Hydrocarbons Chlorobenzenes and PCBs. Lewis Publ., MI, USA, 697p.

McBeath, J.H., 2003. Biological management of snow mold. Golf Course Manage. 71, 124–126.

Metcalfe, T.L., Dillon, P.J., Metcalfe, C.D. Transport of toxic substances from golf courses into watersheds in the Precambrian Shield region of Ontario, Canada, Environ. Toxicol. Chem., in press.

Metcalfe, T.L., Metcalfe, C.D., Bennett, E.R., Haffner, D., 2000. Distribution of toxic organic contaminants in water and sediments in the Detroit River. J. Great Lakes Res. 26, 55–64.

PAN Pesticides DataBase, <http://www.pesticideinfo.org>. Accessed, November 2005.

Sadaghiani, B., Crawford, B.J., Vielkind, J.R., 1994. Changes in the distribution of extracellular matrix components during neural crest development in *Xiphophorus* spp. embryos. Can. J. Zool. 72, 1340–1353.

Vargas, J.M., 1994. Management of Turfgrass Diseases, second ed. Lewis Publ., Ann Arbor, MI, USA, 123p.

Verrin, S.M., Begg, S.J., Ross, P.S., 2004. Pesticide use in British Columbia and the Yukon: an assessment of types, applications and risks to aquatic biota. Canadian Technical Report of Fisheries and Aquatic Sciences No. 2517, 209p.

Vincelli, P., 2004. Simulations of fungicide runoff following applications for turfgrass disease control. Plant Dis. 88, 91–396.

Wisk, J.D., Cooper, K.R., 1990. Comparison of the toxicity of several polychlorinated dibenzo-*p*-dioxins and 2,3,7,8-tetrachlorodibenzofuran in embryos of the Japanese medaka (*Oryzias latipes*). Chemosphere 20, 361–377.

Yamada, K.M., 1991. Fibronectin and other interactive glycoproteins. In: Hay, E.D. (Ed.), Cell Biology of the Extracellular Matrix, second ed. Plenum Press, NY, pp. 23–36.