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Effects of Ethylene and Propylene Glycol on Development and Hatching Success in the Medaka, *Oryzias latipes*

E. L. Bass

Department of Natural Sciences, University of Maryland Eastern Shore, Princess Anne, MD 21853, USA

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Ethylene glycol (EG) and Propylene glycol (PG) are major ubiquitous components of commonly used anti-icing and deicing mixtures and are used in a number of industrial processes. Their low volatility and high affinity for water dictate that their degradation in the environment will depend in part on their reactions in aquatic systems (Nielsen, et al. 1993). Although not considered a hazardous substance, EG has been classified by the USEPA as a "toxic chemical" based on its oral toxicity while PG, by contrast, has been recognized as safe even for human consumption (Sittig 1985). Commercial formulations containing these materials are used in copious amounts for ice removal and prevention at airports as well as by the general public for protection of internal combustion engines and fresh water systems in motor vehicles and recreational and commercial vessels. Advertising, which represents PG as "non-toxic" has often led to disposal in a rather cavalier fashion. Through runoff, snow removal, and improper disposal (e.g. in municipal sewage systems or overboard discharge) these substances can enter waterways and surface waters (Sills and Blakeslee 1992). Here, they may pose a danger to wildlife as well as to human health. Fishes and amphibians may be particularly vulnerable since the eggs of these oviparous organisms are directly exposed during critical early developmental stages especially in stagnant or poorly flushed bodies of water. EG has been reported to be estrogenic in rainbow trout (Ren et al. 1996).

Toxicological studies using EG and PG have usually determined the vulnerability of young organisms or adults, using mortality as the end point. Often, an EC_{50} (concentration of the material in question which produces a particular effect in 50% of the organisms tested) is established. Several investigators have shown that the toxicity of these glycols themselves to aquatic animals, plants and micro-organisms is relatively low and that anti-icing and deicing formulations appear to have a greater toxicity due to compounds other than the glycols included in the mixtures (Pillard 1995; Hartwell et al. 1995; Pillard and DuFresne 1999). Acute LC_{50} values and short term chronic IC_{25} values for EG and PG ranged from a low of 0.898 ml/L EG in an alga, *Selenastrum capricornutum*, (Ministers Expert Advisory Panel 1995) to a high of 65.45 ml/L EG in the fathead minnow, *Pimephales promelas* (Pillard 1995). One must also bear in mind that

biodegradation of glycols can result in a high BOD and lead to secondary mortality as a result of oxygen depletion. A variety of studies have shown that glycols can be degraded relatively rapidly in natural ecosystems. This seems to be temperature dependent process, proceeding at a faster rate in warmer conditions (Pillard 1995).

Understanding the manner in which these compounds may affect the development and/or hatching of aquatic vertebrates, particularly fish, is critical to assessing their potential impact on the ecosystem. The medaka (*Oryzias latipes*), is a small freshwater fish that has frequently been used to evaluate the embryotoxic or teratogenic effects of various pollutants (Holcombe et al. 1995). Fertilized eggs are easy to obtain and, thanks to a large body of literature, the stages of development are well documented and readily observable in the laboratory (Benoit et al. 1991; Kirchen and West 1976).

MATERIALS AND METHODS.

Fertilized medaka eggs as well as the embryo rearing solution were obtained from Carolina Biological Supply (Burlington, NC). EG and PG were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Concentrations of 1, 10, and 100 ml/L EG or PG in embryo rearing solution were used in initial range finding studies. Based on a review of the appropriate literature and these preliminary studies initial test concentrations of 4, 8, 16, 32, and 64, ml/L EG and PG were selected for determination of effects on hatching success and development. A number of investigators have determined that the glycols can be expected to be rapidly degraded (EG faster than PG) under laboratory conditions as well as in the wild, primarily as a result of bacterial action. This process is also temperature dependent and lowered temperatures seem to retard degradation (Pillard 1995, ARCO 1990, Bridie et al. 1979). There is no reason to believe that degradation would not occur under the conditions maintained during this study. Therefore, initial concentrations indicated here are just that, and would be expected to decline at an unknown rate during the course of the study. In each case embryo rearing solution served as the control medium. All experiments were conducted in a walk-in environmental chamber at $23^{\circ} \pm 0.5$ °C and a 14L 10D photoperiod.

Upon arrival fertilized medaka eggs were examined and found to be between stages 17 to 21 of Kirchen and West (1976). Any eggs that were not developing properly at that time were discarded. Embryos were allowed to acclimate to the conditions in the chamber for 8 to 12 hr prior to beginning the study. Three embryos, stage 21 to 22, were transferred to each compartment of a four-compartment acrylic Petri dish which contained 5.5 mL of the control medium or the appropriate concentration of EG or PG. Three replicates were used for each concentration as well as for controls. Dishes were illuminated from above and below by a bank of 40 watt cool white fluorescent bulbs. Each morning

thereafter the embryos were observed with a dissecting microscope for developmental abnormalities and/or mortality. Cessation of heartbeat and circulation as well as accumulation of methylene blue dye provided end points for assessing mortality. Larva which hatched successfully were observed for an additional 72 hr and any anatomical or behavioral abnormalities were noted. Except during observation periods dishes were kept covered to retard evaporation.

Successful hatching of the larvae was used as the end point in the study for statistical purposes. Correlation and linear regression were used to evaluate the relationship between the concentration of EG or PG and hatching success as well as to calculate an estimate of the EC_{50} values and the 95% confidence intervals (Excel 97, Microsoft Corp., Redmond, WA). The test for parallelism was applied to the least squares regression lines as described in Lee and Lee (1982).

RESULTS AND DISCUSSION

In preliminary studies there was no mortality, teratology, or hatching failure noted either in control medium dishes or at concentrations of 1ml/L EG or PG. Concentrations of 10 ml/L showed a mean hatching failure of 60% and 48% for EG and PG respectively. Of those embryos that hatched successfully none exhibited any notable abnormalities in development or behavior. None of the eggs in either the 100 ml/L EG or PG hatched successfully.

Table 1. Hatching failure in glycol exposed embryos

	Nominal Concentration (ml/L)	% Hatching Failure (mean ± SD)	95% Confidence Limits
ETHYLENE GLYCOL	4	32 ± 2.89	7.17
	8	40 ± 5	12.42
	16	58 ± 2.89	7.17
	32	85 ± 5.13	12.74
	64	100	-
PROPYLENE GLYCOL	4	0	-
	8	13 ± 5.77	14.34
	16	30 ± 10	24.84
	32	43 ± 5.77	14.34
	64	88 ± 2.89	7.17

Table 1 shows the mean per cent hatching failure in the various concentrations of EG or PG. None of the embryos succeeded in hatching in 64 ml/L EG and all hatched successfully in 4 ml/L PG. Of the organisms that hatched successfully

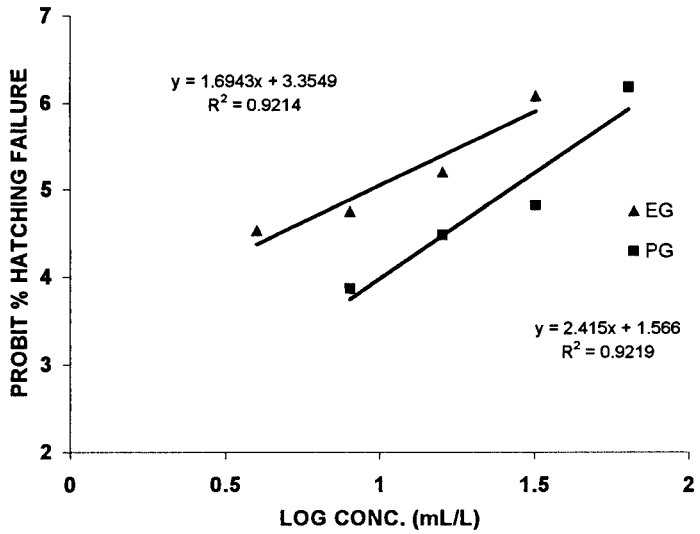

Figure 1. Log-probit plot of glycol concentration versus medaka embryo hatching failure.

none died during the 72 hr post hatching observation period. In addition, none of the organisms which hatched successfully exhibited any gross anatomical or behavioral abnormalities. All showed normal coordinated swimming and breathing movements and the liver, spleen and bladder exhibited no anomalies. Organisms which died during the exposure period without hatching exhibited a variety of generalized developmental abnormalities including failure of eye and fin development and necrosis of head and tail regions. Some of these persisted for several days until the embryo died. Bentivegna and Piatkowski (1998) noted that toxicity in medaka can be stage related. In my study, mortality and teratology occurred frequently at nine to ten days post-fertilization for both EG and PG, regardless of concentration. This period of apparent susceptibility can be quite close to the time of hatching, as most of the organisms in my experiments hatched by day 12 and none hatched after day 18. This time interval for hatching was consistent for control medium as well as glycol exposed organisms. Glycol exposure had no apparent effect on the actual time to hatching. All control medium organisms hatched successfully and exhibited no abnormalities either during development or during the post-hatching observation period.

Figure 1 represents the log-probit transformed data of hatching failure resulting from exposure to EG and PG. Correlation coefficients of 0.96 for both EG and PG indicate a highly linear relationship between concentration of the glycols and failure of embryos to survive to the point of hatching. Though somewhat equivocal, the test for parallelism (Lee and Lee 1982) indicates that the regression lines are statistically parallel and that any differences in the slopes of the lines

may have occurred by chance (t=2.03, P=.1). If parallel, this would suggest that these compounds might be acting by a similar mechanism to produce their effects. The validity of the four point assay for comparison of the toxicity of these two compounds can be verified by computing the value of g, Finney's significance of regression (Lee and Lee 1982). Here g=0.0172. A value of g less than 1 indicates that the assay is valid

Calculated EC_{50} values for EG and PG were 9.33 and 26.42 ml/L, respectively. These values are in the expected range when compared with EC_{50}'s for these glycols in other biological systems, which ranged from 0..54 ml/L to 65.5 ml/L in tests utilizing a variety of experimental protocols and end points as well as an assortment of organisms (Nielsen et al. 1993; Pillard 1995). The EC_{50} values for EG and PG in my study are also above the estimated no effect values (ENEV's) of 2 ml/L for EG and 5 ml/L for PG published by the Canadian Council of Ministers of the Environment for protection of freshwater aquatic organisms (1999). Studies by ARCO (1990) and Pillard (1995) have reported chronic LC_{50}'s for adult fish that range from 28.7 to 44.9 ml/L for EG and <11.1 to 49.2 ml/L for PG. The latter numbers are of interest because they show an equivalent or possibly greater toxicity for PG than for EG. In my study, at any given concentration, embryotoxicity for PG was less than for EG. I also observed a lower EC_{50} for EG disruption of embryological development and hatching than has been observed for toxicity in adult fish. Thus, embryotoxicity may provide a more sensitive test to assess the potential environmental impacts of this compound.

Although the mode of action of EG and PG in medaka embryos is not clear, it seems apparent that the membranes associated with the embryo during development do not protect it from the deleterious effects of these compounds and that these effects are clearly concentration dependent.

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