Fish Embryos are Damaged by Dissolved PAHs, not Oil Particles

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Purpose: distinguish the toxicity of whole oil droplets from the toxicity of polynuclear aromatic hydrocarbons (PAHs) dissolved in water.

Method: compare response of zebrafish (*Danio rerio*) embryos exposed to particle-laden, mechanically dispersed Alaska North slope crude oil (MDO) to responses in embryos isolated from particulate oil by agarose, a size-exclusion matrix (Fig. 1).

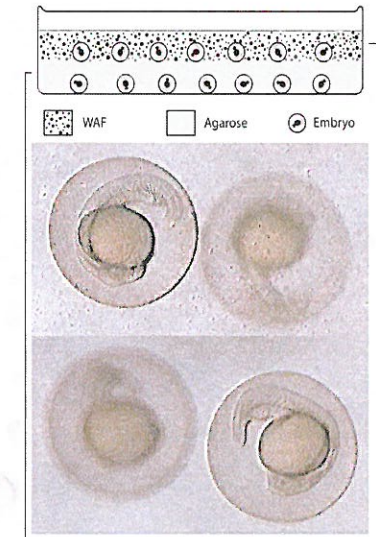

Fig. 1. Comparison of an embryo in MDO (at left) and an embryo in agarose (at right). Top: conditions in MDO with oil droplets visible (top). Bottom: no oil droplets were present in the underlying agarose.

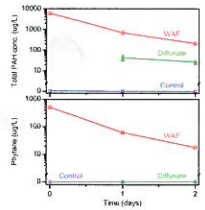

Fig. 2. PAHs diffused through agarose; the oil particles (droplets) were excluded as demonstrated by the lack of phytane.

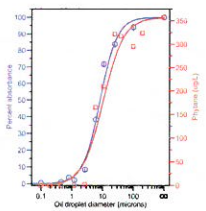

Fig. 3. Oil droplet diameter, estimated spectrophotometrically (at 350 nm) and chemically, using phytane as a tracer of particulate oil.

Chemical results: Most polycyclic aromatic hydrocarbons (PAHs) in MDO were contained in oil droplets; about 16% were dissolved (estimated by filtration through 7 µm glass fiber filters; Fig. 4). The agarose matrix blocked passage of all particulate oil but allowed passage of dissolved PAHs (Fig. 2). Diffusion limited PAH availability in agarose; PAH concentrations reached about 80% of saturation in 4 h. Estimated over the 48 h assay period, the mean total PAH (TPAH) concentration in agarose was about 16% less than the dissolved TPAH concentration in MDO and much less than the mean dissolved + particulate TPAH concentration in MDO. The composition of PAHs in agarose and dissolved PAHs in MDO were very similar (< 2% difference). Both profiles were dissimilar from whole MDO, which contained a larger proportion of higher molecular weight PAHs.

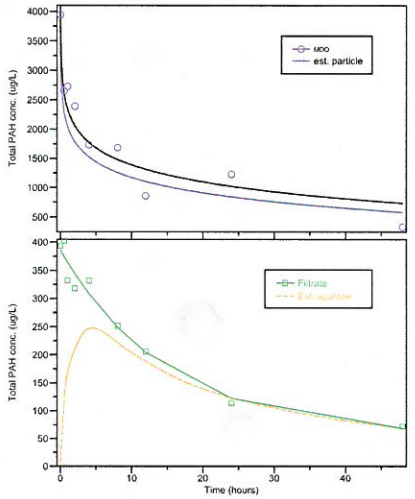

Fig. 4. Estimated relationship among dissolved and particulate TPAH concentrations in MDO and agarose. The MDO concentration is the sum of all TPAH in water. The dissolved concentration was measured by passing MDO through 0.7 um glass fiber filters; the difference is the estimated TPAH concentration due to particulate oil. The relationship between TPAH dissolved concentration in water and in agarose was determined in a separate experiment (to avoid contamination of the agarose surface by particulate oil) and used to model the relationship in this time series (which approximates conditions experienced by zebrafish embryos). The estimated uptake in agarose is based on Michaelis-Menton kinetics ($r^2 = 0.988$).

Biological results: The physical isolation of zebrafish embryos from particulate oil did not protect them against MDO-induced developmental toxicity. The incidence of edema, hemorrhaging, and cardiac abnormalities in embryos was dose-dependent in both MDO and agarose and the biological effects in these treatments were identical in character (Fig. 5). The extent of toxic injury among embryos embedded in agarose averaged 17% lower than in embryos exposed directly to MDO (the 95% confidence bounds were 7 to 28%) . These differences were often significant for abnormal heart looping (asterisks), but generally not in other measures.

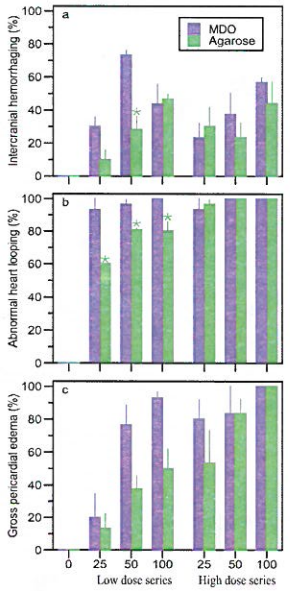

Fig. 5. Incidence of response of embryos in MDO and agarose (means ± standard error). The most sensitive response was abnormal heart looping; this was verified quantitatively by measuring cardiac angles (see Fig. 6g). Significant paired differences between responses in agarose and WAF are indicated with asterisks.

Combined results: Total dissolved PAH concentrations in MDO and in agarose were estimated for the zebrafish assay from the supplementary chemical experiments (e.g., Fig. 4). The resultant response curves were highly similar (Fig. 6) and median responses were not significantly different (see table below).

Response	Treat	n	Mean	95% Confidence bounds		p
Hemorrhaging	MDO	3	245	106	386	
	Agarose	3	413			
Abnormal heart	MDO	2	6	0	28	0.959
	Agarose	2	8	-152	149	
Edema	MDO	3	40	1	79	0.129
	Agarose	2	72	-91	263	

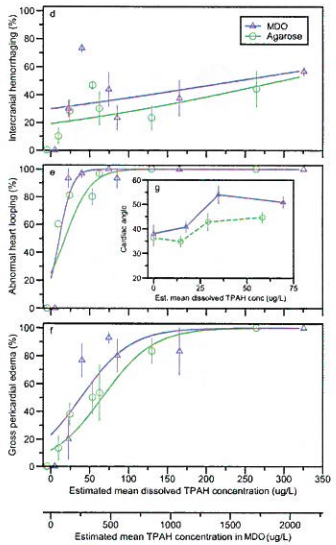

Fig. 6. Relationship between embryo responses in MDO and agarose (means ± standard error) based on estimated mean dissolved TPAH concentrations as modeled in Fig. 4. For comparison, estimated mean TPAH concentrations in whole MDO (including both the dissolved and particulate fractions) are indicated on a separate scale; particulate oil did not enter agarose, thus this scale does not apply to embedded embryos. Fitted curves are logit regressions. The inset graph (g) illustrates quantitative differences in the cardiac angle (with respect to body axis) for the initial portion of the response curve.

Discussion: Particulate oil is not directly toxic to fish embryos, rather, PAHs must enter solution to be biologically available. This is demonstrated directly by a dose-dependent increase in cardiac-related developmental abnormalities in zebrafish embryos isolated from particulate oil via a size-exclusion agarose matrix. The observed effects in agarose-embedded embryos and embryos in direct contact with MDO were identical in character and were consistent with the established cardiovascular impacts of tricyclic PAHs. If oil droplets had direct physical effects on fish embryos, they would not be expected to produce the same phenotype. For example, if oil droplets blocked or reduced oxygen transport across the chorion, embryos might exhibit signs of hypoxia. However, the effects of hypoxia in zebrafish are clearly distinct from and have little overlap with the effects of petrogenic PAH exposure[1-3]. The ratio between mean TPAH concentrations in whole MDO and agarose (5 to 9) was too large to explain the smaller embryo response ratio (1.5) between these compartments (geometric mean; 95% confidence bound 1.0 to 2.1, $n = 39$). However, the dissolved TPAH concentration in MDO was similar to that in agarose, albeit 16% higher when averaged over 48 h. This explains the relatively small differences in embryo response (17%) between the two exposure compartments. It also demonstrates that the contribution of particulate oil to the observed toxicity was negligible.

The role that particulate oil plays in embryo toxicity is apparently primarily that of a reservoir, at least for micron-scale oil particles. Dissolved PAH concentrations in water drop more slowly when particulate oil is present than when it is not. Fish chorions or vitelline envelopes are largely an extracellular matrix material composed of secreted glycoproteins[4-5] and apparently are not particularly hydrophobic. Consistent with this, most oil particles in MDO drifted past chorions without adhering to them, thus the toxic reservoir is particulate oil in water, not whole oil in contact with embryos.

Our conclusion that dissolved PAHs cause toxicity in the absence of particulate oil corroborates and extends the findings of previous studies. Our group has repeatedly demonstrated that dissolved PAHs (<1 to 18 ug/L TPAH) are toxic to fish embryos[6-10]. In these experiments, the amount of particulate oil in oiled rock column effluent was negligible, as indicated by a clear effluent with no visible oil sheens. Moreover, the effluent was enriched with PAHs and nearly devoid of phytane, a highly insoluble tracer of particulate oil. Phytane levels in oiled treatments were 0.04 ± 0.01 ug/L ($n = 168$) compared to 0.01 ± 0.01 ug/L in controls ($n = 41$)[6-10]. Furthermore, phytane and visible oil were never detected in (or on) eggs in these experiments ($n = 59$). In building on these earlier published results and others[11-13], our current study unambiguously refutes the recent assertion that particulate oil is directly involved in the toxicity of PAHs to fish embryos[14].

Our results point to the importance of distinguishing between oil effects on wildlife due to physical processes (coating) and oil effects due to chemical toxicity.

At macroscopic scales, the adverse impacts of coating are well known for situations where oil adheres to feathers or fur or smothers large adult organisms (e.g., bivalves). These physical effects do not appear to extend to fish embryos exposed to oil particles in the micron size range. At these smaller scales the chemical toxicity associated with dissolved-phase oil is the predominant form of injury, at least for eggs. This is in agreement with the conclusions of others that chemicals in true solution are more bioavailable than chemicals in solid or adsorbed forms[15-17]. Studies that do not distinguish particulate and dissolved PAH toxicity may substantially underestimate PAH toxicity (e.g., compare the concentration scales above).

Although not toxic until PAHs dissolve, particulate oil may threaten a variety of marine organisms. Whether mechanically or chemically dispersed in the water column, the large surface volume droplet-to-water ratio promotes dissolution of PAHs. Droplets are thus a toxic reservoir that will persist until PAHs are depleted, diluted, or advected away from sensitive species. Although direct particulate effects are not important for fish embryos, this cannot simply be generalized to organisms that might collect, ingest, or otherwise be fouled by particulate oil. Continued study, including tests with chemical oil dispersants and a broader range of planktonic organisms, is recommended. Such study is prudent because use of chemical dispersants as post-spill clean-up measures in U.S. waters remains under active debate and these dispersants promote formation of particulate oil.

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1. Kajimura, S.; Aida, K.; Duan, C. M., Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. *Proceedings of the National Academy of Sciences of the United States of America* **2005,** 102, (4), 1240-1245.
2. Padilla, P. A.; Roth, M. B., Oxygen deprivation causes suspended animation in the zebrafish embryo. *Proceedings of the National Academy of Sciences U. S. A.* **2001,** 98, (13), 7331-7335.
3. Shang, E. H.; Wu, R. S., Aquatic hypoxia is a teratogen and affects fish embryonic development. *Environmental Science and Technology* **2004,** 38, (18), 4763-4767.
4. Darie, C. C.; Biniossek, M. L.; Jovine, L.; Litscher, E. S.; Wassarman, P. M., Structural characterization of fish egg vitelline envelope proteins by mass spectrometry. *Biochemistry* **2004,** 43, (23), 7459-7478.
5. Monné, M.; Han, L.; Jovine, L., Tracking down the ZP domain: From the mammalian zona pellucida to the molluscan vitelline envelope. *Seminars in Reproductive Medicine* **2006,** 24, (4), 204-216.
6. Carls, M. G.; Rice, S. D.; Hose, J. E., Sensitivity of fish embryos to weathered crude oil: Part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval Pacific herring (*Clupea pallasi*). *Environmental Toxicology and Chemistry* **1999,** 18, (3), 481-493.
7. Heintz, R. A.; Rice, S. D.; Wertheimer, A. C.; Bradshaw, R. F.; Thrower, F. P.; Joyce, J. E.; Short, J. W., Delayed effects on growth and marine survival of pink salmon *Oncorhynchus gorbuscha* after exposure to crude oil during embryonic development. *Marine Ecology Progress Series* **2000,** 208, 205-216.
8. Heintz, R. A.; Short, J. W.; Rice, S. D., Sensitivity of fish embryos to weathered crude oil: Part II. Increased mortality of pink salmon (*Oncorhynchus gorbuscha*) embryos incubating downstream from weathered *Exxon Valdez* crude oil. *Environmental Toxicology and Chemistry* **1999,** 18, (3), 494-503.
9. Marty, G. D.; Short, J. W.; Dambach, D. M.; Willits, N. H.; Heintz, R. A.; Rice, S. D.; Stegeman, J. J.; Hinton, D. E., Ascites, premature emergence, increased gonadal cell apoptosis, and cytochrome P4501A induction in pink salmon larvae continuously exposed to oil-contaminated gravel during development. *Canadian Journal of Zoology* **1997,** 75, (6), 989-1007.
10. Carls, M. G.; Heintz, R. A.; Marty, G. D.; Rice, S. D., Cytochrome P4501A induction in oil-exposed pink salmon Oncorhynchus gorbuscha embryos predicts reduced survival potential. *Marine Ecology Progress Series* **2005,** 301, 253-265.
11. Farwell, A.; Nero, V.; Croft, M.; Bal, P.; Dixon, D. G., Modified Japanese medaka embryo-larval bioassay for rapid determination of developmental abnormalities. *Archives of Environmental Contamination and Toxicology* **2006,** 51, (4), 600-607.
12. Rhodes, S.; Farwell, A.; Hewitt, L. M.; MacKinnon, M.; Dixon, D. G., The effects of dimethylated and alkylated polycyclic aromatic hydrocarbons on the embryonic development of the Japanese medaka. *Ecotoxicology and Environmental Safety* **2005,** 60, (3), 247-258.
13. Kiparissis, Y.; Akhtar, P.; Hodson, P. V.; Brown, R. S., Partition-controlled delivery of toxicants: A novel in vivo approach for embryo toxicity testing. *Environmental Science and Technology* **2003,** 37, (10), 2262-2266.
14. Brannon, E. L.; Collins, K. M.; Brown, J. S.; Neff, J. M.; Parker, K. R.; Stubblefield, W. A., Toxicity of weathered *Exxon Valdez* crude oil to pink salmon embryos. *Environmental Toxicology and Chemistry* **2006,** 25, (4), 962-972.
15. NRC, *Oil in the sea III: inputs, fates, and effects.* National Acadamy Press: Washington DC, 2002.
16. Beckles, D. M.; Chen, W.; Hughes, J. B., Bioavailability of polycyclic aromatic hydrocarbons sequestered in sediment: microbial study and model prediction. *Enviromental Toxicology and Chemistry* **2007,** 26, (5), 878-883.
17. Golding, C. J.; Gobas, F. A. P. C.; Birch, G. F., Characterization of polycyclic aromatic hydrocarbon bioavilability in estuarine sediments using thin-film extraction. *Enviromental Toxicology and Chemistry* **2007,** 26, (5), 829-836.