Response to Brannon et al. 2006

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This document is designed to provide a detailed review of Brannon et al. [2006; Environmental Toxicology and Chemistry 25(4):962-972]. The Brannon et al. (2006) paper was specifically directed at one of our published studies (Auke Bay Laboratory, National Marine Fisheries Service); we take issue with numerous details and conclusions. The procedures used by Brannon et al. to reproduce our results were flawed as were their interpretations of their study and ours. Despite the experimental inconsistencies, Brannon et al. have confirmed our basic findings, though this may not be apparent to many readers because Brannon et al. characterize their doses mainly in terms of nominal oil loadings and compare these with our measured polynuclear aromatic hydrocarbon (PAH) doses in water. This web site contains our full response to Brannon et al. In Part I, we include the letter submitted to the editors of Environmental Toxicology and Chemistry summarizing our disagreements. In Part II, we document in detail the disagreements with the Brannon et al. paper; these were too numerous to include in an efficient response to the journal. Statements by Brannon et al. are in black; our responses are in blue; numbers in brackets, e.g., {1} refer to the assigned statement numbers.

Response to Brannon et al. 2006

Part I

Published communication with the editor of Environmental Toxicology and Chemistry

To the editor,

We take issue with an article by Brannon et al. (2006) [1] in *Environmental Toxicology and Chemistry* questioning the validity of our work on the toxicity of petroleum-derived polycyclic aromatic hydrocarbons (PAHs) to the early life stages of fish. Beginning in 1997, we published a series of articles demonstrating adverse effects in response to PAH exposure concentrations in the low parts per billion [2-6]. Based on their attempt to reproduce our results, Brannon et al. [1] argue the effects we described were an artifact caused by contact with PAH-laden oil microdroplets instead of dissolved PAHs, and therefore our results are not likely applicable to most field situations. The arguments advanced by Brannon et al. [1] are flawed for a number of reasons and we stand by our published work.

This letter focuses only on our fundamental concerns about the Brannon article [1]. We have posted a detailed review at http://www.afsc.noaa.gov/ABL/Habitat/pdfs/review-3.pdf

Any scientist should welcome independent efforts at confirmation of their work, if only as an indication of its potential significance, and indeed we do. But equally, any scientist who attempts to confirm the results of another has an obligation to faithfully duplicate the important aspects of experimental conditions employed. Failure to do so may produce results at odds with the original work.

We designed our dosing apparatus to mimic PAH desorption from oil-coated rock substrate into interstitial water of beaches, where the PAH-contaminated water might be transported to developing salmon embryos [4, 7, 8], using oil from the same source (Alaska North Slope, USA) as that released during the 1989 *Exxon Valdez* spill. We suspected this exposure pathway may have been important during the years immediately following the 1989 *Exxon Valdez* oil spill, where elevated salmon embryo mortality was measured in oil-contaminated streams for several years after the spill [9, 10].

When we conducted our experiments, we were keenly aware that introduction of oil microdroplets by the dosing apparatus might seriously confound interpretation of the results. We described in detail the steps taken to prevent microdroplet formation, and performed chemical measurements to evaluate their efficacy [2-6, 11]. To suppress production of oil microdroplets, we sprayed an oil aerosol onto continuously tumbling rock for at least 90 seconds to minimize oil film thickness and hence promote adhesion. Because oil losses to the mixing container walls were obvious, we reported the oil loadings on the basis of direct measurement of oil adhered to the rock, which was only approximately 40% of the oil sprayed during application. To assess contributions from oil microdroplets during dosing, we used the large difference in the oil-water partitioning behavior of phytane (a branched aliphatic hydrocarbon) compared to PAHs. An absence of phytane in water samples indicates an absence of bulk-phase oil in the dosing water [12], and the ratio of PAH:phytane in the dosing water indicated that PAH contributions from bulk phase oil were negligible.

By their own admission, Brannon et al. [1] took none of these precautions. Instead of spraying the oil onto their rock as an aerosol, they simply added the oil as a single aliquot, minimizing adhesion, and making their preparation more prone to droplet formation. Instead of measuring the oil that actually adhered to the rock at the end of their mixing process, they simply assumed complete adhesion, explaining away the interpretive discrepancies this causes with incorrect statements. Instead of actually measuring PAH contributions from oil microdroplets in their dosing water, they provide a rationale supported by a single visual observation in their highest dose – the one being most prone to droplet formation – despite clear chemical evidence in their data that contributions from oil droplets cannot have been substantial (see web site).

While we accept the observation by Brannon et al. [1] of visible oil droplets in their highest exposure dose at face value, the ensuing speculation is unwarranted. If oil microdroplets were present in their highest dose, perhaps they played some part in the toxic effects observed for the embryos exposed to that dose, although based on their chemical data we doubt it. In any case, this does not necessarily imply that microdroplets were present in their lower doses, where toxic effects were also observed. Furthermore, because Brannon's dosing method was more prone to droplet formation than ours, their observation does not imply the presence of microdroplets in any of our doses; we have strong chemical evidence that microdroplets were not present [2-6]. Brannon et al. [1] simply have no basis for their claim that microdroplets affected our experiments beyond their speculative extrapolation from their less carefully executed experiment.

Despite these experimental inconsistencies, we note that Brannon et al. [1] nonetheless have confirmed our basic findings. This may not be apparent because they characterize their doses mainly in terms of nominal oil loadings (i.e. the amount of oil added per unit mass of rock) and compare these with our measured PAH doses in water. However, when viewed in terms of PAH concentrations measured in their dosing water (their Tables 1 and 2), the toxicity endpoints they monitored appear at aqueous PAH concentrations that are comparable with results of our studies and others [2-6, 13-15]. So, at the very least, perhaps we can agree that every study that has subjected fish embryos to aqueous PAH concentrations in the low part-per-billion range has detected adverse effects, provided the embryos are monitored for their delayed manifestation.

Part I, continued

The experimental finding that would cast doubt on the toxicity role we have ascribed to PAHs would demonstrate an absence of toxic effects following exposure to dissolved PAHs only. Neither Brannon et al. [1], nor anyone else to our knowledge, has reported such a result. Until this finding is persuasively presented in the scientific literature, we will stand by our published interpretations regarding the embryotoxicity of dissolved PAHs in the low part-per-billion range [2-6].

In conclusion, although the procedures used by Brannon et al. [1] were more prone to droplet formation than ours, their chemical and biological evidence demonstrates the toxicity of oil droplets was negligible, corroborating our multiple studies [2-6]. Aqueous PAH concentrations, including hypothetical oil droplets, were damaging at <8 ìg/L (ascites) in the Brannon et al. [1] study, which is also consistent with our results. What distinguishes the Brannon et al. [1] report from ours is that they base their interpretations on dose added (nominal oiling) instead of dose measured (aqueous TPAH concentration).

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Part II.
Comments

Terminology

Oil = AWEVC.

Brannon et al. (2006) describe oil weathered in the laboratory as 'artificially weathered *Exxon Valdez* crude oil (AWEVC).' Because this laboratory oil is indistinguishable from typical *Exxon Valdez* oil collected in Prince William Sound (Bence and Burns 1995), the emphasis given by Brannon et al. as 'artificial' is not an appropriate distinction and we simply describe weathered oil as 'oil' in this review.

Mousse = NWEVC.

Brannon et al. (2006) refer to mousse as 'naturally weathered *Exxon Valdez* crude oil (NWEVC).' Naturally weathered oil has multiple forms and the specific form Brannon et al. refer to is mousse, which was about 1/3 oil and 2/3 water in their experiment. Lower viscosity, weathered oil was more frequent in Prince William Sound than mousse (Payne et al. 1991; Irvine et al.2006). The crucial difference between crude oil and mousse in this experiment is not how the oil was weathered, rather it is the difference in the amount of oil present in the respective treatments. Accordingly, the terminology in this review emphasizes the difference in oil content.

Part II.
Comments

1. "Research was conducted at the University of Idaho (Moscow, ID, USA) on the toxicity of weathered *Exxon Valdez* crude oil to embryos of pink salmon from 2001 to 2003 for the purpose of comparing these data with those from the National Oceanic and Atmospheric Administration Fisheries Laboratory at Auke Bay (AK, USA)." *Brannon et al, page 962, abstract, first sentence*

The stated purpose of the Brannon paper is to replicate our experiment (Heintz et al. 1999), one of a series of studies based on the same dosing method, passage of water through oiled-rock columns (Marty et al. 1997; Heintz et al. 1999, 2000; Carls et al. 1998, 1999, 2005). The purpose of our comments is to evaluate how accurately Brannon et al. replicated our experiments, identify the detailed differences for readers, and to take issue with Brannon et al. on their interpretations of our published work. We stand by our research, conclude that our interpretations are valid, and that interpretations of our work by Brannon et al. and of their own is flawed.

2. "Eggs incubating in tidal reaches of streams in the spill path (14% of salmon streams affected, or ~3% of total PWS pink salmon eggs [2] may have been vulnerable to oil that reached the shoreline." *page 962, column 1, paragraph 1, line 6*

Most salmon streams affected by oil were in the southwestern fishing district of Prince William Sound (PWS); 31% were contaminated by oil (Geiger et al. 1996). Instead of describing impacts to wild pink salmon in the spill area, Brannon et al. include all PWS streams and hatchery salmon in their assessment. Wild salmon, which accounted for 19 to 29% of all salmon between 1989 and 1991 (Rice et al. 2001), represent all of the natural genetic variation in the system.

3. "However, no effects of oil on incubating pink salmon embryos (i.e., deformities or mortality) were found in streams immediately after the spill (spring of 1989) [8], and no lingering effects (genetic damage or reduced adult returns) were observed years later [8,10,11]." *page 962, column 1, paragraph 1, line 11*

Contradictory studies with greater statistical power are ignored, a problem previously pointed out to Brannon et al. (Rice et al. 2001). Because of insufficient power, Brannon et al. (1995) risk a large type II error, failure to detect real differences between oiled and reference embryo survival; these differences were detected in the more powerful study by Bue et al. (1996, 1998).

4. "A very weathered oil (VWO) that had been storred under controlled conditions for at least a year apparently was the most toxic." *page 962, column 2, paragraph 1, line 2*

Measured by total polynuclear aromatic hydrocarbon (PAH) concentration, PAHs in water passed through very weathered oil (VWO) were more toxic per unit mass than those in effluent from less weathered oil. The toxicity of the source oil did not increase because toxicity is a function of composition and concentration; total PAH (TPAH) concentrations in source oil can only decline with time (increases are thermodynamically impossible).

5. "Exposure concentrations were reported as total PAH [TPAH] concentrations dissolved in the column effluent. These concentrations are two or three orders of magnitude lower than the petroleum TPAH concentrations reported previously by ABL researchers as being lethal to pink salmon embryos [13]." *page 962, column 2, paragraph 1, line 4*

Brannon et al. compare doses in our modern long-term, flow-through assays (Marty et al. 1997; Heintz et al. 1999, 2000; Carls et al. 1999, 2005) with doses in research completed in our laboratory in the 1970s (Rice et al. 1975) and state that TPAH concentrations in the modern experiments were 2 to 3 orders of magnitude less than in the earlier experiment. The 1970 studies were short-term, acute bioassays and the mode of action was likely narcotic; toxicity in the modern studies was likely due to the pharmacological activity of specific PAHs. Brannon et al. mischaracterize the dosing units in the 1975 paper as TPAH; instead, total oil in water concentrations were reported, and PAHs would likely comprise about 1% of the total hydrocarbons. In the modern studies, we report concentrations in the low parts per billion of aqueous TPAH [as measured by gas chromatography / mass spectroscopy (GC/MS)]; in the 1970s we measured total oil in the water by infrared techniques and reported LC50s as parts per million of whole oil (not PAH).

6. "The inconsistency between the ABL results that have alleged continuing risk to pink salmon in PWS and the field investigations showing that only low, nontoxic concentrations of oil reached the spill-path salmon streams [8,9] requires reanalysis of weathered oil toxicity..." *page 962, column 2, paragraph 2, line 3*

We do not claim continuing risk to pink salmon in PWS, only that oil impacts lasted longer than expected. Contrary to their statement, "ABL" (Auke Bay Laboratory) results are corroborated by field studies (Bue et al. 1996, 1998; Wiedmer et al. 1996). The claim that "only low, nontoxic concentrations of oil reached the spill path streams…" is at odds with multiple studies (Gundlach et al. 1990; Bue et al. 1996; Murphy et al. 1999), including those of industry researchers (Boehm et al. 1995; Neff et al. 1995).

7. "For reasons explained below, the UI experiments did not employ the alternating cycle of fresh water and saltwater as used in the design described by Heintz et al. [6]." *page 963, column 1, paragraph 6, line 10*

The objective of Brannon et al. was to replicate our experiment; such modifications contradict this objective. Brannon et al. failed to replicate an alternating seawater - freshwater exposure, a condition that replicates the Prince William Sound exposure scenario for pink salmon that spawn in the intertidal reaches of small streams. Alternating seawater exposure is important to both the biology/physiology of the salmon embryos and the chemistry of oil dissolution into water.

8. "A preweighed amount of AWEVC or NWEVC was added to 10.8 kg of gravel in a rotating mixer and mixed for 5 min to coat the gravel with oil as uniformly as possible." *page 963, column 1, paragraph 2, line 2*

We were aware that introduction of oil microdroplets by our dosing apparatus might seriously confound interpretation of the results, thus a procedure was developed to prevent microdroplet formation (an oil aerosol was sprayed onto continuously tumbling rock for at least 90 s to minimize oil film thickness and hence promote adhesion). Our technique is consistent with recommendations that generator columns be used to solubilize sparingly soluble chemicals without confounding emulsifications or particulate solute (Billington et al. 1988). Brannon et al. took none of these precautions. Instead of spraying the oil onto their rock as an aerosol, they simply added the oil as a single aliquot, potentially minimizing adhesion, and making their preparation more prone to droplet formation.

9. "Both AWEVC *[oil]* and NWEVC *[mousse]* were tested at four nominal EVC-on-gravel loadings…" *page 963, column 2, paragraph 1, line 3*

Nominal oil concentrations fail to accurately describe exposure conditions within the Brannon et al. experiment. **A)** Instead of measuring the amount oil that actually adhered to the rock, Brannon et al. assume complete adhesion. In contrast, the oil loading reported by Heintz et al. (1999) was based on direct measurement of oil adherent on gravel. The data in Table 1 of Brannon et al. can be used to calculate the actual amount of oil adherent to gravel. As Brannon et al. correctly indicate, unweathered *Exxon Valdez* oil contains about 1.3% by weight of TPAH. Dividing their initial TPAH concentrations by the nominal oil dose times 0.013 yields estimates of the fraction of oil that adhered to the gravel; estimates range from 18% in the lowest dose to about 42% in the others. Discounting water, mousse adherence was similar, 6% in the lowest dose and about 43% in the others. These discrepancies between nominal and actual oil loadings are comparable with our experience; about 15% adhered in the lowest doses through 44% in the highest (Heintz et al. 1999). **B)** Brannon et al. recognize that 2/3 of the mousse applied to rock was water, yet ignore this later **{**comments **19,28,29,52,59}** when relying on nominal doses instead of measured exposure concentrations. **C)** Nominal oiling does not account for variation caused by other experimental factors, such as flow rate, temperature, and salinity, and **D)** nominal oiling does not account for partial, timed PAH release (as illustrated in Fig. 2 of Brannon et al.), nontoxic, and biologically unavailable oil constituents.

10. "… and all tests run in triplicate." *page 963, column 2, paragraph 1, line 6*

The statistical power in the Brannon et al. experiment (2 oil types, 5 doses/type, 3 replicates/dose, 250 eggs/replicate) was less than in ours. **A)** The number of doses (including controls) in our pink salmon experiments varied depending on objectives. To maximize the statistical power of growth effects, 7 dose levels were examined with about 10000 eggs per dose (1993 brood year); to maximize the statistical power to resolve differences in marine survival between doses, 3 dose levels were examined with about 120000 eggs per dose (Heintz et al. 1999, 2000). Marty et al. (1997) and Carls et al. (2005) each included 5 dose levels. **B)** The number of replicates in our pink salmon experiments was generally substantially greater than in the Brannon experiment (8 replicates per dose by Heintz et al. 1999, except 15 replicates in the highest dose, 7 replicates per dose in the marine survival experiment of Heintz et al. 2000, and 8 replicates per dose except 2 replicates in the highest dose by Carls et al.

2005). Marty et al. (1997) included 3 control and low dose replicates, 2 median dose replicates, and 4 replicates in the 2 highest doses **{12}**.

11. "Tissue and effluent subsequently were sampled on days 11, 23, 40, and 83." *page 963, column 2, paragraph 1, line 8*

Observation time in the Brannon et al. experiment was considerably shorter than in our experiments, reducing the sensitivity of their experiment. The sampling times of Brannon et al. were all within 83 d; our sampling continued for 180 to 240 d and continued after exposure for 0.5 to 1.3 y in some tests (Marty et al. 1997; Heintz et al. 1999, 2000; Carls et al. 2005). As did we, Brannon et al. observed that mortality increased with time.

12. "Approximately 250 eggs were placed at each of two locations in each incubator on September 18, 2001." *page 963, column 2, paragraph 2, line 1*

The number of pink salmon eggs we examined per replicate was greater than the number examined by Brannon et al., resulting in greater statistical power. Brannon et al. assayed approximately 250 eggs per replicate. Marty et al. (1997) added roughly 750 eggs per replicate (300 ml eggs were added; the estimate provided assumes a void volume of 36% for randomly packed spheres). Heintz et al. (1999) added 200 ml aliquots per replicate (about 500 eggs); Heintz et al. (2000) had 10000 eggs per replicate release time, and Carls et al. (2005) included approximately 2700 eggs per replicate.

13. "One-liter water samples were extracted with liquid/liquid techniques [16]." *page 964, column 1, paragraph 1, line 5*

The effluent water samples extracted by Brannon et al. represent the sum of all dissolved PAH (c_d) and hypothetical particulate oil (c_p). No attempt was made to quantify dissolved and particulate phases separately. Summarized, measured aqueous dose (c_w) is the sum of these two phases:

(Eq. 1) $$c_w = c_d + c_p$$

14. "The concentrations of the alkyl PAH were determined from the response factors of the corresponding parent PAH." *page 964, column 1, paragraph 4, line 13*

Our PAH calibration curves for alkylated PAH homologues (as at Environment Canada; Wang and Fingas 2003) are based on the most similar available standard (e.g., the calibration curve for 2,3,5-trimethylnaphthalene is used to quantify all the C3-naphthalenes), whereas the calibration curve for the unsubstituted PAHs is used for all the related alkyl-homologues by Brannon et al. Thus, our alkyl- naphthalenes and phenanthrenes estimates are about 30% higher than in the Brannon study.

15. When blue sac disease (ascites) became apparent, the proportion of alevins infected was similarly tested with the equation." *page 964, column 2, paragraph 1, line 11*

Ascites is a typical direct embryonic response to aromatic hydrocarbons and has been observed in embryos of multiple species for decades (e.g., Linden 1976; Pearson et al. 1985; Incardona et al. 2004, 2005). Ascites is not evidence of an infection caused by a pathogen as suggested here by Brannon et al., rather it refers distension of the peritoneal cavity by fluid. Membrane disruption by PAHs is the most likely reason fluid is abnormally lost from tissue.

16. "Nonweathered EVC contains approximately 13,000 ppm (μg/g; 1.3% by wt) of total resolved PAHs, 96% of which are two- and three-ring PAHs and dibenzothiophenes (sulfure heterocyclics), including their alkyl homologues [22]." *page 964, column 2, paragraph 4, line 1*

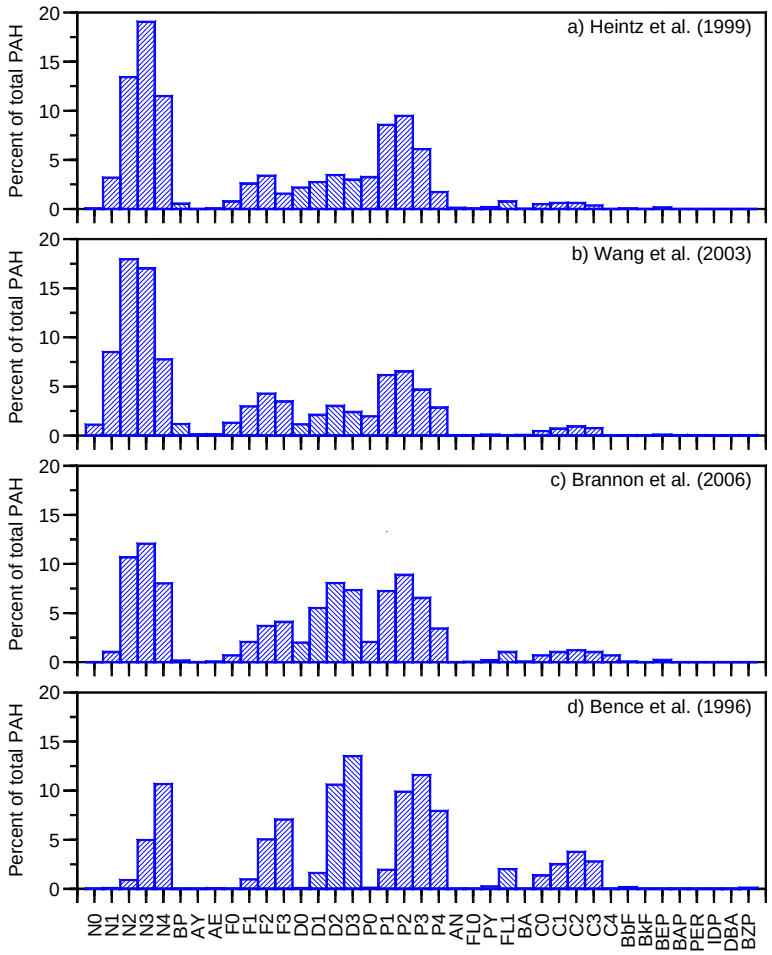


Fig. R1. Comparison of relative PAH distribution of weathered Alaska North Slope crude oils: (a) in oil evaporatively weathered to 20% mass loss as used at the beginning of our pink salmon exposure experiment (Heintz et al. 1999); (b) in oil evaporatively weathered to 30.5% mass loss as reported by Wang and Fingas (2003); (c) in oil evaporatively weathered to 20% mass loss as used at the beginning of the Brannon et al. experiment; (d) after weathering in PWS for one year (Bence et al. 1996), as cited by Brannon et al.

The composition of PAH on oiled rock reported by Brannon et al. is consistent with our observations **{Fig. R1}**, given the calibration differences in the hydrocarbon analysis methods between laboratories **{14}**.

17. "A greater loss of low-molecular weight, two- and three-ring PAHs was found compared with that of high-molecular-weight, four- and five-ring PAHs, as shown by PAH profiles in the gravel (Fig. 1) and the effluent (Fig. 2). The concentration of total naphthalenes (two-ring PAHs) on gravel containing 2,250 ppm of NWEVC decreased by 65% during the 83-d exposure period; the concentration of total chrysenes (four-ring PAHs) decreased by 32% in the same time period." *page 964, column 2, paragraph 5, line 4*

The evidence presented here contradicts the role assigned to oil droplets by Brannon et al. in their experiment. Rather, their aqueous hydrocarbon data demonstrate that most PAHs were dissolved and the proportion associated with ablated oil droplets was negligible. Whereas losses of the most soluble PAHs from rock were >50% in 83 d, losses of C_4-chrysenes and benzo[e]pyrene, the least soluble PAHs presented, were negligible (their Fig. 1). Unchanging concentrations of the least soluble PAHs indicates that ablation losses of bulk oil from the gravel used by Brannon et al. were correspondingly negligible, demonstrating that nearly all of the PAHs were lost from gravel through dissolution. In particular, their results indicate the visible traces of oil noted in the effluent of their highest dose was a negligible fraction of oil remaining on gravel. Expressed mathematically,

(Eq. 2) $$c_p \approx 0$$

Combining equations 1 and 2 we find that aqueous dose is equivalent to the dissolved TPAH concentration.

(Eq. 3) $$c_w \approx c_d$$

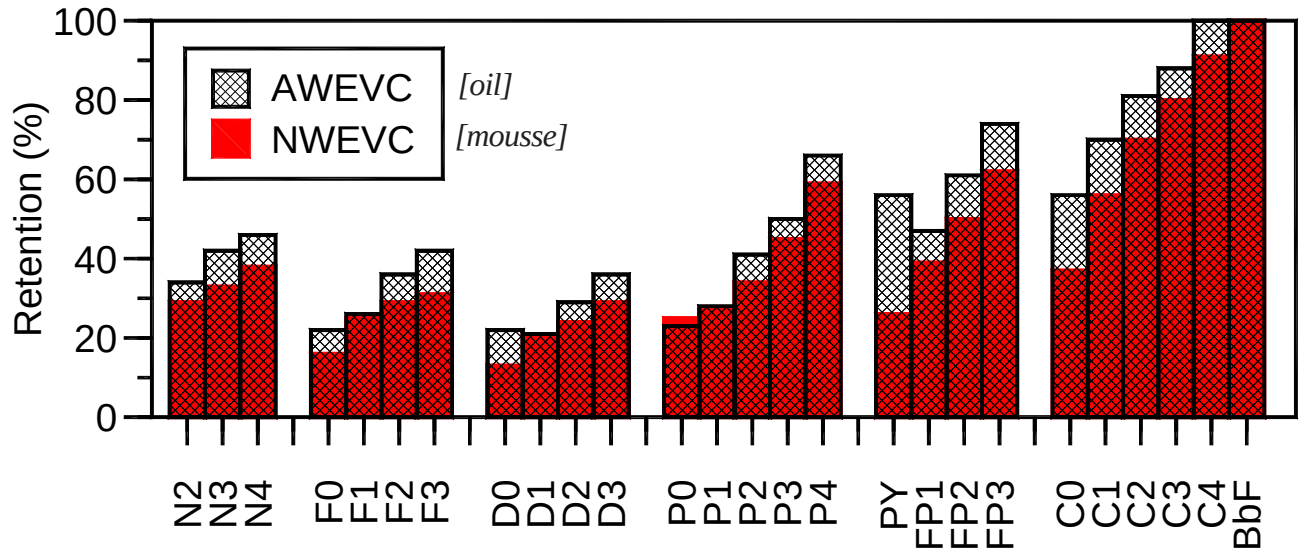


Fig. R2. Retention of PAH after 83 d in the mousse and oiled rock columns of Brannon et al.

The PAH composition changes on the oiled rock of Brannon et al. also demonstrate that mousse and oil were highly similar at the beginning of their experiment (their Fig. 1) and that PAHs transferred slightly *faster* from mousse to water (their Fig. 1 and **Fig. R2** derived from it). At the end of the Brannon et al. study, 35% of the mousse remained compared to 41% of the PAH in oil (calculated from the TPAH concentrations listed in their Fig. 1); these differences were not tested statistically.

18. *page 965, Table 1*

	EVC on gravel	Gravel TPAH	water TPAH	On gravel			Above gravel		
Oil type	(ppm)	(ppb)	(ppb)	M	SD	BS	M	SD	BS
Control	0	<1	0.01	16.9	4.6	3.1	16.5	3.0	0.8
AWEVC	200	460	1.46	12.6	2.2	5.7	12.4	2.2	0.2
[oil]	750	4200	7.43	13.3	2.2	2.8	17.2	2.7	0.7
	1500	8300	7.84	23.1	5.1	5.8	19.4	11.7	2.3[f]
	2250	12100	16.39	59.7[f]	14.0	11.9[f]	41.8[f]	12.0	3.8[f]
NWEVC	200	53	0.84	14.2	7.1	3.1	9.5	1.0	0.9
[mousse]	750	1400	4.34	10.9	5.3	1.3	10.5	3.4	0.5
	1500	3300	4.88	13.7	8.0	3.3	13.5	5.7	0.7
	2250	4400	8.27	14.8	2.3	5.8	16.8	6.8	1.3

[f] Significantly from control mortality (p = 0.05).

Table 1 of Brannon et al. is revealing because it demonstrates that the lowest effective doses were <10 parts-per-billion. Embryos above gravel were significantly damaged (ascites at 8 μg/L measured aqueous TPAH concentration) or killed by exposure (16 μg/L oil); exposure to particulate oil was negligible in these groups **{17}**. Previously established, nominal oiling fails to accurately describe exposure conditions and TPAH on rock merely represents dose added **{9}**. Rather, aqueous TPAH concentration is the demonstrable measure of dose delivered to embryos in effluent and to those on rock **{17,23,24,31}.** Several important observations are summarized in this table:

A) Measured aqueous doses are related to the nominal doses Brannon et al. use throughout their paper. This is important because it permits a true comparison of exposures within Brannon et al.'s experiment **{28}** and to ours **{35}**.

B) Mortality in embryos in contact with oiled gravel was about the same as in those above gravel ($P = 0.922$) **{27}** and PAH bioaccumulation was also about the same **{23}**.

C) Ascites increased with dose. The incidence of ascites in embryos in contact with oil was higher than in for those in water (within a factor of <5 with the exception of one ineffective dose, 1.46 μg/L). The reason for differences in ascites apparently involved factor(s) unrelated to oil; control ascites in gravel (3.1%) was nearly as high as significant oil-related ascites in

the highest above-gravel treatment (3.8%, **{28}**) and nearly 4 times greater than the corresponding above-gravel control (0.8%).
D) Aqueous TPAH concentrations in the maximum mousse dose (8.3 μg/L) were nearly the same as in the penultimate oil dose (7.8 μg/L); ascites was significantly elevated by this dose (as noted on p. 967 of Brannon et al. **{28}**).

19. "The TPAH concentrations at the start of the exposure were approximately twice as high in effluents from columns containing AWEVC *[oil]* as those containing NWEVC *[mousse]* at all loading levels tested (Table 1)." *page 965, column 2, paragraph 2, line 1*

Recall that 64% of the mousse was water, explaining why aqueous TPAH concentrations from mousse were less than those in corresponding nominal oil loadings (mean 57%, range 50 to 62%).

20. "The PAH profiles in effluent water showed that the dominant PAH in the water collected during the first 15 (4 + 11) days of column irrigation were low-molecular-weight PAHs, which is consistent with a predominantly dissolved PAH fraction (Fig. 2). Although the concentrations were low, the relative contributions of low-solubility, high-molecular-weight PAHs increased in effluent water after day 15. These high-molecular-weight PAHs were the dominant components of the PAH assemblage in effluent water samples collected on days 40 and 83 of egg exposure." *page 965, column 2, paragraph 3, line 1*

The time-dependent change in PAH composition in water from naphthalene toward phenanthrene dominance is consistent with our results.

21. "At the highest concentration (2,250 ppm) of AWEVC oil on gravel, we observed oil residue on the inside walls of the incubator columns above the gravel and on the walls of the tubing downstream in the effluent, which indicated that oil droplets were entrained in the water passing through the oil-contaminated gravel. The presence of oil droplets was confirmed by observation of small sheen spots on the incubator water surface, which indicated that the droplets were minute and remained in the effluent stream." *page 965, column 2, paragraph 4, line 1*

The only evidence offered by Brannon et al. in support of oil droplets is visual and this is confined to a single treatment. Although their oil application method increased the likelihood of oil droplets (relative to our experiment) **{8}**, the chemical evidence provided by Brannon et al. demonstrated the *absence* of droplets in effluent water **{17}**. (See **{31}** for further discussion.)

22. "If oil droplets were the source of the toxicity in these short-term column experiments, then they would not be expected to be introduced into PWS pink salmon streams through tidal flushing of adjacent gravels in the years following the spill." *page 965, column 2, paragraph 4, line 9*

Brannon et al. provide no basis for this statement.

23. "In general, for loadings of AWEVC or NWEVC on gravel, embryos positioned in direct contact with oiled gravel in the columns accumulated slightly more TPAH than did embryos exposed to the effluent above the gravel. Tissue PAH concentrations were similar at both locations, because oil droplets contaminated the effluent and coated the eggs. Consequently, with these data, it is not possible to differentiate PAH from residual oil coating the egg surfaces from the soluble fraction assimilated in embryo tissues." *page 966, column 1, paragraph 2, line 3*

Similarity in PAH bioaccumulation between embryos in contact with oil and those isolated from it demonstrates oil was equally available in both situations **{27}**. Because droplet concentrations were negligible in effluent water **{17}**, PAHs in whole oil were not biologically available to embryos until dissolved into water. Furthermore, expected bioaccumulation of dissolved

PAH is about 10^3 times the aqueous concentration (e.g., Neff and Burns 1996; de Voogt et al. 1991), consistent with the data of Brannon et al. For example, in the highest Brannon et al. dose, the initial aqueous TPAH concentration was 16.4 µg/L and was ≤2.2 µg/L when the tissue was collected for analysis (13200 µg/kg), i.e., the geometric mean aqueous TPAH concentration was 6 µg/L and the bioaccumulation factor was roughly 2200. Thus, the evidence of Brannon et al., including point **{24}**, demonstrates that eggs in effluent water were not coated by oil.

24. "The pattern of maximum relative concentrations of different PAHs in embryo tissues was roughly similar to the PAH pattern in the gravel, but not to the PAH composition in the effluent at the times tissue samples were collected (Figs. 1–3)." *page 966, column 2, paragraph 2, line 1*

Contrary to the statement of Brannon et al., the PAH profiles presented for eggs 23 and 83 d after the beginning of exposure (their Fig. 3) are almost identical with the profile presented for the aqueous PAHs at the beginning of the exposure (their Fig. 2) and are not consistent with PAH composition on rock (their Fig. 1). Chrysenes are absent from these profiles in eggs, precluding involvement of whole oil, and the preponderance of the less alkyl-substituted phenanthrenes and dibenzothiophenes reflects the pattern expected from water-washed oil.

Given that the bioconcentration factors of the accumulated PAHs exceed 10^3, and considering these factors as ratios of the uptake and depuration rate constants implies that nearly all of the PAH burden accumulated by the eggs occurred within the first few days of exposure when PAH concentrations were high, explaining the correspondence with initial aqueous exposure. By day 23, aqueous TPAH in their highest dose dropped to <2% of the initial concentration, so the eggs collected at day 23 were essentially depurating the burden accumulated during the first two weeks of exposure. Concentration changes in tissue can be related to those in water with first order kinetics: $dC_N / dt = k_u C_w - k_r C_N$, where C_N = TPAH concentration in tissue, C_W = TPAH concentration in water, k_u = TPAH uptake rate, and k_r = TPAH depuration rate (Heintz et al. 1999).

25. "However, cumulative mortality increased during the exposure period to an overall mean of 23.1% of the embryos and alevins exposed directly to 1,500 ppm of AWEVC on gravel and 59.7% mortality at 2,250 ppm of oil on gravel, compared with 16.9% mortality among controls (Table 1)." *page 967, column 1, paragraph 3, line 5*

Brannon et al. are inappropriately using nominal oil concentrations to describe embryo response, rather than measured aqueous exposure concentrations **{9}**. The important result here is that dose-dependent mortality was significant at 16 µg/L dissolved TPAH in embryos in effluent water and in embryos in contact with oil.

26. "The alevins in direct contact with the oil on gravel showed the highest incidence, which implies that direct contact with the oil concentration causes stress, at least at the higher concentrations [5]." *page 967, column 2, paragraph 1, line 1*

The stress Brannon et al. note in embryos in contact with oiled rock was caused by factors other than oil, demonstrated by increased response in the gravel control **{18}**.

27. "The temporal pattern and final cumulative mortality of embryos and alevins suspended in the effluent stream above the gravel and at all oil-on-gravel loadings in the columns were not significantly different from those among embryos and alevins in direct contact with the oil on gravel (p = 0.922). This indicates that the compositions and concentrations of the toxic fractions of dissolved and dispersed AWEVC were approximately the same at the different exposure locations (in gravel and above gravel)." *page 967, column 2, paragraph 2, line 1*

These are important experimental results because Brannon et al. demonstrate that **A)** TPAH concentration and composition were the same in oil-exposed and effluent-exposed embryos and **B)** embryo mortality was the same for these two groups.

28. "Percentage mortality among embryos, even those exposed to effluents from the highest concentration of NWEVC *[mousse]* on gravel (2,250 ppm; 14.8%), was not significantly different from that of controls (16.9%). Blue sac disease was observed in alevins exposed to NWEVC (Table 1) but was significantly higher only at the highest oil concentration (2,250 ppm)." *page 967, column 2, paragraph 3, line 5*

The important result here is that ascites was significantly higher in the highest mousse treatment. Brannon et al. are again using nominal oil concentration inappropriately to describe embryo response to mousse **{9}**. The highest mousse dose did not elicit mortality because it corresponded to a sublethal oil dose. This discrepancy is evident for every dose metric presented by them (TPAH on rock, in water, and in tissue, **Fig. R3**) except for nominal oiling. Significant sublethal mousse toxicity, ascites at 8 µg/L TPAH, in parallel with significant ascites at 8 µg/L in oil, demonstrates that mousse was biologically active and toxic.

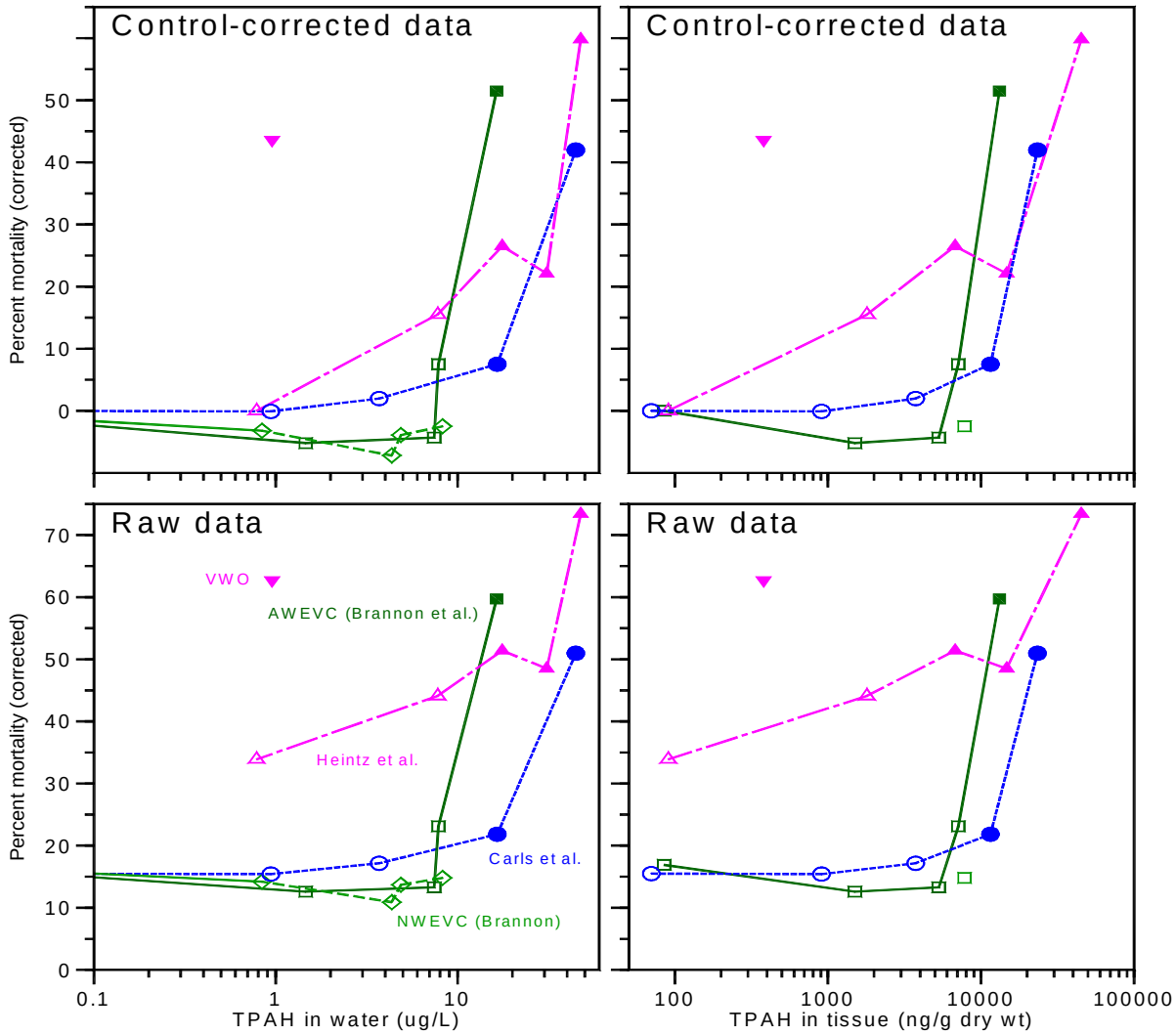


Fig. R3. Relationship between dosing and mortality in the pink salmon embryo experiments of Brannon et al. (2006), Heintz et al. (1999) and Carls et al. (2005). Data in the upper two panels were corrected by control mortality (100 * (P_i – P_c) / (100 – P_c), where P_i = percent response in the i^{th} treatment and P_c = percent response in the control. Control mortality in the Brannon and Carls experiments were highly similar (16.9% and 15.5%, respectively). Mortality in the experiments of Heintz and Carls was measured at emergence (about 180 d after fertilization); mortality in the Brannon experiment was measured after 83 d. Embryos in the Brannon and Heintz experiments were in contact with oiled gravel; embryos in the Carls experiment were exposed to effluent water only for the first 54 to 58 d, then allowed access to oiled substrate. Solid symbols indicate statistical significance. VWO is the very weathered oil treatment of Heintz et al. (1999).

The chemical data of Brannon et al. demonstrate why toxicity of mousse and oil was about the same. Although PAH in mousse apparently moved slightly faster from rock to water, rate differences were small **{17}**. The bioaccumulation factor in eggs exposed to mousse (943) was comparable to that in eggs exposed to mousse (713 to 1027). Thus, PAH transfer was about the same from oiled rock to water to tissue; the only substantive difference between treatments was that 2/3 of the mousse applied to rock was water.

29. "In the present investigation, pink salmon embryo mortality significantly greater than that for controls only occurred during direct exposure to oiled gravel and effluents from columns containing 2,250 ppm of AWEVC *[oil]* on gravel (Table 1). Initial TPAH exposure concentration resulting in mortality was 12,100 ppb on gravel. In contrast, the NWEVC *[mousse]* removed from

the shore of PWS did not result in salmon embryo mortality significantly greater than that for controls at any NWEVC loading on gravel (Table 1)." *page 967, column 2, paragraph 6, line 1*

The use of nominal dose **{9}**, truncated monitoring time **{11}**, exclusion of sublethal responses **{18,28}**, and low statistical power **{10,12}** combine to obscure detection of toxic effects in oil and mousse.

30. "The dispersion of water in the oil *[mousse]* increases its viscosity, thus decreasing the partitioning of petroleum hydrocarbons into ambient water [27]. These results demonstrate that oil reaching the shores of PWS in the form of mousse rendered it less toxic per unit mass than would be estimated from laboratory-weathered oil." *page 968, column 1, paragraph 1, line 14*

The conjecture of Brannon et al. that mousse is less toxic than oil because "the dispersion of water in the oil increases its viscosity, thus decreasing the partitioning of petroleum hydrocarbons into ambient water" is refuted by their own data; PAH from mousse and oil entered water at about the same rate, toxicity was the same when compared with appropriate dose metrics, and bioaccumulation in embryos exposed to mousse was indistinguishable from that in oil **{17,28, Fig. R2}**. Thus, contrary to their conclusion, Brannon et al. have demonstrated that the oil in mousse stranded on PWS shoreline has the same toxic potential as equally weathered whole oil.

31. "The relative PAH composition in embryo tissues remained fairly constant over the exposure period, despite the marked changes in the profile and concentrations of the aqueous PAH over the same time period (Figs. 2 and 3). The relationships among TPAH profiles in gravel, effluent, and tissue suggest that the initial high TPAH concentrations associated with eggs is from direct contact with oil, oil films, or droplets adsorbed on the chorion and may have partitioned directly from the oil phase into the hydrophobic chorion and yolk. Toxicity of dissolved TPAH alone could not be determined, because dispersed oil droplets were present in the effluents. The TPAH profile suggests that the dissolved fraction played a lesser role in oil toxicity to the embryos compared to the dispersed oil. If the PAH residues in salmon embryos were derived primarily or exclusively from dissolved PAHs in the column effluents, and given the lag in tissue uptake, tissue concentrations should have been in quasiequilibrium with concentrations in solution in the water [28,29], which was not the case. The data suggest, therefore, that the toxicity of weathered EVC in the present study was caused primarily by direct contact with oil or droplets that adhere to the eggs during the initial 11 to 20 d of exposure, with mortality gradually increasing in the developing embryo population over successive weeks." *page 968, column 1, paragraph 2, line 1*

Brannon et al. incorrectly identify oil droplets as the cause of embryo response (ascites and death) in their experiment. Instead of actually measuring PAH contributions from oil microdroplets to their dosing water, Brannon et al. only provide a single visual observation in their highest dose, the one most prone to droplet formation **{21}**. The chemical evidence they provide demonstrates that the incidence of oil droplets was negligible throughout their experiment, including the highest dose **{17}**. Their biological data demonstrate that embryos in contact with oil responded at about the same rate as those isolated from oil **{18}**.

The data of Brannon et al. demonstrate that composition of PAH in eggs is determined by dissolved PAH composition in water at the beginning of exposure **{24}**. The fact that PAH burden in tissue did not appear to be in "quasiequilibrium" with the exposure water at day 23 and later merely reflects the fact that the exposures were almost always far from equilibrium (there is an instant during the PAH accumulation phase of the eggs when the declining exposure concentration passes through the concentration that would be in equilibrium with the accumulated PAHs, but this instant varies for the different PAH).
Thus, the data of Brannon et al. demonstrate that dissolved PAH are toxic and contact with whole oil or oil droplets is inconsequential.

32. "The presence of the disease among the control lots indicates that oil only exacerbated the condition, but the mechanism could not be discerned. Pink salmon eggs and alevins incubated in hatcheries often show blue sac disease when exposed to poor water circulation, and in the present study, oil may have inhibited respiration across the yolk membrane." *page 968, column 1, paragraph 3, line 3*

The claim by Brannon et al. that the mechanism for ascites could not be discerned and that oil may have inhibited respiration, thus causing the effect, is not consistent with other studies and ignores other research **{15}**. Although we agree that these stressors may interact when present simultaneously, the embryos in our experiment, as in the Brannon et al. experiment, were apparently well aerated.

33. "Embryos exposed to the only toxic dose of AWEVC (2,250 ppm on gravel) bioaccumulated PAHs to a maximum concentration of 13,200 ppb (Table 2)." *page 968, column 1, paragraph 4, line 1*

Brannon et al. discount sublethal toxicity (ascites) and its implications throughout their discussion **{15,18,28}**. Other defects are typically associated with ascites such as spinal defects, retarded development, and altered heart morphology and function [Carls et al. 1999; Incardona et al. 2004, 2005]. Fish with ascites are unlikely to survive, because impaired swimming, circulatory

problems, and other secondary abnormalities render them ecologically dead. The survival strategy of pink salmon is rapid growth (Parker 1971; Healey 1982; Hargreaves and LeBrasseur 1985); post-exposure growth is impaired even in embryos that are superficially normal, resulting in reduced ocean survival (Heintz et al. 2000; Carls et al. 2005). By narrowly defining the term "toxicity," Brannon et al. focus their discussion entirely on the least sensitive measure of toxic response possible. Low statistical power, reliance on nominal doses, and truncated monitoring time also obscure toxic effects **{29}**.

34. "Only traces of four- and five-ring PAHs were present in the embryo tissues. This suggests that low-molecular-weight PAHs were the main cause of mortality. Such a conclusion is consistent with the observation of Page et al. [30] that the toxicity threshold (based on laboratory bioassays with benthic amphipods) for NWEVC in intertidal sediments from oiled shorelines in PWS is 2,600 ppb of TPAH. Sediments containing greater than 2,600 ppb of TPAH and high relative concentrations of naphthalenes were more toxic compared with sediments containing greater than 2,600 ppb of TPAH and high relative concentrations of chrysenes. The high-molecular-weight PAHs, although more toxic than low-molecular-weight PAHs [31], were not present in bioavailable forms at sufficient concentrations in either the column effluents in the present study or in oiled sediments on the shores of PWS to cause chronic toxicity. Barron et al. [32] evaluated the results of the herring and pink salmon embryo toxicity studies performed at ABL and concluded that the toxicity of weathered EVC was caused primarily by alkyl phenanthrenes. Our results and those of Page et al. [30] indicate that alkyl naphthalenes also contributed to weathered EVC toxicity." *page 968, column 1, paragraph 4, line 5*

Brannon et al. are speculating that because 4- and 5-ring PAH concentrations were low they contributed little to toxicity. To support this argument they refer to a short-term, acute adult amphipods assay where low molecular weight PAH resulted in narcotic toxicity (Page et al. 2002). In so doing, they are confusing toxicity mechanisms; PAHs in crude oils operate through a variety of non-narcotic toxicity mechanisms in embryos (Incardona et al. 2004, 2005, 2006), often with effects that may be considerably delayed (e.g., Baumann and Harshbarger 1998). Our embryo studies suggests that PAH toxicity increases with molecular size (Carls et al. 1999), consistent with other studies (e.g., Anderson et al. 1974, Black et al. 1983, Neff 1985, Neff et al. 2000) and models (Di Toro et al. 2000; Neff et al. 2005). The relative contributions of individual PAH to embryo toxicity remain unknown; because PAH toxicity mechanisms vary, biological effects cannot simply be predicted from hydrophobicity (Incardona et al. 2006), explaining why model results based on hydrophobicity (Di Toro et al. 2000; Neff et al. 2005) underpredict toxicity.

35. "The cumulative mortalities of eggs and embryos in the UI study were much lower than those reported by Heintz et al. [6], even for the controls (16.9% vs 33.9%)." *page 968, column 2, paragraph 2, line 2*

Brannon et al. incorrectly claim that "the cumulative mortalities of eggs and embryos in the UI study were much lower than those reported by Heintz et al." **A)** Reliance on nominal dosing is the primary problem here; different procedures between laboratories preclude nominal doses as appropriate comparators **{9}**. **B)** Instead of comparing dose added, mortality as a function of dose delivered (aqueous TPAH concentrations) or TPAH load accumulated by embryos can legitimately be compared between experiments (in treatments where weathering was about the same). Both the Brannon et al. experiment and ours demonstrate that dissolved PAH are damaging, not particulate oil **{17,23,24,31}**, confirming the appropriateness of aqueous TPAH concentrations as a common inter-experimental factor. Comparison of the response curves reveals that the dose-dependent cumulative mortality

increased rapidly at a lower aqueous TPAH concentration in the Brannon et al. experiment (after 83 d) than in the Heintz et al. experiment (after 190 d) **{Fig. R3},** demonstrating that the median lethal concentration observed by Brannon et al. was somewhat greater than that observed by Heintz et al. (1999) and Carls et al. (2005). **C)** Correction for control response, which differs substantially between studies, is also advisable (upper panels of **Fig. R3**).

36. "Initial AWEVC *[oil]* threshold tissue TPAH concentrations at which mortality was significantly greater than that among controls in the two laboratory studies were similar (ABL, =6,000 ppb; UI, >7.100 ppb) (Table 2), but not when based on TPAH loading on gravel (ABL, 3,800 ppb; UI, 12,100 ppb). Initial gravel TPAH concentrations in the ABL columns were well over twofold those in the UI columns at approximately the same oil-on-gravel loadings, and initial TPAH concentrations in the effluent were more than 2- to 12-fold higher in the ABL columns (Table 2). The most likely explanation for these differences is that the AWEVC used by ABL was fresher and less weathered than the AWEVC used by UI. As discussed below, the lightly weathered oil used by ABL contained substantially more naphthalenes than would be expected for a crude oil weathered to 80% of its original mass.

Total PAH represents 1.3% by weight of the AWEVC weathered at ABL at the 2,450 ppm loading on gravel reported by Heintz et al. [6], compared to 0.5% by weight of AWEVC in the 2,250 ppm loading on gravel in the present study. Fresh, nonweathered EVC contains approximately 1.2% TPAH by weight [22], and the TPAH concentration decreases with weathering. For example, EVC that had weathered in PWS for one year contained 0.47% TPAH by weight [22]. The 2,450 ppm of AWEVC oil on gravel used by Heintz et al. [6] had a weathering parameter (w) of zero [33], indicating that the oil was essentially unweathered by that criterion. The weathering parameter increased progressively with decreasing AWEVC loadings on gravel in the ABL study, indicating that these lower doses were subject to water-washing in the 4 d between the initiation of column irrigation and the start of egg exposure.

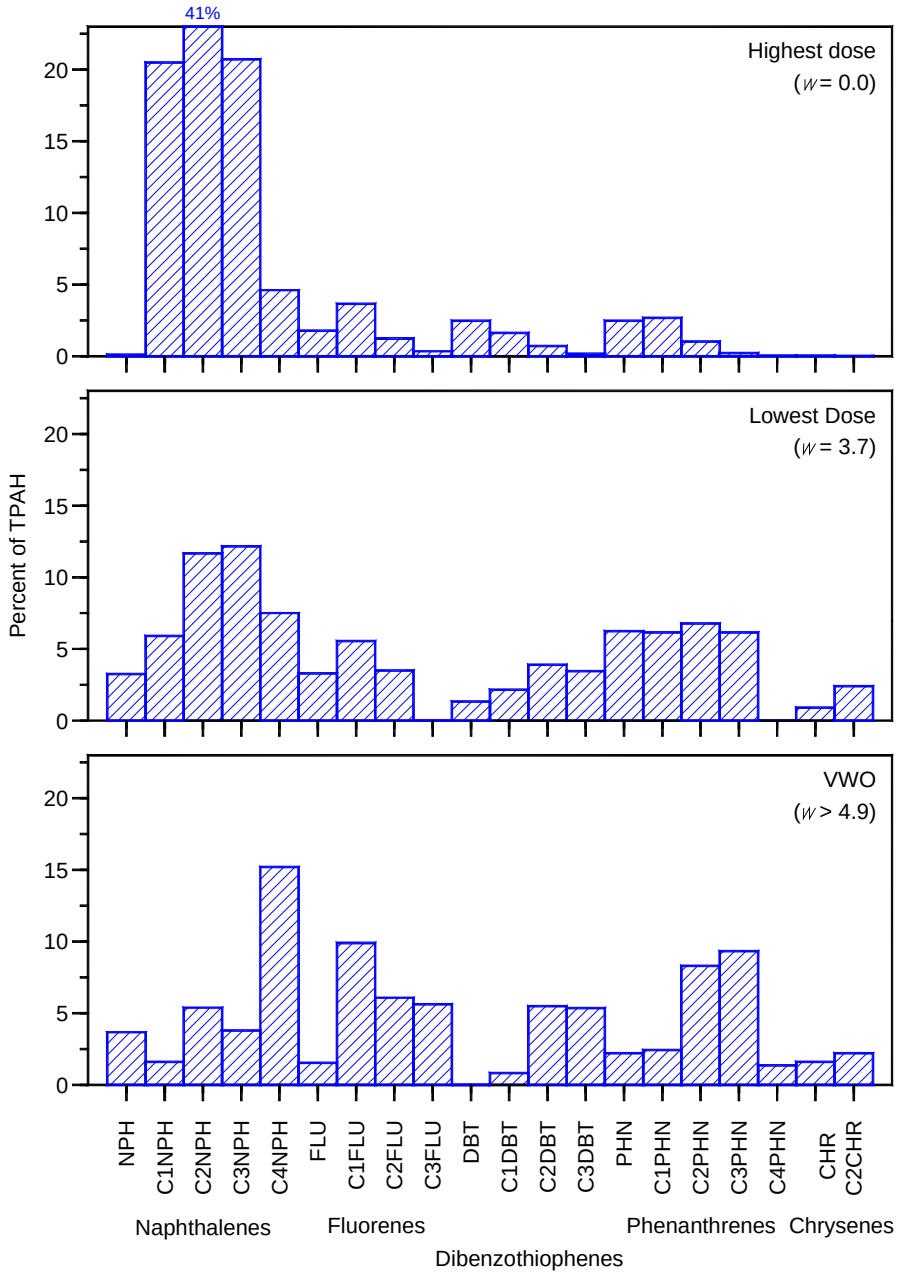
Heintz et al. [6] stated that naphthalenes represented approximately 80% by weight of the TPAH fraction in the AWEVC used in the ABL studies, ''in agreement with the composition of weathered EVC from PWS as reported by Bence and Burns'' [34]. However, Bence and Burns [34] reported that the naphthalenes comprise 83% by weight of the calculated equilibrium water-soluble fraction of fresh EVC. The PAH fraction of fresh EVC contains approximately 50% naphthalenes by weight, and the PAH fraction for the average of 71 shoreline oils collected from PWS one year after the spill is 16.6% naphthalenes by weight [22]. Total naphthalenes represent nearly 48% of TPAH by weight in the AWEVC at an oil-on-gravel loading of 2,450 ppm in the ABL study, which is a value similar to that of fresh oil. Consequently, the PAH composition of the AWEVC employed by Heintz et al. [6] is not consistent with either NWEVC or EVC topped to 80% of its mass by heating to 70ºC. In the present study, total naphthalenes represented 31% of TPAH by weight in the AWEVC at an oil-on-gravel loading of 2,250 ppm. The high concentration of naphthalenes in the ABL study may be part of the reason why their toxicity findings are inconsistent with the present results and with those of previous toxicity studies. Consequently, the ABL results should not be considered as indicative of the toxicity of weathered crude oil in the laboratory or in the field." *page 968, column 2, paragraph 3, line 1*

The claim by Brannon et al. that the oil in our experiments was less weathered than in theirs is in error. Again reliance on nominal oiling is part of the problem **{9}**. To account for discrepencies between amounts of oil added and subsequently measured TPAH concentrations, Brannon et al. assumed that pre-treatment weathering reduces TPAH concentrations in oil. This is not true; weathering in air *increases* TPAH concentration by a factor of about 1.3 because of evaporative losses of components more volatile than PAHs. This concentration increase was independently documented by Wang and Fingas (2003) (Environment Canada); the PAH distribution for 30.5%-weathered oil is nearly identical to the distribution in our 20%-weathered oil **{Fig. R1}**. Wang and Fingas (2003) observed that the proportion of TPAH increased from 1.05 to 1.48% in weathered oil even when the evaporative mass loss was 30.5%; the only PAH that decreased in concentration was unsubstituted naphthalene, the most volatile PAH. Also recall that PAH calibration methods are different between laboratories **{14}.** These factors account for the apparent higher proportion of naphthalenes in our studies rather than evidence of 'unusually fresh' *Exxon Valdez* oil.

In support of their claim that TPAH concentrations decline as pre-treatment weathering proceeds, Brannon et al. misleadingly cite weathering in an EVO sample from PWS which contained only 0.47% TPAH (Bence et al. 1996). They fail to mention that this oil was much more weathered than the oil they applied to their gravel; > 85% of the naphthalenes were gone, as well as considerable proportions of less-substituted alkyl homologues of other PAHs **{Fig. R1}**. In contrast, naphthalenes were by far the most abundant PAHs in the oil Brannon et al. applied to their gravel **{Fig. R1}**. These results show clearly that the evaporative process used in our studies and by Brannon et al. did not reduce the concentration of TPAH in the oil from ~1.3% to 0.47%, but was likely somewhat greater than 1.3%.

Brannon et al. are correct to question our assertion that naphthalenes comprised 80% of TPAH in oil in the methods section of Heintz et al. (1999). The correct percentage is 58%, as depicted in Fig. 1 of that study. We regret the error, and we thank Brannon et al. for bringing this to our attention. However, the PAH distribution depicted in Fig. 1 of Heintz et al. (1999) is

Fig. R4. Composition of PAH in embryos varies depending on the weathering of oil to which they are exposed (from Heintz et al. 1999).



consistent with that expected following 20% evaporative mass loss, given the calibration differences in the hydrocarbon analysis methods between laboratories. In particular, abnormally high concentrations of naphthalenes may be discounted as a reason why our toxicity results are not identical to those of Brannon et al.

37. "The VWO used by Heintz et al. [6] and the NWEVC *[mousse]* used in the present study also are markedly different from one another." *page 969, column 1, paragraph 2, line 1*

The VWO (highly weathered) and mousse (slightly weathered) treatments differed markedly, as correctly noted by Brannon et al. The VWO contained 0.2% TPAH, whereas the mousse only appears to because Brannon et al. use nominal doses, ignoring application loss and the 64% water content. Correction for these errors reveals the TPAH content mousse, about 1.35%. Given the major differences in exposure composition, PAH accumulation in embryos exposed to VWO and mousse was likely very different. Although Brannon et al. do not present PAH profiles in embryos exposed to mousse, they should be highly similar to their oil profiles (their Fig. 1), indicative of a slightly weathered source. In sharp contrast, few naphthalenes were present in the

tissue of embryos exposed to VWO and larger PAH dominated (**Fig. R4**). Because toxicity increases with molecular size (as acknowledged by Brannon et al; **{34}**), damage to VWO-exposed embryos at lower concentrations is plausible. The toxicity of mousse was obscured by low statistical power, reliance on nominal doses, exclusion of sublethal responses, and truncated monitoring time **{29}**.

38. "The composition of the PAH assemblage in the VWO does not resemble that of NWEVC *[mousse]* in PWS shoreline sediments." *page 969, column 1, paragraph 3, line 4*

Although Brannon et al. claim that neither the VWO nor the oil in our experiments are comparable with oil on the shorelines of PWS, direct comparison demonstrates otherwise. Short and Heintz (1997) used the 20% evaporative mass loss of their oil to define the state of zero weathering (their $w = 0$ state); nearly all of the occurrences of EVC in sediments and tissues collected by government agencies during the first three years following the *Exxon Valdez* oil spill conformed with model predictions regarding PAH content. Although the VWO was even more weathered than the weathering range spanned by the model, the distribution of persistent PAHs was entirely consistent with it. For example, the PAH composition in VWO is virtually identical with that of a sediment sample collected in 1995 from a stream delta in Herring Bay, an embayment heavily oiled by the *Exxon Valdez* spill **{Fig. R5}**; other similarly weathered PWS sediment samples have been analyzed by our group. Weathering patterns in oil are also consistent with the natural weathering evident in multiple industry publications (e.g., Bence and Burns 1995; Boehm et al. 2004, 2005; Neff et al. 2006). An *Exxon Valdez* oil detection model by Exxon researchers (Bence & Burns 1995)

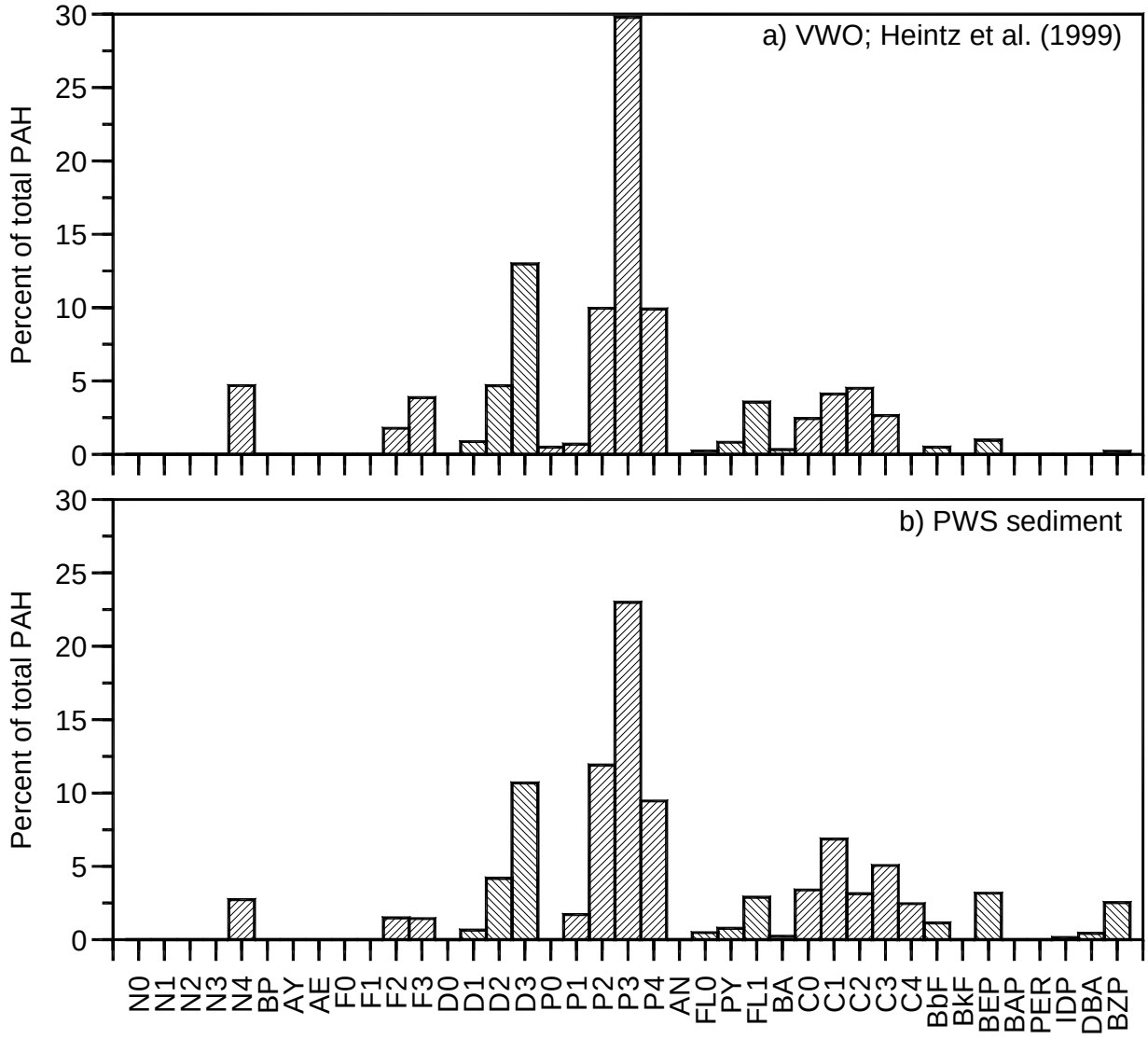


Fig. R5. Comparison of PAH distributions in (a) VWO used in the Heintz et al. experiment (1999) and (b) PWS sediments contaminated by *Exxon Valdez* oil collected in 1995 from Herring Bay (Short et al. 1996).

produces much the same result as the Short and Heintz (1997) model, with the caveat that the latter is more conservative (Carls 2006), further authenticating the commonality of laboratory-weathered oil, VWO, mousse, and other *Exxon Valdez* oil in Prince William Sound. Moreover, as previously demonstrated, the results of Brannon et al. clearly support the same PAH loss mechanism and consequent shifts in composition as weathering proceeds (their Figs. 1 and 2). Combined, chemical and toxicological evidence demonstrates that laboratory oil and VWO accurately reflect weathering and toxicity in natural systems.

39. "Embryos exposed directly to gravel and effluent from the column containing 2,860 ppm of VWO on gravel experienced elevated mortality when tissue TPAH reached 470 ppb [6]. Embryos exposed directly to gravel in the column containing 2,250 ppm of NWEVC*[mousse]* in the UI study contained 7,800 ppb of TPAH in their tissues but did not experience a mortality rate significantly greater than that of controls." *page 969, column 1, paragraph 3, line 6*

Nominal doses are not appropriate for inter-laboratory comparisons **{9}** and Brannon et al should not expect mousse and VWO tissue to cause equal embryo response because of major differences in PAH composition **{37}**. In addition, the statistical power of the Brannon et al. observation was smaller than that of Heintz et al. (1999) **{10}** and truncated monitoring time and exclusion of sublethal responses combine to obscure detection of toxic effects in mousse **{29}**.

40. "Heintz et al. [6] attributed the high toxicity of the VWO to alkyl phenanthrenes." *page 969, column 1, paragraph 3, line 13*

Heintz et al. (1999) did not attribute the high toxicity of VWO solely to alkyl-phenanthrenes but did note that the concentrations of phenanthrenes and chrysenes declined relatively little during their 191 d exposure period and concluded that smaller PAHs (e.g., naphthalenes) contribute relatively little to observed toxicity. Carls et al. (1999) reached exactly the same conclusion with consecutive Pacific herring embryo exposures. The results of Brannon et al. do not contradict these studies.

41. "Other factors associated with the VWO, such as inorganic by-products of oil biodegradation (ammonia and sulfide) [35] and oxidation products of microbial hydrocarbon degradation [36], appear likely to have contributed more than the PAHs to the observed toxicity." *page 969, column 1, paragraph 3, line 22*

Although Brannon et al. accept PAH toxicity in their own experiment **{34}**, they speculate that factors other than PAH may have been responsible for VWO toxicity rather than agreeing that toxicity increases with molecular size, thus explaining why VWO was more toxic per unit mass **{37}**. The alternative factors suggested by Brannon et al. include microbial metabolites, ammonia, and sulfide. These issues, raised previously by Brannon's coauthors and others, fail to explain toxicity in our experiments (Pearson 2005; Carls et al. 2002) and also fail to explain the results in the experiment of Brannon et al.

42. "This interpretation is supported by the relationship between tissue TPAH concentrations and mortality. If PAHs are causing the toxicity to salmon embryos, there should be good correlation between tissue TPAH concentration and the observed toxic effects. However, percentage mortality of salmon embryos that accumulated 470 ppb of TPAH during exposure to effluents from 2,860 ppm of VWO on gravel was similar to that among embryos that accumulated 71,000 ppb of TPAH during exposure to effluents from 2,450 ppm of AWEVC on gravel, and concentrations of alkyl phenanthrenes and chrysenes were lower in embryos exposed to VWO than in embryos exposed to AWEVC in the study by Heintz et al. [6]." *page 969, column 1, paragraph 4, line 1*

Brannon et al. are misrepresenting our results. Correlation between aqueous TPAH concentration, mortality, and sublethal responses has been repeatedly demonstrated in our studies (Marty et al. 1997; Heintz et al. 1999, 2000; Carls et al. 1998, 1999, 2005). By singling out a response to one weathered dose and comparing it to embryo responses to less weathered oil based solely on TPAH, Brannon et al. are ignoring the effects of weathering on PAH composition and toxicity. Total PAH concentration alone does not adequately summarize the data. Carls et al. (1999) demonstrated two complete toxicity curves from less- and more-weathered oil; these were displaced with respect to TPAH concentration because of weathering-dependent composition changes. Most of the difference in toxicity curve position (with respect to TPAH) appeared to be the relatively smaller toxicity

of lower molecular weight PAH, particularly naphthalenes **{40}**. This observation corroborates multiple studies that have demonstrated that toxicity increases with increasing ring number and alkyl-substitution (Moore and Dwyer 1974; Rice et al. 1977; Hutchinson et al. 1980; Black et al. 1983) including those of Brannon's coauthor Neff (1985; 2002); Brannon et al. recognize this relationship **{34}**.

43. "The UI study showed by observation and hydrocarbon chemistry that eggs positioned above the oiled gravel, downstream in the effluent stream of the columns, were exposed to minute oil droplets as well as to dissolved PAHs. Chemical analysis of PAHs in effluent water for those experiments in which elevated embryo mortality was observed indicates that the effluent contained a mixture of dissolved and particulate oil, being particularly obvious during the later part of the exposure period. This is consistent with the results of Payne and Driskell [37], who determined that the sources of PAH (dissolved or oil droplet) could be determined by the tissue TPAH signatures. The presence of minute oil droplets in column effluents of similar study design also was disclosed through visual and chemical analysis by Pearson [35]. Small oil droplets adsorb readily to the hydrophobic chorion of fish eggs [38] and contribute to the PAH body burdens in the embryos.

These results indicate that the 4 d of irrigation of the oil/gravel mixture in the columns before starting the UI study were insufficient to eliminate oil droplets from appearing in the column flow thereafter. In essence, the pink salmon eggs in the effluent were exposed to a mixture of both dispersed and dissolved oil rather than just to the dissolved fraction alone." *page 969, column 2, paragraph 2, line 1*

Although Brannon et al. claim their experiment was confounded by oil droplets, their data demonstrate otherwise; oil droplets were essentially absent, thus embryos in effluent water were exposed only to dissolved PAH **{17}**. The results of Pearson et al. (1985) concerning embryos and oil droplets have yet to be formally published in a peer-reviewed journal. While Brannon et al. describe the Pearson study as "of similar design," exposure techniques were not similar (addition of oil directly to water). We repeatedly looked for evidence of droplets associated with embryos in our experiments, both visually and chemically (by comparing PAH and aliphatic profiles in tissue to those in water and in source oil) and found no evidence of contamination by this route **{44}**. One of the specific reasons for this intense scrutiny was the original Pearson et al. (1985) report (which is not comparable with our experimental conditions or those of Brannon et al.)

44. "The problem of oil-droplet contamination in the water column is viewed differently by the ABL and UI researchers. We suggest that hydrocarbon concentrations diffusing across the egg membrane when in direct contact with oil are much higher than those of the dissolved fraction in the water around eggs not in contact with oil. Heintz et al. [6] assumed that even if the eggs were in direct contact with oil, the bioavailability and, thus, the toxicity was only in the form of the dissolved fraction measured in the water. That viewpoint was based on two oil loadings on gravel (74 and 717 ppm), for which bioaccumulation of TPAH was similar whether embryos were exposed just to dissolved PAH or were in direct contact with oil. Because the prestudy flushing of the gravel columns in the UI study was ineffective in preventing subsequent dispersal of oil, it cannot be assumed that the effluent in the Heintz et al. [6] study was free of minute oil droplets. Thus, the dissolved hydrocarbon levels measured in that study may not represent the actual TPAH concentrations experienced by the eggs." *page 969, column 2, paragraph 3, line 1*

Possible oil droplet movement in the Brannon et al. experiment does not imply that our experiments were confounded by whole oil movement, particularly because the more complete chemical data in our studies demonstrate negligible particulate oil in water and the absence of oil on embryos (Marty et al. 1997; Heintz et al. 1999, 2000; Carls et al. 1998, 1999, 2005). The dosing apparatus was specifically designed to produce an effluent stream containing dissolved PAHs **{8}**. After a 4 d flushing period, effluent water was clear and no slicks were visible, indicating an absence of whole oil ablated from the coated gravel (Heintz et al. 1999).

To assess contributions from oil droplets in our experiments, we exploited the large difference in the oil-water partitioning behavior of phytane (a branched aliphatic hydrocarbon) compared with PAH. An absence of phytane in water samples indicates an absence of bulk-phase oil in the dosing water, and the ratio of PAH:phytane in the dosing water indicated that PAH contributions from bulk phase oil were negligible. For example, the maximum proportion of TPAH associated with bulk-phase oil droplets in the experiments reported by Heintz et al. (1999) may be estimated as the product of the phytane concentration in the sampled medium and the ratio of TPAH to phytane in the oil when applied to the gravel, normalized to the TPAH concentration of the sampled medium. Initial phytane concentrations in the exposure water were <40 ng/L, and the ratio of TPAH to phytane in the oil was <8.4. The product of these is 335 ng/L, or 0.335 μg/L. This is less than 2% of the initial TPAH concentration of any dose producing significant mortality. Phytane was consistently below the method detection limit of 60 ng/g (dry weight basis) in the embryos, even at TPAH concentrations exceeding 70,000 ng/g. Hence, >99% of the TPAH accumulated by embryos during the experiment reported by Heintz et al. (1999) was from dissolved PAH. Similarly, particulate oil was not present in (or on) embryos in other oiled rock column experiments (Marty et al. 1997; Heintz et al. 2000; Carls et al. 1998, 1999, 2005).

Furthermore, the data of Brannon et al. also demonstrate that oil droplets were absent in water **{17}**, bioaccumulation was as expected from dissolved PAH, and uptake and response in embryos in contact with oiled rock was about the same as in embryos in water only **{23,24,27}**. Accumulated PAH demonstrated that the source was dissolved **{24}**. Thus, with droplets

absent, the most straightforward explanation for why embryos in contact with oil in both the Brannon and Heintz experiments responded at the about same rate as those in effluent water is that dissolved PAH are toxic and that they are toxic only when dissolved **{23}**. Therefore, Brannon et al. have no basis to reinterpret our results.

45. "Moreover, at all other oil concentrations tested by Heintz et al. [6], the eggs were not exposed just to the dissolved TPAH fraction but were actually in direct contact with oil-on-gravel mixtures. Therefore, rather than reporting dissolved TPAH concentrations, the more meaningful criteria is to use the total oil-on-gravel PAH concentrations with which the eggs were actually in contact." *page 969, column 2, paragraph 4, line 1*

The conclusion of Brannon et al. that "the more meaningful criteria is to use the total oil-on-gravel PAH concentrations with which eggs were actually in contact" is in error. Foremost is the assumption that oil droplets were present in effluent water; this is not supported by their data **{17}** and it is not supported by our experiments. Second, none of the embryos in effluent water were in contact with whole oil; measured aqueous TPAH concentrations, which included hypothetical oil droplets, ranged from 8 to 16 μg/L, constraining actual exposure to TPAH concentrations several orders of magnitude smaller than those on rock.

46. "Although egg mortality in PWS streams following the oil spill was reported by the Alaska Department of Fish and Game [3,4], the mortality subsequently was found to have been caused by physical shock of the eggs from sampling too early rather than from oil [9,39]." *page 970, column 1, paragraph 1, line 1*

Brannon et al. misrepresent this issue by only citing their own study and rejecting the statistically more powerful Alaska Department of Fish and Game study and ignore biochemical evidence of PAH exposure. Significantly increased cytochrome P4501A (CYP1A) induction in alevins from 1989 through 1991 (the last year of study) confirmed exposure to PAH (Wiedmer et al. 1996), a detail omitted from the Brannon et al. argument. Oil was not eliminated as the cause of embryo mortality when run timing was included in the analyses (Craig et al. 2002), countering the egg shock hypothesis of Brannon et al. **{47-48}.** A study where embryos from oiled and reference streams were incubated in a common environment confirmed increased mortality in oiled streams absent possible confounding natural factors (Bue et al. 1998).

47. "Because oil concentrations were too low in PWS streams to be toxic [8,14], it was proposed that the hypothetical source was the highly weathered oil deposits on the shorelines adjacent to pink salmon streams away from the flushing freshwater flows [14], a hypothesis that was felt to be supported in the laboratory by the allegedly highly toxic VWO at 1 ppb [6]. The hypothetical transport mechanism was interstitial flow carrying the reputedly highly toxic dissolved hydrocarbons into the incubation substrate [15] and was referred to as the interstitial toxic water hypothesis [14]." *page 970, column 1, paragraph 1, line 7*

Brannon et al. conclude that TPAH concentrations in PWS streams were too low to damage pink salmon embryos without recognizing that their data (and ours) supports possible transfer of toxic PAH from oiled rock (in surrounding banks) to embryos via water. When appropriately expressed as TPAH concentrations in water **{17,23}**, damaging exposures in the Brannon study were a few parts-per-billion, supporting the possibility of embryo damage in PWS at low exposure concentrations.

48. "Several conditions are apparent in the ABL and UI laboratory results, however, that would be inconsistent with the interstitial toxic water hypothesis being the process responsible for any egg mortality in the field. First, embryo death observed in the laboratory accumulated over the length of the study period, whereas the Alaska Department of Fish and Game reported that egg mortality in the stream occurred early in incubation, often within the first few days postspawning [9]. Therefore, the pattern of mortality in the field did not follow the laboratory mortality time frame but was associated with a much more acute event." *page 970, column 1, paragraph 2, line 1*

Brannon et al. argue that because oil related mortality increases with time, PWS surveys were completed too early to detect embryo mortality. **A)** Embryo mortality not only increases with time, it increases with dose and as dose increases, the probability of detecting early death increases. The source of observed contamination was oil stranded in the surrounding intertidal gravel, where average TPAH concentrations (3.1×10^5 ng/g; Murphy et al. 1999) were 25 times greater than those Brannon et al. (2006) observed as lethal (1.2×10^4 ng/g). **B)** Brannon et al. misleadingly claim that eggs were sampled "early in incubation, often within the first few days of postspawning." The estimated mean run time (75% escapement) was Sept. 3 and the mean sample date was Oct. 5, so eggs incubated about 32 d (range 22 to 42 d) before sampling (data from Craig et al. 2002).

49. "Second, the embryos reportedly exposed to a lethal aqueous TPAH dose of 18 ppb for AWEVC and 1 ppb for VWO, as reported in the ABL study [6], were, in fact, incubated in direct contact with 280,000 and 2,860,000 ppb of oil on gravel, respectively, and exposed to gravel TPAH concentrations of 3,800 and 4,600 ppb, respectively." *page 970, column 1, paragraph 3, line 1*

Brannon et al. are incorrectly confusing nominal oiling and TPAH concentrations on rock with measured dose (see **{45}** for a summary of this error).

50. "In contrast, following the spill in 1989, gravel TPAH concentrations in PWS streams did not exceed a mean of 267 ppb [8], or a level 14- to 17-fold less than the lethal doses reported at ABL." *page 970, column 1, paragraph 3, line 6*

Brannon et al. argue that PWS streams were not sufficiently contaminated to cause embryo mortality (see also **{47,48}**). This claim assumes that only the relatively low TPAH concentrations in stream gravels contributed to toxicity without mentioning that flowing stream water precluded direct deposition of whole oil in stream channels (Brannon et al. 1996). Instead, surrounding intertidal gravel was oiled at concentrations thousands of times higher than the estimate provided by Brannon et al. Total PAH concentrations approached and exceeded 10^5 ppb in the mid- and upper intertidal sediment of western PWS (Boehm et al. 1995) and oiled stream deltas (Murphy et al. 1999). Furthermore, Brannon's coauthor Neff (et al. 1995) described island shorelines in western PWS as among the most heavily oiled, consistent with Alaska Department of Fish and Game data (e.g., Bue et al. 1996) and shoreline assessment data (Gundlach et al. 1990). The oiling level cited by Brannon et al. implicitly ignores highly contaminated substrate surrounding the streams.

51. "Third, the VWO, which was the most toxic oil in the ABL study, did not resemble the NWEVC removed from the PWS shoreline and also was unlike the AWEVC used in either the ABL or the present study. Application of VWO data cannot apply to the conditions in oiled PWS streams." *page 970, column 1, paragraph 4, line 1*

Brannon et al. incorrectly claim VWO is not representative of weathered *Exxon Valdez* crude oil **{38}**.

52. "Fourth, the naturally weathered oil *[mousse]* removed from the PWS shoreline was nontoxic at laboratory-tested levels as high as 2,250 ppm of oil on gravel. The solubility of the toxic, high-molecular-weight PAH was very limited in the UI study, which implies a similar lack of bioavailability in the field." *page 970, column 1, paragraph 5, line 1*

These claims by Brannon et al. are refuted by their own data; PAH from mousse entered water at about the same rate as from oil, was biologically available, and was toxic **{28}.** The use of nominal dose, truncated monitoring time, exclusion of sublethal responses, and low statistical power combine to obscure detection of toxic effects in mousse **{29}**.

53. "Fifth, naturally weathered oil *[mousse]* from the shore of PWS is not lethal to pink salmon embryos until tissue TPAH concentrations are in excess of 7,800 ppb, as reported here. Tissue PAH concentrations of embryos sampled from PWS oiled streams in 1990 and 1991 were 63 and 94 ppb, respectively [8]. These levels are at least 80-fold lower than what is associated with toxic concentrations, indicating that interstitial toxic water was not experienced by eggs that were incubating at the height of oil presence on PWS beaches." *page 970, column 1, paragraph 6, line 1*

Brannon et al. conclude that "naturally weathered oil from the shore of PWS is not lethal to pink salmon embryos until the tissue TPAH concentrations are in excess of 7,800 ppb." This is simply an alternative way of saying the embryos exposed to 8 ppb aqueous TPAH (with an uptake factor of about 10^3 **{23}**) did not die immediately. Exclusion of sublethal responses, truncated monitoring time, and low statistical power combine to obscure detection of toxic effects in mousse **{29}.** The conclusion of Brannon et al. also discounts observations of significant pink salmon egg mortality in PWS (Bue et al. 1996, 1998) and does not recognize that concentration comparisons are likely confounded by life stage (laboratory embryos versus wild alevins) and that PAH elimination processes in alevins are well developed (van der Oost et al. 2003).

54. "Sixth, using the ABL experimental design [5,6], the UI study demonstrated that oil droplets were not eliminated from the water that eggs were exposed to downstream of the oil-on-gravel substrate in the incubator columns. Therefore, attributing embryo mortality to the dissolved TPAH concentration in an effluent with oil droplets present would result in misinterpretation of the dissolved toxic dose." *page 970, column 1, paragraph 7, line 1*

The data of Brannon et al. demonstrate the opposite; droplets were not present and did not contribute to toxicity **{17,23}**.

55. "In addition to the laboratory results, the biological and physical conditions in PWS pink salmon streams also eliminate the likelihood of interstitial toxic water. Biologically, spawning occurs in gravel flushed with freshwater from the surface stream during most stages of the tidal cycle [1]. Physically, tidal dilution from the tons of marine water flushing oil deposits twice a day quickly exhaust any toxic potential of the deposits." *page 970, column 2, paragraph 2, line 1*

Studies that Brannon et al. ignore refute their discussion and the CYP1A biomarker results demonstrated that embryos were exposed to PAH for at least 2 y and up to 10 y in one particularly oiled system (Wiedmer et al. 1996; Bue et al. 1996, 1998; Carls et al. 2004). Carls et al. (2005) demonstrated adverse biological responses are predicted by increased CYP1A induction in pink salmon embryos exposed to PAH.

56. "As shown by these experiments, as well as by those of Heintz et al. [6], effluent hydrocarbon toxicity levels drop well below those sufficient to cause harm to salmon embryos over the time frame of a few weeks, regardless of oiling level or extent of oil weathering. These data do not support claims of toxic exposure by salmon to hydrocarbons carried by tidal flushing years after the spill." *page 970, column 2, paragraph 3, line 1*

Brannon et al. did not test for persistent toxicity. The toxic implications of weathering **{42}** and studies that demonstrate persistent toxicity are discounted or ignored by them **{37,46}**.

57. "From the biological perspective of natural spawning in the intertidal reaches of the streams, the fertilization and water-hardening of the eggs, as well as the initial blastula formation, occur primarily in flowing freshwater, and exposure to aqueous PAHs is unlikely before tidal flooding. Therefore, to simulate accurately the field conditions during deposition, fertilization, and filling of the perivitelline space in the water-hardening process, the eggs should not be exposed to test concentrations of oil and saltwater until after initial water-hardening. Exposing eggs to high PAH concentrations during this early developmental phase would be atypical of field conditions and may exaggerate the effects of chronic oil exposure during the later stages of embryonic development." *page 970, column 2, paragraph 4, line 1*

In most of our experiments (Heintz et al. 1999, 2000; Carls et al. 1999, 2005), embryos were fertilized in clean freshwater before exposure began. The only exception was Marty et al. (1997); results of the Marty experiment were consistent with the other studies.

58. "Another laboratory scenario that is atypical of the field is the continuous exposure of eggs to PAH-contaminated effluent. Under field conditions, cycling between brackish water and freshwater occurs in the incubation environment. Dissolved or dispersed hydrocarbons in pore water, hypothetically seeping into salmon streams, would be associated primarily with the seawater phase. The oil-free freshwater phase, flushing the redds during receding tides and diluting saline pore water in the incubation environment, would create alternating cycles in the presence and absence of hydrocarbons and the leaching of hydrocarbons that may have adsorbed to redd substrates during saline water exposure. Therefore, toxic thresholds determined by chronic exposure to laboratory TPAH concentrations should not be applied to conditions of exposure in the field when the temporal variation and high diluting effects of tidal and freshwater flows under natural conditions were not represented in the laboratory." *page 970, column 2, paragraph 5, line 1*

Brannon et al. are speculating. The relationship between dissolved PAH in hyporheic water and salinity is not as simple as they imagine. Dye concentrations used to trace this process varied at low salinities, sometimes increasing, sometimes constant, and sometimes fluctuating (Carls et al. 2003). Secondly, most of the PAHs accumulated by embryos are retained between exposure pulses because PAH readily dissolve in lipid but are only sparingly soluble in water; CYP1A induction in embryos in PWS streams demonstrated significant PAH bioaccumulation (Wiedmer et al. 1996).

59. "The laboratory investigations undertaken at UI on weathered EVC, modeled after the ABL experimental design, helped to resolve inconsistencies between laboratory results on weathered oil toxicity and field investigations on pink salmon eggs and alevins conducted in PWS following the 1989 oil spill. Using AWEVC *[oil]*, we found that mortality of pink salmon eggs was not apparent until TPAH concentrations of oil on gravel exceeded 1,500 ppb or tissue TPAH levels exceeded 7,100 ppb. The incidence of blue sac disease was higher among alevins exposed to the higher oil concentration, which at least implies that the physical presence of oil may reduce respiration efficiency, apart from any toxicity. Analysis of the aqueous phase of the oil suggests that dispersed oil was not eliminated from the water assumed to carry only dissolved TPAH; thus, exposure to the column effluent water was not representative of only dissolved hydrocarbons. Consequently, determinations of aqueous TPAH toxicity made using this experimental design should be reconsidered.

The critical observation in the UI study was that chronic exposure of pink salmon embryos to NWEVC *[mousse]* at concentrations as high as 2,250 ppm on gravel resulted in mortality no greater than that of laboratory controls. Nonlethal tissue burdens were as high as 7,800 ppb, and at least 80-fold higher than what was observed in embryos in 1990 and 1991 from PWS streams in the spill path." *page 970, column 2, paragraph 6, line 1*

Brannon et al. conclude by summarizing their errors:

a. "Effluent water was not representative of only dissolved hydrocarbons." Their experiment demonstrates that quantities of oil droplets were negligible; all chemical evidence indicates a predominance of dissolved PAH **{17}**.

b. "Mortality of pink salmon eggs was not apparent until TPAH concentrations of oil exceeded 1500 ppb." Brannon et al. are apparently referring to the nominal 1500 ppm whole oil on gravel in this statement. Nominal oiling does not accurately describe embryo exposure **{9,23}**. Likewise, TPAH concentrations on rock do not accurately describe embryo exposure **{45}**. The corresponding, measured aqueous exposure was 7.8 μg/L.

c. "Mortality … was not apparent until … tissue TPAH levels exceeded 7100 ppb." True, except that Brannon et al. fail to mention that the bioaccumulation of PAH in tissue is roughly 10^3 times aqueous exposure concentrations, consistent with aqueous uptake from the corresponding exposure of 7.8 μg/L TPAH **{23}**.

d. "The incidence of blue sac disease was higher among alevins exposed to the higher oil concentration, which at least implies that the physical presence of oil may reduce respiration efficiency, apart from any toxicity." Ascites is a common embryo response to aromatic hydrocarbons independent of oxygen demand **{15,32}**. Demonstrated by Brannon et al. and consistent with our experiments, physical oil contact was absent and PAH accumulated in tissue, thus ascites was caused by dissolved PAH **{17,23,32}**.

e. "The critical observation in the UI study was that chronic exposure of pink salmon embryos to NWEVC at concentrations as high as 2,250 ppm on gravel resulted in mortality no greater than that of laboratory controls." Brannon et al. omit two important facts, the sublethal toxicity of mousse (which was significant) and that the maximum mousse exposure corresponded to a sublethal oil exposure **{28}**. Detection of toxic effects are obscured by an insensitive toxicity metric, lower statistical power and shorter observation times (than in our studies), and reliance on nominal dose **{29}**.

h. "Nonlethal tissue burdens were as high as 7,800 ppb, and at least 80-fold higher than what was observed in embryos in 1990 and 1991 from PWS streams in the spill path." Brannon et al. fail to mention that **1)** significant ascites was caused by the cited tissue burden **{28}**, and **2)** they measured TPAH in alevins with well developed PAH elimination enzymes, not embryos **{53}**.

Summary

The results of the Brannon et al. experiment are in strong agreement with a series of studies from our laboratory (embryo toxicity at <10 μg/L aqueous TPAH concentrations; Marty et al. 1997; Heintz et al. 1999, 2000; Carls et al. 1999, 2005).

a. Part-per-billion aqueous TPAH concentrations are toxic to pink salmon embryos (≥8 μg/L in their study) **{28}**.

b. Oil and mousse were highly similar chemically and were both toxic (≥8 μg/L) **{17,28}**.

c. Embryo responses to dissolved PAH included death and ascites **{15,18,28}**.

d. Dissolved, toxic PAH are transferred from oil on rock to embryos via water **{18,23}**.

e. PAH are bioaccumulated from water **{23,24}**.

f. PAH must be dissolved to be toxic **{23}**.

g. Direct embryo contact with whole oil is of little consequence **{23}**.
h. The amount of oil droplets produced by oiled rock columns is negligible **{17}**.
i. Predicted oil droplet toxicity is negligible **{31}**.

Although the results between the Brannon et al. study and ours are similar, conclusions of the two studies are not similar. By relying on nominal dose, Brannon et al. obscure the similarities between experiments. Brannon's failure to accurately replicate our dosing procedure (thereby making their preparation more prone to droplet formation) and other aspects of our experiment is also a problem. Reliance of Brannon et al. on droplets of oil as the crucial exposure mechanism is flawed, as demonstrated by the hydrocarbons measured in tissues and exposure water in their experiment and ours. Our studies were substantially more powerful, having been conducted over a series of years (replication across brood years), had more replicates within specific experiments, with more embryos exposed per dose, and longer observation periods, including post-exposure observation for 0.5 to 1.3 years in some studies. Furthermore, using gas chromatography we repeatedly and rigorously demonstrated that oil droplets were not an important toxic factor in these experiments.

We stand by our results, which are corroborated by others (Rhodes et al. 2005; Farwell et al. 2006) and disagree with the conclusions of Brannon et al. Pink salmon embryos exposed to chronic doses of PAH at low part-per-billion concentrations exhibit a variety of symptoms ranging from ascites to poor growth to increased mortality. The sensitivity demonstrated in our experiments to chronic low level PAH exposure is consistent with the observations of elevated embryo mortalities measured by Bue et al. (1996, 1998) in oiled streams over the period of 1989 to 1993, is consistent with the field evidence of exposure as measured by elevated CYP1A in alevins (Wiedmer et al. 1996), and is consistent with the observations of oil exposure via the contaminated stream banks (Murphy et al. 1999) and the interstitial exposure mechanism demonstrated by Carls et al. (2003). We conclude that the *Exxon Valdez* oil spill had impact on pink salmon embryos in oiled streams for several years after the oil spill.

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