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Contaminants as viral cofactors: assessing indirect population effects

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Abstract

Current toxicological methods often miss contaminant effects, particularly when immune suppression is involved. The failure to recognize and evaluate indirect and sublethal effects severely limits the applicability of those methods at the population level. In this study, the Vitality model is used to evaluate the population level effects of a contaminant exerting only indirect, sublethal effects at the individual level. Juvenile rainbow trout (*Oncorhynchus mykiss*) were injected with 2.5 or 10.0 mg/kg doses of the model CYP1A inducer, β-naphthoflavone (BNF) as a pre-stressor, then exposed to a challenge dose of 10^2 or 10^4 pfu/fish of infectious hematopoietic necrosis virus (IHNV), an important viral pathogen of salmonids in North America. At the end of the 28-d challenge, the mortality data were processed according to the Vitality model which indicated that the correlation between the average rate of vitality loss and the pre-stressor dose was strong: $R^2 = 0.9944$. Average time to death and cumulative mortality were dependent on the BNF dose, while no significant difference between the two viral dosages was shown, implying that the history of the organism at the time of stressor exposure is an important factor in determining the virulence or toxicity of the stressor. The conceptual framework of this model permits a smoother transfer of results to a more complex stratum, namely the population level, which allows the immunosuppressive results generated by doses of a CYP1A inducer that more accurately represent the effects elicited by environmentally-relevant contaminant concentrations to be extrapolated to target populations. The indirect effects of other environmental contaminants with similar biotransformation pathways, such as polycyclic aromatic hydrocarbons (PAH), could be assessed and quantified with this model and the results applied to a more complex biological hierarchy.

Keywords: Toxicity models; Population dynamics; Immunotoxicity; IHNV; Fish; Multiple stressors

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1. Introduction

No one questions the presence of low-level contamination in our aquatic ecosystems, only its significance. Biologists, biochemists, ecologists, toxicologists, policy makers and others wrestle with the potential effects that these contaminants could produce in resident biota and human consumers. Scales or models to help categorize the damage caused by chemical stressors can be useful tools (Moudgal et al., 2003; Bailer et al., 2000; Swartz et al., 1995). Toxicity models that are based on specific endpoints generated by laboratory assays are supposed to predict the risk of exposing wildlife to treated, diluted effluent. Assay data are used to define concentrations below which no effects are expected (i.e. the "no observed effect concentration" (NOEC)), and to predict "effect concentration" (EC_x) (Crane et al., 2000). A potential problem with the NOEC is that standard toxicological assays generally do not treat or observe indirect effects. Consequently, a contaminant that, by itself, would have no measurable effect on an organism in a standard assay could, in combination with another have significant biological effects which could easily be overlooked (Grist et al., 2003; Kooijman and Bedaux, 1996). The EC_x establishes that concentration of a contaminant producing an obvious endpoint, usually mortality, to a stipulated percentage, x, of the test cohort. An example is the median lethal concentration, LC_{50}. Such metrics assess toxicity in the absence of other stressors, and do not incorporate past experiences of the target organisms and, therefore, depart from the reality that aquatic organisms face in polluted environments. A comprehensive review of various strategies and other metrics as well as some of the limitations with these two examples can be found in Kooijman and Bedaux (1996).

Sumpter (1998) describes the lack of information surrounding population-level effects in discussing the environmental impact of xenoestrogens. This lack of information applies to other contaminants and their indirect effects as well. A recent model (Anderson, 2000) provides a vehicle by which the indirect, sublethal (at the individual level) effects of a contaminant can be assessed.

The goal of this research was to apply the Vitality model (Anderson, 2000) to a two-stressor system and assess its utility for evaluating indirect biological effects of doses that more accurately represent the effects elicited by environmentally-relevant contaminant concentrations. To meet these goals required an experimental design using a contaminant and challenge stressor of appropriate doses. β-Naphthoflavone (BNF), an aryl hydrocarbon receptor (AhR) agonist that is widely used, was selected as the contaminant. It is a model compound with relatively low toxicity which is widely used for studying other classes of environmental contaminants, such as polycyclic aromatic hydrocarbons (PAH). Infectious hematopoietic necrosis virus (IHNV) was used as the lethal challenge stressor. In this experiment, we exposed rainbow trout fry to a controlled dose of BNF delivered via a single intraperitoneal (i.p.) injection. Two days later, these trout were given a challenge dose of IHNV, also delivered via i.p. injection. By initiating the induction of CYP1A by injection of BNF and then exposing the trout to an endemic pathogen by the same method, noise can be minimized for a clearer representation of the interaction between contaminant and pathogen. We can determine if this model can be used to quantify this relationship and the cumulative effect(s) on the targets and the populations they represent.

2. Materials and methods

2.1. Vitality model

In this model, every individual begins its life with an amount of "vitality." Vitality, in the parlance of structural equation modeling (Hoyle, 1995), is a latent variable related to the intrinsic ability of an organism to self-organize adaptively, and so avoid death. This vitality fluctuates stochastically over the course of the organism's life, influenced by experiences, both positive and negative. Eventually, at some point in time, it inevitably drops to zero, signaling death. Quantitatively, probability of surviving to age t is given by

$$S(t) = \left(\Phi\left(\frac{1}{s\sqrt{t}}(1-rt)\right) - \exp\left(\frac{2r}{s^2}\right)\Phi\left(-\frac{1}{s\sqrt{t}}(1+rt)\right)\right)e^{-kt} \quad (1)$$

where Φ is the cumulative normal distribution; r, the mean rate of vitality loss; s, the stochastic drift intensity of the vitality loss rate; and k, the rate of accidental

mortality. Accidental refers to death whose cause is independent of the history of the organism. Of particular interest here is that, when $k \ll r$ (accidental death plays a minor role), life expectancy, or mean time to death, is closely approximated by

$$t^* = \frac{1}{r} \tag{2}$$

Studies also suggest that stressors could affect r in a linear or very nearly linear manner (Hamel, 2001; Anderson, 2000). Thus, where r_0 is the value of r in the absence of a stressor, stressor intensity is C, and α characterizes the impact per unit stressor

$$r = r_0 + \alpha C. \tag{3}$$

Thus,

$$\frac{1}{t^*} = r_0 + \alpha C. \tag{4}$$

Challenge experiments based on this model present a new and powerful way to characterize the effects of stressors on organisms' survival in their natural environment. The approach involves exposing organisms to known, sublethal concentrations of a stressor, and applying a lethal secondary challenge, then tracking time to death. Write r as a function of a stressor, S_1 and the lethal challenge S_c, Then, using the Taylor expansion

$$\begin{aligned} r = r(S_1, S_c) &= r(0,0) \\ &+ \left[\left(\frac{\partial r}{\partial S_c}\right) S_c + \left(\frac{\partial^2 r}{\partial S_c^2}\right) S_c^2 + \cdots\right] \\ &+ \left[\left(\frac{\partial r}{\partial_1}\right) S_1 + \left(\frac{\partial^2 r}{\partial S_1^2}\right) S_1^2 + \cdots\right] \\ &+ \left[\left(\frac{\partial^2 r}{\partial S_c \partial S_1}\right) S_c S_1 + \cdots\right], \end{aligned} \tag{5}$$

where all derivatives are evaluated at $S_c = 0$, $S_c = 0$. But if S_1 is a weak stressor, and if the impact of S_1 on r is unaffected by S_c, or vice versa (so that the last term, above, is close to zero), then the above, in accord with Anderson's finding of a linear relation (Eq. (2)), can be closely approximated by

$$r \approx \left[r(0,0) + \left(\frac{\partial r}{\partial S_c}\right) S_c\right] + \left(\frac{\partial r}{\partial S_1}\right) S_1 \equiv r_0 + \alpha S_1 \tag{6}$$

where r_0 now expresses the value of r in the presence of the challenge but not the stressor. That is to say, if the mechanism by which the stressor acts on mortality is not affected by the lethal challenge (the converse need not be true), we can assess the impact of the stressor:

$$\alpha S_1 = \frac{1}{t^*_{S_1}} - \frac{1}{t^*_0}. \tag{7}$$

This laboratory-derived result can now be applied to calculate the (direct) effect of S_1 on a natural population: Note, where δ is the average population-level mortality rate, that t^* is defined by

$$e^{-\delta t^*} = 0.5 \tag{8}$$

Thus, $\delta = \ln 2/t^*$, and the increase in mortality rate due to exposure to the stressor is

$$\Delta\delta = \frac{\ln 2}{t^*_{S1}} - \frac{\ln 2}{t^*_0} = \alpha(\ln 2) S_1 \tag{9}$$

Return to Eq. (4), and now consider n stressors, S_1, S_2, . . ., S_n, in the presence of a lethal challenge. If we presume that stressor impacts are not so strong as to require third and higher order terms, we obtain

$$r = r_0 + \sum_{i=1}^{n} \frac{\partial r}{\partial S_i} S_i + \sum_{i=1}^{n} \sum_{j=1}^{n} \left(\frac{\partial^2 r}{\partial S_i \partial S_j}\right)$$

$$S_i S_j \equiv r_0 + \sum_{i=1}^{n} \alpha_i S_i + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} S_i S_j. \tag{10}$$

Challenge experiments involving a combination of contaminants can now be used to calculate r ($= 1/t^*$), and the results regressed on $\{S_i\}$ and $\{S_i S_j\}$ to obtain the direct effects of each, $\{\alpha\}$, and their interactions $\{\beta\}$.

Finding methods to accurately evaluate a population's response to multiple stressors merits attention. The recent advent of risk assessment in aquatic toxicology has shifted the focus from individual response to predictions and measures of population effects, but with varying degrees of success. This success is further compromised by the complexity resulting from stressor interaction. Substantial under-estimation of stressor effects has resulted when assuming a summation of effects, and these errors are positively related to stressor intensity (Power, 1997). An organism's capacity to adequately respond to an immunological challenge in addition to other stressors is yet another concern. Epidemiological and experimental studies have linked immunosuppression and reduced disease resistance in

fish with exposure to environmental contaminants, and the population level effects of this interaction are being addressed (Arkoosh et al., 1998; Spitzbergen et al., 1988).

3. Stressors

3.1. Infectious hematopoietic necrosis virus (IHNV)

This acute rhabdovirus is the cause of numerous high mortality epizootics in salmonids of the northern Pacific coast where it is endemic and is considered to be one of the most important viral pathogens of salmonids in North America (Bootland and Leong, 1999; LaPatra, 1998; Wolf, 1988). Viral strains exhibit varying degrees of virulence and susceptibility to IHNV differs between the numerous salmonid species (Troyer et al., 2000; Bootland and Leong, 1999). The IHNV isolate used in this research was 220-90, an M genogroup strain from Hagerman Valley, ID, which has been shown to be highly virulent in rainbow trout (LaPatra et al., 1994). This pathogen is an effective challenge model since it reproducibly causes 70–95% mortality to exposed juvenile rainbow trout (*Oncorhynchus mykiss*).

The IHNV challenge strain 220-90 was propagated in the epithelioma papulosum cyprini (EPC) cell line (Fijan et al., 1983) and titered by plaque assay as previously described (LaPatra et al., 1994). Inocula for 50 μl/fish challenge doses were prepared with serial dilutions of virus stock (titer 2×10^8 pfu/ml) for challenge doses of 10^2 pfu/fish and 10^4 pfu/fish, determined in pilot studies to produce similar mortalities and time to death results.

3.2. β-Naphthoflavone and dosage preparation

β-Naphthoflavone (BNF; 5,6-benzoflavone, CAS 6051-87-2) has been extensively used in toxicological investigations at a wide range of administered doses, from 5.0 mg/kg to 200 mg/kg (Meyer et al., 2002; Lemaire et al., 1996). It is a potent inducer of Cytochrome P450 CYP1A, a heme-containing enzyme that catalyzes Phase I oxidative and reductive metabolic reactions of a broad spectrum of substrates, many of which are anthropogenic contaminants such as petrochemicals (Buhler and Wang-Buhler, 1998). BNF shows a similar potency and specificity of induction to the carcinogenic PAHs such as benzo[*a*]pyrene (Scholtz and Segner, 1999), yet it is a preferred inducer since it is widely regarded as being non-carcinogenic (McKillop and Case, 1991).

Chemicals were purchased from Acros Organics, Pittsburgh, Pennsylvania. Doses of BNF were prepared at ambient temperature in corn oil as a carrier, and loaded in 1 ml tuberculin syringes (Becton Dickinson, Franklin Lakes, NJ). The doses were: 10 mg/kg, 2.5 mg/kg, and 0 mg/kg (oil only). Pilot studies confirmed literature that these BNF doses are sublethal and can be transferred to targets of this size via this carrier. β-Naphthoflavone doses for three fish weight classes, ≤3.5 g, 3.5–4.5 g, ≥4.5 g, were prepared for each dose in order to maintain uniform injection volume of 50 μl/fish.

3.3. Animal treatment

Fish were obtained from Clear Springs Foods (Buhl, ID). Healthy, disease-free six-week-old rainbow trout fry were delivered to Seattle, WA and held in aerated tanks of flowing water at 15 °C. The mean weight of the fry upon receipt was 0.8 g. The fry were fed daily with commercial feed (BioDiet, Warrenton, OR; protein, 48.0%, fat, 15.0%, carbohydrate, 7.0%). At injection, the mean weight of individual fish in three groups of 10 randomly selected fish was 4.5 g, ±1.29 g.

Thirteen groups of 44 (22/duplicate container) rainbow trout fry were size-selected for uniformity. From each group, individuals were removed and anesthetized in a solution of ∼50 mg/l tricaine methane sulfonate (MS222; Argent Chemical Laboratories, Redmond, WA), weighed, and injected intraperitoneally with a 25G5/8 needle (Becton Dickinson) to administer BNF/oil according to the test groups shown in Table 1. Appropriate series of groups were injected with phospho-buffered saline (PBS), the carrier for IHNV, as a procedural control, and additional control groups were left unhandled. Following injection and recovery in freshwater, groups of 22 fish were transferred into 4L challenge buckets of flowing water at 15 °C.

Forty-six hours after BNF injection individuals were anesthetized and injected with the IHNV challenge doses and returned to the proper challenge buckets.

Table 1
Rainbow trout test groups with doses of BNF and challenge doses of IHNV shown

	Challenge stressor: IHNV doses following BNF or PBS			
Prestressor: BNF injected before IHNV	0 BNF (oil only), 0 IHNV	0 BNF (oil only), 10^2 pfu/fish IHNV	0 BNF (oil only), 10^4 pfu/fish IHNV	Unhandled
	2.5 mg/kg BNF, 0 IHNV	2.5 mg/kg BNF, 10^2 pfu/fish IHNV	2.5 mg/kg BNF, 10^4 pfu/fish IHNV	
	10.0 mg/kg BNF, 0 IHNV	10.0 mg/kg BNF, 10^2 pfu/fish IHNV	10.0 mg/kg BNF, 10^4 pfu/fish IHNV	
PBS control	PBS, 0 IHNV	PBS, 10^2 pfu/fish IHNV	PBS, 10^4 pfu/fish IHNV	

Each test group was conducted as duplicate buckets of 22 fish each (44 fish per test group).

Fish were fed 1% per body wt. commercial feed (BioDiet) daily. Amounts dispensed were based on an average fish wt of 4 g, and number of fish per container. This quantity was modified to maintain a consistent feed rate as fish were lost due to mortality. The test organisms were monitored and mortalities removed and recorded every 4 h for 28 d. After 28 d, the experiment was terminated, and any surviving fish were euthanized in an excess of tricaine methane sulfonate (MS222).

3.4. Data analysis

Data were entered in Microsoft® Excel 2000. Single factor analyses of variance (ANOVA) were conducted between the two viral dosages (i.e., 10^2 and 10^4 pfu/fish) for each BNF dosage group to determine if there were significant differences between groups. The data were exported to VitalityModelFitting.ssc (Salinger et al., 2003) where they were processed. This program uses S-Plus 2000©, and provides the formula for computing the variance of the parameter estimates, i.e., the Hessian inverse of the negative log-likelihood, evaluated at the parameter estimates. In the event of close correlation between *r*, *s*, and *k*, a standard error approximation method estimates the standard error if the above method fails due to close correlation. Stopping tolerances were adjusted to 0.000001; standard error and Pearson C type test for goodness of fit were calculated in model fitting.

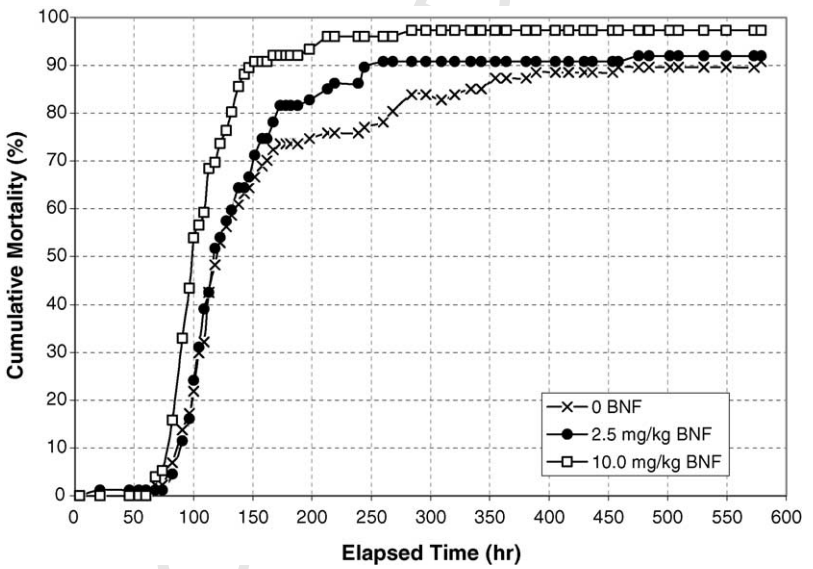

Fig. 1. Cumulative mortalities for three different dosages of β-naphthoflavone for the entire viral challenge (28 d).

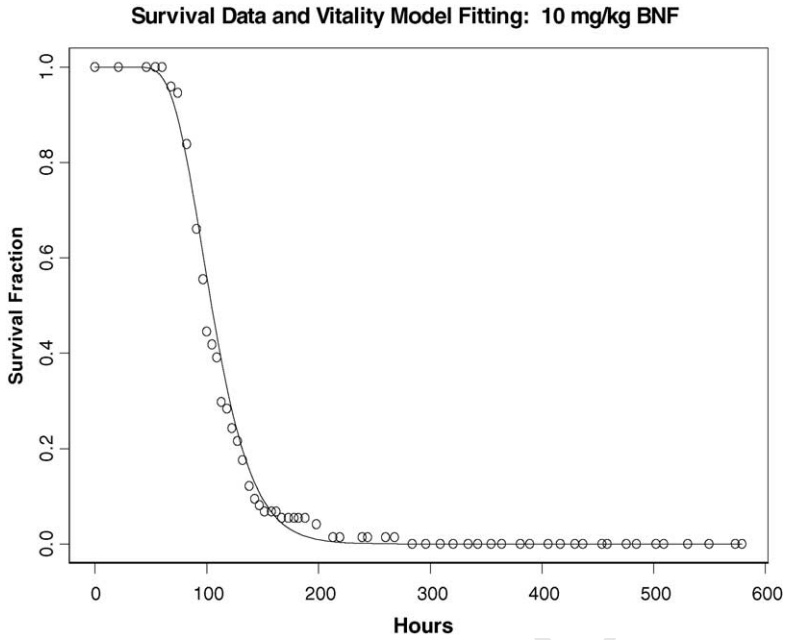

Fig. 2. Survival data after fitting in VitalityModelFitting.ssc for 10 mg/kg β-naphthoflavone and IHNV.

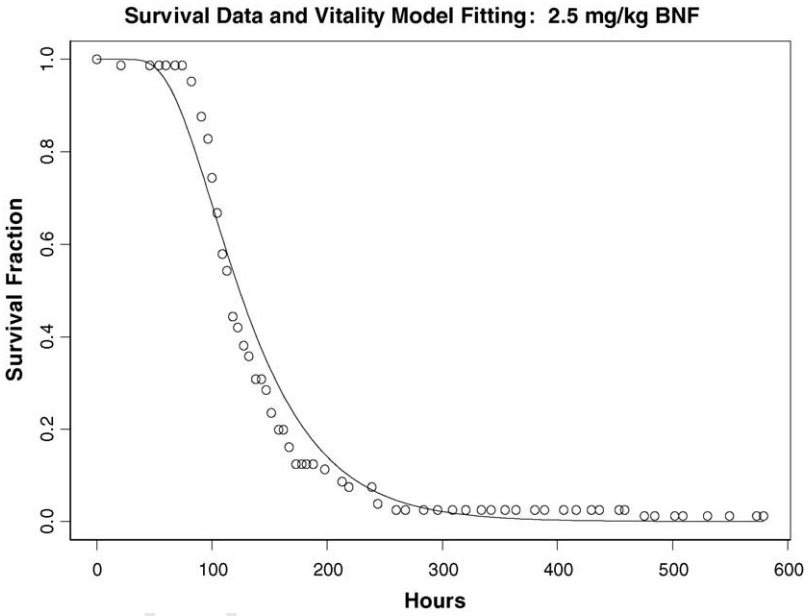

Fig. 3. Survival data after fitting in VitalityModelFitting.ssc for 2.5 mg/kg β-naphthoflavone and IHNV.

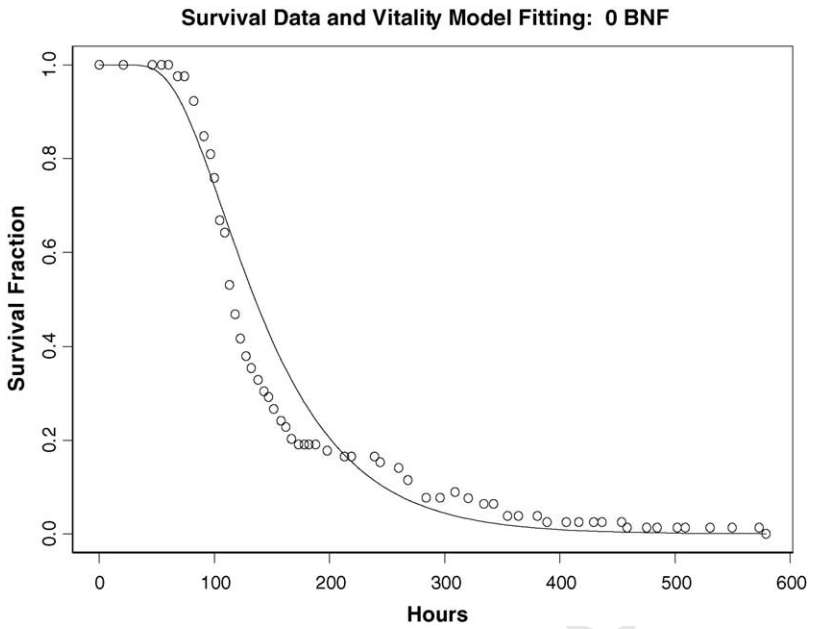

Fig. 4. Survival data after fitting in VitalityModelFitting.ssc for 0 mg/kg β-naphthoflavone (oil) and IHNV.

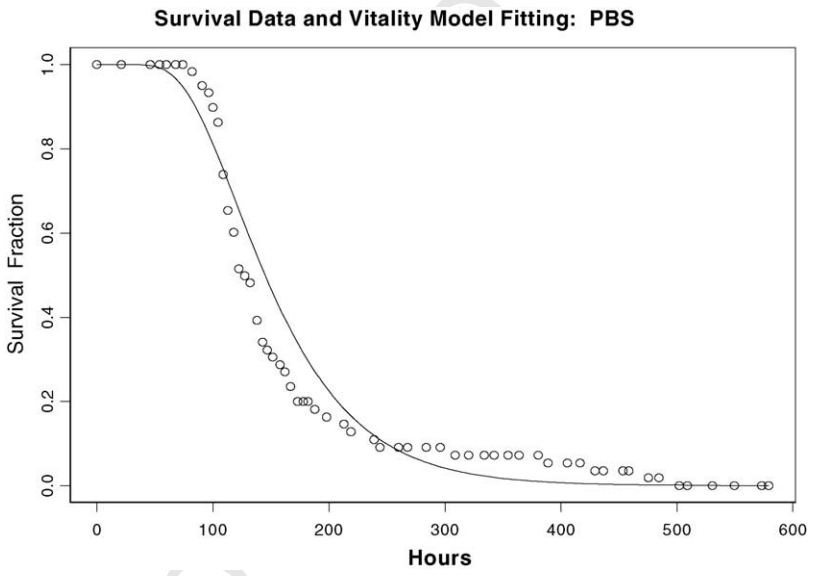

Fig. 5. Survival data after fitting in VitalityModelFitting.ssc for PBS (carrier for IHNV) and IHNV.

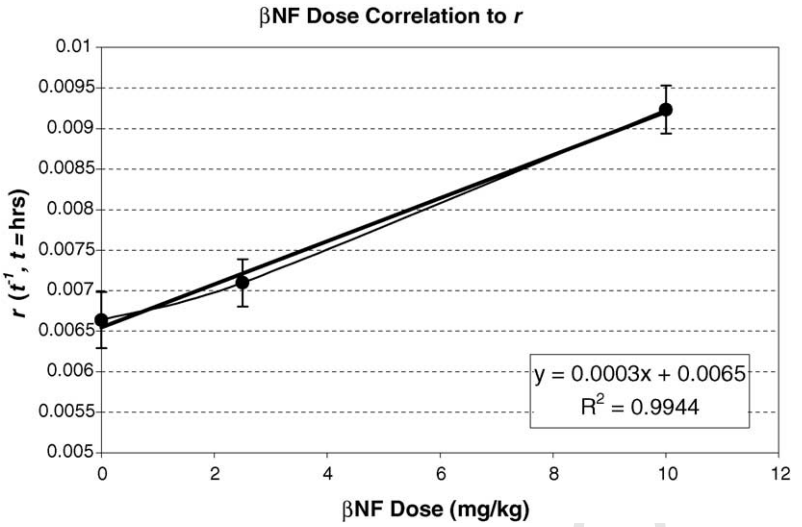

Fig. 6. Correlation of β-naphthoflavone dosage to *r* and trendline (linear regression). Standard error bars represent the standard error generated by VitalityModelFitting.ssc for the total test cohort for that BNF dosage ($n = 88$).

4. Results

No mortalities occurred in control groups with no viral exposure. Cumulative survival (%) for both IHNV dosage groups at the same BNF dosage showed no significant differences ($p < 0.05$). This was not unexpected and confirmed the results of viral dosage pilot studies. Due to the acute nature of IHNV infection, these challenge doses were sufficient to saturate the innate immune response of these younger, more susceptible target organisms. Consequently, results from both IHNV dosage groups were combined

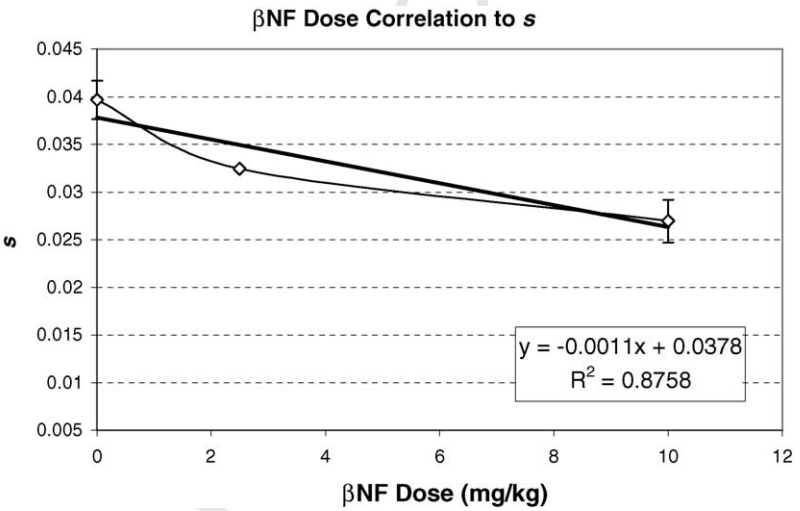

Fig. 7. Correlation of β-naphthoflavone dosage to *s* and trendline (linear regression). Standard error bars represent the standard error generated by VitalityModelFitting.ssc for the total test cohort for that BNF dosage ($n = 88$). The standard error for 2.5 mg/kg was below reporting levels for VitalityModelFitting.ssc.

for each BNF dose to examine the contaminant's effect.

The cumulative mortalities from these groups as well as the timelines along which they occurred can be seen in Fig. 1. During the active phase of the mortality curves, dose-dependent effects of the BNF dosage are evident relative to controls not exposed to BNF. The patterns exhibited by 2.5 mg/kg and 0 mg/kg dosages overlap at the beginning, then diverge at ~150 h.

Results of data fitting in VitalityModelFitting.ssc are shown in Figs. 2–5, and a BNF dose-dependent response is evident The fit to the model prediction for all dosages is close; this alignment becomes closer with increasing dosage showing almost no deviation at 10 mg/kg BNF dosage (Fig. 2). A slight departure from the model is apparent at 2.5 mg/kg BNF (Fig. 3), yet the association is clear and maintained. Of note are the similarities between 0 BNF (Fig. 4) and PBS (Fig. 5), in both degree of disparity from the model and the times at which these incongruities transpire.

There is an unmistakable correlation between the contaminant dose (i.e., BNF) and an increase in the rate of vitality loss, *r*, shown in Fig. 6. The relationship between the stochastic component of the Vitality model, *s*, and the BNF dosage is not as definite, with a lower R^2 and lacking the linearity of that found with *r* (Fig. 7). However, it merits attention since the random element of the model decreases with increasing BNF dosage. As *s* is the variance of the mean rate of vitality decline (*r*), it is important to consider that the variance is a measure of heterogeneity. This is reflected in the dose-dependent decrease in physiological variation from the anticipated response, as indicated in Figs. 2–4 (0, 2.5, 10 mg/kg). That *s* decreases with increasing BNF dose is a measure of how distinct the results were (a measure of immune competence disparity between individuals) and how these responses became more predictable (adhered more closely to predicted values) to increasing concentrations of this CYP1A inducer.

5. Discussion and conclusions

The results of this study clearly indicate that exposure to BNF affects the rate of vitality loss, the variance of this rate, and the average time to death from IHNV. The toxicity and behavior of BNF are well documented both in vitro and in vivo (Gravato and Santos, 2002; Hawkins et al., 2002; Meyer et al., 2002; Navas and Seger, 2000; Râbergh et al., 2000; Weimer et al., 2000; Goksøyer and Förlin, 1992). In rainbow trout, BNF induces CYP1A, uridine diphosphate glucuronosyltransferase (UDPGT), and NAD(P)H-quinone oxidoreductase (also known as DT-diaphorase, or DTD) (Lemaire et al., 1996). BNF stimulates the production of these enzymes with few documented toxic effects such as those exhibited by other accepted inducers such as the PAH benzo[*a*]pyrene or the PCB mixture, Aroclor 1254 (Paolini et al., 1994; Ong et al., 1980). In the absence of other compounds, it manifests few other consequences (NIEHS, 1998). Regarding potential genotoxicity, little information can be found in recent reviews of the literature (NIEHS, 1998). Earlier studies have shown that BNF, over a wide range of concentrations, did not produce mutagenicity in *S. typhimurium* strains TA98, TA100, TA1535, TA 1537, or TA1538 with or without Aroclor 1254-induced S9 (Brown and Dietrich, 1979). As BNF is not used industrially, extrapolations of relevant results to a population require further testing with contaminants of concern to that cohort.

Viral infection leads to potent stimulation of the immune system, particularly with the induction of interferons. The kinetics of this induction in response to IHNV infection have been reported by Hansen and La Patra (2002). The results of their research suggest that IHNV infection in trout can stimulate interferon-mediated responses. O'Farrell et al. (2002) used another rhabdovirus, viral hemorrhagic septicemia virus (VSHV), to study the rainbow trout response to a viral infection. Their research determined that the interferon pathway is the predominant component of the rainbow trout antiviral response, particularly in the disease's early stages. Another protein involved in this immune response is the ubiquitin-like product encoded by *vig-3*. This protein is highly similar to interferon-responsive ISG15, yet its functional homology to this mammalian protein is as yet unknown.

One of the connections between the rainbow trout antiviral innate immune response and the effects of BNF is protein demand. Induced catabolic enzymes are newly synthesized proteins (Swanson and Bradfield, 1993), and the highest levels of CYP1A are found two days following i.p. injection of BNF (Lemaire et al., 1996). Interferon-inducible genes are central to the initial immune response of the rainbow trout challenged

with IHNV (Hansen and La Patra, 2002; Bootland and Leong, 1999) and VHSV (O'Farrell et al., 2002). The elapsed time to death following the viral challenge was greater for groups not previously exposed to BNF; the same groups included fish that survived the viral exposure. One effective difference between the test groups is the quantity of protein generated before the immune response to IHNV was prompted. This suggests that the trout in the 10 mg/kg BNF groups may have lacked the resources necessary to respond to the viral challenge in the same manner as those trout in the 0 mg/kg BNF groups, which is consistent with the rationale of the Vitality model: an organism's experiences affect its ability to survive stresses in the future. In this study, the cost of tolerance of the contaminant (BNF) diminished the exposed groups' capacity to react to a pathogen as effectively as those fish not exposed to BNF. This is also reflected in the rate of vitality loss; the correlation between r and exposure to BNF is highly significant (correlation coefficient = 0.997).

The results of this study reiterate the need for a reassessment of toxicity models. Environmental contaminants form complex mixtures of the initial pollutants, their metabolites, and interactive breakdown products, all with toxicities that can vary between species. Toxicological evaluations of individual components of a complex mixture are incomplete estimates of exposure effects, as indirect effects are not evaluated. In this research, all fish dosed with BNF but not IHNV survived, and so any effects elicited by the contaminant were indirect. Although regulatory agencies consider many germane factors in environmental regulations, indirect effects are not incorporated. For example, BNF at the administered doses meets the criteria proposed by the Scientific Advisory Committee on Toxicity and Ecotoxicity of Chemicals of the European Commission in their definition of a water quality objective (WQO) for the protection of aquatic systems (CTSE, 1994). This objective, intended as an operative no-effect concentration (NEC), would overlook indirect effects by failing to incorporate the condition of the exposed biota at the time of exposure, which compromises the objective's accuracy. When establishing a toxic value, the status of the receptor must be considered. Standardized test models in use at present, which generate the values applied by most regulatory agencies, do not. The relevance of their conceptual integrity, then, can be challenged.

Uncited reference

Harayama et al. (1999).

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