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Dietary exposure to a binary mixture of polybrominated diphenyl ethers alters innate immunity and disease susceptibility in juvenile Chinook salmon (*Oncorhynchus tshawytscha*)

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Abstract

Polybrominated diphenyl ethers (PBDEs) have been used as flame retardants in consumer products and are now found in the aquatic environment. The presence of PBDEs puts the health and survival of aquatic species at risk due to the various toxic effects associated with exposure to these compounds. The effects of a binary dietary mixture of PBDEs on innate immunity and disease susceptibility of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) were examined in the present study. Salmon were fed roughly 1:1 mixtures of two environmentally predominant PBDE congeners, BDE-47 and BDE-99. The six resulting whole body total PBDE concentrations ranged from less than the limit of quantification to 184 ng/g, wet weight (ww). The innate immune system was assessed by using two *in vitro* macrophage function assays. Specifically, assays that examined the ability of head kidney macrophages to: (1) engulf sheep red blood cells (SRBCs); and (2) produce a respiratory burst, as determined by the production of a reactive oxygen species, superoxide anion. Macrophages from salmon fed the BDE-47/99 mixture diets engulfed more SRBCs and produced greater superoxide anion than salmon fed the control diet. An increase in macrophage function was observed in fish with whole body total PBDE concentrations ranging from 2.81 ng/g, ww to 184 ng/g, ww. The mechanism for this increase in macrophage function due to PBDE exposure is currently unknown, but may be due to the ability of PBDEs to act as an endocrine receptor agonist and/or antagonist. Salmon exposed to the BDE-47/99 mixture diets were also challenged with the pathogenic bacteria, *Vibrio (Listonella) anguillarum* to determine disease susceptibility. Kaplan-Meier survival curves of fish exposed to the BDE-47/99 mixture and control diets were significantly different. The Cox proportional hazard risk ratios of disease-induced mortality in juvenile Chinook salmon with whole body concentrations of total PBDEs of 10.9, 36.8, and 184 ng/g, ww were significantly greater than the fish fed the control diet by 1.56, 1.83 and 1.50 times, respectively. Not all concentrations of the binary mixture diets had significant hazard ratios relative to the control diet, due to a non-monotonic concentration response curve. The mixture of PBDE congeners resulted in interactive effects that were generally non-additive and dependent upon the congener concentrations and metric examined. Consequently, predicting the interactive effects in juvenile Chinook salmon exposed to mixtures of PBDE congeners on innate immunity and disease susceptibility cannot be readily determined from the adverse effects of individual PBDE congeners.

Key words: PBDEs, salmon, *Vibrio anguillarum*, innate immunity, disease susceptibility

1. Introduction

Polybrominated diphenyl ethers (PBDEs) have been used as flame retardants in a number of products including plastics, furniture, televisions, computers, mobile phones, and textiles (Alava et al., 2016). Three principal commercial formulations of PBDEs were produced, i.e. Octa-, Deca-, and Penta-BDEs, which differ in the individual PBDE congeners used in the formulations (Manchester-Neesvig et al., 2001). Although the use of these formulations have been either controlled or discontinued, a large 'reservoir' of congeners persist in the environment, allowing for continued environmental exposures for many decades (Santos et al., 2016). In addition, PBDEs are not covalently bound to products and are lipophilic (de Wit et al., 2010). Consequently, PBDEs are continually released into the environment and readily absorbed by aquatic and terrestrial plants and animals. Potential sources of PBDEs in the environment include waste water treatment plants, manufacturing facilities that recycle plastics and electronic waste, and incinerators (Iqbal et al., 2017).

PBDE congeners have been detected in environmental samples worldwide, including in salmon from the Puget Sound, USA (O'Neill et al., 2015; Sloan et al., 2010), the Great Lakes, USA (Gandhi et al., 2017; Gerig et al., 2016; Manchester-Neesvig et al., 2001), the Columbia River, USA (Arkoosh et al., 2011), Huemules and Ñirehuao Rives, Chili (Montory et al., 2010), as well as salmon from European and Canadian waters (Hites et al., 2004; Zacs et al., 2013). A possible 209 PBDE congeners exist, which are named based upon a system developed for polychlorinated biphenyls (Ballschmiter and Zell, 1980). Summed concentrations of PBDEs congeners in salmon have ranged from below the lower limit of quantitation (LOQ) to 188 ng/g, wet weight (ww). Two predominant PBDE congeners detected in salmon whole bodies are BDE-47 (2,2',4,4'-tetrabromodiphenyl ether) and BDE-99 (2,2',4,4'5-pentabromodiphenyl ether) based on their concentrations and frequencies (Hites et al., 2004; Ikonomou et al., 2011; O'Neill et al., 2015; Sloan et al., 2010).

Salmon have critical commercial, recreational, and cultural importance in North America (Quinn, 2005), and a number of species are listed as threatened or endangered in the United States (NOAA, 2017). PBDE exposure can alter physiological processes in fish, including: growth and development, behavior, innate immunity, disease susceptibility, metamorphosis and reproduction (Arkoosh et al., 2010; Arkoosh et al., 2015; Lema et al., 2007; Macaulay et al., 2017; Noyes and Stapleton, 2014; Thornton et al., 2016; Thornton et al., 2018; Torres et al., 2013; Yu et al., 2015).

PBDEs are endocrine disrupting compounds (EDCs) in anadromous (Arkoosh et al., 2017) and freshwater fish (Chen et al., 2012; Noyes and Stapleton, 2014). EDCs can act as endogenous hormones and produce concentration effects that are non-monotonic (Vandenberg et al., 2012). As defined by Vandenberg et al. (2012), a non-monotonic concentration response occurs if the 'slope of the dose-response curve changes sign from positive to negative or vice versa at some point along the range of doses examined.' Juvenile salmon exposed to PBDEs have demonstrated non-monotonic responses with respect to thyroid hormone levels, innate immune functions, and disease susceptibility (Arkoosh et al., 2010; Arkoosh et al., 2015; Arkoosh et al., 2017).

Studies examining the impact of PBDE congeners, specifically on immune function and disease in anadromous fish, are limited. In a previous study, juvenile Chinook salmon fed either an environmentally relevant mixture of five PBDE congeners (BDE-47. BDE-99, BDE-100, BDE-153 and BDE-154) or fed individual congeners (BDE-47 or BDE-99) were found to be more susceptible to disease. Also, the study examining the effects of juvenile salmon fed individual congeners (BDE-47 or BDE-99) investigated the function of the innate immune system and found the system to be impacted by BDE exposure. Both studies determined that the responses produced were non-monotonic and dependent upon the concentration of PBDEs and congeners examined (Arkoosh et al., 2010; Arkoosh et al., 2015). The objective of the present study was to further characterize the effects of a binary mixture of the most predominant PBDEs on the innate immune system and disease susceptibility of an anadromous fish, juvenile Chinook salmon (*Oncorhynchus tshawytscha*). Specifically, *in vitro* macrophage function, i.e. sheep red blood cell engulfment (phagocytosis) and superoxide anion production, and disease susceptibility were examined in salmon exposed to binary mixtures of predominant BDE congeners, BDE-47 and BDE-99. A properly functioning immune system is critical to animal fitness and survival (Segner

et al., 2012). Macrophage cells are important components of both innate and adaptive immunity in fish (Johnson et al., 2014). Disease susceptibility studies, or host resistance assays, in contaminant exposure studies are considered a holistic approach for examining the apex effect of a contaminant, death or survival, on the fish's ability to survive a pathogen exposure (Arkoosh et al., 2005; Segner et al., 2012).

2. Materials and methods

2.1 PBDE Diet Preparation, Feeding and Chemical Analyses

Ocean-type juvenile Chinook salmon that originated from the Garrison Springs hatchery, Washington, USA were held in seawater and exposed to binary mixtures of BDE-47 and BDE-99, hereafter referred to as BDE-47/99 mixture diet, at the National Marine Fisheries Service's Newport Research Station in Newport, Oregon as described in Dietrich et al. (2015). In brief, five BDE-47/99 mixture diets and a control diet were produced. The six diets were designated as control (0) and treatments 1, 2, 3, 4, and 5. A stock concentration of BDE-47 and BDE-99 was dissolved in methylene chloride and mixed with batches of food pellets in stainless steel bowls. The control diet was also mixed with methylene chloride without BDE-47 or BDE-99. Once the methylene chloride evaporated from the food, the batches within each treatment were mixed together and stored in glass jars that were previously fired at 450 °C for 17 to 19 hours to remove any potential contaminants. Five gram samples of the diet preparation were randomly sub-sampled from each treatment for chemical analysis of PBDE concentrations. The jars were covered with aluminum foil, secured with a plastic lid, and stored at 4 °C until fed to fish.

Tanks of fish were fed a daily ration of a BDE-47/99 mixture diet, spread over 3 feedings per day, for 39 days. Each treatment had three replicate feed tanks of 285 fish. Rations were adjusted daily to reflect estimated fish growth and mortalities, in order to consistently feed 2% of fish body mass. Two composites of five fish per feed tank (i.e. six composite samples, or 30 total fish, per treatment) were collected for whole body chemistry analysis one day after the dietary exposure was completed and stored at -80º C until chemical analysis was performed.

Samples of salmon whole bodies and the BDE-47/99 mixture diet food were extracted and analyzed for PBDEs using the gas chromatography/mass spectrometry method described in Sloan et al. (2014; 2004) and reported in Dietrich et al. (2015). The following 11 PBDE congeners were analyzed: BDE-28, BDE-47, BDE-49, BDE-66, BDE-85, BDE-99, BDE-100, BDE-153, BDE-154, BDE-155, and BDE-183. The summed concentration of these 11 congeners was reported as the total PBDE concentration (Table l). Analytes that were less than the lower limit of quantitation (< LOQ) were assigned a value of zero when determining mean and total PBDE concentrations. A method blank and a National Institute of Standards and Technology (NIST) Standard Reference Material (fish tissue, SRM 1947) were analyzed with each set of salmon and food samples as part of a performance-based quality assurance program (Sloan et al., 2006). All quality assurance criteria for the samples were met.

2.2 Innate Immunity — Macrophage Function

Isolation of head kidney (HK) adherent cells (macrophages) and their function as determined by their ability to engulf and undergo respiratory bursts were determined by two *in vitro* assays, as described by Arkoosh et al. (2015). In brief, after exposure to the dietary treatments, subsets of juvenile Chinook salmon were euthanized and composite samples of HKs were collected from each replicate feed tank within 72 hours of the last feeding to assess immune function. The HKs of 3 fish per replicate feed tank were composited on the first sample day; and HKs from 5 fish were composited from each replicate feed tank on the second and third sample days. HKs were aseptically removed and immediately placed on ice in 5 ml polystyrene snap cap tubes containing 1 ml L-15 media (Leibovitz; Sigma) supplemented with 0.1% FBS (fetal bovine serum; Atlanta Biologicals) and 1% penicillin-streptomycin solution (Life Technologies). For each of the two assays, each of the three composite samples (biological replicate) per dietary treatment was divided into 4 wells (technical replicates); i.e. 12 wells per dietary treatment per day. Macrophages were partially isolated from other blood cells by separation with Histopaque 1077 and further purified by their ability to adhere to plastic (Secombes, 1990). Once the cells adhered, their ability to engulf and produce a respiratory burst was determined.

The ability of macrophages to engulf a foreign particle was determined by a colorimetric method

using sheep red blood cells (SRBC, Lampire Biological) as target particles (Arkoosh et al., 2015; Gebran et al., 1992). In brief, after $5x10^5$ HK white blood cells/well were incubated in a 96-well tissue culture plate for 18 hours, the wells were washed once with 1X PBS. After washing, 0.2% SRBC in L-15 media, containing 5.0% FBS, was added to the wells. The plates were placed on a rotating shaker at room temperature for 30 minutes. After the 30-minute incubation, adherent cells were washed twice with cold 1X PBS. The cells were then washed three times for 1 minute in ice cold 11 mM $KHCO_3$ and 152 mM NH_4Cl to lyse and remove non-engulfed SRBCs from the wells, followed by a final wash with cold 1X PBS (Perry et al. 1999). Adherent cells were solubilized by gentle pipette mixing with 100 µl of 0.2 M Tris HCl/6 M urea pH 7.4. After washing, 100 µl of the DAF reagent (2,7-diaminofluorene) was added (Gebran et al. 1992). The plates were incubated for 10 minutes and the absorbance of the wells was measured at 620 nm on a spectrophotometer. The hemoglobin from the lysed SRBC oxidized DAF to produce fluorine blue that had a different absorption spectrum from the unoxidized DAF (Gebran et al. 1992). Engulfment was reported as the mean fold difference in SRBC engulfment. The mean fold difference was calculated by dividing the well absorbance at 620 nm after 10 min of incubation by the background absorbance of adherent cells (~5 x 10^5 cells/well) in the well prior to SRBC addition.

Macrophage respiratory burst activities were determined by monitoring the production of superoxide anion. The production of superoxide anion was determined as the reduction of tetrazolium as per Secombs (1990) with slight modification (Arkoosh et al., 2015). This assay is specific for measuring superoxide anion levels. A water-soluble tetrazolium salt (WST-1; [2-(4-Iodophenyl)-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]; Dojindo) was used, which produced a water-soluble formazan dye upon reduction with the superoxide anion (Peskin and Winterbourn, 2000; Tan and Berridge, 2000). WST-1 at 1 mg/ml in 1X PBS was added either with or without 1µg/ml of soluble phorbol myristate acetate (PMA; Sigma) to stimulate the surface membrane of adherent cells and activate respiratory bust. The cells were then incubated for 60 min at 14° C. The level of superoxide produced was reported as the mean fold difference of the well absorbance at 440 nm divided by the background absorbance of adherent cells (~5 x 10^5 cells/well) in the well prior to WST-PMA addition.

Differences among group means were tested with one-way ANOVA, followed by the Tukey's

Honestly Significant Difference post-hoc analysis test for comparisons among all concentrations using Systat R13 software. All p-values less than or equal to the alpha, 0.05, were considered significant.

2.3 Disease Challenge and Survival Analysis

After exposure to the BDE-47/99 mixture diets for 39 days, juvenile Chinook salmon were challenged with the gram-negative pathogen *Vibrio* (*Listonella) anguillarum* (strain 775, ATCC No. 68554) using disease challenge protocols described by Arkoosh et al. (2015). Details of the disease challenge are provided in Table 2. Briefly, six replicate tanks of 60 fish per BDE-47/99 mixture diet were exposed to *V. anguillarum* by immersion in seawater under static conditions with aeration for 1 hour at an approximate density of 50 g fish/L. A stock concentration of *V. anguillarum* was incubated in tryptic soy broth (TSB), supplemented with 1.5% NaCl, at 22 °C for approximately 15 hours. Thirteen milliliters of the stock was added to 13 liters of seawater for each exposure vessel, for a final challenge concentration of $2.5 \cdot 10^5$ cfu/ml. In addition, two replicate tanks of no-pathogen controls per BDE-47/99 mixture diet were included in the challenge. Each no-pathogen control tank was exposed to sterile TSB, supplemented with 1.5% NaCl, under identical challenge conditions. Following the 1-hour exposure, all the fish were returned to their original flow-through seawater tanks (ca. 300 L) and monitored twice daily for mortalities over a 17-day period. All mortalities were screened for the presence of *V. anguillarum* in kidney tissue using polymerase chain reaction (PCR) as described in Arkoosh et al. (2015).

Survival analysis was performed by generating probabilistic survival curves of the juvenile Chinook in the disease challenges using the non-parametric Kaplan–Meier method (Clark et al., 2003; JMP 13 software, SAS Institute Inc.) across replicate tanks for fish in each pathogen-exposed and BDE-47/99 mixture diet. Two of the 48 challenge tanks were identified as outliers and all fish within them were excluded from analysis due to the low *V. anguillarum* prevalence (< 71%) among mortalities, as determined by PCR. *V. anguillarum* prevalence was 80 to 100% among mortalities in tanks included in the Kaplan-Meier analysis. All fish that survived to 17-days post exposure to the pathogen, as well as fish mortalities that were negative for *V.*

anguillarum presence by PCR, were 'censored' events in the Kaplan-Meier analysis. Censoring, with respect to Kaplan-Meier analysis, occurs when fish have not experienced death due to *V. anguillarum* by the time the study has ended. Therefore, their actual time to death is unknown. The advantage of the non-parametric Kaplan–Meier survival analysis over traditional parametric approaches is that the Kaplan–Meier survival analysis takes in to account fish that have been censored and does not require the data to be normally distributed (Dudley et al., 2016). Differences in survival between fish that were not exposed and fish exposed to the BDE-47/99 mixture diets, irrespective of treatment, were examined with the Mantel method for the log–rank chi-square test. Also, differences in survival among the six individual treatments were determined with the Mantel method for the log–rank chi-square test with the null hypothesis of a common survival curve between treatments. Significance level, alpha (α), was set at 0.05. The survival curves were further characterized by generating mean survival times for each dietary treatment. Mean survival times were estimated as the area under the probabilistic survival curves as in Arkoosh et al. (2015).

Pairwise hazard ratios, generated by the Cox proportional hazards model (Bradburn et al., 2003a; Xia et al., 2015), identified the risk of disease-induced mortality in the PBDE dietary treatments relative to the control diet, determined as follows:

$$Hazard\ ratio = \frac{Hazard_i}{Hazard_0}$$

Where, $Hazard_i$ is the hazard due to a BDE-47/99 mixture diet treatment *i* and $Hazard_0$ is the hazard due to the control diet. JMP 13 was used to calculate hazard ratios and their 95% confidence intervals, as well as p-values, via the Wald Test, which tested the null hypothesis that a BDE-47/99 mixture diet treatment had no effect on disease-induced mortality relative to the control treatment. Two assumptions must be met prior to using the Cox proportional hazards model: (1) proportional hazards, and (2) significant power. The proportionality of the hazards was assessed by plotting the -log(-log(survival probability)) against log(time) as per Bradburn et al. (2003a). The cumulative hazard curves of treatments appeared relatively parallel (data not shown) indicating the first assumption was met. The assumption of significant power requires at

least ten events (or death) for each dietary treatment exposed to the pathogen (Bradburn et al., 2003b). This assumption was also satisfied with 81 to 161 events per treatment.

2.4 Interactive Effects

The directional classification system developed by Piggott et al. (2015) for interactive effects was used to characterize the effects of the BDE-47/99 mixed diet exposure in the present study, relative to the effects produced by exposure to individual BDE-47 or BDE-99 on macrophage function and mean survival time in a previous study (Arkoosh et al., 2015). The observed effects for each exposure treatment were divided by the study's respective control treatment in an attempt to address differences in study years. The directional classification system was used to examine the observed trends produced by the BDE-47/99 mixed diet relative to a predicted modeled additive effect produced by the individual congeners. The modeled additive effect is the sum of the effect produced by the control and the deviations from the control effect produced by the individual congeners:

$$Modeled\ additive\ effect = Control\ effect + BDE\text{-}47\ deviation + BDE\text{-}99\ deviation$$

Where, *deviation* is the observed individual BDE congener effect minus the *Control effect*. This equation can then be modified to account for the observed individual congener and *Control effects* to calculate a predicted additive response.

$$Predictive\ additive\ response = \left(\frac{BDE47_{effect}}{Control\ effect_{BDE47}} + \frac{BDE99_{effect}}{Control\ effect_{BDE99}}\right) - 1$$

3. Results/ Discussion

3.1 PBDE Chemical Analysis

Concentrations of PBDEs in the food and salmon whole bodies from the six dietary treatments are reported in Table 1. Whole body concentrations of total PBDEs in juvenile Chinook salmon ranged from <LOQ to184 ng/g, ww. Individual congener concentrations for each treatment were

reported in detail in Dietrich et al. (2015). The range of whole body total PBDEs in salmon in the present study straddles the whole body PBDE concentration examined in our earlier study (51 ng/g, ww; Arkoosh et al., 2010), and are in the range of whole body PBDE concentrations detected in salmon collected from the environment in either wild or farm-raised settings (<LOQ to188 ng/g, ww; Arkoosh et al., 2011; Bethune et al., 2005; Gandhi et al., 2017; Gerig et al., 2016; Hites et al., 2004; Manchester-Neesvig et al., 2001; Montory et al., 2010; O'Neill et al., 2015; Sloan et al., 2010).

3.2 Innate Immunity — Macrophage Function

Innate immunity was altered in juvenile Chinook salmon exposed to the BDE-47/99 mixture diets. The innate immune response includes engulfing a pathogen by a macrophage and killing the pathogen with reactive oxygen species (ROS), during a respiratory burst (Segner et al., 2012). Macrophages are considered to be the first line of defense of a host against a pathogen. The ability of HK macrophages to engulf SRBCs was significantly greater in PBDE-exposed salmon relative to salmon fed the control diet ($p \leq 0.004$; Figure 1a). However, there was no significant difference in SRBC engulfment among the exposed treatments greater than or equal to 2.8 ng total PBDEs/g, ww ($p \geq 0.05$; Figure 1a). Respiratory burst activities of HK macrophages, as determined by the production of superoxide anion, from juvenile Chinook salmon fed the BDE-47/99 mixture diets were also significantly greater than respiratory burst activities of HK macrophages from fish receiving the control diet ($p \leq 0.007$; Figure 1b). Similar to SRBC engulfment, there was no significant difference in superoxide anion levels among the exposed treatments greater than or equal to 2.8 ng total PBDEs/g, ww ($p \geq 0.05$; Figure 1b).

The observed responses preliminarily indicate that SRBC engulfment and respiratory burst activity of HK macrophages are sensitive to low PBDE mixture concentrations. The lowest BDE-47/99 mixture diet concentration examined, 2.8 ng total PBDEs/g, ww, altered (increased) both HK SRBC engulfment and respiratory burst activities. In our previous study examining individual PBDE congeners, the lowest BDE-99 diet examined, 7.1 ng total PBDEs/g, ww, also altered (reduced) SRBC engulfment while salmon exposed to the lowest BDE-47 diet 10.5 ng/g total PBDEs/g, ww, examined also altered (increased) respiratory burst activity (Arkoosh et al.,

2015). Similar to salmon exposed to the BDE-47/99 mixture diets, salmon exposed to either BDE-47 or BDE-99 also produced significantly greater superoxide anion in HK macrophages than fish receiving the control diet. However, macrophage engulfment was unaltered or suppressed in salmon exposed to either BDE-47 or BDE-99, respectively (Arkoosh et al., 2015), in contrast to the enhancement observed after exposure to BDE-47/99 mixture diets in the present study.

The mechanism responsible for the observed increase in macrophage function due to exposure to a binary mixture of BDE congeners is currently unknown, but may be due to PBDEs' ability to act as endocrine receptor (ER) agonists or antagonists in fish (Noyes and Stapleton, 2014). The immune and endocrine systems in fish are tightly linked. The head kidney is a critical endocrine and haematopoietic-lymphoid organ (Geven and Klaren, 2017). Endocrine receptors and genes have also been found on immune cells in fish (Maule and Schreck, 1991; Quesada-Garcia et al., 2014; Szwejser et al., 2017). ER agonists, such as endosulfan, phthalates, and bisphenol A, also stimulated macrophage engulfment and superoxide anion production in fish (reviewed in Milla et al., 2011). For example, endosulfan not only enhanced macrophage activity, but also inhibited cortisol secretion in fish interrenal cells (Dorval et al., 2003). Cortisol is a hormone produced in the interrenal cells of the head kidney and is responsible for regulating several immunological processes, including innate immunity in fish (Cortes et al., 2013). Cortisol decreases engulfment of leukocytes from common carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus* x *O. aureus*) (Law et al., 2001). A possible mechanistic explanation is that cortisol secretion was inhibited in PBDE-exposed salmon in the present study, thereby enhancing macrophage activity.

Another potential mechanism responsible for increased macrophage function, specifically ROS production, may be due to reduced levels of regulating antioxidants. For example, murine peritoneal macrophages exposed *in vitro* to BDE-47 had increased ROS production, as well as reduced activity of glutathione (Lv et al., 2015). Glutathione is an antioxidant responsible for counteracting oxidative free radicals in order to protect against excess ROS (Vagula and Konieczko, 2012). In theory, an increase in ROS production in macrophage cells due to PBDE exposure may lead to oxidative stress. Oxidative stress, which occurs when there is an imbalance between the production of ROS and the neutralization of ROS, can result in damage to cellular

proteins, DNA, and membrane lipids (reviewed in Valavanidis et al., 2006). Oxidative stress may be expressed as chronic diseases such as cardiovascular diseases, cancer, and obesity in vertebrates (Cheung et al., 2016; Zou and Secombes, 2016).

3.3 Disease Challenge and Survival Analysis

The Kaplan-Meier survival curve of all fish exposed to BDE-47/99 mixture diets, i.e. all exposed treatments pooled, was significantly different from the survival curve of fish exposed to the control diet (data not shown, $p<0.0025$). There were also significant differences in Kaplan-Meier survival curves among the six PBDE individual treatments ($p <0.0001$; Figure 2). In general, the Kaplan-Meier survival curves indicate that mortality began around day 4 post exposure to the pathogen with a steep decline in survival for all treatments until day 10.

Although the log-rank test determined that there was a significant difference among the survival curves, it does not quantify the effect of an individual exposure treatment on survival relative to the control. The Cox proportional hazards regression model estimates the 'strength of effect' (Bradburn et al., 2003a) of the BDE-47/99 mixture diets on disease susceptibility in salmon. The effect, in the present study, refers to increased disease-induced mortality attributed to *V. anguillarum* infection. The risk of disease-induced mortality in juvenile Chinook salmon with whole body concentrations of 10.9 (treatment 2), 36.8 (treatment 3), and 184 ng total PBDEs/g ww (treatment 5), was significantly greater than the fish in the control treatment by 1.56, 1.83 and 1.50 times, respectively (Table 3). However, the risk of disease-induced mortality in juvenile Chinook salmon with whole body concentrations of 2.8 (treatment 1) and 98.6 ng total PBDEs/g ww (treatment 4) was not significantly different ($p \geq 0.639$) from the juvenile Chinook salmon in the control treatment. The lowest whole-body PBDE concentration to increase disease-induced mortality was 10.9 ng total PBDEs/g. In our previous study with individual congener exposures, the lowest PBDE whole-body concentration to produce an increase in disease-induced mortality to *V. anguillarum* was 31.1 ng total PBDEs/g ww in salmon exposed to a BDE-47 contaminated diet, and 7.1 ng total PBDEs/g ww in salmon exposed to a BDE-99 contaminated diet (Arkoosh et al., 2015).

The survival analysis, i.e. Kaplan-Meier survival curves, Cox proportional hazards regression model, and mean survival time, all indicated non-monotonic responses for Chinook salmon exposed to a range of PBDE concentrations. Specifically, mortality (or risk) oscillated from no difference from the control, to greater mortality (risk) than the control, to no differences, and back to greater mortality (risk). This change in slope of the concentration response curve was evident in the estimated mean survival time results (Figure 3). The slope of the mean survival time response is negative from treatment 1 to 2 to 3, but changes signs to positive to treatment 4, and back to negative for treatment 5. Non-monotonic concentration response curves are typical of EDC chemicals (Ribeiro et al., 2017) and were also characteristic of survival curves resulting from disease challenges with juvenile Chinook salmon exposed to either individual PBDE congeners (Arkoosh et al., 2015) or a mixture of five PBDE congeners (Arkoosh et al., 2010).

3.4 Interactive Effects

Juvenile Chinook salmon exposed to binary mixtures of BDE congeners resulted in complex interactive effects from the individual congeners, which deviated from additivity. Classifying interactive effects is less complicated when the outcomes produced by the individual contaminants and the mixtures operate in the same direction. However, classifying the interactive effect produced by the mixture can be complicated if individual contaminants produce countering effects. Specifically, uncertainties arise as to which individual chemical to anchor the interactive effect for classification purposes (Piggott et al., 2015), i.e. a non-additive effect in a binary mixture may appear synergistic with respect to one chemical and antagonistic with respect to the other chemical. The directional classification system developed by Piggott et al. (2015) for interactive effects addresses this problem by anchoring the classification of interactive effects to the predicted additive effect.

Piggott et al. (2015) used the following four classification terms to describe the interactive response relative to the additive model prediction: positive synergism, negative synergism, positive antagonism and negative antagonism (Figure 4a). The predicted additive model responses, based on the summation of effects from the individual BDE-47 and BDE-99 exposures in Arkoosh et al. (2015), and the observed interactive responses, in the present study,

are graphically displayed for phagocytosis (SRBC engulfment, Figure 4b), superoxide anion production (Figure 4c), and mean survival time (Figure 4d). Non-additive synergistic and antagonistic effects can describe the interactive effects produced by BDE-47 and BDE-99 in the mixture exposures, using the classification system defined by Piggott et al. (2015). The most straightforward example of positive synergism, i.e. more positive than the modeled additive interaction, is the increased engulfment of foreign particles by macrophages in juvenile Chinook exposed to any BDE-47/99 diet mixture (Figure 4b). Piggott et al. (2015) further classifies this interaction as mitigating synergism, i.e. individual BDE-47 and BDE-99 exposures produced effects that were both trending negative relative to the control, but the mixture exposure resulted in a positive synergistic interactive effect. Mitigating synergism of macrophage engulfment was observed between approximately 10 and 184 ng total PBDEs/g, ww (Figure 4b).

Positive synergistic responses for superoxide anion production also occurred at whole body concentrations approximately greater than 125 ng total PBDEs/g, ww. In addition, mixtures of the two congeners had a positive antagonistic interactive effect on superoxide anion production at concentrations between 5 and 125 ng total PBDEs /g, ww (Figure 4c). A positive antagonistic interactive effect 'is less positive than predictive additively,' as defined by Piggott et al. (2015). Finally, the mean survival time of fish exposed to BDE-47/99 diet mixtures and *V. anguillarum* demonstrated a negative antagonistic interaction at concentrations less than 184 ng total PBDEs/g, ww. As defined by Piggott et al. (2015), a negative antagonistic interactive effect 'is less negative than predictive additively.' At 184 ng total PBDEs/g, ww, the interactive response appeared to meet the response curve predicted by the additive model (Figure 4d; Arkoosh et al., 2015). In general, these results suggest that the interactive response of binary mixtures of BDE-47 and BDE-99 on salmon disease susceptibility and the innate immune response deviate from additivity, and the direction of deviation is dependent on congener concentration. A review of available literature did not identify any additional studies that examine the interactive effects of PBDE congeners on fish health for comparison to the present study.

4. Conclusion

In conclusion, assessing risk of binary mixtures of environmentally relevant concentrations of predominant PBDE congeners, BDE-47 and BDE-99, on the health and survival of anadromous juvenile Chinook salmon can be complex. A risk assessment is complicated by non-additive effects on Chinook salmon macrophage function and disease-induced mortality. Macrophage engulfment and respiratory burst activities were found to be increased in fish with whole body concentrations greater than or equal to 2.8 ng total PBDEs /g, ww. A no observable adverse effect level (NOAEL) for the binary BDE mixture on macrophage function was not determined. Disease-induced mortality increased in salmon exposed to BDE mixtures with whole body concentrations of 10.9 ng total PBDEs /g, ww, which is similar to the lowest observed effect level in salmon exposed to BDE-99 (7.1 ng total PBDEs /g, ww; Arkoosh et al., 2015). Although the present study did determine that there was no effect on disease-induced mortality in fish exposed to 2.8 ng total PBDEs /g, ww, the non-monotonic nature of the response precludes identifying this as a NOAEL at this time. Finally, an enhanced innate immune response in fish exposed to a binary mixture of BDE congeners does not necessarily associate with an increase in disease resistance. An earlier study also showed that an enhancement or reduction of the innate immune response in fish exposed to either BDE-47 or BDE-99 did not correlate with a decrease or increase, respectively, in disease susceptibility (Arkoosh et al., 2015). Both this current study examining a binary mixture of BDEs and a prior study examining the impact of individual BDE congeners on juvenile Chinook salmon (Arkoosh et al., 2015) suggest that in addition to macrophage function, other immune system components may also be altered by exposure to PBDEs and contribute to the changes observed in disease susceptibility. This study highlights the importance of examining the response of PBDE mixtures on multiple endpoints to assess the impact of environmental levels of PBDEs on salmon health, rather than relying solely on the responses of individual congeners and endpoints.

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FIGURE LEGENDS

Figure 1. (a) Mean phagocytosis activity, as indicated by sheep red blood cell (RBC) engulfment, and (b) mean respiratory burst activity, as indicated by superoxide anion production, of head kidney (HK) macrophages collected from juvenile Chinook salmon exposed to increasing concentrations of a dietary mixture of BDE-47/99. Error bars represent 95% confidence intervals of the mean. Bars that do not have a letter in common are significantly different by Tukey's HSD post–hoc test.

Figure 2. Probabilistic survival curves following disease challenges with *V. anguillarum* in juvenile Chinook salmon exposed to increasing concentrations of a dietary mixture of BDE-47/99. Survival probability curves were calculated using non-parametric Kaplan-Meier methods based on the observed survival of individual fish per PBDE concentration. Differences in survival between fish that were not exposed and fish exposed to the BDE-47/99 mixed congener diets, irrespective of treatments, were examined with the Mantel method for the log–rank chi-square test.

Figure 3. Mean survival times of juvenile Chinook salmon exposed to the BDE-47/99 mixed congener dietary and control treatments and challenged with *V. anguillarum*. Mean survival times were calculated as the area under the probabilistic survival curves (Figure 2).

Figure 4. The directional classification system (a) developed by Piggott et al. (2015) for characterizing interactive effects. The directional classification system compares the observed effect produced by the BDE-47/99 mixed diet to a predicted modeled additive effect produced by the individual congeners. Each metric [i.e. macrophage engulfment (b), macrophage superoxide anion production (c), and mean survival time (d)] is divided by the control treatment of each respective dietary exposure to generate 'control corrected' values, which enables comparison across dietary exposures to a common control line (dash dots) at 1.0. Modeled additive responses (open squares) produced from the effects of the individual congeners were modified from Arkoosh et al. (2015). Interactive responses of the individual congeners in the binary mixtures deviated from additivity and were classified as positive synergism, negative synergism, positive antagonism, or negative antagonism.

Table 1. Mean concentration of targeted (BDE-47 and BDE-99) and total PBDEs in the diets and whole bodies (modified from Dietrich et al.(2015)).

	PBDEs in diets, ng/g food (±SD)				PBDEs in salmon, ng /g fish wet weight (±SD)		
Diets	BDE-47	BDE-99	Total PBDEs [a]	Lipids in salmon, % (±SD)	BDE-47	BDE-99	Total PBDEs [a]
0	0.4	0.1	0.7	2.43 (0.58)	<LOQ[b]	<LOQ	<LOQ
1	4.1	4.2	8.5	2.49 (0.39)	1.5 (0.1)	1.3 (0.1)	2.8 (0.1)
2	16	17	34	2.05 (0.32)	5.6 (0.5)	5.3 (0.6)	10.9 (1.1)
3	50	51	102	1.92 (0.42)	18.2 (0.8)	18.6 (1.7)	36.8 (2.5)
4	160	170	330	2.71 (0.41)	46.7 (2.7)	51.7 (3.1)	98.6 (4.9)
5	330	360	690	1.92 (0.42)	86.0 (3.4)	96.8 (7.1)	184 (9.9)

[a] Total PBDEs equals the sum of all detected BDE congeners (see Methods for a complete list of analytes) with levels greater than the lower limit of quantification.

[b] Less than the lower limit of quantification (LOQ).

Table 2 Experimental conditions during each of the disease challenges following the mixed-congener PBDE exposures.

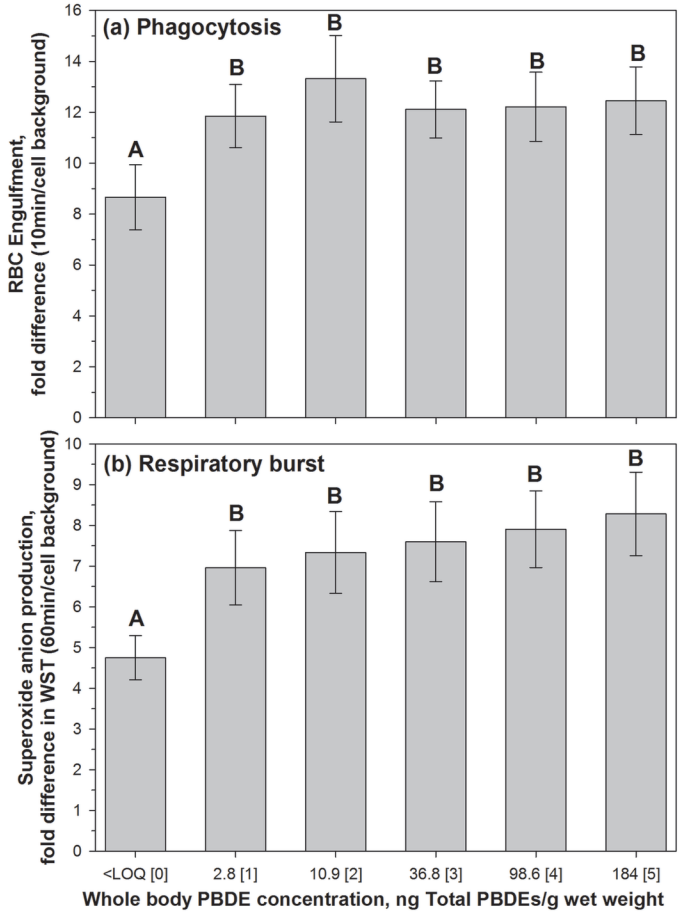
Experimental Conditions	PBDE-47 + PBDE-99
Challenge start, days post last PBDE feed	6
V. anguillarum concentration, cfu/ml	$2.5 \cdot 10^5$
Volume of stock culture, ml	13
Exposure volume, l	13
No. of pathogen exposed tanks per PBDE dose	6
No. of no-pathogen control tanks per PBDE dose	2
No. of fish per tank	60
Monitoring period, days	17
Statistical analysis period, days	17

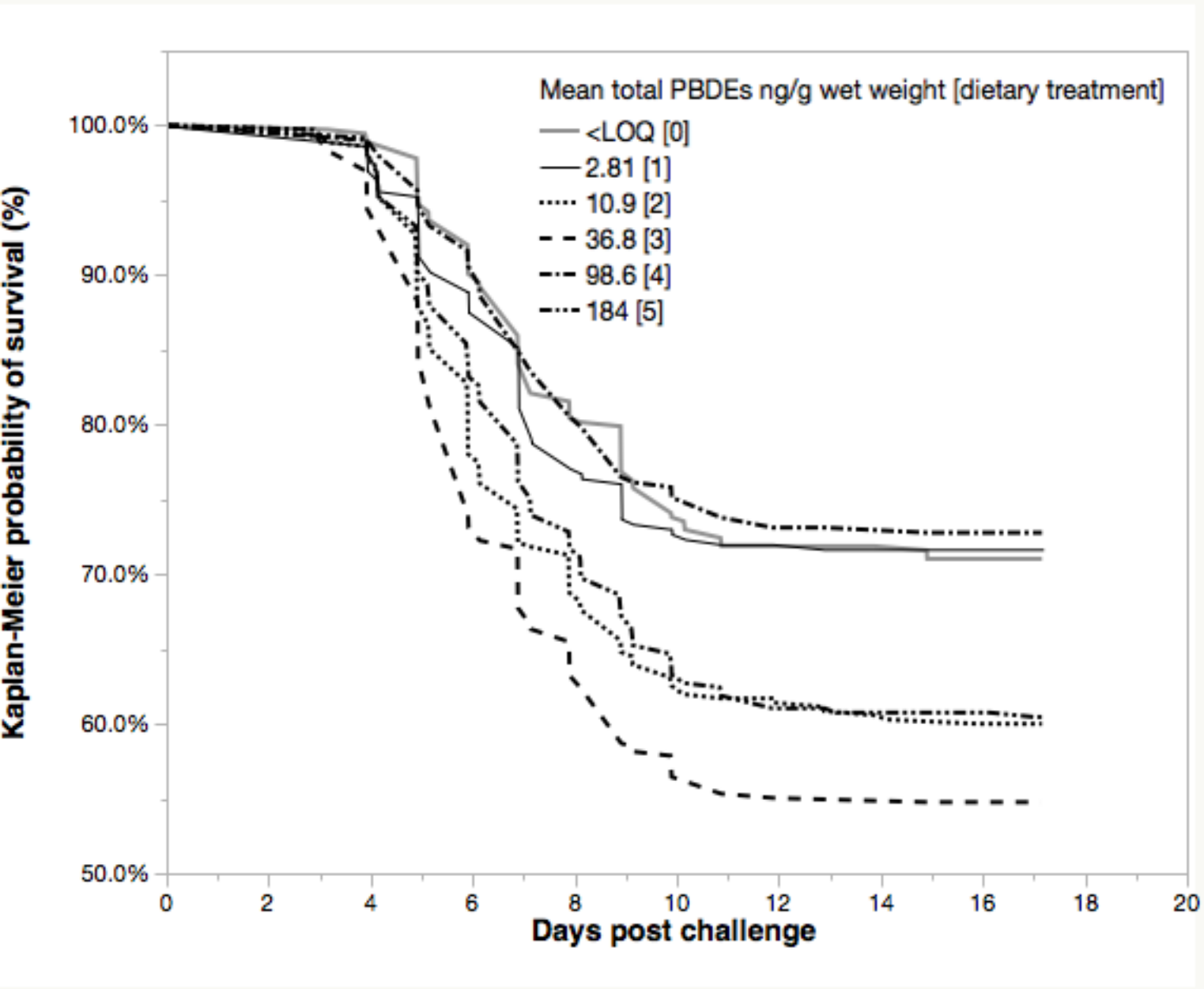
Table 3. Pairwise hazard ratios, p values, and 95% confidence intervals as determined by the Cox Proportional Hazards model, which examines the relative differences in disease-induced mortality between PBDE-exposed dietary treatments and the control dietary treatment.

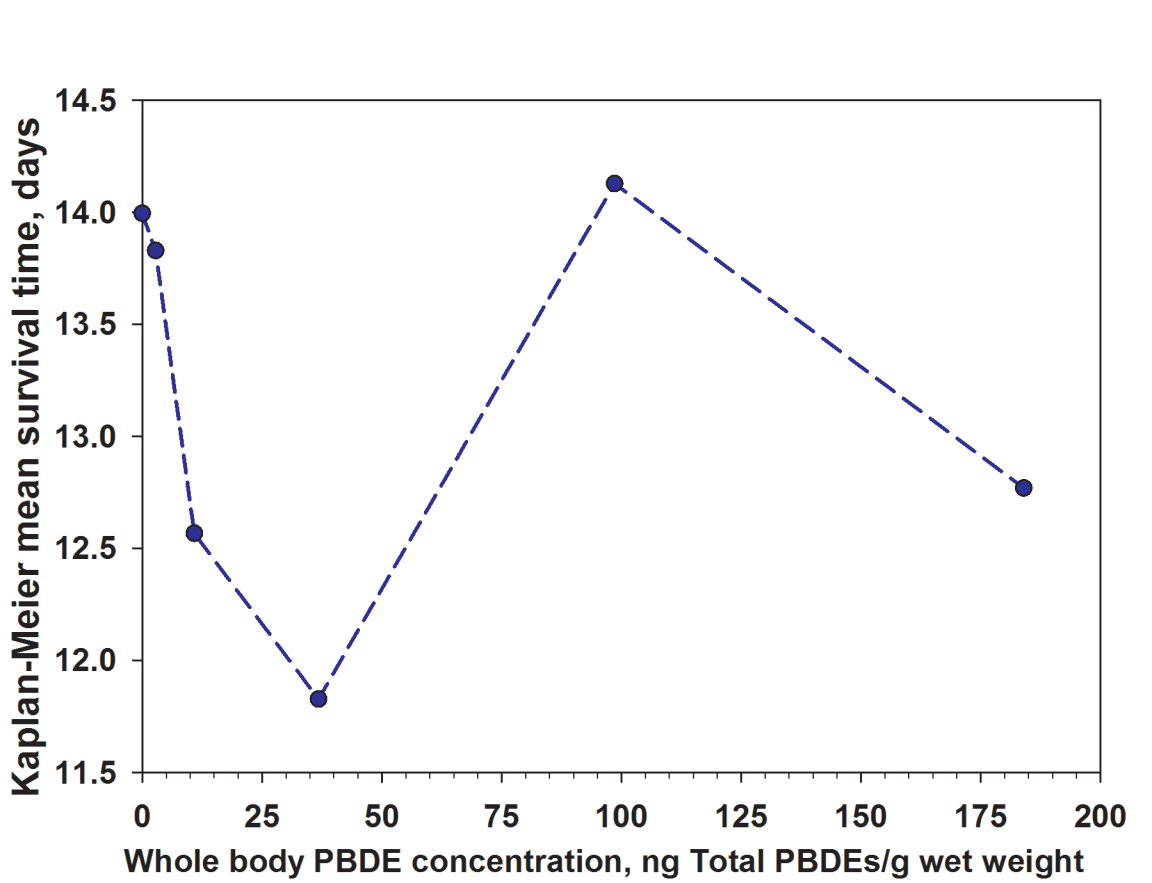
Treatment (PBDEs in salmon, ng /g fish wet weight)[a]	Hazard Ratio [b] (p-value)	95% Confidence Interval
0 (<LOQ)	1.00	
1 (2.8)	1.00 (0.9801)	0.75-1.33
2 (10.9)	1.56 (0.0005)	1.21-2.01
3 (36.8)	1.83 (<0.0001)	1.43-2.34
4 (98.6)	0.93 (0.6389)	0.70-1.25
5 (184)	1.50 (0.0017)	1.16-1.93

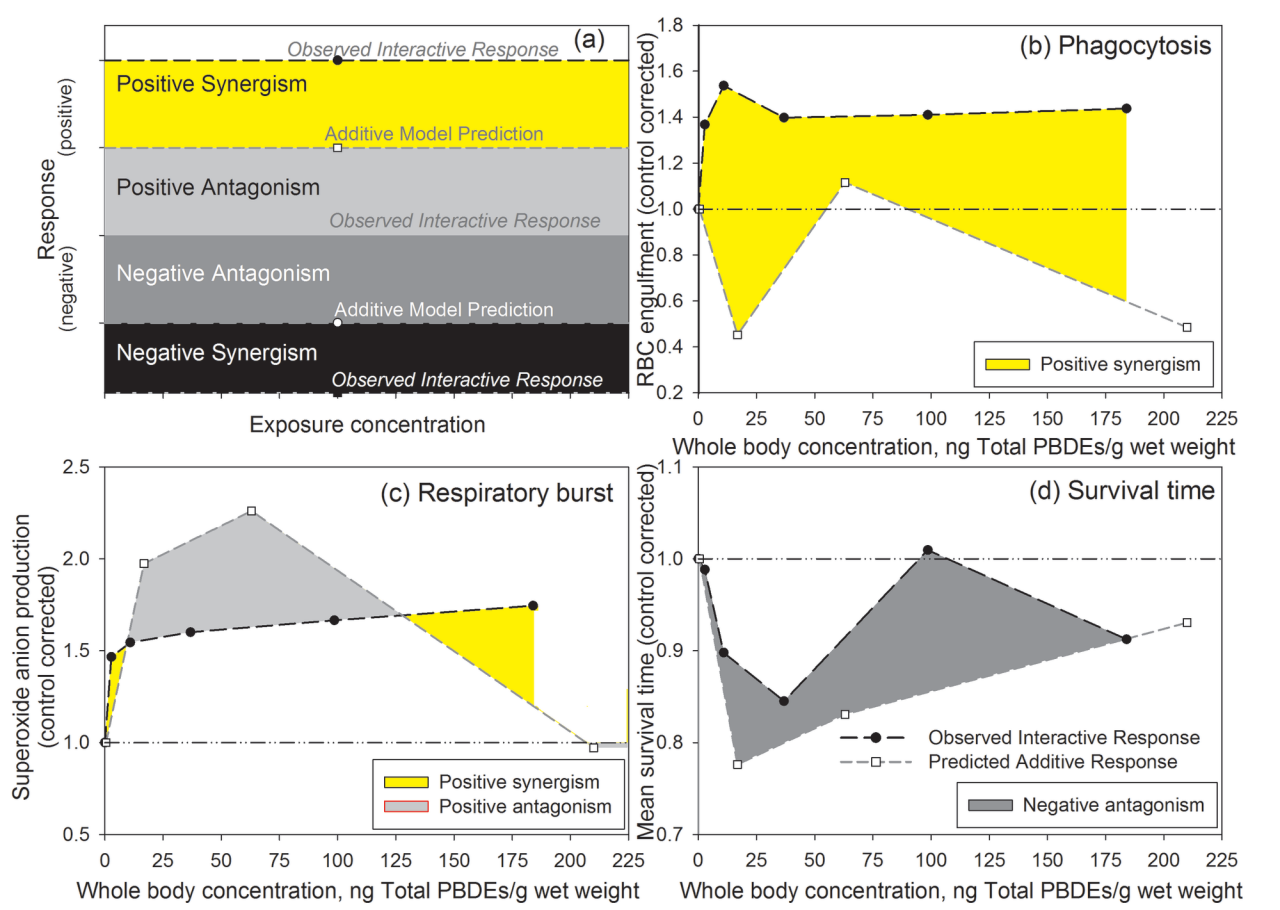
[a] Total PBDEs in the whole bodies modified from Dietrich et al.(2015).

[b] Pairwise hazard ratios, generated by the Cox Proportional Hazards model, quantifies the relative risk of disease-induced mortality in juvenile Chinook salmon exposed to the BDE-47/99 mixture diets relative to juvenile Chinook salmon exposed the control diet.


(a) Phagocytosis
RBC Engulfment, fold difference (10min/cell background)
A
B
B
B
B
B
(b) Respiratory burst
Superoxide anion production, fold difference in WST (60min/cell background)
A
B
B
B
B
B
<LOQ [0]
2.8 [1]
10.9 [2]
36.8 [3]
98.6 [4]
184 [5]
Whole body PBDE concentration, ng Total PBDEs/g wet weight


Mean total PBDEs ng/g wet weight [dietary treatment]
<LOQ [0]
2.81 [1]
10.9 [2]
36.8 [3]
98.6 [4]
184 [5]
Kaplan-Meier probability of survival (%)
100.0%
90.0%
80.0%
70.0%
60.0%
50.0%
0
2
4
6
8
10
12
14
16
18
20
Days post challenge


Kaplan-Meier mean survival time, days
14.5
14.0
13.5
13.0
12.5
12.0
11.5
0
25
50
75
100
125
150
175
200
Whole body PBDE concentration, ng Total PBDEs/g wet weight


(a)
Observed Interactive Response
Positive Synergism
Additive Model Prediction
Positive Antagonism
Observed Interactive Response
Negative Antagonism
Additive Model Prediction
Negative Synergism
Observed Interactive Response
Response
(positive)
(negative)
Exposure concentration
(b) Phagocytosis
RBC engulfment (control corrected)
Positive synergism
Whole body concentration, ng Total PBDEs/g wet weight
(c) Respiratory burst
Superoxide anion production
(control corrected)
Positive synergism
Positive antagonism
Whole body concentration, ng Total PBDEs/g wet weight
(d) Survival time
Mean survival time (control corrected)
Observed Interactive Response
Predicted Additive Response
Negative antagonism
Whole body concentration, ng Total PBDEs/g wet weight