Completion Report

GALLAGHER, Evan

Period: 2/1/2012 - 1/31/2013
Project: R/OCEH-5 - *Effect of salmon Omega-3 Fatty Acids on PBDE toxicity*

:: STUDENTS SUPPORTED

Williams, Chase, crw22@uw.edu, University of Washington, Environmental and Occupational Health Science, status:cont, field of study:Environmental Toxicology, advisor:E. Gallagher, degree type:PhD, degree date:2015-06-01, degree completed this period:No

Student Project Title:
Understanding metal-induced injury and dysfunction in the salmon olfactory system

Involvement with Sea Grant This Period:
Funded graduate student

Post-Graduation Plans:
not applicable

Yeh, Andrew, ayeh3@uw.edu, University of Washington, Environmental and Occupational Health Science, status:cont, field of study:Environmental Toxicology, advisor:E. Gallagher, degree type:PhD, degree date:2015-06-01, degree completed this period:No

Student Project Title:
Biomonitoring of emerging contaminants in the Puget Sound.

Involvement with Sea Grant This Period:
Funded graduate student

Post-Graduation Plans:
not applicable

:: CONFERENCES / PRESENTATIONS

University of Washington Environmental Pathology/Toxicology Training Program (EP/T) biannual seminar - Seattle, WA, USA, public/profession presentation, 25 attendees, 2012-12-03

Society of Environmental Toxicology and Chemistry (SETAC) 33rd Annual Meeting - Long Beach, CA, USA., public/profession presentation, 2000 attendees, 2012-11-14

:: ADDITIONAL METRICS

K-12 Students Reached:	**Acres of degraded ecosystems restored as a result of Sea Grant activities:**
Curricula Developed:	**Resource Managers who use Ecosystem-Based**

	Approaches to Management:
Volunteer Hours:	**HACCP - Number of people with new certifications:**
Cumulative Clean Marina Program - certifications:	

:: PATENTS AND ECONOMIC BENEFITS

Description		Patents	Economic Benefit ($)	Businesses Created	Businesses Retained	Jobs Created	Jobs Retained
	Actual (2/1/2012 - 1/31/2013) :						
	Anticipated (2/1/2013 - 1/31/2014) :						

:: TOOLS, TECH, AND INFORMATION SERVICES

Description		Developed	Used	Names of Managers	Number of Managers
See report for R/OCEH-8	**Actual** (2/1/2012 - 1/31/2013) :				
	Anticipated (2/1/2013 - 1/31/2014) :				

:: HAZARD RESILIENCE IN COASTAL COMMUNITIES

Name of coastal community	County		Number of resiliency trainings / technical assistance services*provided*	Was community hazard resiliency improved (e.g., via changes in zoning ordinances) ?
N/A		**Actual** (2/1/2012 - 1/31/2013) :	0	Yes
		Anticipated (2/1/2013 - 1/31/2014) :	0	Yes

:: ADDITIONAL MEASURES

Safe and sustainable seafood

Number of stakeholders modifying practices
Actual (2/1/2012 - 1/31/2013) :
Anticipated (2/1/2013 - 1/31/2014) :

Number of fishers using new techniques
Actual (2/1/2012 - 1/31/2013) :
Anticipated (2/1/2013 - 1/31/2014) :

Sustainable Coastal Development
Actual (2/1/2012 - 1/31/2013) :
Anticipated (2/1/2013 - 1/31/2014) :

Coastal Ecosystems
Actual (2/1/2012 - 1/31/2013) :
Anticipated (2/1/2013 - 1/31/2014) :

:: PARTNERS

Partner Name: Duke University

Partner Name: National Institute of Environmental Health Sciences, National Institutes of Health, US Department of Health and Human Services (US HHS)

Partner Name: National Marine Fisheries Service (US DOC

Partner Name: University of Washington (UW), Department of Radiology, School of Medicine, type: academic, scale: local

Partner Name: University of Washington (UW), Research Translation and Outreach Core Program, Superfund Research Program, Department of Environmental and Occupational Health Sciences, School of Public Health

Partner Name: University of Washington, Department of Environmental and Occupational Health Sciences, School of Public Health (UW)

Partner Name: US Environmental Protection Agency (US EPA)

:: IMPACTS AND ACCOMPLISHMENTS

Title: **Washington Sea Grant research examines the trade-off between health benefits and pollutant concerns for consumers who eat Puget Sound salmon**

Type: accomplishment

Description:
Relevance: In 2007, the Washington legislature banned polybrominated diphenyl ethers (PBDEs) as flame-retardants and for other uses. However, these toxic organic chemicals are persistent pollutants in Puget Sound sediments and their presence in salmon and other fish has raised public health concerns about seafood safety. Balanced against that concern, salmon also contains beneficial omega-3 polyunsaturated fatty acids in higher concentrations than the PBDEs. Can these antioxidants counteract the effects of oxidant PDBEs? The interaction between these micronutrients and contaminants has not been studied at the molecular level, but any such effect could be important to food safety and public health, and to anxious consumers.
Response: Washington Sea Grant-funded research examined the interaction between antioxidants and PBDEs by comparing two novel, cost-effective approaches: in vitro using human liver cells, and in vivo using zebrafish.
Results: The omega-3s had potent antioxidant action in the cells but provided less than 10 percent protection against PBDE toxicity. Sulforaphane, a compound in broccoli and related vegetables with similar antioxidant properties, afforded only slightly better protection. This suggests that PBDEs have other toxic properties that

antioxidants do not counter. Omega-3s were partially effective at protecting mitochondria from PDBEs; they and sulforaphane improved mitochondrial membrane function in contaminated cells. The zebrafish could not be fed omega-3s but did receive sulforaphane, which protected them somewhat against PBDE-caused heart damage and tail deformation. The approaches tested here could also be used to assess relative benefits and risks of consuming salmon micronutrients together with other chemical contaminants.

Recap:
Washington Sea Grant-supported research assesses the health risks and benefits of eating seafood and shows that antioxidants in salmon and other foods have beneficial effects in cells and may have protective properties against toxic PDBEs in vivo.

Comments:
Primary Focus Area – OCEH (SSSS)

Associated Goals: Reduce toxic, nutrient and pathogen pollutants in water and the marine food web and address their relationships to and impacts on human health (SSSS, Consumers).

Related Partners: Duke University
National Institute of Environmental Health Sciences, National Institutes of Health, US Department of Health and Human Services (US HHS)
National Marine Fisheries Service (US DOC, NOAA, NMFS)
University of Washington (UW), Research Translation and Outreach Core Program, Superfund Research Program, Department of Environmental and Occupational Health Sciences, School of Public Health
University of Washington, Department of Environmental and Occupational Health Sciences, School of Public Health (UW)
University of Washington, Department of Radiology, School of Medicine (UW)
US Environmental Protection Agency (US EPA)

:: PUBLICATIONS

Title: **UW SPH Close Up: Close Up December 2012: Evan Gallagher**

Type: Newsletters Publication Year: 2012
Uploaded File: *none*
URL: http://sph.washington.edu/news/closeup/profile.asp?content_ID=1740

Abstract:
Evan Gallagher tried a variety jobs after college, including playing guitar for touring rock bands. Then he found a niche in environmental toxicology – studying the effects of environmental chemicals. Now, he has become an expert on cells in the tiny noses of salmon, trying to understand how chemicals affect the ability of salmon to locate predators, prey and migrate home. This work can help determine if Superfund sites really are safe for fish such as salmon after site cleanup, and whether other waterways might pose a hazard.

Citation:
University of Washington School of Public Health. 2012. Close Up: Evan Gallagher.

Copyright Restrictions + Other Notes:

Journal Title: *none*

Title: **Of PBDEs and Omega-3s**

Type: Newsletters Publication Year: 2011
Uploaded File: wsg_sea_star_spring_2011.pdf, 801 kb
URL:http://www.wsg.washington.edu/communications/seastar/archives/spring11.pdf

Abstract:
WSG-funded scientist examines the role of Omega-3 fatty acids in protecting our cells against potentially damaging chemicals.

Citation:
Sedlak, R. H. 2011. Of PBDEs and Omega-3s. Washington Sea Grant's quarterly newsletter, Sea Star, Seattle, WA. Spring 2011

Copyright Restrictions + Other Notes:

Journal Title: *none*

Title: **Effect of omega-3 fatty acids on the cellular and mitochondrial toxicity of BDE 47**

Type: Reprints from Peer-Reviewed Journals, Books, Proceedings and Other DocumentsPublication Year: 2013
Uploaded File: *none*
URL: *none*

Abstract:
2,2',4,4'-tetrabromodiphenyl ether (BDE 47) is a toxic flame retardant that despite being banned can be present in food species of fish such as salmon, raising concern over the safety of salmon consumption. However, consumption of salmon is associated with high intakes of omega-3 polyunsaturated fatty acids, i.e. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Because omega-3 fatty acids act as potent antioxidants, and oxidative stress is a mechanism of BDE 47 toxicity, we tested the hypothesis that omega-3s can ameliorate the toxicity of BDE 47. Incubation of HepG2 cells with a mixture of oxidized EPA (oxEPA) and oxidized DHA (oxDHA) 1.5/1 oxEPA/oxDHA induced an antioxidant response, as evidenced by increased glutathione biosynthesis, but only provided marginal protection against BDE 47-induced cellular toxicity. An examination of mitochondrial function revealed that BDE 47 significantly reduced the maximum capacity of electron flux through the electron transport chain (ETC), and State 3 respiratory activity, resulting in a 61% decrease of the respiratory control ratio. oxEPA/oxDHA did not protect against loss of function of the ETC, but did significantly prevent the loss of mitochondrial membrane potential associated with BDE 47. Our results support a beneficial role of omega-3s as antioxidants that protect mitochondrial membranes, but suggest that oxidative stress and injury to mitochondrial membranes play a role in, but are not the primary mechanisms of, BDE 47 toxicity in HepG2 cells. The current study provides mechanistic insight into the biochemical interactions between the beneficial antioxidant micronutrients in fish and PBDEs, filling a key gap in knowledge regarding the risk assessment of the safety of seafood consumption.

Citation:
Yeh A., S.E. Kruse, D.J. Marcinek, and E.P. Gallagher. In submission. Effect of omega-3 fatty acids on the

cellular and mitochondrial toxicity of BDE 47. Journal of Nutritional Biochemistry.

Copyright Restrictions + Other Notes:

Journal Title: Journal of Nutritional Biochemistry

Title: **Role of Nrf2 antioxidant defense in mitigating cadmium-induced oxidative stress in the olfactory system of zebrafish**

Type: Reprints from Peer-Reviewed Journals, Books, Proceedings and Other DocumentsPublication Year: 2012
Uploaded File: wang_2013.pdf, 1614 kb
URL: http://www.ncbi.nlm.nih.gov/pubmed/23174481

Abstract:
Exposure to trace metals can disrupt olfactory function in fish leading to a loss of behaviors critical to survival. Cadmium (Cd) is an olfactory toxicant that elicits cellular oxidative stress as a mechanism of toxicity while also inducing protective cellular antioxidant genes via activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway. However, the molecular mechanisms of Cd-induced olfactory injury have not been characterized. In the present study, we investigated the role of the Nrf2-mediated antioxidant defense pathway in protecting against Cd-induced olfactory injury in zebrafish. A dose-dependent induction of Nrf2-regulated antioxidant genes associated with cellular responses to oxidative stress was observed in the olfactory system of adult zebrafish following 24h Cd exposure. Zebrafish larvae exposed to Cd for 3h showed increased glutathione S-transferase pi (gst pi), glutamate-cysteine ligase catalytic subunit (gclc), heme oxygenase 1 (hmox1) and peroxiredoxin 1 (prdx1) mRNA levels indicative of Nrf2 activation, and which were blocked by morpholino-mediated Nrf2 knockdown. The inhibition of antioxidant gene induction in Cd-exposed Nrf2 morphants was associated with disruption of olfactory driven behaviors, increased cell death and loss of olfactory sensory neurons (OSNs). Nrf2 morphants also exhibited a downregulation of OSN-specific genes after Cd exposure. Pre-incubation of embryos with sulforaphane (SFN) partially protected against Cd-induced olfactory tissue damage. Collectively, our results indicate that oxidative stress is an important mechanism of Cd-mediated injury in the zebrafish olfactory system. Moreover, the Nrf2 pathway plays a protective role against cellular oxidative damage and is important in maintaining zebrafish olfactory function.

Citation:
Wang L., and E.P. Gallagher. 2013. Role of Nrf2 antioxidant defense in mitigating cadmium-induced oxidative stress in the olfactory system of zebrafish. Toxicology and Applied Pharmacology, 226(2) 177-186. DOI: 10.1016/j.taap.2012.11.010

Copyright Restrictions + Other Notes:

Journal Title: Toxicology and Applied Pharmacology

Title: **Cloning, expression and analysis of the olfactory glutathione S-transferases in coho salmon**

Type: Reprints from Peer-Reviewed Journals, Books, Proceedings and Other DocumentsPublication Year: 2013
Uploaded File: espinoza_2013.pdf, 1803 kb
URL: http://www.ncbi.nlm.nih.gov/pubmed/23261526

Abstract:
The glutathione S-transferases (GSTs) provide cellular protection by detoxifying xenobiotics, maintaining redox

status, and modulating secondary messengers, all of which are critical to maintaining olfaction in salmonids. Here, we characterized the major coho salmon olfactory GSTs (OlfGSTs), namely omega, pi, and rho subclasses. OlfGST omega contained an open reading frame of 720bp and encoded a protein of 239 amino acids. OlfGST pi and OlfGST rho contained open reading frames of 627 and 681nt, respectively, and encoded proteins of 208 and 226 amino acids. Whole-protein mass spectrometry yielded molecular weights of 29,950, 23,354, and 26,655Da, respectively, for the GST omega, pi, and rho subunits. Homology modeling using four protein-structure prediction algorithms suggest that the active sites in all three OlfGST isoforms resembled counterparts in other species. The olfactory GSTs conjugated prototypical GST substrates, but only OlfGST rho catalyzed the demethylation of the pesticide methyl parathion. OlfGST pi and rho exhibited thiol oxidoreductase activity toward 2-hydroxyethyl disulfide (2-HEDS) and conjugated 4-hydroxynonenal (HNE), a toxic aldehyde with neurodegenerative properties. The kinetic parameters for OlfGST pi conjugation of HNE were K(M)=0.16±0.06mM and V(max)=0.5±0.1μmolmin(-1)mg(-1), whereas OlfGST rho was more efficient at catalyzing HNE conjugation (K(M)=0.022±0.008mM and V(max)=0.47±0.05μmolmin(-1)mg(-1)). Our findings indicate that the peripheral olfactory system of coho expresses GST isoforms that detoxify certain electrophiles and pesticides and that help maintain redox status and signal transduction.

Citation:
Espinoza H.M., L.M. Shireman, V. McClain, W. Atkins, and E.P. Gallagher. 2012. Cloning, expression and analysis of the olfactory glutathione S-transferases in coho salmon. Biochemical Pharmacology 85(6): 839-848. DOI: 10.1016/j.bcp.2012.11.018

Journal Title: Biochemical Pharmacology

Title: **BDE 49 is a developmental toxicant in zebrafish**

Type: Reprints from Peer-Reviewed Journals, Books, Proceedings and Other DocumentsPublication Year: 2012
Uploaded File: mcclain_2011.pdf, 421 kb
URL: http://www.ncbi.nlm.nih.gov/pubmed/21951712

Abstract:
The polybrominated diphenyl ethers (PBDEs) are a group of brominated flame retardants. Human health concerns of these agents have largely centered upon their potential to elicit reproductive and developmental effects. Of the various congeners, BDE 49 (2,2',4,5'-tetrabromodiphenyl ether) has been poorly studied, despite the fact that it is often detected in the tissues of fish and wildlife species. Furthermore, we have previously shown that BDE 49 is a metabolic debromination product of BDE 99 hepatic metabolism in salmon, carp and trout, underscoring the need for a better understanding of biological effects. In the current study, we investigated the developmental toxicity of BDE 49 using the zebrafish (Danio rerio) embryo larval model. Embryo and larval zebrafish were exposed to BDE 49 at either 5 hours post fertilization (hpf) or 24 hpf and monitored for developmental and neurotoxicity. Exposure to BDE 49 at concentrations of 4iμ-32 μM caused a dose-dependent loss in survivorship at 6 days post fertilization (dpf). Morphological impairments were observed prior to the onset of mortality, the most striking of which included severe dorsal curvatures of the tail. The incidence of dorsal tail curvatures was dose and time dependent. Exposure to BDE 49 caused cardiac toxicity as evidenced by a significant reduction in zebrafish heart rates at 6 dpf but not earlier, suggesting that cardiac toxicity was non-specific and associated with physiological stress. Neurobehavioral injury from BDE 49 was evidenced by an impairment of touch-escape responses observed at 5 dpf. Our results indicate that BDE 49 is a developmental toxicant in larval zebrafish that can cause morphological abnormalities and adversely affect neurobehavior. The

observed toxicities from BDE 49 were similar in scope to those previously reported for the more common tetrabrominated congener, BDE 47, and also for other lower brominated PBDEs, suggest that these compounds may share similarities in risk to aquatic species.

Citation:
McClain V., H.M. Stapleton, F. Tilton, and E.P. Gallagher. 2011. BDE 49 is a developmental toxicant in zebrafish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 155(2) 253-258.

Copyright Restrictions + Other Notes:

Journal Title: Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology

:: OTHER DOCUMENTS

No Documents Reported This Period

:: LEVERAGED FUNDS

Type: influenced Period: 2012-02-01::2013-01-31Amount: $653

Purpose:
Salary support for the research scientist Michael Espinoza and graduate student Chase Williams for work on the project. NOT REPORTED

Source: PIs Murphy chair endowment and Department of Environmental and Occupational Health Sciences

Type: influenced Period: 2012-02-01::2013-01-31Amount: $759

Purpose:
Supplement for supplies needed money for zebrafish husbandry (water system purification contract). NOT REPORTED

Source: PIs indirect cost returned to the investigator by the department from other grants.

Type: influenced Period: 2012-02-01::2013-01-31Amount: $300

Purpose:
Supplement for supplies needed money for laboratory office supplies. NOT REPORTED

Source: PIs indirect cost returned to the investigator by the department from other grants.

Type: influenced Period: 2012-02-01::2013-01-31Amount: $11179

Purpose:
Partial salary support offset for the PI to devote more supervision to researchers and graduate students and project coordination time associated with the project. NOT REPORTED

Source: PIs Murphy chair endowment and Department of Environmental and Occupational Health Sciences

Type: influenced Period: 2012-11-13::2012-11-18Amount: $362

Purpose:
Travel award to Andrew Yeh for conference attendance, Society of Environmental Toxicology and Chemistry

Source: NIEHS Environmental Pathology/Toxicology Training Grant

Type: influenced Period: 2012-02-01::2013-01-31Amount: $8825

Purpose:
NIEHS Environmental Pathology/Toxicology Training Grant Award to Chase Williams

Source: NIEHS Environmental Pathology/Toxicology Training Grant Award

Type: influenced Period: 2012-02-01::2013-01-31Amount: $8825

Purpose:
NIEHS Environmental Pathology/Toxicology Training Grant Award to Andrew Yeh

Source: NIEHS Environmental Pathology/Toxicology Training Grant Award

Section I. A report that describes progress made towards meeting project objectives during **THIS REPORTING PERIOD**, that includes activities carried out, participants, results, challenges encountered, any changes in project direction, etc. **Length**: Two to three pages, include tables and figures as relevant.

WASHINGTON SEA GRANT PROGRESS REPORT

for the period 2/1/2012 – 1/31/2013

1. PROJECT OBJECTIVES.

The overall goal of this project was to use interfacing *in vitro* and *in vivo* biomedical models to investigate if salmon omega-3 polyunsaturated fatty acids may protect against the toxicity and developmental injury of BDE 47 (2,2',4,4'-tetrabromodiphenyl ether). BDE 47 is a major PBDE congener in this group of flame retardants that is often measured in fish and human tissues. We used human liver HepG2 cells as a human cell model to characterize dose-responses and biochemical interactions between PBDEs and omega-3s, as well as to study mechanisms of potential chemoprotection. *In vivo* studies using the zebrafish model were used to further test hypotheses associated with the role of NF-E2 related factor-2 (Nrf2)-dependent pathways in chemoprotection against *in vivo* developmental injury. We tested the following two hypotheses: 1) the toxicity of PBDEs to human HepG2 cells is ameliorated by salmon omega-3s; 2) Nrf2-mediated antioxidant gene and glutathione (GSH) induction by omega-3s and similar dietary antioxidants are mechanisms of chemoprotection against BDE 47 cell injury and *in vivo* developmental toxicity.

Our objectives included work in two specific aims:
Specific Aim 1. Characterize the modulatory effects and dose-response relationship of omega-3s on BDE 47 oxidative injury to human HepG2 cells.
Specific Aim 2. Use *in vitro* and *in vivo* studies with human HepG2 cells and zebrafish to determine if Nrf2-mediated antioxidant gene expression and redox status by omega-3s is a predominant mechanism of omega-3 chemoprotection against BDE 47.

2. PROJECT PROGRESS.

Specific Aim 1 progress and results: The omega-3s in the edible portions of salmon are very labile and quickly undergo a chemical oxidation reaction. Previous studies have also demonstrated that when taken up through the diet, cells naturally modify salmon omega-3s via chemical oxidation, so intake of oxidized omega-3s is relevant to the dietary consumption of salmon. Importantly, it has been shown that only oxidized omega-3s, and not their un-oxidized, unaltered forms, trigger the beneficial antioxidant effects associated with omega-3s observed in the clinic. In the *in vitro* chemoprotection experiments, we chemically-modified the salmon omega-3s by an oxidation process to increase their antioxidant effects in cells. The efficacy of this approach has been demonstrated in other studies. We then tested the hypothesis that treating HepG2 cells directly with oxidized omega-3s would induce a more robust antioxidant response than with un-oxidized omega-3s, and provide the greatest protection against the cellular toxicity

of BDE 47. Some experiments included comparisons with other antioxidant chemoprotective agents.

After assay optimization, we characterized the antioxidant responses induced by oxidized omega-3s, and the modulatory effects of the omega-3s on the cell toxicity by BDE 47. As expected, treatment with oxidized omega-3s caused a potent antioxidant response in cells, as evidenced by the two-fold increase of levels of the protective cellular antioxidant, glutathione (GSH) in cells **[Table 1]**. By contrast, un-oxidized omega-3s induced only a 20% increase in GSH after the same length of exposure as the oxidized forms in HepG2 cells (Data not shown). Additionally, the antioxidant effects of the oxidized omega-3s were somewhat more potent to those observed with the model antioxidant chemoprotectant, sulforaphane (SFN); this finding plays a role in the experiments of **Aim 2**, described in detail in the subsequent section. Following these observations, we conducted several experiments using the oxidized forms of the omega-3s to test their ability to reverse cell injury by BDE 47. We observed only minor protection (<10%) against the toxicity observed under high doses of BDE 47 in cells **[Figure 1]**.

Specific Aim 2 progress and results. Much of our efforts during this reporting period (year 2) were concentrated on Aim 2 in spinoff studies. Aim 2 studies involved more mechanistic approaches to understand the effects of salmon omega-3s at the cellular level, and also used zebrafish as an *in vivo* model to test for protective effects. As discussed in **Aim 1** progress, we observed an increase of cellular glutathione (GSH) indicative of activation of an Nrf2 protective response following treatment with omega-3s, but did not observe chemoprotection against BDE 47 cell injury. In Specific Aim 2 experiments, we probed further into the molecular mechanism underlying the protective effects of antioxidants. Because omega-3s are not relevant to feeding studies in zebrafish, these studies utilized sulforaphane (SFN), a dietary antioxidant molecule with similar cellular effects on Nrf2 as salmon omega-3s. SFN is an anticancer compound in cruciferous vegetables, and experiments from the previous reporting period indicated that this compound is also relevant for experiments in HepG2 cells. As mentioned previously, when we compared SFN to the omega-3s for their antioxidant capacities in cells, SFN and oxidized omega-3s were similarly-potent activators of Nrf2-mediated protective responses **[Table 1]**. Concordantly, in the subsequent *in vitro* chemoprotection experiments, SFN and omega-3s provided similar marginal protection against BDE 47 toxicity to cell viability and membranes of the mitochondria **[Figure 3A and 3B]**.These results suggested that the Nrf2-mediated pathways activated by dietary antioxidants may play only a minor role in protection against the toxicity of BDE 47 *in vitro*.

Several sets of experiments were completed in zebrafish. We conducted *in vivo* experiments investigating the protective effects of dietary antioxidants on the developmental toxicity of BDE 47 in embryonic zebrafish. Treatment with SFN caused an antioxidant response in zebrafish, indicated by an increase in glutamate cysteine ligase catalytic subunit (*GCLC*), an Nrf2-responsive enzyme responsible for GSH biosynthesis **[Figure 4A]**. Interestingly, pretreatment of zebrafish embryos with SFN partially protected against BDE 47-induced developmental toxicity as evidenced by reduced tail curvatures **[Figure 4B]**. Additionally, BDE 47 significantly induced heart defects in larval zebrafish, and co-treatment with SFN partially prevented these abnormalities. However, the effect was not statistically significant likely due to small sample

sizes **[Figure 4C]**. We are following up on these observations in additional zebrafish experimental work via funding from non-Sea Grant mechanisms before we publish.

Summary of results: Our results suggest that salmon omega-3s, while being potent antioxidants, do not ameliorate the toxicity of BDE 47 (the major PBDE congener often measured in fish) in human HepG2 cells. While this is not supportive of our hypothesis, our laboratory cell studies were conducted under acute exposure conditions that do not mimic the scenario of fish consumption in humans, and used higher doses than are relevant for human consumption of fish, for *in vitro* toxicological purposes. This approach using higher doses was necessary due to limits of detection of the cellular endpoints. Furthermore, the HepG2 cells are transformed cell lines that have limitations relative to use of primary human cells, and extrapolations from cell studies do not account for the complex biological processes that occur *in vivo*. It is also possible that while one important component of cell toxicity, may not be the major mechanism of the developmental and mitochondrial toxicity of BDE 47. Interestingly however, the fact that another model antioxidant (i.e. SFN) with some similar characteristics to salmon omega-3s does provide some protection against *in vivo* BDE 47 developmental injury in zebrafish is notable and suggests further work is needed to characterize these effects.

3. ACTIVITIES CARRIED OUT

In addition to completing the laboratory investigations, graduate student Andrew Yeh attended one international and one department-wide conference to present the results of the Sea Grant project. The PI also presented at an international meeting, as well as at a department-wide seminar for faculty and new graduate students. During this reporting period we also conducted laboratory tours for local high school teachers, describing Sea Grant-funded zebrafish study methods. A new collaborative partnership was developed during this reporting period with Dr. David J. Marcinek at the University of Washington, Department of Radiology, as part of the investigation of BDE 47 toxicity to mitochondria (described in detail in Section 5 below).

4. CHALLENGES

No major challenges were encountered in carrying out project tasks during this reporting period.

5. CHANGES IN PROJECT DIRECTION.

As a novel, unexplored area of research, we believed that the mitochondrial aspect of BDE 47 toxicity to mitochondria was a relevant area that needed more detailed investigation. Accordingly, while not part of the original specific aims, we initiated a series of spinoff experiments probing further into the intricacies of the interactions of BDE 47 with cell mitochondria. Mitochondria are cellular organelles often described as the cell’s "power plants" because they generate most of the cell's supply of ATP, which supplies chemical energy. Mitochondria are particularly important in critical cell processes such as cell cycle progression and cell growth regulation, and dysfunctional mitochondria have been implicated in a number of human diseases. Our rationale was that the antioxidant responses under investigation are particularly important for protecting the function of the mitochondrial electron transport chain

(ETC). The mitochondrial ETC is vital for producing the majority of a cell's energy, but in doing so, also inadvertently generates highly toxic reactive oxygen species (ROS). These experiments focused specifically on monitoring injury to mitochondrial membranes, and measuring functional impacts on individual molecular components of the mitochondrial ETC, and were conducted by the graduate student in the laboratory of Dr. David J. Marcinek at the University of Washington, Department of Radiology. We determined that BDE 47 inhibits the function of specific components of the mitochondrial ETC, decreasing mitochondrial function by more than 60% overall **[Figure 2A]**. Importantly, we observed oxidized omega-3s had no protective effect against the injury to mitochondrial ETC function associated with BDE 47 **[Figure 2A, and Table 2]**. However, oxidized omega-3s were significantly beneficial for preserving the integrity of mitochondrial membranes after BDE 47 exposure **[Figure 2B]**. In addition to the mitochondrial studies, two separate but related studies were completed and published during this reporting period with the purpose of better understanding the protective antioxidant mechanisms of the salmon olfactory system.

6. PARTICIPANTS

The majority of project-related activities were performed by 2 graduate students (Chase Williams and Andrew Yeh). Contributions were also made by the laboratory manager (Michael Espinoza), and Dr. Shane E. Kruse, a postdoctoral fellow in the laboratory of Dr. Marcinek.

Section 1 - Tables and Figures:

Table 1. Effect of oxidized EPA and DHA (oxEPA/oxDHA) on glutathione (GSH) levels in HepG2 cells.

	Intracellular GSH (nmoles GSH/mg protein)		
	6 hrs	**12 hrs**	**18 hrs**
Control	6.1 ± 0.3	6.5 ± 0.8	6.1 ± 0.4
oxEPA/oxDHA	11.0 ± 0.7***	13.0 ± 1.1***	13.0 ± 0.3***
SFN	8.0 ± 1.3	12.0 ± 0.9**	12.0 ± 0.7***

After treatment with the carrier (DMSO) or dietary antioxidants, the cells were harvested and GSH levels determined using a fluorometric assay dependent on the conjugation of NDA with GSH, which generates a stable fluorescent product detected at excitation 472 nm/emission 528 nm. GSH concentrations were normalized to total protein. Data are expressed as mean ± SD (n=3). **$p \leq 0.01$, ***$p \leq 0.001$ indicates differences in GSH concentration relevant to controls.

Table 2. Assessment of BDE 47- effects on mitochondrial respiration.

	Mean Oxygen Consumption (pmoles O_2/[sec*mg protein]) ± SD (n=4)		
Respiratory chain parameter (respiratory substrate/inhibitor)	**Control**	**BDE 47**	**oxEPA/oxDHA + BDE 47**
State 3 (Pyruvate, malate, glutamate, ADP)	61 ± 4	28 ± 7***	38 ± 10**
Maximum State 3 (Above conditions, plus succinate)	79 ± 7	36 ± 9***	48 ± 10**
State 4 (Proton Leak) (Above conditions, plus oligomycin)	17 ± 4	13 ± 4	17 ± 1
Uncoupled (Above conditions, plus CCCP)	182 ± 6	70 ± 18***	99 ± 23***
Complex II (Above conditions, plus rotenone)	78 ± 11	53 ± 19	58 ± 15
Non-mitochondrial respiration (Above conditions, plus Antimycin-A)	46 ± 1	30 ± 2*	24 ± 6*
Complex IV (Above conditions, plus TMPD, and ascorbate)	162 ± 28	116 ± 28	156 ± 35

The substrates/inhibitors of the mitochondrial electron transport chain (ETC) used to assess the specific respiratory parameters are indicated, as well as which functional parameter of the mitochondrial ETC they were used to assess. Pretreatment with oxEPA/oxDHA was not protective on any of the tested functional parameters. Data represent mean oxygen consumption ± SD (n=4). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ relative to controls.

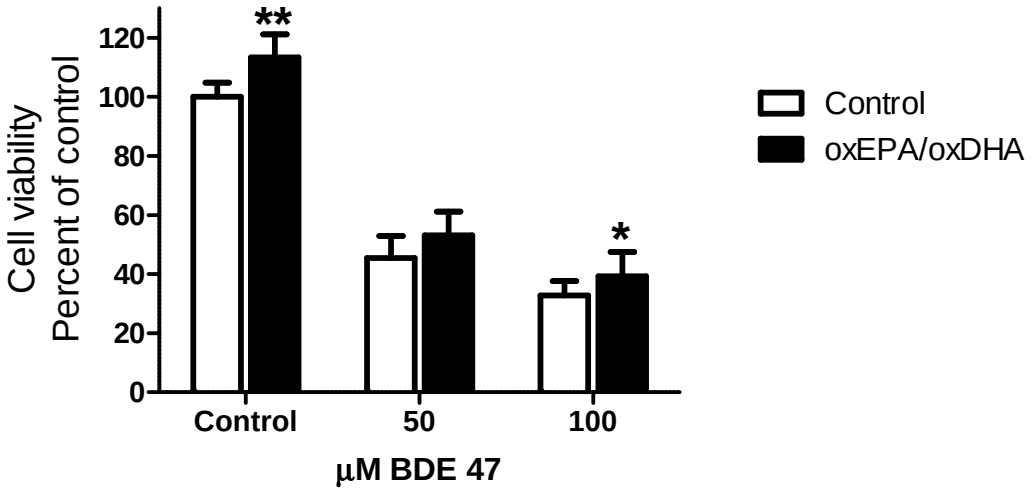


Figure 1. Effect of oxEPA/oxDHA on the cellular toxicity of BDE 47. Cells were dosed with the carrier (DMSO), or a 1.5/1 mixture of omega-3 fatty acids relevant to salmon (60 µM EPA/40 µM DHA). After 12 hrs, cells were exposed to BDE 47 for an additional 24 hours. The viability of cells was then measured by the MTT assay. As observed, pretreatment with the salmon omega-3 mixture significantly increased the overall viability of HepG2 cells (** $p<0.01$) in culture. However, <10% overall protection of the omega-3s against BDE 47 toxicity was observed. Data indicate mean and standard deviation of three experimental replicates, with 3 technical replicates/treatment and experiment.

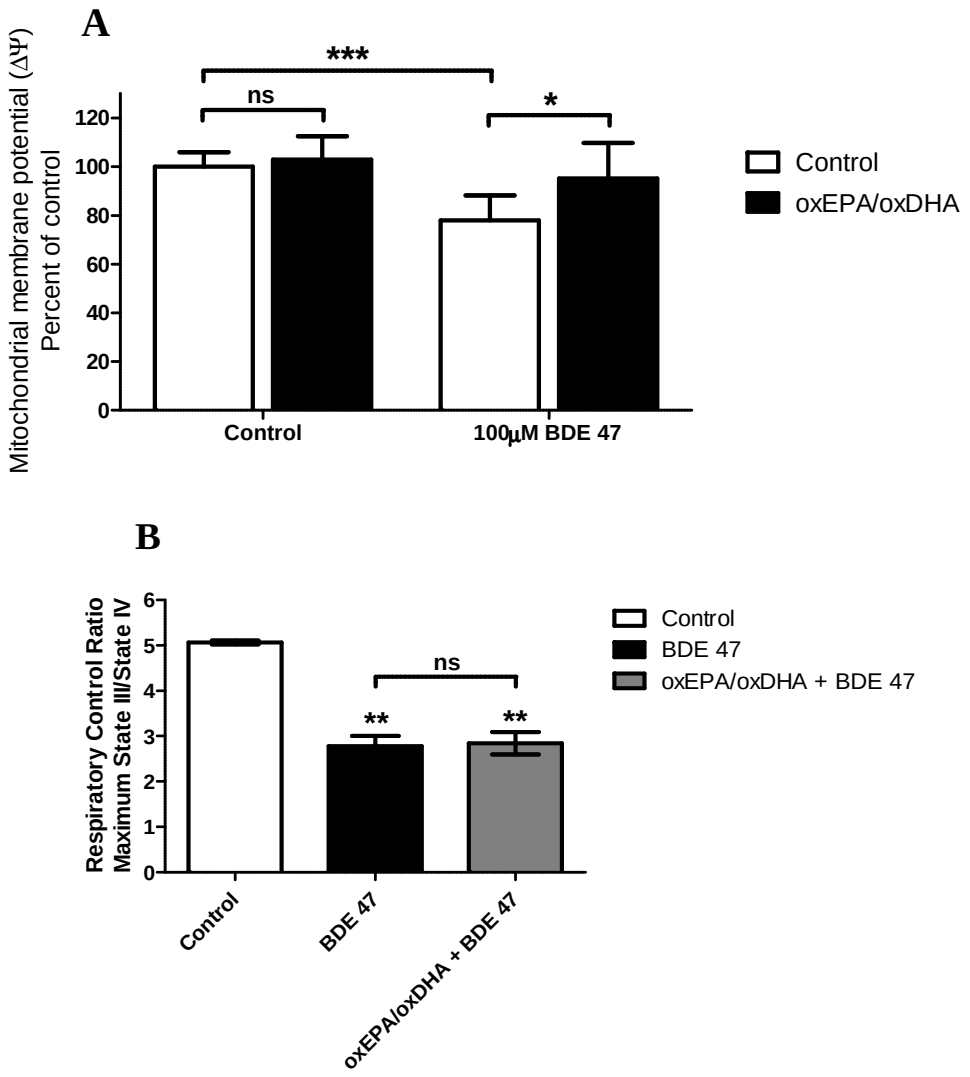
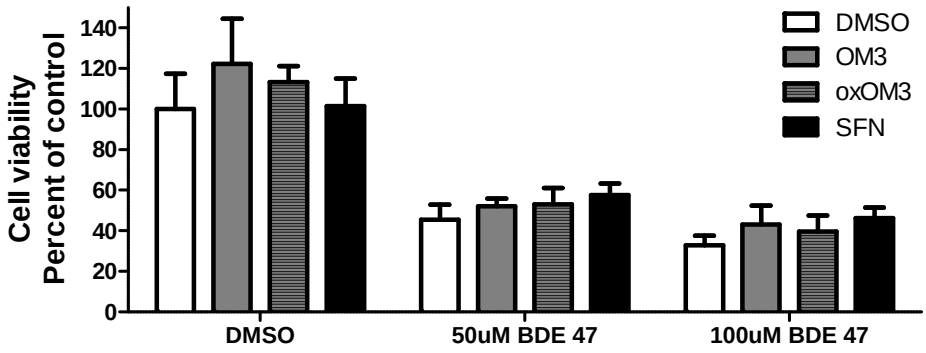


Figure 2. Effect of oxEPA/oxDHA on the mitochondrial toxicity of BDE 47. (A) The oxidized omega-3s (oxEPA/oxDHA) protected the mitochondrial membranes by preventing the loss of membrane potential ($\Delta\Psi_m$) caused by BDE 47. Data are the mean ± SD (n=6). *p≤0.05, ***p≤0.001 relative to controls. **(B)** The Respiratory Control Ratio (RCR) is a measure of mitochondrial function and health and defined as the ratio of oxygen consumption rate during State 3 respiration divided by the rate during State 4 respiration. As observed, BDE 47 significantly decreased the RCR of cells by 60%. Pretreatment with oxEPA/oxDHA had no protective effect. Data are the mean ± SEM (n=4). **p≤0.01 relative to controls.

A.



B.

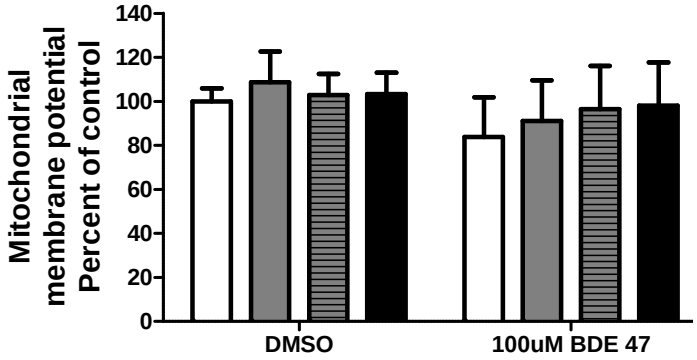


Figure 3. Comparison of the protective effect of omega-3s and SFN against BDE 47 cell toxicity. (A) Omega-3s (whether un-oxidized or oxidized) and SFN offered marginal protection against BDE 47 cell toxicity. **(B)** The protective capacities of omega-3s and SFN on mitochondrial membrane potential after exposure to BDE 47 (100 µM) were also similar. Data are mean ± SD (n=3 experimental replicates).

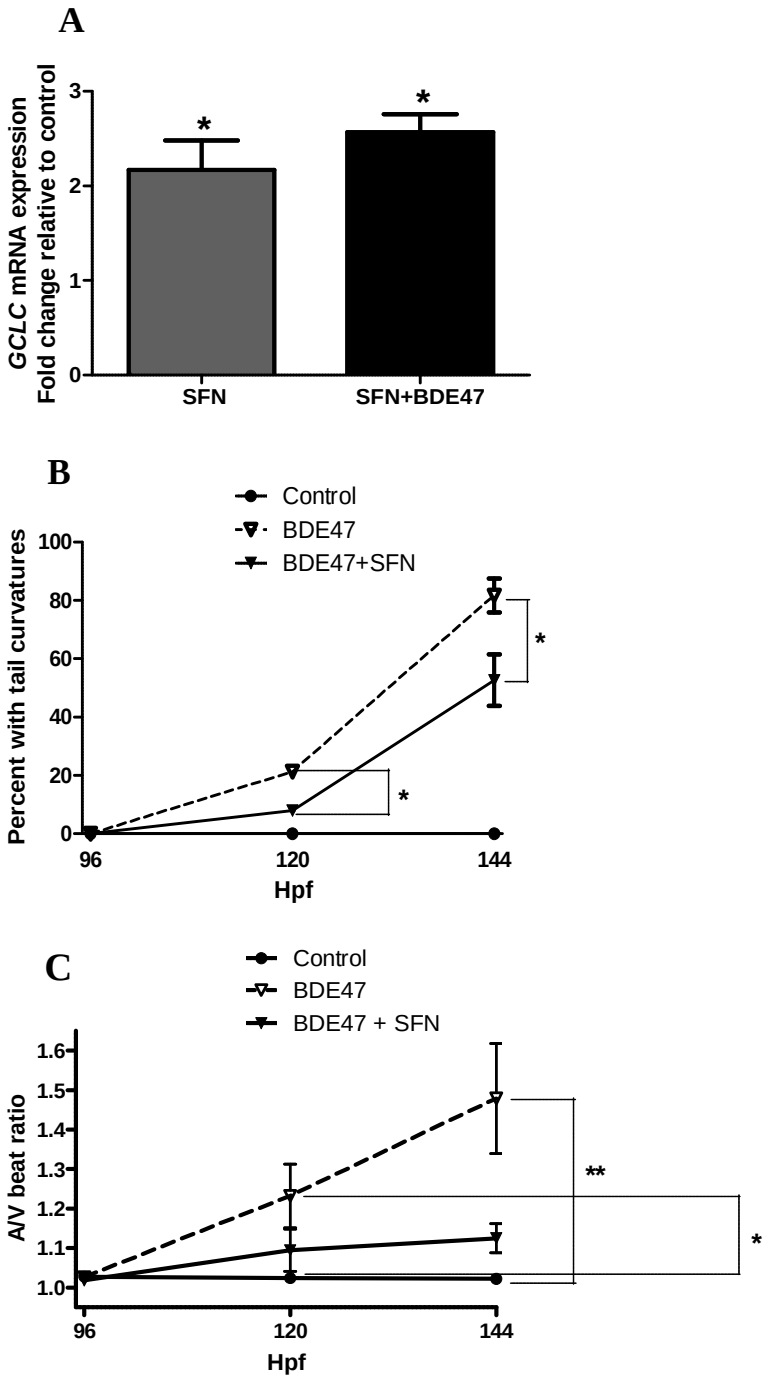


Figure 4. Effect of sulforaphane (SFN) on antioxidant gene expression and developmental toxicity of BDE 47. (A) Treatment of zebrafish with SFN (40µM) for 48 hrs induced the expression of *gclc* as quantified by QPCR. Data represent mean ± SEM (n=6 groups with 15 individuals per group). **(B)** SFN co-treatment significantly, protective against BDE 47-induced tail curvatures in zebrafish larvae, mean ± SEM (n=192 individuals), and **(C)** cardiac abnormalities, mean ± SEM (n=35 individuals). *p≤0.05, **p<0.001 indicate statistical significance relative to control.

Section II. A summary report for the **FULL PROJECT DURATION** that includes project objectives, methodology, rationale, major findings (include figures and tables) and the significance of the results. If relevant, also include students supported (number and degree level), partnerships and outreach and information/technology transfer activities. **Length**: Eight to ten pages, include tables and figures as relevant. Please note that this summary may be made available to the public via the Washington Sea Grant Web site.

WASHINGTON SEA GRANT
PROJECT COMPLETION SUMMARY REPORT

WSG Project Number: **R/OCEH-5**
Project Title: Effect of salmon omega-3 fatty acids on PBDE toxicity

Project Period: 2/1/2010 – 1/31/2013

Principal Investigator and Affiliation:
Evan Gallagher, University of Washington, Department of Environmental and Occupational Health Sciences

PROJECT COMPLETION SUMMARY REPORT

1. RATIONALE.

Polybrominated diphenyl ether (PBDE) flame retardants are environmentally-persistent compounds that are measured in humans and wildlife species. The human health concerns of these agents largely arise from residues detected in serum and in breast milk of women, and the fact that some of these compounds have been shown to be developmental toxicants in animal studies. Although others have shown that household exposures are important for the majority of the population, including exposure to indoor dust, the diet can also be an important route of exposure. Some studies have raised concern regarding PBDE residues in food fish, such as salmon. In the Puget Sound region, Chinook salmon are an important food source, and Chinook from certain regions of the Puget Sound have particularly high levels of PBDEs. This sets up a potential scenario of dietary PBDE exposures, especially in sensitive populations that consume particularly large amounts of salmon. The most prevalent PBDE congener typically detected in Puget Sound salmon is BDE 47 (2,2',4,4'-tetrabromodiphenyl ether), which has been shown to be a developmental and neurological toxicant in animal studies. By contrast, consumption of salmon also has well-documented health benefits, including those due the high content of the antioxidant micronutrients known as omega-3 polyunsaturated fatty acids. On the molecular level, salmon omega-3 fatty acids can activate a robust cellular antioxidant response that includes induction of cellular glutathione (GSH), a cellular pathway that affords protection against many environmental chemicals. Our laboratory has previously demonstrated that the toxicity of BDE 47 occurs through a process termed *oxidative stress*, which is reversed by a model antioxidant compound in human cells. Accordingly, we hypothesized that dietary intake of omega-3s, associated with salmon consumption would protect against the cellular toxicity of BDE 47. Due to the scope of Sea Grant funding, we conducted these studies using an *in vitro* toxicological approach using immortalized human liver hepatoma cells (i.e.

HepG2 cells) and *in vivo* studies using zebrafish, a high-throughput laboratory model and less expensive alternative to relative animal studies.

2. OBJECTIVES.

The overall goal of this project was to use interfacing *in vitro* and *in vivo* models to investigate the mechanistic basis by which salmon omega-3 polyunsaturated fatty acids may protect against the toxicity of, and developmental injury from, PBDEs. As discussed, we used human liver HepG2 cells as a relevant human cell model to characterize dose-responses and biochemical interactions between PBDEs and omega-3s, and to study mechanisms of potential chemoprotection. Our focus was on the particular pathway of chemoprotection involving NF-E2 related factor-2 (Nrf2), a cellular transcription factor that promotes the transcription of protective antioxidant genes, and that is a major drug target for the prevention of cancer. *In vivo* studies using the zebrafish model were used to further test hypotheses associated with the role of Nrf2-dependent pathways in chemoprotection, and its applications to prevention of *in vivo* developmental injury. We tested two hypotheses: 1) the toxicity of PBDEs to human HepG2 cells is ameliorated by salmon omega-3s; 2) Nrf2-mediated antioxidant gene and glutathione (GSH) induction by omega-3s and similar dietary antioxidants are mechanisms of chemoprotection against cell injury and *in vivo* developmental toxicity of PBDEs. This work was accomplished via two specific aims:

Specific Aim 1. Characterize the modulatory effects and dose-response relationship of omega-3s on BDE 47 oxidative injury to human HepG2 cells.

Specific Aim 2. Determine if omega-3 activation of antioxidant mechanisms transcriptionally-regulated by NR-E2-related factor-2 (Nrf2), including modulation of cellular redox status, is a predominant mechanism of omega-3 chemoprotection against BDE 47.

3. METHODOLOGY.

The major omega-3 fatty acids found in fish oil are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Based on the ratio of EPA/DHA in omega-3 dietary supplements, we treated human liver HepG2 cells with a 1.5/1 EPA/DHA mixture before exposure to BDE 47. In the experiments of **Specific Aim 1**, the maximal antioxidant effects of omega-3s in HepG2 cells were established before investigation of the mechanisms of omega-3 antioxidant chemoprotection *in vitro*. Additionally, we altered the chemical structure of EPA and DHA in a process known as oxidation which greatly increases their antioxidant effects in cells. Salmon omega-3s become rapidly oxidized upon exposure to the air, and also occurs naturally in cells during metabolism, so dietary intake of oxidized omega-3s is relevant to the consumption of fish. Thus, HepG2 cells were similarly treated with a 1.5/1 mixture of chemically-oxidized EPA and DHA before exposure to BDE 47. Our approach was to examine the protective effect of the salmon omega-3s using a number of cellular endpoints, including viability, antioxidant status, gene expression by quantitative PCR (qPCR), and mitochondrial health.

In **Specific Aim 2,** we used another model antioxidant, sulforaphane (SFN), which has been shown to be a beneficial anticancer compound found in cruciferous vegetables. SFN also serves as a well-characterized inducer of Nrf2-related antioxidant responses, and is thus a relevant surrogate

compound for the omega-3s in the *in vivo* zebrafish studies. This is due to the fact that it was not appropriate to use dietary omega-3s in dietary studies in fish. Accordingly, our approach was to test the hypothesis of **Specific Aim 2** by exposing larval zebrafish embryos to BDE 47 in the presence and absence of SFN, and analyze expression of antioxidant genes by qPCR to determine an antioxidant response by SFN. We then determined the effect of SFN in protecting against a set of neurodevelopmental injury endpoints, including incidence of tail curvatures and effects on cardiac function.

Related spinoff experiments relevant to the aims of the grant. Several spinoff experiments were conducted through leveraging of non-Sea Grant resources. For example, we carried out developmental studies with BDE 49, another tetrabromodiphenyl ether flame retardant that shares structural similarities with BDE 47 and is a contaminant in salmon that can be produced from the debromination of BDE 99, a common higher molecular PBDE congener. BDE 49 had not been analyzed for its potential to cause developmental injury, which we felt this was an important data gap in the ecological risk assessment of PBDEs. As discussed in the progress report for the final reporting period, we also conducted a study targeting the effects of omega-3s and BDE 47 on components of the mitochondrial electron transport chain. We felt this was an important, yet unexplored area of research and pertinent to characterize the beneficial effects of salmon omega-3s at the cellular level. In addition, leveraging from our NIEHS funding with Sea Grant allowed us to better define the ability of salmon to detoxify environmental chemicals through components of the protective cellular glutathione system, with a focus on glutathione peroxidase and glutathione *S*-transferase enzymes.

4. MAJOR FINDINGS.

1. Salmon omega-3s can induce a robust antioxidant response in human HepG2 cells, as indicated by the activation of Nrf2 reactive proteins and an increase in cellular GSH **[Figures 1 and 2,** and **Table 1]**. This constitutes an important antioxidant effect of omega-3s at the cellular level. Another spinoff study partially supported by Sea Grant with funding leveraged from our NIEHS center showed that Nrf2 was an important pathway for protecting zebrafish against the olfactory toxicity of cadmium, an environmental pollutant of concern in the lower Duwamish waterway, a Superfund site in Seattle undergoing remediation.

2. Contrary to our initial hypothesis, salmon omega-3s provided only marginal protection against BDE 47 cellular toxicity, despite their antioxidant effects **[Figure 3]**.

3. At the mitochondrial level, we discovered that BDE 47 depolarized mitochondrial membranes and significantly decreased mitochondrial function, inhibiting critical molecular components involved in energy production (i.e. the mitochondrial electron transport chain). This has not been reported.

4. The antioxidant effects of oxidized omega-3s did not protect against injury to mitochondrial electron transport chain components **[Figure 4B, Table 2]**, but did prevent BDE 47 injury to mitochondrial membranes **[Figure 4A]**.

5. *In vivo* studies in zebrafish revealed robust antioxidant effects of SFN **[Figure 5A]** similar to observed in cells that elicited minor protective effects in minimizing the occurrence of BDE 47 developmental defects (i.e. tail curvatures, and cardiac dysfunction **[Figure 5B and C]**). We are

following up in more detail to determine the significance, and to investigate the mechanisms, underlying the partial protection of SFN against developmental toxicity. For the most part, our studies in HepG2 cells are in agreement with our zebrafish studies. These studies suggest that oxidative stress is a component of BDE 47 developmental injury in zebrafish, and supports the utility of this model is a low-cost alternative to rodents in future studies evaluating salmon contaminants.

6. Our comparative developmental study in zebrafish showed that BDE 49 was also a developmental toxicant, with roughly similar potency as BDE 47, and a manuscript of our results has been published (*Comparative Physiology and Biochemistry,* 2011).

7. Although the biochemical ability of salmon to detoxify environmental chemicals was not a focus of this project, our spinoff study demonstrated a fairly-extensive glutathione cycle detoxification system in salmon. (*Biochemical Pharmacology*, 2012).

5. SIGNFICANCE OF FINDINGS.

1. Our results support a role of salmon omega-3s as beneficial micronutrients that act of the cellular level by increasing the levels of protective cellular antioxidant molecules, and by maintaining mitochondrial membranes and mitochondrial function. These are significant findings.

2. Currently, the benefit of salmon omega-3s to mitochondria are debated and poorly understood. The novel observations regarding the lack of protection to mitochondrial electron transport chain components by salmon omega-3s, but providing protection of mitochondrial membranes, helps to elucidate the complex interactions of omega-3s with mitochondria at the molecular level. Additionally, this study is the first to determine the toxic effects of BDE 47 on specific components of the mitochondrial electron transport chain, providing novel insight into potential mechanisms underlying the mitochondrial toxicity of BDE 47.

3. A major protective effect of omega-3s against BDE 47 toxicity in immortalized cells was not observed. However, there are several caveats to this observation. As discussed, our laboratory cell studies were conducted under acute exposure conditions that do not mimic the scenario of fish consumption in humans. Furthermore, the HepG2 cells are transformed cell lines that have characteristics that differ from primary human cells, and extrapolations from cell studies do not account for the complex biological processes that occur *in vivo*. Further study is required to understand the complex interactions of omega-3s and BDE 47 with mitochondria, as well as to elucidate the additional mechanisms underlying the toxicity of PBDEs.

4. SFN, a model antioxidant with some similar antioxidant characteristics to the omega-3s, provided partial protection against *in vivo* BDE 47 developmental injury in zebrafish. This is a notable finding that we will follow up upon to clearly discern the significance. We will do this via other funding anticipate to publish an additional manuscript on these results.

5. This study used a novel approach involving transformed human cells and zebrafish as a relatively inexpensive in vitro/in vivo approach to address an issue of relevance to Sea Grant. Specifically, we were able to examine the molecular interactions between the micronutrients in fish and PBDEs, filling a key gap in knowledge regarding the risk assessment of the safety of seafood consumption. A manuscript of these results is currently in submission to the *Journal of Nutritional Biochemistry*.

6. We discovered during the project that another congener that is sometimes detected in salmon, BDE 49, is also a developmental toxicant in zebrafish. These results were published in *Comparative Biochemistry and Physiology* (2012), see reference below.

7. Two additional papers from spinoff studies reported novel findings that were published. We characterized the GSH-utilizing antioxidant enzymes in the olfactory system of coho salmon, and these results were published in a manuscript in *Biochemical Pharmacology* (2013, see reference below*)*. Additionally, we determined that Nrf2-regulated antioxidant responses are critical for protecting the zebrafish olfactory system against another prominent environmental toxicant, cadmium. These results were published in *Toxicology and Applied Pharmacology* (2013).

Publication citations:

1. Wang, L. and E.P. GALLAGHER. 2013. Role of Nrf2 in cadmium-mediated oxidative injury to the zebrafish olfactory system. **Toxicol. Appl. Pharmacol.** 266(2):177-86. PMID: 23174481
2. Espinoza, H.M., Williams, C.R., and E.P. GALLAGHER. 2012. Effect of cadmium on glutathione *S*-transferase and metallothionein gene expression in coho salmon tissues. **Aquat. Toxicol.** 110-111:37-44. PMID: 22257444
3. McClain, V., Stapleton, H.M, Tilton, F., and E.P. GALLAGHER. 2012. BDE 49 is a developmental toxicant in zebrafish. **Comp. Biochem. Physiol. Part C**. 155(2) 253-258. PMID: 21951712

6. STUDENTS SUPPORTED.

Student Name: Andrew Yeh
Department: Department of Environmental and Occupational Health Sciences
Major/Degree field: Environmental Toxicology
Major Professor: Evan Gallagher
Degree (Ph.D., M.S., M.A., B.S., B.A., etc): Ph.D.
Dissertation/Thesis title: Biomonitoring of emerging contaminants in the Puget Sound.
Date of graduation (actual or anticipated): June 2015
Approximate support or affiliation period (e.g., Jan – June 2005): Feb 2010 - Jan 2013
Type of support (RA, research costs, conferences – list all that apply): RA, research costs
Current employment if applicable: N/A

Student Name: Chase R. Williams
Department: Department of Environmental and Occupational Health Sciences
Major/Degree field: Environmental Toxicology
Major Professor: Evan Gallagher
Degree (Ph.D., M.S., M.A., B.S., B.A., etc): Ph.D.
Dissertation/Thesis title: Understanding metal-induced injury and dysfunction in the salmon olfactory system
Date of graduation (actual or anticipated): June 2015
Approximate support or affiliation period (e.g., Jan – June 2005): Feb 2010 - Jan 2013
Type of support (RA, research costs, conferences – list all that apply): RA, research costs
Current employment if applicable: N/A

7. PARTICIPANTS.

Partner	**Specify Type** (Academic, Government, Industry/Business, NGO, SG Program, Other)	**Specify level (**International, Federal, Regional, State, Local)	**Nature of Partnership**
Dr. Heather Stapleton	Academic (Duke University)	Local	Collaborator and colleague.
Dr. Terrance Kavanagh	Academic (UW Analytical cytology laboratory, DEOHS)	Local	Collaborator and colleague.
Dr. David Marcinek	Academic (UW Department of Radiology)	Local	Collaborator and colleague.
Bruce Duncan	Government (USEPA region 10)	Regional	Collaborator
Dr. James Meador	Government (NOAA fisheries)	Federal	Collaborator
Katie Frevert, and Jon Sharpe	Academic (UW community engagement and outreach and translational cores)	Local	Assisting in research translational activities.

8. OUTREACH AND INFORMATION/TECHNOLOGY TRANSFER

a. The PI gave a seminar and participated in a question and answer session at USEPA Region 10 in February 2011 on the use of cellular assays in the zebrafish model for assessing the effects of site remediation in the Puget Sound. This conference was also attended remotely via web interface by other EPA risk assessors, and resource management personnel in the WA Depts. of Ecology and Health.

b. The PI participated in an ATHENA (Academy for Teaching on Health and Environment Associations) workshop sponsored by the UW DEOHS community engagement and research translation core investigators (May 2011). As part of the workshop, the PI gave a presentation on monitoring pollutant effects in salmon and how the zebrafish model can better inform health aspects of pollution.

c. Project updates were continually shared with Katie Frevert of the UW Superfund Basic Sciences research translation core who meets regularly with the Duwamish River Cleanup Coalition and EPA Region 10 partners.

d. The research team answered questions, provided tours of the zebrafish facility, and presented concepts on using zebrafish to address environmental health research questions to high school students and area teachers. This is part of a yearly effort in which our department trains a group of high school science and health teachers in the core concepts of Environmental Health and Ecogenetics through the ATHENA program. These teachers then return to their classrooms and implement a unit of their own design related to what they have learned during the August session. In spring of the following year, they return to the UW for a follow-up workshop and share their work with CEEH staff and investigators.

e. The Sea Grant work was highlighted twice during the project in University of Washington activities, including a feature article in Sea Star published by Washington Sea Grant, and also a recent profile of the PI published on the University of Washington School of Public Health website (December 2012).

COMPLETION REPORT – TABLES AND FIGURES ATTACHMENT.

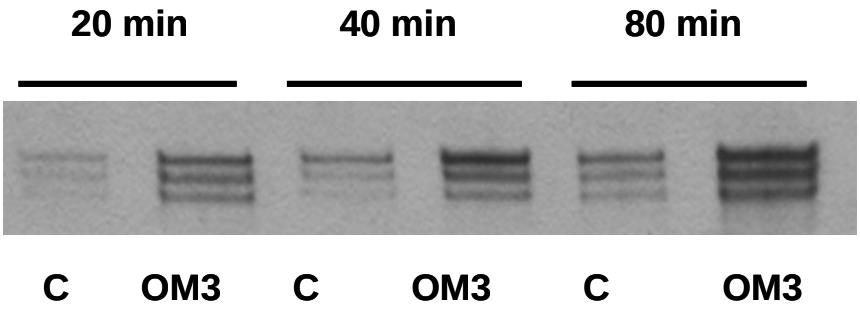


Figure 1. Salmon omega-3s increased cause the accumulation of Nrf2 reactive proteins in the nucleus of Hepg2 cells. HepG2 cells were exposed to a 1.5/1 EPA/DHA mixture of salmon omega-3s (60 µM EPA/40 µM DHA; denoted as OM3 in figure) or the carrier solvent only (C = control) for 20, 40, or 80 minutes. Lysates containing the nuclear fractions were prepared from the cells. An Nrf2 antibody was used to detect the presence of the Nrf2 protein in the nuclear lysates by Western blot analysis.

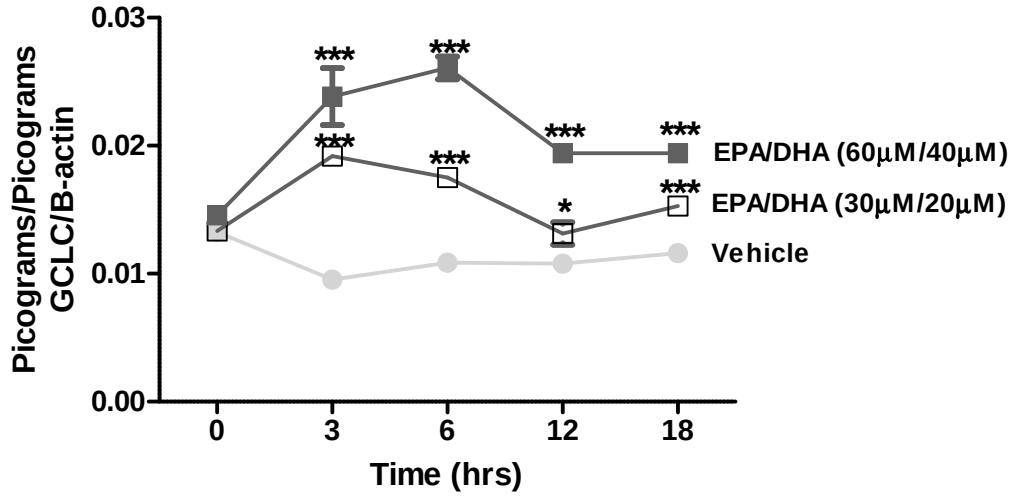


Figure 2. Effect of salmon omega-3s on glutamylcysteine-cysteine ligase (GCLC) gene expression in HepG2 cells. Exposure to a 1.5/1 mixture of EPA/DHA over time dose-dependently increased the expression of *GCLC*, an indicator of an Nrf2 response and mediator of GSH biosynthesis. Data are expressed as mean ± SD (n=3). *P≤0.05. ***P≤0.001 relative to DMSO.

Table 1. Effect of oxidized EPA and DHA (oxEPA/oxDHA) on glutathione (GSH) levels in HepG2 cells.

	Intracellular glutathione concentration nmoles GSH/mg protein		
	6 hrs	**12 hrs**	**18 hrs**
Control	6.1 ± 0.3	6.5 ± 0.8	6.1 ± 0.4
oxEPA/oxDHA	11.0 ± 0.7***	13.0 ± 1.1***	13.0 ± 0.3***
SFN	8.0 ± 1.3	12.0 ± 0.9**	12.0 ± 0.7***

After treatment with the carrier solvent (DMSO) or antioxidants, the cells were harvested and GSH levels determined in a fluorometric assay. Briefly, the assay is dependent on the conjugation of NDA with GSH, which generates a stable fluorescent product detected at excitation 472 nm/emission 528 nm. GSH concentrations were normalized to total protein. Data are expressed as mean ± SD (n=3). **$p \leq 0.01$, ***$p \leq 0.001$ relative to controls.

Table 2. Assessment of BDE 47- effects on mitochondrial respiration.

	Mean Oxygen Consumption (pmoles O_2/[sec*mg protein]) ± SD (n=4)		
Respiratory chain parameter (respiratory substrate/inhibitor)	**Control**	**BDE 47**	**oxEPA/oxDHA + BDE 47**
State 3 (Pyruvate, malate, glutamate, ADP)	61 ± 4	28 ± 7***	38 ± 10**
Maximum State 3 (Above conditions, plus succinate)	79 ± 7	36 ± 9***	48 ± 10**
State 4 (Proton Leak) (Above conditions, plus oligomycin)	17 ± 4	13 ± 4	17 ± 1
Uncoupled (Above conditions, plus CCCP)	182 ± 6	70 ± 18***	99 ± 23***
Complex II (Above conditions, plus rotenone)	78 ± 11	53 ± 19	58 ± 15
Non-mitochondrial respiration (Above conditions, plus Antimycin-A)	46 ± 1	30 ± 2*	24 ± 6*
Complex IV (Above conditions, plus TMPD, and ascorbate)	162 ± 28	116 ± 28	156 ± 35

The substrates/inhibitors of the mitochondrial electron transport chain (ETC) used to assess the specific mitochondrial respiratory parameters are indicated, as well as which functional parameter of the mitochondrial ETC they were used to assess. Pretreatment with oxEPA/oxDHA was not protective on any of the tested functional parameters. Data represent mean oxygen consumption ± SD (n=4). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ relative to controls.

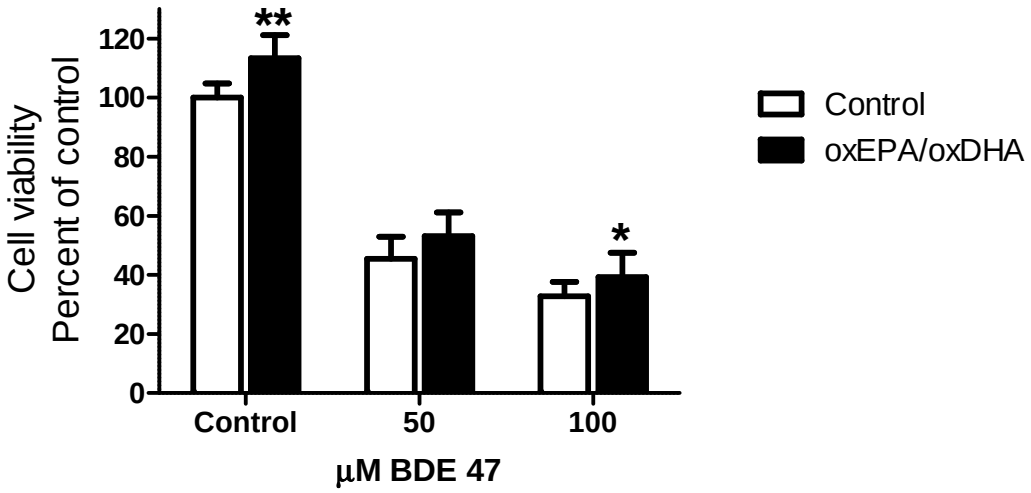


Figure 3. Effect of oxEPA/oxDHA on the cellular toxicity of BDE 47. Cells were dosed with the carrier (DMSO), or a 1.5/1 mixture of omega-3 fatty acids relevant to salmon (60 µM EPA/40 µM DHA). After 12 hr, cells were exposed to BDE 47 for an additional 24 hours. The viability of cells was then measured by the MTT assay. As observed, pretreatment with the salmon omega-3 mixture significantly increased the overall viability of HepG2 cells (**$p<0.01$) in culture. Only <10% protection by the omega-3s against BDE 47 toxicity were observed. Data indicate mean and standard deviation of n=3 experimental replicates, with n=3 technical replicates per treatment per experiment.

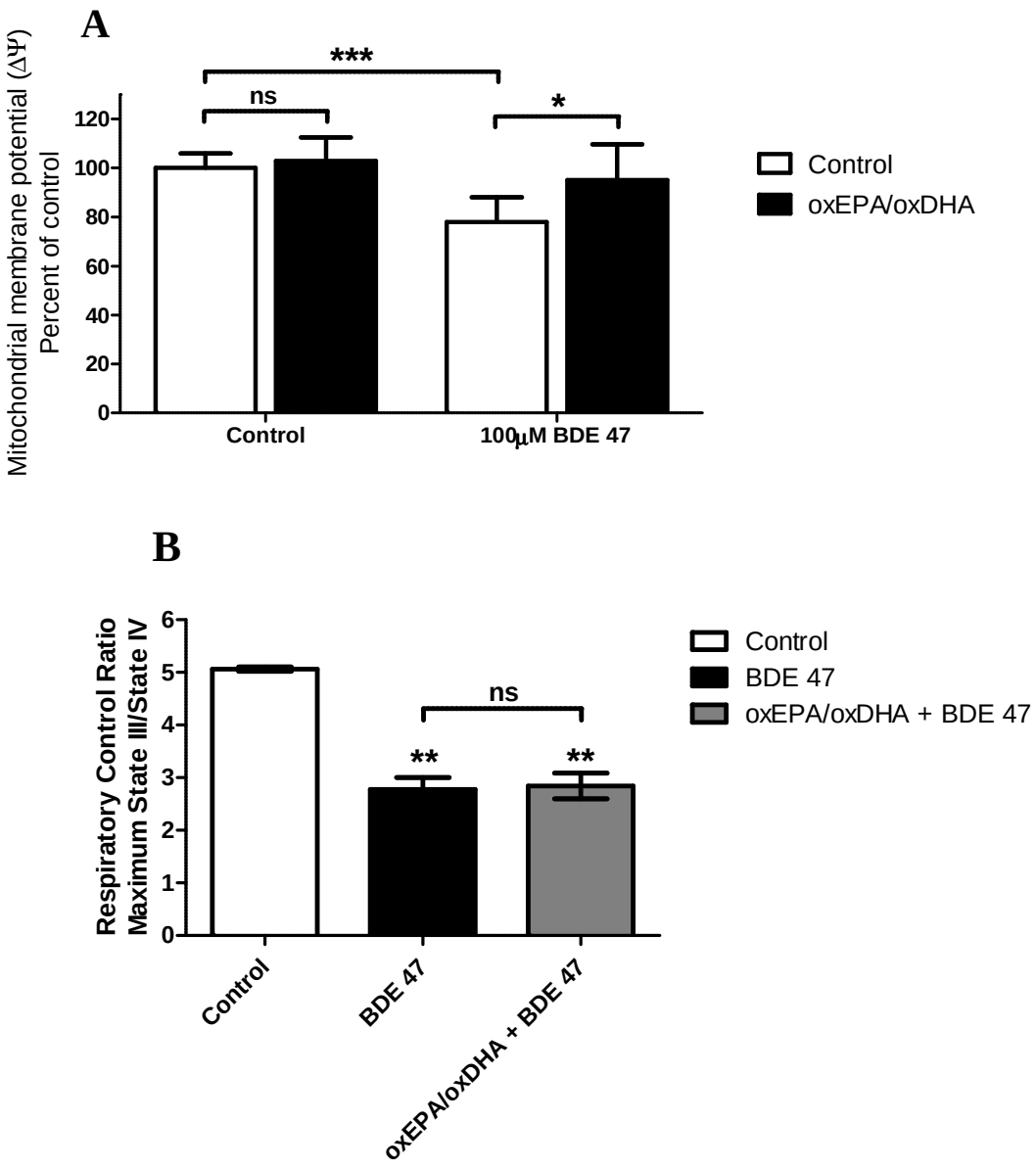


Figure 4. Effect of oxEPA/oxDHA on the mitochondrial toxicity of BDE 47. (A) The oxidized omega-3s (oxEPA/oxDHA) protected mitochondrial membranes by preventing the loss of membrane potential ($\Delta\Psi_m$) caused by BDE 47. Data are the mean ± SD (n=6 technical replicates per treatment). *p≤0.05, ***p≤0.001 relative to controls. **(B)** The Respiratory Control Ratio (RCR) is a measure of mitochondrial function and health and defined as the ratio of oxygen consumption rate during State 3 respiration divided by the rate during State 4 respiration. As observed, BDE 47 significantly decreased the RCR of cells by 60%. Pretreatment with oxEPA/oxDHA had no protective effect. Data are the mean ± SEM (n=4 experimental replicates per treatment). **p≤0.01 relative to controls.

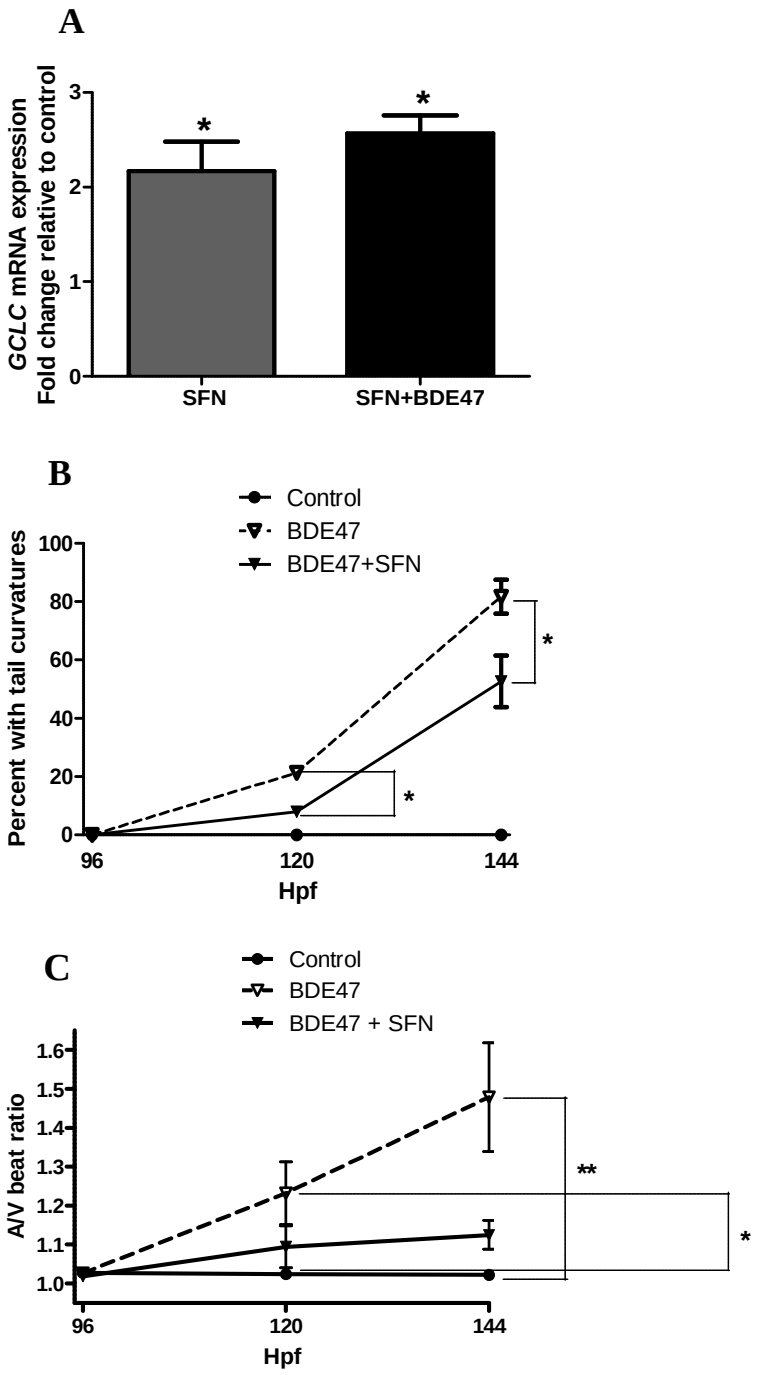


Figure 5. Effect of sulforaphane (SFN) on antioxidant gene expression and developmental toxicity of BDE 47. (A) Treatment of zebrafish with SFN (40µM) for 48 hrs induced the expression of *gclc* as quantified by qPCR. Data represent mean ± SEM (n=6 groups with 15 individuals per group). **(B)** SFN co-treatment was significantly protective against BDE 47-induced tail curvatures in zebrafish larvae, mean ± SEM (n=192 individuals), and **(C)** cardiac abnormalities, mean ± SEM (n=35 individuals). *$p \leq 0.05$, **$p < 0.001$