University of California, Davis

Title of Research:

Developing molecular biomarkers to assess chlorantraniliprole and imidacloprid impacts in aquatic species

Simone Hasenbein[1-2], Erika B. Holland[3], Sarah Stinson[1] & Richard E. Connon[1]

[1]University of California Davis, California, USA
[2]Technical University of Munich, Freising, Germany
[3]California State University Long Beach, California, USA

Project Duration: 01/01/2017 through 12/31/2018

FINAL REPORT January 31, 2018

Department of Pesticide Regulation, Grant # 16-C0084

Working title: Development of molecular biomarkers to assess chlorantraniliprole and imidacloprid impacts in aquatic species

Simone Hasenbein[1-2], Erika B. Holland[3], Sarah Stinson[1] & Richard E. Connon[1]*

[1]University of California Davis, California, USA;

[2]Technical University of Munich, Freising, Germany;

[3]California State University Long Beach, California, USA

*Corresponding author: reconnon@ucdavis.edu

Executive Summary

Chlorantraniliprole (CHL) and imidacloprid (IMI) are emerging insecticides in California with novel modes of toxicity. Specifically, CHL and IMI are known to cause lethality in their target pests by affecting the ryanodine receptor (RyR) or the nicotinic acetylcholine receptor (nAChR), respectively. The RyR and the nAChR are essential to neuromuscular physiology and when altered can lead to hyperneuronal or muscular activity and ultimately paralysis. Little research has been conducted regarding the impact of CHL and IMI on off target species, especially aquatic organisms exposed due to run-off. There has been research highlighting the morphophysiological effects (survival, growth, emergence and behavior) of these insecticides but the sublethal toxic impacts on aquatic macroinvertebrates and fish have not been investigated. There is a pressing need to develop tools that measure early signs of exposure, such as molecular biomarkers, that could be used in laboratory and field based assessments. Such tools would allow the detection of subcellular level effects before they are apparent at higher levels of biological organization, particularly at low environmentally relevant insecticide concentrations.

The overall goal of the current project was to develop novel molecular biomarkers that can evaluate the impact of environmentally relevant CHL and IMI exposure in aquatic invertebrates (*Daphnia magna, Chironomus dilutus*, and *Hyalella azteca*) and a model fish species (fathead minnow; *Pimephales promelas*). A secondary goal was to determine whether changes in subcellular molecular pathways correlate with the insecticides' activity at either the RyR or nAChR in aquatic organisms. Our objectives were 1) to apply novel molecular biomarkers to assess whether environmentally relevant exposures to CHL and IMI alter the subcellular physiology of aquatic organisms and 2) to investigate if changes in subcellular signaling pathways correlate with species sensitivity to CHL- and IMI-induced disruption of the RyR or nAChR, respectively.

Outcomes of this project include molecular biomarkers for CHL and IMI established in common model species used for assessing aquatic health; namely established and validated biomarkers for *Daphnia magna, Chironomus dilutus*, *Hyalella azteca* and the fathead minnow. Molecular biomarker assessments coupled with receptor based *in vitro* screens confirmed that subcellular changes are related to each emerging contaminant's known mode of toxicity. Together these tools will aid biomonitoring practices and will be available for future adaptive management plans set forth by the Department of Pesticide Regulation (DPR). They will represent a unique

opportunity to aid in conserving California's aquatic ecosystems by understanding the impact of environmentally relevant CHL and IMI concentrations.

Introduction

Chlorantraniliprole (CHL) and imidacloprid (IMI) are emerging insecticides in California with novel mode of actions, and unknown sublethal impacts on aquatic invertebrates and fish. CHL is an anthranilic diamide insecticide that causes toxicity by interrupting normal muscle contraction by activating the ryanodine receptor (RyR). Neurotoxicity of the neonicotinoid insecticide IMI is based on a covalent bond to the nicotinic acetylcholine receptor (nAChR), via which continued exposure can lead to cumulative detrimental effects. As such, individuals may be affected at the cell, tissue and whole-organism level, which can contribute to alterations in population dynamics, and even potentially change community compositions (Pörtner 2002). Previous studies have highlighted the morphophysiological effects (survival, growth, emergence and behavior) of these insecticides, while very few studies have assessed sublethal toxic impacts on aquatic macroinvertebrates and fish. There is a pressing need for early warning tools, such as molecular biomarkers for both laboratory and field assessments, as they allow the detection of subcellular level effects before they are apparent at higher levels of biological organization, particularly at low insecticide concentrations.

The overall goal of this project is to develop novel molecular biomarkers that can evaluate the impact of environmentally relevant CHL and IMI exposure in aquatic invertebrates (*Daphnia magna, Chironomus dilutus*, and *Hyalella azteca*) and one fish species (fathead minnow). A secondary goal is to determine whether changes in subcellular molecular pathways correlate to insecticide activity at the corresponding RyR and nAChR receptors. We hypothesize that CHL and IMI exposure will alter subcellular molecular pathways directly related to the chemicals' mode of action.

Our objectives are 1) to apply novel molecular biomarkers to assess whether environmentally relevant exposures to CHL and IMI alter the subcellular physiology of aquatic organisms and 2) to investigate if changes in subcellular signaling pathways correlate with species sensitivity to CHL- and IMI-induced disruption of the RyR or nAChR, respectively.

This research consisted of four tasks: 1) Assessment of subcellular impacts of a range of environmentally relevant concentrations, from low to high concentrations, using specific molecular biomarkers in all four species. 2) Validation of biomarkers using ambient water samples. 3) Protein homogenates enriched in the RyR and nAChR were created from whole invertebrates and larval fish and receptor activity investigated following previously published methods by E. Holland. 4) Dissemination of information and outreach/education.

Material and Methods

Task 1: *Organismal Exposures* and *Development of biomarkers*:

In task 1, we first assessed the subcellular impact of a range of environmentally relevant concentrations, from low to high, using specific molecular biomarkers in all four species.

Organismal exposures followed common chronic toxicity procedures as outlined by the US Environmental Protection Agency (USEPA 2002). Exposure concentrations were chosen to match range-finding experiments and environmentally relevant concentrations (Table 1).

Table 1. *Concentrations of CHL and IMI measured in ambient water samples by DPR and USGS and EPA benchmarks.*

	CHL	*IMI*
Range that DPR measured in ambient water samples (taken throughout California)	*0.102 – 1.68 µg/L*	*0.05-9.86 µg/L*
Range that USGS measured in Delta water	*2 – 30 ng/L*	*2 – 22 ng/L*
EPA benchmark (acute)	*58 µg/L*	*0.385 µg/L*
EPA benchmark (chronic)	*4.5 µg/L*	*0.01 µg/L*
LC50 H. azteca	*>389 µg/L*	*13 µg/L*
LC50 C. dilutus	*85.9 µg/L*	*2.65 µg/L*
LC50 D. magna	*7.1 µg/L*	*6,029 µg/L*
LC50 fathead minnow	*No data*	*No data*

CHL and IMI were purchased from AccuStandard (New Haven, CT, USA) and dissolved in acetone (CHL) or deionized water (IMI). Pesticide-grade acetone was used as a solvent carrier for the CHL treatments, and in solvent controls, to a final concentration of 0.01% in exposure water. Corresponding stock solutions were spiked into control water according to target concentrations, and mixed thoroughly. Organisms were randomly added to each replicate beaker. In total, invertebrate species were exposed to six single concentrations (25, 50, 100, 500, 1000, 10000 ng/L) of each pesticide and three mixture concentrations (25 x 25 ng/L, 500 x 500 ng/L, 10000 x 10000 ng/L), a solvent control (for CHL exposures only), and a negative control. Fish were exposed to three single concentrations (25, 500, 10,000 ng/L) of each pesticide and three mixture concentrations (25 x 25 ng/L, 500 x 500 ng/L, 10,000 x 10,000 ng/L), a solvent control, and a negative control. Fish exposures were conducted in accordance with the University of California Davis, Institutional Animal Care and Use Committee protocol #19690.

Mortality was recorded daily and any dead organisms were removed from the test vessels. In addition, 70% of each test solution was renewed at 24h (fathead minnow*)* or 48h (*C. dilutus, H. azteca, D. magna,*) time intervals. At the time of water renewal, debris was removed and water quality parameters [pH, specific conductance (SC), dissolved oxygen (DO), temperature (T)] of renewal and wastewater were measured. Test vessels were randomly distributed after each water renewal. Mortality was recorded daily and any dead organisms were removed from the test vessels. All surviving animals in a single replicate beaker were pooled into one microcentrifuge tube, immediately snap frozen in liquid nitrogen, and stored at -80 °C for assays of gene transcription (see details of sample processing below).

Chironomus dilutus and *Hyalella azteca* were obtained from Aquatic Research Organisms. 96h-toxicity tests with *C. dilutus* and *H. azteca* were conducted in a temperature-controlled environmental chamber at 23 ± 2ºC with a 16-hr light: 8-hr dark photoperiod. Organisms were

randomly added to each replicate beaker. The 96h toxicity tests were based on U.S. EPA protocols for static sediment toxicity testing (U.S.EPA 2000), with the following modifications for each species. For *C. dilutus*, four replicate 1 L glass beakers, each containing a substrate of 10 g silica sand that was clean and baked (four hours at 450°C), 150 ml of treatment water, and 10 organisms. The *H. azteca* 96h toxicity tests were modified for water column exposures, as described in the Quality Assurance Management Plan for the State of California's Surface Water Ambient Monitoring Program (SWAMP 2002). Briefly, each concentration tested included four replicate 250 ml glass beakers, each containing 150 ml of treatment water, 20 organisms and a 2 cm^2 piece of Nitex® screen as artificial substrate. Organisms were not fed during the exposure.

Daphnia magna were obtained from Aquatic Research Organisms Inc (Hampton, NH, USA), and were cultured in our laboratory at the University of California, Davis for at least six months before testing. Cultures were initiated with third brood offspring from a single female. Groups of 20 daphnids were maintained at 20 ± 2°C and a 16-hr light: 8-hr dark photoperiod in 2L beakers of reconstituted control water which was prepared by dissolving 23.04 g $NaHCO_3$, 14.40 g $CaSO4{\cdot}2H_2O$, 14.40 g $MgSO_4$, and 0.96 g KCl in 120 liter deionized water to achieve a hardness of 160 – 180 mg/L $CaCO_3$ and alkalinity of 110 – 120 mg/L $CaCO_3$. The 96h toxicity test was conducted using control water prepared as described above (OECD 2002; U.S.EPA 2002). Tests were set up using <24h-old third brood neonates. Test exposure temperature was 20 ± 2ºC under fluorescent light with a 16-hr light: 8-hr dark photoperiod. Four replicates per treatments were tested. Twenty individuals were placed into each of the 250-ml replicate beaker containing 200 ml of treatment water. Survival and number of neonates were recorded daily. After 48 h, new treatment waters were prepared by adding 5 mL of a suspension of concentrated (i.e., spun down and rinsed, 3 x 10^7 cells/mL) *P. subcapitata* (both obtained from Aquatic Research Organism Inc) to 200 mL control water. In addition, 1.5 mL of YCT (yeast, cerophyl, trout chow mixture, total solids > 1.9 g solids/L of final YCT mixture) was added as an additional nutrition source per USEPA recommendations (U.S.EPA 2002). Daphnids were then transferred to new replicate beakers.

Fathead minnow larvae were obtained from Aquatic Research Organisms Inc at 7 d post-hatch on the day of arrival. Fish were acclimated to control water at a temperature of 25°C. Control water consisted of deionized water, modified with salts to meet USEPA specifications (specific conductivity (EC): 265–293 µS cm−1; hardness: 80–100 as mg CaCO3 L−1; alkalinity: 57–64 as mg CaCO3 L−1 (USEPA, 2002)). During the acclimation period <1% mortality was observed, and the fish fed and swam normally. During the project period, a routine reference toxicant test was performed using NaCl to ascertain that organism response fell within the acceptable range according to USEPA requirements (USEPA, 2002). Each test consisted of a dilution series (5 test concentrations and a control). All test organisms responded normally (within 95% confidence interval of running mean) and sensitivity was considered typical.
Each treatment consisted of four replicate 600 mL Pyrex beakers containing 250 mL test solution and 10 fish larvae. At test initiation, 10 larvae were transferred from the acclimatization tank to each beaker and exposed to test solutions at a water temperature of 25°C and a 16:8 light–dark ratio. Test vessels were manually distributed in a random manner. Fish were not fed during the exposure period. Fish were fed ad libitum with *Artemia nauplii* 2 times each day. At test termination, surviving fish were euthanized in tricaine methanesulfonate (MS-222), transferred in pairs into 1.5 mL microcentrifuge tubes and immediately flash-frozen in liquid nitrogen.

Organisms exposed to variable sublethal concentrations of CHL and IMI were then utilized for the detection of altered subcellular pathways. Subcellular pathways that were investigated in exposed organisms included RyR and nAChR related molecular pathways known pesticide detoxification pathways.

Sample processing and primer design

Hyalella azteca samples were homogenized for 10 min at 50 vibrations/s in a Tissue Lyser bead mill (Qiagen), and total RNA was extracted using TRIzol Reagent (Ambion RNA, Life Technologies Corporation) according to manufacturer's guidelines. RNA was purified in an RNAeasy Plus spin column (Qiagen) and eluted with RNase free water.
For the fish, we pooled two fish from each treatment replicate, resulting in 20 biological replicates per treatment. For RNA extraction of all other species, each replicate containing all surviving organisms were pooled, resulting in four replicates per treatment. Total RNA was extracted from whole individual organisms, using QIAGEN RNeasy MiniKit according to manufacturer's instructions.

RNA concentrations were determined using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), total RNA 260/280 and 260/230 ratios ranged between 1.86–2.15 and 1.75– 2.05, respectively. Total RNA integrity was verified through electrophoresis on a 1% (wt/vol) agarose gel. 50 ng (*H. azteca*), 500 ng (*C. dilutus, D. magna*), and 1000 ng (fathead minnow) of total RNA were used for cDNA synthesis, respectively. Complementary DNA (cDNA) was synthesized using Superscript III Reverse Transcriptase, a 100 mM dNTP Set, and random primers (Invitrogen, Carlsbad, CA) resulting in 50 µL cDNA. A dilution to a total volume of 160 µL was carried out with nuclease free water to generate sufficient template for qPCR analysis. Primer and probes for qPCR analyses were designed using Roche Universal Probe Library Assay Design Center (https://www.roche-applied-science.com). List of genes of interest and test efficiencies are detailed in Table 2. Designed primers were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com), and TaqMan probes were supplied by Roche.

Table 2. *Genes of interest for which qPCR systems have been designed.*

Gene of Interest	Abbrev.	Forward	Reverse	Probe	Efficiency %
Pimephales promelas					
Aspartoacylase	ASPA	TCTGGTAATGGATGTCCCGATT	GACCTCTATGGAAAAGCCATGC	94	100
apoptosis regulator bcl-2	BCL-2	TAGTGAAATCCTTCAGTGGTGGAG	GTGCATGTTACTAAGAGCCTGACATTT	34	93
Cytochrome P4501A	CYP1a	GCTTCTCGAGGCCTTTATCC	ACAGTGAGGGATGGTGAACG	12	99
CYP3A126	CYP3a	CAACCCAGAGGCCATGAAGA	GGGCCTTATTTGGGAAGGTCT	63	92
Elongation Factor 1-alpha	EF1a	CTCTTTCTGTTACCTGGCAAAGG	TCCCATGATTGATTAGTTTCAGGAT	66	97
Glutathione peroxidase-1	GPx1	AGCTCGTTCATCTGGGTGTAATC	TGGTCCTCATCGAGAATGTCG	131	97
Heat shock protein 90	HSP90	CTGGTCATCCTCCTGTTCGAG	TGTGTCTGAGGATCGTCCAATG	56	103
lactate dehydrogenase beta 4	LDHb4	CCTTTTCCTCAAGACCCCTAAGAT	GTCACCACCACGATACGAGAGTTA	5	95
nicotinic acetylcholine receptor	nAChr	TCAGACTGCGGTGAGAAGATCA	AGCAGCAGGAAGACAGTGAGC	101	107
8-oxoguanine DNA glycosylase	OGG1	AGGAAGAAGTGGGAATGTGCA	CAGTTCCCGTAGAGATCCCCTAA	101	99

Parvalbumin	PVALB	GCTCTGTCTGCTGACAACGTG	CAGCAAAACCCTTCAGCACAA	63	89
Ryanodine Receptor, 1	RyR1	AAGATGACGATGAAGGGTTTGTC	CATGGCAGGTTCCATATATCCAG	65	99
Sarco/Endoplasmic Reticulum ATPase	SERCA1	CAACATTGGCCACTTCAACG	GAGCCACAGCGATCTTFAAGT	92	98
superoxide dismutase	SOD	CATTCCATTATAGGGAGGACCA	TTCATTGCCTCCCTTACCC	88	98
Voltage gated Calcium Channel	VGCC	GATTCTCAGGGTGTTGAGGGTACT	CACTGGACCACGTGCTTTAACC	147	95
L8 ribosomal protein	L8	GGCTAAGGTGGTTTTCCGTGA	CTTCAGCTGCAATGAACAGCTC	35	99
beta-actin	B-ACTIN	CAACACCGTGCTGTCTGGAG	TCTTTCTGCATACGGTCAGCAA	157	93
Daphnia magna					
acetylcholinesterase	AChE	GGGGCCATTTCGTGAGTAT	CGGTGGTTTCAACTGTGCTA	157	105
Calcium-transporting ATPase type 2C	ATP2C	CATGACTGGTGATGGTGTCAATG	GCCCATGGCAATACCGATT	51	102
apoptosis regulator bcl-2	BCL-2	ATCTACCTTCTCGCCTAATGCTTC	CCGACCCGTGAATTCGTG	147	99
Calmodulin	CALM	ATGAGATCGCTTGGACAGAAC	GCGAGCCATCATAGTCAGAAA	156	92
catalase	CAT	GACTATTTCGCCGAAGTCGAAC	GCCTCAATACCCGGAACCATA	26	98
Creatine Kinase	CK	CGAGCTTGGCAAGATCAGC	GACCTTCTGCCCAACCAACTT	44	101
cyp3a40	CYP3a	GAACAATCGGTCCTGAACGTC	CCACAGCCACTACACTCACGAA	72	97
Elongation Factor 1-alpha	EF1a	GAACGTCTCCGTCAAGGAGTT	GGTGGGTTGTTCTTGGAGTC	141	91
Glutathione peroxidase-1	GPx1	CATTCCGTTGAACAGTTTGTCATC	TCCATGTCAAAAGGATTGACGTT	71	96
Glutathione Sulfa-transerase mu	GSTm	TTCACATCAAACCAGCACGATT	CATACACCCAGACCGAGTATGAAG	81	92
Heat shock protein 90	HSP90	CGTAGCCAAGGAGGGAGTTG	GGGTTTTAAGGGCCTCCAATT	133	95
lactate dehydrogenase beta 4	LDHb4	GACCATCGGCTAAAACAACCTT	TTCCACCATGTCTGTTGAGTAAGC	147	102
Ryanodine Receptor, 1	RyR1	CGTCATCACCTTGTGGCATT	ATTCGGGAACTGGAGCACTAAC	26	96
superoxide dismutase	SOD	GTCTGCTGGACCCCACTTTAAC	TCACCAACATGACGAACCTGA	157	99
L8 ribosomal protein	L8	GCGCGATTGTTGGAAGAAAC	ATACCAAGAAGACCCGTGTCAAG	50	99
Beta-actin	B-ACTIN	TAAGGATCTGTACGCCAACACTG	TGCATACGATCAGCAATACCG	157	96
Hyalella azteca					
Acetylcholinesterase	ACHE	CCAGGGACCGGAAGTTTGT	CATTGTGCCCCTGAGGTGA	48	104
Arginine Kinase	AK	TCGCGGTATCTACCACAACG	GAGGTGATCCTCCTCATTGCAC	153	104
Calcium-transporting ATPase type 2C	ATP2C	CGAGGATATTGTCGGCTGCT	CAGCAATGAGGTAGGGGAAGC	14	102
Apoptosis regulator bcl-2	BCL-2	GACGAATGTGAGGGCGACAT	CTTACGCTTTGGCGTCTCCT	17	109
Calmodulin	CALM	GGGACCTTTGGAATGTGGTTC	TGCCCCTACCACACCCTATC	54	105
Elongation Factor 1-alpha	EF1a	ATCGTTCTTAACCACCCTGGTC	TGAGCGGTGTGACAATCCAG	64	92
Glutathione Sulfa-transerase mu	GSTm	TCGCGTACGAGATGTTCGAC	TCCCGAATTGCTTGAGACAGT	62	91
Homer Protein	H1	CCAACGTCGAGGAGTGGAAG	TGTTCTTCATGCGCGTGTTC	50	96
Heat shock protein 70	HSP70	CCTACAGCGTGACTGTCTTGTCC	GACCTGCAGCTTCGTATCCTG	40	102
lactate dehydrogenase beta 4	LDHb4	TCATCCCCAACCTCGTCAA	GGCCACGTACGTCAGGATGT	153	95
Ryanodine Receptor, 1	RYR1	TGCTGTTCCGAGTTCATCGAC	TGCCTGATGCGTATCGTGTT	133	98
Sarco/Endoplasmic Reticulum ATPase	SERCA1	AACCAGTCTCTGCTTGTGATGC	GAAGTGCAGAGTGAGTGAGAGAGC	92	98
Choline transporter 1	SLC5A7	CCTCTGTGGGGAGGTCTTCTG	ACGGACAGTGTCGCTCCTAAG	70	106
L8 ribosomal protein	L8	TTAGTAGCAACCGCGCAATG	AGCGTTGCCAGCCTTGAG	70	102

Note: We have not yet developed qPCR systems for*Chironomus dilutus* due to lack of sequence data accessibility.

Quantitative PCR

TaqMan Universal PCR Mastermix (Applied Biosystems, Carlsbad, CA, USA) was used in qPCR amplifications in a reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 uL of cDNA sample in a final volume of 12 _L. Samples were placed in 384 well plates, and targeted gene fragments were amplified in an automated fluorometer (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, Carlsbad, CA, USA). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95°C and 60 s at 60 °C. Fluorescence of samples was measured every 7 s and signals were considered positive if fluorescence intensity exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle, CT). SDS 2.2.1 software (Applied Biosystems, Carlsbad, CA, USA) was used to quantify transcription.

Differential expression will be analyzed using ANOVA and multivariate analyses, along with unsupervised clustering approaches (e.g., hierarchical clustering and principal components analysis). Gene profiling based on per gene and per exposure replicate response correlation will be conducted, using normalized qPCR data obtained from each replicate (Vandesompele *et al.* 2002; McLoughlin *et al.* 2006; Derveaux *et al.* 2010), which were subjected to hierarchical clustering using Genesis software. Average dot product metric, with complete linkage clustering, will be used to generate a heat-map profile of gene expression (Connon *et al.* 2012). Genes with altered expression were then used as biomarkers for environmentally relevant chemical exposures. Specifically, this approach allowed comparisons of the relative degree of subcellular stress induced by CHL and IMI and will help identify species-specific responses and tolerance thresholds to CHL and IMI exposure. This allows for detecting genes that will be up-regulated in one species, but down-regulated in another species, as well as quantitatively assess the magnitude of expression of the responsive gene set.

Statistical analysis

Levene's test and the Shapiro-Wilk test will be used to test homogeneity of variances and normality, respectively. When data was not normally distributed, ln-transformation was applied to achieve normality. When a significant interaction was detected, one-way ANOVA followed by Tukey multiple comparisons of means was used to determine significant differences between treatments and controls. All analyses were carried out using the statistical software R (R Core Team 2017) with a significance level at $\alpha = 0.05$. All differences discussed below are significant unless otherwise noted.

SDS 2.2.1 software (Applied Biosystems) will be used to quantify gene expression. Data were analyzed using the $Log_2^{-\Delta\Delta Ct}$ Ct method (Livak and Schmittgen 2001) relative to reference genes and negative control samples for each treatment.

Task 1. Results and Discussion

Mean control survival of each species was close or greater than 80% (Tables 3 – 6) meeting test acceptance criteria for each species (SWAMP 2002; U.S.EPA 2000).
Per our test design, overall mortality was low as to achieve a high number of samples for the aPCR analysis.

Table 3. Survival of *Chironomus dilutus* after 48h and 96h of exposure to chloranthriniprole (CHL), imidacloprid (IMI), and binary mixtures of CHL + IMI. Treatments highlighted with the same colors were conducted together.

Treatment (ng/L)	48-hour Survival		96-hour Survival	
	Mean	SE	Mean	SE
DIEPAMHR	81.1%	0.09	75.6%	0.12
Solvent Control	88.7%	0.04	79.8%	0.07
25 CHL	80.0%	0.09	77.5%	0.09
50 CHL	85.0%	0.09	82.5%	0.09
100 CHL	84.4%	0.03	81.7%	0.06
500 CHL	72.5%	0.05	52.5%	0.09
1000 CHL	90.0%	0.06	58.9%	0.12
10000 CHL	52.5%	0.18	20.0%	0.09
DIEPAMHR	84.0%	0.02	67.4%	0.06
Solvent Control	85.4%	0.07	76.0%	0.08
25 IMI	88.1%	0.09	23.1%	0.07
50 IMI	64.4%	0.09	10.6%	0.05
100 IMI	97.5%	0.03	60.0%	0.09
500 IMI	97.5%	0.03	80.8%	0.03
1000 IMI	90.0%	0.04	30.0%	0.09
10000 IMI	40.0%	0.07	0.0%	0.00
25 IMI x 25 CHL	83.5%	0.04	73.2%	0.11
500 IMI x 500 CHL	93.8%	0.06	84.6%	0.08
10000 IMI x 10000 CHL	84.5%	0.07	57.9%	0.13

Table 4. Survival of *Hyalella azteca* after 48h and 96h of exposure to chloranthriniprole (CHL), imidacloprid (IMI), and binary mixtures of CHL + IMI. Treatments highlighted with the same colors were conducted together.

Treatment (ng/L)	48-hour Survival		96-hour Survival	
	Mean	SE	Mean	SE
DIEPAMHR	78.8%	0.10	70.0%	0.09
Solvent Control	84.3%	0.07	76.4%	0.12
25 CHL	98.8%	0.01	89.5%	0.02
50 CHL	81.3%	0.01	66.3%	0.03

100 CHL	88.8%	0.01	85.0%	0.02
500 CHL	91.3%	0.04	86.3%	0.09
1000 CHL	92.4%	0.03	79.8%	0.08
10000 CHL	84.4%	0.04	76.6%	0.04
DIEPAMHR	87.5%	0.05	85.0%	0.05
25 IMI	82.5%	0.03	65.0%	0.03
50 IMI	100.0%	0.00	85.0%	0.06
100 IMI	95.0%	0.05	87.5%	0.05
500 IMI	85.0%	0.10	62.5%	0.05
1000 IMI	87.5%	0.07	66.9%	0.04
10000 IMI	0.0%	0.00	0.0%	0.00
25 IMI x 25 CHL	93.8%	0.02	87.5%	0.03
500 IMI x 500 CHL	92.5%	0.03	87.5%	0.06
10000 IMI x 10000 CHL	92.2%	0.03	31.8%	0.16

Table 5. Survival of *Daphnia magna* after 48h and 96h of exposure to chloranthriniprole (CHL), imidacloprid (IMI), and binary mixtures of CHL + IMI. Treatments highlighted with the same colors were conducted together.

Treatment (ng/L)	48-hour Survival		96-hour Survival	
	Mean	SE	Mean	SE
DIEPAMHR	97.5%	0.03	97.5%	0.03
Solvent Control	100.0%	0.00	100.0%	0.00
25 CHL	100.0%	0.00	100.0%	0.00
50 CHL	100.0%	0.00	100.0%	0.00
100 CHL	95.0%	0.03	90.0%	0.07
500 CHL	100.0%	0.00	90.0%	0.07
1000 CHL	100.0%	0.00	100.0%	0.00
10000 CHL	0.0%	0.00	0.0%	0.00
DIEPAMHR	91.3%	0.06	83.1%	0.08
25 IMI	97.5%	0.03	97.5%	0.03
50 IMI	100.0%	0.00	100.0%	0.00
100 IMI	100.0%	0.00	100.0%	0.00
500 IMI	100.0%	0.00	100.0%	0.00
1000 IMI	100.0%	0.00	100.0%	0.00
10000 IMI	100.0%	0.00	100.0%	0.00
DIEPAMHR	100.0%	0.00	100.0%	0.00
Solvent Control	100.0%	0.00	100.0%	0.00
25 IMI x 25 CHL	100.0%	0.00	100.0%	0.00
500 IMI x 500 CHL	100.0%	0.00	100.0%	0.00
10000 IMI x 10000 CHL	0.0%	0.00	0.0%	0.00

Table 6. Survival of fathead minnow after 24h, 48h, 72h and 96h of exposure to chloranthriniprole (CHL), imidacloprid (IMI), and binary mixtures of CHL + IMI. Treatments highlighted with the same colors were conducted together.

Treatment (ng/L)	24-hour Survival		48-hour Survival		72-hour Survival		96-hour Survival	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
DIEPAMHR	97.5%	0.03	94.4%	0.03	89.4%	0.04	84.4%	0.09
Solvent Control	100.0%	0.00	97.5%	0.03	92.5%	0.05	92.5%	0.05
25 CHL	100.0%	0.00	100.0%	0.00	100.0%	0.00	100.0%	0.00
500 CHL	100.0%	0.00	100.0%	0.00	100.0%	0.00	100.0%	0.00
10000 CHL	100.0%	0.00	100.0%	0.00	97.2%	0.03	92.2%	0.05
DIEPAMHR	97.5%	0.03	97.5%	0.03	95.0%	0.03	92.5%	0.03
25 IMI	100.0%	0.00	100.0%	0.00	92.5%	0.05	92.5%	0.05
500 IMI	97.5%	0.03	100.0%	0.00	100.0%	0.00	97.5%	0.03
10000 IMI	100.0%	0.00	100.0%	0.00	97.5%	0.03	92.5%	0.05
25 IMI x 25 CHL	100.0%	0.00	92.2%	0.03	84.7%	0.03	84.7%	0.03
500 IMI x 500 CHL	100.0%	0.00	95.0%	0.03	95.0%	0.03	95.0%	0.03
10000 IMI x 10000 CHL	97.5%	0.03	97.5%	0.03	90.0%	0.06	82.2%	0.05

Quantitative PCR assays are being completed for IMI and CHL exposed organisms. An addendum to this report will be submitted to DPR as soon as the data has been analyzed. Data will be evaluated in contrast to observed mortality.

Task 2 Validation of biomarkers using ambient water samples:

As part of an on-going monitoring project of DPR at 6 sites in Salinas where samples were collected in September 2017 to validate the developed biomarkers by exposing *Daphnia magna, Chironomus dilutus*, *Hyalella azteca*, and fathead minnow to for 96h as following US Environmental Protection Agency (USEPA 2002) protocols. Analytical water chemistry of the ambient water samples was provided by DPR. At test termination, organisms were snap-frozen and stored at -80°C for subsequent RNA extraction and qPCR.

Task 2. Results and Discussion

Exposures to Ambient Waters conducted in September 2017 (DPR test 304; six sites) highlighted acute toxicity (100% mortality) to *Daphnia magna* at sites 3043617 and 3043637. All *P. promelas* had ⩾90% survival, with lowest survival at site 3043637. Controls for Hyalella and Chironomids exposures did not pass minimum survival requirements, thus were removed from the study. Specific results are shown below:

Species: *P. promelas*
Age: 7 dph at start
Test setup date: 09/12/2017
Control survival: 95% at 96h.
Pass/Fail: PASS

Sample site:

3043617	92.5%	Non-Toxic to Fathead Minnow
3043627	100%	Non-Toxic to Fathead Minnow
3043637	90%	Non-Toxic to Fathead Minnow
3043647	95%	Non-Toxic to Fathead Minnow
3043657	92.5%	Non-Toxic to Fathead Minnow
3043667	95%	Non-Toxic to Fathead Minnow

Species: *Daphnia Magna*
Age: 24hph at start
Test setup date: 09/12/2017
Control survival: 100% at 96h.
Pass/Fail: PASS
Sample Number and Site:

3043617 (Quail Creek at HWY 101)	**0.0%**	**Toxic to Daphnia magna**
3043627 (Chualar Creek at Chualar River Rd.)	92.5%	Non-Toxic to Daphnia magna
3043637 (Alisal Slough at Hartnell Rd.)	**0%**	**Toxic to Daphnia magna**
3043647 (Salinas River at Davis Rd.)	97.5%	Non-Toxic to Daphnia magna
3043657 (Reclamation Ditch at Son Jon Rd.)	97.5%	Non-Toxic to Daphnia magna
3043667 (Tembladero Slough at Haro St.)	97.5%	Non-Toxic to Daphnia magna

Gene expression data (qPCR) from 2017 ambient water exposed organisms are presented in **Figure**s 1 (*P.* promelas) and 2 (*D. magna*).

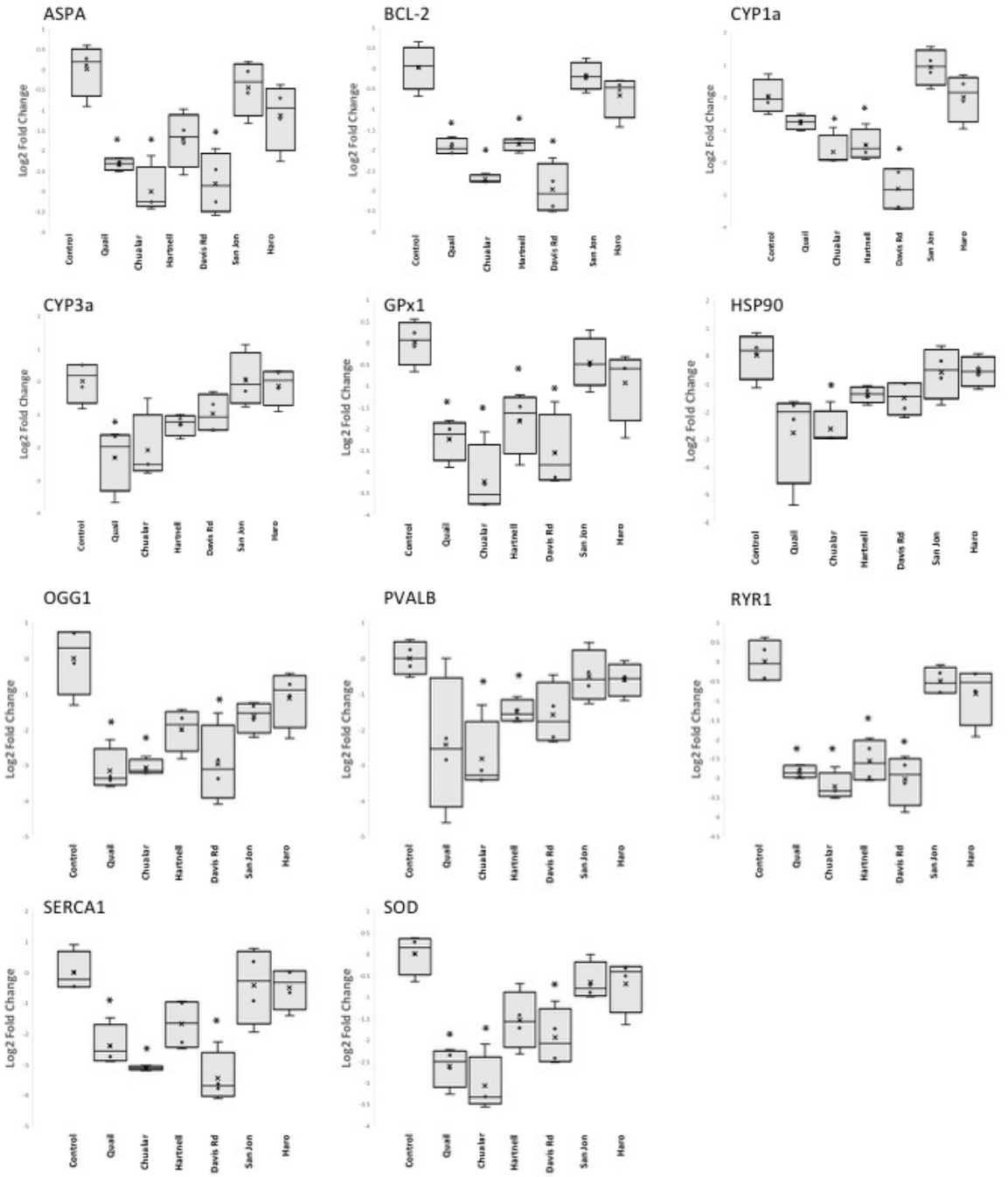

Figure. 1 *Changes in gene expression in the fathead minnow <u>Pimephales promelas</u> after 96h exposure to ambient field water as determined by quantitative PCR. Log2 fold change are plotted on the y-axis. On the x-axis, abbreviations for site locations correspond to Quail Creek at Highway 101 (3043617), Chualar Creek at Chualar River Rd. (3043627), Alisal Slough at Hartnell Rd. (3043637), Salinas River at Davis Rd. (3043647), Reclamation ditch at San Jon Rd. (3043657), and Tembladero Slough at Haro St. (3043667). * indicates significance at $p<0.05$.*

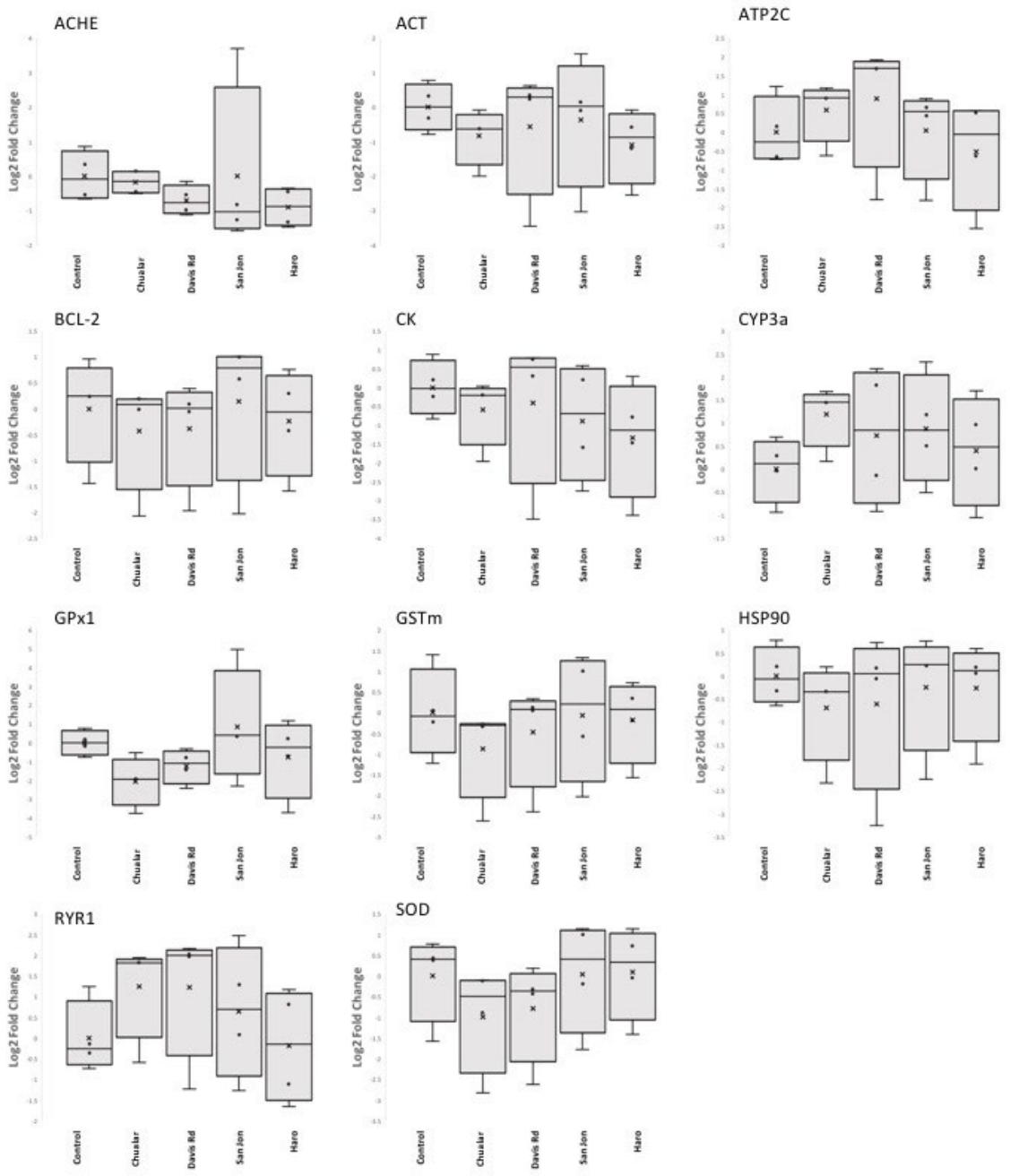

Figure 2. *Changes in gene expression in the waterflea <u>Daphnia magna</u> after 96h exposure to ambient field water as determined by quantitative PCR. Log2 fold changes are plotted on the y-axis. On the x-axis, abbreviations for site locations correspond to Chualar Creek at Chualar River Rd. (3043627), Salinas River at Davis Rd. (3043647), Reclamation ditch at San Jon Rd. (3043657), and Tembladero Slough at Haro St. (3043667).*

Exposures to Ambient Waters were conducted in September 2018 (DPR test 304; five sites), using a geometric dilution series. Acute toxicity (100% mortality) to Daphnia magna was observed at sites 3043617 and 3043637 for 100% concentrations of ambient water (**Figure** 3). Acute toxicity (100% mortality) was also observed at site 3043617 for 60% concentration of ambient water, and 72.5% mortality was observed at the lowest concentration (35%) of ambient water. Controls for Hyalella and Chironomids exposures did not pass minimum survival requirements, thus were removed from the study. There appears to have been issues with control water used for this species. The Delta smelt control water had a different constitution as per USEPA protocols.

Specific results are shown below:

Species: Daphnia Magna
Age: 24hph at start
Test setup date: 09/19/2018
Control survival: 100% at 96h.
Pass/Fail: PASS

	Concentration of ambient water			
Sample Number and Site	**100%**	**60%**	**35%**	
3043617 (Quail Creek at HWY101)	0%	0%	27.5%	**Toxic to D. magna**
3043627 (Alisal Slough at Hartnell Rd.)	0%	62.5%	100%	**Toxic to D. magna**
3043647 (Salinas River at Davis Rd.)	100%	100%	100%	Non-Toxic D. magna
3043657 (Reclamation Ditch at San Jon Rd.)	97.5%	100%	100%	Non-Toxic D. magna
3043667 (Tembladero Slough at Haro St.)	100%	100%	100%	Non-Toxic D. magna

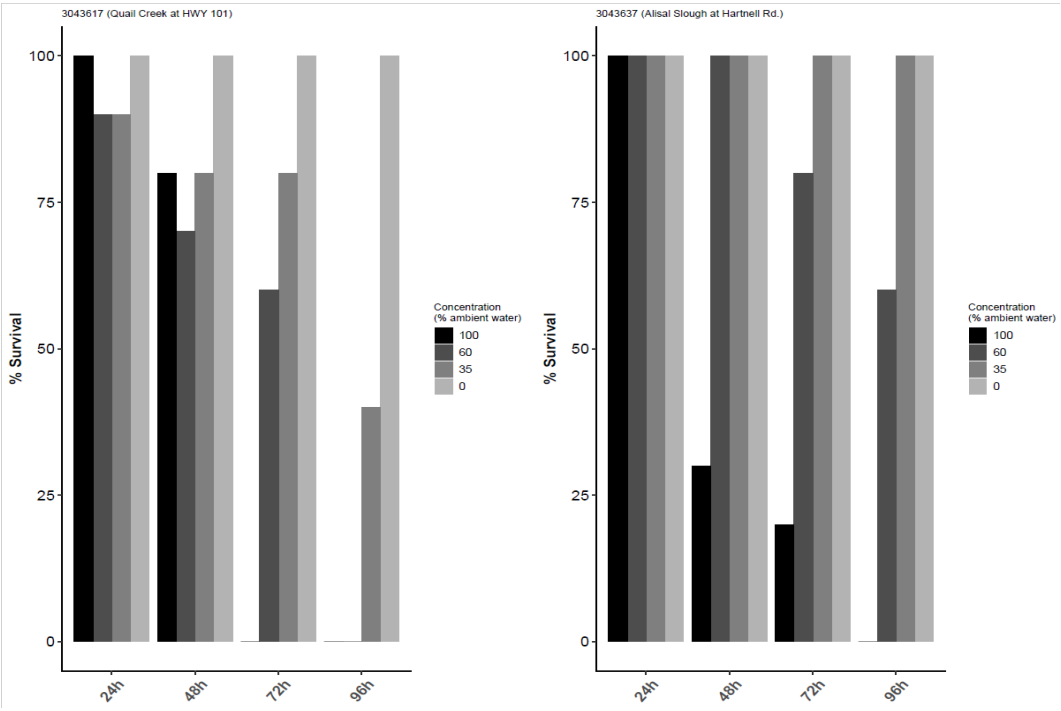

Figure 3. *Daphnia magna Percentage survival following exposures to serial dilutions (100, 60 and 35%, and controls, after 24, 48, 72 and 96h. Depicted here are results from the two toxic sites; 304617 and 304637.*

Notes: Quantitative PCR assays are being completed for the field exposed organisms. An addendum to this report will be submitted to DPR as soon as the data has been analyzed. Data will be evaluated in contrast to observed mortality. A separate report will be submitted for this dataset.

Task 3 In Vitro Assessments

Protein Preparations: Non-exposed invertebrates and whole larval fish (7-14dph) used in *in vitro* assays were purchased from Aquatic Research Organisms Inc, and acclimated and cultured as described under Task 1. For each species separately, whole individuals (n>50), including the carapace, were pooled into 15 mL conical tubes and immediately flash frozen in liquid nitrogen until use in molecular analyses. The pooled tissue was then used to create crude microsomal protein homogenates enriched in RyR or nAChR following previously published methods (Bass *et al.* 2011; Holland-Fritsch *et al.* 2013; Qi and Casida 2013, Wiesner and Kayser, 2000). Briefly, tissue was placed into a homogenization buffer consisting of 300mM Sucrose, 20mM Hepes, leupeptin (2µg ml-1) phenylmethanesulfonyl fluoride (PMSF,1mM), sodium orthovanadate (0.5mM) NaF (10mM), β-glycerol (2mM) and NaP_2O_7 (5mM) adjusted to a pH of 7.2. Tissue was then homogenized, on ice, utilizing a Polytron 1200 E (Kinematica, Bohemia, NY) for 2 bursts of 20s with 2 minutes on ice between bursts. The homogenate underwent centrifugation at 8000RPM for 10min at 4ºC and supernatant collected into an ultracentrifugation tube. The pellet was re-suspended in 5 ml of homogenization buffer and the homogenization and centrifugation steps repeated. Supernatants were combined and underwent ultracentrifugation at 33,000RPM for 1h at 4ºC. The microsomal pellet was then suspended in a 300mM Sucrose 20mM Hepes buffer (pH=7.2) and placed into 100 µl aliquots to avoid multiple freeze thaw cycles after storage at -80°C. Protein concentrations were determined in triplicate using a BCA assay (Pierce, Rockford, IL).

Radio-ligand Binding Assays: To measure the activity of CHL at the RyR, microsomal preparations were incubated in the presences of varying concentrations of CHL together with tritiated ryanodine ([^{3}H]Ry; Bass *et al.* 2011; Holland-Fritsch *et al.* 2013; Qi and Casida 2013). Here, 100 µg ml^{-1} microsomal preparation, from a given species, was incubated in a binding buffer consisting of 140 mM KCL, 20 mM Hepes, and 15 mM NaCl (pH=7.1) with 10nM [^{3}H]Ry and 0.5% DMSO or 0.01-100 µM CHL in 0.5% DMSO. Non-specific binding was run under the same assay conditions but also included 10µM unlabeled ryanodine and 200 µM EGTA Each treatment was run in 300µl of buffer, in triplicate, and assays incubated in a shaking water bath held at 25ºC for 16h . After incubation, samples were filtered using Whatman GF/B filters and washed three times with 5 ml ice cold buffer containing 140 mM KCl, 10 mM Hepes and 0.1 mM $CaCl_2$ adjusted to pH=7.3. The filters were exposed to 5 ml of a scintillation cocktail, stored overnight and radioactivity measured in a liquid scintillation counter. Assays for CHL RyR activity, were tested at least twice and were run on two separate protein homogenates. For the activity of IMI at the nAChR, we assessed the pesticide's ability to displace tritiated IMI ([^{3}H]IMI) in competitive binding assays following methods of Wiesner and Kayser (2000). Here, 100 µg ml^{-1} microsomal preparation, from a given species, was incubated in a binding buffer consisting of 20 mM Na_2HPO_4, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF and 2 µg ml^{-1} (pH=7.0) that contained 1nM [^{3}H]IMI and 0.5% DMSO or 0.01-100 µM IMI in 0.5% DMSO. Non-specific binding was run under the same assay conditions but also included 10µM unlabeled IMI. Assays were run in a total of 300 µl, in triplicate, and were incubatedin a shaking waterbath

at 20ºC for 3h. After incubation, samples were filtered using Whatman GF/B filters and washed three times with 5 ml ice cold buffer containing 20 mM Na_2HPO_4, 150 mM NaCl, 1 mM EDTA adjusted to pH=7.0. The filters were exposed to 5 ml of a scintillation cocktail, stored overnight and radioactivity measured in a liquid scintillation counter. Assays for IMI competitive inhibition, were conducted at least twice on two separate protein homogenates.

Radio-ligand Statistical Analysis: Specific binding was calculated by subtracting the non-specific binding from the total observed binding in a given assay. Specific binding due to chemical concentration, in disintegrations per minute (DPM), was then represented as percent binding relative to control binding. Direct impacts of CHL or IMI on the RyR and nAChR were then determined using sigmoidal-dose response curves or a one-way ANOVA if necessary (Prism 5.0; Graphpad Software). For activity of CHL at the RyR, we calculated an effective concentration that would cause 50% of the maximum response (EC_{50}) and for IMI activity at the nAChR we calculated an inhibition concentration to 50% of control binding (IC_{50}).Due to the nature of the RyR binding assay (see results and discussion), we also calculated the CHL concentration needed to cause a 200% (2-fold change, EC_{2X}) over activation at the RyR of a given species.

Task 3. Results and Discussion

The plant alkaloid ryanodine, for which the RyR is named, binds preferentially to the open state of the RyR. Therefore, increased [^{3}H]Ry binding in the presence of CHL would signify increased activity due to chemical perturbation. Here, CHL was found to activate the RyR present in all invertebrate species and the vertebrate fish species assessed in the current study (**Figure** 4).

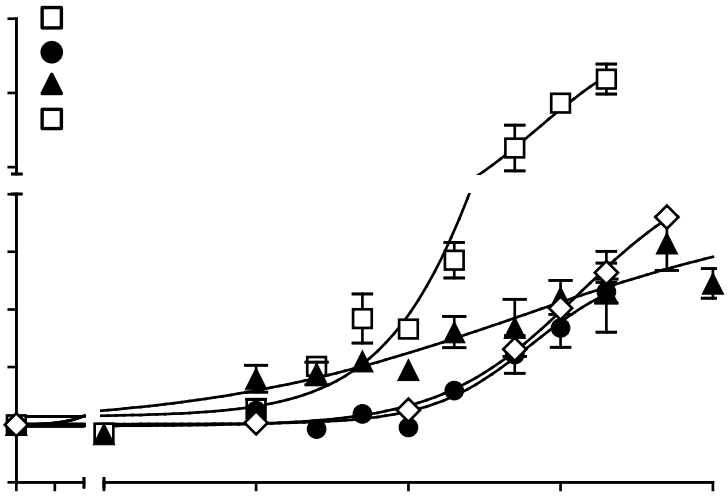

Figure 4. *[^{3}H]Ry binding in aquatic invertebrate and vertebrate model species in the presence of chlorantraniliprole. Specific binding shown as a percentage of DMSO solvent control; mean ± SEM, n=3-15.*

Overall, CHL was the most efficacious in *H. Azteca,* where it caused a maximum response of 1627% relative to that observed under control conditions and this effect was considerably higher than that observed in the other species. For all species, CHL response curves did not reach a plateau even after pushing the CHL concentration to saturation, and thus the curves had wide EC_{50} 95% confidence intervals (**Figure** 4; Table 7). As a better predictor of CHL potency toward the receptor, we calculated the EC_{2X}, or the concentration causing 200% over activation of the RyR, which has been found to be a relevant level of toxicity in mammalian studies (Holland et al. 2017). Both *H. azteca* and *D. magna* displayed the highest sensitivity to CHL with an EC_{2X} of 0.47 µM and 0.48 µM, respectively, relative to *C. diltus* and the vertebrate *P. promelas.* However, we did observed significant activity in the fish species with an EC_{2X} of 3.61 µM; however, binding results were highly variable between fish protein preparations (data not shown) and additional data is currently being collected.

Table 7. *Chlorantraniliprole activation of the ryanodine receptor found in aquatic invertebrate and vertebrate model species*

Species	Maximum Response (%)	EC50 (µM)	EC50 95%CI (µM)	EC_{2X} (µM)	EC_{2X} 95% CI (µM)
H. azteca	1627.0	7.04	1.56-31.87	0.47	0.0 - 1.189
C. dilutus	377.8	6.46	0.98-42.75	4.13	1.59 - 7.27
D. magna	477.9	5.45	0.17-252	0.48	0.18 - 0.89
P. promelas	556.7	13.2	8.08-21.60	3.61	2.93 - 4.28

The diamide insecticides, which include CHL, have been found to display high affinity for invertebrate species with significantly reduced affinity in vertebrates, namely mammals (Cordova et al. 2005; Lahm et al. 2007; Qi and Casida 2013). Published work has demonstrated that concentrations as low of 10 nM of CHL can cause a 200% overactivation of RyR channels found in several insects including the honey bee (*Apis mellifera*) or 100 nM in the common house fly (*Musca domestica*). Conversely, no observed affects in rabbit or lobster (*Homarus americanus*) tissue at 10 or 100 nM CHL concentrations (see Qi and Casida 2013). Similarly, studies comparing responses in cell lines have shown that insects are 300-fold more sensitive to CHL than mammals including mouse, rat and human cell lines (Lahm et al. 2007). The current study is the first to address CHL activity at the RyR of the crustaceans *H. azteca,* and *D. magna,* insect *C. diltus* and the vertebrate fish model *P. promelas*. We observed high CHL affinity for *H. azteca,* and *D. magna* RyR (*,* which was not observed in the other crustacean tested to date (i.e. lobster) suggesting differences in sensitivity. Notably, we also observed significant activation of RyR found in *P. promelas* but this was observed in the µM range. Current data in mammalian cell lines suggests that activity would occur above 14 µM (rat) or > 100 µM (mouse, humans) of CHL in vertebrates. Current data suggest that the fish species may be more sensitive to CHL but more data is needed.

The competitive binding assays for [^{3}H]IMI displacement from the nAChR demonstrated a high affinity of IMI for the nicotinic receptor found in *H. azteca* and *C. diltus* (**Figure** 5) where a IC_{50} values of 8.86 nM and 8.04 nM were observed respectively (Table 8). Interestingly, 1 nM [^{3}H]IMI did not display binding to the nicotinic receptors found in *D, magna* and *P.promelas*

(data not shown) after several assay assessments. These assays are currently be repeated under slightly differing assay conditions.

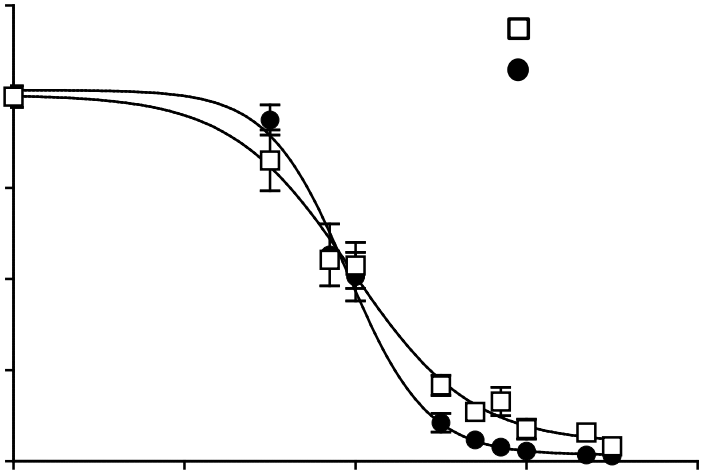

Figure 5. *Competitive inhibition of [^{3}H]IMI binding in aquatic invertebrate and vertebrate model species in the presence of imidicloprid. Specific binding shown as a percentage of DMSO solvent control; mean ± SEM, n=6.*

Table 8*. Imidacloprid competitive inhibition of 1 nM [^{3}H]Imidacloprid binding to the n-acteyl choline receptor found in aquatic invertebrate and vertebrate model species*

Species	IC50 (nM)	IC50 95%CI (nM)
H. azteca	8.86	5.67-13.99
C. dilutus	8.04	5.89-10.99
D. magna	-	-
P. promelas	-	-

Task 4 Dissemination of information and outreach/education:

Presentations:

- Holland, E.B. Tools for assessing toxic endpoints. Invited presentation at the Southern California Coastal Water Research Project (SCCWRP), April 2018, Costa Mesa, CA

- Alejo, J. S. and E.B. Holland. Defining freshwater invertebrate and vertebrate species sensitivity to the pesticides imidacloprid and chlorantraniliprole using ligand binding

assays: Poster presentation at the Southern California Chapter Society of Environmental Toxicology and Chemistry Annual Meeting, April 2018, Los Angeles, CA.

Note: Jordan Alejo is from an underrepresented racial category and is an undergraduate researcher, those stats are always important for our reports at CSULB

- Stinson, S., Deng, X. & Connon R. E. Effect-based analyses of complex contaminant mixtures present in agricultural water, using Daphnia magna and *Pimephales promelas.* Poster presentation at the Society of Environmental Toxicology and Chemistry's North America 39th Annual Meeting held on 4–8 November 2018, in Sacramento, California.

Overall Comments:

Molecular biomarkers for CHL and IMI were developed for *Daphnia magna, Hyalella azteca* and fathead minnow, which will be available to improve biomonitoring and provide additional tools for DPR to evaluate potential effects of CHL and IMI contamination to aquatic organisms in surface water. . We have been unable to develop quantitative PCR systems for *Chironomus dilutus* within the timeframe of this study, however, we will continue to do so as part of ongoing studies in the Connon Lab. A new graduate student is taking over this work.

Integration of *in vitro* assessments and genomic profiling to assess ecosystem health fills a missing gap in environmental monitoring efforts. This integrative approach allowed the quantification of physiological responses of such scenarios and represented a unique opportunity to aid in conserving California's aquatic ecosystems. Further, *in vitro* assessments confirmed that the emerging contaminants act on the corresponding receptors in aquatic organisms and correlate biomarker responses with chemical mode of action in diverse species.

References cited:

Bass, C., Puinean, A.M., Andrews, M., Cutler, P., Daniels, M., Elias, J., Paul, V.L., Crossthwaite, A.J., Denholm, I., Field, L.M., Foster, S.P., Lind, R., Williamson, M.S. and Slater, R. (2011). "Mutation of a nicotinic acetylcholine receptor β subunit is associated with resistance to neonicotinoid insecticides in the aphid myzus persicae." BMC Neuroscience 12(1): 1-11.

Connon, R.E., D'Abronzo, L.S., Hostetter, N.J., Javidmehr, A., Roby, D.D., Evans, A.F., Loge, F.J. and Werner, I. (2012). "Transcription profiling in environmental diagnostics: Health assessments in columbia river basin steelhead (oncorhynchus mykiss)." Environmental Science & Technology 46(11): 6081-6087.

Derveaux, S., Vandesompele, J. and Hellemans, J. (2010). "How to do successful gene expression analysis using real-time pcr." Methods 50(4): 227-230.

Holland-Fritsch, E.B., Connon, R.E., Werner, I., Davies, R.E., Beggel, S., Feng, W. and Pessah, I.N. (2013). "Triclosan impairs swimming behavior and alters expression of excitation-contraction coupling proteins in fathead minnow (pimephales promelas)." Environmental Science & Technology 47(4): 2008-2017.

McLoughlin, K., Turteltaub, K., Bankaitis-Davis, D., Gerren, R., Siconolfi, L., Storm, K., Cheronis, J., Trollinger, D., Macejak, D., Tryon, V. and Bevilacqua, M. (2006). "Limited

dynamic range of immune response gene expression observed in healthy blood donors using rt-pcr." Molecular Medicine 12(7-8): 185-195.
Pörtner, H.O. (2002). "Climate variations and the physiological basis of temperature dependent biogeography: Systemic to molecular hierarchy of thermal tolerance in animals." Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 132(4): 739-761.
Qi, S. and Casida, J.E. (2013). "Species differences in chlorantraniliprole and flubendiamide insecticide binding sites in the ryanodine receptor." Pesticide Biochemistry and Physiology 107(3): 321-326.
USEPA (2002). Short-term methods for estimating the chronic toxicity of effluents and receiving waters to organisms. U. O. o. Water. Washington DC.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). "Accurate normalization of real-time quantitative rt-pcr data by geometric averaging of multiple internal control genes." Genome Biology 3(7): research0034.0031-research0034.0011.

Budget:

Total Research Funds from CDPR: $106,250

Award Dates: 1/1/2017 to 12/31/2018 (100%)

	Budgeted	Expenses	Balance	Distribution
Total Salaries and Benefits	$ 53,826	$ 58,216	$ (4,390)	108%
Subcontracts	$ 11,000	$ 11,000	$ -	100%
Supplies and Expense	$ 19,674	$ 15,303	$ 4,371	78%
Travel	$ 500	$ 481	$ 19	96%
Total Direct Costs	$ 85,000	$ 85,000	$ -	100%
Indirect Costs	$ 21,250	$ 21,250	$ -	100%
Total Costs	$ 106,250	$ 106,250	$ -	100%

Note: Additional funds, approximating $5,000 were made available through Dr. Connon's discretionary accounts towards completing these studies.