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Anthropogenic Impacts on Freshwater Organisms: Bioassessments from the Molecular to Community Levels

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Anthropogenic Impacts on Freshwater Organisms: Bioassessments from the Molecular to Community Levels

by

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DISSERTATION

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"Rerum natura nusquam magis quam in minimis tota sit...
et in contemplatione naturae nihil possit videri supervacuum."

"Nature is the most complete in her smallest things...
and in the contemplation of nature nothing can seem insignificant."

(Gaius Plinius Secundus, Naturalis Historia)

"The river moves from land to water to land, in and out of organisms,
reminding us what native peoples have never forgotten:
that you cannot separate the land from the water, or the people from the land."

(Lynn Noel, *Voyages: Canada's Heritage Rivers*)

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I wrote this dissertation in Sacramento, California, on the traditional lands of the Nisenan people. I conducted most of the laboratory work at the University of California at Davis, which exists on the lands of the Patwin people. My field research was conducted in the Central Coast Region and Salinas Valley of California, which are the traditional lands of the Salinan, Esselen, Yokut, Amah-Mutsun and Ohlone tribes. The Indigenous peoples of California are committed to sustainable stewardship, as they have been for countless centuries. I am honored and grateful to be here today on their traditional lands.

Abstract

This dissertation explores subcellular, organismal and community level effects induced by pesticides of concern in agricultural surface water, and evaluates the use of molecular methods; i.e., environmental DNA (eDNA) metabarcoding, in watershed-wide, multitrophic assessments of freshwater biodiversity. I sought to characterize the impacts of chemical disturbance events occurring at multiple levels of biological organization in anthropogenically impacted freshwater ecosystems.

I first evaluated the lethality and sublethal effects of two pesticides: imidacloprid (IMI) and chlorantraniliprole (CHL), as single compounds and binary mixtures, on invertebrates (*Daphnia magna*) and fish (*Pimephales promelas*). To explore the effects of complex mixtures, I also conducted exposures as described above on contaminated surface water samples collected near agricultural fields associated with the Salinas River Watershed CA (USA). Analytical chemistry data from surface water samples showed chemicals of emerging concern as common analytes at levels expected to cause detrimental effects on aquatic life. I measured acute toxicity in invertebrates exposed to field-collected surface water, and fish exposed to these water samples had significant changes in expression of genes (RT-qPCR) involved with detoxification and neuromuscular function. Exposure of fish to single compounds or binary mixtures of IMI and CHL led to increased relative gene expression of ryanodine receptors (RyR) in fish. Furthermore, IMI targeted the postsynaptic nicotinic acetylcholine receptor (nAChR) in aquatic invertebrates and CHL caused overactivation of RyR in invertebrates and fish. Overall, high levels of invertebrate toxicity and impacts to neuromuscular health in fish are occurring, and pesticides of emerging concern result in detrimental effects in both invertebrates and fish.

Based on these findings, I examined behavioral endpoints in the fathead minnow (*Pimephales promelas*) after the surface water exposures outlined above. I detected differences in both light-induced startle responses and average total distance moved (mm/s), as well as the duration and/or frequency of cruising, bursting and freezing endpoints. These behaviors directly relate to factors influencing survival, feeding and growth, as well as potential for predator avoidance, and thus changes induced by chemical exposure contribute to ecological risk. I detected sublethal and environmentally relevant effects from exposure to contaminated surface waters, which would likely be missed in standard toxicology assessments based on mortality, illustrating the importance of incorporating sublethal endpoints in risk assessments.

I then examined behavioral effects of exposure to contaminated surface water before and after a disturbance event (a "first flush" rainstorm at the end of a dry period). I postulated that the swimming behavior of *D. magna* would be a sensitive bioindicator of exposure to environmentally relevant concentrations of pesticides of concern (IMI and CHL) under laboratory conditions as well as within complex mixtures in contaminated surface waters. I determined that average total distance moved is a sensitive endpoint for pesticide exposure. *Daphnia magna* response to light stimulus was the most sensitive endpoint measured. In exposures conducted before the first flush event, I detected strong dose-response patterns, with exposed organisms showing a significantly reduced response compared to controls. After first flush, I measured hypoactivity for all sites. I detected different response patterns to light stimulus for each site tested: negative dose-response, non-monotonic, and positive dose-response patterns, with significantly different responses from controls at all concentrations tested.

Having determined sublethal and toxic effects from organismal exposure to water samples collected from the Salinas River Watershed, I sought to evaluate how aquatic

biodiversity may be distributed across a chemically impacted watershed, and how diversity estimates obtained from both eDNA metabarcoding and morphological identification would correlate with biotic index scores. I collected eDNA from sediment at sites throughout the Salinas River Watershed, across a range of habitat qualities, and compared the resulting taxonomy with morphological data from a subset of high-diversity sites. I detected sensitive invertebrate taxa (Ephemeroptera, Plecoptera, Trichoptera; EPT) from morphology and eDNA, with significant overlap (> 76.67%) between methods, but some taxa were missing from the sequence database, highlighting the importance of taxonomic database development. Sequencing detected more benthic macroinvertebrate taxa than morphology when compared at the genus and species levels of taxonomic resolution. Metabarcoding of sampled eDNA detected rare species of concern and invasive species. Impacted sites contained greater numbers of species known to be tolerant to poor water quality, whereas I only detected several sensitive EPT taxa from least impacted reference sites. Hydrologic distance (waterbody) and biotic index score both accounted for > 27% of the dissimilarity in taxa measured between sites. These findings suggest that biotic indices obtained from eDNA metabarcoding data can be effectively incorporated into watershed-wide, multitrophic assessments of freshwater biodiversity.

Freshwater ecosystems in urban and agriculturally developed watersheds are simultaneously exposed to chemical mixtures often include new and emerging contaminants of concern, for which toxicological data may be limited. As the complexity of mixtures increases, non-targeted, effect-based evaluations become necessary for determining potential detrimental outcomes. Through my dissertation work I demonstrate that subcellular, organismal and community level effects are induced by pesticides of concern present in surface water.

Glossary of Terms

Term	Description
Amplicon	A targeted, amplified region of DNA
Amplicon Sequence Variant (ASV)	An exact DNA sequence, confirmed from error modeling, obtained from HTS sequencing
Anacapa Toolkit	A pipeline of bioinfomatics tools used to process multilocus sequencing data typical of metabarcoding studies (see Curd et al. 2019)
Benthic Macroinvertebrate (BMI)	Freshwater invertebrate taxa which inhabit the benthos during at least one life stage, and are large enough be seen without magnification
Bioinformatic pipeline	A set of computational, mathematical, and statistical tools used sequentially to collect, organize, and analyze genetic sequencing and related biological data
Biological assessment (bioassessment)	The evaluation of the condition of a waterbody based on the organisms living within it
Biomarker of effect	The quantifiable changes that an individual endures, which indicates an exposure to a compound and may indicate a resulting health effect
California Stream Condition Index (CSCI)	A standardized bioassessment index developed to evaluate habitat condition across the diverse freshwater aquatic ecosystems of California (USA)
Contaminant of emerging concern (CEC)	Pollutants that are detected in contaminated water, for which there is limited information on ecological or human health impacts, and typically are not regulated under current environmental laws
Creating Reference libraries Using eXisting tools (CRUX)	CRUX is the first module of the Anacapa Toolkit and is used to construct custom reference databases for user-defined primers by querying public databases
Differential gene expression	Variation in the abundances of RNA transcripts between organisms from different treatment groups
DNA Barcode	A short, standardized DNA sequence that contains enough genetic variation to differentiate between species

Term	Description
DNA Barcoding	The taxonomic identification of species based on single specimen sequencing of informative regions of DNA (e.g., CO1)
Environmental DNA (eDNA)	Extra-organismal DNA obtained from an environmental sample, which originated from feces, mucus, skin cells, organelles, gametes., etc. Environmental DNA can be sampled from sources including seawater, freshwater, soil, sediment, air, ice, or permafrost
Environmental risk assessment	A process for evaluating how likely it is that an environment will be impacted by an environmental stressor, such as a chemical, as a result of exposure
EPT Taxa	Invertebrates which belong to one of three Orders: Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies), which are known to be indicators of water quality
High-Throughput Sequencing (HTS)	Sequencing techniques that allow for simultaneous analysis of millions of sequences, that are high-throughput when compared to the Sanger sequencing method of processing one sequence at a time
Light-induced startle response	A behavioral response to changing light conditions, measured as the change in mean (± SE) distance traveled after the initiation of a photoperiod
Metabarcoding	The taxonomic identification of multiple species extracted from a mixed sample (community DNA or eDNA) which have been PCR-amplified and sequenced on a high-throughput platform (e.g., Illumina, Ion Torrent)
Mitochondrial cytochrome c oxidase subunit 1 (CO1)	A region of mitochondrial DNA that contains sufficient variation to allow species-level differentiation for many taxa, can be PCR amplified from most animals and the associated database now boasts millions of taxonomically verified DNA sequences
Mode of action	Physiological or functional changes resulting from the exposure of a living organism to a substance
Rarefaction	The statistical technique used to evaluate species richness from sequencing data

Term	Description
Reference Sequence Database	A collection of DNA sequence data and annotations which are maintained in the public databases
Sequence read	A length of base pairs sequenced from a DNA fragment
Surface Water Ambient Monitoring Program (SWAMP)	The State of California Water Resources Control Board's Surface Water Ambient Monitoring Program (SWAMP) is tasked with assessing water quality in all of California's surface waters
EPA Aquatic Life Benchmark	The EPA Office of Pesticide Programs' Aquatic Life Benchmarks for freshwater species used to estimate risk to freshwater organisms from exposure to pesticides and their degradates, based on toxicity values from scientific studies

Table of Contents

Introduction

Freshwater ecosystems are some of the most biodiverse, yet most endangered, habitats on the planet. While only occupying < 1% of Earth's total surface area, they support > 10% of all known species, including between 25-33% of all vertebrate species, and roughly 40% of global fish diversity (Dudgeon et al., 2006; Strayer and Dudgeon, 2010). Many highly biodiverse "hotspots" occur within freshwater systems and, due to the difficulty of inventorying species in these systems, they are likely to contain many as-yet undiscovered species (Lévêque et al., 2008; Strayer and Dudgeon, 2010). The Millennium Ecosystem Assessment, organized by the United Nations Environment Programme, estimated that in just 30 years (1970 - 2002) global freshwater ecosystems experienced a 55% decline in biodiversity (Reid et al., 2005). In 2005, freshwater biodiversity was identified as the predominant conservation priority during the International Decade for Action 'Water for Life' (Dudgeon et al., 2006). In 2020, the Convention on Biological Diversity (CBD) and Biodiversity Strategy of the European Union (EU) identified freshwater biodiversity as disproportionately threatened and underprioritized (van Rees et al., 2021). Biodiversity loss is an imminent threat affecting the performance of many ecosystems, across trophic levels, and an increasingly urgent concern for freshwater ecosystems (Hector et al., 2001; Naeem et al., 1994).

Freshwater environments are imperiled by anthropogenic impacts including poor water quality, invasive species, effects of climate change, low flows and poor management practices (Reid et al., 2019; van Rees et al., 2021). A recent synthesis of > 44,000 articles published in the past decade to assess the research focus on global drivers of loss demonstrated that freshwater systems are disproportionately impacted by pollution (Mazor et al., 2018). The intensification of

agricultural activities is accelerating this threat. Aquatic environments located near high use agricultural and urban areas are frequently impacted by pesticides, which are often detected as complex mixtures (Tang et al., 2021). Pesticide impacts are especially damaging in low-economic regions where there are limited resources available for regulation, monitoring or restoration efforts (Curl et al., 2020; Stehle and Schulz, 2015).

Despite their ecological importance, freshwater ecosystems are understudied in comparison to terrestrial systems. This bias is reflected in a recent meta-analysis showing that freshwater ecosystems only account for just 18.1% of published biodiversity-related studies, most of which focused on habitats located in wealthy, western/global north nations (Tydecks et al., 2018). A meta-analysis of 134,321 biodiversity-related publications reported systematic biases towards research conducted in wealthy countries, while regions with disproportionately high biodiversity as well as a high share of threatened species were underrepresented (Tydecks et al., 2018). Research addressing human-induced habitat change is underrepresented in the literature for freshwater systems (Mazor et al., 2018). There are significant knowledge gaps, both in geographical coverage and lack of taxonomic information for many freshwater ecosystems (Balian et al., 2008).

1.2 Study System: The Salinas River Watershed

The Salinas River Watershed is the largest riparian corridor for California's Central Coast, providing riparian habitat crucial for threatened and endangered species (Clemow et al., 2018; Howell et al., 2010). These species rely on the Salinas River, its tributaries and their associated riparian zones for reproduction, food and habitat (Croll et al., 1986; River, 2002). The river also supplies irrigation water to over 200,000 acres of highly productive agricultural land including some of the most intensively farmed land in the United States (Goh et al., 2019; Hunt

et al., 2003). Urban and agricultural runoff impacts water quality in the Watershed (Anderson et al., 2003; Hunt et al., 2003; Kuivila et al., 2012), resulting in frequent detections of chemicals of concern at levels that are toxic to sensitive organisms (Anderson et al., 2006; Anderson et al., 2003; Deng et al., 2019). As a result, the Salinas River was placed on the US federal Clean Water Act 303(d) list of impaired water bodies (Hunt et al., 2003).

This system is ideal for examining the effects of pesticide mixtures, as they occur in the environment, on aquatic organisms across multiple trophic levels. California contains highly diverse ecosystems which are hotspots for biodiversity (Meyer et al., 2021). The Salinas River Watershed includes a wide range of habitat types and quality (Mazor et al., 2016). Additionally, agencies such as the California Department of Pesticide Regulation (CDPR) and the California State Water Resources Control Board (CSWRCB) under their Surface Water Ambient Monitoring Program (SWAMP), have conducted extensive pesticide monitoring and bioassessments throughout the Salinas River Watershed for more than a decade (Deng et al., 2019; Goh et al., 2019; Sandstrom et al., 2021). Together, these factors and extensive historical data, make the Salinas River Watershed an ideal location to explore and evaluate the impacts of anthropogenic activities on aquatic ecosystems.

1.3 Pesticides: Mixture Toxicity and Risk Assessment

Detrimental impacts on environmental health, along with the development of pesticide resistance will continue to drive the discovery of new insecticides, and concurrently drive the environmental impacts of overuse (Bass et al., 2015; Weston et al., 2013; Wolfram et al., 2018; Zhang, 2018). Novel pesticides are being developed and applied at an increasingly rapid pace worldwide, and these trends are expected to accelerate with global climate change (Bernhardt et al., 2017; Pisa et al., 2021). Pesticides are frequently detected as components of complex

chemical mixtures in aquatic environments, particularly those located near high use agricultural and urban areas (Bradley et al., 2021; Sandstrom et al., 2021; Tang et al., 2021). In the United States and many other countries, some level of risk assessment is required for new pesticides to determine their potential risk to non-target organisms (vertebrates) and to the environment prior to use authorization (Handford et al., 2015). Few standardized assessments currently exist for mixtures, however (Hernández et al., 2017; Reffstrup et al., 2010). Assessing the environmental and health risks of mixtures is complicated by their nonlinear and often synergistic toxicity. There is limited information on the potential toxicity of many pesticides to non-target organisms (Tang et al., 2021).

The use of pesticides with novel modes of action are increasing worldwide (Spurgeon et al., 2010), yet there is limited information on the potential toxicity of many novel pesticides to non-target organisms. Examples of these pesticides of concern include neonicotinoids and anthranilic diamides, for which there are clear global trends showing an increase in use (Bentley et al., 2010; Wolfram et al., 2018) due to their effective action against many insect pests (Teixeira and Andaloro, 2013). Neonicotinoids are the fastest growing class of insecticides world-wide (Mitchell et al., 2017; Simon-Delso et al., 2015), with 81% of global surface water studies reporting neonicotinoid concentrations that exceeded threshold values expected to affect sensitive aquatic organisms (reviewed in Morrissey et al. 2015). Neonicotinoids are authorized for use in over 120 countries worldwide and have been detected in the environment since their introduction (Jeschke et al., 2011). Imidacloprid (IMI) is a neonicotinoid pesticide which interacts agonistically with the postsynaptic nicotinic acetylcholine receptor (nAChR) causing toxic effects to the central nervous system (Li et al., 2021). Application of anthranilic diamides is also rapidly increasing, and currently represents 12% of the global insecticide market (Jeschke,

2021). Chlorantraniliprole (CHL), like other anthranilic diamides, activates and competitively binds to ryanodine receptors (RyRs) (Bentley et al., 2010; Cordova et al., 2007), effectively altering calcium signaling and muscle movement (Bentley et al., 2010; Cordova et al., 2007). Imidacloprid (IMI) and Chlorantraniliprole (CHL) are detected in surface waters around the world (Pisa et al., 2021; Wolfram et al., 2018).

As pesticides with novel modes of action continue to be developed, the complexity of the resulting mixtures which may enter aquatic habitats also increases. In many habitats across the globe, mixtures of chemical contaminants, including pesticides, are present at concentrations expected to cause detrimental effects on the abundance and diversity of aquatic life (Brusseau and Artiola, 2019). A recent study examining the global risk of pesticide pollution found that 74.8% of agricultural land (approximately 28.8 million km^2) is at some risk of pesticide pollution, with 31.4% (approximately 12.1 million km^2) of this land at high risk (Tang et al., 2021). The United States Geological Survey (USGS) recently evaluated freshwater ecosystems across the United States and detected close to 400 unique organic analytes (pharmaceutical, pesticide, organic wastewater indicators), over 300 of which were present at concentrations above US Environmental Protection Agency (EPA) thresholds for aquatic life (Bradley et al., 2021). Their findings indicate that simultaneous exposure to multiple organic contaminants (mixtures) is the norm rather than the exception for habitats located in urban and agriculturally developed areas, which are extremely vulnerable to impacts from contaminant mixtures.

As the complexity of mixtures increases, non-targeted, effect-based evaluations become necessary for determining toxicological impacts. This is especially relevant for mixtures that include new and emerging contaminants of concern, where data on their toxicological effects may be limited to acute exposures on target and model organisms. To assess the potential

toxicity of surface waters, many regulatory organizations evaluate the survival, growth and/or reproduction of sensitive model species after an acute exposure period (Goh et al., 2019). These endpoints risk underestimating sublethal impacts of exposure on aquatic organisms (Connon et al., 2019; Spurgeon et al., 2010). Sublethal molecular and behavioral assays provide sensitive endpoints to assess subcellular and organismal level effects induced by exposure to pesticides and other chemicals, at environmentally relevant concentrations (Beggel et al., 2011; Connon et al., 2009; Hasenbein et al., 2019; Hussain et al., 2020; Mundy et al., 2021, 2020; Steele et al., 2018). The development of gene expression assays for use as monitoring and diagnostic tools is well established (Beggel et al., 2011; Connon et al., 2008; Forbes et al., 2006; Geist et al., 2007; Kaviraj and Gupta, 2014; Kostich et al., 2019; Vandenberg et al., 2012). These assays depend on a clear understanding of the mechanisms underlying a molecular response, and more research is needed particularly for chemicals of emerging concern and their specific mechanisms of activity (Connon et al., 2012). Swimming behavior is also a well-established endpoint in pharmacology and toxicology (Wolter & Arlinghaus, 2003, Kristofco et al., 2016, Colón-Cruz et al., 2018, Steele et al., 2018). Behavioral assessments are effective for capturing underlying physiological or biochemical conditions, which manifest themselves on an organismal level (Yuan et al., 2021), and for determining ecological risk if the behavior directly relates to factors influencing survival, predator avoidance, feeding and growth, or reproduction (Ford et al., 2021). Previous studies have demonstrated that the swimming behavior of the invertebrate *Daphnia magna* is a sensitive endpoint for exposures to pesticides and other classes of contaminants (Bownik, 2017; Bownik et al., 2019; Chevalier et al., 2015; Tkaczyk et al., 2021). Pesticides have also been shown to alter fish behavior, with several behavioral responses in fishes being described as

highly sensitive, sublethal endpoints for evaluating their toxicity (Delcourt et al., 2013; Hong and Zha, 2019).

1.4 Morphology-Based Taxonomic Bioassessments and Biomonitoring

Invertebrate organisms are a key component of the aquatic food web, consuming phytoplankton and detritus, and providing a vital food source for organisms at higher trophic levels (Balian et al., 2008; Merritt and Cummins, 2008). In habitats that are periodically impacted by multiple stressors including poor water quality, the altered abundance or absence of sensitive invertebrates can cause changes in community structure (Thompson et al., 2020a; Thompson et al., 2020b). Declining biodiversity across trophic levels can reduce ecosystem function and alter ecosystem performance at multiple scales (Naeem et al., 1994). The presence/absence and abundance of sensitive groups of invertebrates are routinely used in biomonitoring surveys as a rapid assessment of water quality (Mazor et al., 2010; Ode et al., 2016). Benthic macroinvertebrate (BMI) assemblages are the most commonly used group for conducting bioassessments of freshwater habitat and water quality worldwide due to their taxonomic diversity, abundance, and responsiveness to stressors (Resh, 2008). The patchy distribution of BMI across temporal and spatial scales can result in an underestimation of species richness without appropriate sampling design, however, and significant taxonomic expertise is required for morphological identifications (Lenat and Resh, 2001; Rehn et al., 2007). Many studies only identify individuals to the family level, which can result in an underestimation of species richness, or arguably worse, grouping species with different stressor tolerances together and overestimating site condition or quality (Jones, 2008; Lenat and Resh, 2001). Sampling efforts are often constrained by practical considerations including the availability of expertise,

funds, and time. As a result of these limitations, watershed-wide, multitrophic assessments of freshwater biodiversity are scarce (Mächler et al., 2014)

1.5 Environmental DNA Metabarcoding

Organisms continually shed their DNA into their environment, which can be detected from environmental samples such as water, soil, sediment or air. Environmental DNA (eDNA) is the complex mixture of genomic DNA originating from many organisms, that can be detected in these environmental samples (Taberlet, 2018). To determine which organisms are present in an environment, eDNA can be analyzed and matched with taxonomy in reference sequence databases (Epp et al., 2012; Hajibabaei et al., 2011). To analyze eDNA samples, DNA is first extracted from the sample, then specific fragments of DNA are targeted that contain sufficient variation to differentiate between closely related species, while being highly conserved/present in a wide range of taxa (Taberlet et al., 2012). To capture a broad representation of taxonomic diversity, one or more fragments of DNA are sequenced simultaneously through a multilocus metabarcoding approach using standard markers for animals, plants, fungi, bacteria, etc. (e.g., mitochondrial 12s ribosomal RNA (12S), internal transcribed spacer of nuclear ribosomal DNA (ITS2), Cytochrome Oxidase 1 (CO1), and 18s ribosomal RNA (18S)) (Curd et al., 2019; Meyer et al., 2021). This technique has been shown to improve identification of cryptic species, juvenile life stages, and rare taxa (Mächler et al., 2014).

1.6 Molecular Bioassessments and Biomonitoring

Genetic techniques can greatly enhance traditional biodiversity monitoring, increasing the coverage of species presence-absence data and providing a rapid assessment of a wide range of biodiversity to understand community condition (Deiner et al., 2020, 2017; Ficetola et al., 2010; Hajibabaei et al., 2011; Mächler et al., 2014; Taberlet et al., 2012). Metabarcoding can provide

baseline data across a wide range of taxa - information that is crucial for understanding and sustaining biodiversity. In anthropogenically impacted systems, the detection of sensitive biomonitoring species also provides important habitat quality data and information on how surrounding land use might influence aquatic community composition. While extensive research has demonstrated the utility of eDNA for providing presence-absence data on invasive or endangered species, few studies have applied this technique to landscape-wide assessments of beta diversity (e.g., Altermatt, 2013; Bush et al., 2020).

1.6 Chapter Summary

In Chapter One I evaluated the toxicity of two pesticides, imidacloprid (IMI) and chlorantraniliprole (CHL) as single compounds and binary mixtures, and surface water collected near agricultural fields (Salinas River Watershed, CA), after acute exposures using invertebrates (*Daphnia magna*) and fish (*Pimephales promelas*). In addition to determining acute toxicity, my secondary goal was to assess whether changes in select subcellular molecular pathways correspond to the insecticides' mechanisms of activity in aquatic organisms. To determine this, I conducted acute (96 h) exposures using environmentally relevant concentrations of single and binary mixtures of IMI and CHL, and a geometric dilution series of surface water. I then evaluated survival for invertebrates and fish, and differential expression (RT-qPCR) of target genes for fish.

In Chapter Two I assessed whether exposure to surface water collected from urban and agriculturally developed waterways (Salinas River Watershed, CA) impacted multiple behavioral endpoints in the fathead minnow (*Pimephales promelas*), a model species in toxicology. I collected water samples at monitoring stations downstream from agricultural fields, and screened

them for a suite of pesticides. After acute exposures (96 h) to surface water, I used locomotor assays to assess several behavioral responses of larval fish under light:dark conditions.

In Chapter Three, I evaluated whether the swimming behavior of *D. magna* is a sensitive bioindicator of exposure to two chemicals of concern, CHL and IMI, performed at environmentally relevant concentrations. I also examined the behavioral effects of exposure to contaminated surface water before and after the first rain following an extended dry period, also known as a "first flush" rain event. To determine this, I conducted 96 h exposures using IMI and CHL, and surface water from polluted waterways known to contain chemicals of concern, both before and after a first flush rain event. I then used locomotor assays to assess several behavioral responses of *D. magna* under light:dark conditions.

In Chapter Four I sought to understand how biodiversity varies across the Salinas River Watershed, and whether diversity estimates obtained from eDNA metabarcoding correlated with (previously calculated) biotic index scores using the California Stream Condition Index (CSCI). To test this, I collected eDNA from sediment at sites throughout the Salinas River Watershed, across a range of habitat qualities, amplified the mitochondrial Cytochrome Oxidase 1 (CO1) region, and sequenced samples using a metabarcoding approach. I also compared the resulting taxonomy with morphological data from a subset of high-diversity sites.

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Chapter 1
Agricultural surface water, imidacloprid and chlorantraniliprole, result in altered gene expression and receptor activation in *Pimephales promelas*

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Abstract

The toxicity of single pesticides is likely underestimated when considering complex pesticide mixtures found in agricultural runoff and this is especially true for newer pesticides with little toxicity data on non-target species. The goal of our study was to compare the toxicity of two newer pesticides, imidacloprid (IMI) and chlorantraniliprole (CHL), when an invertebrate and fish were exposed to single compounds, binary mixtures or surface water collected near agricultural fields. A secondary goal was to determine whether changes in select subcellular molecular pathways correspond to the insecticides' mechanisms of activity in aquatic organisms. We conducted acute (96 h) exposures using a dilution series of field water and environmentally relevant concentrations of single and binary mixtures of IMI and CHL. We then evaluated survival, gene expression and the activity of IMI toward the n-acetylcholine receptor (nAChR) and CHL activity toward the ryanodine receptor (RyR). Both IMI and CHL were detected at all sampling locations for May 2019 and September 2019 sampling dates and exposure to field water led to high invertebrate but not fish mortality. Fish exposed to field collected water had significant changes in the relative expression of genes involved with detoxification and neuromuscular function. Exposure of fish to single compounds or binary mixtures of IMI and CHL led to increased relative gene expression of RyR in fish. Furthermore, we found that IMI targets the nAChR in aquatic invertebrates and that CHL can cause overactivation of the RyR in invertebrates and fish. Overall, our finding suggests that IMI and CHL may impact neuromuscular health in fish. Expanding monitoring efforts to include sublethal and molecular assays would allow the detection of subcellular level effects due to complex mixtures present in surface water near agricultural areas.

1. Introduction

The diversity and quantity of pesticides being applied globally are increasing at a rapid pace (Bernhardt et al., 2017; Pisa et al., 2021; Stehle and Schulz, 2015). As the variety of pesticides being applied increases, so does the complexity of the resulting mixtures. Runoff enters waterways from agricultural and urban areas, resulting in complex chemical mixtures that have the potential to cause rapid changes in water quality. These dynamic mixtures often include chemicals of concern that are known to have adverse biological effects in single chemical laboratory exposures. In fact, a recent meta-analysis reported that pesticides exceeded aquatic life benchmarks in 63.5% of agricultural stream sites surveyed across the U.S. (Wolfram et al., 2018). Evaluating the survival of sensitive model species after an acute exposure is a common benchmark for assessing toxicity of surface waters (Goh et al. 2019); however, this may not represent ecologically relevant impacts seen in runoff-impacted waterways (Connon et al., 2019; Spurgeon et al., 2010). Studies of multiple stressors demonstrate complex, nonlinear and often synergistic effects (Spurgeon et al., 2010; Todgham and Stillman, 2013), suggesting that the effects of multiple stressors are often worse than that predicted from results obtained from single stressor studies (Crain et al., 2008; Yang et al., 2007). Therefore, using single stressor data to infer physiological effects occurring in the natural environment may underestimate toxicity.

Pesticide resistance will continue to drive the discovery of new insecticides, and concurrently drive the environmental impacts of overuse (Bass et al., 2015; Weston et al., 2013; Wolfram et al., 2018; Zhang, 2018). Recent shifts in the use trends for various classes of insecticides include phasing out first-generation insecticides in favor of new cost-efficient, effective chemicals. There is a clear global trend showing an increase in the use of

neonicotinoids, as well as chemicals with novel mechanisms of action like anthranilic diamides (Bentley et al., 2010; Wolfram et al., 2018). Two such chemicals of emerging concern are imidacloprid (IMI) and chlorantraniliprole (CHL). Imidacloprid is a neonicotinoid pesticide with a mechanism of action on postsynaptic nicotinic acetylcholine receptors (nAChR), impacting the nervous system (Duke et al., 1993). Neonicotinoids display lower nAChR activity in vertebrates as compared to invertebrates (Liu and Casida, 1993) but have been suggested to cause neurotoxicity in zebrafish as evidenced by changes in expression of the key neurotoxic genes c-fos and Brain Derived Neurotrophic Factor (BDNF; Ozdemi et al. 2018). Additionally, neonicotinoids have been suggested to cause neurotoxicity in mammals, which may be due to neonicotinoid metabolites (see review by Zhao et al., 2020). Chlorantraniliprole is an anthranilic diamide insecticide that increases the activity of the ryanodine receptor (RyR) impacting muscle contraction (Bentley et al., 2010; Cordova et al., 2007). Diamide insecticides were developed to display high affinity for invertebrate species with significantly reduced affinity in vertebrates species (Cordova et al., 2007; Lahm et al., 2007; Qi and Casida, 2013). However, more recent research suggests that CHL may also target the RyR in mammals (Truong and Pessah, 2019) supporting potential impacts in vertebrates. Taken together the mechanisms of action of IMI and CHL suggest that they would exert toxicity on sensitive aquatic invertebrate and potentially vertebrate species.

Both IMI and CHL are now being utilized across the globe (Teikeira and Andaloro 2013; Bakker et al. 2020). One such example is the Central Coast region of California, which contains some of the most intensively farmed agricultural land in the United States (Hunt et al., 2003). Recent data from the CA Department of Pesticide (CDPR) Pesticide Use Report (PUR) database (https://calpip.cdpr.ca.gov/main.cfm) show that approximately 97,026 and 20,620 pounds of IMI

and CHL were applied in the Central Coast region (Monterey and San Luis Obispo counties) between 2017 – 2019, respectively. This has led to increased detections of these pesticides in waterways that surround agricultural areas in the Central Coast, namely, the Salinas River, its tributaries, and other associated waterbodies. These waterways transect the Central Coast receiving runoff from nearby agricultural fields and urbanized areas. The detection of pesticides in these waterways leads to potentially harmful impacts on water quality where chemicals of concern, including IMI and CHL (Table S1.1), are frequently detected in the region at levels that may be toxic to sensitive organisms (Anderson et al., 2003; Goh et al., 2019; Hunt et al., 2003; Kuivila et al., 2012). As a result, the Salinas River was placed on the U.S.A. Federal Clean Water Act 303(d) list of impaired water bodies (Hunt et al., 2003).

Poor water quality threatens the vast number of species present in the Salinas region, including many species of economic and conservation concern. For example, the river and its tributaries have been designated by the National Marine Fisheries Service as critical habitat for southern steelhead trout (*Oncorhynchus mykiss*) serving as a migration corridor and spawning habitat (Anderson et al., 2003). Additionally, pink salmon (*O. gorbuscha*), a commercially harvested and abundant species in the North Pacific are considered imperiled in California and spawn in the Salinas River (Skiles et al., 2013). Toxicity studies have also shown that water collected in the Salinas River and its tributaries causes high rates of mortality in sensitive invertebrate species (Anderson et al., 2006, 2003; Hunt et al., 2003). Invertebrate community structure was also highly impacted downstream of monitoring sites that receive runoff from nearby agricultural fields (Anderson et al., 2006). Together these studies support the impact of agricultural runoff on nearby receiving water. While survival of model invertebrate or fish species is an established endpoint for ecotoxicology assessments, sublethal endpoints are more

sensitive and have greater ecological relevance, revealing a more complete picture of site toxicity (Beggel et al., 2011; Hasenbein et al., 2019).

The goal of our study was to compare the toxicity of IMI and CHL single and binary exposures to that elicited by agricultural surface water collected from the Salinas, CA area in both a sensitive invertebrate and a model fish species. A secondary goal was to determine whether changes in select subcellular molecular pathways correspond to the insecticides' mechanisms of activity in aquatic organisms. We collected water samples at monitoring stations near agricultural fields in Salinas waterways and tributaries, then screened them for pesticides and used standard toxicity assays to evaluate effects in *Daphnia magna* and the fathead minnow (*Pimephales promelas*). We confirmed the insecticides' mechanism of action using radioligand binding in the fathead minnow and three invertebrates (*Daphnia magna, Chironomus dilutus, Hyalella azteca)*. We then evaluated differential gene responses for specific pathways in fish to determine if organisms exposed to agricultural water display similar signs of disruption as those exposed to single or binary mixtures of pure IMI and CHL. This work is the first to address IMI and CHL toxicity in ecologically relevant aquatic organisms, helping to determine the impacts of their use near important waterways.

2. Materials and Methods

2.1 Field Water Sampling

2.1.1 Study Sites

Chemical monitoring sites have been established throughout the Salinas River, nearby tributaries and other waterbodies as previously described (Deng et al. 2019, Goh et al. 2019).

Chemical detection data have been collected from these sites for over a decade (Deng et al., 2019; Goh et al., 2019). Long-term monitoring sites near Salinas, CA were initially chosen based on reported nearby pesticide use, detections from previous monitoring (often determined to be out-of-compliance with water quality levels), and proximity to ecologically sensitive areas (Luo et al., 2018). We sampled water from select, existing long-term monitoring sites (Table S1.2). The sampling sites for this study included six sites in or around Salinas, CA that included four sites that directly receive surface water runoff from adjacent agricultural fields: Quail Creek (Sal_Quail), Chualar Creek (Sal_Chualar), Alisal Creek (Sal_Hartnell), and a reclamation ditch (Sal_SanJon); the main channel of Tembladero Slough (Sal_Haro) and the Salinas River (Sal_Davis). These sites are located immediately downstream of high use agricultural areas, where there is an increased risk of contamination from agricultural runoff.

2.1.2 Water Sampling

We collected water samples from six sites (listed above) on May 14th 2019 and from a subset of those sites (Quail Creek, Alisal Creek and the Salinas River) on September 17th 2019, following standard sampling protocols (Jones, 1999). In brief, we collected samples from well-mixed, wadable waters using 1-liter amber glass bottles certified to meet current US EPA guidelines then sealed with Teflon-lined lids. Immediately after collection, we placed samples in coolers on wet ice for transportation, then refrigerated them at 4°C upon arrival in the lab. We measured water quality parameters *in situ* using a YSI EXO1 multi-parameter water quality Sonde (Doo and He 2008), where parameters recorded including ambient water pH, specific conductance, dissolved oxygen, temperature, total dissolved solids, salinity, and total suspended solids. Results of water quality parameters are shown in Table S1.3.

2.1.3 Geometric Dilution Series for Field Water Treatments

Based on high invertebrate mortality from several previous, preliminary exposure studies from the same field sites (CDPR Technical Report Hasenbein et al. 2018, Grant # 16-C0084), we created a geometric dilution series to better capture sublethal effects. We mixed field water with standard US EPA control water for each test species (see Methods section 2.4.1) to create the dilution series. For our exposures conducted in May, we included 100%, 60% and 35% field water, where dilutions were conducted using control water for a given species (see Methods section 2.2). Based on the high levels of invertebrate mortality observed in our first exposure event, we added additional lower concentrations (20%, 12%) to the subsequent sampling event in September. Immediately before initiating the test, we thoroughly mixed each sample by agitation to homogenize and distribute any remaining sediment particles, then diluted it into control water to obtain the desired concentrations. Once aliquoted into beakers, we allowed the dilutions to reach the desired test temperature for each organism prior to loading organisms into beakers. We repeated this procedure on day 2 of the test to prepare each treatment for the 80% water change. Acute exposure test conditions were identical for both single/binary and field exposures (See Methods section 2.4.2).

2.2 Single/Binary Chemical Treatments

We purchased chemicals (IMI and CHL; >97.5% purity) from AccuStandard (New Haven, CT, USA) and dissolved them in deionized water (IMI) or acetone (CHL). Pesticide-grade acetone (Fisher Chemical, USA) was used as a solvent carrier for the CHL treatments, and in solvent controls, to a final concentration of 0.01% in exposure water. Our stock solutions were then spiked into control water according to target concentrations, keeping acetone at 0.01%, and mixed thoroughly. Our exposure concentrations matched range-finding experiments and

environmentally relevant concentrations (Table S1.1). In total, *D. magna* were exposed to six single concentrations (25, 50, 100, 500, 1000, 10,000ng/L) of each pesticide and three mixture concentrations (25 x 25 ng/L, 500 x 500 ng/L, 10,000 x 10,000ng/L), a solvent control (for CHL exposures only), and a negative control (control water only). *P. promelas* were exposed to three single concentrations (25, 500, 10,000ng/L) of each pesticide and three mixture concentrations (25 x 25ng/L, 500 x 500ng/L, 10,000 x 10,000ng/L), a solvent control, and a negative control. Acute exposure test conditions were identical for both single/binary and field exposures (See Methods section 2.4.2).

2.3 Chemical Analyses

Chemical analysis was completed at the Center for Analytical Chemistry, California Department of Food and Agriculture (Sacramento, CA) using multi-residue liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography–mass spectrometry (GC-MS/MS) methods. For field water, 47 pesticides were included for screening based on the procedures described in the Monitoring Prioritization Model (Luo et al., 2018). For single and binary chemical treatments, IMI and CHL concentrations were measured to confirm target exposure concentrations. Laboratory QA/QC followed CDPR guidelines provided in the Standard Operating Procedure CDPR SOP QAQC012.00 (Teerlink and DaSilva, 2017). Extractions included laboratory blanks and matrix spikes (method detection limit and reporting limit for each analyte available upon request).

2.4 Toxicity Testing

2.4.1 Test Organisms

We obtained *D. magna* from Aquatic Research Organisms Inc. (Hampton, NH, USA), and cultured them in our laboratory at the University of California, Davis (USA). Groups of 20 individuals were maintained at 20 ± 2°C and a 16-hr light: 8-hr dark photoperiod in 2L beakers of reconstituted control water (USEPA, 2002), which was prepared by dissolving 23.04 g $NaHCO_3$, 14.40 g $CaSO_4{\cdot}2H_2O$, 14.40 g $MgSO_4$, and 0.96 g KCl in 120 L of deionized water to achieve a hardness of 160 – 180 mg/L $CaCO_3$ and alkalinity of 110 – 120 mg/L $CaCO_3$. We obtained *P. promelas* larvae from Aquatic Biosystems, Inc. (Ft. Collins, Colorado, USA) at 7 days post-hatch on the day of arrival. We habituated the fish to control water at a temperature of 25°C over a period of 8 hours. Control water consisted of deionized water, modified with salts to meet USEPA specifications (specific conductivity (EC): 265–293 µS/cm; hardness: 80–100 mg/L $CaCO_3$; alkalinity: 57–64 mg/L $CaCO_3$ (USEPA, 2002). During the habituation period <1% mortality was observed, and the fish fed and swam normally. We conducted all studies in accordance with national and institutional guidelines for animal welfare and are described under the University of California Davis, Institutional Animal Care and Use Committee protocol #19690.

2.4.2. Acute Exposure Conditions

Organismal exposures followed acute toxicity procedures outlined by the US Environmental Protection Agency (USEPA, 2002). For 96 h acute exposures we used third brood *D. magna* neonates (< 24h-old) and *P. promelas* larvae (7 days post hatch; dph). Test exposure temperatures were maintained in separate environmental chambers under fluorescent light with a 16-hr light: 8-hr dark photoperiod, at 20ºC/25ºC for *D. magna* neonates and *P. promelas* larvae,

respectively. For *D. magna,* we placed twenty individuals into each of the 250-mL replicate beakers containing 200 mL of treatment water, with four replicates per treatment. For *P. promelas*, each treatment consisted of four replicate 600 mL beakers containing 500 mL test solution and 10 fish larvae. At test initiation, we gently added organisms to each replicate beaker and treatment in a random order. Beaker locations were then randomized within the environmental chamber. We fed *D. magna* at test initiation and at water renewal, using a suspension of concentrated (i.e., 3 x 107 cells/mL) *Raphidocelis subcapitata* (obtained from Aquatic Research Organism Inc), and YCT (yeast, cerophyl, trout chow mixture, total solids > 1.9 g solids/L of final YCT mixture) (USEPA, 2002). We fed fish larvae *ad libitum* with newly hatched *Artemia franciscana*, twice daily.

We recorded mortality daily for all test species, and immediately removed any dead organisms from the test vessels. After 48h, new treatment waters were prepared, and an 80% water change was performed. At the time of water renewal, we measured water quality parameters using a YSI EXO1 multi-parameter water quality Sonde (Doo and He 2008), where parameters recorded including pH, specific conductance, dissolved oxygen, temperature. Test vessels were randomly distributed after each water renewal. At test termination we euthanized surviving fish from each replicate beaker in an overdose of tricaine methanesulfonate (500mg/L MS-222, buffered with 500mg/L sodium bicarbonate). We then pooled remaining fish within each replicate beaker into 1.5mL microcentrifuge tubes, and immediately froze them in liquid nitrogen for subsequent gene expression analysis (See Methods section 2.6).

2.5 Confirmation of IMI and CHL Mechanism of Action in Aquatic Model Species

2.5.1 Protein Preparations

We obtained non-exposed invertebrates and larval fish (7-14 dph) used in *in vitro* assays from Aquatic Research Organisms Inc. (Hampton, NH, USA) and cultured or habituated as described previously. For each species separately, we pooled whole individuals (n > 50) 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; Fritsch and Pessah, 2013; Qi and Casida, 2013; Wiesner and Kayser, 2000). Briefly, we placed tissue into a homogenization buffer consisting of 300mM Sucrose, 20mM Hepes, leupeptin (2µg ml-1), phenylmethanesulfonyl fluoride (PMSF,1 mM), sodium orthovanadate (0.5 mM) NaF (10 mM), β-glycerol (2 mM) and NaP_2O_7 (5 mM) 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 20 s with 2 min on ice between bursts. The homogenate underwent centrifugation at 8000 rpm for 10 min at 4ºC and we collected supernatant into an ultracentrifugation tube. We re-suspended the pellet in 5 mL of homogenization buffer and repeated the homogenization and centrifugation steps. Supernatants were combined and underwent ultracentrifugation at 100,000 g for 1h at 4ºC. The microsomal pellet was then suspended in a 300mM Sucrose 20mM Hepes buffer (pH=7.2) and we it placed into 100 µl aliquots to avoid multiple freeze thaw cycles after storage at -80°C. We determined protein concentrations in triplicate using a BCA assay (Pierce, Rockford, IL).

2.5.2 Radioligand Binding Assays

To measure the activity of CHL at the RyR, we incubated microsomal preparations in the presence of varying concentrations of CHL together with tritiated ryanodine ([^{3}H]Ry; Bass et al., 2011; Fritsch & Pessah, 2013; Qi & Casida, 2013). Here, 100 µg/mL microsomal preparation

from a given species was incubated in a binding buffer consisting of 140mM KCL, 20mM 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. We ran each treatment in 300 µl of buffer, in triplicate, and incubated assays in a shaking water bath held at 25ºC for 16h. After incubation, we filtered samples using Whatman GF/B filters and washed three times with 5mL 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 5mL of a scintillation cocktail, stored overnight and radioactivity measured in a liquid scintillation counter. We tested assays for CHL RyR activity at least twice and ran them 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, we incubated 100 µg/mL microsomal preparation from a given species 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 (pH=7.0) that contained 1 nM [^{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 10 µM unlabeled IMI. We ran assays in a total of 300 µl, in triplicate, and incubated them in a shaking water bath at 20ºC for 3h. After incubation, we filtered samples using Whatman GF/B filters and washed them three times with 5mL ice cold buffer containing 20 mM Na_2HPO_4, 150 mM NaCl, 1mM EDTA adjusted to pH = 7.0. The filters were exposed to 5mL of a scintillation cocktail, stored overnight and radioactivity measured in a liquid scintillation counter. We conducted assays for IMI at least twice on two separate protein homogenates. Due to our findings in *D. magna* and *P. promelas* (see Results section 3.4), we conducted additional studies to investigate

the mechanism of action of CHL and IMI in other important aquatic model species *Hyalella azteca* and *Chironomus dilutus.* We conducted protein preparations, binding conditions and analysis as described for *D. magna* and *P. promelas*.

2.6 Evaluation of Relative Gene Expression

We extracted total RNA from ten pooled fish larvae per replicate (n=4) using a Qiacube system (Qiagen, Hilden, Germany) and QIAGEN RNeasy Plus Mini Kits according to manufacturer's instructions. We confirmed RNA concentrations using a Qubit 4 fluorimeter/broad range RNA assay kit (Invitrogen, Carlsbad, CA), then verified total RNA quality and integrity through nanodrop (Invitrogen, Carlsbad, CA) and electrophoresis on a 1% (wt/vol) agarose gel, respectively. We synthesized complementary DNA (cDNA) from 1 µg of total RNA using Superscript III Reverse Transcriptase, a 100mM dNTP set, and random primers (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Next, we carried out a 1:16 dilution with nuclease free water to generate sufficient template for qPCR analysis, following dilution series analysis during primer validation. We used primer pairs designed for a suite of target genes of interest and three reference genes (Table 1.1). This suite of target genes were selected because they are involved in detoxification, neurological function, or are related to the presumed mechanisms of action for IMI or CHL (Soderlund, 2012; Zanger and Schwab, 2013).

We obtained lyophilized primers from IDT (Integrated DNA Technologies, Inc., Germany) and rehydrated them to 100µmol with RNase-free water. We performed all PCR reactions using QuantiTect SYBR® Green PCR Kit 2× concentration, (Bio-Rad, California, USA) per the manufacturer's protocol, using 5 µL of cDNA in a final reaction volume of 12 µL. Fluorescence was detected (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, Carlsbad, CA,) over 40 cycles, with cycling conditions of 15 min initial heat inactivation at

95°C, 15 sec denaturation at 94°C, 30 sec at an annealing temperature of 55°C, and extension at 72°C. Fluorescence of samples was measured every 7s 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. Using the computational algorithm geNorm (Vandesompele et al., 2002), we assessed the expression stability of each gene. Based on the standard curves, all primer pair efficiencies were within acceptable range, from 92% (*Cyp3a*) to 101% (*AChE*). We examined melt curves for each sample to verify single product amplification and consistency among samples.

Table 1.1: *Genes of interest and reference genes for qPCR analyses. Primers for qPCR analyses for target genes of interest designed using Roche Universal Probe Library (UPL) Assay Design Center.*

Reference Genes	**Abbrev.**	**Forward**	**Reverse**	**Primer Efficiency %**
Elongation Factor 1-alpha	EF1a	CTCTTTCTGTTACCTGGCAAAGG	TCCCATGATTGATTAGTTTCAGGAT	97
L8 ribosomal protein	L8	GGCTAAGGTGGTTTTCCGTGA	CTTCAGCTGCAATGAACAGCTC	99
beta-actin	B-ACTIN	CAACACCGTGCTGTCTGGAG	TCTTTCTGCATACGGTCAGCAA	93
Gene of Interest	**Abbrev.**	**Forward**	**Reverse**	**Primer Efficiency %**
Acetylcholinesterase	AChE	ATGACCAATAGGCCAAAGCATT	ACGGAAAATTCCATCGATCTCA	101
Aspartoacylase	ASPA	TCTGGTAATGGATGTCCCGATT	GACCTCTATGGAAAAGCCATGC	100
Cytochrome P4501A	CYP1a	GCTTCTCGAGGCCTTTATCC	ACAGTGAGGGATGGTGAACG	99
CYP3A126	CYP3a	CAACCCAGAGGCCATGAAGA	GGGCCTTATTTGGGAAGGTCT	92
Ryanodine Receptor, 1	RyR1	AAGATGACGATGAAGGGTTTGTC	CATGGCAGGTTCCATATATCCAG	99
Ryanodine Receptor, 2	RyR2	CCACCTTCTCGAGGTCAGGTT	CCGCCTCAGTGACGGATAATAA	99
Sarco/Endoplasmic Reticulum ATPase	SERCA1	CAACATTGGCCACTTCAACG	GAGCCACAGCGATCTTFAAGT	98

2.7 Statistical Analysis

2.7.1 Mortality

For the dilution series of field water and IMI/CHL single/binary treatments, acute toxicity was defined as a statistically significant difference ($P < 0.05$) in mortality compared to the laboratory control water within 96 h of test initiation. We determined significance by Analysis of Variance (ANOVA) followed by Dunnett's test for multiple comparisons using GraphPad Prism software (version 8.0). For single/binary exposures, we calculated the median lethal concentration (LC) toxicity thresholds (96 h LC_{50} values) with 95% confidence intervals (CI) for single/binary exposures using Probit Analysis in the 'ecotox' package of RStudio statistical software (version 1.3.1073, R Core Team 2020). We also generated dose-response plots to display treatment effects using RStudio (R Core Team 2020).

2.7.2 Radioligand Binding

We calculated specific binding 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. We then determined direct impacts of IMI or CHL on the RyR and nAChR using sigmoidal-dose response curves or a one-way ANOVA if necessary (GraphPad Prism version 8.0). For activity of CHL at the RyR, we calculated an effective concentration that would cause 50% of the maximum response (EC_{50}; relative EC_{50} where maximum effects are not scaled to 100%). 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, $EC_{2X;}$ an absolute value) over activation at the RyR of a given species. For IMI activity at the nAChR we calculated an inhibition concentration to 50% of control binding (IC_{50}).

2.7.3 Relative Gene Expression

For gene expression analyses, we used the mean cycle threshold (Ct) of triplicate technical replicates to calculate relative quantification using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) relative to three reference genes and control samples for each treatment. For single and binary mixture treatments requiring acetone solvent controls, we calculated the mean ΔCt using the solvent treatment control group. We analyzed differential expression using one-way ANOVA followed by Dunnett's multiple comparisons test. To test homogeneity of variances and normality, we used Levene's test and the Shapiro-Wilk test, respectively. When data were not normally distributed, we applied a ln-transformation to achieve normality. When a significant interaction was detected, we used one-way ANOVA followed by Dunnett's multiple comparisons test to determine significant differences between treatments and controls. All analyses were performed using the statistical software GraphPad Prism (version 8.0) with a significance level at $\alpha = 0.05$.

3. Results

3.1 Chemical Analysis of Field Collected Water Samples

Out of the 47 pesticides that were screened for, 17 were detected in the surface waters sampled in May 2019, with a minimum of 11 pesticides detected at each site (Table S1.4). IMI and CHL were detected at all six sites (Table 1.2). IMI ranged in concentration from 0.019 µg/L to 1.19 µg/L. Concentrations of IMI exceeded the EPA benchmarks for acute invertebrate (0.385 µg/L) and/or chronic (0.01 µg/L) exposure in all six sites, with higher concentrations detected at Sal_Hartnell (1.01 µg/L), Sal_Chualar (1.19 µg/L) and Sal_Quail (0.759 µg/L). Additionally, several pyrethroids were detected in the May 2019 samples and were often found at levels at, or

above, EPA benchmarks. This included permethrin, lambda cyhalothrin and bifenthrin, analytes of particular concern (Table S1.4). CHL ranged in concentration from trace detection to a max of 10.2 µg/L. The concentration of CHL detected at Sal_Hartnell (10.2 µg/L) exceeded both the LC_{50} for a sensitive invertebrate species, *D. magna* (7.1 µg/L), and the EPA benchmark for aquatic life (USEPA, 2020) for acute invertebrate exposure (5.8 µg/L).

Overall, 18 of the 47 analyzed pesticides were detected in the surface water samples collected in September 2019, 7 of which were detected at each sampling site (Table S1.5). IMI and CHL were detected at all sites (Table 1.2). IMI concentrations were above the EPA benchmark for chronic invertebrate exposure (0.01 µg/L), and above the acute invertebrate level (0.385 µg/L) at Sal_Hartnell (0.513 µg/L). CHL concentrations were below the acute lethality benchmarks for invertebrate species exposure (LC_{50} =7.1 µg/L; EPA benchmark for acute, 5.8 µg/L, and chronic, 4.47 µg/L). Several other chemical detections exceeded threshold values. Notably, methomyl was detected at Sal_Quail (29.9 µg/L) at nearly three times the limit for chronic fish exposure (12 µg/L), and above the EPA benchmark for chronic invertebrate exposure (0.7 µg/L) at all sites. Thiamethoxam (neonicotinoid) was present in Sal_Quail (3.99 µg/L) and Sal_Hartnell (0.827 µg/L) at levels exceeding the EPA benchmark for chronic invertebrate exposure (0.74 µg/L) and was detected below EPA thresholds at Sal_Davis (0.064 µg/L). Additionally, several pyrethroids were detected in the September 2019 samples and were often found at levels at or above EPA benchmarks. This included permethrin, lambda cyhalothrin and bifenthrin, which are analytes of particular concern (Table S1.5).

Table 1.2: *Chemical Analysis of agricultural surface water samples collected 5/14/2019 and 9/17/2019 from CDPR long-term monitoring sites in Salinas, CA. liquid chromatograph multi-analyte and pyrethroid screen were performed at the Center for Analytical Chemistry, California Department of Food and Agriculture, Sacramento, CA. Laboratory QA/QC followed CDPR guidelines, and Laboratory blanks and matrix spikes were included in each extraction set. Samples from 9/17/2019 for Sal_SanJon, Sal_Chualar and Sal_Haro (shown in gray) were screened for pesticides as part of CDPR's routine monitoring but, these sites were not included in the biological assessments for the September exposures.*

		Sal_Quail	Sal_Hartnell	Sal_Davis	Sal_SanJon	Sal_Chualar	Sal_Haro
05/14/2019	**Chlorantraniliprole (µg/L)**	0.466	10.2	Trace	0.634	0.236	0.258
	Imidacloprid (µg/L)	0.759	1.01	0.019	0.495	1.19	0.292
09/17/2019	**Chlorantraniliprole (µg/L)**	0.35	0.504	0.021	0.368	0.159	0.156
	Imidacloprid (µg/L)	0.293	0.513	0.014	2.10	4.05	0.697

3.2 Mortality of Fish and Invertebrates Exposed to Dilutions of Field Collected Water Samples

No significant mortality occurred for *P. promelas* for any samples. For *D. magna* May 2019 exposures, significant mortality ($p < 0.001$) occurred for the [100] and [60] exposure dilutions. For *D. magna* no significant mortality occurred in any dilution for sites Sal_Haro, Sal_Chualar or Sal_Davis. For Sal_SanJon [100], 100% mortality of *D. magna* occurred at 96 h. For Sal_SanJon [60], 25% mortality occurred. For Sal_Quail [100], 97.5% mortality occurred, and 100% mortality occurred in [60] and 60% in [35]. For Sal_Hartnell, 100% mortality occurred at all dilutions of field water (Table S1.6).

Due to the high mortality of *D. magna* exposed to water samples collected at Salinas monitoring stations in May 2019, two additional dilutions ([20], [12]) were added to the September 2019 exposure study. Exposures targeted two previously toxic sites (Sal_Hartnell and Sal_Quail) and one non-toxic site located downstream in the main Salinas River (Sal_Davis) (Table S1.7). For Sal_Quail, all dilutions had significant ($p < 0.001$) levels of mortality: 100%

was observed at all dilutions of field water except [12], which had 87.5% mortality. For Sal_Hartnell, all dilutions had significant ($p < 0.001$) levels of mortality, except the lowest dilution [12], with: 100% mortality was observed at [100] and [60], 62.5% mortality at [35], 28% at [20]. For Sal_Davis, no significant mortality was observed in any dilution (Table S1.7). All water quality parameters (pH, specific conductance (SC), dissolved oxygen (DO), temperature (T)) of renewal and wastewater for May and September acute exposures fell within acceptable ranges (USEPA, 2002).

3.3 Mortality of Fish and Invertebrates Exposed to IMI and CHL

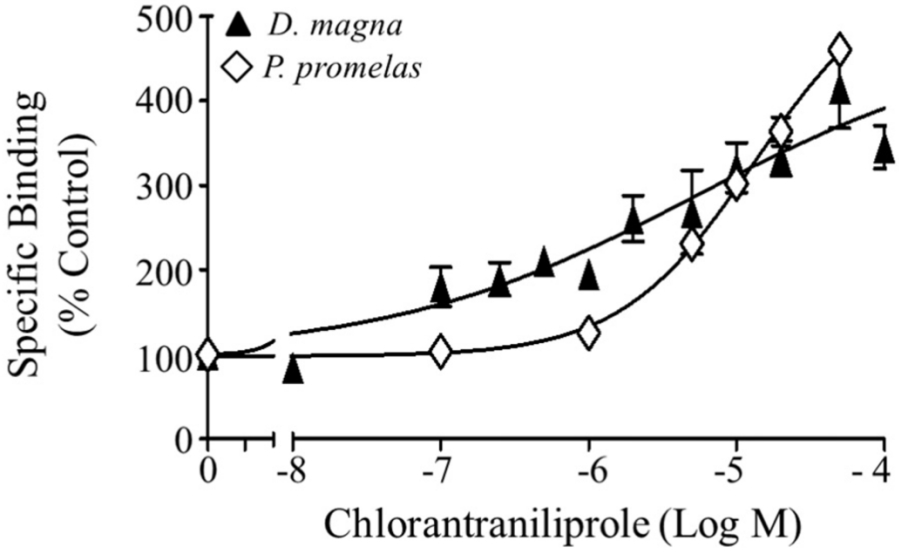
Single and binary exposures to IMI and CHL did not cause mortality of *P. promelas* for any treatment (Table S1.8). For *D. magna*, the highest treatment concentrations of CHL (10,000ng/L) resulted in significant mortality ($p < 0.0001$), with 100% mortality (Figure S1.1, Table S1.9). No significant *D. magna* mortality was observed for IMI for any concentration tested. Mortality for the two highest binary mixture concentrations (500ng/L and 10,000ng/L IMI/CHL) was also significant ($p = 0.0001$, $p < 0.0001$, respectively). Analytical chemistry data of nominal test concentrations for IMI/CHL exposures are shown in Table S1.10.

3.4 IMI and CHL Receptor Binding in Model Aquatic Species

The plant alkaloid ryanodine, for which the RyR is named, binds preferentially to the open state of the RyR (Meissner, 1986). Therefore, increased [^{3}H]Ry binding in the presence of CHL would signify increased activity due to chemical perturbation. Here, we found that CHL

activated the RyR present in the invertebrate model *D. magna* and the fish model *P. promelas* (Figure 1.1) causing an approximate 500% maximal response in both species. The RyR in *D. magna* displayed a higher sensitivity to CHL experiencing a 200% overactivation (EC2x) at 0.48 µM compared to the EC2x seen in fish at 3.61 µM. We also saw that CHL activates the RyR in the important ecotoxicology species *H. azteca* and *C. dilutus* (Figure S1.2). We observed insignificant binding of [^{3}H]IMI at the nAChR in *D. magna* and *P. promelas* where total binding was equal to radioligand binding under non-specific binding conditions (data not shown). This was observed under a wide array of assay conditions including those experiments run with protein preparations created under different homogenization techniques and with varying binding assay conditions including altered buffers, temperature, and incubation periods. Interestingly, despite the lack of binding in *D. magna* and *P. promelas* we did find that [^{3}H]IMI displays a high affinity for the nicotinic receptor found in *H. azteca* and *C. dilutus* (Figure 1.2), with IC_{50} values of 8.86nM and 8.04nM, respectively.

A)

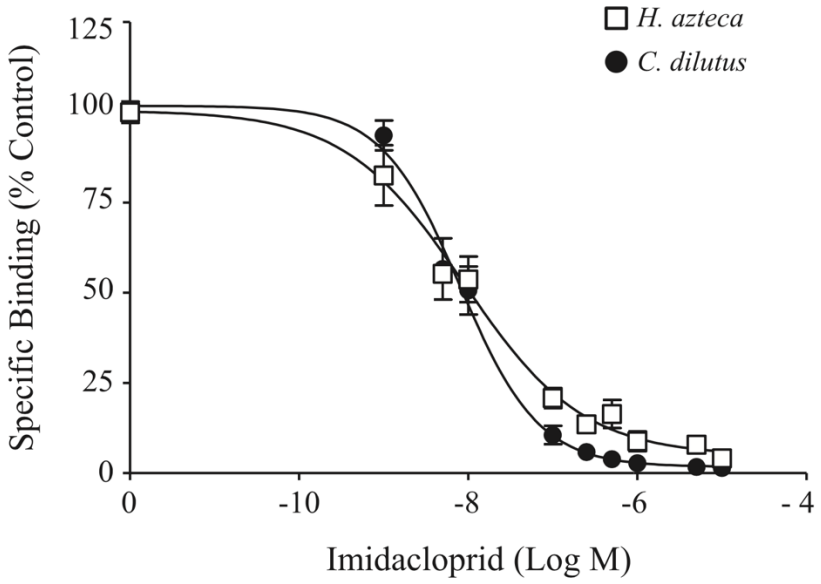


B)

Species	Maximum Response (%; ±SEM)	EC50 (µM)	EC50 95%CI (µM)	EC_{2X} (µM)	EC_{2X} 95% CI (µM)
D. magna	477.9 ± 140	5.45	0.12-252.0	0.48	0.18-0.89
P. promelas	556.7 ± 139	13.21	8.08-21.16	3.61	2.93-4.28

Figure 1.1. Binding of [^{3}H]Ry to *D. magna* and *P. promelas* ryanodine receptors in the presence of chlorantraniliprole. A) Binding curves with specific binding relative to DMSO control (100%); mean ±SEM, n=3-6. B) Potency and efficacy of chlorantraniliprole observed by species. Abbreviations; EC50, Effect Concentration to 50% maximal; EC_{2X}, concentration needed to cause 200% overactivation.

A)



B)

Species	IC50 (nM)	IC50 95%CI (nM)
H. azteca	8.04	5.89-10.99
C. dilutus	8.86	5.61-13.99

Figure 1.2. [^{3}H]IMI binding in *H. azteca* and *C. dilutus* protein preparations in the presence of competitive concentrations of non-labeled imidacloprid. A) Binding curves with specific binding relative to DMSO control (100%); mean ±SEM, n=6-9. B) Inhibitory concentrations to 50% of maximal inhibition (IC50) observed by species.

3.5 Relative Gene Expression of Fish Exposed to IMI and CHL

Differential expression of target genes involved with detoxification response and neuromuscular signaling pathways, comparing treated to non-treated control fish after 96 h exposures to single and binary mixtures of IMI and CHL is shown in Figure 1.3. Gene expression (GE) was determined after 96 h exposure to low (25 ng/L), medium (500 ng/L), and high (10,000 ng/L) concentrations of IMI and CHL individually and as binary mixtures.

Acetylcholinesterase (*AChE*) was upregulated in fish exposed to IMI, CHL, and binary mixtures at the lowest concentration, although changes did were not significantly from controls. *Aspartoacylase* (*ASPA*) was significantly upregulated in CHL exposed fish for all concentrations, and for the highest concentration of the binary mixture. *Cytochrome P4501A* (*Cyp1a*) and *Cytochrome P4503A126* (*Cyp3a*) displayed a non-monotonic change in expression in fish exposed to CHL and the binary CHL/IMI mixtures and a log-linear dose response in IMI exposed fish. *Ryanodine receptor 1* (*RyR1*) and *Ryanodine receptor 2* (*RyR2*) were upregulated at the low and mid concentrations for both CHL and binary mixtures, although this was only significant for *RyR2* at the CHL medium concentration (500 ng/L) and at lowest mixture concentration (25 ng/L). *Sarco(endo)plasmic reticulum 1* (*SERCA1*) showed minor changes in expression in CHL, IMI and CHL/IMI exposed fish but these changes were not significantly different from the controls.

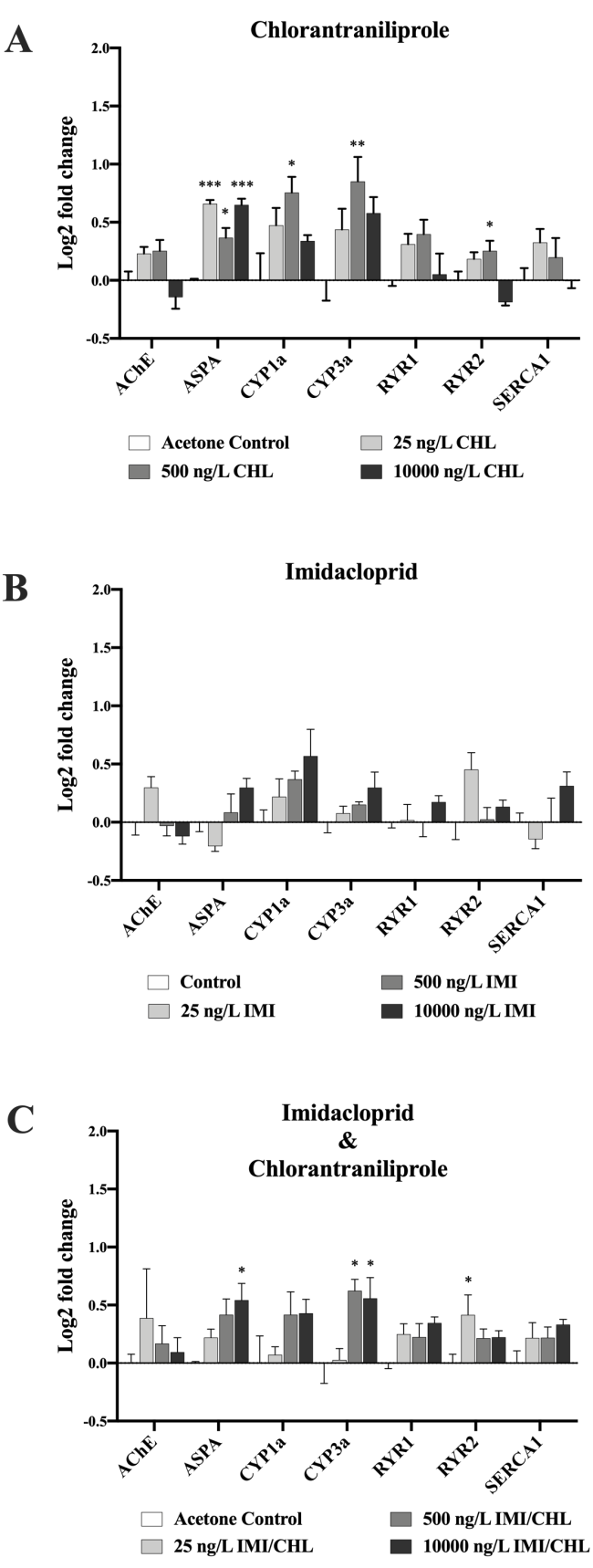


Figure 1.3. Log2 Fold-change of gene expression in *P. promelas* after exposure to chlorantraniliprole (2A), imidacloprid (2B), and binary mixtures (2C) for genes of interest: *Acetylcholinesterase* (*AChE*), *Aspartoacylase* (*ASPA*), *Cytochrome P4501A* (*Cyp1a*), *Cyp3A126* (*Cyp3a*), *Ryanodine receptor 1* (*RyR1*), *Ryanodine receptor 2* (*RyR2*) and *Sarco/Endoplasmic Reticulum ATPase* (*SERCA1*). P-values are reported as * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

3.6 Relative Gene Expression of Fish Exposed to Field Collected Water Samples

Differential expression for target genes (detoxification and neuromuscular pathways) in fish after 96 h exposures to a geometric dilution of field water collected in May 2019 are shown in Figure 1.4. Relative to controls, expression of *Cyp1a* was upregulated for Sal_Quail, Sal_Hartnell and Sal_Davis in a log-linear dose-response, increasing with increasing concentration of field water. *Cyp1a* was significantly upregulated for all sites at [100], Sal_Quail and Sal_Hartnell at [60], and for Sal_Quail at [35]. Expression of *Cyp3a* also followed a log-linear dose-response curve, increasing with increasing concentration of field water for each site. *Cyp3a* was significantly upregulated for Sal_Quail and Sal_Hartnell at all concentrations but was not significant for Sal_Davis at any concentration. Interestingly, in fish exposed to water collected in the field, *Cyp1a* and *Cyp3a* were upregulated in the two field sites that demonstrated high invertebrate mortality. We also observed significant upregulation of *Cyp1a* in the highest concentration of water from Sal_Davis in May 2019, which is considered a non-toxic site based on repeated assessments (CDPR Technical Report Hasenbein et al. 2018, Grant # 16-C0084) and the mortality data from this study.

Differential expression for target genes (detoxification and neuromuscular pathways) of fish after 96 h exposures to a geometric dilution of field water collected in September 2019 are shown in Figure 1.5A-C. *Cyp1a* was upregulated at all sites but differential expression was not statistically significant. *Cyp3a* was significantly upregulated for Sal_Quail at [100]. *SERCA1* was strongly downregulated for Sal_Hartnell and Sal_Davis at all concentrations and downregulated for Sal_Quail at [60]. Fish exposed to water collected in Sept. 2019 displayed a

significant downregulation of *SERCA1* relative gene expression, especially at the Sal_Hartnell and Sal_Davis locations.

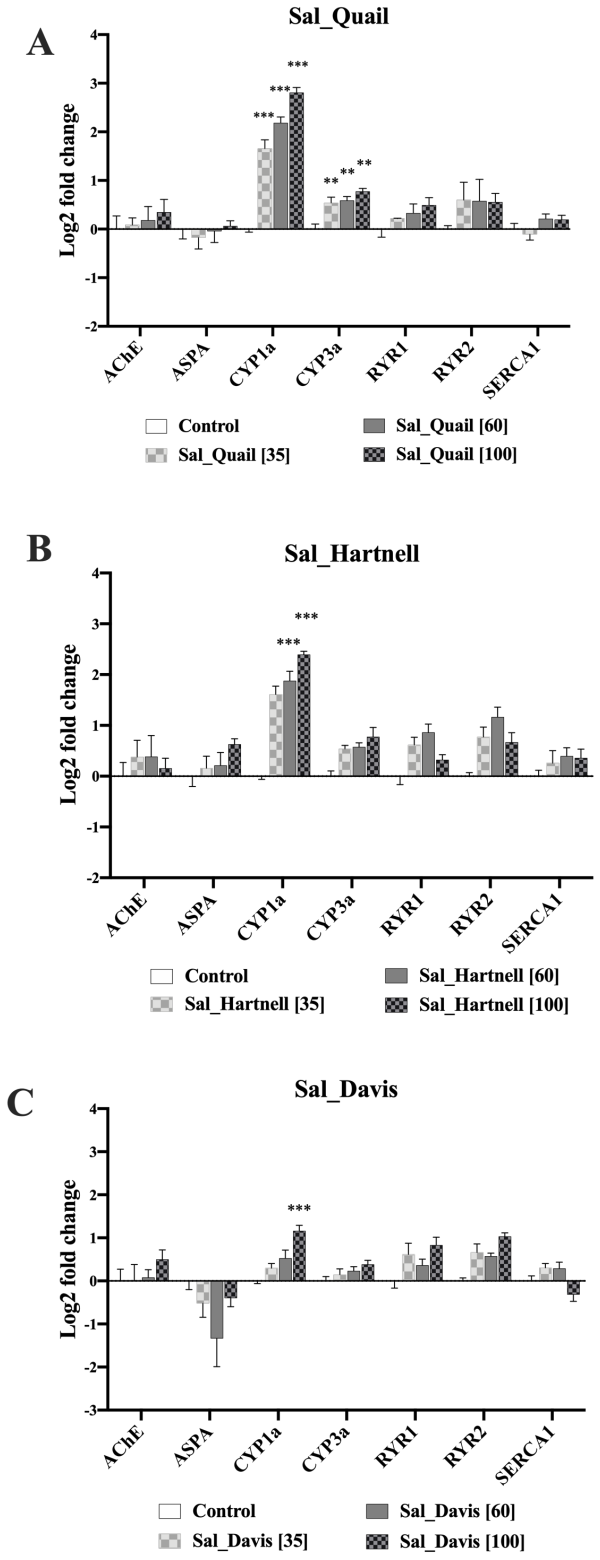


Figure 1.4. Log2 Fold-change of gene expression in *P. promelas* after acute exposure to a geometric dilution series of agricultural surface water ([100], [60] and [35]) collected in May 2019. Target genes of interest are: *Acetylcholinesterase* (*AChE*), *Aspartoacylase* (*ASPA*), *Cytochrome P4501A* (*Cyp1a*), *Cyp3A126* (*Cyp3a*), *Ryanodine receptor 1* (*RyR1*), *Ryanodine receptor 2* (*RyR2*) and *Sarco/Endoplasmic Reticulum ATPase* (*SERCA 1*). Field sites shown: Sal_Quail (2A), Sal_Hartnell (2B) and Sal_Davis (2C). P-values are reported as * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

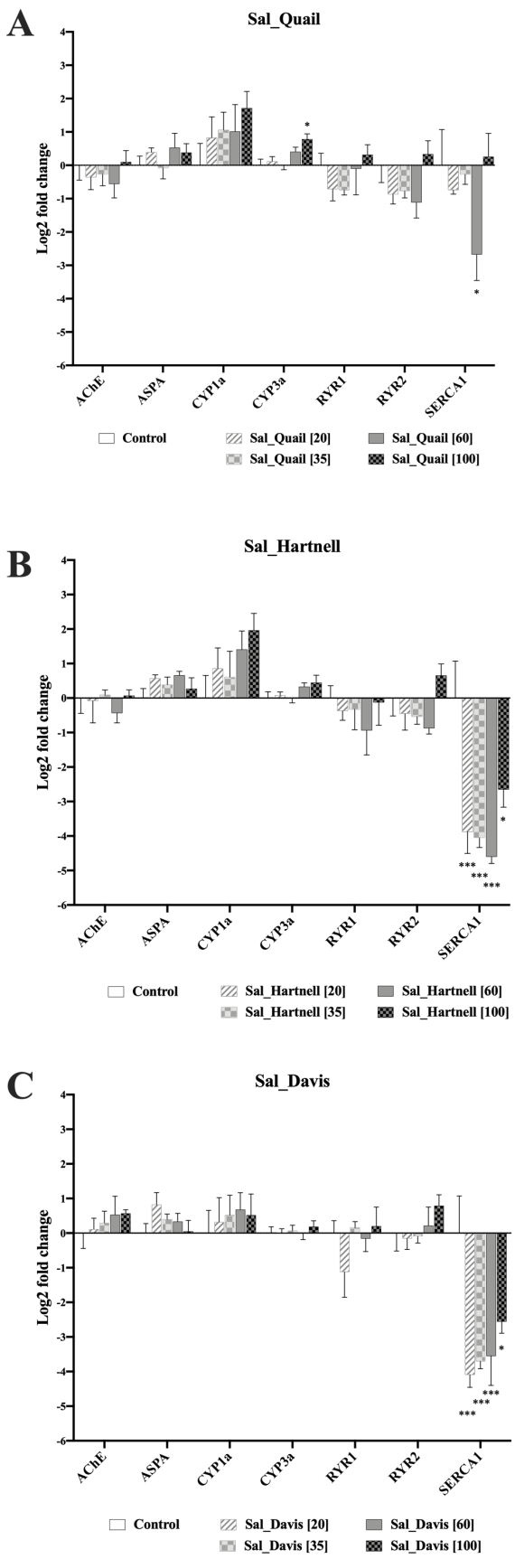


Figure 1.5. Log2 Fold change of gene expression in *P. promelas* after acute exposure to a geometric dilution series of agricultural surface water ([100], [60], [35] and [20]) collected in Sept.2019. Target genes of interest are: *Acetylcholinesterase* (*AChE*), *Aspartoacylase* (*ASPA*), *Cytochrome P4501A* (*Cyp1a*), *Cyp3A126* (*Cyp3a*), *Ryanodine receptor 1* (*RyR1*), *Ryanodine receptor 2* (*RyR2*) and *Sarco/Endoplasmic Reticulum ATPase* (*SERCA1*). Field sites shown: Sal_Quail (3A), Sal_Hartnell (3B) and Sal_Davis (3C). P-values are reported as * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

4. Discussion

We compared the effects of the insecticides IMI and CHL in single and binary mixtures and as components in field water exposures, on *D. magna* and *P. promelas,* two commonly used aquatic toxicology model species. Exposure to surface water collected near high use agricultural areas resulted in high invertebrate mortality even at the most diluted field waters. We did not observe any changes to survival of *P. promelas* exposed to surface water and single or binary mixtures containing the insecticides IMI and/or CHL, which suggests low acute toxicity to the model fish. However, exposed fish had significant changes in the relative expression of genes involved in detoxification and neuromuscular function, showing potential sublethal impacts. We also investigated the activity of IMI and CHL at the nAChR and RyR, respectively. Taken together, the survival, gene expression and binding activity data suggest that CHL and mixtures containing CHL have biologically important effects in both invertebrates and fish.

Chemical analyses of field water samples show repeated detections of IMI and CHL. Imidacloprid had the highest detection frequency among all the pesticides monitored between 2007 - 2016, making it a main pesticide of concern in Salinas and throughout California (Deng et al., 2019). Imidacloprid along with other neonicotinoids are used ubiquitously in over 120 countries worldwide and have been detected in the environment since their introduction (Jeschke et al., 2011). Chlorantraniliprole is an emerging chemical of concern that has proven to be extremely effective against many insect pests, and has subsequently experienced a rapid increase in use around the world (Teixeira and Andaloro, 2013). In many of the evaluated samples, both pesticides were present at concentrations that would be expected to affect sensitive species, where September 2019 chemistry data had lower concentrations of IMI and CHL than those seen

in May 2019. Additionally, several other pesticides of concern exceeded benchmark levels and/or LC_{50s} for sensitive species likely contributing to invertebrate mortality and sublethal effects in fish. Notably, methomyl a carbamate pesticide, was detected at concentrations many times the level expected to impact fish and is likely contributing to the toxicity for these samples (Van Scoy et al., 2013).

There was considerable overlap in the pesticides detected during both sampling periods, with few exceptions. The neonicotinoid thiamethoxam was only detected in the September sampling event and has been shown to increase toxicity of CHL and esfenvalerate (Jones et al., 2012). Previous studies on thiamethoxam have shown to that acute exposure can alter locomotor activity in zebrafish larvae (Liu et al., 2018) and cause neurotoxicity in catfish (Baldissera et al., 2018), albeit at concentrations above those detected in our sites. In September, there were also several pyrethroids present at higher levels, compared to that detected in May 2019, including lambda cyhalothrin, permethrin and malathion. This finding is consistent with a recent study examining the lag time between pesticide application during the growing season and subsequent detections in California surface water due to the pattern of dry summers followed by winter rain events typical of this region (DeMars et al., 2021). In addition to the 47 pesticides included in our analysis, it is possible that other, untargeted pesticides could be contributing to the observed toxicity. Pesticide use patterns in the area surrounding Salinas waterways and tributaries have been shifting away from organophosphate pesticides towards pyrethroid and neonicotinoid pesticides (Anderson et al., 2003), emphasizing the importance of monitoring a wide variety of pesticides at regular intervals. Previous toxicity studies using field water from multiple sites in the Salinas waterways and tributaries have shown high rates of mortality in sensitive invertebrate species including *D. magna* (unpublished data; Anderson et al., 2006). In these studies,

macroinvertebrate community structure was also highly impacted downstream of the sampling sites, suggesting that multitrophic assessments are crucial to understanding the ecological impacts of contaminants on a larger geographical scale (Anderson et al., 2006).

The current study is the first to address CHL activity at the RyR in model organisms commonly used in aquatic ecotoxicology. We show that CHL activates the RyR in the crustaceans *H. azteca,* and *D. magna,* insect *C. dilutus* and the vertebrate fish model *P. promelas*. The high CHL affinity for *H. azteca* and *D. magna* RyR was not observed in the other crustacean (Maine lobster; *Homarus americanus*) tested to date (Qi and Casida, 2013), suggesting differences in sensitivity in diverse crustacean species. Notably, we also observed significant activation of RyR found in the vertebrate fish model *P. promelas* suggesting CHL may impact neuromuscular health in fish. This is in line with more recent data regarding the impact of CHL, and related pesticides, on mice. Specifically, CHL caused a 200% over activation of RyR in *P. promelas* at 3.61μM (current study) and was found to cause a ~200% over activation of RyR in wildtype mice at 1 μM (Truong and Pessah, 2019) showing similar levels of vertebrate sensitivity. It should be noted, however, that the fish binding assays completed in the current study were run in crude microsomal preparations compared to the junctional sarcoplasmic reticulum preparations run in mice (Truong and Pessah, 2019). We also observed high IMI affinity toward the nAChR in the aquatic toxicology model species *H. azteca* and *C. dilutus* at 8.86nM and 8.04nM, respectively, which is similar to that seen in other invertebrates such as the house fly (*Musca domestica;* 1.2nM; Liu and Casida 1993). The lack of binding in *D. magna* was surprising, which may have been due to the binding conditions utilized in the current study. However, there are conflicting results of IMI affinity across closely related invertebrate species, where there are still many questions regarding the interaction of IMI and related compounds with

the nAChR (Crosswaithe et al. 2017). For example, insects, mainly hemipteran species that are particularly sensitive to neonicotinoids, display numerous IMI binding sites on the nAChR including a very high affinity site sensitive to sub-nM concentrations of IMI. Other insect species may lack the very high affinity site, possibly explaining lower neonicotinoid whole organism toxicity (Crosswaithe et al. 2017). The lack of IMI binding toward the nAChR in *P. promelas* is consistent with the lack of binding seen in other vertebrate species including the electric eel electric organ and numerous mammalian species (Liu and Casida, 1993, Tomizawa et al. 2000). The current work is one of the few studies looking at the direct interaction of IMI with the nAChR in a fish species and future research with varying assay conditions and the inclusion of IMI metabolites may better explain neurotoxic effects in neonicotinoid exposed fish.

We also observed changes in the expression of genes involved in target pathways after acute exposure to agricultural surface water and environmentally relevant chemical mixtures of IMI and CHL. In both field and single/binary exposures, genes in the Cytochrome P450 (Cyp450) family were differentially expressed, including *Cyp1a* and *Cyp3a*, which are involved in the metabolism of diverse chemicals as a first line of detoxification (De Montellano, 2005; Stegeman, 1994; Zanger and Schwab, 2013). In the single/binary exposures, *Cyp1a* and *Cyp3a* expression was consistent with responses of Cyp450 family proteins in other studies (Vandenberg et al., 2012). We did not observe changes in Cyp450 genes in IMI exposed fish. IMI displays low acute toxicity to fish, although it has been shown to cause immune system suppression and neurobehavioral impairment in larval zebrafish exposed to mg/L concentrations (Crosby et al., 2015). Our exposure concentrations did not approach the mg/L scale and could have been too low in single compound exposures to observe differential expression for Cyp450 markers. Upregulation of Cyp family genes is well-documented after exposure to several

pesticides present in our field samples. The Cyp450 family proteins can be induced by a wide variety of xenobiotics making them particularly useful indicators for mixtures containing multiple classes of pesticides (Crain et al., 2008).

A gene involved in neurologic function was differentially expressed in both field and single/binary exposures. *ASPA* specifically maintains myelin sheet integrity in nerve cells (Baslow, 2002). Differential expression of *ASPA* has been measured in delta smelt (*Hypomesus transpacificus*) and *P. promelas* after sub-lethal exposure to insecticides, and may be implicated in impairing neurological function (Beggel et al., 2011; Connon et al., 2009). Physiological changes to the myelin-like structure (medullary sheath) of target pest invertebrates after exposure to CHL have also been observed (Ma et al., 2017). To our knowledge, no literature exists on the mechanism by which CHL may affect expression of *ASPA*.

Genes related to cellular Ca^{2+} homeostasis and signaling were altered in *P. promelas* exposed to water collected in the field and single/binary IMI and CHL exposures. Specifically, we investigated changes in relative gene expression in *RyR1*, *RyR2* and *SERCA1*, an ATPase that pumps Ca^{2+} into the sarco(endo)plasmic reticulum (SR/ER) to restore SR/ER Ca^{2+} stores needed for muscle contraction and neuronal signaling. We saw changes in *RyR2* gene expression when fish were exposed to CHL alone as would be suggested by CHL's mechanism of action. We also found increased *RyR2* in the IMI and CHL binary mixtures. IMI and its metabolites affect intracellular Ca^{2+} concentrations through their action at voltage-gated Ca^{2+} channels (VGCCs) (Jepson et al., 2006; Simon-Delso et al., 2015), which are well-known signaling partners of RyR. The combination of CHL with IMI may have led to altered Ca^{2+} homeostasis contributing to changes in *RyR2* expression. Interestingly, we saw a large decrease in *SERCA1* gene expression in fish exposed to field waters from Sal_Hartnell and Sal_Davis collected in September 2019.

Pyrethroids have been documented to change Ca^{2+} homeostasis via interactions with VGCCs and a high affinity to the SERCA pump (Cao et al., 2011; Dusza et al., 2018). Pesticides that cause SERCA pump inhibition can further enhance the effect of compounds that cause an opening of the RyR by decreasing SR/ER Ca^{2+} stores (Dusza et al., 2018; Yao et al., 2011). CHL is more toxic when used in combination with some pyrethroids (Jones et al., 2012), and could have an increased contribution to site toxicity when present in combination with pyrethroids. Together, these findings support the conclusion that the observed mixture toxicity exceeded predictions based on single chemical assessments, and that altered gene expression could potentially impact fish.

Acute single chemical exposure assessments have been an integral part of the regulatory framework but cannot predict organismal responses to environmentally relevant mixtures. Synergistic effects of complex chemical mixtures are well documented in previous studies (Crain et al., 2008; Todgham and Stillman, 2013). Furthermore, the interaction of contaminants in combination with other environmental stressors can result in synergistic, additive and/or antagonistic effects. This illustrates the limitations of extrapolating toxicity from single stressor studies for comparison to environmentally relevant mixtures. As the complexity of mixtures increases, non-targeted, effect-based evaluations become necessary for determining biological outcomes. This is especially relevant for mixtures that include new and emerging contaminants of concern, where data on their biological effects may be limited to acute exposures on target and model organisms. The development of gene expression assays for use as monitoring and diagnostic tools depend on a clear understanding of the mechanisms underlying a molecular response, and more research is needed particularly for chemicals of emerging concern and their specific mechanisms of activity.

Climate change is expected to influence pest dynamics and pesticide applications globally (Wolfram et al., 2018). There is a pressing need to expand monitoring efforts to include effects-based assays to determine the biological effects of complex mixtures (e.g., binding assays, gene expression, Connon et al., 2019, 2012; Mehinto et al., 2021; Schuijt et al., 2021). Such efforts would allow the detection of subcellular level effects before they are apparent at higher levels of biological organization, particularly at low but environmentally relevant insecticide concentrations. Furthermore, additional endpoints such as development and behavior would provide for greater understanding of the consequences of pesticide exposure on invertebrate and fish populations (Ford et al., 2021; von Hellfeld et al., 2020; Wlodkowic and Campana, 2021).

5. Conclusions

In this study we targeted subcellular molecular pathways known to coincide with insecticides' mechanisms of activity in aquatic organisms, then compared the relative degree of subcellular stress induced by IMI and CHL with responses to environmental mixtures. This combined approach helped evaluate species-specific responses and tolerance thresholds to IMI and CHL exposure. We demonstrated that CHL activates RyR in fathead minnow and several model invertebrates commonly used in aquatic ecotoxicology. This finding is important for understanding how CHL may impact neuromuscular health in fish. Exposure to agricultural surface waters resulted in invertebrate toxicity that exceeded predictions based on single chemical assessments, and elicited detoxification responses and impacted neuromuscular function pathways in fish. In the absence of sublethal endpoints, our findings would have excluded important effects on fish. By conducting geometric dilution series and examining

differential gene expression, we obtained a more comprehensive understanding of the sublethal effects of agricultural surface water on aquatic life. Pesticide contamination is a serious issue in agricultural and urban areas worldwide, and particularly in the central coast region of California. The implications of the current study may serve to inform management efforts and highlight the importance of continued research on chemicals of emerging concern.

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Chapter 2

Exposure to pesticide contaminated surface water impacts swimming behaviors and light-induced startle response of fathead minnows (*Pimephales promelas*)

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Abstract

Freshwater ecosystems in urban and agriculturally developed watersheds are extremely vulnerable to impacts from pesticides. When multiple pesticides from runoff enter aquatic ecosystems, the resulting mixtures can negatively impact aquatic species. Little is known about the potential toxicity of many pesticides to non-target organisms, particularly the effects of chemicals of emerging concern (CEC). Additionally, CEC contributions to the toxicity of complex mixtures are often unknown. Behavioral responses of aquatic species are sensitive, effects-based approaches to assess sublethal toxicity. Our goal in this study was to assess whether exposure to surface water collected from urban and agriculturally developed waterways (Salinas River Watershed, CA) impacted multiple behavioral endpoints in the fathead minnow (*Pimephales promelas*), a model species in toxicology. We collected water samples at monitoring stations downstream from agricultural fields, and screened them for a suite of pesticides. Analytical chemistry data showed chemicals of emerging concern as common analytes, including neonicotinoids (thiamethoxam, imidacloprid), pyrethroid insecticides (bifenthrin), and the carbamate insecticide methomyl, present at levels exceeding US Environmental Protection Agency benchmarks for Aquatic Life. We used locomotor assays to assess several behavioral responses of larval fish after acute exposure (96 h) to surface water. We detected significant differences in light-induced startle responses and average total movement, as well as the duration and/or frequency that fish swim at cruising, bursting and freezing velocities. The most sensitive endpoint was the light-induced startle response, which was significantly different from controls for all water samples tested. Results from this study show sublethal and environmentally relevant effects from exposure to contaminated surface waters, which would likely be missed through the use of standard toxicology assessments based on mortality.

1. Introduction

Pesticides are detected ubiquitously in the environment, and frequently pose significant threats to water quality, biodiversity and human health (Morrissey et al., 2015; Tang et al., 2021). In addition, new pesticides are being developed and applied at an increasingly rapid pace worldwide, and these trends are expected to accelerate with global climate change (Bernhardt et al., 2017; Pisa et al., 2021). Aquatic environments located near high use agricultural and urban areas are frequently impacted by pesticides, which are often detected as complex mixtures. Assessing the environmental and health risks of mixtures is complicated by their nonlinear and often synergistic toxicity (Tang et al., 2021), and there is limited information on the potential toxicity of many pesticides to non-target organisms. In the United States and many other countries, some level of risk assessment is required for new pesticides to determine their potential risk to non-target organisms (vertebrates) and to the environment prior to use authorization (Handford et al., 2015). To assess the potential toxicity of surface waters, many regulatory organizations evaluate the survival of sensitive model species after an acute exposure period (Goh et al., 2019). However, determining survival alone can underestimate the true impact of exposure on aquatic organisms and does not consider sublethal, ecologically relevant impacts (Connon et al., 2019; Spurgeon et al., 2010). Additionally, few standardized assessments currently exist for mixtures, which are often assumed to produce no adverse effects at very low doses (when none of the compounds in the mixture have any toxic effect) (Hernández et al., 2017; Reffstrup et al., 2010).

As pesticides with novel modes of action continue to be developed, the complexity of the resulting mixtures which may enter aquatic habitats also increases. In many habitats across the globe, mixtures of chemical contaminants including pesticides are present at concentrations

expected to cause detrimental effects on the abundance and diversity of aquatic life present (Brusseau and Artiola, 2019). A recent study examining the global risk of pesticide pollution found that 74.8% of agricultural land (approximately 28.8 million km^2) is at some risk of pesticide pollution, with 31.4% (approximately 12.1 million km^2) of this land at high risk (Tang et al., 2021). Neonicotinoids are the fastest growing class of insecticides world-wide, with 81% of global surface water studies reporting neonicotinoid concentrations that exceeded threshold values expected to affect sensitive aquatic organisms (reviewed in Morrissey et al. 2015). Neonicotinoids are authorized for use in over 120 countries worldwide and have been detected in the environment since their introduction (Jeschke et al., 2011). There is a clear global trend showing an increase in the use of neonicotinoids, as well in the use of anthranilic diamides (Bentley et al., 2010; Wolfram et al., 2018) which are proven to be extremely effective against many insect pests (Teixeira and Andaloro, 2013).

Organisms in natural environments are commonly exposed to mixtures of pesticides, rather than single chemicals. The United States Geological Survey (USGS) recently evaluated freshwater ecosystems across the United States and detected close to 400 unique organic analytes (pharmaceutical, pesticide, organic wastewater indicators), over 300 of which were present at concentrations above US Environmental Protection Agency (EPA) thresholds for aquatic life (acute toxicity value = the lowest 48- or 96-hour EC_{50} or LC_{50} in a standardized test, chronic toxicity value = the lowest NOEAC from a lifecycle or early life stage test) (Bradley et al., 2021). Their findings indicate that simultaneous exposure to multiple organic contaminants (mixtures) is the norm rather than the exception for developed-watershed streams, and that freshwater ecosystems in urban and agriculturally developed watersheds are extremely vulnerable to impacts from contaminant mixtures.

Exposure to surface water containing mixtures of pesticides can potentially reduce fish ecological fitness by changing behaviors that are key for their survival. Behaviors that are enacted to avoid predation, ensure reproductive success, locate/obtain food, or navigate the environment often determine individual survival, and are therefore expected to be evolutionarily fine-tuned. Therefore, any changes in normal swimming behavior, or in the ability of an organism to respond to stimuli, can have detrimental effects.

Pesticides can alter fish behavior, and several behavioral responses are described as sensitive, sublethal endpoints for evaluating the toxicity of pesticides (Delcourt et al., 2013; Hong and Zha, 2019). A light and dark locomotion test can evaluate behavioral endpoints under alternating light:dark conditions to screen numerous compounds for potential neuroactive properties (Kokel et al., 2010; Legradi et al., 2015; Mundy et al., 2020; Segarra et al., 2021; Steele et al., 2018). Acute exposures to a wide variety of target chemicals with various modes of action are known to cause behavioral changes in fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*) larvae under alternating light:dark conditions (Kalueff et al., 2016; Steele et al., 2018). Larval fish may respond to stimuli that they perceive as threatening by engaging in escape and avoidance behaviors such as freezing, jumping, moving erratically, or displaying a startle response. A sudden decrease in movement (e.g., freeze response) under changing light conditions can occur as a passive fear response or a predator avoidance behavior (Rennekamp et al., 2016). When an imminent threat is detected, however, fish will often rapidly change the speed, angle and direction of motion (Gazzola et al., 2012). These “startle” responses can also be induced when light conditions change abruptly and is generally characterized by a brief period of increased movement/velocity (Colwill and Creton, 2011; van den Bos et al., 2017). In a recent study using zebrafish larvae, changes in response to light stimulus could be detected at

concentrations one order of magnitude below concentrations causing mortality for several neuroactive chemicals (Leuthold et al., 2019). Measuring the disruption of normal swimming behavior is a sensitive test for measuring the effects of pesticides in fish, at concentrations expected to occur in the environment.

The Salinas River is the main riparian corridor for California's Central Coast region, which transects both urban and agricultural land, then empties into a marine sanctuary near Salinas, CA. For more than ten years, state agencies have collected data from several chemical monitoring sites located in the Salinas River, tributaries, and other nearby water bodies to collect data for a suite of chemicals of concern (Deng et al., 2019; Goh et al., 2019). Many pesticides are routinely detected at these sites above EPA benchmarks for aquatic life, highlighting the importance of continued monitoring efforts. While chemical monitoring data for these sites is extensive, biological impact data are more limited both in number and scope and have mainly consisted of acute exposures to sensitive aquatic invertebrate species (Anderson et al., 2006, 2003; Hunt et al., 2003). While evaluating the survival of model invertebrate species is an established method for ecotoxicology assessments, sublethal endpoints such as behavior are more sensitive and have greater ecological relevance, revealing a more complete picture of site toxicity (Beggel et al., 2011; Hasenbein et al., 2019). Previous assessments of Salinas Valley waters frequently resulted in high levels of invertebrate toxicity (Amweg et al., 2005; Anderson et al., 2018; Hunt et al., 2003). In a recent study by our group conducted on Salinas Valley surface waters (Stinson et al., 2021), we initially found high mortality of a sensitive invertebrate species (*Daphnia magna*), even for concentrations of surface water as low as 12%. We found no mortality of fathead minnow larvae, and measured changes in expression of genes associated with neuromuscular function and detoxification. Our previous study also showed that water from

contaminated sites caused changes in expression of genes for neuromuscular function and detoxification in fathead minnow larvae. Based on these findings, we aimed to determine whether larval fathead minnows exposed to Salinas Valley waters would show behavioral changes. By examining sublethal exposure effects (such as gene expression and behavior changes) of fish, we can obtain additional information about biological effects in aquatic systems.

In this study, we hypothesized that 1) exposure to surface waters in urban and agriculturally developed areas (Salinas Valley, CA) would elicit behavioral changes in fathead minnow larvae, and 2) swimming behaviors of fathead minnow larvae are sensitive endpoints for determining the sublethal effects of acute (96 h) exposures to surface water known to contain complex mixtures of pesticides. To test these assumptions, we exposed larval fish to water samples collected at existing monitoring stations near agricultural fields where previous assessments have detected several pesticides of concern, then measured their swimming behaviors and response to changing light conditions.

2. Materials and Methods

2.1 Study Sites & Water Sampling

We selected a subset of established sites in the Salinas Valley that have been monitored for contaminants for over a decade, and also occasionally for invertebrates (Figure S2.1, described in detail in Stinson et al. 2021). Briefly, these provide over a decade of chemical detection data on pesticide use in the surrounding area and are located near ecologically sensitive areas (Deng et al., 2019; Goh et al., 2019; Luo et al., 2018). We sampled surface water from two sites that directly receive runoff from adjacent agricultural fields: Quail Creek (waterway) and

Alisal Creek (reclamation ag. ditch). A third site, considered to be less impacted, is located in the downstream reach of the Salinas River at Davis Rd. These sites are located immediately downstream of high use agricultural areas, where there is an increased risk of contamination from runoff.

We collected surface water samples on September 17th, 2019, following standard protocols (Jones, 1999). In brief, we sampled water from wadable waters using 1-liter amber glass bottles (Cole-Parmer Vernon Hills, Illinois, USA) certified to meet current US EPA guidelines then sealed with Teflon-lined lids. We immediately placed sample bottles in coolers on wet ice for transportation and refrigerated them at 4 °C upon arrival in the lab. We measured water quality parameters *in situ* using a calibrated YSI 6920 V2 multiparameter water quality sonde (YSI Incorporated, Yellow Springs, OH, USA), including ambient water temperature, pH, specific conductance, total dissolved solids, turbidity, and dissolved oxygen. We initiated all acute exposure tests within 24 h of collection.

2.2 Chemical Analyses

We conducted chemical analyses as described in Stinson et al. (2021). In brief, the Center for Analytical Chemistry, at the California Department of Food and Agriculture (Sacramento, CA) conducted chemical analyses using multi-residue liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography–mass spectrometry (GC-MS/MS) methods. A total of 47 target pesticides were evaluated based on the procedures described in the Monitoring Prioritization Model for pesticide screening (Luo et al., 2018). Laboratory QA/QC followed California Department of Pesticide Regulation guidelines provided in the Standard Operating Procedure CDPR SOP QAQC012.00 (Teerlink and DaSilva, 2017). Extractions

included laboratory blanks and matrix spikes, and the method detection limit and reporting limit for each analyte were reported.

2.3 Acute (96 h) Exposures

2.3.1 Test Organisms

We obtained fathead minnow larvae (< 7 days post hatch; dph) from Aquatic Biosystems, Inc. (Ft. Collins, Colorado, USA). On arrival, prior to exposures, we habituated the fish to control water at a temperature of 25 °C over a period of 8 hours. We made synthetic, medium hard control water (hereafter referred to as control water) consisting of deionized water modified with salts to meet US EPA freshwater specifications (specific conductivity (EC): 265–293 µS/cm; hardness: 80–100 mg/L $CaCO_3$; alkalinity: 57–64 mg/L $CaCO_3$ (USEPA 2002), which was aerated >24 hours prior to use. Upon arrival, we fed fish larvae *ad libitum* with newly hatched brine shrimp (*Artemia franciscana*). During the habituation period <1% mortality was observed, and the fish fed and swam normally. We conducted this research in accordance with national and institutional guidelines for animal welfare under the University of California Davis, Institutional Animal Care and Use Committee protocol #19690.

2.3.2. Exposures

We followed acute toxicity exposure procedures outlined by the EPA (USEPA 2002). We used 7 dph fathead minnow larvae, maintaining test exposure temperature (25 ºC) within +/-1 ºC in a temperature controlled, illuminated chamber (Precision 818, Thermo Fisher Scientific, CA, USA) under full spectrum fluorescent light with a 16-hr light: 8-hr dark photoperiod. For each sample and controls, we used four replicate 600 mL beakers containing 500 mL test solution and 10 fish larvae for a total of 40 individuals per treatment. At test initiation, we randomly added

organisms to each replicate beaker and treatment. We then randomized the location of beakers in the environmental chamber to minimize any chance of positional bias. We fed fish larvae *ad libitum* with newly hatched brine shrimp twice daily. We performed an 80% water change after 48 h using well-mixed, aerated water samples. We measured water quality parameters at the water change using a YSI EXO1 Multi-parameter Water Quality Sonde (Doo and He 2008), where parameters recorded included pH, specific conductance, dissolved oxygen, and temperature. We performed titrations to measure alkalinity and hardness in CaCO3 mg/L.

2.4 Behavioral Assays

2.4.1 Behavioral Assay Conditions

After 96 h exposure to agricultural surface water, we placed ten (11 dph) larvae from each technical replicate per treatment (n=40), individually into each well of a 24-well cell culture plate (Thermo Fisher Scientific, CA, USA) containing 2 mL of control water. We gently transferred larvae using 1 mL plastic pipettes and allowed them to habituate to the plate conditions, in the temperature-controlled chambers used for the exposures, for > 1 hour prior to commencing video recordings. We randomly assigned larvae from each treatment group to minimize plate effects, while ensuring at least three individuals from each treatment and controls were represented in each plate. We then placed the plate into a DanioVision Observation Chamber (Noldus, Wageningen, Netherlands) and allowed an additional 5 min adjustment period prior to initiating video recording (Figure S2.2). We recorded larval movement using a top-mounted Basler Gen1 camera located directly above the plate and tracked movement using EthoVision XT software (version 14.0; Noldus, Wageningen, Netherlands). This software is specifically designed to simultaneously track larval fish movement from multiple wells. Larval fish movement in each plate was recorded for a total of 50 min in alternating 10 min periods of

light and dark conditions. The chamber was illuminated during light cycles with a programmable light located beneath the plate, set at 10,000 lx for each light cycle. Light cycles consisted of an initial 10 min dark period (Dark 1), followed by 10 min light (Light 1), 10 min dark (Dark 2), 10 min light (Light 2), and 10 min dark period (Dark 3). The temperature of the plate was maintained at 20 °C ± 0.5 °C during the test via a recirculating water system attached to a chiller (TECO-US, Terrell, TX, USA). We ran a total of 7 x 24-well plates to encompass all samples. Each video recording was assessed visually to confirm software tracking accuracy prior to analyses. Behavior was assessed between 7am and 7pm to reduce diurnal effects on activity.

We evaluated multiple behavioral endpoints following protocols described for larval fathead minnow and zebrafish (Steele et al., 2018), and delta smelt (*Hypomesus transpacificus*) (Mundy et al., 2021, 2020; Segarra et al., 2021). Behavioral endpoints included Total Distance moved (mm), mean Velocity (mm/s), Freezing Duration and Frequency, Cruising Duration and Frequency, Bursting Duration and Frequency, Turn Angle, and Angular Velocity. The measured velocities were categorized as Freezing (< 5 mm/s), Cruising (≥ 5 mm/s and < 20 mm/s), and Bursting (≥ 20 mm/s). We measured duration (time spent in the respective velocity range, s), and Frequency (number of times the larvae initiated/terminated movement in a respective velocity range, count number) for each velocity threshold. We also measured light-induced startle response (hereafter referred to as startle response), determined as the change in mean (± SE) distance traveled between the last 1 min of a photoperiod and the first min of the following period. Two dark-to-light and two light-to-dark startle responses were measured. We assessed the startle response to measure larval behavior immediately following a sudden change in light condition, following calculations by Steele et al. (2018). Briefly, for each light transition (e.g., transition from light cycle Dark 1 to Light 1 at 10:00 min), we calculated the change in mean

distance traveled (in mm) between the last min of a photoperiod (e.g., 09:00-10:00 min) and the first min of the following period (e.g., 10:00-11:00 min). After we completed the behavioral assays, we rapidly euthanized the larvae in an overdose of tricaine methanesulfonate (500 mg/L MS-222, buffered with 500 mg/L sodium bicarbonate).

2.4.2 Statistical Analysis

We first evaluated normality and equivalence of variance assumptions using Levene and Shapiro-Wilk tests. We then used a Kruskal-Wallis analysis of variance to analyze changes in behavioral parameters using the kruskal_test function in the statistical software RStudio (version 1.3.1073) (Kassambara, 2020). We then conducted Dunn's multiple comparisons test as a post-hoc analysis to compare distance moved between cycles (at $\alpha < 0.05$ with Holm's sequential Bonferroni p-value adjustment), using the dunn_test function in RStudio (Kassambara, 2020). To compare control versus treatment contrasts, we used emmeans multiple comparison test in RStudio (Lenth et al., 2018) with the contrast method trt. vs. ctrl ($\alpha < 0.05$). The p-value was adjusted using the dunnetx method (Dunnett's test) to account for multiple testing. For data not meeting ANOVA assumptions, we performed a log transformation. Following Segarra et al. (2021) and Mundy et al. (2020), we summarized behavioral parameters visually via radar plots using RStudio (Kassambara, 2020), and plotted Z-scores to increase visual clarity while presenting multiple parameters having different units (cm/s, s, %) on the same figure, and normalized to controls. To evaluate startle response, assumptions of normality and variance were evaluated as above, then we performed an analysis of variance (ANOVA) followed by a Dunnett's multiple comparisons test in GraphPad Prism (version 8.0, San Diego, CA, USA). We visualized light-induced startle responses as bar graphs using GraphPad Prism (version 8.0, San Diego, CA, USA).

3. Results

3.1 Physicochemical and Chemical Analysis

Physicochemical water parameters for fathead minnow culture water (measured on arrival), laboratory control water, and surface water are listed in Table S2.2. Temperature, dissolved oxygen, and pH of surface water samples were comparable to controls. Conductivity, hardness, alkalinity, and salinity of surface water from Quail Creek and Alisal Creek exceeded values for control water. Upon collection, total suspended solids (TSS) were highest for Quail Creek (593.8 mg/L) compared to Alisal Creek (449.2 mg/L) and the Salinas River (45.58 mg/L).

Several pesticides were detected at levels exceeding an EPA benchmark for aquatic life in each site tested (Table S2.1). Of 47 pesticides analyzed, 17 were detected, and each site contained a minimum of seven target pesticides. Neonicotinoids (imidacloprid, thiamethoxam) were present at all sites above EPA benchmarks. The carbamate insecticide methomyl was present in all samples, at levels exceeding EPA benchmarks, most notably in Quail Creek where levels were three-fold higher than EPA benchmarks for chronic exposure in fish. Additional pyrethroid insecticides (bifenthrin, lambda-cyhalothrin, esfenvalerate), organophosphates (malathion) and neonicotinoids (permethrin, clothianidin) were detected at Quail Creek and Alisal Creek. Overall, the Salinas River site contained the fewest total number of chemicals, and at the lowest concentrations of the three sites we examined.

3.2 Acute Toxicity

Following 96 h exposures to contaminated surface water, no fish mortality occurred within any group. Fish mortality data pertaining to this study and the companion study, Stinson et al. (2021), are presented in Table S2.3.

3.3 Behavioral Responses

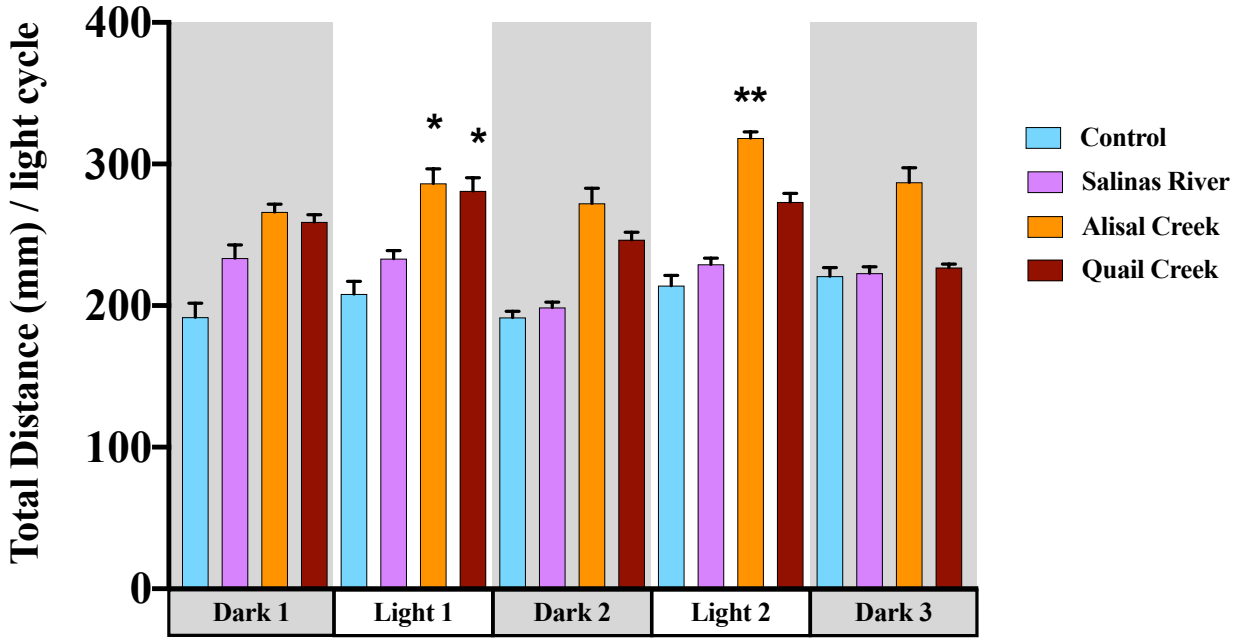


Figure 2.1. Average (±SE) Total Distance (mm) moved per 10 min time bin for fathead minnow larvae following 96h ambient field water exposures from three sites in Salinas, CA in September 2019. Larvae were exposed to three dark and two light cycles of 10 min durations. P-values are reported as * = $p \leq 0.05$, ** = $p \leq 0.01$. Normality and equivalence of variance assumptions were first evaluated using Levene and Shapiro-Wilk tests, then an analysis of variance (ANOVA) was performed followed by a Dunnett's multiple comparison's post hoc test.

3.3.1 Total Distance Moved

The average Total Distance (mm) moved for fathead minnow larvae is shown in Figure 2.1. Larvae exposed to water from Quail Creek and Alisal Creek demonstrated significant hyperactivity compared to controls during Light cycle 1 (increased Total Distance moved per light:dark cycle period). Larvae exposed to water from Alisal Creek demonstrated significant hyperactivity compared to controls during all Light cycles. The average Total Distance moved for control groups was comparable between the first and second light cycles.

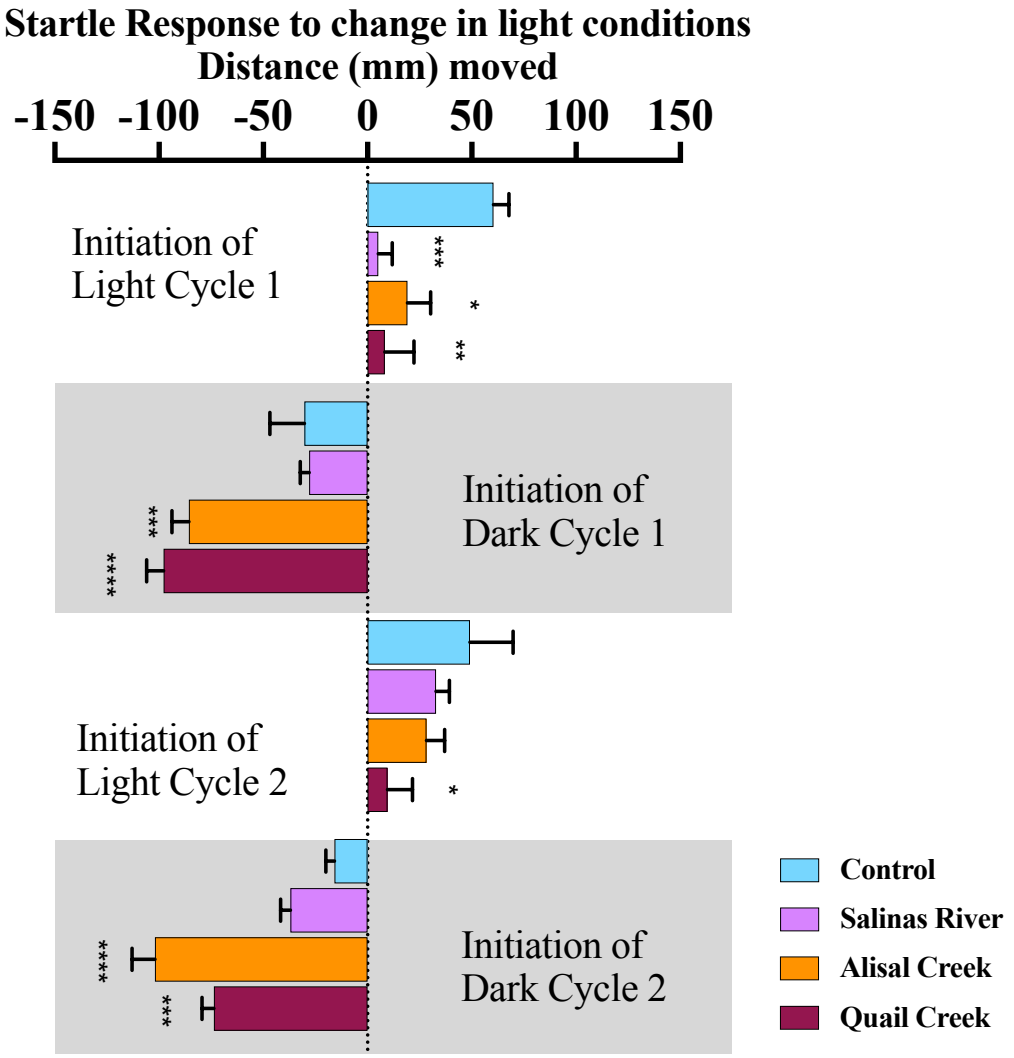
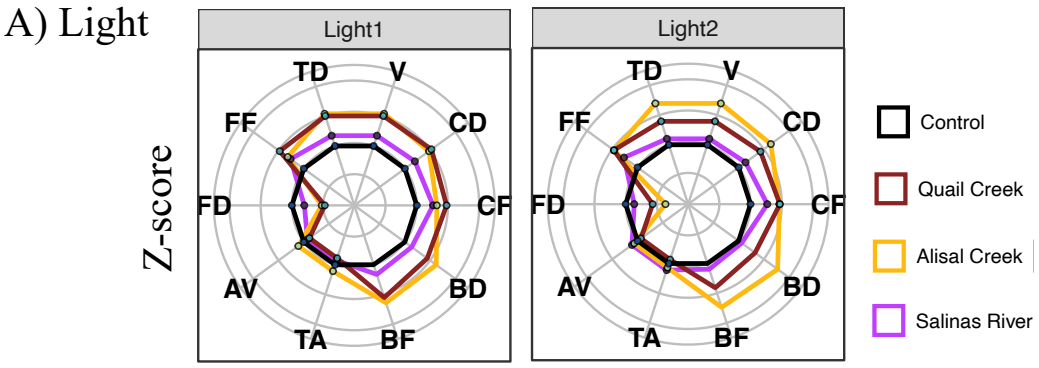


Figure 2.2. Light stimuli-induced startle responses of fathead minnow larvae following 96h ambient field water exposures from three sites in Salinas, CA in September 2019. Startle response was measured as the change in mean (±SE) distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. P-values are reported as * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$. Normality and equivalence of variance assumptions were first evaluated using Levene and Shapiro-Wilk tests, then an analysis of variance (ANOVA) was performed followed by a Dunnett's multiple comparison's post hoc test.

3.3.2 Light-Induced Startle Response

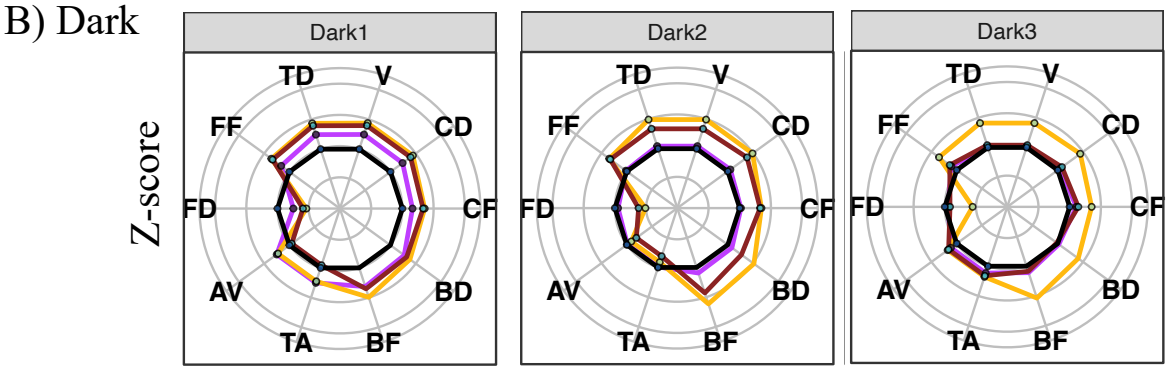
The light-induced startle response of fathead minnow larvae is shown in Figure 2.2. All treatment groups displayed altered light-induced startle response patterns during at least one

change in light conditions compared to controls. Fish exposed to surface water from all sites had reduced responses to light stimuli and had greater freeze responses in dark conditions when compared with controls. At the initiation of the first Light cycle (first dark-to-light transition), exposed fish had reduced responses to light stimuli compared to controls. Quail Creek produced a 7.5-fold decrease, Alisal Creek produced a 3.5-fold decrease, and fish exposed to water from the Salinas River varied the most significantly from controls, demonstrating minimal startle response (12-fold decrease) to light stimuli. At the initiation of the first Dark cycle (the first light-to-dark transition, fish exposed to water from Quail Creek and Alisal Creek both decreased their movement approximately 3-fold more than controls, while larvae from Salinas River showed the same response as controls. Fish from all groups increased their Total Distance moved in response to the second dark-to-light transition (consistent with the previous dark-to-light transition) but exposed groups showed smaller magnitudes of change compared with controls. Larvae exposed to water from Quail Creek demonstrated a 5-fold decrease in the magnitude of response, compared with controls. For the second light-to-dark transition, the same response patterns were observed (4.8-fold decrease from Quail Creek, 6.7-fold decrease from Alisal Creek).



	V	CD	BF	BD	FD	TD
Quail Creek	* ↑	* ↑	* ↑	–	* ↑	* ↑
Alisal Creek	* ↑	–	* ↑	* ↑	–	* ↑
Salinas River	–	–	–	–	–	–

	V	CD	BF	BD	FD	TD
Quail Creek	–	–	–	–	–	–
Alisal Creek	** ↑	** ↑	** ↑	** ↑	** ↑	** ↑
Salinas River	–	–	–	–	–	–



	V	CD	BF	BD	FD	TD
Quail Creek	–	–	–	–	–	–
Alisal Creek	–	–	–	–	–	–
Salinas River	–	–	–	–	–	–

	V	CD	BF	BD	FD	TD
Quail Creek	–	–	–	–	–	–
Alisal Creek	–	–	** ↑	** ↑	–	–
Salinas River	–	–	–	–	–	–

	V	CD	BF	BD	FD	TD
Quail Creek	–	–	–	–	–	–
Alisal Creek	–	–	** ↑	–	–	–
Salinas River	–	–	–	–	–	–

Figure 2.3. Radar plot of behavioral response parameters for fathead minnow larvae (n = 40) following acute (96h) exposure to surface water collected from three sites in Salinas, CA. Behavior was measured under alternating 10 min light (3A) and dark (3B) cycles. Measured parameters included Velocity (V), Cruising Duration (CD), Cruising Frequency (CF), Turn Angle (TA), Angular Velocity (AV), Freezing Duration (FD), Freezing Frequency(FF) and Total Distance (TD). Z-score for each parameter was normalized to controls (Z-score = 0). The measured velocities were categorized as Freezing (< 5 mm/s), Cruising (≥ 5 mm/s and < 20 mm/s), and Bursting (≥ 20 mm/s). Duration (time spent in the respective velocity range, s), and Frequency (number of times the larvae initiated/terminated movement in a respective velocity range, count number) were measured for each velocity threshold. * $p < 0.05$; ** $p < 0.005$; Kruskal–Wallis test followed by Dunn's test, comparing all concentrations to control within each cycle per treatment.

3.3.3 Locomotor Assay

In addition to Total Distance and Light-induced startle response, we measured significant changes across multiple behavioral endpoints under alternating light:dark conditions (Figure 2.3). Significant endpoints are shown in Table S2.4, and all results from the Kruskal-Wallis analysis of variance are shown in Table S2.5. During Light cycle 1, fish exposed to water from Alisal Creek and Quail Creek increased their Total Distance, Bursting Frequency and Velocity. Fish from Quail Creek showed increased Cruising Duration and a decrease in Freezing Duration (Table S2.4). Fish exposed to water from Alisal Creek also demonstrated increased Bursting Duration. For Dark cycle 2, the Alisal Creek fish showed increased Bursting Duration and Frequency. For Light cycle 2, Alisal Creek exposed fish showed increased Total Distance, Bursting Duration and Frequency, Cruising Duration, and Velocity, and a decrease in Freezing Duration. During Dark cycle 3, fish exposed to water from Alisal Creek showed increased Bursting Frequency, as compared with controls. During all periods without light stimulus, interindividual variation in response variables was high, resulting in fewer significant differences from controls, although we observed the same directional changes as seen during Light cycles.

4. Discussion

In this study, we hypothesized that swimming behaviors of fathead minnow (*Pimephales promelas*) larvae are sensitive endpoints for determining the sublethal effects of acute (96 h) exposures to surface water known to contain complex mixtures of pesticides. We evaluated the behavioral response profiles of fathead minnow larvae following exposure to contaminated surface water from an urban and agriculturally developed watershed (Salinas Valley, CA), and detected significant differences in light-induced startle response, average Total Distance moved,

Freezing, Cruising and Bursting (Frequency and Duration), and Velocity as compared with unexposed controls. Unexposed fish (controls) showed increased movement in light conditions and decreased movement in dark conditions, which is consistent with other studies on fathead minnows (Colón-Cruz et al., 2018; Steele et al., 2018). We found hyperactivity in fish exposed to contaminated surface water under light conditions compared with controls. This finding is consistent with previous assessments of fathead minnow behavior after exposure to many chemical classes present in our water samples (Steele et al., 2018). The affected behaviors measured in our study are biologically and ecologically important. Prey schooling behavior, predator activity, the detection of predators and evasive responses of prey, and the defensive use of structure in the habitat by prey are all light-dependent behaviors (Brown and Warburton, 1997; Cerri, 1983; Magurran et al., 2010).

The light-induced startle response assay is a sensitive test to measure the effects of contaminant exposure. In recent studies, changes to the startle response of fathead minnow larvae have been observed after exposure to single pesticides with various modes of action (Ankley and Villeneuve, 2006; Steele et al., 2018). Responses to changing light conditions that we observed in this study are also consistent with evolutionary-linked adaptive responses in zebrafish larvae observed in other studies (Burgess and Granato, 2007; Colwill and Creton, 2011). Startle responses may be species specific, thus determining the level of sensitivity, direction of response (dark-to-light; light-to-dark), and response timing is essential for interpretation and comparison of responses across species (Steele et al., 2018). Hyperactivity combined with a diminished freeze response may make the fish more susceptible to predation.

For many species, early life stages are known to more sensitive to the effects of pesticide toxicity (Mu et al., 2019), but this is not universal, and responses may be pesticide-, age- and species-dependent. Zebrafish larvae, which are frequently used in behavioral assessments and for which the behavioral assay was originally developed, increase their activity under dark conditions whereas fathead minnows have an opposite response (Dach et al., 2019; Jin et al., 2015; Steele et al., 2018). A recent study found that adult rare minnows (*Gobiocypris rarus*) are more sensitive to binary and tertiary mixtures of pesticides with different modes of action, potentially a result of reduced metabolic action in larval minnows resulting in lower concentrations of toxic metabolites (Yang et al., 2021). While imidacloprid induced hyperactivity in adult carp, exposures conducted on zebrafish larvae during early developmental stages resulted in decreased swimming activity (Crosby et al., 2015). Our study found clear evidence of hyperactivity in fathead minnow larvae after exposure to contaminated surface water containing neuroactive chemicals. Behavioral responses including the startle response are age- and species- dependent (Ankley and Villeneuve, 2006; Voesenek et al., 2018). A recent study comparing larval and adult fathead minnow behavior reported that 16 dph larvae have a preference for light while mature fish preferred dark conditions, reflecting age-specific habitat preference, feeding strategy and predator avoidance behavior (Vignet and Parrott, 2017). These findings emphasize that choice of test species and age range should be considered when conducting toxicity assessments on environmental mixtures. Detection of adverse effects at any life stage is a cause for concern, assuming that normal behaviors are adapted to ecological conditions. Our findings suggests that the light-induced startle response is a sensitive endpoint for 7-11 dph fathead minnow larvae.

Many of the pesticides detected in our samples are known to be neurotoxic to invertebrates and also affect the behavior of fishes, during single chemical acute or chronic assessments. Considering the high concentrations of the carbamate insecticide methomyl present in our samples, it is likely that this chemical is driving some of the observed behavioral effects. Methomyl was detected in Quail Creek at levels three times above EPA aquatic life benchmarks for chronic fish exposure, and also in Alisal Creek and the Salinas River at levels above invertebrate thresholds. Methomyl is known to induce oxidative stress, reduce muscle maintenance, and reduce swimming strength in fish (Ren et al., 2021; Yoon et al., 2016) by altering cholinesterase activity (Moser et al., 2015; Yoon et al., 2016). Carbamate insecticides affect acetylcholinesterase via irreversible inactivation by inhibiting cholinesterase, which results in behavioral changes in fish (Ren et al., 2021). We also detected several pyrethroid insecticides in our samples, including bifenthrin, lambda-cyhalothrin and permethrin. Lambda-cyhalothrin and permethrin were present in all samples above EPA invertebrate thresholds and at levels exceeding chronic fish exposures in Quail Creek. Pyrethroid insecticides induce hyperexcitability, tremors, convulsions, and death in fish via disruption of normal voltage-gated sodium channel function (Beggel et al., 2011; Clark and Symington, 2011; Connon et al., 2009; Mundy et al., 2021, 2020; Ullah et al., 2019). In addition, several pyrethroids are also known to disrupt endocrine pathways and alter immune function in larval fish, which may also impact behavior (Brander et al., 2016). Bifenthrin has been shown to alter predatory avoidance behaviors in juvenile Chinook salmon, which are known to inhabit the Salinas River during the spawning season (Giroux et al., 2019; Hunt et al., 2003). Overall, the results from single chemical assessments support our findings, although many were conducted at concentrations

higher than would be expected in environmental samples generally, and higher than the concentrations we found in our samples.

We detected three neonicotinoids that are considered contaminants of emerging concern for surface waters (Sousa et al., 2019; Thompson et al., 2020): imidacloprid, thiamethoxam and clothianidin. Several neonicotinoids have been shown to alter anti-predator behaviors of larval fish after acute exposure to environmental concentrations (Faria et al., 2020). We measured thiamethoxam in all samples, and measured concentrations exceeded invertebrate thresholds in Alisal Creek and Quail Creek. Thiamethoxam alters locomotor activity (hyperactivity and reduced startle response) in zebrafish larvae (Liu et al., 2018), which is consistent with the behavioral effects we observed in our study. Imidacloprid was present in all samples and is known to induce hyperactivity in carp via effects on nicotinic acetylcholine receptors (Bhardwaj and Tyor, 2021). A recent study determined that exposure to clothianidin at levels comparable to Alisal Creek (0.15 µg/L) caused a significant (4.7-fold) increase in whole body 17β-estradiol levels in wild sockeye salmon (*Oncorhynchus nerka*) fry (Marlatt et al., 2019), and oxidative stress and liver damage were also reported for juvenile trout after acute exposures to low concentrations (3 µg/L) of clothianidin (Dogan et al., 2021). The startle response of zebrafish larvae was increased after exposure to clothianidin, but only at the highest concentration tested (30 µg/L) (Faria et al., 2020). These findings suggest that the neonicotinoids we detected are able to affect the behavior and stress response systems of fish.

Predictions of toxicity obtained from single chemical studies inherently ignore the effects of mixtures, environmental conditions, choice of test species and age range, and synergistic effects which can be difficult to predict quantitatively (Hernández et al., 2017). Thiamethoxam demonstrated synergistic toxicity when combined in binary and tertiary mixtures with a

fungicide (tetraconazole), organophosphate (chlorpyrifos) and pyrethroid (cypermethrin), after acute exposures in rare minnows (*Gobiocypris rarus*) (Yang et al., 2021). Mixtures of organophosphate and carbamate pesticides inhibit the activity of acetylcholinesterase (AChE) synergistically and thus have potential to interfere with behaviors that may be essential for salmon survival (Laetz et al., 2009). Binary mixtures of the organophosphate chlorpyrifos and two triazine herbicides elicited synergistic responses on the swimming behavior of zebrafish larvae, despite the fact that the herbicides were not effective acetylcholinesterase inhibitors on their own (Pérez et al., 2013).

There is increasing evidence that human populations living in proximity to agricultural land disproportionately experience negative health impacts from pesticide exposure (Garí et al., 2018; Han et al., 2018; Piel et al., 2019). Although neonicotinoids pose a lower exposure risk to humans and other mammals relative to many other insecticides, studies show that many populations are environmentally exposed to neonicotinoids, organophosphates, and pyrethroids in their daily lives (Han et al., 2018; Osaka et al., 2016). Osaka et al. (2016) found organophosphates and pyrethroids in the urine from > 79.8% of young children (under 3 years old) tested, and levels were higher among children who lived with adults working in pesticide-related occupations. A 2014 study conducted in the San Joaquin Valley of California (USA) found that the mothers' residential proximity to imidacloprid usage was positively related to an increased risk for anencephaly, and proximity to methomyl usage increased risk of neural tube defects (NTDs) (Yang et al., 2014). Subsistence fishing can also expose individuals to increased levels of many classes of chemicals, including chemicals of emerging concern such as lambda-cyhalothrin and chlorantraniliprole, which have been shown to bioaccumulate in fish tissue (Clasen et al., 2018). A recent survey conducted in the San Francisco-Bay Delta region of

California (USA) showed that over 90% of 206 survey respondents who reside in the Delta indicated that they or their family eat fish from the Delta four or more times per week (Ag Innovations, 2021). Together, these findings link the deleterious effects of pesticide contamination in freshwater ecosystems to vulnerable human populations.

Many pesticides lack sufficient mixture toxicity and environmental risk assessment data (Bopp et al., 2019; Steele et al., 2018). Pesticides play an integral role in urban and agricultural environments by helping to mitigate the spread of vector-borne diseases, increase crop yield and improve food security (Sharma et al., 2020; Tang et al., 2021). While their utility is undeniable, so too is their impact on the environment. In the ongoing battle against pest species, new pesticides are continually being formulated with novel modes of action (Cordova et al., 2007; Dayan et al., 2019; Frank et al., 2019; Umetsu and Shirai, 2020). As the quantity and diversity of synthetic chemicals increase, so do the complexity of mixtures that can enter aquatic habitats. When combined, the vast ever-growing number of pesticides with diverse modes of action can result in an exponentially greater number of potential mixtures. Worryingly, these include many pesticides of environmental concern, for which toxicological effects remain unknown (Bopp et al., 2019). Understanding the potential toxicity of chemical mixtures and predicting their effects in ecosystems is an increasingly urgent task for environmental toxicologists (Spurgeon et al., 2010; Tang et al., 2021). Sensitive assessments are vital to mitigate the impacts of pesticide pollution on urban and agriculturally developed watersheds, and to protect the health of aquatic ecosystems.

5. Conclusions

Our study provides additional evidence that acute exposure to environmental samples containing mixtures of chemicals of concern can reduce fish ecological fitness by altering their behavior. We measured changes in light-induced startle response, average Total Distance moved, Freezing Cruising and Bursting (Frequency and Duration), and Velocity. We detected changes to the startle responses of fish exposed to surface water from all three sites when compared with controls. The most significant differences in behavioral response were observed during the dark-to-light transitions, measured as light-induced startle responses. The inability to respond normally to light stimuli suggests negative effects on the fitness of individuals may occur in these aquatic ecosystems. Behaviors that are enacted to avoid predation, such as freeze response and startle response are strongly linked with individual survival. Resultingly, any changes in normal swimming behavior, or in the ability of an organism to respond to stimuli, are highly environmentally relevant endpoints. Startle response represented the most sensitive behavioral endpoint that we tested. Studies that incorporate sensitive endpoints such as startle responses, serve to better inform on the risk posed by contaminants in aquatic ecosystems. Further research is needed to optimize behavioral assays to target the most sensitive life stage for measuring this endpoint, and to understand species-specific responses for a wider range of organisms. Our study findings suggest that light-induced startle response, total movement and freezing response are sensitive endpoints to measure the effects of exposure to surface water often containing complex mixtures of environmentally relevant concentrations of pesticides. Taken together, our findings demonstrate that acute exposure to surface water samples from urban and agriculturally developed areas causes adverse, environmentally relevant effects on the behavior of fathead minnow larvae.

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Chapter 3
Alterations in swimming behaviors of *Daphnia magna* provide sensitive endpoints for determining toxicological effects of chemicals of concern in contaminated surface water

A similar version of this chapter is in preparation for submission to the journal ECOTOXICOLOGY & ENVIRONMENTAL SAFETY with the following co-authors:

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Abstract

Aquatic ecosystems receive periodic influxes of runoff that often contain complex chemical mixtures. These dynamic mixtures include chemicals known to have adverse effects in single chemical laboratory tests but understanding their effects in mixtures is less straightforward. Many pesticides are frequently detected in surface waters worldwide, at concentrations that may elicit sublethal effects in invertebrates, but toxicological data are scarce. We evaluated swimming behaviors of *D. magna* as sensitive endpoints in the assessment of exposure to two chemicals of concern; chlorantraniliprole (CHL) and imidacloprid (IMI), performed at environmentally relevant concentrations (1.0 µg/L and 5.0 µg/L). We also evaluated these behaviors after exposure to a dilution series of surface water collected from an agricultural region associated with the Salinas Valley Watershed (Salinas, CA), before and after the first rain following an extended dry period, also known as a "first flush" rain event. We measured behavior by calculating Total Distance moved per time period under light and dark conditions, and as organismal response to light stimulus. For CHL and IMI tests, at least one behavioral response was significantly affected in all treatments. We detected CHL and IMI as components of complex mixtures from surface water at all sites, at both sampling events. During our first sampling event (prior to first flush), we detected 17 target chemicals in the surface water samples, and each site contained a minimum of 7 target pesticides. During our second sampling event (24h after first flush), we detected a total of 27 chemicals, and each site contained a minimum of 21 target pesticides. During both sampling events, we detected neonicotinoids, pyrethroid insecticides, organophosphates, carbamate insecticides and others. Exposure to undiluted and less-diluted surface water from two of these sites (Quail Creek, Hartnell Rd) resulted in high invertebrate mortality, for both sampling events. For our initial sampling event,

water from Quail Creek resulted in close to 100% mortality for all concentrations of surface water as low as 6%. Acute exposure to a geometric progression dilution series of surface water caused changes in *D. magna* swimming behavior, and changes differed across sites and sampling dates. We detected strong dose-response patterns for all sites and concentrations, with controls showing the largest decrease in total movement and the highest concentrations of surface water showing the most divergent responses from controls. Prior to first flush, we observed hyperactivity in our Davis Rd treatments and hypoactivity in Hartnell Rd compared to controls. *Daphnia magna* response to light stimulus was the most sensitive endpoint measured for both sampling events. After first flush, we measured hypoactivity for all sites during at least one time period, in at least one concentration. We detected different response patterns to light stimulus for each site tested. For Quail Creek, *D. magna* behavior followed a negative dose-response pattern. For Hartnell Rd, *D. magna* were hypoactive under dark conditions and followed a weak non-monotonic pattern under light conditions. For Davis Rd., *D. magna* behavior followed a positive dose-response pattern, with significantly different responses from controls at all concentrations tested. These findings are relevant for understanding the impacts of complex chemical mixtures on fish prey. Both CHL and IMI are chemicals found ubiquitously in many watersheds, and there are limited data on behavioral effects. Swimming behavior is a sensitive endpoint to assess the effects of complex chemical mixtures that may impact freshwater ecosystems.

1. Introduction

Aquatic ecosystems are threatened by anthropogenic pollution. For many novel chemicals, there is insufficient information available regarding potential risks to water quality, biodiversity and human health (Morrissey et al., 2015; Tang et al., 2021). Pesticides are frequently detected as components of complex chemical mixtures in aquatic environments, particularly those located near high use agricultural and urban areas (Bradley et al., 2021; Sandstrom et al., 2021; Tang et al., 2021). First flush events occur when the first major rain event occurs after an extended dry period (e.g., in areas with Mediterranean climates), flushing accumulated surface contaminants into surrounding waterways (Peter et al., 2020). These events are known to result in higher detections and abundance of pesticides and other chemicals, and rapid decreases in water quality (Olsson et al., 2013). Infrequent, low-intensity precipitation events characteristic of drought conditions can allow pesticides and other pollutants to accumulate on surfaces, thus increasing their toxicity when they enter aquatic systems at higher-than-normal concentrations (Peter et al., 2020). California (USA) reports high pesticide usages, in part due to intensive agricultural practices (Deng et al., 2019). A study examining first flush toxicity in California found that the concentration of pollutants (including pesticides) was between 1.2 and 20 times higher at the start of the rain season versus the end (Lee et al., 2004). Climate change is altering rainfall patterns in many areas of the world and understanding how these changes may impact sensitive aquatic systems is crucial for monitoring water quality.

The use of pesticides with novel modes of action, such as neonicotinoids and anthranilic diamides, are increasing worldwide (Spurgeon et al., 2010), yet there is limited information on the potential toxicity of many novel pesticides to non-target organisms. Neonicotinoids are currently the most commonly used insecticide worldwide (Mitchell et al., 2017; Simon-Delso et

al., 2015). Application of anthranilic diamides is rapidly increasing, and currently represents 12% of the global insecticide market (Jeschke, 2021). Imidacloprid (IMI) is a neonicotinoid pesticide which interacts agonistically with the postsynaptic nicotinic acetylcholine receptor (nAChR) causing toxic effects to the central nervous system (Li et al., 2021). Chlorantraniliprole (CHL), like other anthranilic diamides, activates and competitively binds to the RyR (Bentley et al., 2010; Cordova et al., 2007), effectively altering calcium signaling and muscle movement (Bentley et al., 2010; Cordova et al., 2007). Chlorantraniliprole (CHL) and imidacloprid (IMI) are detected in surface waters around the world (Pisa et al., 2021; Wolfram et al., 2018). Despite being relatively less environmentally persistent than many older pesticide classes such as organophosphates, CHL and IMI are frequently detected at levels shown to cause adverse effects on non-target aquatic organisms (Cui et al., 2017; Van Dijk et al., 2013).

To assess the potential toxicity of surface waters, many regulatory organizations evaluate exposure impacts on survival, growth and/or reproduction of sensitive model species (Goh et al., 2019). These endpoints risk underestimating sublethal effects resulting from exposure, in aquatic organisms (Connon et al., 2019; Spurgeon et al., 2010). Behavioral assessments performed after exposure to sublethal concentrations of pesticides are effective in capturing underlying physiological or biochemical conditions, which manifest themselves at an organismal level (Yuan et al., 2021). They further serve to determine ecological risk if the behavioral alterations directly relate to factors influencing survival, predator avoidance, feeding/growth, or reproduction (Ford et al., 2021). Locomotor assays to evaluate swimming can show adverse effects at much lower chemical concentrations than other toxicological endpoints (Yuan et al., 2021), and this makes them ideal for analyzing low levels of environmental chemicals, such as pesticides (Beggel et. al., 2010).

Swimming behavior is a well-established endpoint in pharmacology and toxicology (Colón-Cruz et al., 2018; Kristofco et al., 2016; Steele et al., 2018). Previous studies have demonstrated that altered swimming behavior of the invertebrate *Daphnia magna* is a sensitive endpoint for exposures to metals, pesticides and pharmaceuticals under single chemical assessments (Bownik, 2017; Bownik et al., 2019; Chevalier et al., 2015; Tkaczyk et al., 2021). *D. magna* are an ideal test organism due to their demonstrated sensitivity to many chemicals of concern, rapid reproduction, and ease of cultivation (Tkaczyk et al., 2021). They are frequently used in aquatic toxicology and water quality testing, and have well defined acute toxicity testing parameters (USEPA, 2002). *D. magna* swimming behavior is characterized by fractal, irregular locomotion (Seuront et al., 2004), and changes to this natural swimming behavior affect the overall fitness of the organisms. Despite their record of extensive use in toxicology, there is little data on the effects of complex environmental mixtures on *D. magna* swimming behavior. The few studies available in the literature show that changes to *D. magna* locomotor behavior in both light and dark periods might be more sensitive to measure exposure to sublethal chemicals than zebrafish behavior under the same conditions (Hussain et al., 2020).

The Central Coast region of California, USA, where we conducted sampling for this study, is a highly productive agricultural region where heavy agricultural use and dense urban centers result in run-off containing complex mixtures of many pesticides classes. Chemical analysis of water surface samples from this area shows that the range of chemicals encompass many global chemicals of concern, including CHL, IMI, other neonicotinoids, pyrethroids, among others (California Department of Pesticide Regulation, 2020). Little is known about how these chemicals of concern interact in mixtures at environmentally relevant concentrations, or the risk they pose to aquatic ecosystem health. In this study, we evaluated mortality and swimming

behavior of *D. magna* after exposure to chlorantraniliprole and imidacloprid, two known neurotoxicants that are frequently found in waterways at levels exceeding the EPA benchmarks for aquatic life (Deng, 2016; Deng et al., 2019; Goh et al., 2019). We also examined the behavioral effects of surface water samples from two time points: before and after a first flush storm event. We used a geometric dilution series in order to observe a wide range of toxicological outcomes from mortality to sublethal changes in behavior. Invertebrate swimming behavior has the potential to be a sensitive endpoint when assessing the effects of environmental chemicals, in both single chemical assessments and in environmentally relevant mixtures.

2. Materials and Methods

2.1 Imidacloprid and Chlorantraniliprole

We evaluated low (1.0 µg/L) and high (5.0 µg/L) exposure concentrations for both chlorantraniliprole and imidacloprid, based on environmentally relevant concentrations found in monitored waterways, as well as experimental median effect and lethal concentrations (EC50/LC50) (Environmental Protection Agency, 2008, US Environmental Protection Agency, 2016, Deng et al., 2019, California Department of Pesticide Regulation, 2020). We purchased both pesticides (> 97.5% purity) from AccuStandard (New Haven, CT, USA). We used pesticide-grade acetone (Fisher Chemical, USA) as a solvent carrier for the CHL treatments, and in solvent controls, to a final concentration of 0.01% in exposure water, and deionized water for the IMI treatments. We spiked our stock solutions into culture water (described in Methods Section 2.4.1) according to target concentrations, keeping acetone at 0.01% for CHL exposures, and mixed thoroughly. Acute exposure approaches were identical for both IMI/CHL and field exposures (See Methods section 2.4.2).

2.2 Field Water Sampling

2.2.1 Study Sites

Chemical detection data from monitoring sites established throughout the Salinas River, nearby tributaries and other waterbodies (Deng et al. 2019, Goh et al. 2019) have been collected for over a decade (Deng et al., 2019; Goh et al., 2019). Long-term monitoring sites near Salinas, CA are located near areas of high pesticide use, and detections from previous monitoring were often determined to be out-of-compliance with water quality levels. Importantly, these sites are also located near ecologically sensitive areas known to support many species of concern (Luo et al., 2018). We sampled water from three select, existing long-term monitoring sites (Table S3.1) near Salinas, CA: the Salinas River (Sal_Davis) and two sites that directly receive surface water runoff from adjacent agricultural fields; Quail Creek (Sal_Quail), and Alisal Creek (Sal_Hartnell). These sites are at increased risk of contamination from agricultural runoff, as they are located downstream of high use agricultural areas.

2.1.2 Water Sampling

We collected water samples from sampling sites prior to first flush (September 17th 2019) and within 24h of the first flush event (November 26th 2019) from Quail Creek, Alisal Creek and the Salinas River, following standard sampling protocols (Jones, 1999). At each site, we collected 1 L of water into amber glass bottles certified to meet current US EPA guidelines then sealed with Teflon-lined lids, then stored them on ice for transportation, where we refrigerated them at 4 °C. We initiated all exposures within 24 h of sample collection. We measured water quality parameters (ambient water pH, specific conductance, dissolved oxygen, temperature, total dissolved solids, salinity, and total suspended solids) in situ using a YSI EXO1 multi-parameter water quality Sonde (Doo and He 2008). Results of water quality parameters are

shown in Table S3.2.

2.2.3 Geometric Dilution Series of Surface Water

During previous exposure studies from our group (Stinson et al., 2021), conducted on water samples collected at the same field sites, we observed high invertebrate mortality. To better capture and understand the extent of the toxicity at these sites we created a geometric progression dilution series for our surface water samples. We mixed surface water with standard US EPA control water (see Methods section 2.4.1) to create the dilution series, which initially included 100%, 60%, 35%, 20% and 12% surface water for our September sampling event. Based on the high levels of invertebrate mortality observed in this first exposure event, we added an additional lower concentration (6%) to the subsequent sampling event in November. We diluted each sample into control water to obtain the desired concentrations after mixing thoroughly to distribute any sediment particles. We then aliquoted the treatment concentrations into 250 mL beakers, brought the water temperature to 20 °C then loaded the organisms. We repeated the dilution procedure on day 2 of the test and conducted an 80% water change. Acute exposure test conditions were identical for both IMI/CHL and field exposures (See Methods section 2.4.2).

2.3 Chemical Analysis

Chemical analysis was conducted by the Center for Analytical Chemistry, California Department of Food and Agriculture (Sacramento, CA) using multi-residue liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography–mass spectrometry (GC-MS/MS) methods. For chemical treatments, CHL and IMI concentrations were measured to confirm target exposure concentrations. Laboratory QA/QC followed CDPR guidelines provided in the Standard Operating Procedure CDPR SOP QAQC012.00 (Teerlink

and DaSilva, 2017). For field water, 47 pesticides were included for screening based on the procedures described in the Monitoring Prioritization Model (Luo et al., 2018). Extractions included laboratory blanks and matrix spikes (method detection limit and reporting limit for each analyte available upon request).

2.4 Toxicity Testing

2.4.1 Test Organisms

Daphnia magna neonates (< 48h) were provided to us by Aquatic Biosystems (Hampton, NH, USA). We maintained *D. magna* at 20 ± 2°C under a 16-hr light: 8-hr dark photoperiod in EPA synthetic control water (US Environmental Protection Agency, 2002), which was prepared by dissolving 23.04 g NaHCO3, 14.40 g CaSO4.2H2O, 14.40 g MgSO4, and 0.96 g KCl in 120 L of deionized water to achieve a hardness of 160 – 180 mg/L CaCO3 and alkalinity of 110 – 120 mg/L CaCO3. On arrival, we fed all organisms a mixture of suspended *Raphidocelis subcapitata* (obtained from Aquatic Research Organism Inc) and YCT (yeast, cerophyl, trout chow mixture). *Daphnia magna* were acclimated to control water for > 8 h prior to exposures, and swan and fed normally during that time period.

2.4.2 Acute Exposure Conditions

We exposed organisms in 20 mL scintillation vials (n = 6), with 6 replicates per treatment for a total of n = 36 individuals per time point (48h and 96h). We randomly selected 24 individuals per treatment group to use in behavioral assays at two time points: 48h and 96h. For field exposures, we placed twenty individuals into each of the 250 mL replicate beakers containing 200 mL of treatment water, with four replicates per treatment for each time point (n = 40). We used larger exposure volumes for the field water to reduce the potential influence of

sediment on organism toxicity, and to follow EPA guidelines for acute exposures to effluent (US EPA 2002). A recent study comparing *D. magna* acute toxicity tests using various exposure volumes (down to 48-well plates) demonstrated that exposures performed in smaller volumes produced equivalent results as traditional test configurations, for different chemicals (Grintzalis et al., 2017). We conducted all exposures in temperature-controlled chambers kept at 20± 2°C, with a 16h:8h dark:light cycle to maintain optimal conditions for our test organisms (US Environmental Protection Agency, 2002). Every day, during the exposure, we recorded the number of organisms per beaker and the mortality, while removing any dead individuals from the tests. At the 48h mark we performed 50% water changes in the chlorantraniliprole and imidacloprid exposure, and 80% water changes for the water sampling exposure studies (larger volumes were exchanged in water sample exposures to account for suspended solids and additional bacterial activity seen in field samples). We tested temperature, total alkalinity, hardness, pH and dissolved oxygen at both test initiation and 48h to ensure that the water remained within the acceptable ranges for *D. magna.* We fed all organisms a mixture of suspended *Raphidocelis subcapitata* (obtained from Aquatic Research Organism Inc) and YCT (yeast, cerophyl, trout chow mixture) at both the test initiation and after 48h water renewals (US Environmental Protection Agency, 2002).

2.5 Behavioral Assays and Data Analysis

2.5.1 Behavioral Assay Conditions

We performed behavioral assays after acute (48h and 96h) exposures to the geometric progression dilution series of agricultural surface water, and to single and binary mixtures of imidacloprid and chlorantraniliprole. We randomly placed *D. magna* from each technical replicate per treatment individually into each well of a 24-well cell culture plate (Thermo Fisher

Scientific, CA, USA) containing 2 mL of control water. Randomization minimized plate effects, but we also ensured that at least three individuals from each treatment and controls were represented in each plate. We gently transferred individuals using 1 mL plastic pipettes and allowed them to habituate to the plate conditions, in the temperature-controlled chambers used for the exposures prior to commencing video recordings. We then placed the plate into a DanioVision Observation Chamber (Noldus, Wageningen, Netherlands) and allowed an additional 5 min adjustment period prior to initiating video recording. The temperature of the plate was maintained at 20 °C ± 0.5 °C during the test via a recirculating water system attached to a chiller (TECO-US, Terrell, TX, USA).

2.5.2 Video Data Tracking and Analysis

We recorded movement using a top-mounted Basler Gen1 camera located directly above the plate and tracked movement using EthoVision XT software (version 14.0; Noldus, Wageningen, Netherlands). This software is specifically designed to simultaneously track movement from multiple wells. D. magna movement in each plate was recorded for a total of 30 min in dark:light conditions; having excluded the first 5 min of video tracking as a habituation period. The chamber was illuminated during light cycles with a programmable light located beneath the plate, set at 10,000 lux for each light cycle. Light conditions included an initial 10 min dark period, followed by a 20 min light period. Each video recording was assessed visually to confirm software tracking accuracy prior to analyses.

2.5.3 Statistical Analysis

We evaluated Total Distance moved (mm), mean Velocity (mm/s), and response to light stimulus. We measured response to light stimulus by determining the change in mean (±SE) distance traveled between the last 1 min of the dark photoperiod and the first min of the

following light period, following Steele et al. (2018). The software we used for video tracking is specifically designed to quantify measurements of the organisms' behavior including both horizontal distance moved and speed (Noldus et al., 2001). Assessing horizontal movement over time is a common approach for determining changes in locomotor ability after exposure to pesticides (Bownik, 2017). We then exported summary statistics from Ethovision XT per 1 min intervals for each treatment and analyzed the data in GraphPad Prism (Version 9.0, San Diego, CA, USA). Prior to analysis, we checked that all data met the assumptions of an ANOVA by performing Shapiro-Wilk tests for normality. We determined significance of mortality data by Analysis of Variance (ANOVA) followed by Dunnett's test for multiple comparisons. For distance moved and photomotor response data we performed two-way ANOVAs with Tukey's post hoc tests in order to test the significance between treatments.

3. Results

3.1 Chemical Analysis

3.1.1 Chlorantraniliprole and Imidacloprid

Physicochemical water parameters for laboratory control water and IMI/CHL treatments are listed in Table S3.2. Analytical confirmations of test concentrations were determined for chlorantraniliprole and imidacloprid stock solutions (Table S3.3). Temperature, dissolved oxygen, and pH of surface water samples were comparable to controls. Conductivity, hardness, alkalinity, and salinity of surface water from Quail Creek and Alisal Creek exceeded values for control water. Upon collection, total suspended solids (TSS) were highest for Quail Creek (593.8 mg/L) as compared to Alisal Creek (449.2 mg/L) and the Salinas River (45.58 mg/L).

3.1.2 September 2019 Surface Water Exposures

Chemicals detected at levels exceeding an EPA benchmark for aquatic life are shown in Table S3.4. Of 47 pesticides analyzed, 17 were detected in our surface water samples, and each site contained a minimum of 7 target pesticides. Chlorantraniliprole was present at all sites at concentrations below the acute lethality benchmarks for invertebrate species exposure (LC50 =7.1 µg/L; EPA benchmark for acute, 5.8 µg/L, and chronic, 4.47 µg/L). The neonicotinoid imidacloprid was present above the EPA benchmark for chronic invertebrate exposure (0.01 µg/L), and above the acute invertebrate level (0.385 µg/L) at Sal_Hartnell (0.513 µg/L). Neonicotinoids were present at all sites. Organophosphates were present at two of the sites: Sal_Quail and Sal_Hartnell. Several pyrethroids were present at levels at or above an EPA benchmark, including permethrin, lambda-cyhalothrin, and bifenthrin (analytes of particular concern). Several other chemical detections exceeded threshold values. Notably, methomyl was present at Sal_Quail (29.9 µg/L) at nearly three times the limit for chronic fish exposure (12 µg/L), and above the EPA benchmark for chronic invertebrate exposure (0.7 µg/L) at all sites. Overall, Sal_Davis contained the fewest total number of chemicals at the lowest concentrations of the three sites we examined.

3.1.3 November 2019 Surface Water Exposures

We detected several chemicals at levels exceeding an EPA benchmark for aquatic life (exceedances are listed in Table S3.5). Of 47 pesticides analyzed, 27 were present in our surface water samples, and each site contained a minimum of 21 target pesticides. Neonicotinoids were present at all sites. The neonicotinoid imidacloprid was present above the EPA benchmark for chronic invertebrate exposure (0.01 µg/L) at Sal_Davis (0.03068 µg/L), and above the acute invertebrate level (0.385 µg/L) at Sal_Hartnell (0.29254 µg/L) and Sal_Quail (0.30697 µg/L). Thiamethoxam was also present at all sites, at levels below the EPA benchmark for chronic

invertebrate exposure (0.74 µg/L). Clothianidin was present above the acute invertebrate benchmark (0.05 µg/L) at Sal_Quail (0.89898 µg/L) and Sal_Hartnell (0.09285 µg/L). Organophosphates were present at all sites. Malathion was present above the EPA benchmark for chronic invertebrate exposure (0.049 µg/L) at Sal_Quail (0.99858 µg/L) and Sal_Hartnell (0.07848 µg/L). Chlorpyrifos was present at Sal_Hartnell (0.12826 µg/L) above the EPA benchmark for chronic invertebrate exposure (0.05 µg/L). Several pyrethroids were present at levels at or above an EPA benchmark, including analytes of particular concern. Bifenthrin was present at all three sites (Sal_Quail: 0.0665 µg/L, Sal_Hartnell: 0.0308 µg/L, Sal_Davis: 0.0197 µg/L) above the EPA benchmark for chronic invertebrate exposure (0.0013 µg/L). Cyfluthrin was present at Sal_Hartnell (0.0162 µg/L) above the EPA benchmark for chronic fish exposure (0.01 µg/L). Lambda cyhalothrin was present at Sal_Quail (0.0324 µg/L) and Sal_Hartnell (0.0761 µg/L) above the EPA benchmark for chronic fish exposure (0.031 µg/L), and at Sal_Davis (0.00496 µg/L) above the EPA benchmark for acute invertebrate exposure (0.0035 µg/L). Fenpropathrin was present at Sal_Quail (0.0989 µg/L) and Sal_Hartnell (0.0297 µg/L) above the EPA benchmark for chronic fish exposure (0.06 µg/L). Esfenvalerate/fenvalerate was present at Sal_Hartnell (0.0219 µg/L) above the EPA benchmark for chronic invertebrate exposure (0.017 µg/L). Permethrin was present at all three sites (Sal_Quail: 0.0328 µg/L, Sal_Hartnell: 0.0493 µg/L, Sal_Davis: 0.0140 µg/L) above the EPA benchmark for chronic invertebrate exposure (0.0014 µg/L). Chlorantraniliprole was present at all sites below benchmarks. Overall, Sal_Davis contained the fewest total number of chemicals at the lowest concentrations of the three sites we examined.

3.1.4 Relative Change in Chemical Concentration after First Flush

Table 3.2 shows the relative change in water chemistry before and after a first flush rain event, for all chemicals that exceeded an EPA benchmark for aquatic life value during at least one sampling event. Analytes which increased in concentration from September to November are shown in yellow and increased values that also exceeded EPA acute invertebrate aquatic life benchmarks are shown in red. The absolute change in µg/L is noted, unless one detection was below LOQ and listed as "trace." We annotated these instances as greater than or less than zero, depending on the direction of change.

Table 3.2. *Change in concentration (µg/L) of analytes of concern, from 9/17/2019 versus 11/26/2019. Analytes which increased in concentration from September to November are shown in yellow and values that increased and exceeding EPA acute invertebrate aquatic life benchmarks are shown in red. For analytes that were initially detected at Trace levels in September then increased/decreased in November, change value is listed as > 0 or < 0, respectively.*

Analyte	Δ Quail Creek	Δ Hartnell Rd.	Δ Davis Rd.
Acetamiprid	-0.12	-0.09	0
Atrazine	0	0	0
Azoxystrobin	0.78	0.06	0
Bensulide	-1.4	0.39	<0
Chlorantraniliprole	0.01	0.01	0.02
Chlorpyrifos	0	0.11	0
Clothianidin	0.82	-0.08	>0
Cyprodinil	0.164	0.028	>0
Dimethoate	0	-0.052	0
Diuron	>0	0	0.224
Fenamidone	-0.17	-0.11	>0
Fenhexamid	0	0	0
Fludioxonil	-0.12	0.027	>0
Imidacloprid	0.01	-0.22	0.02
Indoxacarb	-0.09	0.027	0
Malathion	0.97	-0.27	0.02
Methomyl	-29.65	-0.89	-0.31
Methoxyfenozide	0.054	0	>0
Prometryn	0	0	>0
Pyraclostrobin	0.07	0.08	>0
Quinoxyfen	>0	>0	0
Simazine	0	0	0
S-Metolachlor	0	0	0
Thiamethoxam	-3.7	-0.49	-0.04

Trifloxystrobin	0.034	>0	0
Bifenthrin	0.064	0.028	0.0197
Fenpropathrin	0.0989	0.0297	0
Lambda Cyhalothrin	-0.0473	0.0634	0.00496
Permethrin Cis	-0.0898	0.0019	0.00608
Permethrin Trans	-0.1114	-0.0112	0.00789
Cyfluthrin	0	0.0103	0.00554
Cypermethrin	0	0	0
Esfenvalerate/Fenvalerate	-0.0224	0.029	0
Permethrin Total	-0.2012	-0.0093	0.014

3.2 Mortality and Behavioral Assays

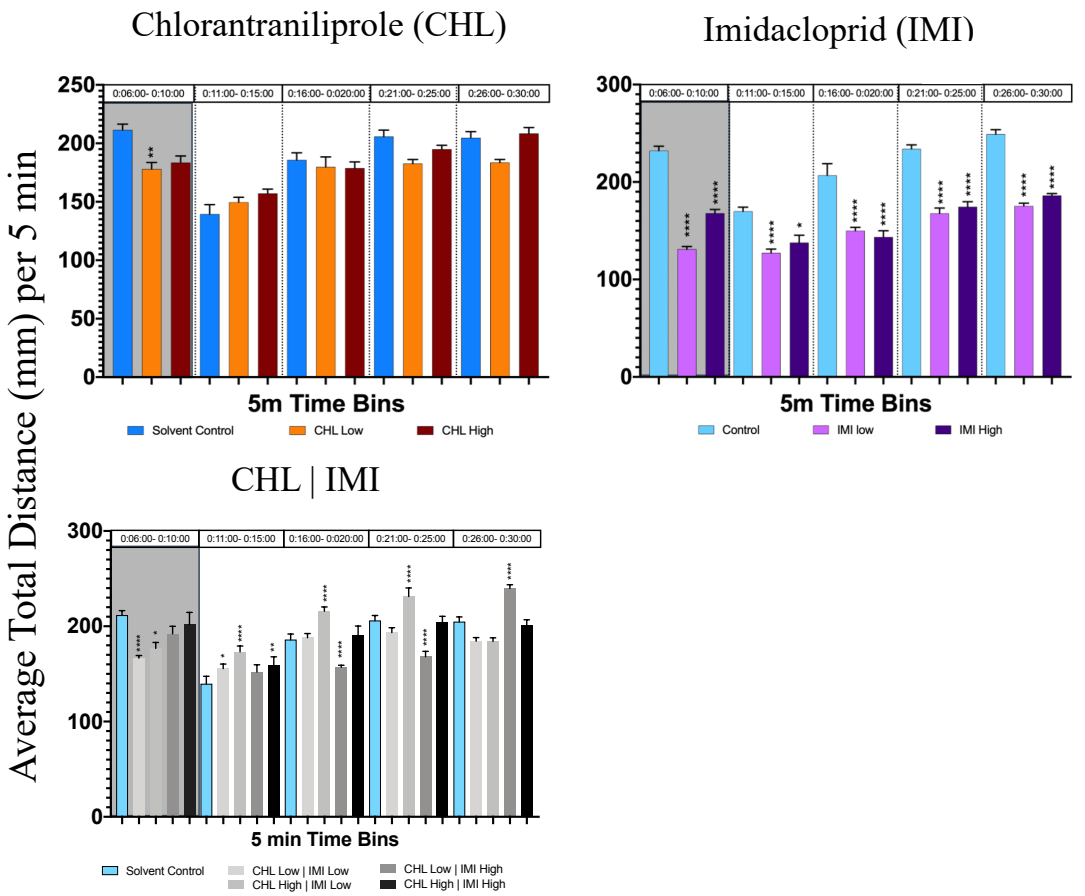

Figure 3.1. Average (±SE) Total Distance (mm) moved for *Daphnia magna* following 96h acute exposures to Low (1.0 µg/L) and High (5.0 µg/L) concentrations of imidacloprid, chlorantraniliprole, and binary mixtures. Daphnia were exposed to an initial 5 min dark period (shown as a dark background) and one 20 min light cycle. P-values are reported as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

3.2.1 Chlorantraniliprole and Imidacloprid Exposures

We found no significant mortality in *D. magna* exposed to IMI, CHL or binary mixtures (Table S3.6). We calculated the average Total Distance moved (mm/s) for CHL, IMI, binary mixtures, and controls, in dark and light conditions (Figure 3.1, Table S3.7). During the initial dark photoperiod, organisms exposed to low levels (1.0 µg/L) of CHL were hypoactive, showing a ~ 17% decrease in average Total Distance moved (mm/s) compared with controls. No significant differences were observed for either CHL treatment during the light photoperiod. For *D. magna* exposed to low (1.0 µg/L) and high (5.0 µg/L) levels of IMI were hypoactive for the duration of the locomotor assay under both dark and light conditions. For binary mixtures, both treatments with low IMI concentrations were hypoactive in the dark. Under light conditions, the low/low and high/high treatment groups were initially hyperactive during the first 5 min time bin, then were not significantly different from the control group for the duration of the locomotor assay. The high CHL/ low IMI treatment group was consistently hyperactive under light conditions. The low CHL/high IMI group were increasingly hyperactive for the duration of the locomotor assay under light conditions.

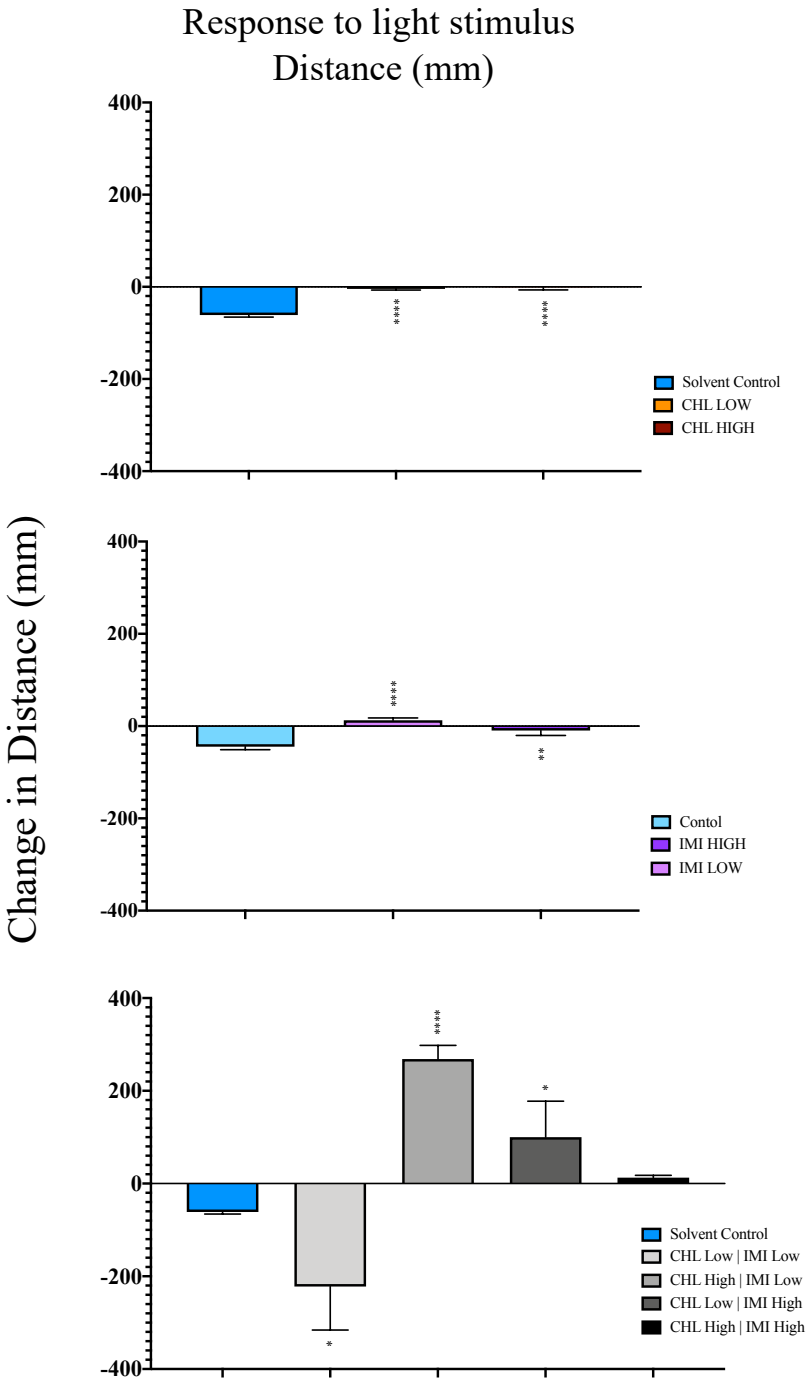

Figure 3.2. Response to light stimulus of *Daphnia Magna* following 96h exposures to Low (1.0 µg/L) and High (5.0 µg/L) concentrations of imidacloprid, chlorantraniliprole, and binary mixtures. Light response was measured as the change in mean (±SE) distance traveled between the last minutes of the initial dark photoperiod and the first minute of the following light period. Daphnia were exposed to one 5 min dark and one 20 min light cycle. P-values are reported as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

We measured significant changes in response to light stimulus, measured as the mean (±SE) distance traveled between the last 1 min of the initial dark photoperiod and the first 1 min of the light photoperiod, following Steele et al. (2018). The change in distance moved during the dark:light transition is shown in Figure 3.2 (results from statistical analyses are reported in Table S3.7). Negative values represent rapid reduction in Total Distance moved in response to changing light stimuli. In response to light stimuli, both the control and solvent control groups exhibited a large reduction in Total Distance moved consistent with a freeze response. For both CHL treatments, organisms exhibited no change in Total Distance moved in response to light stimulus, representing a nearly 60-fold difference in response from the control group. Organisms exposed to 1.0 µg/L IMI had an inverse response to light stimulus when compared to the control group, increasing their Total Distance moved in response to light stimulus. Organisms exposed to 5.0 µg/L IMI exhibited a reduction in their average Total Distance moved, but this response was 5-fold smaller than controls. Mixtures of CHL and IMI resulted in the most divergent responses to light stimulus. *D. magna* exposed to low CHL/low IMI responded more negatively to light, reducing their Total Distance by more than 3-fold that of individuals in the control group. *Daphnia magna* in the high CHL/low IMI treatment group had an opposite response to light, increasing their Total Distance more than 4-fold compared with the magnitude of response we observed from controls. The low CHL/high IMI exposures resulted in a similar Total Distance moved magnitude of response to controls. High CHL/high IMI exposed *D. magna* did not show a significantly different response from controls, however the direction of response varied.

3.2.2 September 2019 Exposure

For *D. magna* exposed to water from Quail Creek, we observed high levels of mortality at all concentrations (Table S3.7) and as a result, we were unable to run behavioral assays for this sampling site. Due to elevated mortality in the undiluted (100%) Alisal Creek treatment, we only evaluated behavior from higher concentrations (35%, 20%, and 12%) for the remaining sites. *Daphnia magna* exposed to the 20% concentration of water from Alisal Creek exhibited hypoactivity during the dark and light photoperiods, compared with the control group. At the highest concentration of surface water tested (35%) from this site, we observed an initial period of hyperactivity during the light photoperiod, followed by hypoactivity after 15 min (Figure 3.3, Table S3.8). *Daphnia magna* exposed to water from Davis Rd. demonstrated hyperactivity during the light photoperiod (Figure 3.3).

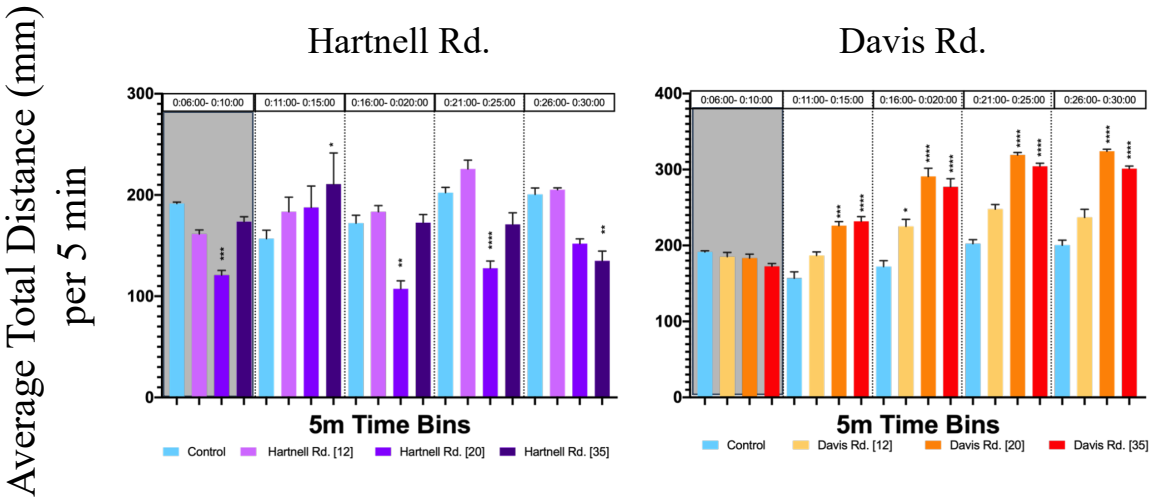

Figure 3.3. Average (±SE) Total Distance (mm) moved for *Daphnia magna* following 96h ambient field water exposures from two sites in Salinas, CA on 09/17/2019: Hartnell Rd. and Davis Rd. Bar charts show Average Total Distance per 5 min time bin. Daphnia were exposed to an initial 5 min dark period (shown as a dark background) and one 20 min light cycle. P-values are reported as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

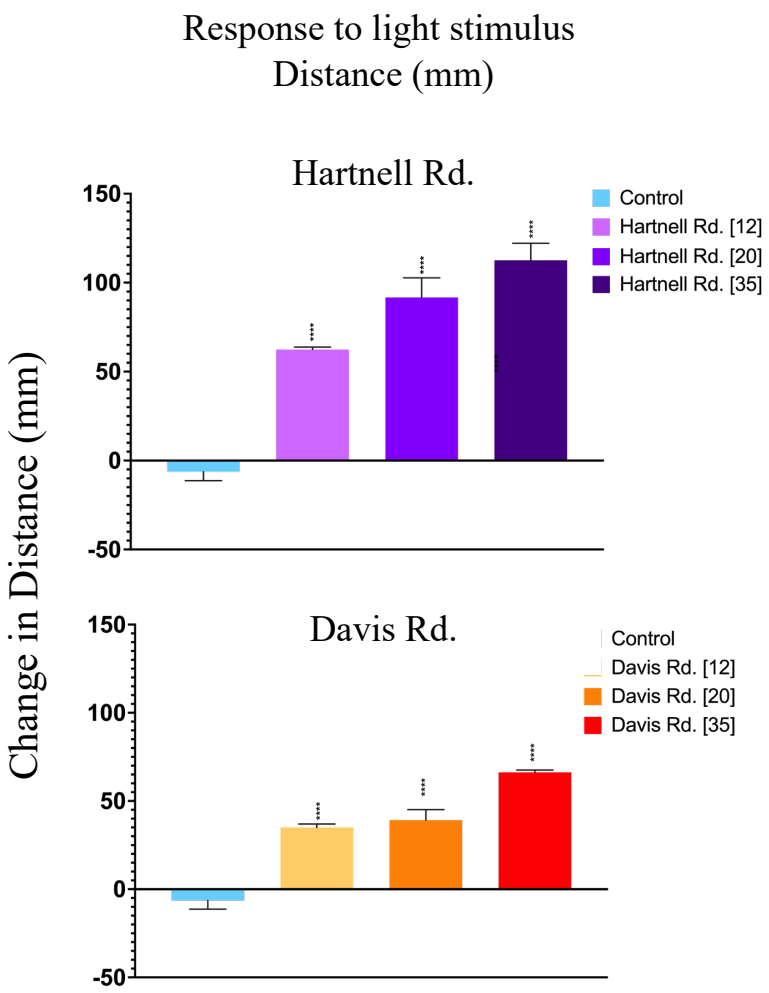

Figure 3.4. Response to light stimulus of *Daphnia Magna* following 96h ambient field water exposures from two sites in Salinas, CA on 09/17/2019: A) Hartnell Rd. and B) Davis Rd. Light response was measured as the change in mean (±SE) distance traveled between the last minutes of the initial dark photoperiod and the first minute of the following light period. Daphnia were exposed to one 5 min dark and one 20 min light cycle. P-values are reported as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

All treatment groups were significantly different from controls and demonstrated increased movement in response to light stimulus. Organisms exposed to water from both Hartnell Rd. and Davis Rd. followed clear dose-response patterns (Figure 3.4, Table S3.8), with increasing concentrations positively correlating with hyperactivity.

3.2.3 First Flush Storm Event (November 2019) Exposure

For *D. magna* exposed to undiluted (100%) water from Quail Creek and Hartnell Rd., we observed high levels of mortality (Table S3.8). As a result, we were only able to evaluate behavior for the 20%, 12% and 6% dilution treatments for these sites. Undiluted water from Davis Rd. did not result in significant mortality, and so we analyzed *D. magna* behavior for all concentrations for this site.

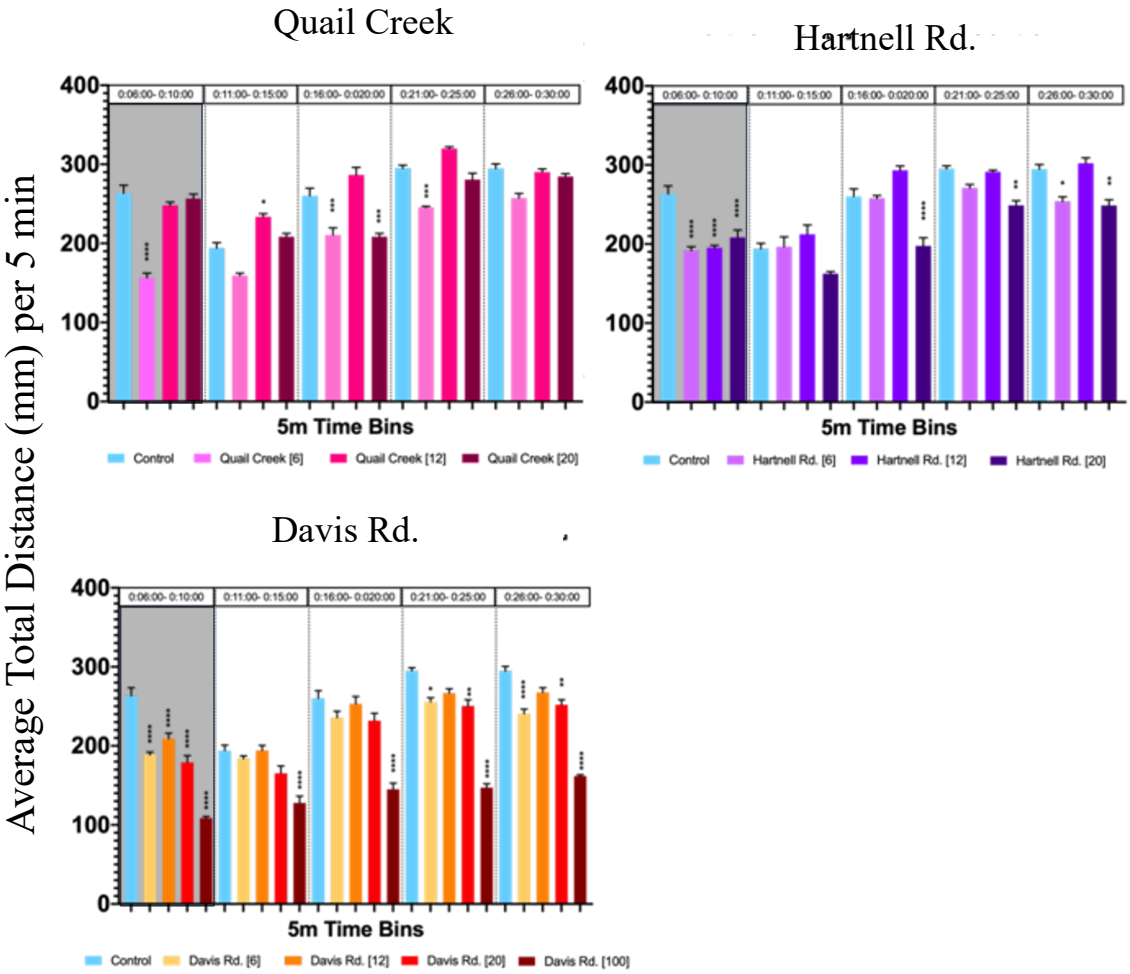

Figure 3.5. Average (±SE) Total Distance (mm) moved for *Daphnia magna* following 96h ambient field water exposures from two sites in Salinas, CA on 11/26/2019: Hartnell Rd. and Davis Rd. Bar charts show Average Total Distance per 5 min time bin. Daphnia were exposed to an initial 5 min dark period (shown as a dark background) and one 20 min light cycle. P-values are reported as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

Daphnia magna exposed to water from all three sites were hypoactive under dark and light conditions, for at least one treatment concentration (Figure 3.5, Table S3.9). For Quail Creek treatments, *Daphnia magna* behavior followed non-monotonic responses; the lowest (6%) and highest (20%) concentrations tested showed hyperactivity compared with controls. Only one treatment group (Quail Creek 12%) exhibited significantly hyperactivity compared to controls at any time point examined. Organisms exposed to 6% water from Quail Creek were the most hypoactive compared to controls in both dark and light conditions throughout most of the behavioral assay. *Daphnia magna* exposed to water from Hartnell Rd. were hypoactive under dark conditions and followed a weak non-monotonic pattern under light conditions. Organisms exposed to the highest concentration (20%) of water from Hartnell Rd. exhibited the most significant hypoactivity. Hypoactivity of *D. magna* exposed to water from Davis Rd. was positively correlated with treatment concentration. The average Total Distance moved for organisms exposed to undiluted water from Davis Rd. was 50% that of the controls.

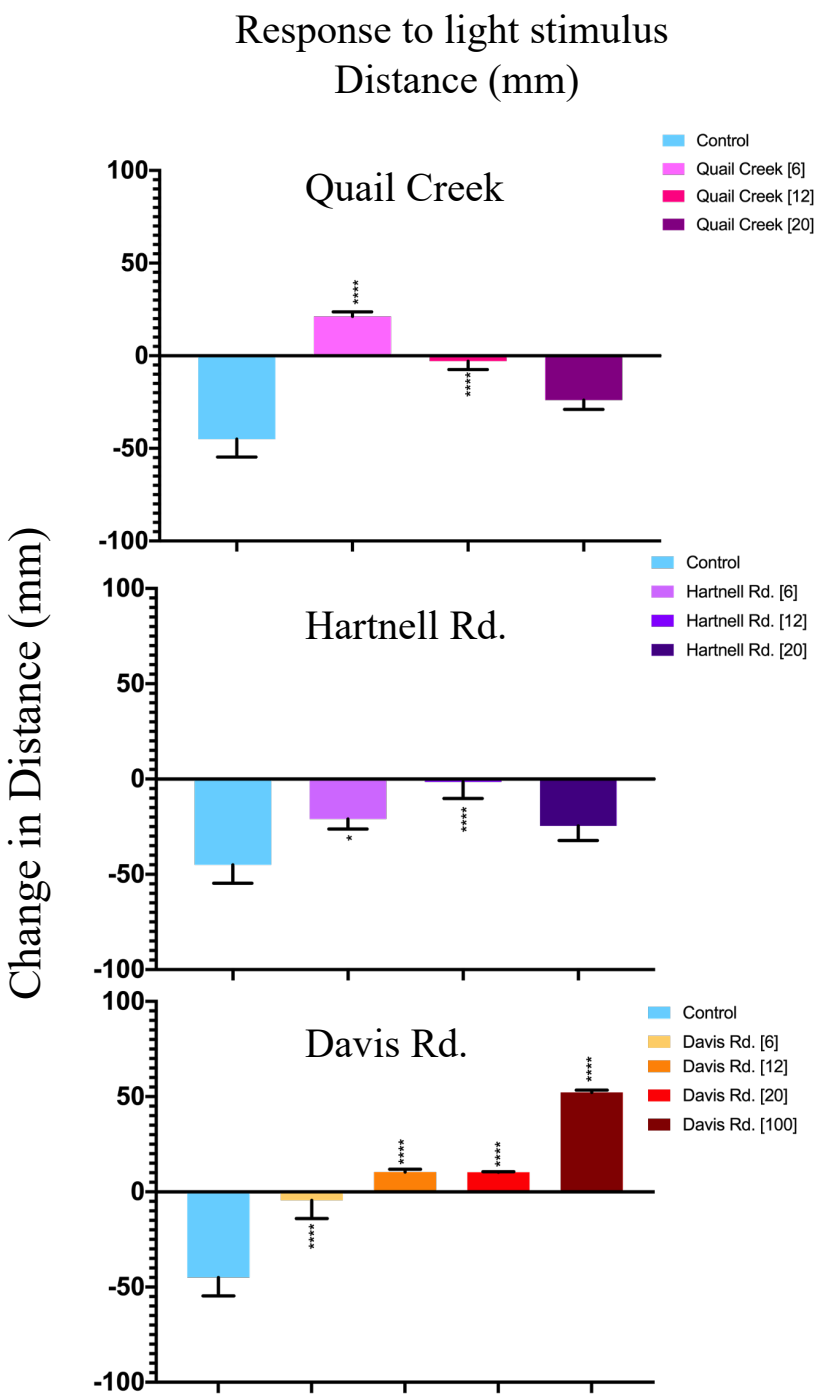

Figure 3.6. Response to light stimulus of *Daphnia Magna* following 96h ambient field water exposures from two sites in Salinas, CA on 11/26/2019: A) Hartnell Rd. and B) Davis Rd. Light response was measured as the change in mean (±SE) distance traveled between the last minutes of the initial dark photoperiod and the first minute of the following light period. Daphnia were exposed to one 5 min dark and one 20 min light cycle. P-values are reported as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

In November, we found unique response patterns to light stimulus from each site tested (Figure 3.6, Table S3.9). *Daphnia magna* exposed to water samples from Quail Creek site demonstrated an inverse dose response pattern, where exposure to the lowest concentration of surface water gave the most significant change in response (increase), and exposure to the highest concentration of surface water was not significantly different from control groups. Organisms in the Hartnell Rd treatment exhibited a non-monotonic dose response, with organisms exposed to 12% surface water showing a reduced response to light stimulus (smaller decrease) compared with controls. Organisms exposed to 6% surface water had a significantly lessened photomotor response pattern, and the highest concentration was not significantly different from the control group. *Daphnia magna* behavioral responses differed significantly from controls after exposure to water from Davis Rd. at all concentrations, following a positive dose-response pattern (increasing). For Davis Rd., organisms exposed to undiluted surface water responded by increasing their Total Distance an equal magnitude in the opposite direction from controls which decreased their Total Distance moved.

Discussion

We assessed effects of two emerging chemicals of concern, in single/binary exposures, and as components of contaminated surface water, on the swimming behavior of *D. magna*. We detected CHL and IMI as components of complex mixtures from surface water at all sites, where we also detected other neonicotinoids, pyrethroid insecticides, organophosphates, carbamate insecticides and other chemicals of concern, both before and after a first flush rain event. We found significant impacts on *D. magna* swimming behavior for all treatments. We determined

that average Total Distance moved and response to light stimulus are both sensitive endpoints for the sublethal assessments of IMI/CHL, and for surface water exposure.

In September, we detected strong dose-response patterns for both sites. *Daphnia magna* exposed to all concentrations of surface water responded in the opposite direction from controls by increasing their Total Distance. This response may have implications for survival in natural populations. Individuals who cannot respond to predator cues, or who's activity is altered by exposure, may have an increased risk of predation (Dodson, 1988). After first flush (November), we measured hypoactivity for all sites during both dark:light conditions, in at least one concentration. In natural populations, *D. magna* exhibit patterns of diel vertical migration and horizontal distribution which may be linked to predator presence (Dodson, 1988). Hypoactivity may reduce their capacity to follow these patterns, thus increasing predation risk. We detected different response patterns to light stimulus for these sites. Exposure to surface water from Quail Creek and Hartnell Rd, resulted in dose-dependent biphasic response patterns. For Hartnell Rd, *D. magna* were hypoactive under dark conditions and followed a weak non-monotonic pattern under light conditions. For Davis Rd., *D. magna* behavior followed a positive dose-response pattern, with significantly different responses from controls at all concentrations tested.

We detected changes in *D. magna* locomotion which were chemical-specific. We observed hypoactivity for both low and high concentrations of IMI, across both dark and light conditions. This is consistent with a recent study examining Total Distance moved of *D. magna* after single chemical exposure to CHL and IMI, among other chemicals, at low (1.0 μg/L) concentrations (Hussain et al., 2020). In this study, they observed similar hypoactivity under dark and light conditions after IMI exposure. IMI is known to inhibit acetylcholinesterase (AChE), which can negatively impact nerve conduction and alter swimming behavior in *D.*

magna (Ren et al., 2017), and our results are consistent with these studies. Exposure to IMI altered response to light stimulus following a dose-response pattern. In a recent study examining the effects of IMI on the amphipod *Gammarus fossarum*, IMI stimulated locomotor activity at low exposure concentrations (0.1 µg/L) and inhibited activity at higher concentrations (1.0 µg/L) (Lebrun et al., 2020). Chlorantraniliprole exposures resulted in significant hypoactivity only under dark conditions. Response to light stimulus was reduced in *D. magna* exposed to both concentrations of CHL. Low (µg/L) levels of CHL exposure have been shown to produce dose-dependent inhibition of swimming, and decreased responses to both light stimulation in a recent study (Yuan et al., 2021), which are consistent with our findings. For binary mixtures of CHL and IMI containing higher levels of IMI, we observed hyperactivity compared to controls. We also detected hyperactivity under dark conditions for our CHL single chemical exposure, at the Low concentration. Increased activity under dark conditions could suggest a possible disruption of signal transmission in the vision or nervous systems, and has been observed for IMI exposures at low (µg/L) exposure levels in other studies (Yuan et al., 2021). Our finding is inconsistent with the Hussain et al. (2020) study, where investigators determined hyperactivity under light conditions and no significance under dark conditions (Hussain et al., 2020). It is possible that increased replication could have reduced inter-individual variation and improved our ability to observe small changes in Total Distance moved. Hussain et al. (2020) used one exposure vessel containing 50 Daphnids per treatment group, whereas we used fewer Daphnia (n = 24) but had greater replication of technical replicates (6 exposure vessels per treatment). *Daphnia magna* locomotion is inherently irregular when compared with fish larval movement patterns (Seuront et al., 2004), and presents additional challenges for analysis. Our replication exceeded many previously published studies, however, and the high significance observed from other treatments

and endpoints suggests that our experimental design was significant to detect sublethal effects (Ren et al., 2017; Rivetti et al., 2016).

Comparing locomotor responses before and after a first flush event, we observed the largest changes for Davis Rd. Out of the three sites examined, we detected the fewest number of chemicals at Davis Rd. in September (5 chemicals detected) at the lowest concentrations (no EPA benchmark exceedances). Prior to first flush (September sampling) *D. magna* exposed to water from Davis Rd. were hyperactive, particularly at the higher concentrations. For this site/date, only IMI and methomyl exceeded EPA invertebrate benchmarks. In our second sampling event from this site, we observed the largest influx of chemicals from the first flush event (17 chemicals detected), most of which were present below EPA chronic or acute invertebrate benchmarks (four exceedances). Following the first flush event, Hartnell Rd. received the highest concentrations of chemicals with eight exceeding EPA benchmarks. *D. magna* exposed to water from Hartnell Rd. were hypoactive, with the most significantly different Total Distance moved measurements observed for the 20% concentration. Analytical chemistry from this treatment detected several chemicals known to have sublethal effects on *D. magna*, including IMI, CHL, bifenthrin, clothianidin, malathion, methomyl, and lambda-cyhalothrin (Bownik et al., 2019; Bownik and Szabelak, 2021; Brausch et al., 2010; Hussain et al., 2020). Due to the high mortality observed for Quail Creek in September, we were unable to make any behavioral comparisons. It is notable that the level of methomyl detected at this site was greater than three times the EPA chronic fish exposure level, and it is likely that methomyl represents a main driver of the toxicity for this site. It is possible that additional contaminants are present at this site, which were not included in our analysis. Many pharmaceuticals are known to cause hyperactivity and have been detected in waste water at other sites in California (Brodin et al.,

2014; Loraine and Pettigrove, 2006; Tkaczyk et al., 2021). Davis Rd. has been used by our group and by CA DPR as a least-impacted reference site in previous toxicity studies (Deng et al., 2019; Stinson et al., 2021). Taken together, these findings illustrate the importance of conducting sublethal assessments to link physiological responses to chemical monitoring data.

Sublethal impacts can result in ecologically relevant effects on individual fitness, populations, and communities. In pesticide contaminated aquatic environments, overall invertebrate biomass and diversity are reduced as sensitive individuals and species decline. Neonicotinoids are known to induce community-level changes by altering the abundance of invertebrate predator species in a mesocosm study (Miles et al., 2017). As aquatic systems continue to experience pesticide influxes from agricultural and urban sources, invertebrates which may have bioaccumulated pesticides may represent a greater risk to their predators. A recent study demonstrated that field-collected pyrethroid-resistant *H. azteca* bioaccumulated several pyrethroids (bifenthrin, permethrin and cyhalothrin) and organophosphate pesticides (e.g. chlorpyrifos) and consequently, individuals containing higher loads of several chemicals represent the majority of prey biomass for this species (Fuller et al., 2021; Huff Hartz et al., 2021). There is increased risk of bioaccumulation and trophic transfer for pesticide-resistant invertebrates highlighted by a study confirming that permethrin-resistant *H. azteca* fed to fish can produce detectable concentrations of permethrin in fish tissues (Derby et al., 2021; Fuller et al., 2021; Muggelberg et al., 2017). Many of the chemicals we detected in surface water samples are known to affect the behavior of *D. magna*. Specifically, several classes of chemicals can cause reduced swimming speed and distance traveled in a concentration- and time-dependent manner, at concentrations relevant to those detected in our samples (Bownik et al., 2019; Bownik and Szabelak, 2021).

Conclusions

We demonstrated that response to light stimulus and average Total Distance moved are sensitive behavioral endpoints suitable for determining pesticide exposure effects, specifically for exposures performed at environmentally relevant concentrations. *Daphnia magna* response to light stimulus was the most sensitive endpoint measured for all treatments tested. In addition to IMI and CHL, we detected neonicotinoids, pyrethroid insecticides, organophosphates, carbamate insecticides and others from surface water collected from an agricultural region (Salinas, CA), before and after a "first flush" rain event. We detected strong dose-response patterns for all surface water samples and concentrations, with the highest concentrations of surface water showing the most divergent responses from controls. The site which produced the lowest mortality produced the greatest changes in behavior (Davis Rd.). The sublethal changes to behavior that we measured in our study suggest that this site is not appropriate for use as a "least impacted reference site," as has been previously reported. These findings highlight the importance of incorporating sublethal endpoints into toxicity assessments. Sublethal impacts can result in ecologically relevant effects on individual fitness, populations, and communities via bioaccumulation of pesticides. Swimming behavior is a sensitive endpoint to assess the effects of complex mixtures that may impact Freshwater ecosystems across multiple trophic levels.

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Chapter 4
Bioassessment in an impacted watershed: Environmental DNA metabarcoding reveals differences in aquatic community structure along a gradient of habitat quality

A similar version of this chapter is in preparation for submission to the journal ENVIRONMENTAL DNA, with the following co-authors:

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Abstract

Freshwater biodiversity is extremely vulnerable to anthropogenic impacts from climate change, habitat loss and pollution, and many species face rapid population declines and extinction. Biomonitoring can detect anthropogenic impacts in freshwater ecosystems by using indices of biological condition to generate a score for the habitat quality of a given site. Traditional bioassessment methods are limited by the patchy distribution of bioindicator groups, and low detection probabilities for rare species. The California Stream Condition Index (CSCI) is used to assess the integrity of freshwater habitat in California (USA). We measured biodiversity across the Salinas River watershed (CA), using environmental DNA (eDNA) metabarcoding, at sites with a wide range of CSCI scores. We detected sensitive bioindicator taxa (Ephemeroptera, Plecoptera, Trichoptera; EPT taxa), a rare species of conservation concern (Steelhead trout; *Oncorhynchus mykiss*), as well as invasive species (e.g., the New Zealand mud snail; *Potamopyrgus antipodarum*). We found significant overlap (> 76.67%) between benthic macroinvertebrate families identified morphologically that were also represented in sequences detected from the CO1 marker. Two genera were missing from the NCBI nucleotide sequence database, highlighting the importance of reference sequence database development. At the family level, we were able to identify a higher number of EPT taxa morphologically (29) than with sequencing (24), but at the genus level, sequencing (52) detected more diversity than morphology (32). Plecopterans were only detected at sites with CSCI > 0.79, and Trichopterans were only detected at sites CSCI > 0.63, with the majority of detections occurring in sites with the highest CSCI scores. We found high beta diversity between sites with differing CSCI scores for sensitive taxa, and alpha diversity was positively correlated with habitat quality. Sites in closest proximity to, or hydrologically influenced by, impacted sites (CSCI < 0.63) contained the

most divergent community composition as compared to least impacted reference sites (CSCI > 0.92). Hydrologic distance (waterbody) and CSCI score both accounted for dissimilarity in taxa between sites. Our analyses revealed greater dissimilarity when evaluated at the species level than at the family level. Our study supports the hypothesis that biotic indices to estimate habitat quality can be generated from eDNA metabarcoding data across a wide range of taxa. Metabarcoding of eDNA was more sensitive and effective for detecting macroinvertebrates than traditional net sampling when evaluated below taxonomic family level (genus and species). Bioassessments that incorporate eDNA metabarcoding can be effectively used for watershed-wide, multitrophic assessments of freshwater biodiversity to improve biomonitoring.

1. Introduction

Freshwater ecosystems are some of the most biodiverse, yet most endangered, habitats on the planet. While only occupying < 1% of Earth's total surface area, they support > 10% of all known species, including between 25-33% of all vertebrate species, and roughly 40% of global fish diversity (Dudgeon et al., 2006; Strayer and Dudgeon, 2010). Species inhabiting freshwater ecosystems are extremely vulnerable to anthropogenic impacts from climate change, habitat loss and pollution (Dudgeon et al., 2006; Reid et al., 2019; Stehle and Schulz, 2015; Tang et al., 2021). As a result, freshwater organisms are facing rapid population declines and extinction at nearly four times the rates of terrestrial organisms (Reid et al., 2019). According to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, freshwater vertebrate populations around the world declined by over 80% from 1970 to 2014, and as of 2020, nearly 30% of all assessed freshwater species were considered as threatened with extinction (Tickner et al., 2020). As biodiversity loss becomes an increasingly pressing concern, comprehensive monitoring efforts are needed to rapidly identify and conserve vulnerable taxa.

Many countries have established biomonitoring programs to detect anthropogenic impacts in freshwater ecosystems, yet implementing these programs effectively poses many challenges and limitations (Barbour et al., 1999; Buss et al., 2015; Smith et al., 1999). Benthic macroinvertebrate (BMI) assemblages are the most commonly used group for conducting bioassessments of freshwater habitat and water quality worldwide due to their taxonomic diversity, abundance, and responsiveness to stressors (Resh, 2008). The patchy distribution of BMI across temporal and spatial scales can result in an underestimation of species richness without the appropriate sampling design, however, and significant taxonomic expertise is required for morphological identifications (Lenat and Resh, 2001; Rehn et al., 2007). As a result,

many monitoring programs only perform taxonomic resolution to the level deemed necessary to satisfy the objective of a given study (Lenat and Resh, 2001). This approach saves time and resources but could also result in an underestimation of species richness, or arguably worse, grouping species with different stressor tolerances together and overestimating site condition or quality (Jones, 2008; Lenat and Resh, 2001). Monitoring programs that are focused on rare or endangered species face several additional challenges. Rare species can occur at low densities across a large area or may occur in high local abundance but in low occupancy across a landscape (Mackenzie and Royle, 2005; Piggott et al., 2020). These taxa may have lower sampling probabilities and detection rates when compared with more common species (Mackenzie and Royle, 2005). Obtaining species-level identifications for protected species can be difficult, often requiring additional permitting and at the risk of stressing or injuring the organism (Piggott et al., 2020). Sampling efforts are often constrained by practical considerations including the availability of expertise, funds, and time. As a result of these limitations, watershed-wide, multitrophic assessments of freshwater biodiversity are scarce (Mächler et al., 2014).

To characterize the ecological condition of waterways and quantify the severity of biological degradation in impacted sites, a biotic index "score" is calculated using biotic and abiotic data (Buss et al., 2015). The parameters used to calculate biotic index scores vary between indices, which are often developed for specific geographic regions (Buss et al., 2015). Multimetric indices use a combination of individual metrics that, together, represent a range of assemblage responses to human impact to generate a score value. Response factors can include taxonomic richness, percentage of sensitive taxa, and abiotic conditions (e.g., levels of dissolved oxygen, temperature, pH) (Buss et al., 2015; Karr, 1981; Resh, 2008). An example of a recently

developed multimetric bioassessment index is the California Stream Condition Index (CSCI), which was developed to identify reference benchmark conditions of biological diversity for the range of natural environmental conditions found throughout California, USA (Mazor et al., 2016, 2010). The CSCI employs a combination of two indices of biological condition (a ratio of observed-to-expected taxa and a multimetric index) into a single index to ensure accuracy across the heterogeneous environment of California, USA (Mazor et al., 2016). The CSCI categorizes site quality as: streams that are likely to be "intact," i.e., undisturbed (CSCI $\geq$ 0.92), possibly altered (CSCI $\geq$ 0.79), likely to be altered (CSCI $\geq$ 0.63), and very likely to be altered (CSCI < 0.63). CSCI and many other indices rely on BMI for score calculations, and as such are subject to the limitations of BMI detection and identification listed above.

Genetic techniques can greatly enhance traditional biodiversity monitoring, increasing the coverage of species presence-absence data by providing a rapid assessment of a wide range of biodiversity to understand community condition (Deiner et al., 2020, 2017; Ficetola et al., 2010; Hajibabaei et al., 2011; Mächler et al., 2014; Taberlet et al., 2012). Using genetic-based approaches, taxa are detected from the DNA they shed into their environment. In the process of environmental DNA (eDNA) metabarcoding, specific fragments of DNA are targeted which contain sufficient variation to differentiate between closely related species, while being highly conserved/present in a wide range of taxa (Taberlet et al., 2012). To capture a broad representation of taxonomic diversity, a multilocus metabarcoding approach targeting standard markers for animals and plants (e.g., CO1, 12S, 18S, and ITS) can be used (Curd et al., 2019; Meyer et al., 2021). This technique has been shown to improve identification of cryptic species, juvenile life stages, and rare taxa (Mächler et al., 2014). Metabarcoding provides baseline data across a wide range of taxa, which is crucial for understanding and sustaining biodiversity. In

anthropogenically impacted systems, the detection of sensitive biomonitoring species also provides important habitat quality data and information on how surrounding land use might influence aquatic community composition. While extensive research has demonstrated the utility of eDNA for providing presence-absence data on invasive or endangered species, few studies have applied this technique to landscape-wide assessments of beta diversity (e.g., Altermatt, 2013; Bush et al., 2020).

The Salinas River watershed is the largest riparian corridor for California's Central Coast, providing riparian habitat crucial for several species of concern, such as the red-legged frog (*Rana aurora draytonii*) and the least Bell's vireo (*Vireo bellii pusillus*) (Clemow et al., 2018; Howell et al., 2010). The river and its tributaries act as a migration corridor for the southern steelhead trout (*Oncorhynchus mykiss*) and have been designated by the National Marine Fisheries Service as critical habitat for steelhead (Anderson et al., 2003). The river ultimately empties into both an estuarine National Wildlife Refuge and a National Marine Sanctuary. Several threatened and endangered species rely on the Salinas River for reproduction, food and habitat including steelhead (*Oncorhynchus mykiss*), bald eagles (*Haliaeetus leucocephalus*), and Smith's blue butterfly (*Euphilotes enoptes smithi*) (Croll et al., 1986; River, 2002). The river supplies irrigation water to over 200,000 acres of highly productive agricultural land including some of the most intensively farmed land in the United States (Goh et al., 2019; Hunt et al., 2003). Urban and agricultural runoff impacts water quality in the watershed (Anderson et al., 2003; Hunt et al., 2003; Kuivila et al., 2012), resulting in frequent detections of chemicals of concern at levels that are toxic to sensitive organisms (Anderson et al., 2006; Anderson et al., 2003; Deng et al., 2019). As a result, the Salinas River was placed on the US federal Clean Water Act 303(d) list of impaired water bodies (Hunt et al., 2003). The California

Department of Pesticide Regulation (CDPR) and the California State Water Resources Control Board (CSWRCB) under their Surface Water Ambient Monitoring Program (SWAMP) has conducted bioassessments at sites throughout the Salinas River watershed, and chemical monitoring of sites at high-risk locations based on reported pesticide use, detections from previous monitoring, previous detections determined to be out-of-compliance with water quality levels, and proximity to ecologically sensitive areas (Deng et al., 2019; Goh et al., 2019; Sandstrom et al., 2021). Understanding connectivity and exchange across watersheds has far-reaching implications for functional, community, and genetic structure of lotic ecosystems (Altermatt, 2013).

In this study, we sought to understand if and how biodiversity may vary across the Salinas River watershed, and whether beta diversity correlates with habitat quality as determined by CSCI score. We hypothesized that 1) the watershed contains high beta diversity, and that species richness will be positively correlated with water quality, and 2) sites that are in closest proximity to, or hydrologically influenced by, known impacted sites will show the lowest overall numbers of taxa, lowest CSCI score, and the most divergent community composition (as compared to least impacted reference sites). Our objectives were to 1) characterize community composition at sites throughout the Salinas River watershed, across a range of habitat qualities and surrounding land uses, and 2) to identify sites representing sources of regional biodiversity.

2. Methods

2.1 Sampling

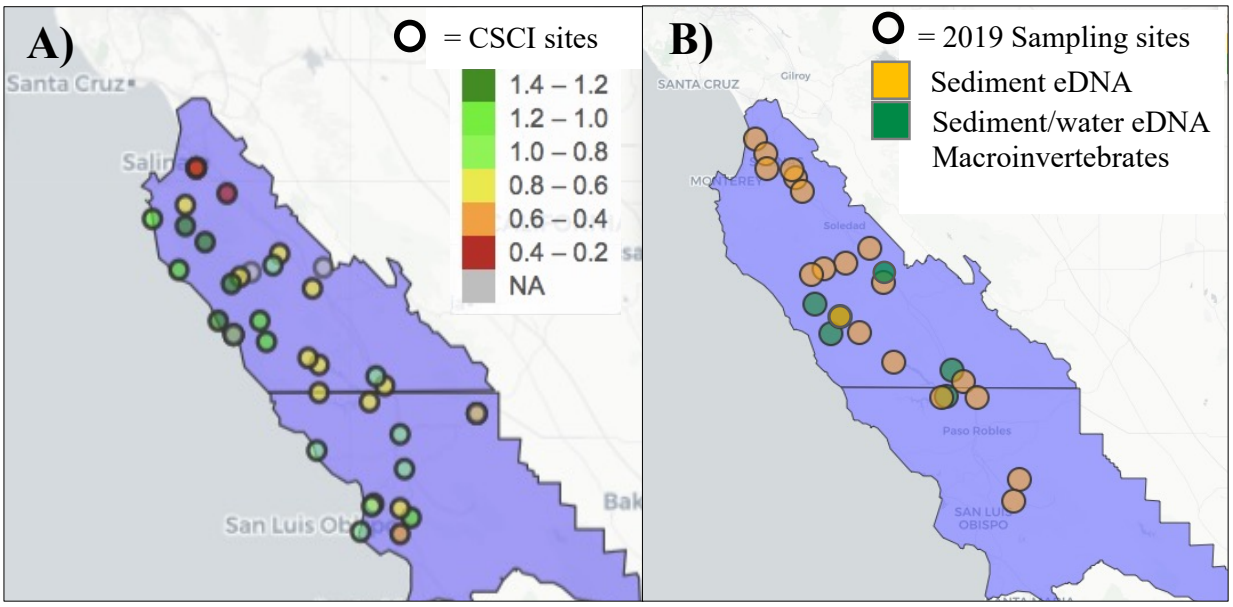

Figure 4.1. Maps of Monterey and San Luis Obispo counties in the Central Coast Region of California (USA). A) Historic sampling locations from SWAMP biomonitoring assessments, color coded by California Stream Condition Index (CSCI), a habitat quality index designed specifically for aquatic macroinvertebrate diversity of wadable rivers and streams in California. B) The 22 eDNA biomonitoring sampling sites were chosen to overlap with historic sampling sites, in order to represent a gradient of habitat quality. Least impacted reference sites where additional sampling was performed are shown in green.

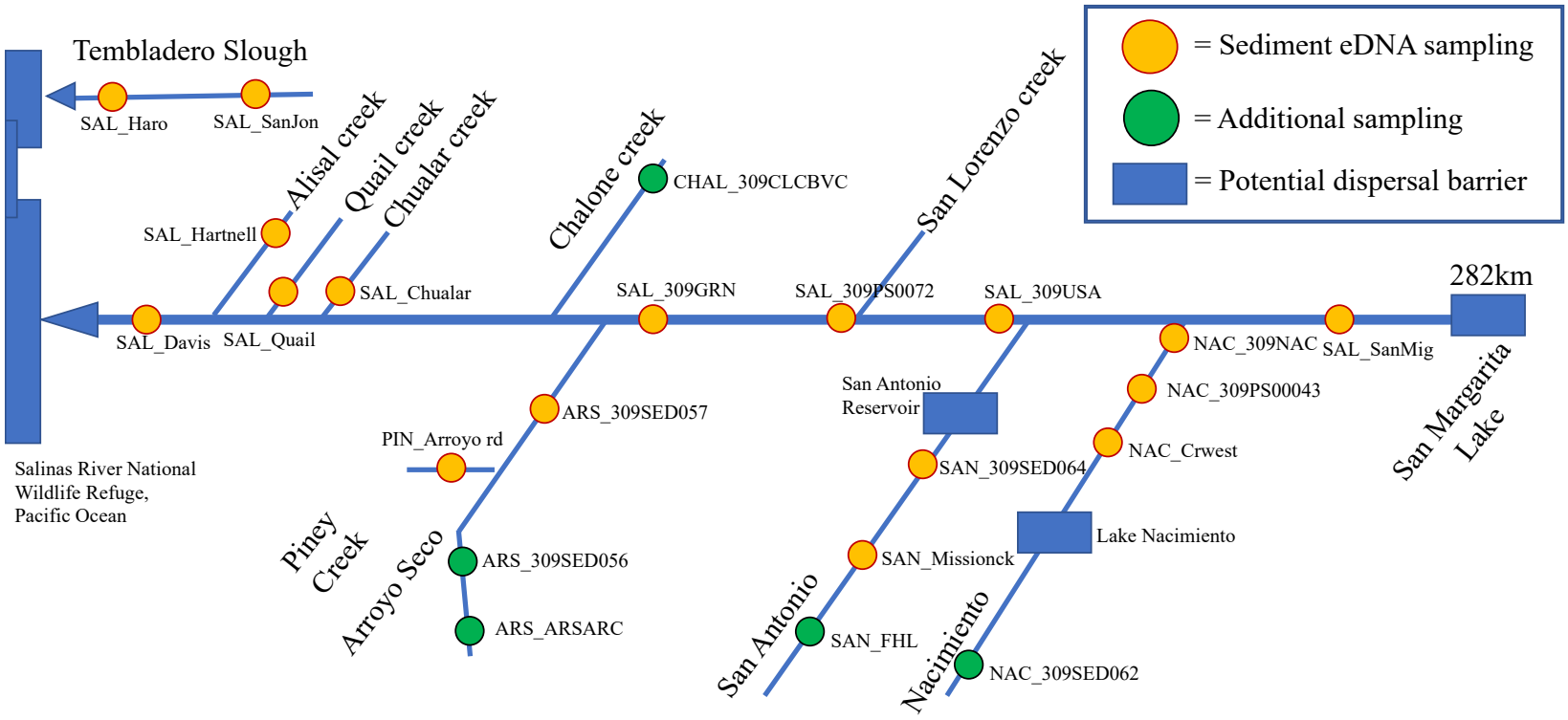

Figure 4.2. Linearized map (not shown to scale) of sampling sites located within the Salinas River watershed in the Central Coast Region of California (USA). The 22 sampling sites used in this study were chosen to overlap with previous bioassessment sampling sites within the Salinas River and major tributaries. Least impacted reference sites where additional sampling was performed are shown in green.

2.1.1 Sites

We first accessed the SWAMP bioassessment database (https://www.waterboards.ca.gov/water_issues/programs/swamp/bioassessment/csci_scores_map.html) to identify all bioassessment sites with CSCI scores located in the Central Coast region of California, sampled between 1994-2018 (n = 499), then selected only those sites located in the Salinas River and surrounding tributaries. We chose these sites to represent a range of stream order, hydrologic connectivity, habitat and water. The CSCI categorizes site quality as: streams that are likely to be "intact," i.e., undisturbed (CSCI ≥ 0.92), possibly altered (CSCI ≥ 0.79), likely to be altered (CSCI ≥ 0.63), and very likely to be altered (CSCI < 0.63). We selected sites located in wadable streams and rivers within the Salinas River watershed, resulting in a total of 22 sites (Figure 4.1, Table 4.1), including the mainstem of the Salinas River and all major tributaries (Nacimiento, San Antonio, Arroyo Seco, Chalone Creek). Hydrological connectivity between sites is shown as a linearized map in Figure 4.2. Downstream sites included the Tembladero Slough (TEM) which parallels the furthest downstream reach of the Salinas River and historically supported wetland habitat but has been channelized to receive agricultural and urban runoff (Anderson et al., 2018). In addition to the main tributaries, we also sampled a higher order stream that feeds the Arroyo Seco (ARS), Piney Creek (PIN). Several long-term chemical monitoring sites used by the CA Department of Pesticide Regulation were also included in the study (categorized as SAL_DPR) where previous detections of chemicals of concern and high invertebrate mortality were measured in several studies (Deng et al., 2019). Three of these sites did not have previous CSCI scores but are expected to score very poorly, based on their established history of invertebrate toxicity and the CSCI scores obtained from

adjacent sites (all CSCI < 0.63). As a result, we ranked these sites with other sites that are very likely to be altered (CSCI < 0.63). All sites are located in Monterey and San Luis Obispo counties and span the length of the Salinas River (175 miles). Sites within the Salinas River watershed that are located downstream from urban or agricultural land use are known to be affected by multiple stressors including water diversion, habitat loss due to channelization and land use, as well as influxes of effluent and pesticides (Anderson et al., 2018; Anderson et al., 2003; Deng et al., 2019; Goh et al., 2019; Hunt et al., 2003). We recorded physicochemical data (water chemistry, substrate type, depth and flow rate), habitat type, flow, depth, turbidity and GPS location for each site. To minimize the risk of contaminating our eDNA samples with DNA from kick-net sampling, we conducted all sampling from downstream to upstream in the order listed in Sections 2.2.2 – 2.2.4.

Table 4.1. *Sampling location data. Date sampled, site ID, abbreviation, waterbody, description, site type, latitude and longitude for all sampling locations.*

date sampled	site ID	abbreviation	waterbody	description	site type	lat	lon
4/29/19	NAC_309SED062	NAC062	NAC	Nacimiento Creek - Below campground 62	Reference/ SWAMP Bioassessment site	36.00339	-121.39141
4/30/19	ARS_309ARSARC	ARSARC	ARS	Arroyo Seco ~0.3mi above Rosevelt Cr.	Reference/ SWAMP Bioassessment site	36.1198069	-121.46888
5/13/19	DPR_SAL_Haro (309TEH)	SALHARO	TEM	Tembladero Slough at Haro Street	DPR monitoring site	36.7596	-121.75433
5/13/19	DPR_SAL_SanJon (309JON)	SALSANJ	SAL	Rec Ditch at San Jon Road	DPR monitoring site	36.7049	-121.70506
5/13/19	DPR_SAL_Quail (309QUI)	SALQUAIL	SAL	Quail Creek at HWY 101, btwn Spence and Potter Roads	DPR monitoring site/ SWAMP Bioassessment site	36.6092	-121.56269
5/13/19	DPR_SAL_Chualar	SALCHU	SAL	Chualar Creek at Chualar River Rd., near 309SAC	DPR monitoring site/ SWAMP Bioassessment site	36.5584	-121.52964
5/13/19	DPR_SAL_Hartnell	SALHART	SAL	Alisal Creek at Hartnell Rd	DPR monitoring site	36.6435	-121.57836
5/13/19	DPR_SAL_Davis (309DAVxxx)	SALDAVIS	SAL	Salinas River at Davis Road	DPR monitoring site/ SWAMP Bioassessment site	36.646449	-121.7018
5/22/19	Chal_309CLCBVC	CHAL	CHAL	Chalone Creek @ Old Pinnacles/Balconies Cave trail near 309CLCBVC	SWAMP Bioassessment site	36.496887	-121.17566
5/27/19	SAL_SanMig	SALSANMG	SAL	Salinas @ San Miguel, CA. between Estrella and Nacimiento	SWAMP Bioassessment site	35.753227	-120.68864

5/27/19	SAL_309USA	SALBRAD	SAL	Salinas @ Bradley, CA. downstream of San Antonio	SWAMP Bioassessment site	35.864222	-120.80946
5/27/19	SAL_309PS0072	SALSANLO	SAL	Salinas @ King City, CA. near 309PS0072	SWAMP Bioassessment site	36.202728	-121.14296
5/27/19	SAL_309GRN	SALGRN	SAL	Salinas @ Greenfield, CA. near 309PS0040	SWAMP Bioassessment site	36.338144	-121.20479
6/6/19	NAC_309PS00043	NAC043	NAC	Nacimiento @ River Rd. in Camp Roberts	SWAMP Bioassessment site	35.758927	-120.8399
6/6/19	NAC_Crwest	CAMPROB2	NAC	Nacimiento @ Camp Roberts west boundary	New Site	35.756291	-120.85885
6/6/19	NAC_309NAC	309NAC	NAC	Nacimiento @ Hwy 101 in Camp Roberts	SWAMP Bioassessment site	35.819827	-120.75698
6/7/19	SAN_FHL	SANFHL	SAN	San Antonio River @ Fort Hunter Liggett	New Site	36.069947	-121.34687
6/7/19	SAN_MissionCk/ 309CAW194	FHL2	SAN	San Antonio River @ Mission Creek	SWAMP Bioassessment site	36.0104	-121.25389
6/7/19	SAN_309SED064	FHL064	SAN	San Antonio River @ Interlake Rd	SWAMP Bioassessment site	35.89444	-121.09051
6/13/19	ARS_309SED057	SALELM	ARS	Arroyo Seco @ Elm St Bridge	SWAMP Bioassessment site	36.280487	-121.32257
6/13/19	PIN_ArroyoRd	PINCRK	PIN	Piney Creek @ Arroyo Seco Rd	New Site	36.257464	-121.4268
6/14/19	ARS_309SED056	ARS56	ARS	Arroyo Seco River @ upstream of Day Use area	SWAMP Bioassessment site	36.22549	-121.48767

2.1.2 Environmental DNA Sampling

The abundance and diversity of groups known to be sensitive to poor habitat quality tend to be greater in shallow, oxygenated riffle habitat (Merritt and Cummins, 2008; Rehn et al., 2007; Resh, 2008). To maximize the potential for detecting DNA from sensitive macroinvertebrates, we selected transects immediately downstream from riffle habitats, where eDNA may accumulate (described in Section 2.1.4). To target contemporary biodiversity, we looked for areas with recently deposited sediment and gently scraped the top (< 2 cm) layer into collection tubes. Based on pilot analysis, we determined that 10 x 5 mL subsamples of sediment were an appropriate sampling depth for detecting common benthic invertebrates from Salinas River watershed sites (Figure S4.1). We collected 10 subsamples of sediment into sterile 50 mL falcon tubes from all 22 sites. For each site, we collected sediment across 3 transects spaced > 1 m apart. We combined 10 subsamples into each 50 mL falcon tube and homogenized them by gently inverting the tube. For two of our sampling sites that are classified as undisturbed based on CSCI sores (≥ 0.92) from previous SWAMP assessments, we also collected water filtrate samples. Water samples were filtered onsite using a custom portable peristaltic pump system. Following established protocols, we collected three replicate water (1 L) samples per location into sterile plastic bags (Nasco Whirl-Pak B01447WA Sample Bag, 5441-mL Capacity) for environmental DNA filtration (Miya et al., 2016). We filtered 1 L of sample water from three sites, at three transects per site (total volume = 3 L) using Sterivex HV 0.45 μm filters (EMD Millipore Corp., Burlington, MA USA). For water sample field blanks, we transported molecular grade water to field sites in sterile falcon tubes and filtered them in the field alongside our water samples. For sediment field blanks, we transported molecular grade water to field sites in sterile falcon tubes and then used this in place of sediment samples in our lab extractions, alongside

sediment samples. For extraction blanks, we replaced the environmental sample type with molecular grade water.

All collection and sampling equipment were sterilized between sites, and sampling at each site was conducted from downstream to upstream to avoid cross-contamination. Immediately after collection, all eDNA samples were placed on dry ice (-60 °C) for transport back to the laboratory (UC Davis, Davis CA). Upon arrival, we transferred all samples into -80 °C freezers. This was done to reduce degradation of DNA and preserve rare sequences for analysis.

2.1.3 Physicochemical Parameters

We measured water quality parameters *in situ*, including ambient water temperature, pH, specific conductance, total dissolved solids, turbidity, and dissolved oxygen (using a YSI multiparameter sonde; Yellow Springs, OH, USA), flow rate (m/s) (Hanna Instruments Multiparameter System model 9829; Woonsocket, RI, USA) and depth.

2.1.4 Kick-net Sampling

For a subset of our sampling sites with CSCI scores > 0.92, we collected BMI following modified SWAMP protocols (Ode et al., 2016). Briefly, we chose three transects per site based on the location of suitable riffle habitat within the reach. Starting with the downstream transect we identified three cross-reach points: a point that is 25% of the stream width from the left bank. We then placed a 500-µm D-frame net into the water, and visually defined a 1-foot square (0.09 m^2) sampling plot on the stream bottom upstream of the net opening. We then vigorously disturbed the substrate within the sampling plot by kicking and dislodging loose sediment at a depth of 5 cm with a rubber boot for 60 seconds. If rocks or other objects larger than 4.0 cm^3

occurred in the sampling plot, we scrubbed them by hand to collect any organisms that might still be attached. After sampling, we removed the D-frame net from the water column and transferred all collected organisms into sample jars containing > 90% EtOH. This sampling approach was repeated for points located 50% and 75% from the left bank, and then repeated across the remaining two transects for a total of 9 kicks per site. We conducted kick-net sampling following the collection of all eDNA samples and recorded physicochemical parameters, so as to prevent potential sediment disturbance or contamination.

2.2 Morphological Assessments

To "ground truth" our sequence data and compare species detections obtained across methods, we conducted morphological identifications of BMI for two sites located upstream in major tributaries to the Salinas River. We selected sites expected to contain high biodiversity, located upstream in the two largest tributaries for the Salinas River (San Antonio River; SANFHL1, Nacimiento River; 309SED062), hereafter referred to as our ground truthing sites. These sites are surrounded by undeveloped land and were not expected to be impacted by poor water quality, based on previous SWAMP assessments and field observations (Deng et al., 2019). For these reasons, as well as due to the physical characteristics of each site (flow, depth, temperature, percent embeddedness) we expected to find a high diversity of BMI taxa. We first sorted specimens to order, then individuals within each order were identified to the lowest possible taxonomic level under a dissecting microscope using dichotomous keys (Merritt and Cummins, 2008; Stewart and Stark, 1988; Wiggins, 1996). To ensure the identification of rare taxa, we sorted and identified all specimens collected from these sites, rather than a random

subset containing up to 500 individuals which is typical of traditional bioassessments (Ode et al., 2016).

For our ground truthing sites, we evaluated the overlap between morphological identification and taxonomy assigned from eDNA-derived sequences. To do this, we searched the Taxon Tables generated from the eDNA bioinformatics pipeline for family, genus and species names of morphologically ID'd specimens from the same sites (Section 2.7). To account for differences in spelling or misidentification of taxonomic level (i.e., suborder listed as family) which may occur in NCBI data, we sorted our Taxon Tables alphabetically, searched for only the preface (e.g., "Epheme*" for Ephemeroptera), and visually checked the list to ensure that all taxa within that group were accounted for. We then calculated the overlap as the percentage of taxa found morphologically that were not represented in Taxon Tables generated from sequence data.

2.3 Environmental DNA Method Optimization

To ensure that our primers (see Section 2.4) would effectively target California invertebrate taxa, we conducted pilot assessments. First, we collected invertebrates from freshwater habitats near Davis, CA, preserved them in > 90% EtOH, then identified them to the lowest practical taxonomic level using dichotomous keys (Merritt and Cummins, 2008). Specimens included common invertebrate taxa known to be present at our sampling sites (Amphipoda, Anisoptera, Astracoidea, Chironomidae, Corixidae, Dytiscidae, Ephemeroptera). We extracted DNA from all tissue samples (methods outlined in Section 2.4), then conducted standard PCR using CO1 primers (methods outlined in Section 2.5). We then visualized amplification products on a 1.5% agarose gel to confirm target amplicon presence and size.

To determine the appropriate sediment eDNA subsampling depth and to optimize our laboratory extraction/amplification methods (see Sections 2.4 – 2.5), we collected 16 replicate

samples (Section 2.1.2) from one transect located in the least impacted site of our study, (site id 309SED062; Nacimiento River), following collection protocols outlined in Section 2.1.3. We extracted DNA from sediment samples following methods outlined in Section 2.4 then conducted an initial library preparation and sequencing (following methods in Sections 2.4 – 2.6). We used the results from this initial sequence run to inform our main study.

Previous studies have debated whether water filtrate or sediment yield more reliable estimates of biodiversity (Sakata et al., 2020; Turner et al., 2015). To compare detections obtained from sediment with water filtrate, we conducted additional sampling from a subset of sites used in our main study. We collected and filtered 3 L of sample water on site using Sterivex HV 0.45 μm filters (EMD Millipore Corp., Burlington, MA USA). We extracted DNA from water filters following methods outlined in Section 2.4 then included these samples in our final library preparation and sequencing run (following methods in Sections 2.4 – 2.6).

2.4 DNA Extraction

To extract DNA from tissue samples, we first isolated tissue (a single leg or a whole organism, depending on specimen size) then homogenized the samples with a Qiagen TissueLyser LT (Qiagen Inc., Germantown, MD). We used the QIAGEN Dneasy kit (Qiagen Inc., Germantown, MD) and protocols, then stored extracted DNA at -20 °C in 2.0 mL LoBind tubes (#86-922; Genesee Scientific, San Diego CA). To extract organismal DNA from sediment, we followed a modified QIAGEN PowerSoil Pro (Qiagen Inc., Germantown, MD) protocol (Sx). In brief, we defrosted sediment samples on ice, then removed excess water and large pieces of inorganic material by centrifugation (15,000 x g for 1 min). We then transferred 10 subsamples (250 mg each) into QIAGEN PowerSoil Pro Powerbead Pro tubes. Next, we followed the QIAGEN PowerSoil Pro protocol with the addition of a phenol:chloroform:isoamyl alcohol step

to remove PCR inhibitors, and an additional ethanol-based solution rinse step to remove additional residual salt, humic acid and other contaminants. To extract DNA from water filtrate captured on Sterivex filters, we used a modified QIAGEN Dneasy protocol following Miya et al. (2016) and Spens et al. (2017). To promote the lysis of cell membranes, denaturation of proteins and other macromolecules, we increased the amount of proteinase-K solution (40 µl) and buffer AL (400 µl) used, pipetted directly into the Sterivex filter cartridge to reduce sample contamination risk, then capped and incubated the filters for 12 h at 56 °C in a rotary incubator to maximize the total amount of DNA extracted. To collect the filtrate, we pipetted 400 µl of molecular grade ethanol into the Sterivex filter housing, vortexed, then centrifuged at 5,000 x g for 1 min into a LoBind 2.0 mL collection tube. The rest of the extraction process then followed QIAGEN DNeasy protocols. We stored all extracted DNA samples in 1.5 mL DNA/RNA LoBind Tubes at -80 °C until library preparation. To confirm DNA quality, we ran 1.5% w/v agarose gels and quantified all DNA extractions using a Qubit 4 with a HS DNA Kit (Thermofisher Scientific, Carlsbad, CA, USA).

2.5 HTS Library Preparation

For the preparation of our HTS libraries, we followed protocols established by the University of California Conservation Genomics Consortium for their eDNA monitoring program, the CAL eDNA Project (https://ucedna.com/methods-for-researchers), as outlined in Meyer et al. (2021). In brief, we performed initial PCR reactions using primers designed to target taxonomically informative regions of DNA. We obtained primers from Integrated DNA Technologies (San Diego CA USA). To capture a wide range of taxonomy, we used well-established primers targeting the mitochondrial CO1 (Leray et al., 2013) described in Table 4.1. For each barcode region we amplified three technical replicates to reduce reaction bias. We

checked PCR products on a 1.5% w/v agarose gel, then pooled the three technical replicates of each marker. Next, we performed a bead purification (0.8x, Magbio Genomics, Inc., Gaithersburg, MD USA) to remove primer dimers and dNTPs. We quantified PCR products using a Qubit 4. These quantifications were then used to pool an equal number of copies of each marker by sample/site. Once pooled, we performed a second PCR to add dual indices to identify sequences from each sample/site. After running another confirmation gel, we performed a final bead purification and pooled our cleaned, indexed PCR products for sequencing. The UC Davis Genome Center checked DNA quality using a Bioanalyzer to verify the amplicon size and that there were no contaminating adapter-dimers, and then performed an additional bead purification prior to sequencing. All PCRs included a negative control (no template control) which used molecular grade nuclease-free water in place of DNA.

Table 4.1. *Primer names and sequences (5' to 3') for metabarcoding.*

Name	Primer Sequence (5' to 3')	Source
COI_F_mlCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC	Leray *et al*. 2013
CO1_R_jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA	Leray *et al.* 2013

2.6 Sequencing

All sequencing was carried out by the DNA Technologies and Expression Analysis Cores at the UC Davis Genome Center using an Illumina MiSeq that generates paired reads 2 x 300 base pairs to produce paired end sequences up to 600 base pairs. We aimed to sequence a minimum of 25,000 paired reads for each CO1 barcoding region for each sample. PhiX controls were spiked into the run, representing ~2.1% of final reads. An initial sequencing run was completed to generate pilot data and evaluate the appropriate sequencing depth to detect rare taxa. This was done by extracting and sequencing 16 subsamples of sediment collected from a

single location, following the protocols outlined in Sections 2.2 - 2.4 above. We chose a site predicted to contain a higher taxonomic diversity and abundance, based on California Stream Condition Index (CSCI) scores from previous habitat assessments performed by SWAMP. The site (309SED062) is located in the upstream reach of the Nacimiento River and is used as a least-impacted reference site by SWAMP. Sequencing resulted in >15.6 M reads passing quality filters, with > 71.4% above quality thresholds (Q30). Quality Scores (Q) for individual base pairs obtained from raw reads followed normal patterns of distribution (Figure S4.2). Sequencing quality scores measure the probability that a base is called incorrectly. The sequencing quality score of a given base, Q, is defined by the following equation: $Q = -10\log_{10}(e)$, where e is the estimated probability of the base call being wrong. As read length increases, it is typical for the quality score to decrease, with lowest quality reads located at the last cycles (approaching 300). We removed all quality scores below Q30 (99.9% accuracy) during filtration steps.

2.7 Bioinformatics

To create custom primer-specific reference databases, we used CRUX (Creating Reference libraries Using eXisting tools) (Curd et al., 2019; Meyer et al., 2021). CRUX runs *in silico* PCR using ecoPCR (Ficetola et al., 2010), generates an EMBL seed library using Obitools (Boyer et al., 2016), then runs the Basic Local Alignment Search Tool (BLAST; NCBI) using blastn. Lastly, CRUX assigns taxonomy in Qiime (Caporaso et al., 2010) to create a Bowtie2 database which is then used in downstream analyses. We used the *Anacapa Toolkit* which is specifically designed to determine community composition from multilocus datasets (Curd et al., 2019). We processed sequence data using the default parameters and assigned taxonomy for *Anacapa Toolkit* using the custom reference databases for each primer pair (https://ucedna.com/reference-databases-for-metabarcoding) that were created using CRUX. The

Anacapa Toolkit performs quality control of raw sequences using Cutadapt (Martin, 2011) and FastX-Toolkit (Gordon and Hannon, 2010) and makes inferences of Amplicon Sequence Variants (ASVs) with DADA2 (Callahan et al., 2016). We matched sequence data to taxonomy for each ASV using Bowtie2 (Langmead and Salzberg, 2012) and the Bayesian Lowest Common Ancestor algorithm (BLCA; Gao et al., 2017).Taxonomy assignments with a bootstrap confidence cutoff score over 0.6 were kept for each ASV. The Illumina raw sequence data and all scripts used for analyses will be made available on Dryad and GitHub, and datasets will be made publicly available on Dryad.

2.8 Analysis

To determine community composition, we used the *Anacapa Toolkit* to conduct quality control, ASV parsing, and taxonomic assignment using user-generated custom reference databases (Curd et al., 2019). To confirm whether our sampling depth was sufficient to fully capture BMI community diversity, we created species rarefaction curves using TaxonTableTools (Macher et al., 2021). To evaluate how taxonomic level influenced variation in alpha diversity for each site across a range of CSCI scores, we visualized boxplots at the ASV, family and species levels. We then repeated this for each site across each waterbody to evaluate how hydrologic connectivity influenced overall alpha diversity at each taxonomic level. Next, we compared BMI taxa detected in sites with CSCI scores ranging from 0.2-1.4 to identify shared and unique BMI taxa. To do this, we generated beta diversity heat maps and visualized dissimilarity using Principal Coordinates Analysis (PCoA) multidimensional scaling plots in TaxonTableTools. We generated PCoA plots to evaluate the taxonomic distribution between waterbodies (ARS=Arroyo Seco, CHAL=Chalone Creek, NAC=Nacimiento Rivers, SAL=Salinas, SAL_DPR=Salinas DPR monitoring sites, SAN=San Antonio, TEM=Tembladero

Slough), and the taxonomic distribution between CSCI score categories (undisturbed; CSCI ≥ 0.92, possibly altered; CSCI ≥ 0.79, likely to be altered; CSCI ≥ 0.63, and very likely to be altered; CSCI < 0.63). We also determined the most abundant sequences from each site (identified to family level) by sorting Taxon Tables in the command line. Statistical significance of dissimilarity (ANOSIM) and visualization (PCoA, Venn diagrams) were performed using RStudio (RStudio Team 2020; Version 1.3.1073), rANACAPA (Kandlikar et al., 2018) and TaxonTableTools.

3. Results

3.1 Method Optimization

3.1.1 CO1 Primer Bias Evaluation

The CO1 primers used in our study are well-established and are frequently applied to freshwater assessments (Leray et al., 2013). Primer choice plays an important role in downstream analysis, however, and omission of any sensitive BMI taxa due to primer bias could potentially affect estimates of habitat quality. We therefore sought to evaluate any primer bias for major BMI taxonomic groups, and invertebrates known to occur in our study area. In our assessment of primer suitability, we successfully amplified a wide range of invertebrate taxa. PCR products were ~450 bp mitochondrial CO1 amplicons from all tissue-derived and environmental DNA in our pilot study (Figure S4.3).

3.1.2 Sampling and Sequencing Depth

To determine the appropriate sampling and sequencing depth needed to discover rare taxa, we performed an initial library preparation and sequencing of 16 sediment eDNA

subsamples, obtained from a single site, and results from these pilot data are reported in Figures S4.1 – 4.4. Our initial sequencing run detected common taxa (e.g., Chironomidae) in all subsamples. For this site, we counted 302 Chironomidae individuals from morphological identifications. Some sensitive benthic macroinvertebrate taxa (e.g., Amelitidae, Ephemerellidae and Heptageniidae) were only detected in two out of 16 subsamples. In our kick-net samples, we identified an average of ~1 individual per kick from the Amelitidae family. Based on these findings, we selected a sampling depth of 10 subsamples per site for the full set of samples. This approach increased our chances for detecting rare taxa while adhering to practical constraints of sample number per sequencing run.

Species richness (measured as number of unique Amplicon Sequence Variants) rarefaction curves from our initial sequencing run suggested that the minimum sequencing depth at which most rare taxa would be detected was ~30,000 reads per sample for most samples (Figure S4.4). One sample contained a higher diversity of reads, however, and all rare taxa were not discovered despite the high number of total reads (> 110,000) that were assessed/allocated, based on the slope of the rarefaction curve. The slope of the rarefaction curve models the probability that each additional sampling event will result in new species detections. As the slope approaches zero, the likelihood of discovering new taxa diminishes. For a single subsample, the rarefaction curve did not plateau after > 110,000 reads, so we can assume that rare taxa are present but were not detected in this sample.

3.1.3 Sample Type

We compared family-level detections between sample types (10 x 5 g sediment versus 3 L water filtrate) for the subset of sites predicted to be undisturbed or slightly altered (CSCI > 0.79), and found that while there was overlap in detections, many taxa were only identified from

one sample type (Figure S4.5). We detected an overall higher number of taxa in water filtrate, with more vertebrate (fish) detections than in sediment. We identified a higher diversity of BMI in sediment than water filtrate. Across all samples, BMI represented a higher percentage of total reads from sediment than from water filtrate.

3.1.4 Morphological Assessments of BMI

A summary of BMI taxa identified from our ground truthing sites from morphological identification are shown in Table 4.2. From the San Antonio River site, we sorted and identified a total of 1861 macroinvertebrates, of which 1423 were from the three orders most commonly used in biotic indices (Ephermeroptera, Plecoptera, Trichoptera; abbreviated as EPT). We identified 1179 Ephemeropteran, 52 Plecopteran, and 192 Trichopteran individuals representing a total of 34 unique genera and species within 13 families. From the Nacimiento River, we sorted and identified 1003 individual macroinvertebrates, including 650 EPT individuals. Individuals represented 32 unique genera and species, within 14 unique families. Across both sites, we detected a total of 29 EPT families including 33 genera from morphological identification.

We then searched the Taxon Table generated from our sequence data from these two sites to compare the percent overlap of family-level taxonomy between the morphological and sequence approaches. From the San Antonio River site, 76.67% of BMI families identified morphologically were also represented in sequences detected from the CO1 marker. For the Nacimiento River site, 88.68% of BMI families identified morphologically were also represented in sequences detected from the CO1 marker.

***Table 4.2**. Taxonomy obtained from DNA sequence data and morphological identification for ground truthing sites in the San Antonio and Nacimiento Rivers expected to be highly biodiverse in comparison to other sites. Species for which detections may be representative of incorrect taxonomy are indicated with an asterisk (*).*

DNA				Morphology		
Order	**Family**	***Genus***	***Species***	**Order**	**Family**	***Genus***
Ephemeroptera	Ameletidae	*Ameletus*	*Ameletus amador*	Ephemeroptera	Ameletidae	*Ameletus*
Ephemeroptera	Ameletidae	*Ameletus*	*Ameletus andersoni*	Ephemeroptera	Baetidae	*Baetis*
Ephemeroptera	Baetidae	*Baetis*	*Baetis tricaudatus*	Ephemeroptera	Baetidae	*Baetis*
Ephemeroptera	Baetidae	*Baetodes*	*Baetodes sp. gmycM19*	Ephemeroptera	Baetidae	*Diphetor*
Ephemeroptera	Baetidae	*Callibaetis*	*Callibaetis ferrugineus*	Ephemeroptera	Baetidae	*Procleon*
Ephemeroptera	Baetidae	*Fallceon*	*Fallceon sp. BOLD:AAL8084*	Ephemeroptera	Baetidae	
Ephemeroptera	Baetidae	*Procloeon*	*Procloeon fragile*	Ephemeroptera	Ephemerelidae	*Serratella*
Ephemeroptera	Dipteromimidae*	*Dipteromimus**	*Dipteromimus tipuliformis**	Ephemeroptera	Ephemerellidae	*Drunella*
Ephemeroptera	Ephemerellidae	*Drunella*	*Drunella flavilinea*	Ephemeroptera	Ephemerellidae	*Ephemerella*
Ephemeroptera	Ephemerellidae	*Ephemerella*	*Ephemerella dorothea*	Ephemeroptera	Ephemerellidae	*Serratella*
Ephemeroptera	Ephemerellidae	*Serratella*	*Serratella micheneri*	Ephemeroptera	Ephemerellidae	
Ephemeroptera	Heptageniidae	*Notonurus*	*Notonurus matitensis**	Ephemeroptera	Heptagenidae	
Ephemeroptera	Heptageniidae	*Rhithrogena*	*Rhithrogena nuragica*	Ephemeroptera	Heptageniidae	*Cinygmula*
Ephemeroptera	Leptophlebiidae	*Paraleptophlebia*	*Paraleptophlebia debilis*	Ephemeroptera	Heptageniidae	*Ecdyonurus*
Plecoptera	Capniidae	*Capnura*	*Capnura manitoba*	Ephemeroptera	Heptageniidae	*Epeorus*
Plecoptera	Capniidae	*Eucapnopsis*	*Eucapnopsis brevicauda*	Ephemeroptera	Heptageniidae	*Nixe*
Plecoptera	Capniidae	*Mesocapnia*	*Mesocapnia frisoni*	Ephemeroptera	Leptohyphidae	*Tricorythodes*
Plecoptera	Capniidae		*Capniidae sp. BOLD:AAP1278*	Ephemeroptera	Leptohyphidae	
Plecoptera	Chloroperlidae	*Alloperla*	*Alloperla serrata*	Ephemeroptera	Leptophlebiidae	*Paraleptophlebia*
Plecoptera	Chloroperlidae	*Plumiperla*	*Plumiperla diversa*	Ephemeroptera	Leptophlebiidae	
Plecoptera	Chloroperlidae	*Suwallia*	*Suwallia sp. BIOUG22893-F02*	Ephemeroptera	Serratella	
Plecoptera	Gripopterygidae*	*Trinotoperla**	*Trinotoperla sp. JMH1731**	Plecoptera	Chloroperlidae	*Haploperla*
Plecoptera	Gripopterygidae*	*Zelandobius**	*Zelandobius truncus**	Plecoptera	Chloroperlidae	*Plumiperla*
Plecoptera	Gripopterygidae*	*Zelandobius**	*Zelandobius uniramus**	Plecoptera	Chloroperlidae	*Suwallia*
Plecoptera	Leuctridae	*Leuctra*	*Leuctra biloba*	Plecoptera	Chloroperlidae	*Sweltsa*
Plecoptera	Perlidae	*Calineuria*	*Calineuria californica*	Plecoptera	Chloroperlidae	
Plecoptera	Perlodidae	*Isoperla*	*Isoperla dicala*	Plecoptera	Immature	

Plecoptera	Perlodidae	*Isoperla*	*Isoperla petersoni*	Plecoptera	Nemouridae	Zapada
Plecoptera	Perlodidae	*Isoperla*	*Isoperla sp. AMI 1*	Plecoptera	Nemouridae	
Plecoptera	Pteronarcyidae	*Pteronarcys*	*Pteronarcys californica*	Plecoptera	Perlodidae	Cultus
Plecoptera	Taeniopterygidae	*Taenionema*	*Taenionema pallidum*	Plecoptera	Perlodidae	Isoperla
Trichoptera	Brachycentridae	*Amiocentrus*	*Amiocentrus aspilus*	Plecoptera	Perlodidae	Kogotus
Trichoptera	Glossosomatidae	*Agapetus*	*Agapetus celatus*	Plecoptera	Perlodidae	
Trichoptera	Glossosomatidae	*Agapetus*	*Agapetus sp. DER 1*	Trichoptera	Brachycentridae	Amniocentrus
Trichoptera	Helicopsychidae	*Helicopsyche*	*Helicopsyche sp. BOLD:AAA4321*	Trichoptera	Brachycentridae	Micrasema
Trichoptera	Hydropsychidae	*Ceratopsyche*	*Ceratopsyche oslari*	Trichoptera	Glossomatidae	Agapetus
Trichoptera	Hydropsychidae	*Cheumatopsyche*	*Cheumatopsyche mickeli*	Trichoptera	Glossosomatidae	Glossosoma
Trichoptera	Hydropsychidae	*Hydropsyche*	*Hydropsyche occidentalis*	Trichoptera	Helicopsychidae	Helicopsyche
Trichoptera	Hydropsychidae	*Parapsyche*	*Parapsyche almota*	Trichoptera	Helicopsychidae	
Trichoptera	Hydroptilidae	*Hydroptila*	*Hydroptila arctia*	Trichoptera	Hydropsychidae	Ceratopsyche
Trichoptera	Lepidostomatidae	*Lepidostoma*	*Lepidostoma canthum*	Trichoptera	Hydropsychidae	Cheumatopsyche
Trichoptera	Lepidostomatidae	*Lepidostoma*	*Lepidostoma podagrum*	Trichoptera	Hydropsychidae	Hydropsyche
Trichoptera	Lepidostomatidae	*Lepidostoma*	*Lepidostoma sp. DRCAD13-87*	Trichoptera	Hydropsychidae	
Trichoptera	Lepidostomatidae	*Lepidostoma*	*Lepidostoma canthum*	Trichoptera	Hydroptilidae	Paleagapetus
Trichoptera	Lepidostomatidae	*Lepidostoma*	*Lepidostoma sp. SCCWRP0083005*	Trichoptera	Lepidostomatidae	Lepidostoma
Trichoptera	Lepidostomatidae	*Lepidostoma*	*Lepidostoma unicolor*	Trichoptera	Lepidotostomatidae	Lepidostoma
Trichoptera	Odontoceridae	*Marilia*	*Marilia flexuosa*	Trichoptera	Odontoceridae	Marilia
Trichoptera	Odontoceridae		*Odontoceridae sp. BIOUG07750-D02*	Trichoptera	Philopotamidae	Wormaldia
Trichoptera	Philopotamidae	*Chimarra*	*Chimarra utahensis*	Trichoptera	Philopotamidae	
Trichoptera	Philopotamidae	*Wormaldia*	*Wormaldia gabriella*			
Trichoptera	Philopotamidae	*Wormaldia*	*Wormaldia sp. DRCAD13-4*			
Trichoptera	Psychomyiidae	*Tinodes*	*Tinodes consuetus*			
Trichoptera	Uenoidae	*Oligophlebodes*	*Oligophlebodes ruthae*			
Trichoptera			*Trichoptera sp. INB0004337956*			

3.2 ASV detection and distribution

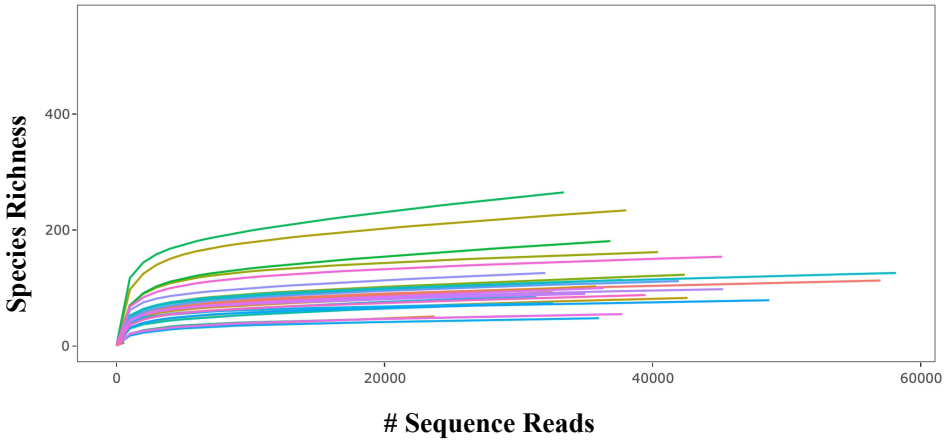

Figure 4.3. Salinas River Watershed eDNA biomonitoring Species Richness (measured as number of unique Amplicon Sequence Variants) of samples for C01 marker. Raw sequence data was analyzed using the ANACAPA bioinformatic pipeline and visualized in RANACAPA.

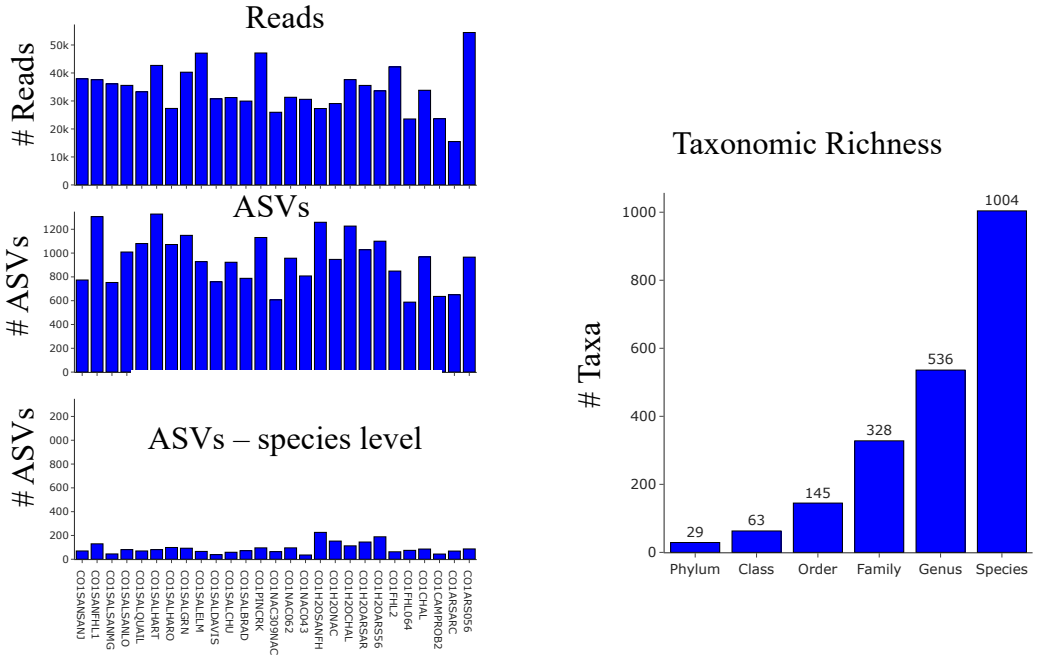

Figure 4.4. Salinas River Watershed eDNA biomonitoring A) Number of reads, Amplicon Sequence Variants (ASVs) and species-level matches from ASVs for C01 primers. B) Taxonomic richness, i.e., the total number of taxonomic assignments at multiple levels, across the dataset. Raw sequence data was analyzed using the ANACAPA bioinformatic pipeline and visualized in TaxonTableTools.

Overall, we found that our sampling strategy was sufficient to detect rare sequences, based on rarefaction curves (Figure 4.3). The total number of reads varied between markers and samples, but the lowest number of reads was still > 22,000 for any sampling site after quality filtering. Field blanks and negative controls resulted in a low number of reads (< 1,000) after quality filtering and the fewest Amplicon Sequence Variants (ASVs). ASVs varied between samples and markers and were positively correlated with the number of reads (Figure 4.4A). Field blanks, extraction blanks and no template controls had between one and four ASVs with the exception of one sediment extraction blank, which contained a higher amount of human DNA assigned to multiple ASVs (determined by BLASTing sequences).

3.3 Taxonomic Assignment and Detection from eDNA

We detected a total of 1,004 species, from 536 genera, 328 families, etc., as determined from matching sample ASVs to known sequences from the NCBI nucleotide database, as outlined in Methods Section 2.6 (Figure 4.4B).

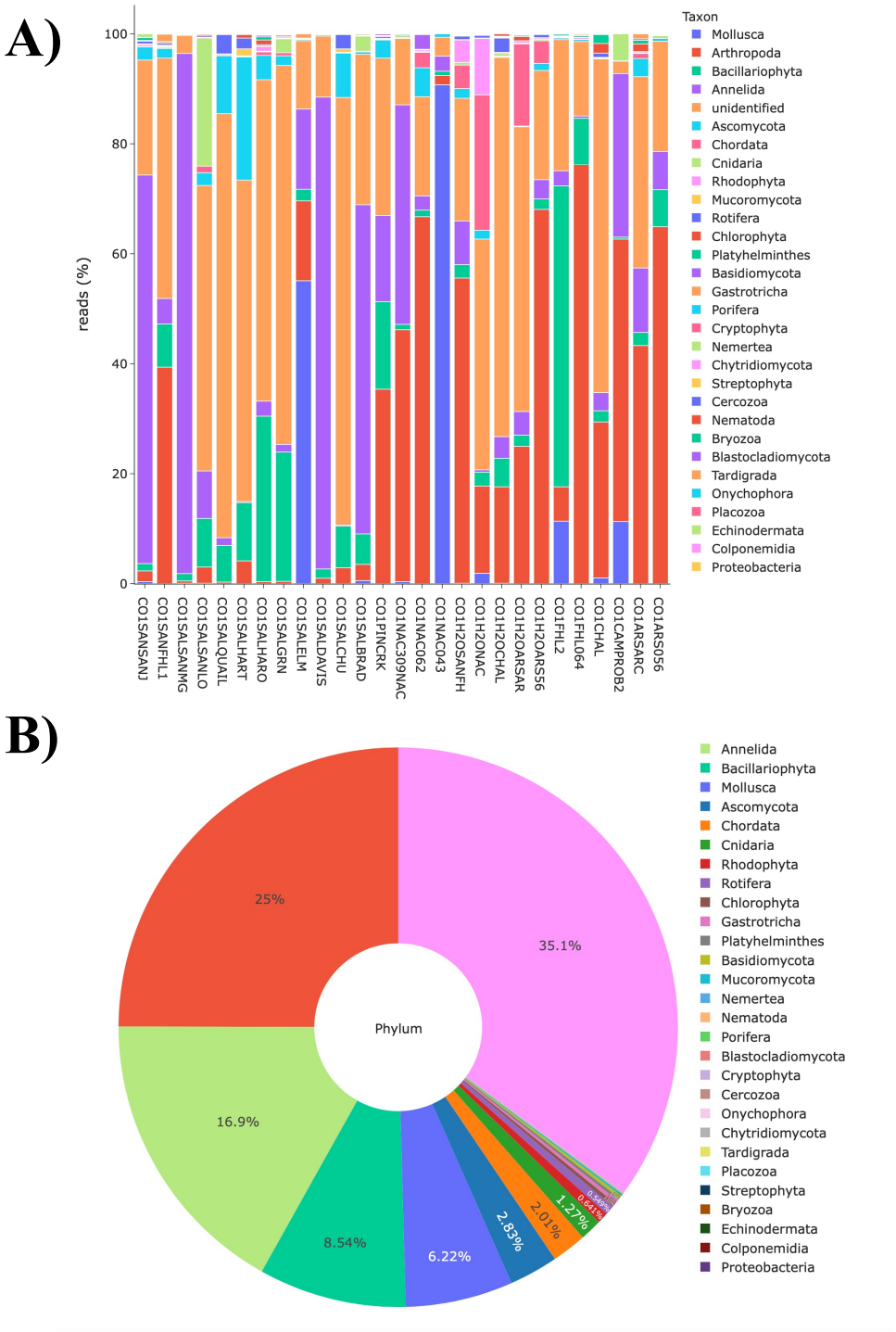

Figure 4.5. Salinas River Watershed eDNA biomonitoring A) Percentage of reads assigned to phylum level for C01 primers, shown per sample. B) Percent of total reads assigned to various taxonomic groups across the dataset. Raw sequence data was analyzed using the ANACAPA bioinformatic pipeline and visualized in TaxonTableTools.

The percentage of reads assigned to each taxonomic group varied between samples (Figure 4.5A). Major taxonomic groups corresponded to organisms typically found in sediment communities, with Arthropoda, Annelida, Bacillariophyta and Mollusca accounting for the majority of reads. Across all samples, 64.9% of sequence reads corresponded to known sequences available in the NCBI nucleotide database, and 35.1% were not able to be identified from available reference sequences (Figure 4.5B). Of the identified sequences, Arthropoda accounted for the highest percentage of reads (25%), followed by Annelida (16.9%) and Bacillariophyta (8.54%).

3.3.1 Comparisons Between eDNA-derived and Morphological Taxonomy

For the two ground truth sites where additional sampling occurred, we compared taxonomic identification from morphology with taxonomic assignments from the NCBI nucleotide sequence database. For this comparison we only considered taxonomy that matched reference sequences above 60% similarity. For the San Antonio and Nacimiento River sites, we detected 779 and 948 unique ASVs respectively that had sequences matching reference sequences above 60% similarity. Across both sites, we detected 24 EPT families including 52 genera from eDNA samples, and 29 EPT families including 34 genera from morphology. To further refine genus and species level taxonomic assignment, we filtered 34 genera and 39 species with > 90% similarity.

Some detections were unexpected. For example, we obtained 20 ASVs with a 97% match to the mayfly *Notonurus matitensis*, which is not known to occur in California. When we re-BLASTed this sequence (11/11/2021), however, we found a 100% match to *Ecdyonurus simplicioides* (GenBank: MG383351), the reference sequence for this species was uploaded after our CRUX libraries were created. We found similar results for *Zelandobius uniramus* (100%

match; 2870 ASVs) and *Zelandobius truncus* (100% match; 57 ASVs), which when re-BLASTed resulted in a single 100% match to *Isoperla difformis* (GenBank: MZ627347.1). This could be the result of an incorrect taxonomic assignment, or lack of resolution at the CO1 region for these species. These results emphasize the importance of creating contemporary reference libraries, and highlight the continual improvement of sequence databases.

3.3.2 Taxonomic Distribution

We identified the most abundant sequences detected for each site, with ASVs assigned at the family level (Table 4.3, Figure S4.6). Common aquatic macroinvertebrate families (Chironomidae, Simuliidae) were predominant in many sites. The largest family of beetles (Staphylinidae) were also the most common family observed at the two least impacted sites in the Nacimiento River (NAC309), and the least impacted site in the Arroyo Seco (CAMPROB2). This family is known to occur in riparian habitat near water margins but is not considered an aquatic group (Klimaszewski et al., 2018). Staphylinidae sequences were assigned at 100% similarity to the *Philonthus* genus, of which several species are known to occur in California. A gastropod (Tateidae) was the dominant family collected at two downstream sites in the Nacimiento and Arroyo Seco Rivers (SALELM, 309NAC042), and present in several others. Freshwater jellyfish (Olindiidae) was the most common family detected at SALSANLO and this group includes another invasive: the common freshwater jellyfish (*Craspedacusta sowerbii*). Oligochaeta and fungi known to thrive in sites with poor habitat quality were commonly detected at downstream sites in the Salinas River and associated tributaries (SALQUAIL, SALCHU, SALSANLO). These sites are considered highly impacted based on previous SWAMP water chemistry assessments. The most common family of Oligochaetes (Naididae) were also detected in SALDAVIS, a site commonly referenced as a least-impacted nearby site in SWAMP

assessments. Common families of Diatoms (Bacillariaceae, Thalassiosiraceae) were the most common ASVs for two sites in the Salinas River (SALGRN, SALHARO). The most common family detected for DPR monitoring sites (SALCHU, SALQUAIL, SALHART) was the family of fungi Nectriaceae, with sequence matching the species *Fusarium oxysporum* at 93.11% similarity.

For one site located upstream in the Nacimiento River (309NAC062), Salmonidae was the most common family detected in water filtrate, matched to *O. mykiss* at 98.89% similarity. *O. mykiss* sequences also occurred in sediment from that site, although the most abundant sequences were assigned to Chironomidae (genus: *Tanytarsus*; 99.25% similarity).

In addition to the most abundant family-level detections, several other detections were correlated with CSCI score. Known pollution-tolerant taxa were detected more often at sites with low CSCI scores. The diatom *Navicula cryptocephala* was detected in DPR monitoring sites (SAL_DPR) and other sites with low CSCI scores in the mainstem of the Salinas River (SALQUAIL, SALSANJ, SALHART, SALGRN). The tubicifid worm *Limnodrilus hoffmeisteri* was detected at one DPR monitoring site (SALQUAIL). Another tolerant tubicifid *Tubifex tubifex* was detected at SALSANMG. At the species level, we detected the invasive New Zealand mud snail, *Potamopyrgus antipodarum* (100% similarity) in several sites in the Salinas, Nacimiento, and San Antonio rivers (CAMPROB2, FHL2, NAC043, 309NAC, SALBRAD, SALELM), but not in Chalone Creek or Pine Creek, or in our field/lab blanks.

Many taxa were detected across all waterbodies, although a few groups were site-specific. For example, Bryozoa were only detected at SALHARO. SALHARO is the downstream sampling location for the Tembladero Slough and does not directly connect to the Salinas River. Species of annelids, arthropods, fungi, diatoms, flatworms, vertebrates, mollusks

and rotifers were detected in all sites. The Salinas River contained the highest number of sites sampled (n = 8) and also the highest coverage of taxa overall.

Table 4.3. *Most abundant sequences per sample, assigned at family level. Numbers represent ASVs per family. Sequence data was analyzed using phyloseq in the command line.*

Sample	waterbody	Chironomidae	Staphylinidae	Cleridae	Sellaphoraceae	Simuliidae	Naididae	Salmonidae	Tateidae	Nectriaceae	Bacillariaceae	Thalassiosiraceae	Olindiidae	Hominidae	Cyprinidae	Most common family
ARS056	ARS	35852	0	0	856	0	3936	0	0	60	1287	0	0	0	1	***Chironomidae***
ARSARC	ARS	10931	0	0	27	10	3019	0	0	168	141	0	1	0	0	***Chironomidae***
H2OARS56	ARS	7948	1528	0	38	12234	1276	1036	0	70	674	0	0	0	355	***Simuliidae***
H2OARSARC	ARS	9222	0	0	0	343	1300	0	0	16	753	0	0	0	5975	***Chironomidae***
PINCRK	ARS	14331	0	0	991	15	8014	0	0	578	3456	0	0	0	0	***Chironomidae***
SALELM	ARS	808	5814	0	395	0	7091	0	26826	54	184	0	0	0	0	***Tateidae***
CHAL	CHAL	6346	0	0	65	274	1192	0	0	0	248	0	0	0	0	***Chironomidae***
H2OCHAL	CHAL	7222	0	0	17	15	1660	0	0	0	2038	0	0	0	0	***Chironomidae***
CAMPROB2	NAC	57	11872	0	2	1	7011	0	2676	20	51	0	0	0	0	***Staphylinidae***
H2ONAC62	NAC	1901	0	0	12	56	155	9039	685	117	747	0	0	0	0	***Salmonidae***
NAC043	NAC	268	0	0	80	0	591	0	27873	173	104	0	0	0	0	***Tateidae***
NAC062	NAC	18036	0	0	56	4939	67	1014	0	293	292	0	0	0	0	***Chironomidae***
NAC309NAC	NAC	27	11774	0	69	0	10406	0	91	13	95	0	102	0	0	***Staphylinidae***
SALBRAD	SAL	877	0	0	274	0	18208	0	149	69	906	2	874	0	0	***Naididae***
SALGRN	SAL	72	0	0	350	0	585	0	0	580	7675	1	1001	0	1	***Bacillariaceae***
SALSANLO	SAL	924	88	0	344	0	2663	0	0	669	1941	0	8394	0	0	***Olindiidae***
SAL_SANMG	SAL	51	0	0	172	0	33822	0	1	0	243	0	0	0	0	***Naididae***
SALCHU	DPR	39	0	0	4	0	0	0	0	2027	552	0	0	0	0	***Nectriaceae***
SALDAVIS	DPR	345	0	0	8	0	25651	0	0	68	580	0	0	0	0	***Naididae***
SALHART	DPR	24	0	0	37	33	94	0	0	9329	2770	0	0	0	0	***Nectriaceae***

SALQUAIL	DPR	**11**	**0**	**0**	**0**	**0**	**473**	**0**	**0**	**2970**	**320**	**0**	**0**	**0**	**0**	***Nectriaceae***
FHL064	SAN	**9993**	**4186**	**102 97**	**605**	**0**	**126**	**0**	**0**	**32**	**1760**	**0**	**0**	**0**	**0**	***Cleridae***
FHL2	SAN	**2577**	**0**	**0**	**22433**	**0**	**1171**	**0**	**4817**	**45**	**195**	**0**	**0**	**0**	**0**	***Sellaphoraceae***
H2OSANFHL	SAN	**5821**	**371**	**0**	**27**	**7643**	**2489**	**252**	**1**	**30**	**729**	**0**	**0**	**3**	**879**	***Simuliidae***
SANFHL1	SAN	**16224**	**0**	**0**	**536**	**750**	**2096**	**0**	**0**	**146**	**1356**	**0**	**0**	**0**	**0**	***Chironomidae***
SALHARO	TEM	**0**	**0**	**0**	**0**	**0**	**832**	**0**	**0**	**724**	**209**	**6956**	**0**	**0**	**58**	***Thalassiosirac***
SANSANJ	TEM	**18**	**0**	**0**	**22**	**0**	**20401**	**0**	**0**	**474**	**56**	**1**	**0**	**0**	**0**	***Naididae***
H2OEB	EB	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	***Chironomidae***
SEDEB	EB	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**403**	**0**	***Hominidae***
H2OFB	FB	**1**	**0**	**0**	**0**	**0**	**2**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	***Naididae***
SEDFB	FB	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	***Cyprinidae***
NTC	NTC	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	***Staphylinidae***

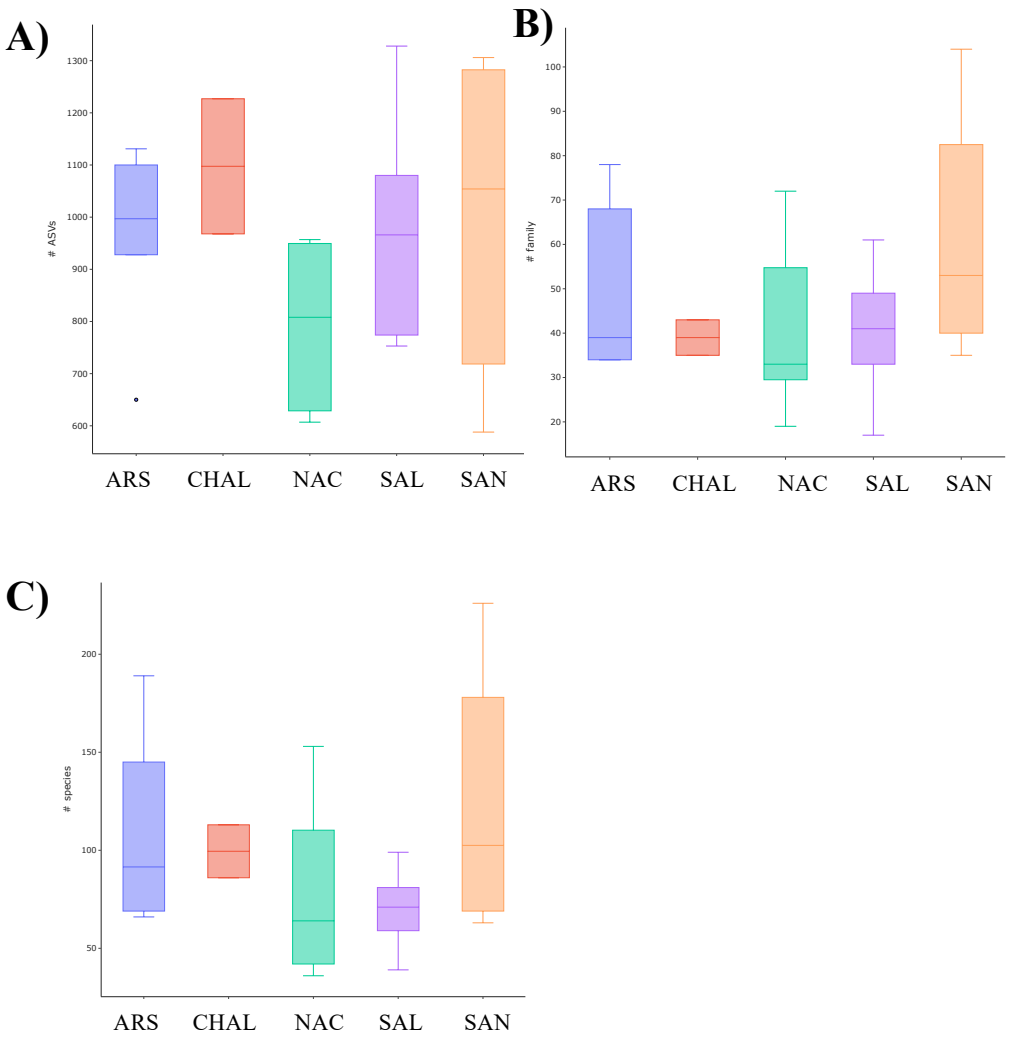

Figure 4.6. Salinas River Watershed eDNA biomonitoring alpha diversity per waterbody for the CO1 marker by A) ASV level, B) family level, and C) species level.

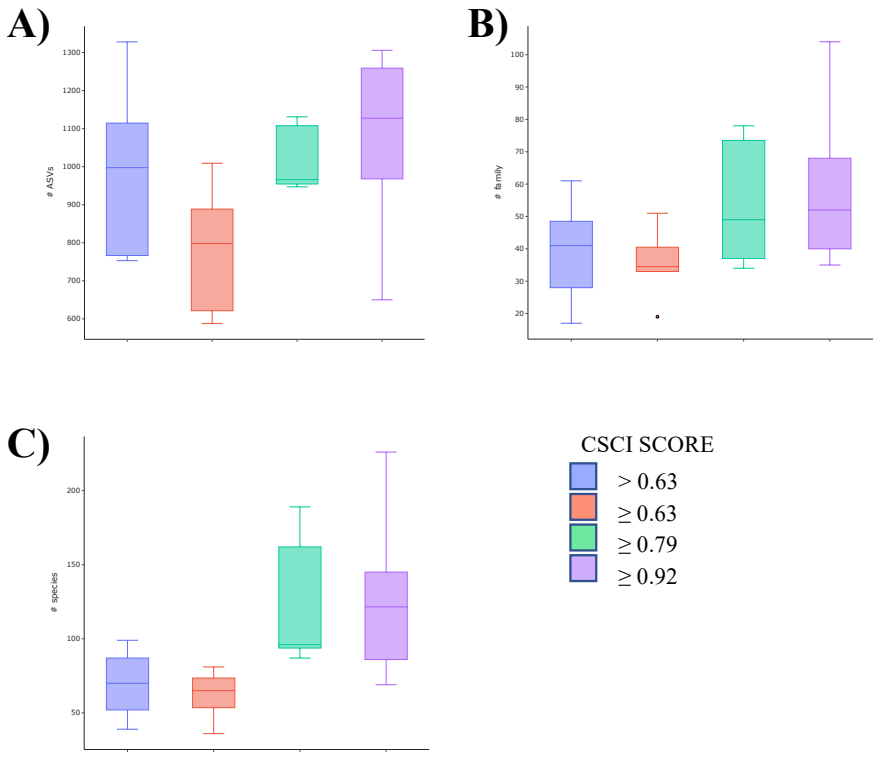

Figure 4.7. Salinas River Watershed eDNA biomonitoring alpha diversity per CSCI score for the CO1 marker by A) ASV level, B) family level, and C) species level.

Alpha diversity per waterbody is shown in Figure 4.6, at the ASV, family, and species levels. The highest number of sampling sites were located within the Salinas River, which may have resulted in a sampling bias resulting in a higher number of ASV detections. Overall, the largest variation in the number of ASVs detected within a waterbody was seen for the San Antonio River. Alpha diversity per CSCI score category is shown in Figure 4.7 at the ASV, family and species levels. At the family and species levels, there is a positive correlation between increased taxonomic diversity and CSCI score. At the species level, sites less likely to be altered (CSCI > 0.79) contain higher diversity than sites with lower CSCI scores.

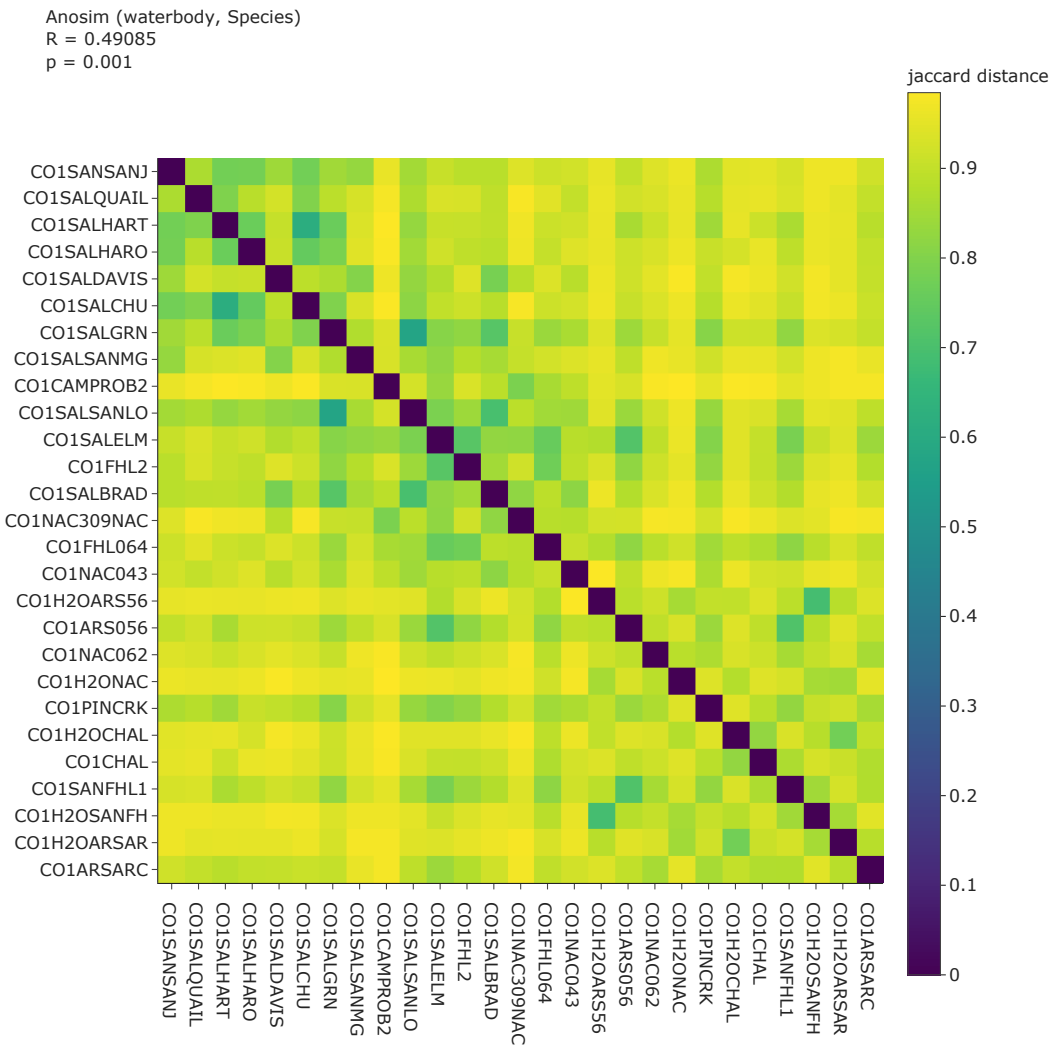

Figure 4.8. Salinas River Watershed eDNA biomonitoring beta diversity per waterbody for the CO1 marker at the species level. Raw sequence data were analyzed using the ANACAPA bioinformatic pipeline and visualized in TaxonTableTools.

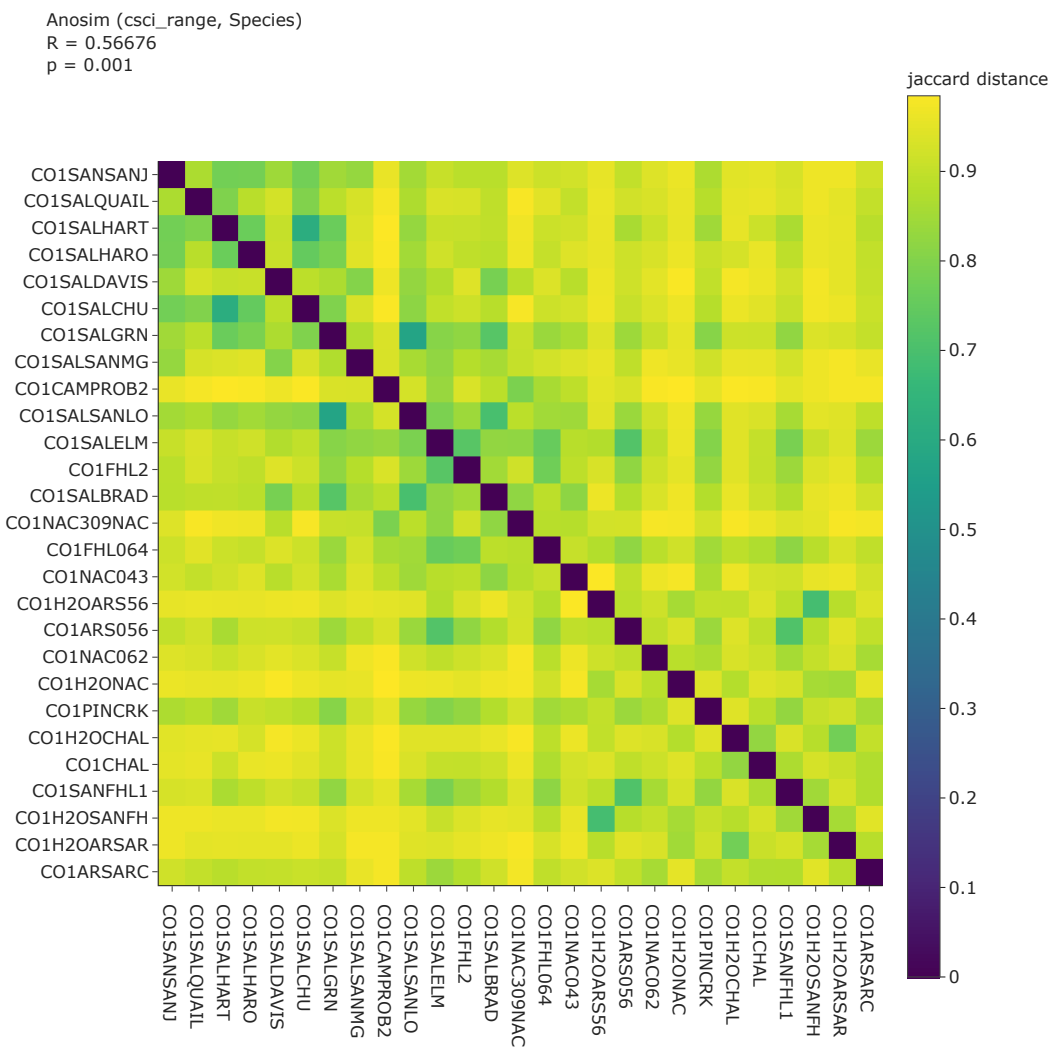

Figure 4.9. Salinas River Watershed eDNA biomonitoring beta diversity (Jaccard distance) per CSCI score category for the CO1 marker at the species level. Raw sequence data were analyzed using the ANACAPA bioinformatic pipeline and visualized in TaxonTableTools.

To visualize beta diversity between sites, we calculated Jaccard distances explained by hydrologic connectivity (Figure 4.8) and CSCI score (Figure 4.9), and visualized the differences as heat maps. Beta diversity (Jaccard distance) was high for most site pairs within waterbodies. Site pairs that were located in the same water body and were closer together generally had lower beta diversity (more similar species composition). For example, NAC309 and CAMPROB2 are both sites within the Nacimiento River downstream from Lake Nacimiento, located

approximately 5 miles apart. The low beta diversity between these sites is unsurprising considering their connectivity and physical proximity. Jaccard distances were greater when we used species level data ($R = 0.49085$, $p = 0.001$) to evaluate site pairs as compared with family level data ($R = 0.36131$, $p = 0.001$). We also generated heat maps to examine Jaccard distance of taxa which could be explained by CSCI score, at the family ($R = 0.49108$, $p = 0.001$) and species ($R = 0.56676$, $p = 0.001$) levels (Figure 4.9). Jaccard distances were greater when we used species level data versus family level data.

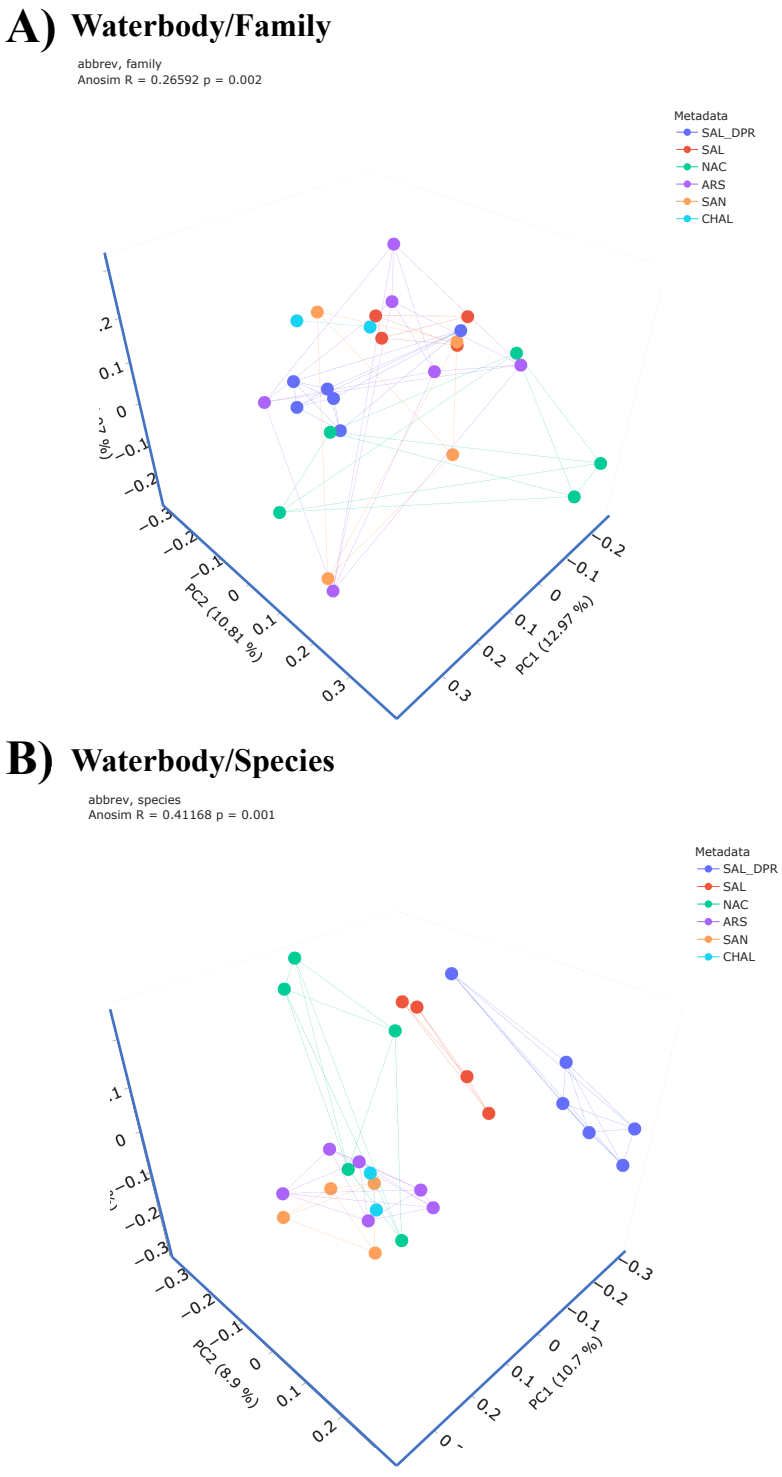

Figure 4.10. Salinas River Watershed eDNA biomonitoring Principal Coordinates Analysis (PCoA) per waterbody for CO1 A) at the family level (R = 0.26592, p = 0.002), and B) at the species level (R = 0.41168, p = 0.001). Raw sequence data was analyzed using the ANACAPA bioinformatic pipeline and visualized in TaxonTableTools.

To visualize the percent dissimilarity of taxa which could be explained by hydrologic connectivity, we conducted Principal Coordinates Analysis (PCoA) per waterbody at the family level and at the species level (shown in Figure 4.10) and generated three orthogonal axes to capture the variation between sites. The Anosim R statistic calculated at the species level (R= 0.41168, p = 0.001) revealed higher dissimilarity between sites than the family level (R = 0.26592, p = 0.002). Eigenvalues for the first three axes analyzed at the family level (PC1 = 12.97%, PC2 = 10.81%, PC3 = 6.97%) explained slightly higher percentages of variation in taxa observed than species level analysis (PC1 = 10.7%, PC2 = 8.9%, PC3 = 6.81%). The eigenvalues for the first three axes did not represent a high percentage of the variation, however, suggesting that waterbody does not explain the majority of variation in taxa observed. Notably, the sites located within the Salinas River were split between highly impacted sites used in SWAMP water chemistry long-term monitoring efforts (SAL_DPR) and other, less impacted sites located further upstream (SAL). The dissimilarity between these two groups is consistent with previous chemistry and habitat quality assessments.

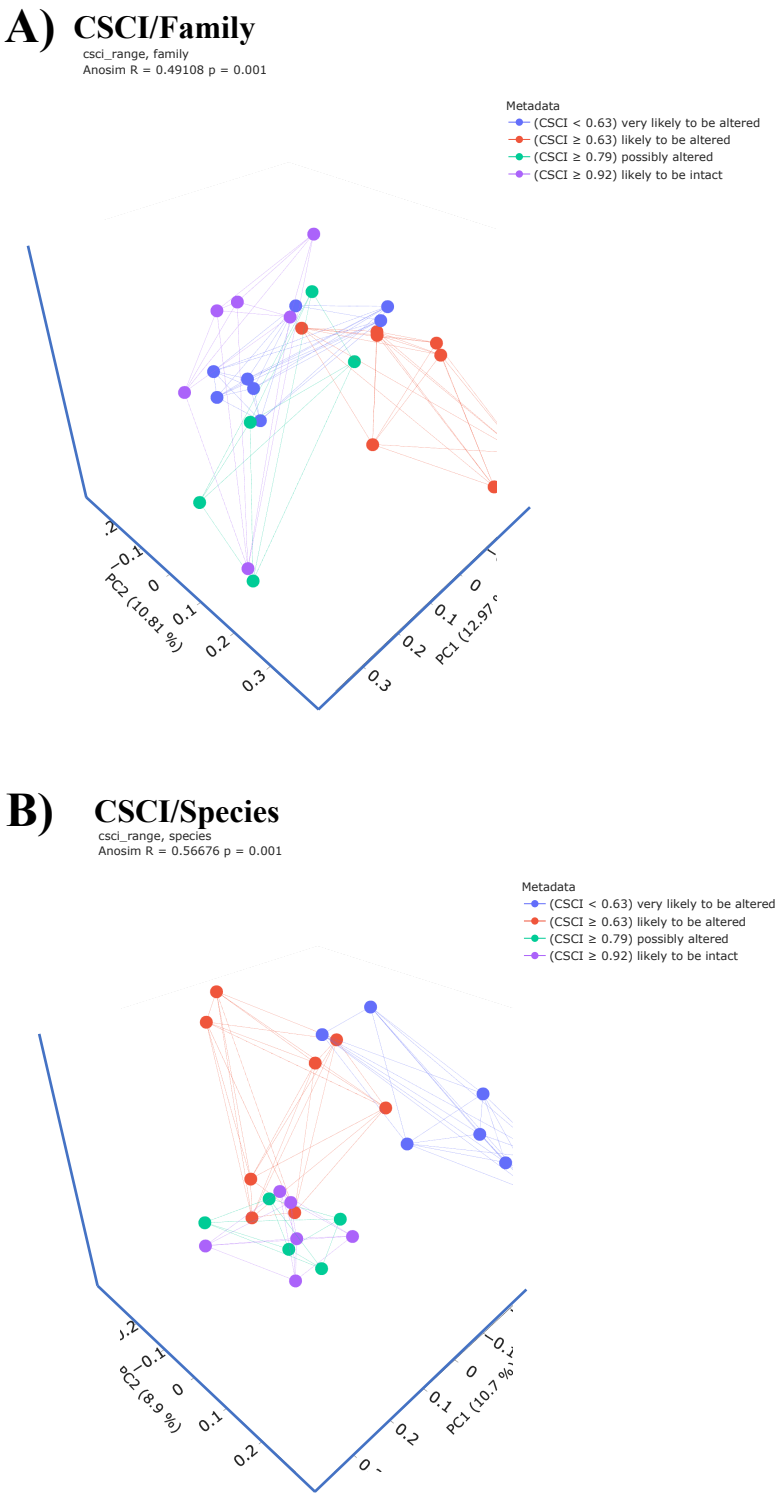

Figure 4.11. Salinas River Watershed eDNA biomonitoring Principal Coordinates Analysis (PCoA) per CSCI for CO1 at A) the family level (R = 0.49108, p = 0.001) and B) the species level (R = 0.56676, p = 0.001). Raw sequence data was analyzed using the ANACAPA bioinformatic pipeline and visualized in TaxonTableTools.

To visualize the percent dissimilarity of taxa which could be explained by CSCI score, we conducted Principal Coordinates Analysis (PCoA) per CSCI score range at the family level and at the species level (shown in Figure 4.11) and generated three orthogonal axes to capture the variation between sites. The Anosim R statistic calculated at the species level ($R = 0.56676$, $p = 0.001$) revealed higher dissimilarity between sites than the family level ($R = 0.49108$, $p = 0.001$). Eigenvalues for the first three axes analyzed at the family level (PC1 = 12.97%, PC2 = 10.81%, PC3 = 6.97%) explained slightly higher percentages of variation in taxa observed than species level analysis (PC1 = 10.7%, PC2 = 8.9%, PC3 = 6.81%).

4. Discussion

We analyzed biodiversity across an urban and agriculturally impacted watershed from sites representing a wide range of habitat and water quality, to determine how estimates of biotic integrity obtained from molecular biomonitoring compared to those obtained from traditional biomonitoring assessments. We evaluated site occupancy for sensitive biomonitoring indicator taxa and species of concern (invasive and threatened) to understand how habitat quality might impact metacommunity composition in this system. For sensitive taxa, we detected high beta diversity between sites with differing CSCI scores, and family-level richness was positively correlated with habitat quality. Impacted sites considered “very likely to be altered” (CSCI < 0.63) and least impacted “intact” sites (CSCI > 0.92) had the highest beta diversity of EPT and across all taxa. We found that biodiversity in the Salinas River watershed was similar between sites that were located in proximity to one other, within the same water body. Taxonomic similarity between sites varied by waterbody and CSCI score.

A comparison of family-level taxonomy obtained from sediment and water filtrate showed different biodiversity observed from each sample type, with significant overlap. Generally, more organisms that would be expected to be in contact with sediment were detected in sediment samples, while more pelagic organisms were detected in water samples. This finding suggests that future studies aiming to capture comprehensive site taxonomy may benefit from employing a combined sampling approach, collecting water and sediment at multiple transects per site.

From eDNA, we detected a broad range of biodiversity (ASVs matching sequences with known taxonomy from the NCBI nucleotide database). This broad diversity is expected considering the degenerate nature of the primers themselves and because the target region of the mitochondrial CO1 gene is highly conserved across phylogenies. The Salinas River contained the highest coverage of taxa overall. This may be due to sampling bias, since the highest number of sampling sites from our study were located in the Salinas River. Further sampling is needed to determine whether the taxonomic coverage from other waterbodies would increase with the addition of more sampling sites.

We observed variation between replicates in our pilot data, where one subsample contained a higher diversity of sequences, and still contained undiscovered ASVs after > 110,000 reads. For many taxonomic groups, individuals are patchily distributed across ecosystems. It is therefore unsurprising that environmental DNA from these taxa might also be patchily distributed across sites, and DNA abundance will vary between subsamples (Barnes and Turner, 2016). Thus, some variation in read number is expected from environmental samples. Although we standardized our sampling in terms of the number of subsamples and sample volume, natural variation in substrate type, organic matter composition and other factors affect the quantity of

extracted DNA. Estimates of species diversity in a community can depend on how deeply extracted DNA is sequenced (i.e., the number of sequence reads produced from each sample). Common invertebrate and vertebrate taxa represented the most abundant sequences in most of our sediment samples. This is expected, given that these taxa generally represent a higher proportion of the biomass in benthic communities. The high percentages of sequences from targeted taxonomic groups support the conclusion that we successfully detected organisms within the sediment communities sampled, and that DNA from those organisms are present in sufficient abundance to be detected using this approach (10 subsamples of 250 mg homogenized sediment per site). Sequence abundance does not directly correspond to biomass or number of individual organisms, however, due to several factors including primer bias, amplification bias, and effects of sampling design (Taberlet, 2018), and so we have refrained from making any inference about abundance of individual taxa from our findings.

From our ground truthing sites, we found significant overlap (> 76.67%) between taxonomic identifications obtained from sequence data and morphology for BMI taxa, with some unique identifications from each method. We morphologically identified taxa that eDNA methods failed to detect, and vice versa. For the taxa that we identified morphologically but failed to find from our eDNA sequence data, we searched the CO1 CRUX library to confirm that they would be expected to be amplified our primers. We also searched the NCBI nucleotide sequence database to ensure that these taxa were represented. Of the 6 families (14 genera) that were not detected from eDNA but were identified morphologically, two were not represented in the sequence database at the genus level, but all were present at the family level. Interestingly, we detected a higher number of EPT families from morphology versus sequencing (29 versus 24, respectively), but a higher number of genera from sequencing versus morphological

identification (52 versus 33). We initially retained all ASVs above 60% similarity for our analysis, but a high percentage of reads matched reference taxonomy well above this threshold. A high percentage of ASVs (70.5%) matched a reference sequence above 90% similarity, and 60.2% matched above 97%. In studies using OTU clustering methods, it is typical to cluster at 97% similarity for species-level identifications (Callahan et al., 2017). An analysis of CO1 sequence diversity for a well-studied genus (Baetis) of Ephemeroptera found a large degree of CO1 sequence diversity (average genetic distance: 16.2%), resulting from the high species diversity and early evolutionary divergence of this group (Curry et al., 2018). Additionally, most EPT taxa were identified from multiple ASV matches, where only a subset of ASVs matched at low similarity cutoff values. This does not account for sequences with incorrect taxonomy present in the sequence database. Ultimately, our ability to identify unknown taxa using DNA barcodes is determined by the quality and comprehensiveness of reference sequence databases (Porter and Hajibabaei, 2020). Efforts should be made to improve the quality and completeness of these databases to maximize the utility of sequence data.

Many of the morphological specimens we collected from the San Antonio River site were early instars, and some were inevitably damaged in the collection process. These factors made identification more difficult for this site. As a result, fewer individuals were identified below family level, and none were identified to species. Additionally, we collected sediment immediately downstream from riffle habitat, whereas we sampled invertebrates from within riffles. It is possible that the patchiness of DNA distribution in the environment caused some of the variation we observed between the two methods. There is debate over the tradeoff between the increased effort required for finer scale identification and the resulting information gained, and as a result many assessments are only done to family level (Lenat and Resh, 2001). If genus

and species level identification can be aided with genetic techniques, we may increase the accuracy of habitat quality assessments, and even be able to detect deleterious changes before major shifts in community composition occur. Together, these findings highlight the importance of comprehensive taxonomic database development in eDNA studies. To increase the relevance of sequence data in ecological management, databases need to incorporate site-specific biodiversity, ideally at the species level. Researchers should prioritize global efforts to improve taxonomic representation from biodiverse regions where funding and resources may be limited.

We detected EPT taxa at 15 out of 22 sites. Plecopterans were only detected at sites with CSCI > 0.79, and Trichopterans were only detected at sites CSCI > 0.63, with the majority of detections occurring in site with the highest CSCI scores. SAL_SanMig was the only site with a CSCI score below 0.63 containing an EPT taxon. However, this taxon (the mayfly *Caenis latipennis*), has relatively a high pollution tolerance of 7/10 (Hilsenhoff, 1988). The last time the CSCI score was calculated for this site was in 2012, so it is possible that conditions have changed. It is also the furthest site upstream in the Salinas River, and thus it has fewer potential runoff inputs than the other low-quality sites.

In additional to BMI, we detected other taxonomic groups which are routinely used in bioassessments. Freshwater diatom assemblages are an important indicator group used for water quality assessments in many countries and there is emerging interest in their use for bioassessments with metabarcoding (Chonova et al., 2019; Kelly, 1998; Zimmermann et al., 2015). Aquatic oligochaete communities are valuable indicators of sediment condition in streams and lakes (Vivien et al., 2020) and are the bases for indices such as the Oligochaete Index of Sediment Bioindication for streams and the Oligochaete Index of Lake Bioindication used in Europe (Lafont et al., 2012). Many of the taxa detected in our samples are currently used as

bioindicators of habitat quality. Rove beetles (Staphylinidae; *Philonthus spp.*) were the most common family observed at the two least impacted sites in the Nacimiento River (NAC309), and the least impacted site in the Arroyo Seco (CAMPROB2). Although not considered an aquatic species, these beetles have been used as an indicator of good habitat quality in boreal forest due to their sensitivity to changes in environmental conditions, and because this group may include a large number of potentially significant species in biological control efforts (Klimaszewski et al., 2018).

Pollution tolerant taxa are commonly found in degraded sites and are used as a determinant in many biotic indices, including the CSCI (Mazor et al. 2016). For example, previous work has shown strong correlations between poor water quality (nutrient loading, effluent discharge) and the presence of tolerant diatom groups (Bharathi et al., 2018). The diatom *Navicula cryptocephala* was detected in sites with poor water quality (SALQUAIL). This diatom has been classified as highly pollution-tolerant and is a widely distributed species (Kalyoncu and Akkoz, 2009). In environments with low dissolved oxygen concentrations and water bodies receiving heavy sewage pollution, tubicifid worms such as *Limnodrilus hoffmeisteri* and *Tubifex tubifex* have been shown to become the predominant species (Aston, 1973). The tubicifid worm *Limnodrilus hoffmeisteri* was detected at SALQUAIL, a site that has reported benchmark exceedances of many pesticides, and is highly toxic to invertebrates (Stinson et al., 2021). Another tolerant tubicifid *Tubifex tubifex* was detected downstream from a densely populated urban area in the Salinas River (SALSANMG). We detected these and other taxa known to thrive in sites with poor habitat quality at downstream sites in the Salinas River and associated tributaries (SALQUAIL, SALCHU, SALSANLO). These sites are considered highly impacted based on previous SWAMP water chemistry assessments. The detection of tolerant taxa is

unsurprising, considering that many of these sites are channelized, lack substrate to support sensitive macroinvertebrate species, and are known to be impacted by pesticides. While sequence abundance does not directly correlate to individual abundance for many taxa, future studies might explore whether the ratio of tolerant to sensitive sequences is informative as a metric off habitat quality in eDNA assessments.

By including primers that target a wide range of taxa, we detected the presence of invasive species and disease-causing organisms in our samples. The fungus *Fusarium oxysporum,* known to cause Fusarium wilt in lettuce, strawberry, cilantro and cucumber crops (Koike et al., 2009; Koike and Gordon, 2005; Wang et al., 2015), was detected at SALQUAIL, a highly impacted site located adjacent to agricultural fields outside of Salinas, CA. The disease was first detected in the United States during the 1980s in the San Joaquin Valley of California and has since spread to other lettuce production areas (Koike and Gordon, 2005). At the species level, we detected the New Zealand mud snail *Potamopyrgus antipodarum* in several sites in the Salinas, Nacimiento, and San Antonio Rivers (CAMPROB2, FHL2, NAC043, 309NAC, SALBRAD, SALELM), but not in Chalone Creek or Pine Creek, or in our field/lab blanks. The New Zealand mud snail is a species of Tateidae and is an invasive species of concern in California (Cooper et al., 2013). This family of gastropod (Tateidae) was the dominant family collected at two downstream sites in the Nacimiento and Arroyo Seco Rivers, that are frequently used for recreation (SALELM, 309NAC042). Aquatic mollusks were observed at these sites, and at several others. We would have expected that sites occupied by the US Military (Camp Roberts and Fort Hunter Liggett) and thus with limited access from the public, would be at lower risk for the spread of invasive species, but this was not the case. We detected *Corbicula fluminea* at only one site within the Salinas River (SALBRAD), immediately downstream from Camp Roberts

military base and a state recreational area popular with hunters. Despite these advantages, some species remain elusive from eDNA detection. For example, the invasive crayfish *Procambarus clarkii* has proven difficult to detect with the degenerate primers used in other eDNA metabarcoding studies, despite success with species-specific primers (Tréguier et al., 2014) highlighting the importance of understanding the limitations of this approach.

We detected an important species of concern, steelhead trout (*O. mykiss)* at several sites, illustrating the utility of metabarcoding for monitoring rare taxa. For one site located upstream in the Nacimiento river (309NAC062), steelhead (Salmonidae) was the most common group detected in water filtrate. Steelhead was also detected in sediment at this site, but at lower sequence number than Chironomidae. Salmonids are known to occur in the Nacimiento River, which is popular with anglers, and during sampling at this site, we observed anglers and fish carcasses in the water (S. Stinson, pers. observation). Thus, it is not surprising that fish DNA would be readily detected from water at this site.

Environmental DNA metabarcoding complements traditional methods by targeting a broad range of taxa, sampling greater diversity and increasing the resolution of taxonomic identifications (Deiner et al., 2017). With all bioassessment techniques, the patchy distribution of key indicator species such as BMI combined with the low abundance and/or occupancy of rare taxa are challenging. By incorporating eDNA into bioassessments, we can widen the net to sample broader taxonomic diversity at increased sampling depth. No method is without limitations. Bioassessments using only eDNA may never yield ecologically relevant measures of abundance for all taxa, due to the complex dynamics of DNA in the aquatic environment (Barnes and Turner, 2016). Yet biotic indices obtained from eDNA metabarcoding data can be effectively used for watershed-wide, multitrophic assessments of freshwater biodiversity. As

biodiversity continues to decline globally, we must identify hotspots of biodiversity and safeguard them through monitoring and conservation efforts.

5. Conclusion

We detected EPT taxa from morphology and eDNA, with significant overlap (> 76.67%) between methods. Some taxa identified morphologically were missing from the reference sequence database, highlighting the importance of taxonomic database development. Sequencing detected more BMI than morphology when compared at the genus and species levels of taxonomic resolution. For EPT taxa, richness was positively correlated with CSCI. Overall taxonomic richness was also positively correlated with CSCI. We found high beta diversity between sites with differing CSCI scores for sensitive taxa, and alpha diversity was positively correlated with habitat quality. Sites in closest proximity to, or hydrologically influenced by, impacted sites (CSCI < 0.63) contained the most divergent community composition as compared to least impacted reference sites (CSCI > 0.92). Hydrologic distance (waterbody) and CSCI score both accounted for dissimilarity in taxa between sites. Our analyses revealed greater dissimilarity among sites when evaluated at the species level than at the family level. Metabarcoding of eDNA is useful for detecting species of concern and invasive species. As biodiversity continues to decline globally, we must identify hotspots of biodiversity and safeguard them through monitoring and conservation efforts. Biotic indices obtained from eDNA metabarcoding data can be effectively used for watershed-wide, multitrophic assessments of freshwater biodiversity.

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Summary

My dissertation research demonstrates that behavioral and molecular assays can detect subcellular, organismal and community level effects induced by chemicals of concern, either individually or as components of complex mixtures present in surface water near agricultural areas. I found community-level impacts of water quality occurring on a watershed scale, using multitrophic assessments of freshwater biodiversity.

In Chapter One I compared the toxicity of two pesticides, imidacloprid (IMI) and chlorantraniliprole (CHL) as single compounds and binary mixtures, to the toxicity of surface water collected near agricultural fields, after acute exposures using invertebrate and fish. In addition to acute toxicity, my secondary goal was to determine whether changes in select subcellular molecular pathways correspond to the insecticides' mechanisms of activity in aquatic organisms. To determine this, I conducted acute (96h) exposures using a dilution series of surface water and environmentally relevant concentrations of single and binary mixtures of IMI and CHL. I then evaluated survival and gene expression. In the published article, my co-authors contributed related research regarding the activity of IMI toward the n-acetylcholine receptor (nAChR) and CHL activity toward the ryanodine receptor (RyR). Analytical chemistry data showed chemicals of emerging concern as common analytes, including neonicotinoids (thiamethoxam, imidacloprid), pyrethroid insecticides (bifenthrin), and the carbamate insecticide methomyl, present at levels exceeding US Environmental Protection Agency benchmarks for Aquatic Life. I found that IMI and CHL were detected at all sampling locations and exposure to surface water led to high invertebrate but not fish mortality. Fish exposed to surface collected water had significant changes in the relative expression of genes involved with detoxification and neuromuscular function. Exposure of fish to single compounds or binary mixtures of IMI

and CHL led to increased relative gene expression of RyR in fish. Furthermore, I found that IMI targets the nAChR in aquatic invertebrates and that CHL can cause overactivation of the RyR in invertebrates and fish. Overall, these findings suggest that IMI and CHL may impact neuromuscular health in fish. Expanding monitoring efforts to include sublethal and molecular assays would allow the detection of subcellular level effects due to complex mixtures present in surface water near agricultural areas.

In Chapter Two I assessed whether exposure to surface water collected from urban and agriculturally developed waterways (Salinas River Watershed, CA) impacted multiple behavioral endpoints in the fathead minnow (*Pimephales promelas*), a model species in toxicology. I collected water samples at monitoring stations downstream from agricultural fields, and screened them for a suite of pesticides. I used locomotor assays to assess several behavioral responses of larval fish after acute exposure (96 h) to surface water. I detected significant differences in light-induced startle responses and average total movement, as well as the duration and/or frequency that fish swim at cruising, bursting and freezing velocities. The most sensitive endpoint was the light-induced startle response, which was significantly different from controls for all water samples tested. Results from this study show sublethal and environmentally relevant effects from exposure to contaminated surface waters, which would likely be missed through the use of standard toxicology assessments based on mortality.

In Chapter Three, I evaluated whether the swimming behavior of *D. magna* could be used as a sensitive bioindicator of exposure to two chemicals of concern, CHL and IMI, performed at environmentally relevant concentrations. I also examined the behavioral effects of exposure to contaminated surface water before and after the first rain following an extended dry period, also known as a “first flush” rain event. To determine this, I conducted 96h exposures using IMI and

CHL, and surface water from polluted waterways known to contain chemicals of concern, both before and after a first flush rain event. I determined that average Total Distance (mm) is a sensitive biomarker of exposure for IMI in single and binary chemical exposures, and for CHL albeit to a lesser extent. From surface water, analytical chemistry showed CHL and IMI as components of complex mixtures from surface water at all sites, at both sampling events, in addition to neonicotinoids, pyrethroid insecticides, organophosphates, and carbamate insecticides. Acute exposure to a geometric dilution series of surface water caused changes in Daphnia swimming behavior, and changes differed across sites and sampling dates. Daphnia response to light stimulus was the most sensitive endpoint measured for both sampling events. Before first flush, I detected strong dose-response patterns with controls showing the largest decrease in total movement and the highest concentrations of surface water showing the most divergent responses from controls. After first flush, I measured hypoactivity for all sites during at least one time period, in at least one concentration. I detected different response patterns to light stimulus for each site tested: a negative dose-response, non-monotonic pattern, and a positive dose-response pattern, with significantly different responses from controls at all concentrations tested.

In Chapter Four I sought to understand how biodiversity varies across the Salinas River Watershed, and whether diversity estimates obtained from eDNA metabarcoding correlated with (previously calculated) biotic index scores using the California Stream Condition Index (CSCI). To test this, I collected eDNA from sediment at sites throughout the Salinas River Watershed, across a range of habitat qualities, and compared the resulting taxonomy with morphological data from a subset of high-diversity sites. I detected sensitive invertebrate taxa (EPT) from morphology and eDNA, with significant overlap (> 76.67%) between methods. Some taxa

identified morphologically were missing from the sequence database, highlighting the importance of taxonomic database development. Sequencing detected more benthic macroinvertebrate taxa than morphology when compared at the genus and species levels of taxonomic resolution. For EPT taxa, richness was positively correlated with CSCI. Metabarcoding of eDNA is useful for detecting species of concern and invasive species. Sites in closest proximity to, or hydrologically influenced by, impacted sites (CSCI < 0.63) contained the most divergent community composition as compared to least impacted reference sites (CSCI > 0.92). Hydrologic distance (waterbody) and CSCI score both accounted for dissimilarity in taxa between sites. These analyses revealed greater dissimilarity among sites when evaluated at the species level than at the family level. As biodiversity continues to decline globally, we must identify hotspots of biodiversity and safeguard them through monitoring and conservation efforts. Biotic indices obtained from eDNA metabarcoding data can be effectively used for watershed-wide, multitrophic assessments of freshwater biodiversity.

Taken together, these studies show that sublethal behavioral and molecular assays can detect subcellular and organismal level effects induced by chemicals of concern, either individually or as components of complex mixtures present in surface water near agricultural areas. This research also demonstrates that enhanced biomonitoring across a wide range of biodiversity can detect sensitive, rare, and invasive taxa, and provide valuable information to assess habitat quality. As biodiversity continues to decline globally, we must identify hotspots of biodiversity and safeguard them through monitoring and conservation efforts. Biotic indices obtained from eDNA metabarcoding data may be effectively used for watershed-wide, multitrophic assessments of freshwater biodiversity.

There is growing public concern regarding pesticide misuse and overuse (Schaub et al. 2020), and as with many other environmental issues, underserved communities bear a disproportionate burden of exposure risk. It is known that low-income communities of color experience greater vulnerability to the health impacts of environmental chemical exposure (Johnston and Cushing, 2020). Worldwide, agricultural workers are among the most vulnerable populations due to risk factors associated not only with location and job duties, but also due to disparities stemming from immigration status, language barriers and lack of access to healthcare (Curl et al. 2020). Fishes share many physiological pathways with humans, making them excellent models for environmental risk assessment (Clasen et al., 2018; Hernández et al., 2017). By understanding the subcellular impacts of exposure, we can better understand potential human risk. As biodiversity continues to decline worldwide, we require a better understanding the resilience and persistence of freshwater ecosystems in response to rapid environmental change or disturbance. To safeguard the biological integrity of freshwater ecosystems, research is urgently needed.

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Appendix A: Chapter 1
Supplemental Figures and Tables

Chapter 1 Supplemental Figures

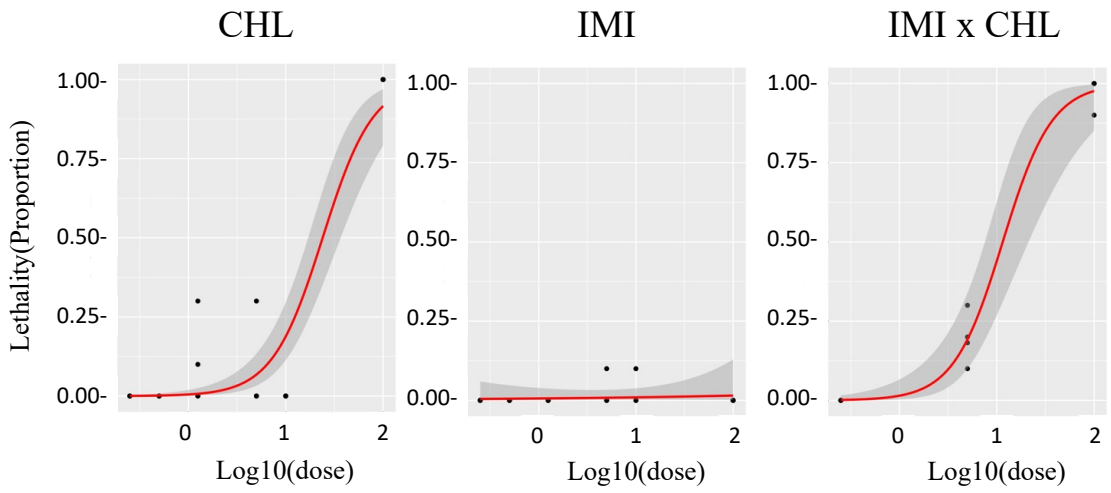
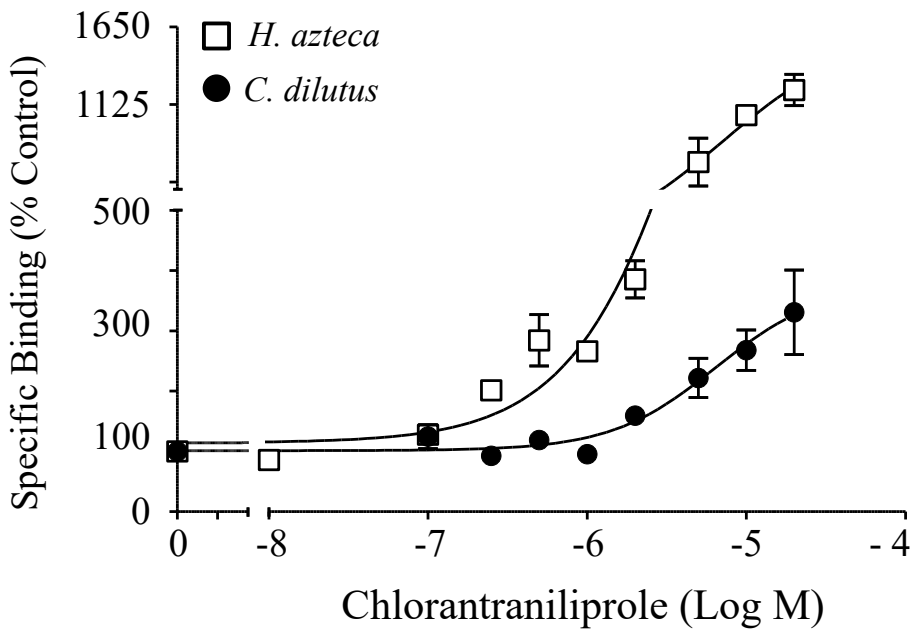


FIGURE S1. Lethal concentration thresholds (96 h LC_{50} values) for single and binary exposures of imidacloprid and chlorantraniliprole to *D. magna*. Plots are shown with 95% confidence intervals (CI) and were produced using Probit Analysis in the 'ecotox' package of R statistical software (R Core Team 2020).

A)



B)

Species	Maximum Response (%; ±SEM)	EC50 (µM)	EC50 95%CI (µM)	EC_{2X} (µM)	EC_{2X} 95% CI (µM)
H. azteca	1627 ± 519	7.04	1.56-31.87	0.47	0.0-1.89
C. dilutus	377.8 ± 145	6.46	0.98-42.75	4.13	1.59-7.27

FIGURE S2. Binding of [^{3}H]Ry to *H. azteca* and *C. dilutus* ryanodine receptors in the presence of chlorantraniliprole. A) Binding curves with specific binding relative to DMSO control (100%); mean ±SEM, n=6-9. B) Potency and efficacy of chlorantraniliprole observed by species. Abbreviations; EC50, Effect Concentration to 50% of maximal; EC_{2X}, concentration needed to cause 200% overactivation.

Chapter 1 Supplemental Tables

Table S1.1. *Concentrations of CHL and IMI (µg/L) measured in ambient water throughout California and within Salinas, CA (2016 – present) compared to EPA Aquatic Life Benchmarks and Ecological Risk Assessments for Registered Pesticides.*

	CHL	**IMI**
[a]Ambient water samples (California)	0.01 - 10.2 µg/L	0.01 - 41.1 µg/L
[a]Ambient water samples (Salinas, CA)	0.01 - 10.2 µg/L	0.01- 41.1 µg/L
[b]EPA benchmark (acute invertebrate)	4.7 µg/L	0.385 µg/L
[b]EPA benchmark (chronic invertebrate)	5.8 µg/L	0.01 µg/L
[b]LC_{50} *H. azteca*	>389 µg/L	13 µg/L
[b]LC_{50} *D. magna*	7.1 µg/L	6,029 µg/L
[b]LC_{50} *P. promelas*	No data	No data

[a]Concentration data collected from California Environmental Data Exchange Network (CEDEN) from 03/22/2016 to 03/17/2020

[b]Benchmarks represent EPA Aquatic Life Benchmarks and Ecological Risk Assessments for Registered Pesticides updated 09/28/2020

Table S1.2. *Sampling site descriptions and locations for ambient field water collection on 5/13/2019 and 9/14/2019.*

Site ID	Watershed	Water body Type	Site Description	Latitude	Longitude	[a]CDPR ID
Sal_Chualar	Salinas River	Ag Ditch	Chualar Creek at Chualar River Rd., ca. 1.2 mi. from HWY 101 (trib. to Salinas R.)	36.5584	-121.52964	27_8
Sal_Quail		Waterway	Quail Creek at HWY 101, between Spence and Potter Roads (trib. to Salinas R.).	36.6092	-121.56269	27_7
Sal_Davis		Waterway	Salinas River at Davis Rd	36.647	-121.70219	27_13
Sal_SanJon	Tembladero Slough	Ag Ditch	Rec Ditch at San Jon Road	36.7049	-121.70506	27_12
Sal_Haro		Waterway	Tembladero Slough at Haro Street	36.7596	-121.75433	27_66
Sal_Hartnell		Ag Ditch	Alisal Creek at Hartnell Rd	36.6435	-121.57836	27_70

[a] California Department of Pesticide Regulation (CDPR) Surface Water Database site identification number (SURF loc_cd1).

Table S1.3. *Water quality parameters measured in situ using a YSI EXO1 multi-parameter water quality Sonde. Parameters recorded including ambient water pH, specific conductance, dissolved oxygen, temperature, total dissolved solids, salinity, and total suspended solids.*

May 2019							
SiteID	pH (units)	Cond (ms/cm)	DO (mg/L)	Temp (°C)	TDS (mg/L)	Salinity (ppt)	TSS (mg/L)
Sal_Haro	6.82	2.286	13.52	21.2	1486	1.18	293.4
Sal_SanJon	6.77	1.329	10.65	21.5	863	0.66	77.4
Sal_Quail	7.19	0.902	8.54	25.2	587	0.44	593.8
Sal_Chualar	7.63	1.524	8.39	25.9	991	0.76	268.4
Sal_Hartnell	7.79	1.195	7.6	26.1	777	0.59	183.8
Sal_Davis	7.92	0.464	9.2	23.1	301	0.22	71.6
September 2019							
SiteID	pH (units)	Cond (ms/cm)	DO (mg/L)	Temp (°C)	TDS (mg/L)	Salinity (ppt)	TSS (mg/L)
Sal_Quail	8.29	0.986	8.43	24.3	641	0.49	1703.27
Sal_Hartnell	8.2	1.186	7.68	24.1	771	0.59	449.02
Sal_Davis	8.39	0.419	9.6	23.2	273	0.2	45.58

Table S1.4. *Pesticides detected in ambient field water collected from CDPR long-term monitoring sites in Salinas, CA on 5/14/2019. Trace values are below the MDL and ND represents analytes that were not detected.*

Analyte	Sal_Quail µg/L	Sal_Hartnell µg/L	Sal_Davis µg/L	Sal_SanJon µg/L	Sal_Chualar µg/L	Sal_Haro µg/L
Atrazine	ND	ND	ND	ND	ND	ND
Azoxystrobin	0.101	0.047	Trace	0.181	Trace	0.109
Bensulide	16.9	18.8	0.351	14.8	0.299	2.37
Carbaryl	Trace	Trace	Trace	Trace	Trace	Trace
Chlorantraniliprole	0.466	10.2	Trace	0.634	0.236	0.258
Chlorpyrifos	ND	ND	ND	ND	ND	ND
Cyprodinil	Trace	0.048	ND	Trace	Trace	Trace
Diazinon	ND	0.069	ND	0.040	ND	Trace
Diflubenzuron	ND	ND	ND	ND	ND	ND
Dimethoate	ND	Trace	ND	ND	ND	0.135
Diuron	Trace	Trace	ND	Trace	Trace	0.029
Hexazinone	ND	ND	ND	ND	ND	ND
Imidacloprid	0.759	1.01	0.019	0.495	1.19	0.292
Indoxacarb	Trace	0.091	ND	Trace	0.151	ND
Malathion	ND	0.455	ND	ND	ND	Trace
Methomyl	Trace	15.8	0.031	15.6	0.743	1.03
Methoxyfenozide	0.025	0.048	Trace	0.113	0.059	0.120
Oryzalin	ND	ND	ND	0.157	ND	0.028
Prometryn	1.48	0.441	Trace	0.045	0.036	Trace
Pyraclostrobin	0.263	0.060	Trace	0.029	Trace	Trace
Quinoxyfen	0.073	Trace	ND	ND	Trace	ND
Simazine	ND	ND	ND	ND	ND	ND
S-Metolachlor	ND	Trace	ND	ND	ND	ND
Trifloxystrobin	ND	0.087	ND	ND	ND	ND
Atrazine-d5	0.0475	0.0447	0.0467	0.0437	0.0458	0.0406
Imidacloprid-d4	0.0450	0.0429	0.0469	0.0423	0.0459	0.0415
Bifenthrin	0.01110	0.0215	ND	0.00236	ND	0.00230
Fenpropathrin	ND	ND	ND	ND	ND	ND
Lambda Cyhalothrin	0.0115	ND	ND	ND	ND	ND
Permethrin Cis	ND	0.00891	ND	ND	ND	ND
Permethrin Trans	0.00878	0.00655	ND	ND	ND	ND
Cyfluthrin	0.00736	ND	ND	ND	ND	ND
Cypermethrin	ND	ND	ND	ND	ND	ND
Esfenvalerate/ Fenvalerate	ND	0.0112	ND	ND	ND	ND
Permethrin Total	0.0161	0.0155	ND	ND	ND	ND

Table S1.5. *Pesticides detected in ambient field water collected from CDPR long-term monitoring sites in Salinas, CA on 9/17/2019. Trace values are below the MDL and ND represents analytes that were not detected.*

Analyte	Sal_Quail µg/L	Sal_Hartnell µg/L	Sal_Davis µg/L
Acetamiprid	0.314	0.137	Trace
Atrazine	ND	ND	ND
Azoxystrobin	0.056	0.029	Trace
Bensulide	3.9	0.888	0.121
Chlorantraniliprole	0.35	0.504	0.021
Chlorpyrifos	ND	0.02	ND
Clothianidin	0.081	0.177	ND
Cyprodinil	ND	Trace	ND
Dimethoate	ND	0.052	ND
Diuron	ND	Trace	ND
Fenamidone	0.247	0.272	ND
Fenhexamid	ND	ND	ND
Fludioxonil	0.148	Trace	ND
Imidacloprid	0.293	0.513	0.014
Indoxacarb	0.146	Trace	ND
Malathion	0.024	0.349	ND
Methomyl	29.9	1.64	0.386
Methoxyfenozide	Trace	0.065	ND
Prometryn	Trace	Trace	ND
Pyraclostrobin	0.112	0.052	ND
Quinoxyfen	ND	ND	ND
Simazine	ND	ND	ND
S-Metolachlor	ND	ND	ND
Thiamethoxam	3.99	0.827	0.064
Trifloxystrobin	ND	ND	ND
Bifenthrin	0.00254	0.00278	ND
Fenpropathrin	ND	0.00530	ND
Lambda Cyhalothrin	0.0797	0.0127	ND
Permethrin Cis	0.108	0.0296	ND
Permethrin Trans	0.126	0.0290	ND
Cyfluthrin	ND	0.00588	ND
Cypermethrin	ND	ND	ND
Esfenvalerate/Fenvalerate	0.0224	ND	ND
Permethrin Total	0.234	0.0586	ND

TABLE S1.6. *Survival of P. promelas and D. magna after 96h of exposure to a geometric dilution series of ambient water samples collected in May 2019. Concentrations of field water tested were 100%, 60% and 35%. Treatments with significant mortality are shown in bolded text. P-values are as reported and nonsignificant mortality (ns = p > 0.05).*

***P. promelas* percent survival**						
Concentration of field water						
Site ID	100%		60%		35%	
Sal_Haro	90	ns	95	ns	95	ns
Sal_SanJon	95	ns	97.5	ns	95	ns
Sal_Quail	100	ns	100	ns	97.5	ns
Sal_Chualar	92.5	ns	97.5	ns	97.5	ns
Sal_Hartnell	97.5	ns	87.5	ns	90	ns
Sal_Davis	92.5	ns	92.5	ns	97.5	ns

***D. magna* percent survival**						
Concentration of field water						
Site ID	100%		60%		35%	
Sal_Haro	100	ns	97.5	ns	100	ns
Sal_SanJon	**0**	**p < 0.0001**	**75**	**p < 0.001**	97.5	ns
Sal_Quail	**2.5**	**p < 0.0001**	**0**	**p < 0.0001**	**40**	**p < 0.0001**
Sal_Chualar	100	ns	80	ns	92.5	ns
Sal_Hartnell	**0**	**p < 0.0001**	**0**	**p < 0.0001**	**0**	**p < 0.0001**
Sal_Davis	100	ns	97.5	ns	100	ns

Table S1.7. *Survival of P. promelas and D. magna after 96h of exposure to a geometric dilution series of ambient water samples collected in September 2019. Concentrations of field water tested were 100%, 60%, 35% and 20% for P. promelas, and 100%, 60%, 35%, 20% and 12% for D. magna. Treatments with significant mortality are shown in bolded text. P-values are as reported along with nonsignificant mortality (ns = p > 0.05).*

***P. promelas* percent survival**										
Site ID	Concentration of ambient water									
	100%		60%	ns	35%	ns	20%	ns		
Sal_Quail	100	ns	100	ns	100	ns	100	ns		
Sal_Hartnell	97.5	ns	100	ns	97.5	ns	97.5	ns		
Sal_Davis	97.5	ns	97.5	ns	100	ns	97.5	ns		

***D. magna* percent survival**										
Site ID	Concentration of ambient water									
	100%		60%		35%		20%		12%	
Sal_Quail	**0**	**p < 0.0001**	**0**	**p < 0.0001**	**0**	**p < 0.0001**	**0**	**p < 0.0001**	**12.5**	**p < 0.0001**
Sal_Hartnell	**0**	**p < 0.0001**	**0**	**p < 0.0001**	**37.5**	**p < 0.0001**	**72.5**	**p < 0.0001**	85	ns
Sal_Davis	100	ns	100	ns	90	ns	100	ns	90	ns

TABLE S8: Mean survival of *P. promelas* after 24h, 48h, 72h and 96h of exposure to chlorantraniliprole (CHL), imidacloprid (IMI), and binary mixtures of CHL + IMI. All treatments led to nonsignificant mortality (ns = $p > 0.05$).

Treatment	24h Survival		48h Survival		72h Survival		96h Survival	
DIEPAMHR	97.50%	ns	94.40%	ns	89.40%	ns	84.40%	ns
Solvent	100.00%	ns	97.50%	ns	92.50%	ns	92.50%	ns
0.025 CHL	100.00%	ns	100.00%	ns	100.00%	ns	100.00%	ns
0.500 CHL	100.00%	ns	100.00%	ns	100.00%	ns	100.00%	ns
10.000 CHL	100.00%	ns	100.00%	ns	97.20%	ns	92.20%	ns
DIEPAMHR	97.50%	ns	97.50%	ns	95.00%	ns	92.50%	ns
0.025 IMI	100.00%	ns	100.00%	ns	92.50%	ns	92.50%	ns
0.500 IMI	97.50%	ns	100.00%	ns	100.00%	ns	97.50%	ns
10.000 IMI	100.00%	ns	100.00%	ns	97.50%	ns	92.50%	ns
0.025	100.00%	ns	92.20%	ns	84.70%	ns	84.70%	ns
0.500	100.00%	ns	95.00%	ns	95.00%	ns	95.00%	ns
10.000	97.50%	ns	97.50%	ns	90.00%	ns	82.20%	ns

Table S1.9. *Mean survival of D. magna after 48h and 96h of exposure to chlorantraniliprole (CHL), imidacloprid (IMI), and binary mixtures of CHL + IMI. Treatments with significant mortality are shown in bolded text. P-values are as reported along with nonsignificant mortality (ns = p > 0.05).*

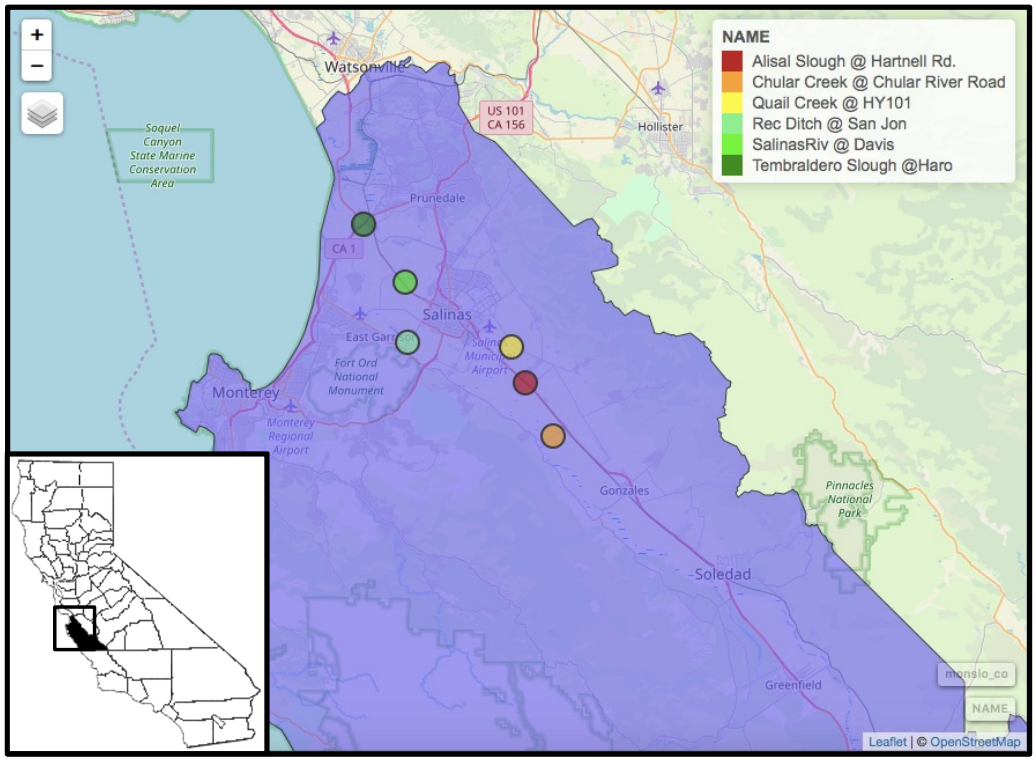
Treatment (µg/L)	**48h Survival**		**96h Survival**	
DIEPAMHR	97.50%	ns	97.50%	ns
Solvent Control	100.00%	ns	100.00%	ns
0.025 CHL	100.00%	ns	100.00%	ns
0.05 CHL	100.00%	ns	100.00%	ns
0.10 CHL	95.00%	ns	90.00%	ns
0.500 CHL	100.00%	ns	90.00%	ns
1.000 CHL	100.00%	ns	100.00%	ns
10.000 CHL	**0.00%**	**< 0.0001**	**0.00%**	**< 0.0001**
DIEPAMHR	91.30%	ns	83.10%	ns
0.025 IMI	97.50%	ns	97.50%	ns
0.05 IMI	100.00%	ns	100.00%	ns
0.10 IMI	100.00%	ns	100.00%	ns
0.500 IMI	100.00%	ns	100.00%	ns
1.000 IMI	100.00%	ns	100.00%	ns
10.000 IMI	100.00%	ns	100.00%	ns
DIEPAMHR	100.00%	ns	100.00%	ns
Solvent Control	100.00%	ns	100.00%	ns
0.025 IMI+CHL	100.00%	ns	100.00%	ns
0.500 IMI+CHL	100.00%	ns	**80.40%**	**0.0001**
10.000 IMI+CHL	**0.00%**	**< 0.0001**	**0.00%**	**< 0.0001**

Table S1.10. Imidacloprid and chlorantraniliprole concentrations detected in experimental solutions created for single/binary exposures.

Final Concentrations (ng/L)			
	78_imidacloprid	400_Chlorantraniprole	Nominal
170427_19_1stbatch_treat2_pos	n.a	0.0	0
170427_20_1stbatch_treat6_pos	n.a	92.1	25
170427_21_1stbatch_treat7_pos	n.a	43.8	50
170427_22_1stbatch_treat8_pos	n.a	435.4	100
170427_23_1stbatch_treat9_pos	n.a	938.9	500
170427_24_1stbatch_treat10_pos	n.a	277.8	1000
170427_25_1stbatch_treat11_pos	n.a	8875.5	10000
170427_28_2ndbatch_treat2_pos	0.0	n.a	0
170427_29_2ndbatch_treat3_pos	216.5	n.a	25
170427_30_2ndbatch_treat4_pos	254.4	n.a	50
170427_31_2ndbatch_treat5_pos	354.1	n.a	100
170427_32_2ndbatch_treat6_pos	835.9	n.a	500
170427_33_2ndbatch_treat7_pos	1332.6	n.a	1000
170427_34_2ndbatch_treat8_pos	9423.6	n.a	10000

Appendix B: Chapter 2
Supplemental Figures and Tables

Chapter 2 Supplemental Figures



Water body	DPR Site ID	Type	Description	Latitude	Longitude	SURF
Quail Creek	Sal_Quail	Waterway	Quail Creek at HWY 101	36.6092	-121.56269	27_7
Salinas River	Sal_Davis	Waterway	Salinas River at Davis Rd	36.647	-121.70219	27_13
Alisal Creek	Sal_Hartnell	Ag Ditch	Alisal Creek at Hartnell Rd	36.6435	-121.57836	27_70

Figure S2.1. Surface water sampling sites for CA California Department of Pesticide Regulation (DPR) located in Monterey County, Ca. Sites used for ambient field water collection on 9/14/2019: Quail Creek (Sal_Quail), Alisal Creek (Sal_Hartnell), and the Salinas River (Sal_Davis). CA California Department of Pesticide Regulation (DPR) site IDs are listed along with associated watershed, waterbody type, site description, latitude/longitude and CA DPR Surface Water Database site identification number (SURF). Sites are located immediately downstream of high use areas, where there is a high potential risk of contamination.

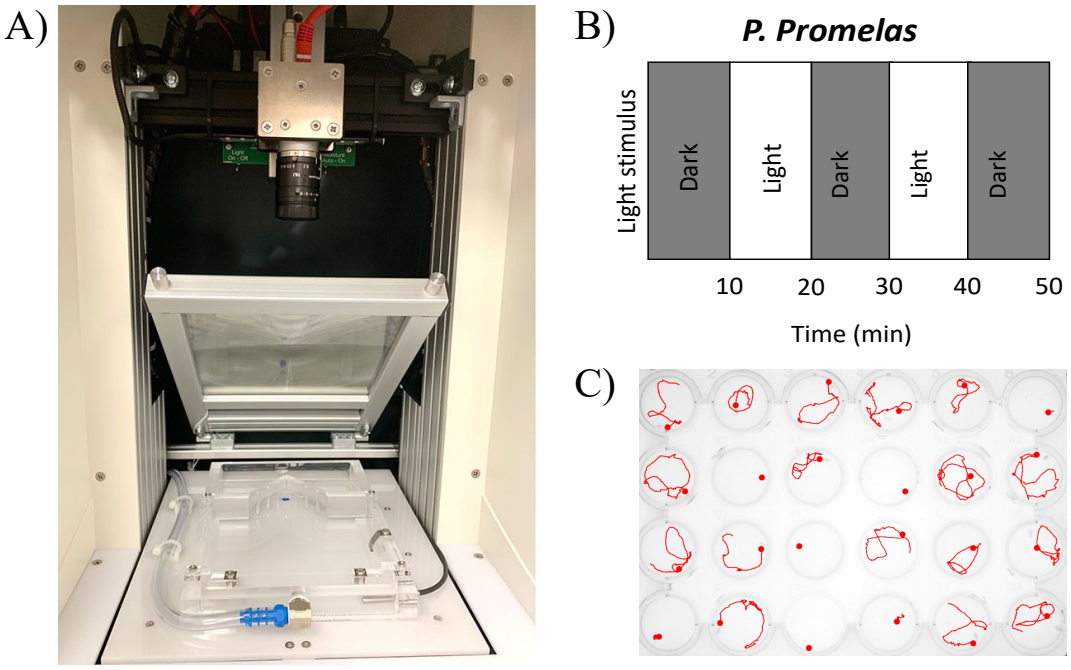


Figure S2.2. Behavioral assay setup and light:dark cycle parameters for 96 h exposures conducted on fathead larvae. After exposures, A) we placed fathead minnow larvae in 24-well plates into DanioVision Observation Chamber (Noldus, Wageningen, Netherlands) equipped with a top-mounted camera, B) larval behavior was recorded under alternating 10 min light:dark conditions, then C) behavioral parameters were tracked and analyzed using EthoVision XT software (version 14.0; Noldus, Wageningen, Netherlands).

Chapter 2 Supplemental Tables

Table S2.1. *Pesticide detections and benchmark values (µg/L), as reported in Chapter 1 (Stinson et al. 2021). A) Pesticides that exceeded one or more US Environmental Protection Agency (EPA) Aquatic Life Benchmark detected in ambient surface water collected from sites in Salinas, CA on 09/17/2019. B) EPA Aquatic Life Benchmarks and Ecological Risk Assessments for Registered Pesticides updated 09/28/2020. Benchmarks for aquatic life for acute toxicity equal the lowest 48- or 96-hour EC50 or LC50 in a standardized test, and chronic toxicity values equal the lowest NOEAC from a lifecycle or early life stage test). Colors shown in Table S2.1A correspond to aquatic life toxicity benchmarks for each chemical, as shown in Table S2.1B.*

A. Chemical detections (µg/L) exceeding one or more Aquatic Life Benchmark 09/17/2019

	Quail Creek	Alisal Creek	Salinas River
Bifenthrin	0.00254	0.00278	ND
Esfenvalerate/ Fenvalerate	0.0224	ND	ND
Imidacloprid	0.293	0.513	0.014
Lambda Cyhalothrin	0.0797	0.0127	ND
Malathion	0.024	0.349	ND
Methomyl	29.9	1.64	0.386
Permethrin Total	0.234	0.0586	ND
Clothianidin	0.081	0.177	ND
Thiamethoxam	3.99	0.827	0.064

B. EPA Aquatic Life Benchmarks for Registered Pesticides (µg/L)

	EPA chronic fish	EPA acute invert	EPA chronic invert
Bifenthrin	0.04	0.8	0.0013
Esfenvalerate/ Fenvalerate	0.035	0.025	0.017
Imidacloprid	9000	0.385	0.01
Lambda Cyhalothrin	0.031	0.0035	0.002
Malathion	8.6	0.049	0.06
Methomyl	12	2.5	0.7
Permethrin Total	0.0515	0.0195	0.0014
Clothianidin	9700	11	0.05

Table S2.2. *Physicochemical parameters measured in fathead minnow larvae culture water on arrival, laboratory control water, and surface water samples collected near Salinas CA on 09/17/2019. Parameters include Temperature (C), Electrical conductivity, Dissolved Oxygen (%), pH, Alkalinity (as CaCO3; mg/L), Hardness Alkalinity (as CaCO3; mg/L), and Salinity (ppm). Parameters were measured on arrival, at test initiation, at the 48h water change, and at test termination (96h).*

Sample	Temp °C	EC	Dissolved Oxygen %	pH	Alkalinity (CaCO3) mg/L	Hardness (CaCO3) mg/L	Sal ppm
FHM Arrival 9/17/2021							
Shipping water	21.7	463	100.7	7.52	98	126	0.2
Control water	20.3	476	82.1	8.45	124	180	0.3
Test initiation 9/17/2019							
Control water	20.3	476	82.1	8.45	124	180	0.3
Quail Creek	20.8	967	89.2	8.29	184	376	0.5
Alisal Creek	20.8	954	85.3	7.77	108	202	0.5
Salinas River	20.2	398.6	102.5	8.38	130	198	0.2
Test termination 9/21/2019							
Control water	20.3	476	82.1	8.45	124	180	0.3
Quail Creek	20.9	841	80.3	8.29	190	362	0.4
Alisal Creek	20.7	915	77.5	7.91	130	280	0.4
Salinas River	20.3	355	80.5	8.32	120	188	0.2

Table S2.3. *Survival of P. promelas and D. magna after 96h of exposure to a geometric dilution series of ambient water samples collected in September 2019. Concentrations of surface water tested were 100%, 60%, 35% and 20% for P. promelas, and 100%, 60%, 35%, 20% and 12% for D. magna. Treatments with significant mortality are shown in bolded text. P-values are reported as* $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$.

P. promelas percent survival										
Site ID	Concentration of ambient water									
	100%		60%	ns	35%	ns	20%	ns		
Quail Creek	100	ns	100	ns	100	ns	100	ns		
Alisal Creek	97.5	ns	100	ns	97.5	ns	97.5	ns		
Salinas River	97.5	ns	97.5	ns	100	ns	97.5	ns		
D. magna percent survival										
Site ID	Concentration of ambient water									
	100%		60%		35%		20%		12%	
Quail Creek	0	p < 0.0001	0	p < 0.0001	0	p < 0.0001	0	p < 0.0001	12.5	p < 0.0001
Alisal Creek	0	p < 0.0001	0	p < 0.0001	37.5	p < 0.0001	72.5	p < 0.0001	85	ns
Salinas River	100	ns	100	ns	90	ns	100	ns	90	ns

Table S2.4. *Significant parameters for fathead minnow larvae following 96 h exposures to surface water collected from three sites in Salinas, CA in September 2019. Measured parameters included Total Distance (TD), Velocity (V), Cruising Duration (CD), Cruising Frequency (CF), Turn Angle (TA), Angular Velocity (AV), Freezing Duration (FD), Freezing Frequency(FF). Larvae were exposed to three dark and two light cycles of 10 min durations. P-values are reported as * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001.*

Light Cycle	Parameter	Treatment	std.error	adj.p.value	significant
Light1	Mean Velocity	Quail Creek	0.50052053	0.0444211	*
Light1	Mean Velocity	Alisal Creek	0.50052053	0.02785951	*
Light1	Cruising Duration	Quail Creek	1.74631471	0.03465775	*
Light1	Bursting Frequency	Quail Creek	7.33090412	0.02231835	*
Light1	Bursting Frequency	Alisal Creek	7.33090412	0.00641762	**
Light1	Bursting Duration	Alisal Creek	0.59275371	0.00906975	**
Light1	Freezing Duration	Quail Creek	1.74631471	0.03465775	*
Light1	Total Distance	Quail Creek	30.014852	0.0444315	*
Light1	Total Distance	Alisal Creek	30.014852	0.02786413	*
Dark2	Bursting Frequency	Alisal Creek	8.28369104	0.01004979	*
Dark2	Bursting Duration	Alisal Creek	0.69622125	0.04049662	*
Light2	Mean Velocity	Alisal Creek	0.51314422	0.00260195	**
Light2	Cruising Duration	Alisal Creek	1.77151792	0.00698065	**
Light2	Bursting Frequency	Alisal Creek	7.60543848	0.00122529	**
Light2	Bursting Duration	Alisal Creek	0.60637501	0.00062156	**
Light2	Freezing Duration	Alisal Creek	1.77151792	0.00698065	**
Light2	Total Distance	Alisal Creek	30.7732694	0.00260148	**
Dark3	Bursting Frequency	Alisal Creek	8.20062619	0.03119442	*

Table S2.5. *Kruskal-Wallis analysis of variance output for all behavioral parameters for fathead minnow larvae following 96 h exposures to surface water collected from three sites in Salinas, CA in September 2019. Measured parameters included Total Distance (TD), Velocity (V), Cruising Duration (CD), Cruising Frequency (CF), Turn Angle (TA), Angular Velocity (AV), Freezing Duration (FD), Freezing Frequency(FF). Larvae were exposed to three dark and two light cycles of 10 min durations. P-values are reported as * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001.*

Label	Variable	Comparison	Est.	SEM	DF	Statistic	adj. p-value	
Dark1	MeanVelocity	T1 100 - Control	1.12902136	0.59969064	164	1.88267296	0.15576839	ns
Dark1	MeanVelocity	T2 100 - Control	1.2512176	0.59969064	164	2.08643841	0.10087965	ns
Dark1	MeanVelocity	T3 100 - Control	0.70366815	0.60839488	164	1.15659775	0.51278252	ns
Dark2	MeanVelocity	T1 100 - Control	0.91562729	0.57295641	164	1.59807494	0.26612513	ns
Dark2	MeanVelocity	T2 100 - Control	1.34205982	0.57295641	164	2.3423419	0.05520681	ns
Dark2	MeanVelocity	T3 100 - Control	0.1182412	0.58127262	164	0.20341781	0.98357714	ns
Dark3	MeanVelocity	T1 100 - Control	0.10387146	0.56773536	164	0.18295753	0.9867968	ns
Dark3	MeanVelocity	T2 100 - Control	1.10543542	0.56773536	164	1.94709632	0.13638762	ns
Dark3	MeanVelocity	T3 100 - Control	0.03594329	0.57597578	164	0.06240418	0.99852933	ns
Light1	MeanVelocity	T1 100 - Control	1.21562508	0.50052053	164	2.42872171	0.0444211	*
Light1	MeanVelocity	T2 100 - Control	1.30420201	0.50052053	164	2.60569134	0.02785951	*
Light1	MeanVelocity	T3 100 - Control	0.41772232	0.50778536	164	0.82263562	0.72523284	ns
Light2	MeanVelocity	T1 100 - Control	0.98399109	0.51314422	164	1.91757221	0.14502607	ns
Light2	MeanVelocity	T2 100 - Control	1.73637492	0.51314422	164	3.38379518	0.00260195	**
Light2	MeanVelocity	T3 100 - Control	0.25039238	0.52059227	164	0.48097598	0.90354116	ns
Dark1	Cruise.freq	T1 100 - Control	20.641	13.1633828	164	1.56806198	0.2801806	ns
Dark1	Cruise.freq	T2 100 - Control	22.2135	13.1633828	164	1.68752215	0.22697181	ns
Dark1	Cruise.freq	T3 100 - Control	10.0923158	13.3544433	164	0.75572718	0.76520972	ns
Dark2	Cruise.freq	T1 100 - Control	20.121	13.0172253	164	1.54572112	0.29093791	ns
Dark2	Cruise.freq	T2 100 - Control	21.416	13.0172253	164	1.64520468	0.24498164	ns
Dark2	Cruise.freq	T3 100 - Control	1.17073684	13.2061644	164	0.08865079	0.99700083	ns
Dark3	Cruise.freq	T1 100 - Control	8.392	12.8688831	164	0.65211564	0.82298373	ns
Dark3	Cruise.freq	T2 100 - Control	21.332	12.8688831	164	1.65764191	0.23959255	ns
Dark3	Cruise.freq	T3 100 - Control	6.3951579	13.0556691	164	0.48983762	0.89990336	ns
Light1	Cruise.freq	T1 100 - Control	31.176	13.7396244	164	2.26905767	0.06602756	ns
Light1	Cruise.freq	T2 100 - Control	20.961	13.7396244	164	1.52558756	0.30084523	ns
Light1	Cruise.freq	T3 100 - Control	17.0623158	13.9390488	164	1.22406601	0.47057401	ns
Light2	Cruise.freq	T1 100 - Control	29.8235	13.3038485	164	2.24171976	0.07049553	ns
Light2	Cruise.freq	T2 100 - Control	29.316	13.3038485	164	2.2035729	0.07714846	ns
Light2	Cruise.freq	T3 100 - Control	17.0496842	13.4969478	164	1.26322517	0.44660893	ns
Dark1	Cruising.dur	T1 100 - Control	3.64862	1.93253175	164	1.88800003	0.15409094	ns
Dark1	Cruising.dur	T2 100 - Control	4.07422	1.93253175	164	2.10822927	0.09606639	ns
Dark1	Cruising.dur	T3 100 - Control	2.21006737	1.96058157	164	1.12725091	0.53142589	ns
Dark2	Cruising.dur	T1 100 - Control	3.39682	1.87029583	164	1.81619397	0.17786909	ns

Dark2	Cruising.dur	T2 100 - Control	4.37192	1.87029583	164	2.33755534	0.05586454	ns
Dark2	Cruising.dur	T3 100 - Control	0.45217263	1.89744233	164	0.23830639	0.97723944	ns
Dark3	Cruising.dur	T1 100 - Control	0.71664	1.81490644	164	0.39486333	0.93552952	ns
Dark3	Cruising.dur	T2 100 - Control	3.83764	1.81490644	164	2.1145112	0.09471383	ns
Dark3	Cruising.dur	T3 100 - Control	0.45373474	1.84124899	164	0.24642769	0.97560985	ns
Light1	Cruising.dur	T1 100 - Control	4.40804	1.74631471	164	2.52419566	0.03465775	*
Light1	Cruising.dur	T2 100 - Control	3.99494	1.74631471	164	2.28764036	0.06312789	ns
Light1	Cruising.dur	T3 100 - Control	1.66668211	1.77166167	164	0.94074514	0.65126464	ns
Light2	Cruising.dur	T1 100 - Control	3.69734	1.77151792	164	2.08710279	0.10073008	ns
Light2	Cruising.dur	T2 100 - Control	5.45554	1.77151792	164	3.07958499	0.00698065	**
Light2	Cruising.dur	T3 100 - Control	1.17272421	1.7972307	164	0.65251735	0.82277125	ns
Dark1	Burst.freq	T1 100 - Control	13.726	8.18327997	164	1.67732255	0.23122803	ns
Dark1	Burst.freq	T2 100 - Control	19.2585	8.18327997	164	2.3533962	0.05371289	ns
Dark1	Burst.freq	T3 100 - Control	12.2196842	8.30205657	164	1.47188641	0.32823574	ns
Dark2	Burst.freq	T1 100 - Control	17.14	8.28369104	164	2.06912594	0.10484039	ns
Dark2	Burst.freq	T2 100 - Control	24.5275	8.28369104	164	2.96093853	0.01004979	*
Dark2	Burst.freq	T3 100 - Control	3.74789474	8.40392506	164	0.44596956	0.9172901	ns
Dark3	Burst.freq	T1 100 - Control	3.3645	8.20062619	164	0.41027355	0.93026308	ns
Dark3	Burst.freq	T2 100 - Control	21.0245	8.20062619	164	2.56376763	0.03119442	*
Dark3	Burst.freq	T3 100 - Control	4.2551579	8.31965457	164	0.51145848	0.89076715	ns
Light1	Burst.freq	T1 100 - Control	19.693	7.33090412	164	2.68629895	0.02231835	*
Light1	Burst.freq	T2 100 - Control	22.773	7.33090412	164	3.10643812	0.00641762	**
Light1	Burst.freq	T3 100 - Control	5.808	7.43730887	164	0.78092763	0.75036272	ns
Light2	Burst.freq	T1 100 - Control	15.1625	7.60543848	164	1.99363916	0.12358285	ns
Light2	Burst.freq	T2 100 - Control	27.395	7.60543848	164	3.60202769	0.00122529	**
Light2	Burst.freq	T3 100 - Control	3.54473684	7.71582798	164	0.45941108	0.91212938	ns
Dark1	Bursting.dur	T1 100 - Control	1.19912	0.78931564	164	1.51918946	0.30403542	ns
Dark1	Bursting.dur	T2 100 - Control	1.43962	0.78931564	164	1.82388379	0.17520081	ns
Dark1	Bursting.dur	T3 100 - Control	1.01648842	0.8007722	164	1.26938526	0.44288062	ns
Dark2	Bursting.dur	T1 100 - Control	0.89696	0.69622125	164	1.2883261	0.43149314	ns
Dark2	Bursting.dur	T2 100 - Control	1.71596	0.69622125	164	2.46467629	0.04049662	*
Dark2	Bursting.dur	T3 100 - Control	0.2693179	0.70632658	164	0.38129373	0.93999776	ns
Dark3	Bursting.dur	T1 100 - Control	-0.06356	0.69337277	164	-0.0916679	0.99678946	ns
Dark3	Bursting.dur	T2 100 - Control	1.33214	0.69337277	164	1.9212465	0.14392878	ns
Dark3	Bursting.dur	T3 100 - Control	0.07057684	0.70343676	164	0.10033147	0.99614115	ns
Light1	Bursting.dur	T1 100 - Control	1.26814	0.59275371	164	2.13940458	0.08950501	ns
Light1	Bursting.dur	T2 100 - Control	1.77514	0.59275371	164	2.99473453	0.00906975	**
Light1	Bursting.dur	T3 100 - Control	0.38847158	0.60135726	164	0.64599133	0.82621103	ns
Light2	Bursting.dur	T1 100 - Control	0.9689	0.60637501	164	1.59785609	0.26622596	ns
Light2	Bursting.dur	T2 100 - Control	2.2983	0.60637501	164	3.79022877	0.00062156	**
Light2	Bursting.dur	T3 100 - Control	0.17970526	0.61517627	164	0.29211996	0.96534975	ns
Dark1	Turn.ang	T1 100 - Control	-0.056072	0.26375153	164	-0.212594	0.98201416	ns
Dark1	Turn.ang	T2 100 - Control	0.28090454	0.26375153	164	1.06503476	0.57131702	ns

Dark1	Turn.ang	T3 100 - Control	0.31234609	0.26757976	164	1.1673009	0.50602066	ns
Dark2	Turn.ang	T1 100 - Control	-0.2199493	0.24835259	164	-0.8856333	0.68619409	ns
Dark2	Turn.ang	T2 100 - Control	-0.1034537	0.24835259	164	-0.4165598	0.9280565	ns
Dark2	Turn.ang	T3 100 - Control	-0.0129268	0.25195731	164	-0.0513054	0.9990105	ns
Dark3	Turn.ang	T1 100 - Control	0.20018353	0.2658424	164	0.75301584	0.76679058	ns
Dark3	Turn.ang	T2 100 - Control	0.22274122	0.2658424	164	0.83786944	0.71590045	ns
Dark3	Turn.ang	T3 100 - Control	0.14349644	0.26970098	164	0.53205756	0.88172575	ns
Light1	Turn.ang	T1 100 - Control	-0.159241	0.30900539	164	-0.5153339	0.88909097	ns
Light1	Turn.ang	T2 100 - Control	0.15550853	0.30900539	164	0.50325505	0.89427678	ns
Light1	Turn.ang	T3 100 - Control	-0.1069478	0.31349047	164	-0.3411515	0.95227742	ns
Light2	Turn.ang	T1 100 - Control	-0.0967852	0.31249514	164	-0.3097174	0.9609033	ns
Light2	Turn.ang	T2 100 - Control	0.10140148	0.31249514	164	0.32448978	0.95695848	ns
Light2	Turn.ang	T3 100 - Control	0.16320622	0.31703087	164	0.51479598	0.88932434	ns
Dark1	Ang.vel	T1 100 - Control	-1.4018067	6.59378874	164	-0.212595	0.98201397	ns
Dark1	Ang.vel	T2 100 - Control	7.02261229	6.59378874	164	1.06503447	0.57131721	ns
Dark1	Ang.vel	T3 100 - Control	7.80863951	6.6894946	164	1.16729887	0.50602194	ns
Dark2	Ang.vel	T1 100 - Control	-5.4987312	6.20881357	164	-0.8856332	0.68619416	ns
Dark2	Ang.vel	T2 100 - Control	-2.5863352	6.20881357	164	-0.4165587	0.9280569	ns
Dark2	Ang.vel	T3 100 - Control	-0.32316	6.29893169	164	-0.0513039	0.99901055	ns
Dark3	Ang.vel	T1 100 - Control	5.0045674	6.6460604	164	0.75301263	0.76679245	ns
Dark3	Ang.vel	T2 100 - Control	5.56851205	6.6460604	164	0.8378666	0.71590219	ns
Dark3	Ang.vel	T3 100 - Control	3.58740021	6.74252496	164	0.5320559	0.88172649	ns
Light1	Ang.vel	T1 100 - Control	-3.981028	7.72513574	164	-0.5153344	0.88909078	ns
Light1	Ang.vel	T2 100 - Control	3.88771491	7.72513574	164	0.50325522	0.89427671	ns
Light1	Ang.vel	T3 100 - Control	-2.673692	7.83726259	164	-0.3411513	0.9522775	ns
Light2	Ang.vel	T1 100 - Control	-2.4196325	7.81237937	164	-0.3097177	0.9609032	ns
Light2	Ang.vel	T2 100 - Control	2.53502767	7.81237937	164	0.32448855	0.95695882	ns
Light2	Ang.vel	T3 100 - Control	4.08015202	7.92577252	164	0.5147955	0.88932454	ns
Dark1	Freeze.freq	T1 100 - Control	20.5545	13.07132	164	1.57248848	0.27807894	ns
Dark1	Freeze.freq	T2 100 - Control	22.052	13.07132	164	1.68705227	0.2271667	ns
Dark1	Freeze.freq	T3 100 - Control	10.0625263	13.2610442	164	0.75880347	0.76341208	ns
Dark2	Freeze.freq	T1 100 - Control	19.963	12.9428377	164	1.54239746	0.29255958	ns
Dark2	Freeze.freq	T2 100 - Control	21.2405	12.9428377	164	1.6411007	0.24677741	ns
Dark2	Freeze.freq	T3 100 - Control	1.14642105	13.1306971	164	0.08730847	0.99709249	ns
Dark3	Freeze.freq	T1 100 - Control	8.331	12.7913093	164	0.65130158	0.82341403	ns
Dark3	Freeze.freq	T2 100 - Control	21.121	12.7913093	164	1.65119922	0.24237422	ns
Dark3	Freeze.freq	T3 100 - Control	6.36178947	12.9769694	164	0.49023692	0.89973797	ns
Light1	Freeze.freq	T1 100 - Control	30.9465	13.6627758	164	2.26502289	0.06667168	ns
Light1	Freeze.freq	T2 100 - Control	20.7065	13.6627758	164	1.51554122	0.30586346	ns
Light1	Freeze.freq	T3 100 - Control	17.0166316	13.8610848	164	1.22765511	0.46835945	ns
Light2	Freeze.freq	T1 100 - Control	29.6905	13.2261424	164	2.24483444	0.06997409	ns
Light2	Freeze.freq	T2 100 - Control	29.0705	13.2261424	164	2.19795758	0.0781703	ns
Light2	Freeze.freq	T3 100 - Control	17.0211579	13.4181139	164	1.2685209	0.44340304	ns

Dark1	Freeze.dur	T1 100 - Control	-3.71918	1.92033432	164	-1.9367357	0.13937274	ns
Dark1	Freeze.dur	T2 100 - Control	-4.22008	1.92033432	164	-2.1975757	0.0782402	ns
Dark1	Freeze.dur	T3 100 - Control	-2.3198695	1.94820711	164	-1.1907715	0.49127428	ns
Dark2	Freeze.dur	T1 100 - Control	-3.39682	1.87029583	164	-1.816194	0.17786909	ns
Dark2	Freeze.dur	T2 100 - Control	-4.37192	1.87029583	164	-2.3375553	0.05586454	ns
Dark2	Freeze.dur	T3 100 - Control	-0.4521726	1.89744233	164	-0.2383064	0.97723944	ns
Dark3	Freeze.dur	T1 100 - Control	-0.71664	1.81490644	164	-0.3948633	0.93552952	ns
Dark3	Freeze.dur	T2 100 - Control	-3.83764	1.81490644	164	-2.1145112	0.09471383	ns
Dark3	Freeze.dur	T3 100 - Control	-0.4537347	1.84124899	164	-0.2464277	0.97560985	ns
Light1	Freeze.dur	T1 100 - Control	-4.40804	1.74631471	164	-2.5241957	0.03465775	*
Light1	Freeze.dur	T2 100 - Control	-3.99494	1.74631471	164	-2.2876404	0.06312789	ns
Light1	Freeze.dur	T3 100 - Control	-1.6666821	1.77166167	164	-0.9407451	0.65126464	ns
Light2	Freeze.dur	T1 100 - Control	-3.69734	1.77151792	164	-2.0871028	0.10073008	ns
Light2	Freeze.dur	T2 100 - Control	-5.45554	1.77151792	164	-3.079585	0.00698065	**
Light2	Freeze.dur	T3 100 - Control	-1.1727242	1.7972307	164	-0.6525173	0.82277125	ns
Dark1	Total.Distance	T1 100 - Control	67.3839194	35.9779462	164	1.8729229	0.15887414	ns
Dark1	Total.Distance	T2 100 - Control	74.3959677	35.9779462	164	2.06782142	0.10514382	ns
Dark1	Total.Distance	T3 100 - Control	41.8416199	36.5001498	164	1.14634105	0.51928209	ns
Dark2	Total.Distance	T1 100 - Control	54.9149405	34.3628905	164	1.59808851	0.26611888	ns
Dark2	Total.Distance	T2 100 - Control	80.4868885	34.3628905	164	2.34226188	0.05521775	ns
Dark2	Total.Distance	T3 100 - Control	7.09263684	34.8616523	164	0.20345097	0.98357163	ns
Dark3	Total.Distance	T1 100 - Control	6.22621505	34.0464679	164	0.18287404	0.98680919	ns
Dark3	Total.Distance	T2 100 - Control	66.2903918	34.0464679	164	1.94705636	0.13639903	ns
Dark3	Total.Distance	T3 100 - Control	2.15313796	34.540637	164	0.06233637	0.99853257	ns
Light1	Total.Distance	T1 100 - Control	72.8949735	30.014852	164	2.42863011	0.0444315	*
Light1	Total.Distance	T2 100 - Control	78.2076055	30.014852	164	2.60563022	0.02786413	*
Light1	Total.Distance	T3 100 - Control	25.05075	30.450504	164	0.82267111	0.72521119	ns
Light2	Total.Distance	T1 100 - Control	59.0119812	30.7732694	164	1.91763768	0.14500647	ns
Light2	Total.Distance	T2 100 - Control	104.132097	30.7732694	164	3.38384899	0.00260148	**
Light2	Total.Distance	T3 100 - Control	15.0185637	31.2199295	164	0.48105694	0.90350821	ns

Appendix C: Chapter 3
Supplemental Tables

Chapter 3 Supplemental Tables

Table 3.1. *Treatment groups for imidacloprid, chlorantraniliprole, and binary exposures are shown. We chose Low (1.0 µg/L) and High (5.0 µg/L) concentrations based environmentally relevant concentrations found in monitored waterways and experimental EC50/LC50 values. Each exposure group contained 6 replicate 20 mL scintillation vials for two time points (48h and 96h), and 6 organisms per vial. We randomly selected 4 individuals per vial for a total of n =24 individuals per treatment, per time point.*

Chlorantraniliprole	Imidacloprid	Binary Mixtures	Solvent Control	Control
High	High	High x High	Acetone + Control water	
High	Low	High x Low	Acetone + Control water	
Low	High	Low x High	Acetone + Control water	
Low	Low	Low x Low	Acetone + Control water	
High			Acetone + Control water	
Low			Acetone + Control water	
	Low			Control water
	High			Control water

Table S3.1. *Surface water sampling sites located in Monterey County, Ca. Sites are located immediately downstream of high use areas, where there is a high potential risk of contamination. Sites used for ambient field water collection on 9/14/2019 and 11/26/2019: Quail Creek (Sal_Quail), Alisal Creek (Sal_Hartnell), and the Salinas River (Sal_Davis). California Department of Pesticide Regulation (DPR) site IDs are listed along with associated watershed, waterbody type, site description, latitude/longitude and CA DPR Surface Water Database site identification number (SURF).*

Water body	***DPR Site ID***	***Type***	***Description***	***Latitude***	***Longitude***	***SURF***
Quail Creek	***Sal_Quail***	***Waterway***	***Quail Creek at HWY 101***	***36.6092***	***-121.56269***	***27_7***
Salinas River	***Sal_Davis***	***Waterway***	***Salinas River at Davis Rd***	***36.647***	***-121.70219***	***27_13***
Alisal Creek	***Sal_Hartnell***	***Ag Ditch***	***Alisal Creek at Hartnell Rd***	***36.6435***	***-121.57836***	***27_70***

Table S3.2. *Physicochemical parameters measured in laboratory control water, solvent control water, and for imidacloprid, chlorantraniliprole, and binary exposure treatment groups for exposures. Parameters include Temperature (°C), Electrical conductivity, Dissolved Oxygen (%), pH, Alkalinity (as CaCO3; mg/L), Hardness Alkalinity (as CaCO3; mg/L), and Salinity (ppm). Parameters were measured at test initiation and at test termination (96h).*

Treatment	Temp °C	EC	Dissolved Oxygen (mg/L)	pH	Alkalinity (CaCO3) mg/L	Hardness (CaCO3) mg/L
Test Initiation 2/12/2020						
Control	20	574	8.84	8.25	124	194
Solvent Control	20	552	8.84	8.33	118	182
CHL LOW	20	550	8.82	8.26	124	184
CHL HIGH	20	584	8.86	8.39	125	192
IMI LOW	20	586	8.67	8.41	125	186
IMI HIGH	20	563	8.88	8.4	115	184
Test Termination 2/15/2020						
Control	21.9	559	8.8	7.76	102	170
Solvent Control	21.1	510	9.1	8.36	100	170
CHL LOW	21.7	492	9.7	8.42	106	162

CHL HIGH	21	505	9.09	8.42	110	168
IMI LOW	21	517	8.97	8.27	106	164
IMI HIGH	21.2	499	8.81	8.34	108	168

Table S3.3. *Analytical confirmation of test concentrations determined for chlorantraniliprole and imidacloprid stock solutions for each treatment group (CHL LOW, CHL HIGH, IMI LOW, IMI HIGH, CHL LOW/IMI LOW, CHL HIGH/IMI LOW, CHL LOW/IMI HIGH, CHL HIGH/IMI HIGH, SOLVENT CONTROL, CONTROL). Chemical analysis was completed at the Center for Analytical Chemistry, California Department of Food and Agriculture (Sacramento, CA) using multi-residue liquid chromatography tandem mass spectrometry (LC-MS/MS).*

Sample Number	Treatment	Chlorantraniliprole Result (ppb)	Imidacloprid Result (ppb)
1	CHL LOW	0.986	ND
2	CHL HIGH	5.71	ND
3	IMI LOW	ND	0.85
4	IMI HIGH	ND	4.8
5	CHL LOW | IMI LOW	1.05	0.85
6	CHL HIGH | IMI LOW	5.26	0.947
7	CHL LOW | IMI HIGH	0.996	5.07
8	CHL HIGH | IMI HIGH	5.81	4.93
9	SOLVENT CONTROL	ND	ND
10	CONTROL	ND	ND

Table S3.4. *Pesticide detections and benchmark values (µg/L) detected in ambient surface water collected from sites in Salinas, CA on 09/17/2019, as reported in Stinson et al. 2021. A) Pesticides that exceeded one or more US Environmental Protection Agency (EPA) Aquatic Life Benchmark. B) EPA Aquatic Life Benchmarks and Ecological Risk Assessments for Registered Pesticides updated 09/28/2020. Benchmarks for aquatic life for acute toxicity equal the lowest 48- or 96-hour EC50 or LC50 in a standardized test, and chronic toxicity values equal the lowest NOEAC from a lifecycle or early life stage test). Colors shown in Table S3.4A correspond to aquatic life toxicity benchmarks for each chemical, as shown in Table S3.4B.*

A. Chemical detections (µg/L) exceeding one or more Aquatic Life Benchmark 09/17/2019

	Quail Creek	Alisal Creek	Salinas River
Bifenthrin	0.0025	0.0028	ND
Esfenvalerate/ Fenvalerate	0.0224	ND	ND
Imidacloprid	0.2930	0.5130	0.0140
Lambda Cyhalothrin	0.0797	0.0127	ND
Malathion	0.0240	0.3490	ND
Methomyl	29.9000	1.6400	0.3860

Permethrin Total	0.2340	0.0586	ND
Clothianidin	0.0810	0.1770	ND
Thiamethoxam	3.9900	0.8270	0.0640

B. EPA Aquatic Life Benchmarks for Registered Pesticides (µg/L)

	EPA chronic fish	EPA acute invert	EPA chronic invert
Bifenthrin	0.0400	0.800	0.0013
Esfenvalerate/ Fenvalerate	0.0350	0.0250	0.0170
Imidacloprid	9000.0000	0.3850	0.0100
Lambda Cyhalothrin	0.0310	0.0035	0.0020
Malathion	8.6000	0.0490	0.0600
Methomyl	12.0000	2.5000	0.7000
Permethrin Total	0.0515	0.0195	0.0014
Clothianidin	9700.0000	11.0000	0.0500

Table S3.5. *Pesticide detections and benchmark values (µg/L) detected in ambient surface water collected from sites in Salinas, CA on 11/26/2019. A) Pesticides that exceeded one or more US Environmental Protection Agency (EPA) Aquatic Life Benchmark. B) EPA Aquatic Life Benchmarks and Ecological Risk Assessments for Registered Pesticides updated 09/28/2020. Benchmarks for aquatic life for acute toxicity equal the lowest 48- or 96-hour EC50 or LC50 in a standardized test, and chronic toxicity values equal the lowest NOEAC from a lifecycle or early life stage test). Colors shown in Table S3.4A correspond to aquatic life toxicity benchmarks for each chemical, as shown in Table S3.5B.*

A. Chemical detections (µg/L) exceeding one or more Aquatic Life Benchmark 09/17/2019

	Sal_Quail	Sal_Hartnell	Sal_Davis
Chlorantraniliprole	0.3646	0.51672	0.04127
Chlorpyrifos	ND	0.1283	ND
Clothianidin	0.8990	0.0929	Trace
Imidacloprid	0.3070	0.2925	0.0307
Malathion	0.9986	0.0785	0.0205
Methomyl	0.2467	0.7522	0.0781
Bifenthrin	0.0665	0.0308	0.0197
Fenpropathrin	0.0989	0.0297	ND
Lambda Cyhalothrin	0.0324	0.0761	0.0050
Cyfluthrin	ND	0.0162	0.0055
Esfenvalerate/Fenvale	ND	0.0219	ND
Permethrin Total	0.0328	0.0493	0.0140

B. EPA Aquatic Life

	EPA chronic fish	EPA acute invert	EPA chronic invert
Chlorantraniliprole	110.0000	5.8000	4.4700
Chlorpyrifos	0.5700	0.0500	0.0400
Clothianidin	9700.0000	11.0000	0.0500
Imidacloprid	9000.0000	0.3850	0.0100

Malathion	8.6000	0.0490	0.0600
Methomyl	12.0000	2.5000	0.7000
Bifenthrin	0.0400	0.8000	0.0013
Fenpropathrin	0.0600	0.2650	0.0640
Lambda Cyhalothrin	0.0310	0.0035	0.0020
Cyfluthrin	0.0100	0.0125	0.0074
Esfenvalerate/Fenvale	0.0350	0.0250	0.0170
Permethrin Total	0.0515	0.0195	0.0014

Table S3.6. *Percent Survival of Daphnia magna after 96h of exposure to Low (1.0 µg/L) and High (5.0 µg/L) concentrations of imidacloprid, chlorantraniliprole and binary mixtures. No treatments resulted in significant mortality.*

Sample Number	Treatment	Survival %	S.D.
1	CHL LOW	97.2	6.8
2	CHL HIGH	94.4	13.6
3	IMI LOW	100	0
4	IMI HIGH	97.2	6.8
5	CHL LOW | IMI LOW	83.3	21.1
6	CHL HIGH | IMI LOW	83.3	14.9
7	CHL LOW | IMI HIGH	100	0
8	CHL HIGH | IMI HIGH	100	0
9	SOLVENT CONTROL	97.2	6.8
10	CONTROL	93.3	9.1

Table S3.7. *Survival of D. magna after 96h of exposure to a geometric dilution series of ambient water samples collected on 09/17/2019. Concentrations of field water tested were 100%, 60%, 35%, 20% and 12% for D. magna. Treatments with significant mortality are shown in bolded text. P-values are reported as p > 0.05, p ≤ 0.05, p ≤ 0.01, p ≤ 0.001, p ≤ 0.0001.*

***D. magna* percent survival 09/17/2019**										
Site ID	Concentration of ambient water									
	100%		60%		35%		20%		12%	
Sal_Quail	**0**	**p < 0.0001**	**0**	**p < 0.0001**	**0**	**p < 0.0001**	**0**	**p < 0.0001**	**12.5**	**p < 0.0001**
Sal_Hartnell	**0**	**p < 0.0001**	**0**	**p < 0.0001**	**37.5**	**p < 0.0001**	**72.5**	**p < 0.0001**	85	ns
Sal_Davis	100	ns	100	ns	90	ns	100	ns	90	ns

Table S3.8. *Survival of D. magna after 96h of exposure to a geometric dilution series of ambient water samples collected on 11/26/2019. Concentrations of field water tested were 100%, 60%, 35%, 20% and 12% for D. magna. Treatments with significant mortality are shown in bolded text. P-values are reported as p > 0.05, p ≤ 0.05, p ≤ 0.01, p ≤ 0.001, p ≤ 0.0001.*

***D. magna* percent survival 11/26/2019**								
Site ID	Concentration of ambient water							
	100%		20%		12%		6%	
Quail Creek (Sal_Quail)	**0**	**p < 0.0001**	77.5	ns	87.5	ns	97.5	ns
Hartnell Rd (Sal_Hartnell)	**0**	**p < 0.0001**	**50**	**p < 0.0001**	75	ns	92.5	ns
Salinas River (Sal_Davis)	85	ns	85	ns	85	ns	95	ns

Table S3.7. *Statistical analysis for Daphnia magna behavior (Total Distance, response to light stimuli) following 96 h exposures to Imidacloprid (IMI) and Chlorantraniliprole (CHL) as single chemicals and binary mixtures. We determined significance of mortality data by Analysis of Variance (ANOVA) followed by Tukey's post hoc tests in order to test the significance between treatments.*

Total Distance IMI/CHL

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	15.72	<0.0001	****	Yes	
Row Factor	37.51	<0.0001	****	Yes	
Column Factor	24	<0.0001	****	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	1704986	261	6533	F (261, 6690) = 17.62	P<0.0001
Row Factor	4068249	29	140284	F (29, 6690) = 378.4	P<0.0001
Column Factor	2602838	9	289204	F (9, 6690) = 780.1	P<0.0001
Residual	2480173	6690	370.7		
Data summary					
Number of columns (Column Factor)	10				
Number of rows (Row Factor)	30				
Number of values	6990				
Number of families	5				
Number of comparisons per family	1225				
Alpha	0.05				

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Summary	Adjusted P Value
Control 6-10 vs. Solvent Control 6-10	19.65	-2.482 to 41.78	ns	0.2046
Control 6-10 vs. CHL High 6-10	54.95	32.82 to 77.08	****	<0.0001
Control 6-10 vs. CHL Low 6-10	47.83	25.70 to 69.96	****	<0.0001
Control 6-10 vs. IMI High 6-10	86.41	64.28 to 108.5	****	<0.0001
Control 6-10 vs. IMI Low 6-10	103.9	81.76 to 126.0	****	<0.0001
Control 6-10 vs. CHL High*IMI High 6-10	47.89	24.71 to 71.08	****	<0.0001
Control 6-10 vs. CHL High*IMI Low 6-10	44.26	21.64 to 66.87	****	<0.0001

Control 6-10 vs. CHL Low*IMI High 6-10	39.71	17.58 to 61.84	****	<0.0001
Control 6-10 vs. CHL Low*IMI Low 6-10	75.64	53.51 to 97.77	****	<0.0001
Control 11-15 vs. Solvent Control 11-15	19.89	-2.243 to 42.01	ns	0.1818
Control 11-15 vs. CHL High 11-15	5.863	-16.27 to 27.99	ns	>0.9999
Control 11-15 vs. CHL Low 11-15	14.7	-7.428 to 36.83	ns	0.8817
Control 11-15 vs. IMI High 11-15	12.92	-9.211 to 35.05	ns	0.9812
Control 11-15 vs. IMI low 11-15	37.8	15.67 to 59.93	****	<0.0001
Control 11-15 vs. CHL High*IMI High 11-15	-5.492	-28.68 to 17.69	ns	>0.9999
Control 11-15 vs. CHL High*IMI Low 11-15	-18.91	-41.52 to 3.706	ns	0.3405
Control 11-15 vs. CHL Low*IMI High 11-15	-3.33	-25.46 to 18.80	ns	>0.9999
Control 11-15 vs. CHL Low*IMI Low 11-15	21.23	-0.8976 to 43.36	ns	0.087
Control 16-20 vs. Solvent Control 16-20	15.46	-6.669 to 37.59	ns	0.7978
Control 16-20 vs. CHL High 16-20	13.17	-8.958 to 35.30	ns	0.9741
Control 16-20 vs. CHL Low 16-20	12.42	-9.712 to 34.55	ns	0.9906
Control 16-20 vs. IMI High 16-20	49.09	26.97 to 71.22	****	<0.0001
Control 16-20 vs. IMI low 16-20	31.06	8.929 to 53.19	****	<0.0001
Control 16-20 vs. CHL High*IMI High 16-20	-5.638	-28.82 to 17.55	ns	>0.9999
Control 16-20 vs. CHL High*IMI Low 16-20	-21.26	-43.87 to 1.360	ns	0.1117
Control 16-20 vs. CHL Low*IMI High 16-20	22.34	0.2074 to 44.46	*	0.0437
Control 16-20 vs. CHL Low*IMI Low 16-20	-14.63	-36.76 to 7.495	ns	0.8879
Control 21-25 vs. Solvent Control 21-25	28.13	5.998 to 50.26	***	0.0004
Control 21-25 vs. CHL High 21-25	26.71	4.579 to 48.84	**	0.0016
Control 21-25 vs. CHL Low 21-25	38.92	16.79 to 61.05	****	<0.0001
Control 21-25 vs. IMI High 21-25	54.45	32.32 to 76.57	****	<0.0001
Control 21-25 vs. IMI low 21-25	63.88	41.75 to 86.01	****	<0.0001
Control 21-25 vs. CHL High*IMI High 21-25	20.55	-2.634 to 43.74	ns	0.2078
Control 21-25 vs. CHL High*IMI Low 21-25	32.9	10.28 to 55.51	****	<0.0001
Control 21-25 vs. CHL Low*IMI High 21-25	71.43	49.30 to 93.56	****	<0.0001
Control 21-25 vs. CHL Low*IMI Low 21-25	43.48	21.35 to 65.61	****	<0.0001
Control 26-30 vs. Solvent Control 26-30	52.16	30.03 to 74.29	****	<0.0001
Control 26-30 vs. CHL High 26-30	33.13	11.00 to 55.25	****	<0.0001
Control 26-30 vs. CHL Low 26-30	55.98	33.85 to 78.11	****	<0.0001
Control 26-30 vs. IMI High 26-30	56.86	34.73 to 78.98	****	<0.0001
Control 26-30 vs. IMI low 26-30	81.02	58.90 to 103.2	****	<0.0001
Control 26-30 vs. CHL High*IMI High 26-30	33.02	9.835 to 56.21	****	<0.0001
Control 26-30 vs. CHL High*IMI Low 26-30	-1.686	-24.30 to 20.93	ns	>0.9999
Control 26-30 vs. CHL Low*IMI High 26-30	73.83	51.70 to 95.96	****	<0.0001
Control 26-30 vs. CHL Low*IMI Low 26-30	76.82	54.69 to 98.95	****	<0.0001
Solvent Control 6-10 vs. CHL High 6-10	35.31	13.41 to 57.20	****	<0.0001
Solvent Control 6-10 vs. CHL Low 6-10	28.18	6.290 to 50.07	***	0.0003

Solvent Control 6-10 vs. IMI High 6-10	66.76	44.87 to 88.65	****	<0.0001
Solvent Control 6-10 vs. IMI Low 6-10	84.24	62.35 to 106.1	****	<0.0001
Solvent Control 6-10 vs. CHL High*IMI High 6-10	28.25	5.287 to 51.21	***	0.001
Solvent Control 6-10 vs. CHL High*IMI Low 6-10	24.61	2.227 to 46.99	*	0.0107
Solvent Control 6-10 vs. CHL Low*IMI High 6-10	20.06	-1.828 to 41.96	ns	0.1482
Solvent Control 6-10 vs. CHL Low*IMI Low 6-10	55.99	34.10 to 77.89	****	<0.0001
Solvent Control 11-15 vs. CHL High 11-15	-14.02	-35.91 to 7.869	ns	0.9246
Solvent Control 11-15 vs. CHL Low 11-15	-5.185	-27.08 to 16.71	ns	>0.9999
Solvent Control 11-15 vs. IMI High 11-15	-6.968	-28.86 to 14.92	ns	>0.9999
Solvent Control 11-15 vs. IMI low 11-15	17.92	-3.975 to 39.81	ns	0.3961
Solvent Control 11-15 vs. CHL High*IMI High 11-15	-25.38	-48.34 to -2.418	**	0.0097
Solvent Control 11-15 vs. CHL High*IMI Low 11-15	-38.8	-61.18 to -16.41	****	<0.0001
Solvent Control 11-15 vs. CHL Low*IMI High 11-15	-23.22	-45.11 to -1.324	*	0.0201
Solvent Control 11-15 vs. CHL Low*IMI Low 11-15	1.345	-20.55 to 23.24	ns	>0.9999
Solvent Control 16-20 vs. CHL High 16-20	-2.289	-24.18 to 19.60	ns	>0.9999
Solvent Control 16-20 vs. CHL Low 16-20	-3.043	-24.93 to 18.85	ns	>0.9999
Solvent Control 16-20 vs. IMI High 16-20	33.63	11.74 to 55.53	****	<0.0001
Solvent Control 16-20 vs. IMI low 16-20	15.6	-6.294 to 37.49	ns	0.7572
Solvent Control 16-20 vs. CHL High*IMI High 16-20	-21.1	-44.06 to 1.862	ns	0.1441
Solvent Control 16-20 vs. CHL High*IMI Low 16-20	-36.72	-59.10 to -14.33	****	<0.0001
Solvent Control 16-20 vs. CHL Low*IMI High 16-20	6.876	-15.02 to 28.77	ns	>0.9999
Solvent Control 16-20 vs. CHL Low*IMI Low 16-20	-30.09	-51.99 to -8.202	****	<0.0001
Solvent Control 21-25 vs. CHL High 21-25	-1.419	-23.31 to 20.47	ns	>0.9999
Solvent Control 21-25 vs. CHL Low 21-25	10.79	-11.10 to 32.68	ns	0.9994
Solvent Control 21-25 vs. IMI High 21-25	26.32	4.426 to 48.21	**	0.0017
Solvent Control 21-25 vs. IMI low 21-25	35.76	13.86 to 57.65	****	<0.0001
Solvent Control 21-25 vs. CHL High*IMI High 21-25	-7.575	-30.54 to 15.39	ns	>0.9999
Solvent Control 21-25 vs. CHL High*IMI Low 21-25	4.772	-17.61 to 27.16	ns	>0.9999
Solvent Control 21-25 vs. CHL Low*IMI High 21-25	43.31	21.41 to 65.20	****	<0.0001
Solvent Control 21-25 vs. CHL Low*IMI Low 21-25	15.36	-6.537 to 37.25	ns	0.79
Solvent Control 26-30 vs. CHL High 26-30	-19.04	-40.93 to 2.857	ns	0.2478
Solvent Control 26-30 vs. CHL Low 26-30	3.822	-18.07 to 25.71	ns	>0.9999
Solvent Control 26-30 vs. IMI High 26-30	4.696	-17.20 to 26.59	ns	>0.9999
Solvent Control 26-30 vs. IMI low 26-30	28.86	6.972 to 50.76	***	0.0002
Solvent Control 26-30 vs. CHL High*IMI High 26-30	-19.14	-42.10 to 3.821	ns	0.3482
Solvent Control 26-30 vs. CHL High*IMI Low 26-30	-53.85	-76.23 to -31.46	****	<0.0001
Solvent Control 26-30 vs. CHL Low*IMI High 26-30	21.67	-0.2199 to 43.56	ns	0.0576
Solvent Control 26-30 vs. CHL Low*IMI Low 26-30	24.66	2.765 to 46.55	**	0.0068

Table Analyzed	pmr

Data sets analyzed	A-J

ANOVA summary	
F	8.924
P value	<0.0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0.2648

Brown-Forsythe test	
F (DFn, DFd)	
P value	
P value summary	
Are SDs significantly different (P < 0.05)?	

Bartlett's test

Bartlett's statistic (corrected)	556.9
P value	<0.0001
P value summary	****
Are SDs significantly different (P < 0.05)?	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	3151024	9	350114	F (9, 223) = 8.924	P<0.0001
Residual (within columns)	8748630	223	39232		
Total	11899654	232			

Data summary	
Number of treatments (columns)	10
Number of values (total)	233
Number of families	1

Number of comparisons per family	9
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	A-?	
Contol vs. Solvent Control	16.51	-139.9 to 173.0	No	ns	0.9996	B	Solvent Control
Contol vs. CHL High	-44.44	-200.9 to 112.0	No	ns	0.9788	C	CHL High
Contol vs. CHL Low	-41.04	-197.5 to 115.4	No	ns	0.9879	D	CHL Low
Contol vs. IMI High	-35.33	-191.8 to 121.1	No	ns	0.9936	E	IMI High
Contol vs. IMI Low	-57.29	-213.7 to 99.16	No	ns	0.9108	F	IMI Low
Contol vs. CHL low IMI low	177.3	20.86 to 333.8	Yes	*	0.018	G	CHL low IMI low
Contol vs. CHL high IMI low	-313	-472.9 to -153.1	Yes	****	<0.0001	H	CHL high IMI low
Contol vs. CHL low IMI high	-144.9	-301.3 to 11.56	No	ns	0.0833	I	CHL low IMI high
Contol vs. CHL high IMI high	-57.29	-221.2 to 106.6	No	ns	0.9297	J	CHL high IMI high

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Contol vs. Solvent Control	-44.6	-61.1	16.51	57.8	23	24	0.2856	223
Contol vs. CHL High	-44.6	-0.161	-44.44	57.8	23	24	0.7689	223
Contol vs. CHL Low	-44.6	-3.562	-41.04	57.8	23	24	0.71	223
Contol vs. IMI High	-44.6	-9.27	-35.33	57.8	23	24	0.6113	223
Contol vs. IMI Low	-44.6	12.7	-57.29	57.8	23	24	0.9913	223
Contol vs. CHL low IMI low	-44.6	-221.9	177.3	57.8	23	24	3.068	223
Contol vs. CHL high IMI low	-44.6	268.4	-313	59.07	23	22	5.3	223
Contol vs. CHL low IMI high	-44.6	100.3	-144.9	57.8	23	24	2.507	223
Contol vs. CHL high IMI high	-44.6	12.7	-57.29	60.56	23	20	0.9461	223

Table S3.8. *Statistical analysis for Daphnia magna behavior (Total Distance, response to light stimulus) following 96 h exposures to contaminated surface waters prior to a first flush rain event (September 17th, 2019). We determined significance of mortality data by Analysis of Variance (ANOVA) followed by Tukey's post hoc tests in order to test the significance between treatments.*

Table Analyzed	September TD DM				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	5.272	<0.0001	****	Yes	
Row Factor	2.66	<0.0001	****	Yes	
Column Factor	14.14	<0.0001	****	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	546432	24	22768	F (24, 939) = 2.828	P<0.0001
Row Factor	275690	4	68922	F (4, 939) = 8.560	P<0.0001
Column Factor	1464941	6	244157	F (6, 939) = 30.33	P<0.0001
Residual	7560107	939	8051		
Data summary					
Number of columns (Column Factor)	7				
Number of rows (Row Factor)	5				
Number of values	974				
Within each row, compare columns (simple effects within rows)					
Number of families	5				
Number of comparisons per family	21				
Alpha	0.05				

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Summary	Adjusted P Value

Dark 06:00 - 10:00

Control vs. Hartnell Rd. [12]	30.16	-32.78 to 93.09	ns	0.7933
Control vs. Hartnell Rd. [20]	70.7	-28.11 to 169.5	ns	0.345
Control vs. Hartnell Rd. [35]	17.97	-85.66 to 121.6	ns	0.9987
Control vs. Davis Rd. [12]	6.463	-57.43 to 70.36	ns	>0.9999
Control vs. Davis Rd. [20]	8.123	-52.43 to 68.68	ns	0.9997
Control vs. Davis Rd. [35]	19.02	-44.87 to 82.92	ns	0.9755
Hartnell Rd. [12] vs. Hartnell Rd. [20]	40.54	-58.55 to 139.6	ns	0.8909
Hartnell Rd. [12] vs. Hartnell Rd. [35]	-12.19	-116.1 to 91.71	ns	0.9999
Hartnell Rd. [12] vs. Davis Rd. [12]	-23.7	-88.03 to 40.63	ns	0.9316
Hartnell Rd. [12] vs. Davis Rd. [20]	-22.04	-83.05 to 38.98	ns	0.9375
Hartnell Rd. [12] vs. Davis Rd. [35]	-11.13	-75.46 to 53.20	ns	0.9987
Hartnell Rd. [20] vs. Hartnell Rd. [35]	-52.73	-181.6 to 76.10	ns	0.8906
Hartnell Rd. [20] vs. Davis Rd. [12]	-64.23	-163.9 to 35.47	ns	0.4783
Hartnell Rd. [20] vs. Davis Rd. [20]	-62.57	-160.2 to 35.02	ns	0.4845
Hartnell Rd. [20] vs. Davis Rd. [35]	-51.67	-151.4 to 48.03	ns	0.7258
Hartnell Rd. [35] vs. Davis Rd. [12]	-11.5	-116.0 to 92.98	ns	>0.9999
Hartnell Rd. [35] vs. Davis Rd. [20]	-9.844	-112.3 to 92.63	ns	>0.9999
Hartnell Rd. [35] vs. Davis Rd. [35]	1.057	-103.4 to 105.5	ns	>0.9999
Davis Rd. [12] vs. Davis Rd. [20]	1.66	-60.34 to 63.66	ns	>0.9999
Davis Rd. [12] vs. Davis Rd. [35]	12.56	-52.71 to 77.83	ns	0.9976
Davis Rd. [20] vs. Davis Rd. [35]	10.9	-51.10 to 72.91	ns	0.9986

Light 10:00 - 15:00

Control vs. Hartnell Rd. [12]	-26.57	-89.51 to 36.36	ns	0.8752
Control vs. Hartnell Rd. [20]	-30.73	-129.5 to 68.08	ns	0.9696
Control vs. Hartnell Rd. [35]	-53.9	-157.5 to 49.73	ns	0.7225
Control vs. Davis Rd. [12]	-30	-93.90 to 33.89	ns	0.8087
Control vs. Davis Rd. [20]	-69.24	-129.8 to -8.684	*	0.0134
Control vs. Davis Rd. [35]	-74.92	-138.8 to -11.02	**	0.01
Hartnell Rd. [12] vs. Hartnell Rd. [20]	-4.159	-103.2 to 94.93	ns	>0.9999
Hartnell Rd. [12] vs. Hartnell Rd. [35]	-27.33	-131.2 to 76.57	ns	0.9871
Hartnell Rd. [12] vs. Davis Rd. [12]	-3.429	-67.76 to 60.90	ns	>0.9999
Hartnell Rd. [12] vs. Davis Rd. [20]	-42.67	-103.7 to 18.35	ns	0.3738
Hartnell Rd. [12] vs. Davis Rd. [35]	-48.35	-112.7 to 15.98	ns	0.2853
Hartnell Rd. [20] vs. Hartnell Rd. [35]	-23.17	-152.0 to 105.7	ns	0.9984
Hartnell Rd. [20] vs. Davis Rd. [12]	0.7306	-98.97 to 100.4	ns	>0.9999

Hartnell Rd. [20] vs. Davis Rd. [20]	-38.51	-136.1 to 59.09	ns	0.9067
Hartnell Rd. [20] vs. Davis Rd. [35]	-44.19	-143.9 to 55.51	ns	0.8476
Hartnell Rd. [35] vs. Davis Rd. [12]	23.9	-80.58 to 128.4	ns	0.9939
Hartnell Rd. [35] vs. Davis Rd. [20]	-15.34	-117.8 to 87.13	ns	0.9994
Hartnell Rd. [35] vs. Davis Rd. [35]	-21.02	-125.5 to 83.46	ns	0.997
Davis Rd. [12] vs. Davis Rd. [20]	-39.24	-101.2 to 22.76	ns	0.501
Davis Rd. [12] vs. Davis Rd. [35]	-44.92	-110.2 to 20.35	ns	0.3941
Davis Rd. [20] vs. Davis Rd. [35]	-5.679	-67.68 to 56.32	ns	>0.9999

Light 16:00 - 20:00

Control vs. Hartnell Rd. [12]	-11.28	-74.22 to 51.65	ns	0.9984
Control vs. Hartnell Rd. [20]	64.8	-34.01 to 163.6	ns	0.4556
Control vs. Hartnell Rd. [35]	-0.4936	-104.1 to 103.1	ns	>0.9999
Control vs. Davis Rd. [12]	-52.95	-116.8 to 10.94	ns	0.1797
Control vs. Davis Rd. [20]	-118.7	-179.3 to -58.14	****	<0.0001
Control vs. Davis Rd. [35]	-105.1	-169.0 to -41.21	****	<0.0001
Hartnell Rd. [12] vs. Hartnell Rd. [20]	76.08	-23.01 to 175.2	ns	0.2604
Hartnell Rd. [12] vs. Hartnell Rd. [35]	10.79	-93.11 to 114.7	ns	>0.9999
Hartnell Rd. [12] vs. Davis Rd. [12]	-41.67	-106.0 to 22.66	ns	0.4714
Hartnell Rd. [12] vs. Davis Rd. [20]	-107.4	-168.4 to -46.40	****	<0.0001
Hartnell Rd. [12] vs. Davis Rd. [35]	-93.82	-158.2 to -29.49	***	0.0004
Hartnell Rd. [20] vs. Hartnell Rd. [35]	-65.29	-194.1 to 63.53	ns	0.7464
Hartnell Rd. [20] vs. Davis Rd. [12]	-117.8	-217.5 to -18.05	**	0.0091
Hartnell Rd. [20] vs. Davis Rd. [20]	-183.5	-281.1 to -85.91	****	<0.0001
Hartnell Rd. [20] vs. Davis Rd. [35]	-169.9	-269.6 to -70.21	****	<0.0001
Hartnell Rd. [35] vs. Davis Rd. [12]	-52.46	-156.9 to 52.02	ns	0.7547
Hartnell Rd. [35] vs. Davis Rd. [20]	-118.2	-220.7 to -15.73	*	0.0121
Hartnell Rd. [35] vs. Davis Rd. [35]	-104.6	-209.1 to -0.1306	*	0.0495
Davis Rd. [12] vs. Davis Rd. [20]	-65.74	-127.7 to -3.741	*	0.0295
Davis Rd. [12] vs. Davis Rd. [35]	-52.15	-117.4 to 13.12	ns	0.2166
Davis Rd. [20] vs. Davis Rd. [35]	13.59	-48.41 to 75.60	ns	0.9951

Light 20:00 - 25:00

Control vs. Hartnell Rd. [12]	-23.35	-86.75 to 40.05	ns	0.9316
Control vs. Hartnell Rd. [20]	74.76	-24.05 to 173.6	ns	0.2774
Control vs. Hartnell Rd. [35]	31.34	-72.29 to 135.0	ns	0.9736
Control vs. Davis Rd. [12]	-45.64	-109.5 to 18.26	ns	0.3472

Control vs. Davis Rd. [20]	-116.9	-177.4 to -56.31	****	<0.0001
Control vs. Davis Rd. [35]	-101.8	-165.7 to -37.94	****	<0.0001
Hartnell Rd. [12] vs. Hartnell Rd. [20]	98.11	-1.277 to 197.5	ns	0.0557
Hartnell Rd. [12] vs. Hartnell Rd. [35]	54.69	-49.49 to 158.9	ns	0.7136
Hartnell Rd. [12] vs. Davis Rd. [12]	-22.29	-87.07 to 42.50	ns	0.9503
Hartnell Rd. [12] vs. Davis Rd. [20]	-93.52	-155.0 to -32.02	***	0.0002
Hartnell Rd. [12] vs. Davis Rd. [35]	-78.49	-143.3 to -13.70	**	0.0066
Hartnell Rd. [20] vs. Hartnell Rd. [35]	-43.42	-172.2 to 85.41	ns	0.9549
Hartnell Rd. [20] vs. Davis Rd. [12]	-120.4	-220.1 to -20.70	**	0.0069
Hartnell Rd. [20] vs. Davis Rd. [20]	-191.6	-289.2 to -94.03	****	<0.0001
Hartnell Rd. [20] vs. Davis Rd. [35]	-176.6	-276.3 to -76.90	****	<0.0001
Hartnell Rd. [35] vs. Davis Rd. [12]	-76.97	-181.5 to 27.51	ns	0.3091
Hartnell Rd. [35] vs. Davis Rd. [20]	-148.2	-250.7 to -45.73	***	0.0004
Hartnell Rd. [35] vs. Davis Rd. [35]	-133.2	-237.7 to -28.69	**	0.0033
Davis Rd. [12] vs. Davis Rd. [20]	-71.23	-133.2 to -9.228	*	0.0127
Davis Rd. [12] vs. Davis Rd. [35]	-56.2	-121.5 to 9.068	ns	0.1449
Davis Rd. [20] vs. Davis Rd. [35]	15.03	-46.97 to 77.03	ns	0.9916

Light 26:00 - 30:00

Control vs. Hartnell Rd. [12]	-4.663	-67.60 to 58.27	ns	>0.9999
Control vs. Hartnell Rd. [20]	48.68	-50.12 to 147.5	ns	0.7709
Control vs. Hartnell Rd. [35]	65.59	-38.03 to 169.2	ns	0.5007
Control vs. Davis Rd. [12]	-36.41	-100.3 to 27.49	ns	0.6273
Control vs. Davis Rd. [20]	-123.5	-184.0 to -62.93	****	<0.0001
Control vs. Davis Rd. [35]	-100.6	-164.4 to -36.66	****	<0.0001
Hartnell Rd. [12] vs. Hartnell Rd. [20]	53.35	-45.74 to 152.4	ns	0.6884
Hartnell Rd. [12] vs. Hartnell Rd. [35]	70.26	-33.64 to 174.2	ns	0.4165
Hartnell Rd. [12] vs. Davis Rd. [12]	-31.74	-96.07 to 32.59	ns	0.7696
Hartnell Rd. [12] vs. Davis Rd. [20]	-118.8	-179.8 to -57.81	****	<0.0001
Hartnell Rd. [12] vs. Davis Rd. [35]	-95.89	-160.2 to -31.56	***	0.0002
Hartnell Rd. [20] vs. Hartnell Rd. [35]	16.91	-111.9 to 145.7	ns	0.9997
Hartnell Rd. [20] vs. Davis Rd. [12]	-85.09	-184.8 to 14.61	ns	0.1526
Hartnell Rd. [20] vs. Davis Rd. [20]	-172.2	-269.8 to -74.58	****	<0.0001
Hartnell Rd. [20] vs. Davis Rd. [35]	-149.2	-248.9 to -49.54	***	0.0002
Hartnell Rd. [35] vs. Davis Rd. [12]	-102	-206.5 to 2.481	ns	0.0609
Hartnell Rd. [35] vs. Davis Rd. [20]	-189.1	-291.6 to -86.61	****	<0.0001

Hartnell Rd. [35] vs. Davis Rd. [35]	-166.1	-270.6 to -61.67	****	<0.0001
Davis Rd. [12] vs. Davis Rd. [20]	-87.08	-149.1 to -25.08	***	0.0007
Davis Rd. [12] vs. Davis Rd. [35]	-64.15	-129.4 to 1.121	ns	0.0577
Davis Rd. [20] vs. Davis Rd. [35]	22.93	-39.07 to 84.94	ns	0.9303

Test details	Predicted (LS) mean 1	Predicted (LS) mean 2	Predicted (LS) mean diff.	SE of diff.	N1	N2	q	DF
Row 1								
Control vs. Hartnell Rd. [12]	191.6	161.4	30.16	21.3	36	35	2.002	939
Control vs. Hartnell Rd. [20]	191.6	120.9	70.7	33.44	36	9	2.99	939
Control vs. Hartnell Rd. [35]	191.6	173.6	17.97	35.07	36	8	0.7245	939
Control vs. Davis Rd. [12]	191.6	185.1	6.463	21.62	36	33	0.4227	939
Control vs. Davis Rd. [20]	191.6	183.5	8.123	20.49	36	41	0.5605	939
Control vs. Davis Rd. [35]	191.6	172.6	19.02	21.62	36	33	1.244	939
Hartnell Rd. [12] vs. Hartnell Rd. [20]	161.4	120.9	40.54	33.54	35	9	1.71	939
Hartnell Rd. [12] vs. Hartnell Rd. [35]	161.4	173.6	-12.19	35.16	35	8	0.4903	939
Hartnell Rd. [12] vs. Davis Rd. [12]	161.4	185.1	-23.7	21.77	35	33	1.539	939
Hartnell Rd. [12] vs. Davis Rd. [20]	161.4	183.5	-22.04	20.65	35	41	1.509	939
Hartnell Rd. [12] vs. Davis Rd. [35]	161.4	172.6	-11.13	21.77	35	33	0.7233	939
Hartnell Rd. [20] vs. Hartnell Rd. [35]	120.9	173.6	-52.73	43.6	9	8	1.71	939
Hartnell Rd. [20] vs. Davis Rd. [12]	120.9	185.1	-64.23	33.74	9	33	2.692	939
Hartnell Rd. [20] vs. Davis Rd. [20]	120.9	183.5	-62.57	33.03	9	41	2.679	939
Hartnell Rd. [20] vs. Davis Rd. [35]	120.9	172.6	-51.67	33.74	9	33	2.166	939
Hartnell Rd. [35] vs. Davis Rd. [12]	173.6	185.1	-11.5	35.36	8	33	0.4601	939
Hartnell Rd. [35] vs. Davis Rd. [20]	173.6	183.5	-9.844	34.68	8	41	0.4014	939
Hartnell Rd. [35] vs. Davis Rd. [35]	173.6	172.6	1.057	35.36	8	33	0.04227	939
Davis Rd. [12] vs. Davis Rd. [20]	185.1	183.5	1.66	20.98	33	41	0.1119	939
Davis Rd. [12] vs. Davis Rd. [35]	185.1	172.6	12.56	22.09	33	33	0.8042	939
Davis Rd. [20] vs. Davis Rd. [35]	183.5	172.6	10.9	20.98	41	33	0.7347	939
Row 2								
Control vs. Hartnell Rd. [12]	156.9	183.5	-26.57	21.3	36	35	1.764	939
Control vs. Hartnell Rd. [20]	156.9	187.6	-30.73	33.44	36	9	1.3	939
Control vs. Hartnell Rd. [35]	156.9	210.8	-53.9	35.07	36	8	2.173	939
Control vs. Davis Rd. [12]	156.9	186.9	-30	21.62	36	33	1.962	939

Control vs. Davis Rd. [20]	156.9	226.1	-69.24	20.49	36	41	4.778	939
Control vs. Davis Rd. [35]	156.9	231.8	-74.92	21.62	36	33	4.9	939
Hartnell Rd. [12] vs. Hartnell Rd. [20]	183.5	187.6	-4.159	33.54	35	9	0.1754	939
Hartnell Rd. [12] vs. Hartnell Rd. [35]	183.5	210.8	-27.33	35.16	35	8	1.099	939
Hartnell Rd. [12] vs. Davis Rd. [12]	183.5	186.9	-3.429	21.77	35	33	0.2227	939
Hartnell Rd. [12] vs. Davis Rd. [20]	183.5	226.1	-42.67	20.65	35	41	2.922	939
Hartnell Rd. [12] vs. Davis Rd. [35]	183.5	231.8	-48.35	21.77	35	33	3.14	939
Hartnell Rd. [20] vs. Hartnell Rd. [35]	187.6	210.8	-23.17	43.6	9	8	0.7515	939
Hartnell Rd. [20] vs. Davis Rd. [12]	187.6	186.9	0.7306	33.74	9	33	0.0306 2	939
Hartnell Rd. [20] vs. Davis Rd. [20]	187.6	226.1	-38.51	33.03	9	41	1.649	939
Hartnell Rd. [20] vs. Davis Rd. [35]	187.6	231.8	-44.19	33.74	9	33	1.852	939
Hartnell Rd. [35] vs. Davis Rd. [12]	210.8	186.9	23.9	35.36	8	33	0.9558	939
Hartnell Rd. [35] vs. Davis Rd. [20]	210.8	226.1	-15.34	34.68	8	41	0.6256	939
Hartnell Rd. [35] vs. Davis Rd. [35]	210.8	231.8	-21.02	35.36	8	33	0.8407	939
Davis Rd. [12] vs. Davis Rd. [20]	186.9	226.1	-39.24	20.98	33	41	2.644	939
Davis Rd. [12] vs. Davis Rd. [35]	186.9	231.8	-44.92	22.09	33	33	2.876	939
Davis Rd. [20] vs. Davis Rd. [35]	226.1	231.8	-5.679	20.98	41	33	0.3827	939
Row 3								
Control vs. Hartnell Rd. [12]	172.2	183.5	-11.28	21.3	36	35	0.7491	939
Control vs. Hartnell Rd. [20]	172.2	107.4	64.8	33.44	36	9	2.741	939
Control vs. Hartnell Rd. [35]	172.2	172.7	-0.4936	35.07	36	8	0.0199	939
Control vs. Davis Rd. [12]	172.2	225.2	-52.95	21.62	36	33	3.463	939
Control vs. Davis Rd. [20]	172.2	290.9	-118.7	20.49	36	41	8.191	939
Control vs. Davis Rd. [35]	172.2	277.3	-105.1	21.62	36	33	6.874	939
Hartnell Rd. [12] vs. Hartnell Rd. [20]	183.5	107.4	76.08	33.54	35	9	3.208	939
Hartnell Rd. [12] vs. Hartnell Rd. [35]	183.5	172.7	10.79	35.16	35	8	0.4339	939
Hartnell Rd. [12] vs. Davis Rd. [12]	183.5	225.2	-41.67	21.77	35	33	2.707	939
Hartnell Rd. [12] vs. Davis Rd. [20]	183.5	290.9	-107.4	20.65	35	41	7.357	939
Hartnell Rd. [12] vs. Davis Rd. [35]	183.5	277.3	-93.82	21.77	35	33	6.094	939
Hartnell Rd. [20] vs. Hartnell Rd. [35]	107.4	172.7	-65.29	43.6	9	8	2.118	939
Hartnell Rd. [20] vs. Davis Rd. [12]	107.4	225.2	-117.8	33.74	9	33	4.935	939
Hartnell Rd. [20] vs. Davis Rd. [20]	107.4	290.9	-183.5	33.03	9	41	7.857	939
Hartnell Rd. [20] vs. Davis Rd. [35]	107.4	277.3	-169.9	33.74	9	33	7.121	939
Hartnell Rd. [35] vs. Davis Rd. [12]	172.7	225.2	-52.46	35.36	8	33	2.098	939
Hartnell Rd. [35] vs. Davis Rd. [20]	172.7	290.9	-118.2	34.68	8	41	4.82	939

Hartnell Rd. [35] vs. Davis Rd. [35]	172.7	277.3	-104.6	35.36	8	33	4.184	939
Davis Rd. [12] vs. Davis Rd. [20]	225.2	290.9	-65.74	20.98	33	41	4.431	939
Davis Rd. [12] vs. Davis Rd. [35]	225.2	277.3	-52.15	22.09	33	33	3.339	939
Davis Rd. [20] vs. Davis Rd. [35]	290.9	277.3	13.59	20.98	41	33	0.916	939
Row 4								
Control vs. Hartnell Rd. [12]	202.3	225.7	-23.35	21.46	36	34	1.539	939
Control vs. Hartnell Rd. [20]	202.3	127.6	74.76	33.44	36	9	3.162	939
Control vs. Hartnell Rd. [35]	202.3	171	31.34	35.07	36	8	1.264	939
Control vs. Davis Rd. [12]	202.3	248	-45.64	21.62	36	33	2.985	939
Control vs. Davis Rd. [20]	202.3	319.2	-116.9	20.49	36	41	8.065	939
Control vs. Davis Rd. [35]	202.3	304.2	-101.8	21.62	36	33	6.66	939
Hartnell Rd. [12] vs. Hartnell Rd. [20]	225.7	127.6	98.11	33.64	34	9	4.125	939
Hartnell Rd. [12] vs. Hartnell Rd. [35]	225.7	171	54.69	35.26	34	8	2.193	939
Hartnell Rd. [12] vs. Davis Rd. [12]	225.7	248	-22.29	21.93	34	33	1.437	939
Hartnell Rd. [12] vs. Davis Rd. [20]	225.7	319.2	-93.52	20.81	34	41	6.355	939
Hartnell Rd. [12] vs. Davis Rd. [35]	225.7	304.2	-78.49	21.93	34	33	5.062	939
Hartnell Rd. [20] vs. Hartnell Rd. [35]	127.6	171	-43.42	43.6	9	8	1.408	939
Hartnell Rd. [20] vs. Davis Rd. [12]	127.6	248	-120.4	33.74	9	33	5.046	939
Hartnell Rd. [20] vs. Davis Rd. [20]	127.6	319.2	-191.6	33.03	9	41	8.205	939
Hartnell Rd. [20] vs. Davis Rd. [35]	127.6	304.2	-176.6	33.74	9	33	7.402	939
Hartnell Rd. [35] vs. Davis Rd. [12]	171	248	-76.97	35.36	8	33	3.079	939
Hartnell Rd. [35] vs. Davis Rd. [20]	171	319.2	-148.2	34.68	8	41	6.044	939
Hartnell Rd. [35] vs. Davis Rd. [35]	171	304.2	-133.2	35.36	8	33	5.326	939
Davis Rd. [12] vs. Davis Rd. [20]	248	319.2	-71.23	20.98	33	41	4.801	939
Davis Rd. [12] vs. Davis Rd. [35]	248	304.2	-56.2	22.09	33	33	3.598	939
Davis Rd. [20] vs. Davis Rd. [35]	319.2	304.2	15.03	20.98	41	33	1.013	939
Row 5								
Control vs. Hartnell Rd. [12]	200.6	205.2	-4.663	21.3	36	35	0.3096	939
Control vs. Hartnell Rd. [20]	200.6	151.9	48.68	33.44	36	9	2.059	939
Control vs. Hartnell Rd. [35]	200.6	135	65.59	35.07	36	8	2.645	939
Control vs. Davis Rd. [12]	200.6	237	-36.41	21.62	36	33	2.381	939
Control vs. Davis Rd. [20]	200.6	324.1	-123.5	20.49	36	41	8.521	939
Control vs. Davis Rd. [35]	200.6	301.1	-100.6	21.62	36	33	6.576	939
Hartnell Rd. [12] vs. Hartnell Rd. [20]	205.2	151.9	53.35	33.54	35	9	2.25	939
Hartnell Rd. [12] vs. Hartnell Rd. [35]	205.2	135	70.26	35.16	35	8	2.826	939
Hartnell Rd. [12] vs. Davis Rd. [12]	205.2	237	-31.74	21.77	35	33	2.062	939

Hartnell Rd. [12] vs. Davis Rd. [20]	205.2	324.1	-118.8	20.65	35	41	8.138	939
Hartnell Rd. [12] vs. Davis Rd. [35]	205.2	301.1	-95.89	21.77	35	33	6.229	939
Hartnell Rd. [20] vs. Hartnell Rd. [35]	151.9	135	16.91	43.6	9	8	0.5485	939
Hartnell Rd. [20] vs. Davis Rd. [12]	151.9	237	-85.09	33.74	9	33	3.566	939
Hartnell Rd. [20] vs. Davis Rd. [20]	151.9	324.1	-172.2	33.03	9	41	7.372	939
Hartnell Rd. [20] vs. Davis Rd. [35]	151.9	301.1	-149.2	33.74	9	33	6.255	939
Hartnell Rd. [35] vs. Davis Rd. [12]	135	237	-102	35.36	8	33	4.079	939
Hartnell Rd. [35] vs. Davis Rd. [20]	135	324.1	-189.1	34.68	8	41	7.71	939
Hartnell Rd. [35] vs. Davis Rd. [35]	135	301.1	-166.1	35.36	8	33	6.645	939
Davis Rd. [12] vs. Davis Rd. [20]	237	324.1	-87.08	20.98	33	41	5.869	939
Davis Rd. [12] vs. Davis Rd. [35]	237	301.1	-64.15	22.09	33	33	4.107	939
Davis Rd. [20] vs. Davis Rd. [35]	324.1	301.1	22.93	20.98	41	33	1.546	939

Table Analyzed	pmr Sept
Data sets analyzed	A-G

ANOVA summary

F	50.76
P value	<0.0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0.6183

Brown-Forsythe test

F (DFn, DFd)	
P value	
P value summary	
Are SDs significantly different (P < 0.05)?	

Bartlett's test

Bartlett's statistic (corrected)	140.2
P value	<0.0001
P value summary	****
Are SDs significantly different (P < 0.05)?	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	181088	6	30181	F (6, 188) = 50.76	P<0.0001
Residual (within columns)	111792	188	594.6		
Total	292880	194			

Data summary	
Number of treatments (columns)	7
Number of values (total)	195
Number of families	1
Number of comparisons per family	6
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	A-?	
Control vs. Hartnell Rd. [12]	-68.74	-83.89 to -53.58	Yes	****	<0.0001	B	Hartnell Rd. [12]
Control vs. Hartnell Rd. [20]	-98.04	-121.8 to -74.25	Yes	****	<0.0001	C	Hartnell Rd. [20]
Control vs. Hartnell Rd. [35]	-118.9	-143.9 to -93.96	Yes	****	<0.0001	D	Hartnell Rd. [35]
Control vs. Davis Rd. [12]	-41.38	-56.76 to -25.99	Yes	****	<0.0001	E	Davis Rd. [12]
Control vs. Davis Rd. [20]	-45.48	-60.06 to -30.90	Yes	****	<0.0001	F	Davis Rd. [20]
Control vs. Davis Rd. [35]	-72.57	-87.95 to -57.18	Yes	****	<0.0001	G	Davis Rd. [35]

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. Hartnell Rd. [12]	6.327	62.41	-68.74	5.789	36	35	11.87	188
Control vs. Hartnell Rd. [20]	6.327	91.71	-98.04	9.088	36	9	10.79	188
Control vs. Hartnell Rd. [35]	6.327	112.6	-118.9	9.531	36	8	12.48	188
Control vs. Davis Rd. [12]	6.327	35.05	-41.38	5.877	36	33	7.041	188
Control vs. Davis Rd. [20]	6.327	39.15	-45.48	5.57	36	41	8.166	188
Control vs. Davis Rd. [35]	6.327	66.24	-72.57	5.877	36	33	12.35	188

Table S3.9. *Statistical analysis for Daphnia magna behavior (Total Distance, response to light stimulus) following 96 h exposures to contaminated surface waters immediately following a first flush rain event (November 26th, 2019). We determined significance of mortality data by Analysis of Variance (ANOVA) followed by Tukey's post hoc tests in order to test the significance between treatments.*

Table Analyzed	Nov TD DM				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary		
Interaction	1.112	0.9808	ns		
Row Factor	5.392	<0.0001	****		
Column Factor	8.032	<0.0001	****		
ANOVA table	SS (Type III)	DF	MS		
Interaction	345884	40	8647		
Row Factor	1677158	4	419290		
Column Factor	2498457	10	249846		
Residual	26488101	1810	14634		
Data summary					
Number of columns (Column Factor)	11				
Number of rows (Row Factor)	5				
Number of values	1865				
Within each row, compare columns (simple effects within rows)					
Number of families	5				
Number of comparisons per family	55				
Alpha	0.05				

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
Row 1					
Control vs. Quail Creek [6]	107	21.90 to 192.0	Yes	**	0.0026
Control vs. Quail Creek [12]	15.46	-71.58 to 102.5	No	ns	>0.9999
Control vs. Quail Creek [20]	6.777	-78.91 to 92.46	No	ns	>0.9999

Control vs. Hartnell Rd. [6]	71.73	-15.31 to 158.8	No	ns	0.2212
Control vs. Hartnell Rd. [12]	68.07	-23.03 to 159.2	No	ns	0.3618
Control vs. Hartnell Rd. [20]	55.05	-53.02 to 163.1	No	ns	0.8642
Control vs. Davis Rd. [6]	74.68	-11.00 to 160.4	No	ns	0.1553
Control vs. Davis Rd. [12]	54.47	-34.08 to 143.0	No	ns	0.6613
Control vs. Davis Rd. [20]	84.43	-9.713 to 178.6	No	ns	0.1268
Control vs. Davis Rd. [100]	154.9	67.84 to 241.9	Yes	****	<0.0001
Quail Creek [6] vs. Quail Creek [12]	-91.49	-182.8 to -0.1497	Yes	*	0.0492
Quail Creek [6] vs. Quail Creek [20]	-100.2	-190.2 to -10.13	Yes	*	0.0152
Quail Creek [6] vs. Hartnell Rd. [6]	-35.22	-126.6 to 56.12	No	ns	0.9775
Quail Creek [6] vs. Hartnell Rd. [12]	-38.88	-134.1 to 56.34	No	ns	0.9662
Quail Creek [6] vs. Hartnell Rd. [20]	-51.9	-163.5 to 59.65	No	ns	0.9203
Quail Creek [6] vs. Davis Rd. [6]	-32.27	-122.3 to 57.77	No	ns	0.9869
Quail Creek [6] vs. Davis Rd. [12]	-52.48	-145.3 to 40.28	No	ns	0.7663
Quail Creek [6] vs. Davis Rd. [20]	-22.52	-120.6 to 75.61	No	ns	0.9997
Quail Creek [6] vs. Davis Rd. [100]	47.93	-43.41 to 139.3	No	ns	0.8403
Quail Creek [12] vs. Quail Creek [20]	-8.686	-100.6 to 83.24	No	ns	>0.9999
Quail Creek [12] vs. Hartnell Rd. [6]	56.27	-36.93 to 149.5	No	ns	0.6867
Quail Creek [12] vs. Hartnell Rd. [12]	52.61	-44.39 to 149.6	No	ns	0.8103
Quail Creek [12] vs. Hartnell Rd. [20]	39.59	-73.49 to 152.7	No	ns	0.9891
Quail Creek [12] vs. Davis Rd. [6]	59.22	-32.71 to 151.1	No	ns	0.5948
Quail Creek [12] vs. Davis Rd. [12]	39	-55.59 to 133.6	No	ns	0.9638
Quail Creek [12] vs. Davis Rd. [20]	68.97	-30.89 to 168.8	No	ns	0.4865
Quail Creek [12] vs. Davis Rd. [100]	139.4	46.22 to 232.6	Yes	****	<0.0001
Quail Creek [20] vs. Hartnell Rd. [6]	64.96	-26.97 to 156.9	No	ns	0.4501
Quail Creek [20] vs. Hartnell Rd. [12]	61.3	-34.49 to 157.1	No	ns	0.6046
Quail Creek [20] vs. Hartnell Rd. [20]	48.27	-63.77 to 160.3	No	ns	0.9513
Quail Creek [20] vs. Davis Rd. [6]	67.9	-22.74 to 158.5	No	ns	0.3578
Quail Creek [20] vs. Davis Rd. [12]	47.69	-45.66 to 141.0	No	ns	0.862
Quail Creek [20] vs. Davis Rd. [20]	77.66	-21.02 to 176.3	No	ns	0.2841
Quail Creek [20] vs. Davis Rd. [100]	148.1	56.18 to 240.0	Yes	****	<0.0001
Hartnell Rd. [6] vs. Hartnell Rd. [12]	-3.659	-100.7 to 93.34	No	ns	>0.9999
Hartnell Rd. [6] vs. Hartnell Rd. [20]	-16.68	-129.8 to 96.40	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [6]	2.949	-88.98 to 94.88	No	ns	>0.9999

Hartnell Rd. [6] vs. Davis Rd. [12]	-17.26	-111.9 to 77.33	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [20]	12.7	-87.16 to 112.6	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [100]	83.15	-10.05 to 176.3	No	ns	0.1317
Hartnell Rd. [12] vs. Hartnell Rd. [20]	-13.02	-129.3 to 103.2	No	ns	>0.9999
Hartnell Rd. [12] vs. Davis Rd. [6]	6.608	-89.18 to 102.4	No	ns	>0.9999
Hartnell Rd. [12] vs. Davis Rd. [12]	-13.61	-112.0 to 84.74	No	ns	>0.9999
Hartnell Rd. [12] vs. Davis Rd. [20]	16.36	-87.06 to 119.8	No	ns	>0.9999
Hartnell Rd. [12] vs. Davis Rd. [100]	86.81	-10.19 to 183.8	No	ns	0.1288
Hartnell Rd. [20] vs. Davis Rd. [6]	19.63	-92.41 to 131.7	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [12]	-0.5816	-114.8 to 113.7	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [20]	29.38	-89.25 to 148.0	No	ns	0.9994
Hartnell Rd. [20] vs. Davis Rd. [100]	99.83	-13.25 to 212.9	No	ns	0.142
Davis Rd. [6] vs. Davis Rd. [12]	-20.21	-113.6 to 73.14	No	ns	0.9998
Davis Rd. [6] vs. Davis Rd. [20]	9.753	-88.93 to 108.4	No	ns	>0.9999
Davis Rd. [6] vs. Davis Rd. [100]	80.2	-11.73 to 172.1	No	ns	0.1543
Davis Rd. [12] vs. Davis Rd. [20]	29.97	-71.21 to 131.1	No	ns	0.9971
Davis Rd. [12] vs. Davis Rd. [100]	100.4	5.815 to 195.0	Yes	*	0.0267
Davis Rd. [20] vs. Davis Rd. [100]	70.45	-29.41 to 170.3	No	ns	0.4527

Row 2

Control vs. Quail Creek [6]	34.75	-49.52 to 119.0	No	ns	0.9638
Control vs. Quail Creek [12]	-39.81	-126.1 to 46.48	No	ns	0.9243
Control vs. Quail Creek [20]	-14.48	-99.39 to 70.44	No	ns	>0.9999
Control vs. Hartnell Rd. [6]	-2.49	-88.07 to 83.09	No	ns	>0.9999
Control vs. Hartnell Rd. [12]	-18.35	-108.7 to 72.03	No	ns	0.9999
Control vs. Hartnell Rd. [20]	31.48	-78.26 to 141.2	No	ns	0.9978
Control vs. Davis Rd. [6]	9.98	-74.29 to 94.25	No	ns	>0.9999
Control vs. Davis Rd. [12]	-0.1306	-87.93 to 87.66	No	ns	>0.9999
Control vs. Davis Rd. [20]	28.71	-63.66 to 121.1	No	ns	0.9957
Control vs. Davis Rd. [100]	66.16	-20.86 to 153.2	No	ns	0.3352
Quail Creek [6] vs. Quail Creek [12]	-74.56	-165.9 to 16.78	No	ns	0.2336
Quail Creek [6] vs. Quail Creek [20]	-49.22	-139.3 to 40.82	No	ns	0.8025
Quail Creek [6] vs. Hartnell Rd. [6]	-37.24	-127.9 to 53.44	No	ns	0.9648
Quail Creek [6] vs. Hartnell Rd. [12]	-53.1	-148.3 to 42.12	No	ns	0.782
Quail Creek [6] vs. Hartnell Rd. [20]	-3.271	-117.0 to 110.5	No	ns	>0.9999

Quail Creek [6] vs. Davis Rd. [6]	-24.77	-114.2 to 64.67	No	ns	0.9984
Quail Creek [6] vs. Davis Rd. [12]	-34.88	-127.6 to 57.89	No	ns	0.9813
Quail Creek [6] vs. Davis Rd. [20]	-6.044	-103.1 to 91.06	No	ns	>0.9999
Quail Creek [6] vs. Davis Rd. [100]	31.41	-60.62 to 123.4	No	ns	0.991
Quail Creek [12] vs. Quail Creek [20]	25.33	-66.60 to 117.3	No	ns	0.9984
Quail Creek [12] vs. Hartnell Rd. [6]	37.32	-55.23 to 129.9	No	ns	0.969
Quail Creek [12] vs. Hartnell Rd. [12]	21.46	-75.55 to 118.5	No	ns	0.9998
Quail Creek [12] vs. Hartnell Rd. [20]	71.28	-43.97 to 186.5	No	ns	0.6538
Quail Creek [12] vs. Davis Rd. [6]	49.79	-41.55 to 141.1	No	ns	0.8054
Quail Creek [12] vs. Davis Rd. [12]	39.68	-54.92 to 134.3	No	ns	0.9594
Quail Creek [12] vs. Davis Rd. [20]	68.51	-30.34 to 167.4	No	ns	0.4809
Quail Creek [12] vs. Davis Rd. [100]	106	12.09 to 199.8	Yes	*	0.0127
Quail Creek [20] vs. Hartnell Rd. [6]	11.99	-79.28 to 103.3	No	ns	>0.9999
Quail Creek [20] vs. Hartnell Rd. [12]	-3.876	-99.66 to 91.91	No	ns	>0.9999
Quail Creek [20] vs. Hartnell Rd. [20]	45.95	-68.28 to 160.2	No	ns	0.9696
Quail Creek [20] vs. Davis Rd. [6]	24.46	-65.59 to 114.5	No	ns	0.9986
Quail Creek [20] vs. Davis Rd. [12]	14.34	-79.00 to 107.7	No	ns	>0.9999
Quail Creek [20] vs. Davis Rd. [20]	43.18	-54.48 to 140.8	No	ns	0.9423
Quail Creek [20] vs. Davis Rd. [100]	80.63	-11.99 to 173.3	No	ns	0.1565
Hartnell Rd. [6] vs. Hartnell Rd. [12]	-15.86	-112.2 to 80.52	No	ns	>0.9999
Hartnell Rd. [6] vs. Hartnell Rd. [20]	33.97	-80.76 to 148.7	No	ns	0.9971
Hartnell Rd. [6] vs. Davis Rd. [6]	12.47	-78.21 to 103.1	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [12]	2.359	-91.60 to 96.32	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [20]	31.2	-67.04 to 129.4	No	ns	0.9949
Hartnell Rd. [6] vs. Davis Rd. [100]	68.65	-24.59 to 161.9	No	ns	0.3847
Hartnell Rd. [12] vs. Hartnell Rd. [20]	49.83	-68.53 to 168.2	No	ns	0.9583
Hartnell Rd. [12] vs. Davis Rd. [6]	28.33	-66.89 to 123.6	No	ns	0.997
Hartnell Rd. [12] vs. Davis Rd. [12]	18.22	-80.13 to 116.6	No	ns	>0.9999
Hartnell Rd. [12] vs. Davis Rd. [20]	47.06	-55.39 to 149.5	No	ns	0.9264
Hartnell Rd. [12] vs. Davis Rd. [100]	84.51	-13.15 to 182.2	No	ns	0.163
Hartnell Rd. [20] vs. Davis Rd. [6]	-21.5	-135.3 to 92.26	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [12]	-31.61	-148.0 to 84.78	No	ns	0.9986
Hartnell Rd. [20] vs. Davis Rd. [20]	-2.773	-122.6 to 117.1	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [100]	34.68	-81.13 to 150.5	No	ns	0.9969
Davis Rd. [6] vs. Davis Rd. [12]	-10.11	-102.9 to 82.66	No	ns	>0.9999

Davis Rd. [6] vs. Davis Rd. [20]	18.73	-78.38 to 115.8	No	ns	>0.9999
Davis Rd. [6] vs. Davis Rd. [100]	56.18	-35.86 to 148.2	No	ns	0.6719
Davis Rd. [12] vs. Davis Rd. [20]	28.84	-71.34 to 129.0	No	ns	0.9977
Davis Rd. [12] vs. Davis Rd. [100]	66.29	-28.98 to 161.6	No	ns	0.4747
Davis Rd. [20] vs. Davis Rd. [100]	37.45	-62.04 to 136.9	No	ns	0.9811

Row 3

Control vs. Quail Creek [6]	49.57	-34.70 to 133.8	No	ns	0.7204
Control vs. Quail Creek [12]	-26.52	-112.8 to 59.77	No	ns	0.9961
Control vs. Quail Creek [20]	9.298	-75.61 to 94.21	No	ns	>0.9999
Control vs. Hartnell Rd. [6]	2.402	-83.18 to 87.98	No	ns	>0.9999
Control vs. Hartnell Rd. [12]	-33.23	-123.6 to 57.15	No	ns	0.9841
Control vs. Hartnell Rd. [20]	62.54	-47.20 to 172.3	No	ns	0.758
Control vs. Davis Rd. [6]	24.13	-60.15 to 108.4	No	ns	0.9978
Control vs. Davis Rd. [12]	6.954	-80.84 to 94.75	No	ns	>0.9999
Control vs. Davis Rd. [20]	28.21	-64.15 to 120.6	No	ns	0.9963
Control vs. Davis Rd. [100]	115.1	28.10 to 202.1	Yes	**	0.0011
Quail Creek [6] vs. Quail Creek [12]	-76.09	-167.4 to 15.25	No	ns	0.2076
Quail Creek [6] vs. Quail Creek [20]	-40.27	-130.3 to 49.77	No	ns	0.9379
Quail Creek [6] vs. Hartnell Rd. [6]	-47.17	-137.8 to 43.51	No	ns	0.8477
Quail Creek [6] vs. Hartnell Rd. [12]	-82.8	-178.0 to 12.42	No	ns	0.1578
Quail Creek [6] vs. Hartnell Rd. [20]	12.98	-100.8 to 126.7	No	ns	>0.9999
Quail Creek [6] vs. Davis Rd. [6]	-25.44	-114.9 to 64.00	No	ns	0.998
Quail Creek [6] vs. Davis Rd. [12]	-42.61	-135.4 to 50.15	No	ns	0.9264
Quail Creek [6] vs. Davis Rd. [20]	-21.36	-118.5 to 75.74	No	ns	0.9998
Quail Creek [6] vs. Davis Rd. [100]	65.55	-26.49 to 157.6	No	ns	0.4375
Quail Creek [12] vs. Quail Creek [20]	35.81	-56.11 to 127.7	No	ns	0.9758
Quail Creek [12] vs. Hartnell Rd. [6]	28.92	-63.63 to 121.5	No	ns	0.9955
Quail Creek [12] vs. Hartnell Rd. [12]	-6.714	-103.7 to 90.29	No	ns	>0.9999
Quail Creek [12] vs. Hartnell Rd. [20]	89.06	-26.19 to 204.3	No	ns	0.3107
Quail Creek [12] vs. Davis Rd. [6]	50.64	-40.70 to 142.0	No	ns	0.7881
Quail Creek [12] vs. Davis Rd. [12]	33.47	-61.13 to 128.1	No	ns	0.9881
Quail Creek [12] vs. Davis Rd. [20]	54.73	-44.12 to 153.6	No	ns	0.7896
Quail Creek [12] vs. Davis Rd. [100]	141.6	47.75 to 235.5	Yes	****	<0.0001
Quail Creek [20] vs. Hartnell Rd. [6]	-6.896	-98.17 to 84.37	No	ns	>0.9999

Quail Creek [20] vs. Hartnell Rd. [12]	-42.53	-138.3 to 53.26	No	ns	0.9407
Quail Creek [20] vs. Hartnell Rd. [20]	53.25	-60.99 to 167.5	No	ns	0.9194
Quail Creek [20] vs. Davis Rd. [6]	14.83	-75.22 to 104.9	No	ns	>0.9999
Quail Creek [20] vs. Davis Rd. [12]	-2.344	-95.69 to 91.01	No	ns	>0.9999
Quail Creek [20] vs. Davis Rd. [20]	18.91	-78.74 to 116.6	No	ns	>0.9999
Quail Creek [20] vs. Davis Rd. [100]	105.8	13.20 to 198.4	Yes	*	0.0108
Hartnell Rd. [6] vs. Hartnell Rd. [12]	-35.63	-132.0 to 60.75	No	ns	0.9835
Hartnell Rd. [6] vs. Hartnell Rd. [20]	60.14	-54.59 to 174.9	No	ns	0.8412
Hartnell Rd. [6] vs. Davis Rd. [6]	21.72	-68.95 to 112.4	No	ns	0.9995
Hartnell Rd. [6] vs. Davis Rd. [12]	4.552	-89.41 to 98.51	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [20]	25.81	-72.43 to 124.0	No	ns	0.999
Hartnell Rd. [6] vs. Davis Rd. [100]	112.7	19.48 to 205.9	Yes	**	0.0048
Hartnell Rd. [12] vs. Hartnell Rd. [20]	95.78	-22.58 to 214.1	No	ns	0.2452
Hartnell Rd. [12] vs. Davis Rd. [6]	57.36	-37.86 to 152.6	No	ns	0.6897
Hartnell Rd. [12] vs. Davis Rd. [12]	40.19	-58.16 to 138.5	No	ns	0.966
Hartnell Rd. [12] vs. Davis Rd. [20]	61.44	-41.00 to 163.9	No	ns	0.6955
Hartnell Rd. [12] vs. Davis Rd. [100]	148.3	50.69 to 246.0	Yes	****	<0.0001
Hartnell Rd. [20] vs. Davis Rd. [6]	-38.42	-152.2 to 75.34	No	ns	0.9917
Hartnell Rd. [20] vs. Davis Rd. [12]	-55.59	-172.0 to 60.80	No	ns	0.9065
Hartnell Rd. [20] vs. Davis Rd. [20]	-34.33	-154.2 to 85.54	No	ns	0.9978
Hartnell Rd. [20] vs. Davis Rd. [100]	52.57	-63.24 to 168.4	No	ns	0.9316
Davis Rd. [6] vs. Davis Rd. [12]	-17.17	-109.9 to 75.60	No	ns	>0.9999
Davis Rd. [6] vs. Davis Rd. [20]	4.085	-93.02 to 101.2	No	ns	>0.9999
Davis Rd. [6] vs. Davis Rd. [100]	90.99	-1.045 to 183.0	No	ns	0.0558
Davis Rd. [12] vs. Davis Rd. [20]	21.26	-78.92 to 121.4	No	ns	0.9998
Davis Rd. [12] vs. Davis Rd. [100]	108.2	12.89 to 203.4	Yes	*	0.0117
Davis Rd. [20] vs. Davis Rd. [100]	86.91	-12.59 to 186.4	No	ns	0.153

Row 4

Control vs. Quail Creek [6]	49.65	-34.62 to 133.9	No	ns	0.7182
Control vs. Quail Creek [12]	-24.83	-111.1 to 61.45	No	ns	0.9977
Control vs. Quail Creek [20]	14.38	-70.54 to 99.29	No	ns	>0.9999
Control vs. Hartnell Rd. [6]	24.23	-61.35 to 109.8	No	ns	0.998
Control vs. Hartnell Rd. [12]	3.84	-86.54 to 94.22	No	ns	>0.9999
Control vs. Hartnell Rd. [20]	46.19	-61.26 to 153.6	No	ns	0.952

Control vs. Davis Rd. [6]	39.33	-44.94 to 123.6	No	ns	0.9188
Control vs. Davis Rd. [12]	28.63	-59.16 to 116.4	No	ns	0.9937
Control vs. Davis Rd. [20]	44.7	-47.66 to 137.1	No	ns	0.8988
Control vs. Davis Rd. [100]	147.8	61.56 to 234.1	Yes	****	<0.0001
Quail Creek [6] vs. Quail Creek [12]	-74.48	-165.8 to 16.86	No	ns	0.2349
Quail Creek [6] vs. Quail Creek [20]	-35.28	-125.3 to 54.77	No	ns	0.9748
Quail Creek [6] vs. Hartnell Rd. [6]	-25.42	-116.1 to 65.25	No	ns	0.9982
Quail Creek [6] vs. Hartnell Rd. [12]	-45.81	-141.0 to 49.41	No	ns	0.9023
Quail Creek [6] vs. Hartnell Rd. [20]	-3.46	-115.0 to 108.1	No	ns	>0.9999
Quail Creek [6] vs. Davis Rd. [6]	-10.32	-99.77 to 79.12	No	ns	>0.9999
Quail Creek [6] vs. Davis Rd. [12]	-21.02	-113.8 to 71.75	No	ns	0.9997
Quail Creek [6] vs. Davis Rd. [20]	-4.955	-102.1 to 92.15	No	ns	>0.9999
Quail Creek [6] vs. Davis Rd. [100]	98.19	6.854 to 189.5	Yes	*	0.0231
Quail Creek [12] vs. Quail Creek [20]	39.21	-52.72 to 131.1	No	ns	0.9545
Quail Creek [12] vs. Hartnell Rd. [6]	49.06	-43.49 to 141.6	No	ns	0.8315
Quail Creek [12] vs. Hartnell Rd. [12]	28.67	-68.33 to 125.7	No	ns	0.9972
Quail Creek [12] vs. Hartnell Rd. [20]	71.02	-42.06 to 184.1	No	ns	0.632
Quail Creek [12] vs. Davis Rd. [6]	64.16	-27.18 to 155.5	No	ns	0.4596
Quail Creek [12] vs. Davis Rd. [12]	53.46	-41.13 to 148.1	No	ns	0.7674
Quail Creek [12] vs. Davis Rd. [20]	69.53	-29.32 to 168.4	No	ns	0.4574
Quail Creek [12] vs. Davis Rd. [100]	172.7	79.48 to 265.9	Yes	****	<0.0001
Quail Creek [20] vs. Hartnell Rd. [6]	9.853	-81.42 to 101.1	No	ns	>0.9999
Quail Creek [20] vs. Hartnell Rd. [12]	-10.54	-106.3 to 85.25	No	ns	>0.9999
Quail Creek [20] vs. Hartnell Rd. [20]	31.82	-80.22 to 143.9	No	ns	0.998
Quail Creek [20] vs. Davis Rd. [6]	24.95	-65.09 to 115.0	No	ns	0.9984
Quail Creek [20] vs. Davis Rd. [12]	14.26	-79.09 to 107.6	No	ns	>0.9999
Quail Creek [20] vs. Davis Rd. [20]	30.32	-67.33 to 128.0	No	ns	0.9958
Quail Creek [20] vs. Davis Rd. [100]	133.5	41.54 to 225.4	Yes	***	0.0002
Hartnell Rd. [6] vs. Hartnell Rd. [12]	-20.39	-116.8 to 75.99	No	ns	0.9998
Hartnell Rd. [6] vs. Hartnell Rd. [20]	21.96	-90.58 to 134.5	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [6]	15.1	-75.58 to 105.8	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [12]	4.404	-89.55 to 98.36	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [20]	20.47	-77.77 to 118.7	No	ns	0.9999
Hartnell Rd. [6] vs. Davis Rd. [100]	123.6	31.07 to 216.2	Yes	***	0.0009
Hartnell Rd. [12] vs. Hartnell Rd. [20]	42.35	-73.88 to 158.6	No	ns	0.9852

Hartnell Rd. [12] vs. Davis Rd. [6]	35.49	-59.73 to 130.7	No	ns	0.9825
Hartnell Rd. [12] vs. Davis Rd. [12]	24.79	-73.56 to 123.1	No	ns	0.9993
Hartnell Rd. [12] vs. Davis Rd. [20]	40.86	-61.59 to 143.3	No	ns	0.9713
Hartnell Rd. [12] vs. Davis Rd. [100]	144	47.00 to 241.0	Yes	****	<0.0001
Hartnell Rd. [20] vs. Davis Rd. [6]	-6.864	-118.4 to 104.7	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [12]	-17.56	-131.8 to 96.68	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [20]	-1.495	-119.3 to 116.3	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [100]	101.7	-11.43 to 214.7	No	ns	0.1246
Davis Rd. [6] vs. Davis Rd. [12]	-10.69	-103.5 to 82.07	No	ns	>0.9999
Davis Rd. [6] vs. Davis Rd. [20]	5.369	-91.73 to 102.5	No	ns	>0.9999
Davis Rd. [6] vs. Davis Rd. [100]	108.5	17.18 to 199.9	Yes	**	0.0062
Davis Rd. [12] vs. Davis Rd. [20]	16.06	-84.11 to 116.2	No	ns	>0.9999
Davis Rd. [12] vs. Davis Rd. [100]	119.2	24.61 to 213.8	Yes	**	0.0025
Davis Rd. [20] vs. Davis Rd. [100]	103.1	4.298 to 202.0	Yes	*	0.0322

Row 5

Control vs. Quail Creek [6]	37.38	-47.91 to 122.7	No	ns	0.9455
Control vs. Quail Creek [12]	4.54	-82.85 to 91.93	No	ns	>0.9999
Control vs. Quail Creek [20]	10.19	-75.77 to 96.14	No	ns	>0.9999
Control vs. Hartnell Rd. [6]	41.07	-45.58 to 127.7	No	ns	0.9107
Control vs. Hartnell Rd. [12]	-7.383	-99.08 to 84.31	No	ns	>0.9999
Control vs. Hartnell Rd. [20]	46.07	-61.68 to 153.8	No	ns	0.9537
Control vs. Davis Rd. [6]	54.09	-31.20 to 139.4	No	ns	0.6179
Control vs. Davis Rd. [12]	27.1	-61.87 to 116.1	No	ns	0.9964
Control vs. Davis Rd. [20]	42.77	-52.17 to 137.7	No	ns	0.9349
Control vs. Davis Rd. [100]	133	45.61 to 220.4	Yes	****	<0.0001
Quail Creek [6] vs. Quail Creek [12]	-32.84	-125.5 to 59.78	No	ns	0.9879
Quail Creek [6] vs. Quail Creek [20]	-27.19	-118.5 to 64.08	No	ns	0.997
Quail Creek [6] vs. Hartnell Rd. [6]	3.694	-88.24 to 95.62	No	ns	>0.9999
Quail Creek [6] vs. Hartnell Rd. [12]	-44.76	-141.5 to 51.93	No	ns	0.9227
Quail Creek [6] vs. Hartnell Rd. [20]	8.692	-103.3 to 120.7	No	ns	>0.9999
Quail Creek [6] vs. Davis Rd. [6]	16.71	-73.93 to 107.4	No	ns	>0.9999
Quail Creek [6] vs. Davis Rd. [12]	-10.28	-104.4 to 83.84	No	ns	>0.9999
Quail Creek [6] vs. Davis Rd. [20]	5.388	-94.38 to 105.2	No	ns	>0.9999
Quail Creek [6] vs. Davis Rd. [100]	95.62	2.997 to 188.2	Yes	*	0.0362

Quail Creek [12] vs. Quail Creek [20]	5.646	-87.59 to 98.88	No	ns	>0.9999
Quail Creek [12] vs. Hartnell Rd. [6]	36.53	-57.35 to 130.4	No	ns	0.9759
Quail Creek [12] vs. Hartnell Rd. [12]	-11.92	-110.5 to 86.63	No	ns	>0.9999
Quail Creek [12] vs. Hartnell Rd. [20]	41.53	-72.11 to 155.2	No	ns	0.9848
Quail Creek [12] vs. Davis Rd. [6]	49.55	-43.07 to 142.2	No	ns	0.8232
Quail Creek [12] vs. Davis Rd. [12]	22.56	-73.46 to 118.6	No	ns	0.9996
Quail Creek [12] vs. Davis Rd. [20]	38.23	-63.34 to 139.8	No	ns	0.9811
Quail Creek [12] vs. Davis Rd. [100]	128.5	33.90 to 223.0	Yes	***	0.0006
Quail Creek [20] vs. Hartnell Rd. [6]	30.89	-61.66 to 123.4	No	ns	0.9925
Quail Creek [20] vs. Hartnell Rd. [12]	-17.57	-114.8 to 79.71	No	ns	>0.9999
Quail Creek [20] vs. Hartnell Rd. [20]	35.89	-76.66 to 148.4	No	ns	0.9948
Quail Creek [20] vs. Davis Rd. [6]	43.9	-47.37 to 135.2	No	ns	0.9024
Quail Creek [20] vs. Davis Rd. [12]	16.92	-77.80 to 111.6	No	ns	>0.9999
Quail Creek [20] vs. Davis Rd. [20]	32.58	-67.76 to 132.9	No	ns	0.9939
Quail Creek [20] vs. Davis Rd. [100]	122.8	29.58 to 216.0	Yes	**	0.0012
Hartnell Rd. [6] vs. Hartnell Rd. [12]	-48.46	-146.4 to 49.44	No	ns	0.8846
Hartnell Rd. [6] vs. Hartnell Rd. [20]	4.998	-108.1 to 118.1	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [6]	13.02	-78.91 to 104.9	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [12]	-13.97	-109.3 to 81.39	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [20]	1.695	-99.25 to 102.6	No	ns	>0.9999
Hartnell Rd. [6] vs. Davis Rd. [100]	91.92	-1.955 to 185.8	No	ns	0.0611
Hartnell Rd. [12] vs. Hartnell Rd. [20]	53.45	-63.53 to 170.4	No	ns	0.9288
Hartnell Rd. [12] vs. Davis Rd. [6]	61.47	-35.22 to 158.2	No	ns	0.6143
Hartnell Rd. [12] vs. Davis Rd. [12]	34.49	-65.47 to 134.4	No	ns	0.9902
Hartnell Rd. [12] vs. Davis Rd. [20]	50.15	-55.15 to 155.4	No	ns	0.908
Hartnell Rd. [12] vs. Davis Rd. [100]	140.4	41.83 to 238.9	Yes	***	0.0002
Hartnell Rd. [20] vs. Davis Rd. [6]	8.019	-104.0 to 120.1	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [12]	-18.97	-133.8 to 95.90	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [20]	-3.303	-122.8 to 116.2	No	ns	>0.9999
Hartnell Rd. [20] vs. Davis Rd. [100]	86.93	-26.72 to 200.6	No	ns	0.326
Davis Rd. [6] vs. Davis Rd. [12]	-26.99	-121.1 to 67.13	No	ns	0.9978
Davis Rd. [6] vs. Davis Rd. [20]	-11.32	-111.1 to 88.45	No	ns	>0.9999
Davis Rd. [6] vs. Davis Rd. [100]	78.91	-13.71 to 171.5	No	ns	0.1805
Davis Rd. [12] vs. Davis Rd. [20]	15.67	-87.27 to 118.6	No	ns	>0.9999
Davis Rd. [12] vs. Davis Rd. [100]	105.9	9.871 to 201.9	Yes	*	0.017

Davis Rd. [20] vs. Davis Rd. [100]	90.23	-11.34 to 191.8	No	ns	0.1359

Test details	Predicted (LS) mean 1	Predicted (LS) mean 2	Predicted (LS) mean diff.	SE of diff.	N1	N2	q	DF
Row 1								
Control vs. Quail Creek [6]	263.5	156.5	107	26.39	47	38	5.731	1810
Control vs. Quail Creek [12]	263.5	248	15.46	27.01	47	35	0.8097	1810
Control vs. Quail Creek [20]	263.5	256.7	6.777	26.59	47	37	0.3605	1810
Control vs. Hartnell Rd. [6]	263.5	191.8	71.73	27.01	47	35	3.756	1810
Control vs. Hartnell Rd. [12]	263.5	195.4	68.07	28.27	47	30	3.405	1810
Control vs. Hartnell Rd. [20]	263.5	208.4	55.05	33.53	47	18	2.322	1810
Control vs. Davis Rd. [6]	263.5	188.8	74.68	26.59	47	37	3.972	1810
Control vs. Davis Rd. [12]	263.5	209	54.47	27.47	47	33	2.804	1810
Control vs. Davis Rd. [20]	263.5	179.1	84.43	29.21	47	27	4.088	1810
Control vs. Davis Rd. [100]	263.5	108.6	154.9	27.01	47	35	8.11	1810
Quail Creek [6] vs. Quail Creek [12]	156.5	248	-91.49	28.34	38	35	4.565	1810
Quail Creek [6] vs. Quail Creek [20]	156.5	256.7	-100.2	27.94	38	37	5.07	1810
Quail Creek [6] vs. Hartnell Rd. [6]	156.5	191.8	-35.22	28.34	38	35	1.757	1810
Quail Creek [6] vs. Hartnell Rd. [12]	156.5	195.4	-38.88	29.55	38	30	1.861	1810
Quail Creek [6] vs. Hartnell Rd. [20]	156.5	208.4	-51.9	34.61	38	18	2.121	1810
Quail Creek [6] vs. Davis Rd. [6]	156.5	188.8	-32.27	27.94	38	37	1.633	1810
Quail Creek [6] vs. Davis Rd. [12]	156.5	209	-52.48	28.79	38	33	2.579	1810
Quail Creek [6] vs. Davis Rd. [20]	156.5	179.1	-22.52	30.45	38	27	1.046	1810
Quail Creek [6] vs. Davis Rd. [100]	156.5	108.6	47.93	28.34	38	35	2.392	1810
Quail Creek [12] vs. Quail Creek [20]	248	256.7	-8.686	28.52	35	37	0.4307	1810
Quail Creek [12] vs. Hartnell Rd. [6]	248	191.8	56.27	28.92	35	35	2.752	1810
Quail Creek [12] vs. Hartnell Rd. [12]	248	195.4	52.61	30.1	35	30	2.472	1810
Quail Creek [12] vs. Hartnell Rd. [20]	248	208.4	39.59	35.09	35	18	1.596	1810
Quail Creek [12] vs. Davis Rd. [6]	248	188.8	59.22	28.52	35	37	2.936	1810
Quail Creek [12] vs. Davis Rd. [12]	248	209	39	29.35	35	33	1.879	1810
Quail Creek [12] vs. Davis Rd. [20]	248	179.1	68.97	30.99	35	27	3.148	1810
Quail Creek [12] vs. Davis Rd. [100]	248	108.6	139.4	28.92	35	35	6.818	1810
Quail Creek [20] vs. Hartnell Rd. [6]	256.7	191.8	64.96	28.52	37	35	3.22	1810
Quail Creek [20] vs. Hartnell Rd. [12]	256.7	195.4	61.3	29.72	37	30	2.917	1810

Quail Creek [20] vs. Hartnell Rd. [20]	256.7	208.4	48.27	34.76	37	18	1.964	1810
Quail Creek [20] vs. Davis Rd. [6]	256.7	188.8	67.9	28.13	37	37	3.414	1810
Quail Creek [20] vs. Davis Rd. [12]	256.7	209	47.69	28.97	37	33	2.328	1810
Quail Creek [20] vs. Davis Rd. [20]	256.7	179.1	77.66	30.62	37	27	3.587	1810
Quail Creek [20] vs. Davis Rd. [100]	256.7	108.6	148.1	28.52	37	35	7.343	1810
Hartnell Rd. [6] vs. Hartnell Rd. [12]	191.8	195.4	-3.659	30.1	35	30	0.1719	1810
Hartnell Rd. [6] vs. Hartnell Rd. [20]	191.8	208.4	-16.68	35.09	35	18	0.6724	1810
Hartnell Rd. [6] vs. Davis Rd. [6]	191.8	188.8	2.949	28.52	35	37	0.1462	1810
Hartnell Rd. [6] vs. Davis Rd. [12]	191.8	209	-17.26	29.35	35	33	0.8318	1810
Hartnell Rd. [6] vs. Davis Rd. [20]	191.8	179.1	12.7	30.99	35	27	0.5797	1810
Hartnell Rd. [6] vs. Davis Rd. [100]	191.8	108.6	83.15	28.92	35	35	4.066	1810
Hartnell Rd. [12] vs. Hartnell Rd. [20]	195.4	208.4	-13.02	36.07	30	18	0.5107	1810
Hartnell Rd. [12] vs. Davis Rd. [6]	195.4	188.8	6.608	29.72	30	37	0.3144	1810
Hartnell Rd. [12] vs. Davis Rd. [12]	195.4	209	-13.61	30.52	30	33	0.6305	1810
Hartnell Rd. [12] vs. Davis Rd. [20]	195.4	179.1	16.36	32.09	30	27	0.721	1810
Hartnell Rd. [12] vs. Davis Rd. [100]	195.4	108.6	86.81	30.1	30	35	4.079	1810
Hartnell Rd. [20] vs. Davis Rd. [6]	208.4	188.8	19.63	34.76	18	37	0.7986	1810
Hartnell Rd. [20] vs. Davis Rd. [12]	208.4	209	-0.5816	35.45	18	33	0.0232	1810
Hartnell Rd. [20] vs. Davis Rd. [20]	208.4	179.1	29.38	36.81	18	27	1.129	1810
Hartnell Rd. [20] vs. Davis Rd. [100]	208.4	108.6	99.83	35.09	18	35	4.024	1810
Davis Rd. [6] vs. Davis Rd. [12]	188.8	209	-20.21	28.97	37	33	0.9869	1810
Davis Rd. [6] vs. Davis Rd. [20]	188.8	179.1	9.753	30.62	37	27	0.4505	1810
Davis Rd. [6] vs. Davis Rd. [100]	188.8	108.6	80.2	28.52	37	35	3.976	1810
Davis Rd. [12] vs. Davis Rd. [20]	209	179.1	29.97	31.39	33	27	1.35	1810
Davis Rd. [12] vs. Davis Rd. [100]	209	108.6	100.4	29.35	33	35	4.838	1810
Davis Rd. [20] vs. Davis Rd. [100]	179.1	108.6	70.45	30.99	27	35	3.215	1810
Row 2								
Control vs. Quail Creek [6]	194	159.2	34.75	26.15	49	38	1.879	1810
Control vs. Quail Creek [12]	194	233.8	-39.81	26.77	49	35	2.103	1810
Control vs. Quail Creek [20]	194	208.5	-14.48	26.35	49	37	0.777	1810
Control vs. Hartnell Rd. [6]	194	196.5	-2.49	26.55	49	36	0.1326	1810
Control vs. Hartnell Rd. [12]	194	212.3	-18.35	28.04	49	30	0.9254	1810
Control vs. Hartnell Rd. [20]	194	162.5	31.48	34.05	49	17	1.307	1810
Control vs. Davis Rd. [6]	194	184	9.98	26.15	49	38	0.5397	1810

Control vs. Davis Rd. [12]	194	194.1	-0.1306	27.24	49	33	0.00678	1810
Control vs. Davis Rd. [20]	194	165.3	28.71	28.66	49	28	1.417	1810
Control vs. Davis Rd. [100]	194	127.8	66.16	27	49	34	3.465	1810
Quail Creek [6] vs. Quail Creek [12]	159.2	233.8	-74.56	28.34	38	35	3.72	1810
Quail Creek [6] vs. Quail Creek [20]	159.2	208.5	-49.22	27.94	38	37	2.492	1810
Quail Creek [6] vs. Hartnell Rd. [6]	159.2	196.5	-37.24	28.14	38	36	1.872	1810
Quail Creek [6] vs. Hartnell Rd. [12]	159.2	212.3	-53.1	29.55	38	30	2.542	1810
Quail Creek [6] vs. Hartnell Rd. [20]	159.2	162.5	-3.271	35.3	38	17	0.1311	1810
Quail Creek [6] vs. Davis Rd. [6]	159.2	184	-24.77	27.75	38	38	1.262	1810
Quail Creek [6] vs. Davis Rd. [12]	159.2	194.1	-34.88	28.79	38	33	1.714	1810
Quail Creek [6] vs. Davis Rd. [20]	159.2	165.3	-6.044	30.13	38	28	0.2837	1810
Quail Creek [6] vs. Davis Rd. [100]	159.2	127.8	31.41	28.56	38	34	1.556	1810
Quail Creek [12] vs. Quail Creek [20]	233.8	208.5	25.33	28.52	35	37	1.256	1810
Quail Creek [12] vs. Hartnell Rd. [6]	233.8	196.5	37.32	28.72	35	36	1.838	1810
Quail Creek [12] vs. Hartnell Rd. [12]	233.8	212.3	21.46	30.1	35	30	1.008	1810
Quail Creek [12] vs. Hartnell Rd. [20]	233.8	162.5	71.28	35.76	35	17	2.819	1810
Quail Creek [12] vs. Davis Rd. [6]	233.8	184	49.79	28.34	35	38	2.484	1810
Quail Creek [12] vs. Davis Rd. [12]	233.8	194.1	39.68	29.35	35	33	1.912	1810
Quail Creek [12] vs. Davis Rd. [20]	233.8	165.3	68.51	30.67	35	28	3.159	1810
Quail Creek [12] vs. Davis Rd. [100]	233.8	127.8	106	29.13	35	34	5.145	1810
Quail Creek [20] vs. Hartnell Rd. [6]	208.5	196.5	11.99	28.32	37	36	0.5985	1810
Quail Creek [20] vs. Hartnell Rd. [12]	208.5	212.3	-3.876	29.72	37	30	0.1844	1810
Quail Creek [20] vs. Hartnell Rd. [20]	208.5	162.5	45.95	35.45	37	17	1.833	1810
Quail Creek [20] vs. Davis Rd. [6]	208.5	184	24.46	27.94	37	38	1.238	1810
Quail Creek [20] vs. Davis Rd. [12]	208.5	194.1	14.34	28.97	37	33	0.7004	1810
Quail Creek [20] vs. Davis Rd. [20]	208.5	165.3	43.18	30.3	37	28	2.015	1810
Quail Creek [20] vs. Davis Rd. [100]	208.5	127.8	80.63	28.74	37	34	3.968	1810
Hartnell Rd. [6] vs. Hartnell Rd. [12]	196.5	212.3	-15.86	29.91	36	30	0.7501	1810
Hartnell Rd. [6] vs. Hartnell Rd. [20]	196.5	162.5	33.97	35.6	36	17	1.349	1810
Hartnell Rd. [6] vs. Davis Rd. [6]	196.5	184	12.47	28.14	36	38	0.6268	1810
Hartnell Rd. [6] vs. Davis Rd. [12]	196.5	194.1	2.359	29.15	36	33	0.1144	1810
Hartnell Rd. [6] vs. Davis Rd. [20]	196.5	165.3	31.2	30.48	36	28	1.447	1810
Hartnell Rd. [6] vs. Davis Rd. [100]	196.5	127.8	68.65	28.93	36	34	3.356	1810
Hartnell Rd. [12] vs. Hartnell Rd. [20]	212.3	162.5	49.83	36.72	30	17	1.919	1810
Hartnell Rd. [12] vs. Davis Rd. [6]	212.3	184	28.33	29.55	30	38	1.356	1810

Hartnell Rd. [12] vs. Davis Rd. [12]	212.3	194.1	18.22	30.52	30	33	0.8444	1810
Hartnell Rd. [12] vs. Davis Rd. [20]	212.3	165.3	47.06	31.79	30	28	2.094	1810
Hartnell Rd. [12] vs. Davis Rd. [100]	212.3	127.8	84.51	30.3	30	34	3.944	1810
Hartnell Rd. [20] vs. Davis Rd. [6]	162.5	184	-21.5	35.3	17	38	0.8613	1810
Hartnell Rd. [20] vs. Davis Rd. [12]	162.5	194.1	-31.61	36.12	17	33	1.238	1810
Hartnell Rd. [20] vs. Davis Rd. [20]	162.5	165.3	-2.773	37.2	17	28	0.1054	1810
Hartnell Rd. [20] vs. Davis Rd. [100]	162.5	127.8	34.68	35.93	17	34	1.365	1810
Davis Rd. [6] vs. Davis Rd. [12]	184	194.1	-10.11	28.79	38	33	0.4967	1810
Davis Rd. [6] vs. Davis Rd. [20]	184	165.3	18.73	30.13	38	28	0.8789	1810
Davis Rd. [6] vs. Davis Rd. [100]	184	127.8	56.18	28.56	38	34	2.782	1810
Davis Rd. [12] vs. Davis Rd. [20]	194.1	165.3	28.84	31.08	33	28	1.312	1810
Davis Rd. [12] vs. Davis Rd. [100]	194.1	127.8	66.29	29.56	33	34	3.171	1810
Davis Rd. [20] vs. Davis Rd. [100]	165.3	127.8	37.45	30.87	28	34	1.716	1810

Row 3

Control vs. Quail Creek [6]	260.1	210.6	49.57	26.15	49	38	2.681	1810
Control vs. Quail Creek [12]	260.1	286.7	-26.52	26.77	49	35	1.401	1810
Control vs. Quail Creek [20]	260.1	250.8	9.298	26.35	49	37	0.4991	1810
Control vs. Hartnell Rd. [6]	260.1	257.7	2.402	26.55	49	36	0.1279	1810
Control vs. Hartnell Rd. [12]	260.1	293.4	-33.23	28.04	49	30	1.676	1810
Control vs. Hartnell Rd. [20]	260.1	197.6	62.54	34.05	49	17	2.598	1810
Control vs. Davis Rd. [6]	260.1	236	24.13	26.15	49	38	1.305	1810
Control vs. Davis Rd. [12]	260.1	253.2	6.954	27.24	49	33	0.361	1810
Control vs. Davis Rd. [20]	260.1	231.9	28.21	28.66	49	28	1.392	1810
Control vs. Davis Rd. [100]	260.1	145	115.1	27	49	34	6.029	1810
Quail Creek [6] vs. Quail Creek [12]	210.6	286.7	-76.09	28.34	38	35	3.797	1810
Quail Creek [6] vs. Quail Creek [20]	210.6	250.8	-40.27	27.94	38	37	2.038	1810
Quail Creek [6] vs. Hartnell Rd. [6]	210.6	257.7	-47.17	28.14	38	36	2.371	1810
Quail Creek [6] vs. Hartnell Rd. [12]	210.6	293.4	-82.8	29.55	38	30	3.963	1810
Quail Creek [6] vs. Hartnell Rd. [20]	210.6	197.6	12.98	35.3	38	17	0.5199	1810
Quail Creek [6] vs. Davis Rd. [6]	210.6	236	-25.44	27.75	38	38	1.296	1810
Quail Creek [6] vs. Davis Rd. [12]	210.6	253.2	-42.61	28.79	38	33	2.094	1810
Quail Creek [6] vs. Davis Rd. [20]	210.6	231.9	-21.36	30.13	38	28	1.002	1810
Quail Creek [6] vs. Davis Rd. [100]	210.6	145	65.55	28.56	38	34	3.246	1810
Quail Creek [12] vs. Quail Creek [20]	286.7	250.8	35.81	28.52	35	37	1.776	1810

Quail Creek [12] vs. Hartnell Rd. [6]	286.7	257.7	28.92	28.72	35	36	1.424	1810
Quail Creek [12] vs. Hartnell Rd. [12]	286.7	293.4	-6.714	30.1	35	30	0.3155	1810
Quail Creek [12] vs. Hartnell Rd. [20]	286.7	197.6	89.06	35.76	35	17	3.522	1810
Quail Creek [12] vs. Davis Rd. [6]	286.7	236	50.64	28.34	35	38	2.527	1810
Quail Creek [12] vs. Davis Rd. [12]	286.7	253.2	33.47	29.35	35	33	1.613	1810
Quail Creek [12] vs. Davis Rd. [20]	286.7	231.9	54.73	30.67	35	28	2.523	1810
Quail Creek [12] vs. Davis Rd. [100]	286.7	145	141.6	29.13	35	34	6.876	1810
Quail Creek [20] vs. Hartnell Rd. [6]	250.8	257.7	-6.896	28.32	37	36	0.3443	1810
Quail Creek [20] vs. Hartnell Rd. [12]	250.8	293.4	-42.53	29.72	37	30	2.024	1810
Quail Creek [20] vs. Hartnell Rd. [20]	250.8	197.6	53.25	35.45	37	17	2.124	1810
Quail Creek [20] vs. Davis Rd. [6]	250.8	236	14.83	27.94	37	38	0.7506	1810
Quail Creek [20] vs. Davis Rd. [12]	250.8	253.2	-2.344	28.97	37	33	0.1144	1810
Quail Creek [20] vs. Davis Rd. [20]	250.8	231.9	18.91	30.3	37	28	0.8827	1810
Quail Creek [20] vs. Davis Rd. [100]	250.8	145	105.8	28.74	37	34	5.207	1810
Hartnell Rd. [6] vs. Hartnell Rd. [12]	257.7	293.4	-35.63	29.91	36	30	1.685	1810
Hartnell Rd. [6] vs. Hartnell Rd. [20]	257.7	197.6	60.14	35.6	36	17	2.389	1810
Hartnell Rd. [6] vs. Davis Rd. [6]	257.7	236	21.72	28.14	36	38	1.092	1810
Hartnell Rd. [6] vs. Davis Rd. [12]	257.7	253.2	4.552	29.15	36	33	0.2208	1810
Hartnell Rd. [6] vs. Davis Rd. [20]	257.7	231.9	25.81	30.48	36	28	1.197	1810
Hartnell Rd. [6] vs. Davis Rd. [100]	257.7	145	112.7	28.93	36	34	5.51	1810
Hartnell Rd. [12] vs. Hartnell Rd. [20]	293.4	197.6	95.78	36.72	30	17	3.688	1810
Hartnell Rd. [12] vs. Davis Rd. [6]	293.4	236	57.36	29.55	30	38	2.745	1810
Hartnell Rd. [12] vs. Davis Rd. [12]	293.4	253.2	40.19	30.52	30	33	1.862	1810
Hartnell Rd. [12] vs. Davis Rd. [20]	293.4	231.9	61.44	31.79	30	28	2.734	1810
Hartnell Rd. [12] vs. Davis Rd. [100]	293.4	145	148.3	30.3	30	34	6.923	1810
Hartnell Rd. [20] vs. Davis Rd. [6]	197.6	236	-38.42	35.3	17	38	1.539	1810
Hartnell Rd. [20] vs. Davis Rd. [12]	197.6	253.2	-55.59	36.12	17	33	2.177	1810
Hartnell Rd. [20] vs. Davis Rd. [20]	197.6	231.9	-34.33	37.2	17	28	1.305	1810
Hartnell Rd. [20] vs. Davis Rd. [100]	197.6	145	52.57	35.93	17	34	2.069	1810
Davis Rd. [6] vs. Davis Rd. [12]	236	253.2	-17.17	28.79	38	33	0.8437	1810
Davis Rd. [6] vs. Davis Rd. [20]	236	231.9	4.085	30.13	38	28	0.1917	1810
Davis Rd. [6] vs. Davis Rd. [100]	236	145	90.99	28.56	38	34	4.506	1810
Davis Rd. [12] vs. Davis Rd. [20]	253.2	231.9	21.26	31.08	33	28	0.9672	1810
Davis Rd. [12] vs. Davis Rd. [100]	253.2	145	108.2	29.56	33	34	5.174	1810
Davis Rd. [20] vs. Davis Rd. [100]	231.9	145	86.91	30.87	28	34	3.981	1810

Row 4								
Control vs. Quail Creek [6]	295.1	245.5	49.65	26.15	49	38	2.685	1810
Control vs. Quail Creek [12]	295.1	319.9	-24.83	26.77	49	35	1.312	1810
Control vs. Quail Creek [20]	295.1	280.7	14.38	26.35	49	37	0.7717	1810
Control vs. Hartnell Rd. [6]	295.1	270.9	24.23	26.55	49	36	1.29	1810
Control vs. Hartnell Rd. [12]	295.1	291.3	3.84	28.04	49	30	0.1937	1810
Control vs. Hartnell Rd. [20]	295.1	248.9	46.19	33.34	49	18	1.959	1810
Control vs. Davis Rd. [6]	295.1	255.8	39.33	26.15	49	38	2.127	1810
Control vs. Davis Rd. [12]	295.1	266.5	28.63	27.24	49	33	1.487	1810
Control vs. Davis Rd. [20]	295.1	250.4	44.7	28.66	49	28	2.206	1810
Control vs. Davis Rd. [100]	295.1	147.3	147.8	26.77	49	35	7.81	1810
Quail Creek [6] vs. Quail Creek [12]	245.5	319.9	-74.48	28.34	38	35	3.717	1810
Quail Creek [6] vs. Quail Creek [20]	245.5	280.7	-35.28	27.94	38	37	1.786	1810
Quail Creek [6] vs. Hartnell Rd. [6]	245.5	270.9	-25.42	28.14	38	36	1.278	1810
Quail Creek [6] vs. Hartnell Rd. [12]	245.5	291.3	-45.81	29.55	38	30	2.193	1810
Quail Creek [6] vs. Hartnell Rd. [20]	245.5	248.9	-3.46	34.61	38	18	0.1414	1810
Quail Creek [6] vs. Davis Rd. [6]	245.5	255.8	-10.32	27.75	38	38	0.5261	1810
Quail Creek [6] vs. Davis Rd. [12]	245.5	266.5	-21.02	28.79	38	33	1.033	1810
Quail Creek [6] vs. Davis Rd. [20]	245.5	250.4	-4.955	30.13	38	28	0.2326	1810
Quail Creek [6] vs. Davis Rd. [100]	245.5	147.3	98.19	28.34	38	35	4.9	1810
Quail Creek [12] vs. Quail Creek [20]	319.9	280.7	39.21	28.52	35	37	1.944	1810
Quail Creek [12] vs. Hartnell Rd. [6]	319.9	270.9	49.06	28.72	35	36	2.416	1810
Quail Creek [12] vs. Hartnell Rd. [12]	319.9	291.3	28.67	30.1	35	30	1.347	1810
Quail Creek [12] vs. Hartnell Rd. [20]	319.9	248.9	71.02	35.09	35	18	2.863	1810
Quail Creek [12] vs. Davis Rd. [6]	319.9	255.8	64.16	28.34	35	38	3.201	1810
Quail Creek [12] vs. Davis Rd. [12]	319.9	266.5	53.46	29.35	35	33	2.576	1810
Quail Creek [12] vs. Davis Rd. [20]	319.9	250.4	69.53	30.67	35	28	3.206	1810
Quail Creek [12] vs. Davis Rd. [100]	319.9	147.3	172.7	28.92	35	35	8.445	1810
Quail Creek [20] vs. Hartnell Rd. [6]	280.7	270.9	9.853	28.32	37	36	0.492	1810
Quail Creek [20] vs. Hartnell Rd. [12]	280.7	291.3	-10.54	29.72	37	30	0.5014	1810
Quail Creek [20] vs. Hartnell Rd. [20]	280.7	248.9	31.82	34.76	37	18	1.294	1810
Quail Creek [20] vs. Davis Rd. [6]	280.7	255.8	24.95	27.94	37	38	1.263	1810
Quail Creek [20] vs. Davis Rd. [12]	280.7	266.5	14.26	28.97	37	33	0.6961	1810
Quail Creek [20] vs. Davis Rd. [20]	280.7	250.4	30.32	30.3	37	28	1.415	1810

Quail Creek [20] vs. Davis Rd. [100]	280.7	147.3	133.5	28.52	37	35	6.617	1810
Hartnell Rd. [6] vs. Hartnell Rd. [12]	270.9	291.3	-20.39	29.91	36	30	0.9642	1810
Hartnell Rd. [6] vs. Hartnell Rd. [20]	270.9	248.9	21.96	34.92	36	18	0.8894	1810
Hartnell Rd. [6] vs. Davis Rd. [6]	270.9	255.8	15.1	28.14	36	38	0.7589	1810
Hartnell Rd. [6] vs. Davis Rd. [12]	270.9	266.5	4.404	29.15	36	33	0.2136	1810
Hartnell Rd. [6] vs. Davis Rd. [20]	270.9	250.4	20.47	30.48	36	28	0.9496	1810
Hartnell Rd. [6] vs. Davis Rd. [100]	270.9	147.3	123.6	28.72	36	35	6.088	1810
Hartnell Rd. [12] vs. Hartnell Rd. [20]	291.3	248.9	42.35	36.07	30	18	1.661	1810
Hartnell Rd. [12] vs. Davis Rd. [6]	291.3	255.8	35.49	29.55	30	38	1.699	1810
Hartnell Rd. [12] vs. Davis Rd. [12]	291.3	266.5	24.79	30.52	30	33	1.149	1810
Hartnell Rd. [12] vs. Davis Rd. [20]	291.3	250.4	40.86	31.79	30	28	1.818	1810
Hartnell Rd. [12] vs. Davis Rd. [100]	291.3	147.3	144	30.1	30	35	6.766	1810
Hartnell Rd. [20] vs. Davis Rd. [6]	248.9	255.8	-6.864	34.61	18	38	0.2804	1810
Hartnell Rd. [20] vs. Davis Rd. [12]	248.9	266.5	-17.56	35.45	18	33	0.7005	1810
Hartnell Rd. [20] vs. Davis Rd. [20]	248.9	250.4	-1.495	36.55	18	28	0.05784	1810
Hartnell Rd. [20] vs. Davis Rd. [100]	248.9	147.3	101.7	35.09	18	35	4.097	1810
Davis Rd. [6] vs. Davis Rd. [12]	255.8	266.5	-10.69	28.79	38	33	0.5254	1810
Davis Rd. [6] vs. Davis Rd. [20]	255.8	250.4	5.369	30.13	38	28	0.252	1810
Davis Rd. [6] vs. Davis Rd. [100]	255.8	147.3	108.5	28.34	38	35	5.415	1810
Davis Rd. [12] vs. Davis Rd. [20]	266.5	250.4	16.06	31.08	33	28	0.7309	1810
Davis Rd. [12] vs. Davis Rd. [100]	266.5	147.3	119.2	29.35	33	35	5.744	1810
Davis Rd. [20] vs. Davis Rd. [100]	250.4	147.3	103.1	30.67	28	35	4.756	1810
Row 5								
Control vs. Quail Creek [6]	294.8	257.4	37.38	26.47	48	37	1.997	1810
Control vs. Quail Creek [12]	294.8	290.3	4.54	27.12	48	34	0.2368	1810
Control vs. Quail Creek [20]	294.8	284.6	10.19	26.67	48	36	0.5401	1810
Control vs. Hartnell Rd. [6]	294.8	253.7	41.07	26.89	48	35	2.16	1810
Control vs. Hartnell Rd. [12]	294.8	302.2	-7.383	28.45	48	29	0.367	1810
Control vs. Hartnell Rd. [20]	294.8	248.7	46.07	33.43	48	18	1.949	1810
Control vs. Davis Rd. [6]	294.8	240.7	54.09	26.47	48	37	2.89	1810
Control vs. Davis Rd. [12]	294.8	267.7	27.1	27.61	48	32	1.388	1810
Control vs. Davis Rd. [20]	294.8	252	42.77	29.46	48	26	2.053	1810
Control vs. Davis Rd. [100]	294.8	161.8	133	27.12	48	34	6.936	1810
Quail Creek [6] vs. Quail Creek [12]	257.4	290.3	-32.84	28.74	37	34	1.616	1810

Quail Creek [6] vs. Quail Creek [20]	257.4	284.6	-27.19	28.32	37	36	1.358	1810
Quail Creek [6] vs. Hartnell Rd. [6]	257.4	253.7	3.694	28.52	37	35	0.1831	1810
Quail Creek [6] vs. Hartnell Rd. [12]	257.4	302.2	-44.76	30	37	29	2.11	1810
Quail Creek [6] vs. Hartnell Rd. [20]	257.4	248.7	8.692	34.76	37	18	0.3536	1810
Quail Creek [6] vs. Davis Rd. [6]	257.4	240.7	16.71	28.13	37	37	0.8402	1810
Quail Creek [6] vs. Davis Rd. [12]	257.4	267.7	-10.28	29.2	37	32	0.4977	1810
Quail Creek [6] vs. Davis Rd. [20]	257.4	252	5.388	30.96	37	26	0.2461	1810
Quail Creek [6] vs. Davis Rd. [100]	257.4	161.8	95.62	28.74	37	34	4.705	1810
Quail Creek [12] vs. Quail Creek [20]	290.3	284.6	5.646	28.93	34	36	0.276	1810
Quail Creek [12] vs. Hartnell Rd. [6]	290.3	253.7	36.53	29.13	34	35	1.774	1810
Quail Creek [12] vs. Hartnell Rd. [12]	290.3	302.2	-11.92	30.58	34	29	0.5514	1810
Quail Creek [12] vs. Hartnell Rd. [20]	290.3	248.7	41.53	35.26	34	18	1.666	1810
Quail Creek [12] vs. Davis Rd. [6]	290.3	240.7	49.55	28.74	34	37	2.438	1810
Quail Creek [12] vs. Davis Rd. [12]	290.3	267.7	22.56	29.8	34	32	1.071	1810
Quail Creek [12] vs. Davis Rd. [20]	290.3	252	38.23	31.52	34	26	1.715	1810
Quail Creek [12] vs. Davis Rd. [100]	290.3	161.8	128.5	29.34	34	34	6.192	1810
Quail Creek [20] vs. Hartnell Rd. [6]	284.6	253.7	30.89	28.72	36	35	1.521	1810
Quail Creek [20] vs. Hartnell Rd. [12]	284.6	302.2	-17.57	30.19	36	29	0.8231	1810
Quail Creek [20] vs. Hartnell Rd. [20]	284.6	248.7	35.89	34.92	36	18	1.453	1810
Quail Creek [20] vs. Davis Rd. [6]	284.6	240.7	43.9	28.32	36	37	2.192	1810
Quail Creek [20] vs. Davis Rd. [12]	284.6	267.7	16.92	29.39	36	32	0.814	1810
Quail Creek [20] vs. Davis Rd. [20]	284.6	252	32.58	31.13	36	26	1.48	1810
Quail Creek [20] vs. Davis Rd. [100]	284.6	161.8	122.8	28.93	36	34	6.004	1810
Hartnell Rd. [6] vs. Hartnell Rd. [12]	253.7	302.2	-48.46	30.38	35	29	2.256	1810
Hartnell Rd. [6] vs. Hartnell Rd. [20]	253.7	248.7	4.998	35.09	35	18	0.2014	1810
Hartnell Rd. [6] vs. Davis Rd. [6]	253.7	240.7	13.02	28.52	35	37	0.6453	1810
Hartnell Rd. [6] vs. Davis Rd. [12]	253.7	267.7	-13.97	29.59	35	32	0.6677	1810
Hartnell Rd. [6] vs. Davis Rd. [20]	253.7	252	1.695	31.32	35	26	0.07652	1810
Hartnell Rd. [6] vs. Davis Rd. [100]	253.7	161.8	91.92	29.13	35	34	4.463	1810
Hartnell Rd. [12] vs. Hartnell Rd. [20]	302.2	248.7	53.45	36.3	29	18	2.083	1810
Hartnell Rd. [12] vs. Davis Rd. [6]	302.2	240.7	61.47	30	29	37	2.898	1810
Hartnell Rd. [12] vs. Davis Rd. [12]	302.2	267.7	34.49	31.02	29	32	1.572	1810
Hartnell Rd. [12] vs. Davis Rd. [20]	302.2	252	50.15	32.67	29	26	2.171	1810
Hartnell Rd. [12] vs. Davis Rd. [100]	302.2	161.8	140.4	30.58	29	34	6.492	1810
Hartnell Rd. [20] vs. Davis Rd. [6]	248.7	240.7	8.019	34.76	18	37	0.3262	1810

Hartnell Rd. [20] vs. Davis Rd. [12]	248.7	267.7	-18.97	35.64	18	32	0.7526	1810
Hartnell Rd. [20] vs. Davis Rd. [20]	248.7	252	-3.303	37.09	18	26	0.1259	1810
Hartnell Rd. [20] vs. Davis Rd. [100]	248.7	161.8	86.93	35.26	18	34	3.486	1810
Davis Rd. [6] vs. Davis Rd. [12]	240.7	267.7	-26.99	29.2	37	32	1.307	1810
Davis Rd. [6] vs. Davis Rd. [20]	240.7	252	-11.32	30.96	37	26	0.5172	1810
Davis Rd. [6] vs. Davis Rd. [100]	240.7	161.8	78.91	28.74	37	34	3.883	1810
Davis Rd. [12] vs. Davis Rd. [20]	267.7	252	15.67	31.94	32	26	0.6936	1810
Davis Rd. [12] vs. Davis Rd. [100]	267.7	161.8	105.9	29.8	32	34	5.026	1810
Davis Rd. [20] vs. Davis Rd. [100]	252	161.8	90.23	31.52	26	34	4.049	1810

Table Analyzed	Novpmr
Data sets analyzed	A-K
ANOVA summary	
F	17.98
P value	<0.0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0.33
Brown-Forsythe test	
F (DFn, DFd)	
P value	
P value summary	
Are SDs significantly different (P < 0.05)?	
Bartlett's test	
Bartlett's statistic (corrected)	415
P value	<0.0001
P value summary	****
Are SDs significantly different (P < 0.05)?	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	260919	10	26092	F (10, 365) = 17.98	P<0.0001

Residual (within columns)	529734	365	1451
Total	790653	375	

Data summary	
Number of treatments (columns)	11
Number of values (total)	376
Number of families	1
Number of comparisons per family	10
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	A-?	
Control vs. Quail Creek [6]	-66.27	-89.12 to -43.41	Yes	****	<0.0001	B	Quail Creek [6]
Control vs. Quail Creek [12]	-42.1	-65.50 to -18.70	Yes	****	<0.0001	C	Quail Creek [12]
Control vs. Quail Creek [20]	-21.1	-44.13 to 1.934	No	ns	0.0924	D	Quail Creek [20]
Control vs. Hartnell Rd. [6]	-24.07	-47.28 to -0.8563	Yes	*	0.0375	E	Hartnell Rd. [6]
Control vs. Hartnell Rd. [12]	-43.51	-68.01 to -19.01	Yes	****	<0.0001	F	Hartnell Rd. [12]
Control vs. Hartnell Rd. [20]	-20.41	-49.50 to 8.690	No	ns	0.3361	G	Hartnell Rd. [20]
Control vs. Davis Rd. [6]	-40.59	-63.45 to -17.73	Yes	****	<0.0001	H	Davis Rd. [6]
Control vs. Davis Rd. [12]	-55.41	-79.21 to -31.60	Yes	****	<0.0001	I	Davis Rd. [12]
Control vs. Davis Rd. [20]	-55.28	-80.32 to -30.25	Yes	****	<0.0001	J	Davis Rd. [20]
Control vs. Davis Rd. [100]	-97.23	-120.6 to -73.84	Yes	****	<0.0001	K	Davis Rd. [100]

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. Quail Creek [6]	45.02	21.25	-66.27	8.272	48	38	8.011	365
Control vs. Quail Creek [12]	45.02	-2.918	-42.1	8.468	48	35	4.972	365
Control vs. Quail Creek [20]	45.02	-23.92	-21.1	8.334	48	37	2.531	365
Control vs. Hartnell Rd. [6]	45.02	-20.95	-24.07	8.399	48	36	2.865	365
Control vs. Hartnell Rd. [12]	45.02	-1.506	-43.51	8.866	48	30	4.907	365

Control vs. Hartnell Rd. [20]	-45.02	-24.61	-20.41	10.53	48	18	1.938	365
Control vs. Davis Rd. [6]	-45.02	-4.426	-40.59	8.272	48	38	4.907	365
Control vs. Davis Rd. [12]	-45.02	10.39	-55.41	8.615	48	33	6.432	365
Control vs. Davis Rd. [20]	-45.02	10.27	-55.28	9.059	48	28	6.103	365
Control vs. Davis Rd. [100]	-45.02	52.22	-97.23	8.468	48	35	11.48	365

Appendix D: Chapter 4

Supplemental Figures and Tables

Supplemental Figures

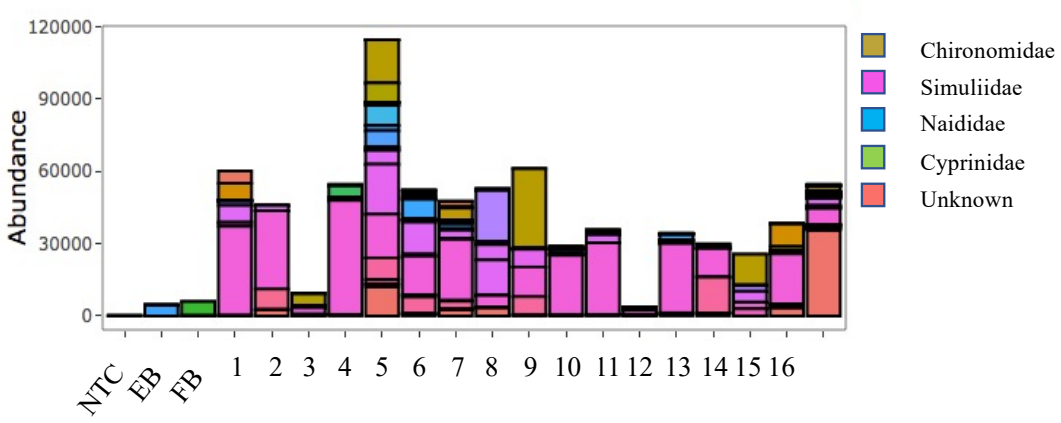


Figure S4.1. Salinas River Watershed eDNA biomonitoring Pilot data showing sequence abundance of 16 replicate sediment samples from Site 309SED062 for CO1 primers. No template control (NTC), extraction blank (EB) and field blank (FB) are shown on the left hand side, and top five taxonomic families detected are listed in the legend. Raw sequence data was analyzed using the ANACAPA bioinformatic pipeline and visualized in RANACAPA.

Figure S4.2. Salinas River Watershed eDNA biomonitoring sequencing output (MiSeq 300 PE) showing the total number of reads and Quality Score (Q) values per sample for raw (unfiltered) sequence data. Visualized in the dada2 package of RStudio.

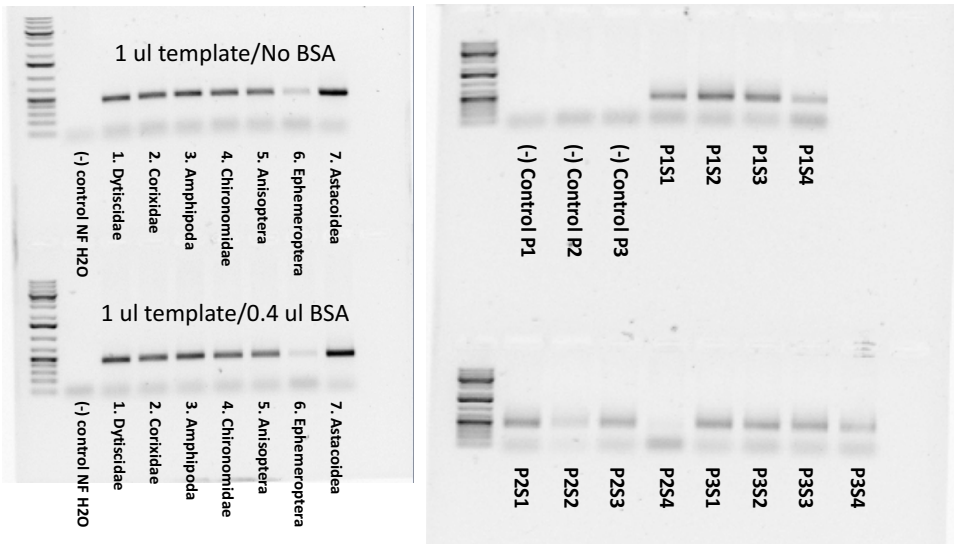


Figure S4.3. Pilot data. Amplification of California taxa using CO1 primers from A) invertebrate tissue and B) 500 mL of water filtrate.

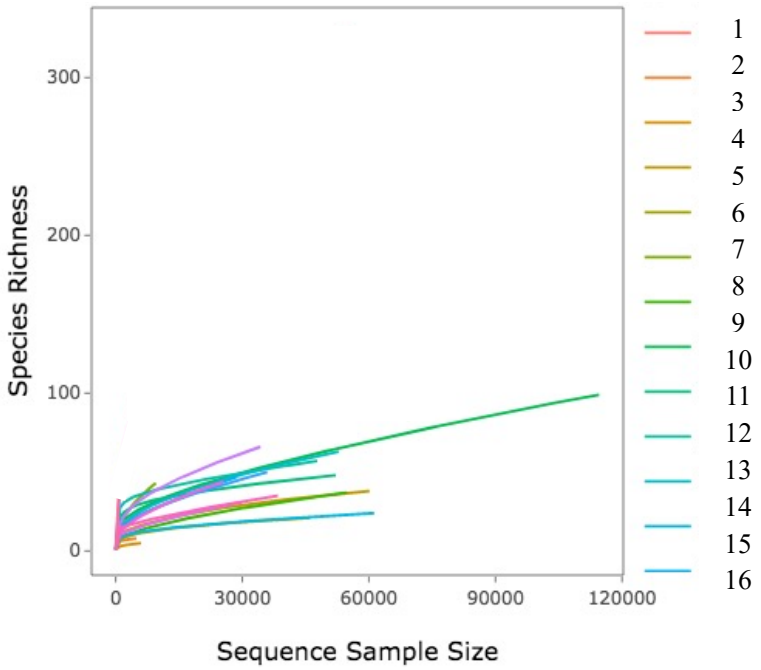


Figure S4.4. Salinas River Watershed eDNA biomonitoring pilot data showing species richness (measured as number of unique Amplicon Sequence Variants) of 16 replicate sediment samples from Site 309SED062 for CO1 primers. Raw sequence data was analyzed using the ANACAPA bioinformatic pipeline and visualized in RANACAPA.

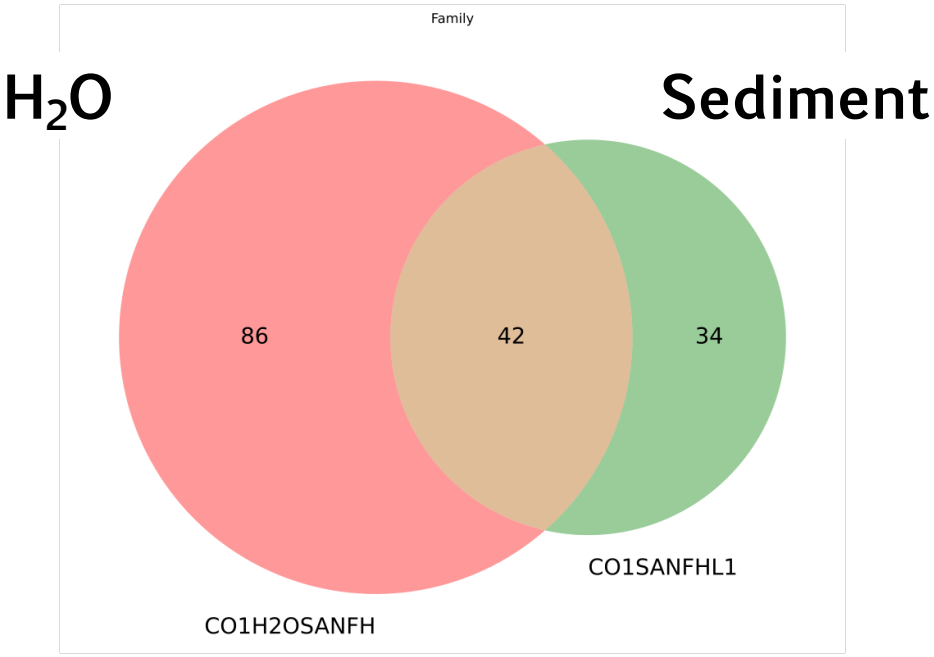


Figure S4.5. Salinas River Watershed eDNA biomonitoring Venn diagram showing family-level identifications for water filtrate versus sediment, from site 309SED062 (CSCI > 1.2) for CO1 primers. Raw sequence data was analyzed using the ANACAPA bioinformatic pipeline and visualized in TaxonTableTools.

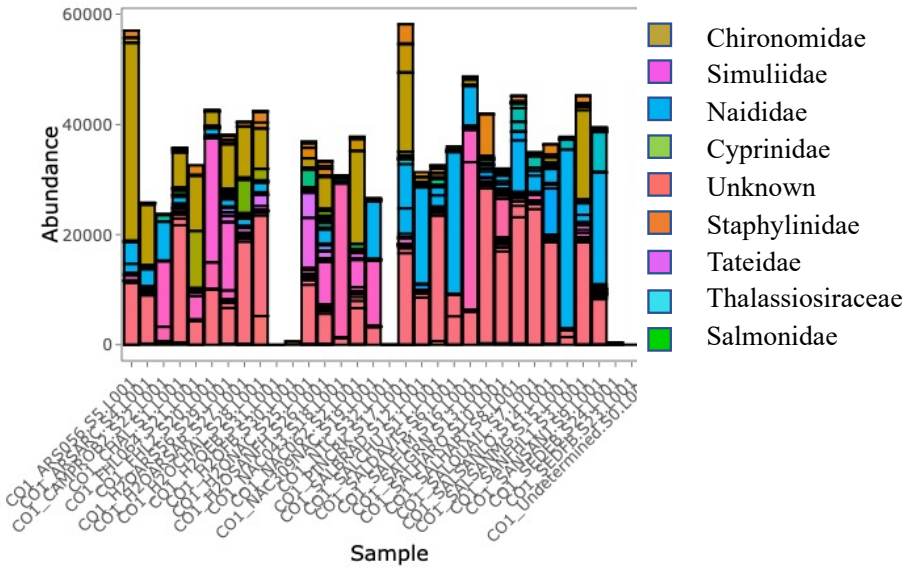


Figure S4.6. Salinas River Watershed eDNA biomonitoring unrarefied abundance of sequence reads assigned to family level for CO1 primers, shown per sample. Raw sequence data was analyzed using the ANACAPA bioinformatic pipeline and visualized in RANACAPA.