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RESEARCH

Sub-Lethal Responses of Delta Smelt to Contaminants Under Different Flow Conditions

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ABSTRACT

The Delta Smelt is a largely zooplanktivorous, endangered fish endemic to the San Francisco Estuary (the estuary). High flows increase the availability of fresh and brackish water habitat for Delta Smelt, but also may mobilize contaminants, potentially increasing toxicological stress. Here, we examine the association between contaminants and Delta Smelt health across contrasting water year types and flow-related management actions. Our study spanned the fall season of three years: 1 dry year (2018) bracketed by 2 wet years (2017 and 2019) and coincided with several management actions meant to benefit Delta Smelt. We collected field water from six sites in the estuary that encompass the freshwater and low-salinity habitat of Delta Smelt and analyzed the water for contaminant concentrations. After a 96-hour exposure to the field water, we assessed cultured Delta Smelt survival and the histopathological condition of the gill and liver. Insecticides, particularly fipronil metabolites, were the most prevalent contaminants detected in 2017 and 2018, and a variety of contaminants associated with the rice harvest were detected in 2019. No acute toxicity was observed during any exposure, but we observed negative effects in the livers of Delta Smelt exposed to agricultural water from the Toe Drain and Cache Slough during a 2019 pulse flow action, which coincided with elevated detections and concentrations of organic pesticides. Other noteworthy sub-lethal effects, likely occurring in response to contaminant mixtures, included severe gill lesions in Delta Smelt exposed to Decker Island water in 2019. In the drier year of 2018, lesions were generally mild or absent. Thus, the trade-offs between increased habitat availability and contaminant loading may provide one explanation for why Delta Smelt abundance does not consistently respond positively to outflow.

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INTRODUCTION

The San Francisco Estuary and adjacent Sacramento–San Joaquin Delta (hereafter the estuary) together comprise the largest estuary on the west coast of the United States. The estuary is a highly altered system, rife with anthropogenic changes resulting from the economic development of California. It receives flow from the Sacramento and San Joaquin rivers, provides irrigation and drinking water to millions of Californians, and serves as a migratory pathway for various anadromous and semi-anadromous fish species.

The Delta Smelt (*Hypomesus transpacificus*) is a small, mainly zooplanktivorous, and largely annual fish that is endemic to the estuary. Its distribution varies according to life stage, with the dominant phenotype rearing in the Low Salinity Zone (LSZ; 0.5 to 6 psu) and later migrating upstream to freshwater areas in the Delta to spawn (MAST 2015; Moyle et al. 2018; Hobbs et al. 2019). The species is listed as threatened by the United States Fish and Wildlife Service (USFWS) and endangered by the California Department of Fish and Wildlife (CDFW) because of its sustained low abundance for the past several decades (MAST 2015; Jin et al. 2018). For example, from 2017 to 2022, zero Delta Smelt were detected by the CDFW's Fall Midwater Trawl, a survey which previously collected thousands of Delta Smelt annually (CDFW 2023).

Because they reside in the freshwater and low-salinity areas of the estuary during summer and fall, the location and extent of Delta Smelt habitat is largely determined by the amount of freshwater flow through the system (Delta Science Program 2020). Thus, increases in freshwater flow may support recovery of the species. Fall outflow is of particular interest because Delta Smelt appears to go through a demographic and condition bottleneck as its available low-salinity habitat shrinks with low flows at the end of the summer dry season (Moyle et al. 1992; Feyrer et al. 2011; Hammock et al. 2021). A number of water-management strategies in the estuary are aimed at the recovery of the species. Specifically, Fall X2 enhances the habitat available for Delta Smelt. The position of "X2" refers to the distance (km) from the Golden Gate Bridge to the salinity isohaline of two psu. Historically, X2 has been a zone of high productivity and turbidity and typically represents the center of Delta Smelt distribution (Jassby et al. 1995; Kimmerer et al. 2013; Delta Science Program 2022). Fall X2 actions require the US Bureau of Reclamation (USBR) and the CDWR to manage exports and reservoir releases (either actively or passively; USFWS 2008) so that Delta outflow maintains specific monthly average locations of X2 in the fall during "Wet" or "Above Normal" water years when there is sufficient water available. The goal of Fall X2 is to push the LSZ westward to increase the overlap with the turbid, cooler environment of Suisun Bay and Suisun Marsh, thereby enhancing availability of high-quality habitat and prey for Delta Smelt (MAST 2015; CNRA 2017).

Food availability, which is intrinsically tied to habitat distribution, is an important factor related to the status of Delta Smelt. Accordingly, specific management actions, such as the North Delta Food Subsidy (NDSF; Sommer et al. 2020), aim to increase resource availability for Delta Smelt. The North Delta and the Yolo Bypass (a managed floodplain) in particular, maintain high levels of phytoplankton and are considered areas of high productivity (Mahardja et al. 2019). However, during the summer and fall, low flows keep this highly productive water within the Yolo Bypass region, and water conveyance can result in reverse flows in the area (Frantzich et al. 2021). In 2011 and 2012, fall phytoplankton blooms were observed downstream of the North Delta for the first time in over 20 years after larger than normal fall agricultural flow pulses (Twardochleb et al. 2021). The NDFS aims to mirror the 2011–2012 pulse flow by re-routing freshwater from agricultural return water or from the Sacramento River through the Yolo Bypass, thereby moving nutrients and phytoplankton downstream of the Yolo Bypass (e.g., lower Cache Slough and lower

Sacramento River). The goal of the NDFS is to reduce negative net flows in the Cache Slough complex and consequently improve food-web productivity and prey availability for Delta Smelt downstream toward the Central Delta and in the more food-limited areas of the upper estuary (Frantzich et al. 2021).

Although freshwater flows may benefit Delta Smelt, in the estuary these flows also contain varying concentrations and types of contaminants from industrial, urban, and agricultural sources (Thompson et al. 2007; Smalling et al. 2013; Orlando et al. 2014; Jabusch et al 2018; De Parsia et al 2018; 2019), resulting in the inclusion of the estuary on the Clean Water Act Section 303(d) and the 305(d) List of Impaired Water Bodies (SWRCB 2018). For instance, effluent from municipal wastewater treatment plants (e.g., Sacramento, Stockton, and Vacaville) and untreated stormwater runoff discharges into the Delta. While some urban effluents (e.g., wastewater) are typically discharged continuously throughout the year, other sources are more seasonal. For example, irrigated agricultural land is linked to seasonal inputs of contaminants, as tail-water is discharged into the estuary during timed pulse flows in the dry summer months, while urban stormwater runoff discharges contaminants during wet winter months. The positive association between high outflow and contaminants (Chen et al. 2019; Commelin et al. 2022; Schönenberger et al. 2022) has important implications for water quality and biota in the estuary.

While the increase of freshwater outflow has long been known to expand physical habitat availability for Delta Smelt (Jassby et al. 1995; Feyrer et al. 2011; Bever et al. 2016), a concurrent increase in contaminants may also negatively affect the species. For example, Teh et al. (2020) described substantial improvement in liver condition of wild-caught Delta Smelt as drought severity increased, suggesting that water quality improved during the drought, improving the liver health of surviving fish. Trade-offs between increased habitat availability and contaminant loading may partially explain why Delta Smelt abundance does not necessarily respond positively with outflow, if exposure to contaminants negatively affects Delta Smelt health and condition (Stevens and Miller 1983; Dege and Brown 2003; Miller et al. 2012; Brown et al. 2020). Thus, understanding the cumulative effects of outflow and contaminants on Delta Smelt viability is integral for developing optimal management practices aimed at recovering Delta Smelt populations.

To examine the influence of regional variation in water quality and potential increases in contaminants associated with increased flow, we evaluated the survival, health, and condition of cultured Delta Smelt exposed to water collected from six locations in the estuary. Our study occurred during the fall season of two wet years (2017 and 2019) and one dry year (2018) and coincided with Fall X2 and NDFS actions. We conducted chemical analyses to determine the presence and concentrations of contaminants in the collected water and to examine whether contaminants were associated with detrimental effects on Delta Smelt. Our assessment of Delta Smelt included survival and histopathology of the liver and gills, a method which can detect a multitude of sub-lethal stressors (Hadi and Alwan 2012; Devi and Mishra 2013; Cao et al. 2018). Our (a priori) hypotheses were that (1) Delta Smelt would exhibit site-specific gill and liver damage associated with contaminant exposure; (2) lesions would be more prevalent and/or severe during the wetter years of 2017 and 2019 compared to the drier year of 2018; and (3) lesions and contaminants would be more prevalent and/or severe during flow actions.

MATERIALS AND METHODS

Sampling Design and Water Collections

We collected water samples every 2 weeks from October to December in 2017 and from September to November in 2018 and 2019. We selected six fixed sampling sites to span freshwater and low-salinity habitat of Delta Smelt (Merz et al 2011; Hammock et al. 2015). We selected the Toe Drain, Cache Slough [Cache], Sacramento River at Isleton [Isleton], Sacramento River at Decker Island [Decker Island], Montezuma Slough [Montezuma],

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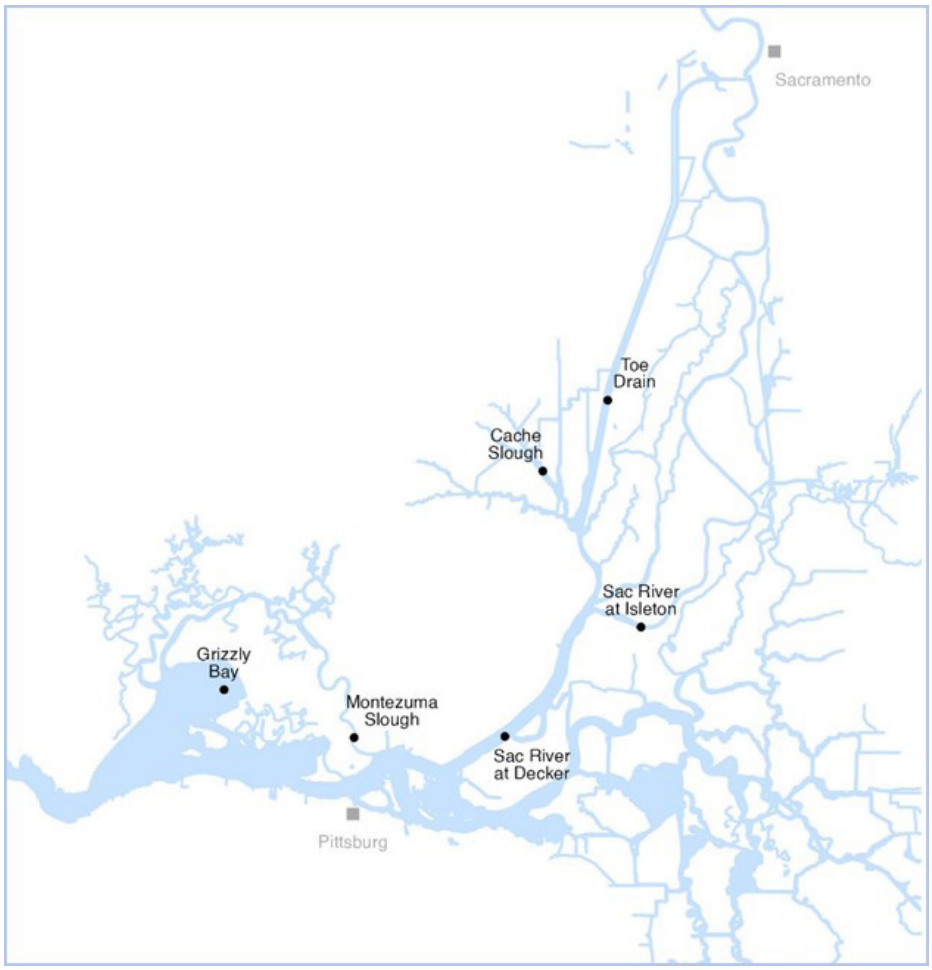

Figure 1 Map of study area and sampling sites in the Sacramento–San Joaquin River Delta and San Francisco Estuary, located in California, USA

Table 1 Summary of sampling events and exposure test initiation dates across the study

Year	Ave Delta Outflow ($m^3 s^{-1}$)	Flow Action	Collection Dates	Test Date	Test Exposure
2017	292.3	Fall X2	Oct. 11, 12	Oct. 13	1
2017	254.9	Fall X2	Oct. 24, 25	Oct. 27	2
2017	255.8	Fall X2	Nov. 8	Nov. 10	3
2017	201.6	Fall X2	Nov. 22	Nov. 24	4
2017	163.8		Dec. 5, 6	Dec. 8	5
2018	167.0	NDFS	Sept. 19, 20	Sept. 21	1
2018	292.3		Oct. 3, 4	Oct. 5	2
2018	117.4		Oct. 17, 18	Oct 19	3
2018	124.0		Oct. 31, Nov. 1	Nov. 2	4
2018	139.9		Nov. 14, 15	Nov. 16	5
2019	376.2	NDFS, Fall X2	Sept. 10, 11	Sept. 13	1
2019	290.1	NDFS, Fall X2	Sept. 24, 25	Sept. 27	2
2019	412.0	Fall X2	Oct. 10	Oct. 11	3
2019	372.5	Fall X2	Oct. 22, 24	Oct. 25	4
2019	203.7	Fall X2	Nov. 5, 6	Nov. 8	5

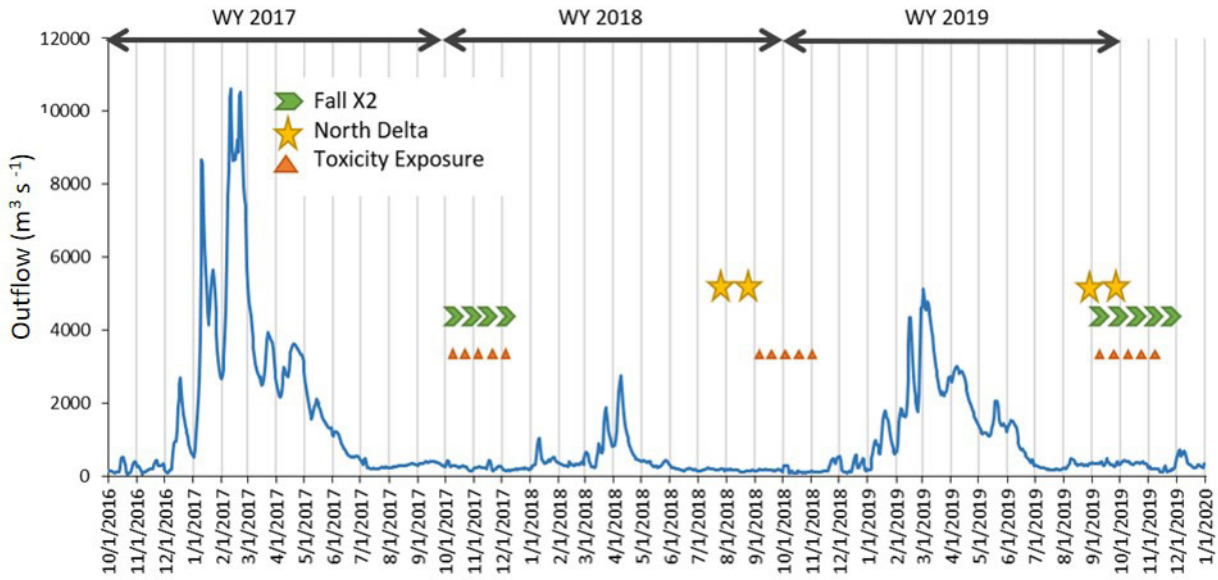

Figure 2 Net Delta outflow ($m^3 s^{-1}$) encompassing the 3-year project period, flow actions, and exposures. Outflow data obtained from Dayflow California Natural Resources Agency Open Data (*https://data.cnra.ca.gov/dataset/dayflow).*

and Grizzly Bay as our study sites (Figure 1, Table 1). In 2017, we did not collect samples at all sites for all toxicity exposures; the Toe Drain, Isleton, and Decker Island sites were not included in Exposure 1, and Grizzly Bay was not included in Exposure 2. We collected water from all site locations for subsequent toxicity exposures in 2018 and 2019.

We collected up to 80 L of ambient water from each site with a bilge pump as sub-surface grabs and stored in four 20-L plastic cubitainers (I-CHEM, Fisher Scientific) for use in Delta Smelt toxicity exposure tests. We collected additional sub-samples in 1-L glass amber bottles (I-CHEM, Fisher Scientific) and 1-L plastic bottles (I-CHEM, Fisher Scientific) for chemical analyses and water-quality measurements, respectively. All samples were kept in cold (0 to 6 °C), dark conditions until use.

Water Years and Outflow Actions

CDWR classified the 2017 water year as "Wet," which triggered an X2 action that coincided with toxicity exposures 1 through 4. Average Delta outflow during this period ranged from a high of 292 $m^3 s^{-1}$ for waters collected for Exposure 1, and subsequently decreased to a low of 164 $m^3 s^{-1}$ for waters collected for Exposure 5 (Table 1, Figure 2; *https://cdec.water.ca.gov).* The 2018 water year was classified as "below normal" and as such, no X2 action was implemented. Delta outflow ranged from 117 to 292 $m^3 s^{-1}$ during these exposures (Figure 2). The NDFS took place in early fall of 2018, where rice drainage agricultural tail-water from Colusa Basin Drain was rerouted through the Yolo Bypass between August 28 and September 26, resulting in peak mean flows in the Toe Drain at 17 $m^3 s^{-1}$ (Twardochleb et al. 2021). This NDFS action coincided with Delta Smelt Exposure 1 (Table 1; Figure 2). The 2019 water year was classified as "Wet," triggering an X2 action that coincided with all Delta Smelt exposures. Delta outflow ranged from 204 to 412 $m^3 s^{-1}$ during these exposures (Table 1; Figure 2), and, in early fall, rice-field-drainage agricultural return water was redirected during the NDFS between August 26 and September 21 (Twardochleb et al. 2021). This NDFS action increased peak mean flows through the Toe Drain to 23 $m^3 s^{-1}$ and coincided with Delta Smelt Exposures 1 and 2 (Figure 2).

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Chemical Analyses

Chemical analyses varied across years. In 2017 and 2019, organic compound analyses included Gas Chromatography coupled with Quadrupole Time-Of-Flight Mass Spectrometry (GC-QTOF-MS; Agilent model 7200) and Liquid Chromatography coupled with Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS; Agilent model 6530) for targeted pesticide analyses (e.g., for synthetic pyrethroid insecticides). Upon receipt, we passed 1-L water samples through solid phase extraction cartridges (Oasis HLB) and sequentially eluted them with solvents to produce two extracts, one for LC and one for GC analysis, following methods outlined in Moschet et al. (2017). Depending on the year, we analyzed extracts on up to four instrument platforms: LC with positive and negative electrospray ionization modes (ESI+ and ESI-) and GC with both electron ionization (EI) and negative chemical ionization (NCI). In 2018 we focused only on GC-NCI-targeted analyses, following the aforementioned methods.

Delta Smelt Toxicity Testing

We used sub-adult Delta Smelt obtained from the UC Davis Fish Conservation and Culture Laboratory (FCCL; Byron, California) in each of the toxicity exposures. FCCL cultures a population of Delta Smelt which is annually outbred with fish collected from the wild, serves as a refuge population, and has recently been used to supplement the wild population (Fisch et al. 2012; Lindberg et al. 2013; Hung et al. 2022). Cultured Delta Smelt are maintained in pre-conditioned surface water from the California Aqueduct that is treated for solids removal and disinfected with UV, but almost certainly contains dissolved contaminants. One day before the start of the exposure tests, we placed Delta Smelt into replicate buckets filled with temperature-controlled FCCL culture water for a 24-hr acclimation period. At the toxicity exposure initiation, we replaced water in the buckets with collected ambient field water, replenished culture water (i.e., control), or high-salinity control (HSC) water. We included the freshwater control (FCCL culture water) as a comparison for the freshwater sites (Toe Drain, Cache, Isleton, Decker Island), while the HSC was included as a comparison for the brackish water sites (Montezuma, Grizzly Bay), thereby accounting for possible salinity stress or benefits to Delta Smelt (See Table A1 in Appendix A for site conductivities). High-salinity control water consisted of FCCL culture water amended with Instant Ocean® (Spectrum Brands) to match the salinity of Grizzly Bay (the site with the highest salinity), which ranged from 2.8 to 10.8 psu. Given that the Delta Smelt in this study were likely exposed to contaminants (i.e., from the California Aqueduct) before their use in the experiments, as well as during the experiments in the control water, we conducted chemical analyses on the control water in 2018 and 2019, although analyses were not conducted in 2017 (Table A2).

All toxicity exposure tests had durations of 96 hours and were conducted indoors at FCCL using a static water system. Experimental replicates consisted of 20-L black plastic buckets with lids (Encore Plastics). We loosely placed lids on the replicate buckets to minimize light while allowing room for constant aeration (Delta Smelt are sensitive to light; Lindberg et al. 2013). Tests in 2017 and 2019 included four replicate buckets containing 8 L of water and five fish each, for a total of 20 fish per treatment, 160 fish per exposure (20 fish for each of eight treatments [six sites and two controls]), and 800 fish per year (five exposures of 160 fish each). Because of limited Delta Smelt availability for toxicity testing in 2018, we reduced experimental replicates from four to three, with five fish per replicate for a total of 15 fish per treatment, 120 fish per exposure, and 600 fish total. We kept test replicates in a temperature-controlled water bath maintained at 16 °C using a chiller and pump system to circulate the water, and test temperatures deviated from 16 °C by no more than +/- 2 °C during acclimation and toxicity exposures. We maintained other water-quality parameters (e.g., pH, DO, ammonia–nitrogen) within optimal physiological ranges. FCCL staff removed and recorded Delta Smelt mortalities daily. At the end of each 96-hour exposure period, we euthanized surviving fish with an overdose of buffered tricaine methane sulfonate and fixed in 10% buffered formalin for histopathology and other sub-lethal analyses.

Histopathology

Following the exposure tests, we performed histopathology on the gills and livers of Delta Smelt. Histological assessment can be used as a biomarker of environmental contamination (Au 2004). Generally, liver and gill histopathological changes are sensitive and responsive but not specific to pollutant exposure. The occurrence of similar lesion types under a wide range of stressors and contaminants (Mallatt 1985) and chemical interactions (e.g., synergism/antagonism) precludes our ability to directly study the cause–effect relationship between specific pollutants and lesions (Au 2004). Because the liver is the primary location for metabolic and detoxification processes and the gills are one of the first exposure routes for waterborne contaminants, these are the primary tissues used for the assessment of morphological alterations. We anticipated that gills would respond more quickly to external stressors than the liver (Teh et al. 2020), making gill histology of particular interest because the toxicity exposures were brief.

We excised and placed the left gill arches and the whole liver in 10% neutral buffered formalin and processed according to Teh at al. (2016). Briefly, tissues were embedded in paraffin, sectioned to 3-µm thickness and stained with hematoxylin and eosin. For liver and gill tissues, we scored lesions qualitatively from 0 to 3, where 0 = lesion not present, 1 = mild, 2 = moderate, and 3 = severe (multiple lesion types were scored per tissue; see descriptions in Teh et al. 2020). To provide an aggregate metric of the liver and gill condition, we calculated a histopathological index by summing the scores for each lesion type for each organ for an individual fish. We then used these aggregate lesion scores from individual fish in our statistical analyses. Thus, for each treatment, the average gill or liver lesion score represents the average degree of damage to each organ, with higher scores indicating more damage (Hammock et al. 2015; Teh et al. 2020).

We statistically compared the lesion scores of liver and gill tissues within individual project years with a Kruskal–Wallis Rank Sum test, with Site and Toxicity Exposure as predictors, followed by a Steel–Dwass multiple comparisons test if the Kruskal–Wallis test was significant. In each project year, we used a subset of fish for histological analyses (n = 368 fish in 2017, n = 236 fish in 2018, and n = 160 fish in 2019). To test for differences among years, we analyzed liver and gill data with a Kruskal–Wallis Rank Sum test with Year as the predictor, followed by a Dunn's multiple comparisons test if the Kruskal–Wallis test was significant.

RESULTS

Analytical Chemistry

Of the 90 water samples collected over the 3-year study, we identified 16 different compounds among 270 detections. The greatest number of detections occurred in 2017 (166), followed by 2019 (74), with 2018 having the fewest detections (30; Figure 3). However, we note that we analyzed for fewer chemical classes in 2018 than in 2017 and 2019. Thus, the lower number of chemical detections observed during 2018 is presumably a direct result of that change. Because of the difference in the analyses conducted, we cannot make annual comparisons across all 3 years, but we can compare 2017 and 2019. Overall, analyte concentrations were higher in 2019 compared to 2017, but the specific pesticides that were detected varied across years (Figure 3).

In 2017, the majority of detected compounds were insecticides (81%). Fipronil and its metabolites made up 66% of all detections during this year (Table A3). Fipronil and its degradates fipronil-desulfinyl, fipronil-sulfide, and fipronil-sulfone were detected consistently at concentrations ranging from 0.05 to 0.29 ng L^{-1}. The herbicide hexazinone and fungicide azoxystrobin were the second-most-frequently-detected compounds. Concentrations of azoxystrobin ranged from 4.67 to 23.67 ng L^{-1}, with the highest concentration detected at Isleton during Exposure 4 (Figure 3A). In 2017, fungicides and herbicides respectively comprised 11% and 12% of all detections. The insecticide methoxyfenozide was detected at least twice at each site throughout this study period, with concentrations ranging from 3.74 to 12.61 ng L^{-1}. Chlorpyrifos was detected in water

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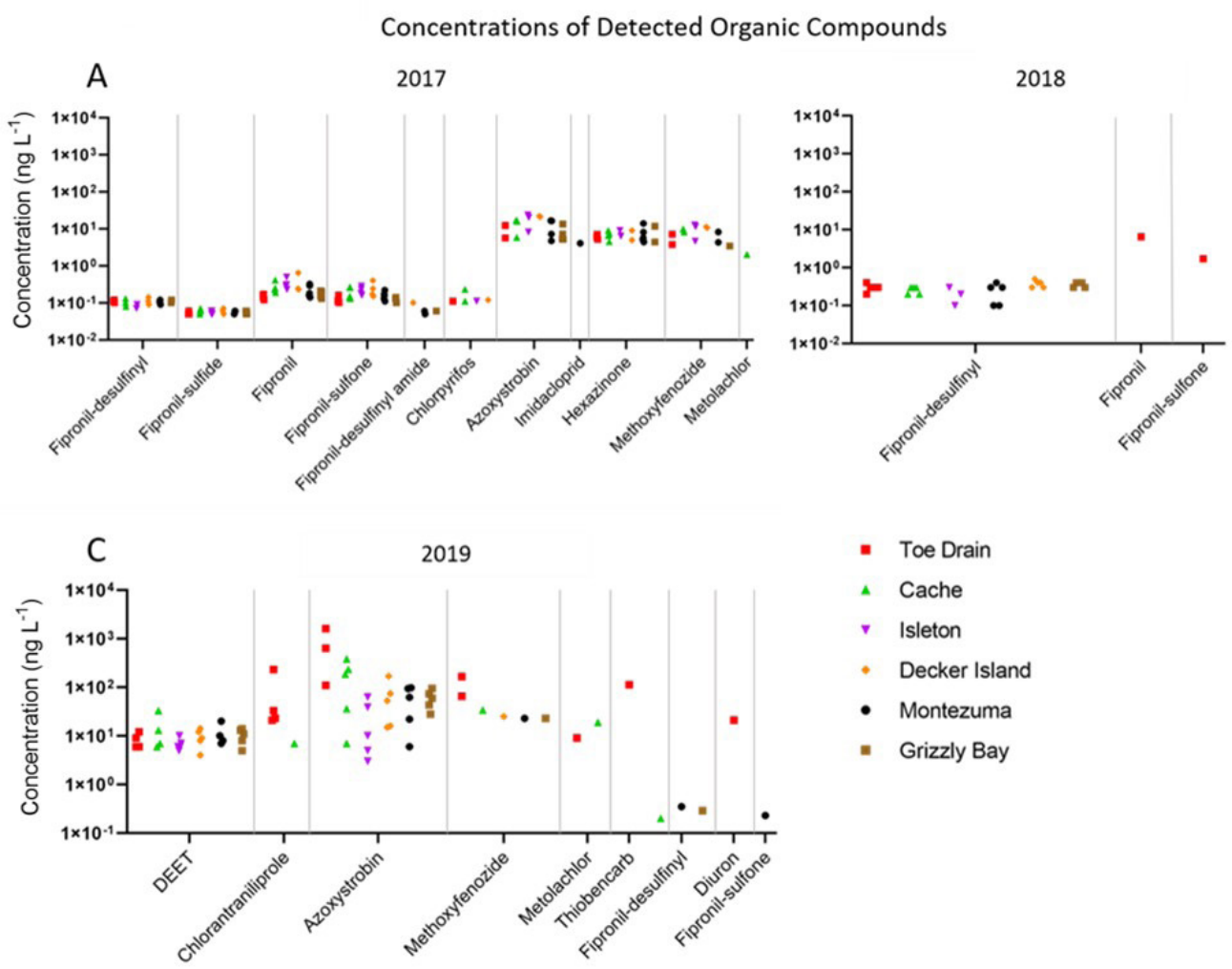

Figure 3 Concentrations of organic compound detections shown for each exposure test from the six sites for (**A**) 2017, (**B**) 2018, and (**C**) 2019. Note the different *y*-axis in Panel **C**.

samples collected from Isleton in Exposure 3 and from Toe Drain, Cache, and Decker Island in Exposure 4, with concentrations ranging from 0.11 to 0.23 ng L^{-1}.

In 2018, we focused only on GC-NCI-targeted analytes; thus, we observed a low number of organic contaminants, with only fipronil and its degradates detected consistently across exposures (Figure 3B). Fipronil-desulfinyl was detected in 93% of samples with concentrations ranging from 0.1 to 0.5 ng L^{-1}. Fipronil and fipronil-sulfone were detected in the Toe Drain during Exposures 2 and 3 at 6.5 ng L^{-1} and 1.7 ng L^{-1}, respectively (Table A4).

In 2019, we saw a difference in the composition of analytes detected, and contaminant concentrations were generally higher when compared to previous project years. For example, fipronil metabolites were only detected four times: during Exposure 1, fipronil-desulfinyl concentrations were 0.20, 0.35, and 0.29 ng L^{-1} at Cache, Montezuma, and Grizzly Bay, respectively; and fipronil-sulfone concentration was 0.23 ng L^{-1} at Montezuma. The fungicide azoxystrobin and the insecticide DEET were particularly prevalent in the 2019 water samples (Figure 3C). Azoxystrobin had a 100% detection frequency, with the highest concentration of 1,601 ng L^{-1} in the Toe Drain during Exposure 1 (Table B5). DEET (N,N-diethyl-m-toluamide) was detected at all sites

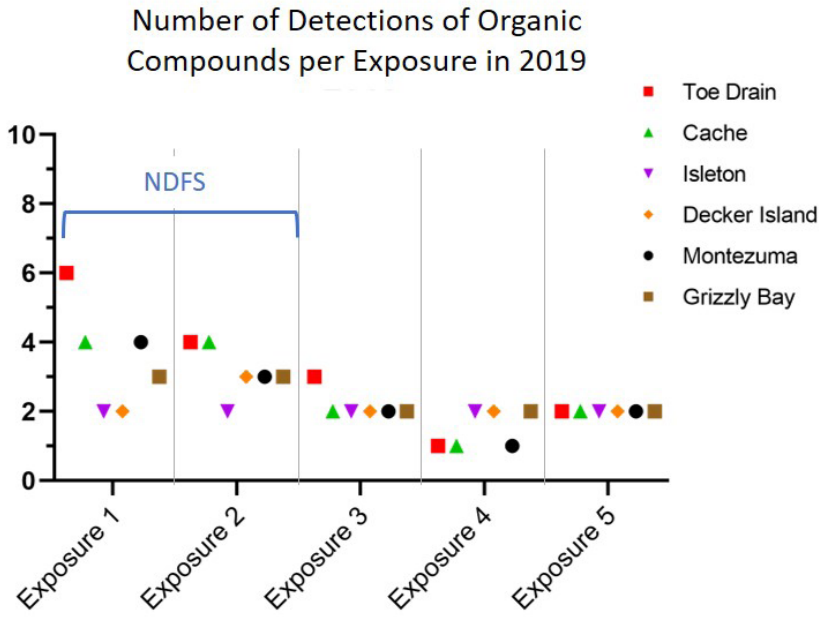

Figure 4 Number of detections of organic compounds per exposure in 2019

in almost all exposures in 2019, with a maximum concentration of 33 ng L^{-1} in Exposure 3 at Cache. Exposures 1 and 2 had the highest concentrations and greatest variety of contaminants, including DEET, chlorantraniliprole, azoxystrobin, methoxyfenozide, metolachlor, and thiobencarb (Figure 3C). The presence of certain contaminants (e.g., chlorantraniliprole, azoxystrobin, metolachlor) was particularly notable in water from the Toe Drain and Cache in Exposures 1 and 2, which also coincided with the NDFS (Figure 4). Methoxyfenozide was also detected in Decker Island during Exposure 2.

Toxicity Testing and Histopathology

No acute toxicity was observed in Delta Smelt exposed to water from any of the six sampling locations across the 3-year study period. Fish survival rates were high, exhibiting at least 96% survival across the 3 years (see Figure B1, panel A, in Appendix B). As detected by histopathology, fish condition was variable, especially in the gills. When combining observations across exposure tests and sites, gill lesion scores differed significantly across years (Kruskal–Wallis χ^2: 72.23, df: 2, $P < 0.0001$; Figure 5; Figure B1 in Appendix B), with higher scores in 2017 compared to 2018 ($P < 0.0001$) and 2019 ($P = 0.0208$). Liver lesion scores also differed across years (Kruskal–Wallis χ^2: 213.9, df: 2, $P < 0.0001$; Figure 5; Figure B1, panel C, in Appendix B), with higher lesion scores in 2017 compared to both 2018 ($P < 0.0001$) and 2019 ($P < 0.0001$). For 2017, the presence of mild gill and liver lesions extended to the controls (Figure 5A and 5D). For 2017, we cannot explain why Delta Smelt in the HSC exhibited elevated gill lesion scores while those in the freshwater control did not. It was apparently unrelated to salinity stress because the difference in salinity between control and HSC was similar in 2017 and 2019 and we did not see elevated gill lesion scores.

Gill lesion scores significantly differed in 2017 across sites (Kruskal–Wallis, χ^2: 32.28, df: 7, $P < 0.0001$). Elevated gill lesions were observed in fish exposed to all ambient sites when compared to the freshwater control, including fish in the HSC (Figure 5A). Chloride cell hyperplasia and mucous cell hyperplasia were the most frequently observed type of gill lesions, especially during Exposures 1 and 3 (Figure 6A). In Exposures 1 and 3 lesions were also observed in HSC fish, indicating the possibility that there was a contaminant present in the ambient water used for culturing the Delta Smelt (i.e., and used for the controls); however, we did not measure analytical chemistry on the control water in 2017, thus we

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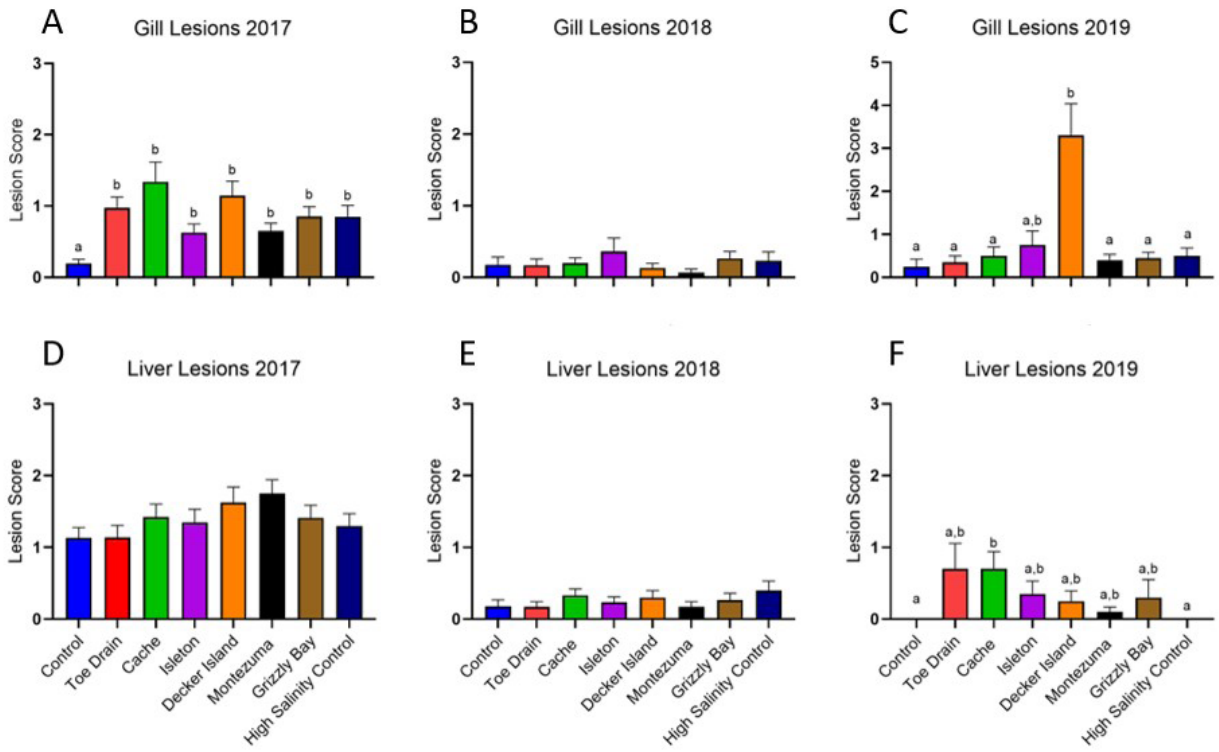

Figure 5 Summary of gill lesion scores for (**A**) 2017, (**B**) 2018, (**C**) 2019, and liver lesion scores for (**D**) 2017, (**E**) 2018, and (**F**) 2019. *Error bars* denote standard error. *Different letters* indicate significant differences in lesion score across sites. Note the *y*-axis in panel **C** differs from other panels.

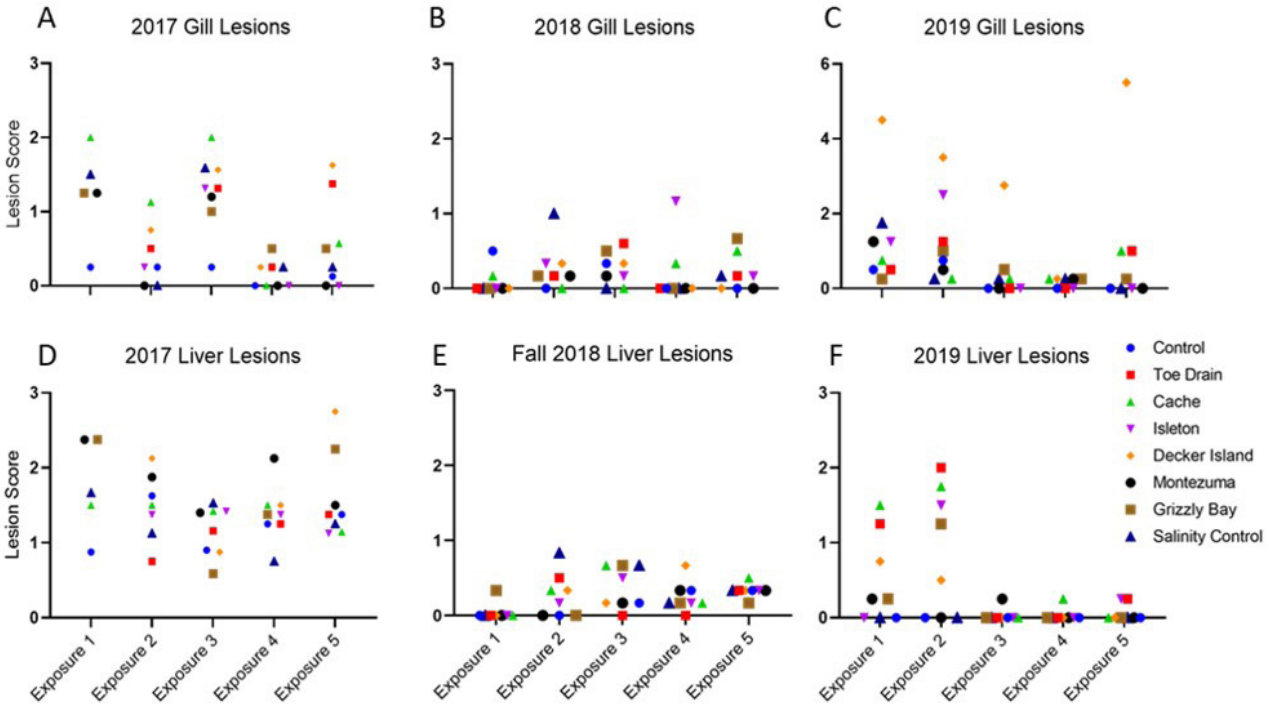

Figure 6 Summary of gill lesion scores for (**A**) 2017, (**B**) 2018, (**C**) 2019, and liver lesion scores for (**D**) 2017, (**E**) 2018, and (**F**) 2019 across toxicity exposures. Note the *y*-axis in panel **C** differs from other panels.

cannot confirm this. Chloride cell hyperplasia was observed in 88% of fish exposed to water from Cache, 21% of fish exposed to the Toe Drain, and in 63% of fish exposed to Montezuma. Mucous cell hyperplasia was observed in 26% of fish exposed to Cache, 38% of fish exposed to Toe Drain, and 20% of fish exposed to Montezuma. In particular, fish exposed to Cache water (Figure B2) tended to have high lesion scores, including half of the replicate fish with severe mucous cell hyperplasia in Exposure 3 (Figure B3, panel B) as well as one fish with severe gill aneurysm or telangiectasia in Exposure 2 (Figure B3, panel C).

Liver lesion scores were elevated in fish exposed to all sites and controls, but did not vary across sites in 2017 (Kruskal–Wallis, χ^2: 8.69, df: 7, P=0.2755). Lipidosis was observed in most Delta Smelt livers, including some control fish, suggesting that cultured fish had liver lesions before their use in the experiments in 2017. In Exposure 1, moderate and severe lipidosis was prevalent in fish exposed to water collected from Grizzly Bay (75%), Montezuma (75%), and Cache (38%), although this severity of lipidosis also occurred with high frequency in fish from the HSC (50%). Severe lipidosis was more prevalent in Delta Smelt exposed to water collected from Montezuma (50%) and Decker Island (63%) in Exposure 5, including one fish with severe lipidosis and moderate sinusoidal congestion.

Overall, gill lesions were mild or absent in 2018, with no differences across sites (Kruskal–Wallis, χ^2: 4.56, df: 7, P=0.7138; Figures 5B and 6B). However, moderate chloride cell hyperplasia was intermittently observed in the gills of fish exposed to field water. In the liver, lesions were generally absent and did not differ across sites (Kruskal–Wallis, χ^2: 4.65, df: 7, P=0.7032; Figures 5E and 6E).

In 2019, gill lesion scores differed across sites (Kruskal–Wallis, χ^2: 26.70, df: 7, P=0.0004). Delta Smelt exposed to Decker Island had higher gill lesion scores than any other site or control (Kruskal–Wallis χ^2: 26.7, df: 7, P=0.0004; Figure 5C). In fish exposed to Decker Island water, lesions such as mucous cell hyperplasia and epithelial cell hyperplasia were prevalent (Figure B4) and most notable in Exposures 1, 2, 3, and 5 (Figure 6C). Mucous cell hyperplasia and epithelial cell hyperplasia were evident in several fish exposed to Isleton and Grizzly Bay during Exposure 2.

Liver lesion scores also differed across sites in 2019 (Kruskal–Wallis χ^2: 18.65, df: 7, P=0.0093). Observed lesions were generally mild, or in the case of both controls, absent. Delta Smelt exposed to water collected from Cache exhibited higher lesion scores than the freshwater control (P=0.0430; Figure 5F), but were not statistically different from the other sites. The highest liver lesion scores in fish exposed to Cache water were observed during Exposures 1 and 2, coinciding with the NDFS (Figure 6F). Moderate to severe lipidosis was observed in 25% of fish exposed to the Toe Drain, Cache, and Decker Island in Exposure 1.

DISCUSSION

This study evaluated the toxicity of estuary waters to cultured sub-adult Delta Smelt under different fall flow conditions. Generally, detected organic contaminants were always present, but type and concentration varied across our six study sites and were dynamic in each site within and across years, suggesting contaminant exposure and therefore hazard risk to freshwater biota varied through space and time. We observed low concentrations of organic compounds similar to those previously reported in the estuary during this season (Orlando et al. 2013; DeParsia 2018; DeParsia 2019; Orlando et al. 2020). Our study did not capture any storm events, which would likely be associated with increased pesticide concentrations. Nonetheless, we observed gill and liver damage in Delta Smelt following 96-hr exposure periods, indicating the potential for mixtures of low levels of contaminants to have sub-lethal effects on our study organism under a short-term exposure.

The chemicals detected in our study have been associated with negative health effects in fish

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at sub-lethal concentrations. For example, exposure to fipronil caused DNA damage in *Rhamdia quelen* (Ghisi et al. 2011) and oxidative damage in *Prochilodus lineatus* (Deiú et al. 2021). Chlorpyrifos has been noted to reduce hepatic glycogen in *Oreochromis niloticus* (Majumder and Kaviraj 2019) as well as gill damage in *Mugil cephalus* (Marigoudar et al. 2018). Azoxystrobin has caused oxidative stress and genotoxicity in *Australoheros facetus* (Crupkin et al. 2021) and endocrine disruption in *Danio rerio* (Jiang et al. 2018). In our study, detected concentrations of these organic compounds were lower than those in the literature; however, we still observed sub-lethal effects. Although the direct effects of contaminant exposure are difficult to measure in the environment, synergistic and/or additive interactions can cause sub-lethal toxic effects to tissues and affect physiological processes, emphasizing the need to identify the scope of mixture effects of these stressors on native fish species (Brooks et al. 2011; Fong et al. 2016).

Hypothesis 1: Site-Specific Responses to Contaminants

We observed numerous instances of site-specific detections of contaminants, some of which coincided with increased gill or liver lesions, partially supporting our first hypothesis. For example, elevated liver lesion scores in fish exposed to water from Toe Drain and Cache in early 2019 (i.e., Exposures 1 and 2) likely correspond to the mixture of insecticides, fungicides, and herbicides that were detected at higher concentrations than at other sites (e.g., azoxystrobin, methoxyfenozide, and thiobencarb). Despite concentrations of certain organic compounds being relatively high at Toe Drain and Cache compared to our other sites, these concentrations are nonetheless below benchmark concern levels (e.g., USEPA Aquatic Benchmark levels for freshwater vertebrates; USEPA 2022). Thus, our results suggest the benchmark concern levels may be too conservative, given the mixture of contaminants detected during our study. Based on our data, it appears that sub-lethal effects on wild Delta Smelt populations in the estuary vary by region and contaminant, and we suspect that mixture effects were a likely contributor to the toxicity observed in our study.

While lesions appeared to be associated with contaminant prevalence in some cases, chemical detections and concentrations did not always correspond to elevated lesion scores. For instance, fish exposed to Decker Island water in 2019 consistently exhibited severe gill lesions, but we did not detect any individual contaminant at a concentration likely to cause such detrimental effects. Additionally, other contaminants not measured by our study may have contributed to the observed toxicity (Fong et al. 2016). However, gill lesions are not necessarily caused by any individual contaminant; rather the types and severity of gill lesions are often determined by exposure to low and moderate contaminant concentrations (Polesksic and Mitrovoic–Tutundzic 1994). For example, lesions such as epithelial hyperplasia with lamellar fusion and telangiectasia are typically attributed to a wide range of contaminants—including organophosphates, carbamates, and herbicides—and mild to moderate mucus secretion is typically a protective response to contaminant exposure (Au 2004; Matey et al. 2011). Such gill lesions were frequently observed in Delta Smelt exposed to Decker Island water in 2019. Therefore, Delta Smelt may have been adversely affected by the interaction of a diverse suite of contaminants present at these sites, rather than the presence of any single chemical.

The prevalence of elevated gill lesions and liver lipidosis in sites from the freshwater region of the estuary (e.g., Toe Drain, Cache, Decker Island) may have implications for dispersal and spawning of Delta Smelt in the wild. Specifically, Decker Island is located downstream of the confluence of the Cache Slough complex and Sacramento watershed and is the main corridor for dispersal for Delta Smelt to and from the North Delta, while Cache is critical spawning habitat for Delta Smelt (Bennett 2005; Sommer et al. 2011; Kurobe et al. 2022). The elevated lesion scores observed in Delta Smelt exposed to Cache, Toe Drain, and Decker Island water in 2019 suggest that these areas may be of elevated hazard risk for Delta Smelt.

Hypothesis 2: Water-Year Effects

The years 2017 and 2019 were considerably wetter than 2018; thus, we expected lesions to be more prevalent in these years than in 2018. This hypothesis was supported, as lesions were either mild or absent in fish in 2018. In contrast, during the 2 wet years we observed moderate to severe lipidosis and moderate sinusoidal congestion in the liver, as well as chloride cell hyperplasia and mucous cell hyperplasia in the gills. Consequently, we suggest that contaminant mobilization during wet years (e.g., Orlando et al. 2020) may offset habitat benefits, providing one explanation for why Delta Smelt abundance does not consistently increase with freshwater flow despite improved access to higher-quality habitat during wetter periods (e.g., Stevens and Miller 1983; Dege and Brown 2003; Miller et al. 2012; Brown et al. 2020; Mahardja et al. 2021).

Fipronil metabolite concentrations and frequency of detection did not follow our expectation of higher contaminant concentrations and detections during wetter years. Fipronil and its metabolites were detected in greater than 66% of samples collected in 2017 and over 90% of samples in 2018, but this detection frequency decreased to 6% in 2019. We believe this was the result of the changing use of fipronil during our study rather than differences in hydrodynamics. Fipronil isn't registered for agricultural use in California (Sadaria et al. 2017); thus, the probable source of fipronil and metabolite detections in 2017 and 2018 was from topical flea products entering municipal wastewater through home and commercial pet grooming (Sadaria et al. 2017; Sutton et al. 2019). As we didn't capture any storm events, this contaminant is largely entering the estuary from wastewater effluent (as opposed to stormwater runoff); thus, its presence is unlikely to be related to water-year type. Instead, the decline in detections of fipronil in 2019 is more likely the result of the label changes enacted by USEPA and CDPR, which considerably restricted fipronil applications after October 2018 (Messenger–Sikes and Windbiel–Rojas, 2018).

Given that we did not measure the full suite of analytes in 2018 that we measured in 2017 and 2019, we cannot compare contaminant presence and concentration between wet and dry years; however, we did observe trends between the 2 wet years. Although fipronil and metabolite detection frequency varied greatly between 2017 and 2019, concentrations were similar in both years, consistently being detected below ng L^{-1}. Other compounds—such as chlorantraniliprole, methoxyfenozide, azoxystrobin, and hexazinone—were detected in similar concentrations and detection frequencies in both wet years. Chlorantraniliprole and methoxyfenozide were the fourth- and fifth-most applied insecticides in California by acreage for both 2017 and 2019, and azoxystrobin was the second-most applied fungicide in California by acreage during these years (CDPR 2017, 2019). Similarities between chemical classes, concentrations, and detection frequencies may be the result of the antecedent conditions at these sites. Wetter conditions provide greater access to water for agriculture, leading to increased crop production and therefore, increased pesticide use and the potential for non-target exposure.

Hypothesis 3: Managed Flows and Contaminant Exposure

Our study overlapped with Fall X2 in 2017 and 2019, and the NDFS in 2018 and 2019. While these actions were intended to benefit Delta Smelt (CNRA 2016) by increasing freshwater flows, habitat access, and food availability in the downstream reaches of the estuary, we were interested in whether they could have unintended detrimental effects on the species through increased exposure to contaminants. During the NDFS action in 2019, we observed elevated contaminant concentrations and sub-lethal effects at sites in the North Delta (i.e., Toe Drain, Cache) compared to the downstream sites. Specifically, during the NDFS (Exposures 1 and 2), we observed a higher prevalence and severity of lipidosis in the livers of Delta Smelt exposed to the Toe Drain water and moderate to severe lipidosis in fish exposed to Cache water (e.g., Figure 6C and 6E). Additionally, we detected the highest number of chemicals at some of the highest concentrations in Exposure 1 (e.g., Figures 3C and 4; Table B3). The waters collected from Toe Drain and Cache contained several contaminants

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associated with rice crops, such as azoxystrobin, methoxyfenozide, and thiobencarb (Orlando et al. 2020). Contaminant detections and liver lesion scores dropped noticeably between Exposures 2 and 3, coinciding with the end of the 2019 NDFS (e.g., Figure 4). Because the elevated liver lesion severity in Exposures 1 and 2 was largely restricted to fish exposed to water from upstream sites, the cause is unlikely to be an estuary-wide or water-year effect. Rather, these sub-lethal effects were likely caused by localized effects of the NDFS. Together, these results suggest that a pulse of contaminated source water may have impaired water quality in the North Delta (i.e., Toe Drain and Cache) during the NDFS, but these effects were transient and localized (i.e., they did not affect downstream sites or Exposures 3 through 5).

Although NDFS actions in 2018 and 2019 both used agricultural tail-water from rice field drainages, we did not detect negative effects on fish from the 2018 NDFS, which coincided with Exposure 1. Gill and liver lesion scores did not vary from Exposures 1 to 2 in the North Delta sites, nor did organic compound detections or concentrations. Moreover, observed gill and liver lesions were mild and, in some cases, absent. This annual difference is likely related to the amount of active agriculture taking place during the year. Water use and availability can vary dramatically between wet and dry water years, resulting in changes in acreage of fallow fields and of crops planted. For instance, between 2018 and 2019, pesticide-treated acreage in California increased 3.3% from 105 to 109 million acres (CDPR 2019), which may partially account for the differences we observed between the 2 years where the NDFS actions occurred. The increase in agriculture and concomitant increase in pesticide use in 2019 may have resulted in increased exposure of Delta Smelt to these contaminants. Thus, the water source used for the NDFS (i.e., agriculture return water vs. Sacramento River water) can significantly affect Delta Smelt health and condition. With respect to the NDFS action, our hypothesis that managed flow pulses would increase contaminants and lesions was partially supported, because NDFS actions were associated with increased sub-lethal effects in 2019 but not in 2018.

Determining whether the Fall X2 actions had adverse effects on Delta Smelt during our study is difficult because Fall X2 occurred in 2017 and 2019, but not in 2018, such that this management action is conflated with water-year type. A recent synthesis of Delta Smelt-related studies determined that high outflow by itself may not be sufficient in providing favorable habitat conditions, because other abiotic factors, such as temperature, can eclipse the habitat benefits provided by Fall X2 and a favorable LSZ location (Brown et al. 2020). We note that the fall study periods all coincided with periods of base flow (i.e., low flow in general). A more thorough assessment of our hypothesis related to Fall X2 would need to include periods that do not conflate a Fall X2 action with a wet water year. However, this may not be possible, because Fall X2—a mandated action—does not occur during dry water years (USFWS 2008). A second possibility would be to examine the toxicity of water sources used for outflow augmentation, (e.g., runoff versus reservoir releases).

CONCLUSION

Our results demonstrate that low levels of contaminants are ubiquitous in estuary waters in the fall, and even short-term exposure to these contaminants can elicit adverse health effects on Delta Smelt. Although our study included a limited number of years for comparison, our results suggest that habitat benefits attributed to wet years may be at least partially offset by elevated sub-lethal toxicity from exposure to increased contaminants. Water year appears to affect Delta Smelt toxicity, because Delta Smelt in the dry year of 2018 exhibited the fewest number of lesions—which were generally mild or absent—and where the NDFS appeared to have no influence on Delta Smelt health and condition. In comparison, we observed negative effects in the livers of Delta Smelt exposed to the Toe Drain and Cache, and elevated organic detections coinciding with the 2019 NDFS pulse flow, likely from a combination of the antecedent agricultural conditions and pulse flow water source. Our results suggest contaminant loading is an important consideration in planning flow-

management actions, especially those actions using agricultural return water. Negative effects of contaminant exposure from freshwater flows outweighing the benefits provided by an increase in habitat availability—or other benefits of flow—suggest that improved contaminant management should be a consideration to reduce the costs of these actions. The inclusion of contaminant effects to Delta Smelt (and other imperiled species) can greatly benefit the conceptual models used in the decisions made by multiple municipal agencies and aid in developing optimal management practices aimed at recovering Delta Smelt populations.

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