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Decline in methylmercury in museum-preserved bivalves from San Francisco Bay, California

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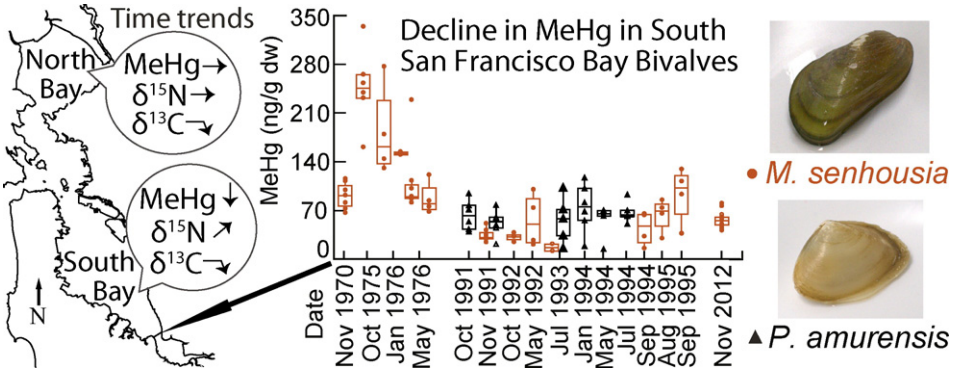
HIGHLIGHTS

- First reported long-term decline in methylmercury in San Francisco Bay biota
- Methylmercury concentration decline in preserved bivalves since mine closure
- Stable isotopes indicate methylmercury trends not attributable to food web changes.
- $\delta^{15}N$ and $\delta^{13}C$ trends likely caused by both natural and anthropogenic drivers

GRAPHICAL ABSTRACT



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ABSTRACT

There are ongoing efforts to manage mercury and nutrient pollution in San Francisco Bay (California, USA), but historical data on biological responses are limited. We used bivalves preserved in formalin or ethanol from museum collections to investigate long-term trends in methylmercury (MeHg) concentrations and carbon and nitrogen isotopic signatures. In the southern reach of the estuary, South Bay, MeHg in the Asian date mussel (*Musculista senhousia*) significantly declined over the study duration (1970 to 2012). Mean MeHg concentrations were highest (218 ng/g dry weight, dw) in 1975 and declined 3.8-fold (to 57 ng/g dw) by 2012. This decrease corresponded with closure of the New Almaden Mercury Mines and was consistent with previously observed declines in sediment core mercury concentrations. In contrast, across all sites, MeHg in the overbite clam (*Potamocorbula amurensis*) increased 1.3-fold from 64 ng/g dw before 2000 to 81 ng/g dw during the 2000s and was higher than in *M. senhousia*. Pearson correlation coefficients of the association between MeHg and $\delta^{13}C$ or $\delta^{15}N$ provided no evidence that food web alterations explained changing MeHg concentrations. However, isotopic composition shifted temporally. South Bay bivalve $\delta^{15}N$ increased from 12‰ in the 1970s to 18‰ in 2012. This increase corresponded with increasing nitrogen loadings from wastewater treatment plants until the late 1980s and increasing phytoplankton biomass from the 1990s to 2012. Similarly, a 3‰ decline in $\delta^{13}C$ from 2002 to 2012 may represent greater utilization of planktonic food sources. In a complimentary 90 day laboratory study to validate use of these preserved specimens, preservation had only minor effects (<0.5‰) on $\delta^{13}C$ and $\delta^{15}N$. MeHg increased following preservation but then stabilized. These are the first documented long-term trends in biota MeHg and stable isotopes in this heavily impacted estuary and support the utility of preserved specimens to infer contaminant and biogeochemical trends.

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1. Introduction

Mercury pollution of estuaries poses a global threat to wildlife and human health. Humans are exposed to the organometallic form, methylmercury (MeHg), primarily through consumption of coastal and estuarine seafood (Sunderland, 2007). Wildlife that depend on the productive estuarine habitat are also exposed, and reproductive impairment has been documented in birds (Eagles-Smith et al., 2009; Scheuhammer et al., 2007). Accordingly, there are local, national, and international efforts to reduce mercury. However, our ability to assess the efficacy of regulatory efforts is limited by lack of long-term studies on MeHg concentrations in biota, especially in coastal ecosystems (Lambert et al., 2012).

To evaluate temporal trends, some studies have relied on total mercury (Hg_T) measurements, which include both inorganic and organic forms, in fish (Bhavsar et al., 2010; Gandhi et al., 2014; Kraepiel et al., 2003). Measuring Hg_T works well at the top of the food chain where >95% of the Hg_T is MeHg (Bloom, 1992). However, one disadvantage of this approach is that fish Hg_T concentrations may be decoupled from mercury inputs. For example, Monson et al. (2011) found that Hg_T concentrations in walleye in Ontario declined from 1970 to 1990, reflecting a general decrease in atmospheric inputs in North America and Europe. However, walleye concentrations subsequently increased from the mid-1990s to 2009, possibly due to changes in the food web (Monson et al., 2011).

At our study site in San Francisco Bay, California, USA (Fig. 1), the temporal trends in fish are particularly perplexing. There has been no decline in Hg_T in striped bass from the 1970s to the present (Davis et al., 2016; Greenfield et al., 2005), despite the closure of the New Almaden Mercury Mining District (hereafter New Almaden) in 1975, and ongoing efforts to understand and control MeHg pollution in San Francisco Bay and its watersheds (Davis et al., 2012). New Almaden was once the nation's largest mercury mining district, and it drained into lower San Francisco Bay via the Guadalupe River (Conaway et al., 2008; Davis et al., 2012). Mercury concentrations recorded in sediment cores do show a decline since the mid-20th century (Conaway et al., 2004; Donovan et al., 2013) and since 1970 (Hornberger et al., 1999), so perhaps fish are not the best biomonitor for long-term trends in this estuary.

Another approach is to focus on invertebrates lower in the food chain, such as bivalves, which efficiently accumulate and transfer metals (Pan and Wang, 2011). Resident bivalves have been successfully used as biosentinels to record long-term declines in other metals (e.g., Cu and Ag) in San Francisco Bay (Hornberger et al., 2000), and are widely and successfully employed for contaminant trend biomonitoring (e.g., Lauenstein and Daskalakis, 1998; Luengen et al., 2004; Melwani et al., 2014). Both resident and transplanted mussels have been central to long-term monitoring efforts in California, and nationwide, through the "Mussel Watch" programs (Melwani et al., 2014). Mussel Watch includes three sites in San Francisco Bay, although reliable data are available only from 1986 to 2009 and only for Hg_T (Melwani et al., 2013). Unlike in fish, MeHg is only a fraction of Hg_T in bivalves, and varies considerably, between 12 and 60% (Francesconi and Lenanton, 1992; Pan and Wang, 2011; R. Stewart, USGS, pers. comm.). Studies with transplanted mussels indicate that that measurements of Hg_T in bivalves are not useful for predicting mercury concentrations in higher trophic levels (Gunther et al., 1999), because only the methylated form biomagnifies. Another reason to focus on the methylated form is that it is difficult to contaminate samples (even preserved ones) with MeHg, unlike Hg_T (Vo et al., 2011).

Unfortunately, there are no historical measurements of MeHg in invertebrates because methods to measure MeHg were not established until 1988 (Bloom and Fitzgerald, 1988) and not widely applied until much later. This lack of historical data leaves the analysis of archived specimens as the only potential avenue for determination of past concentrations. Along those lines, Vo et al. (2011) recently used bird

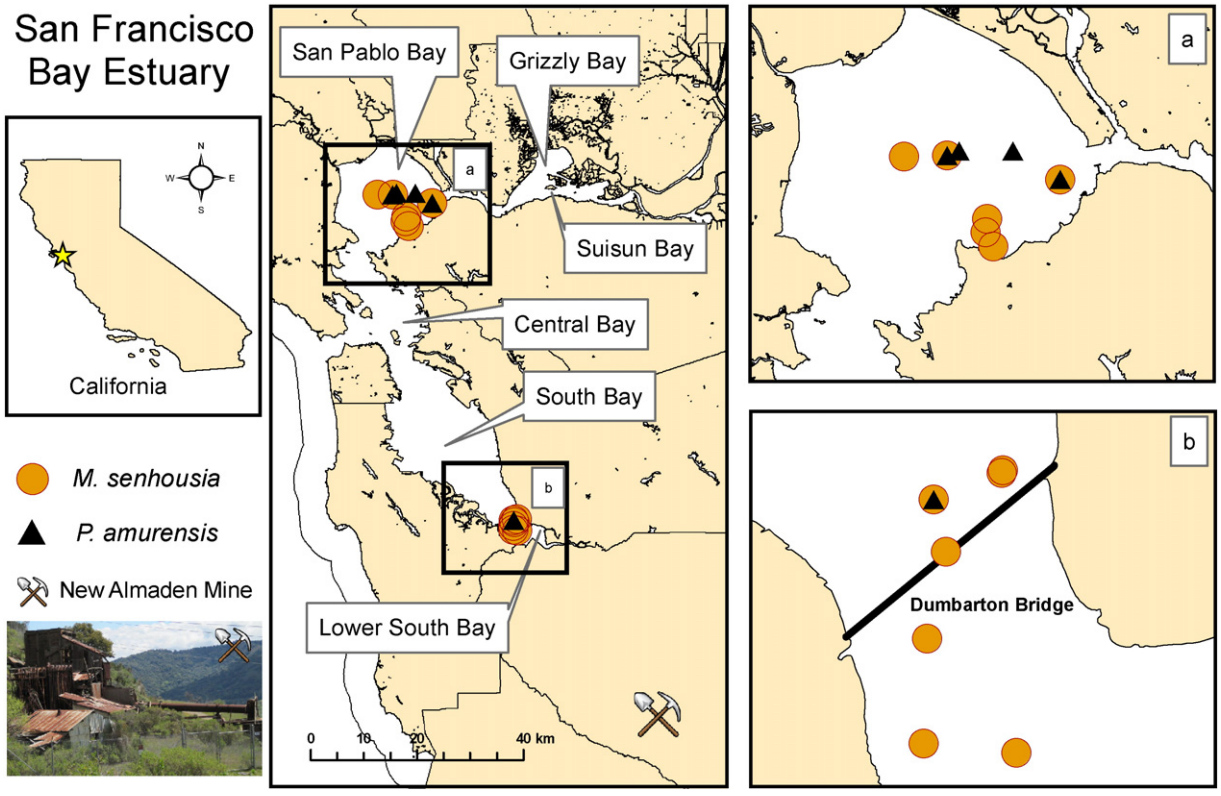

Fig. 1. San Francisco Bay Estuary is divided into distinct hydrological sub-embayments. Bivalves were available in natural history collections from both the well-flushed northern reach of the estuary and the more stagnant southern reach, which also has the former New Almaden mercury mining district in its watershed. Specimens of the Asian date mussel, *Musculista senhousia*, were available from 1970 to 2012. Specimens of the overbite clam, *Potamocorbula amurensis*, were available from 1988 through 2002.

feathers from museum specimens and found an increase in MeHg concentration in black-footed albatrosses from the Pacific Ocean between 1880 and 2002. However, there are no published studies employing archived museum invertebrate collections to collect MeHg data. Museum specimens are a largely untapped source of data; this novel approach has the potential to create a baseline for MeHg in bivalves in a situation where there are no historical data (Campbell and Drevnick, 2015).

Analyzing historical samples also has the benefit of allowing concurrent isotopic analysis. In particular, stable nitrogen and carbon isotopes have been useful in attributing mercury body burden to trophic position (e.g., $\delta^{15}N$ enrichment can indicate higher trophic position) and food source (e.g., $\delta^{13}C$ can reflect carbon source) (France, 1995; Minagawa and Wada, 1984; Pan and Wang, 2011; Stewart et al., 2008). Most bivalves feed opportunistically, switching between phytoplankton, benthic algae, and a detritus-based diet that can include microzooplankton, such as ciliates and flagellates (Greene et al., 2011; Pan and Wang, 2011). Accordingly, this study employed stable isotope measurements to determine if dietary changes could account for any changes in MeHg body burden. Only a few studies, focused on hair and feathers in museum collections (e.g., Bond et al., 2015; Horton et al., 2009; Vo et al., 2011), have tracked both stable isotopes and mercury over decadal time scales.

To assess the validity of using preserved specimens in a long-term study, it is necessary to quantify the effect of preservation on MeHg concentrations in bivalves, which previously has been studied only in fish (e.g., Hill et al., 2010; Kelly et al., 1975; Levengood et al., 2013). In contrast, stable isotope analysis has been validated for use with various species of preserved invertebrates, including bivalves (Rennie et al., 2012) and has been successfully applied during a study to reconstruct historical food web pathways in Lake Tahoe (Vander Zanden et al., 2003). Accordingly, an experimental preservation study was conducted by adding formalin or ethanol to freshly collected bivalves and analyzing MeHg concentrations and stable isotopes over time.

Next, for the historical portion of the study, preserved specimens from museum collections were used to evaluate temporal changes in MeHg concentrations, $\delta^{13}C$, and $\delta^{15}N$ in bivalves from San Francisco Bay from 1970 to 2012. Museum specimens were collected and preserved by the California Academy of Sciences (CAS) and the United States Geologic Survey (USGS). The study focused on two species: the Asian date mussel (*Musculista senhousia*), a mussel widely distributed in the estuary, and the overbite clam (*Potamocorbula amurensis*; also referred to as *Corbula amurensis*), an invasive clam that displaced natives when it was introduced in 1986 (Cloern and Jassby, 2012; Fry, 1999). This voracious clam has been shown to accumulate high selenium concentrations (Lee et al., 2006; Stewart et al., 2004); one goal of the study was to determine whether *P. amurensis* accumulates high MeHg relative to the Asian date mussel, thereby helping to mobilize MeHg to the food web. In addition to species invasions, the Bay has seen a host of ecological changes (Nichols et al., 1986); another study goal was to evaluate whether stable isotope signatures in bivalves could reveal underlying ecological processes that could potentially alter mercury uptake. The overall study hypothesis was that MeHg in bivalves would decrease over time due to reductions in mercury loadings and that stable isotope signatures would help in interpretation of coincident ecological changes.

2. Methods

2.1. Preservation study

The experimental preservation study was designed to evaluate the use of preserved bivalves for MeHg, $\delta^{13}C$, and $\delta^{15}N$. For each species, 80 to 100 individuals were obtained from a single site on a single date. *M. senhousia* were collected from Tomales Bay (Millerton Point, 38°6′28″ N, 122°50′41″ W) on June 19, 2013, and *P. amurensis* were collected from Suisun Bay (38°4′21″ N, 121°58′10″ W) on July 24, 2013. Within a species, individuals had similar mean (± standard deviation, SD) shell lengths: 15.5 ± 2.7 mm for *M. senhousia* and 11.3 ± 2.0 mm for *P. amurensis*. Upon return to the lab, bivalves were either processed immediately (hereafter "fresh" samples) or preserved via two different techniques to determine if the type of fixative influenced MeHg concentrations or stable isotope ratios. Any potential effect of fixative type was of interest because the historical study included some samples that had been preserved with formalin (especially older CAS samples and some USGS samples) and others that had been preserved with ethanol.

The fresh samples were divided into two batches: a control batch that received no treatment and an experimental batch that received $MgCl_2$ to determine if the use of $MgCl_2$ as a relaxant to open the valves affected MeHg concentrations. The control batch of 10 individuals was placed in the freezer for a few minutes to make them unresponsive, then measured, rinsed, pat dried, shucked, weighed (wet weight), and individually lyophilized for later analysis. The experimental batch of 10 individuals was placed in 0.36 M $MgCl_2 \cdot 6H_2O$ made up with water from the collection site (Tomales Bay for *M. senhousia* and Suisun Bay for *P. amurensis*). This anesthetization method was described in Williams and Van Syoc (2007) and employed by CAS when appropriate (E. Kools, CAS, pers. comm). When the bivalves were unresponsive to touch, they were removed from the $MgCl_2$ solution, measured, rinsed, pat dried, shucked, weighed, and prepared for lyophilization.

Fresh samples were compared to samples fixed with either formalin (following USGS preservation techniques, J. Crauder, USGS, pers. comm.) or 95% ethanol (following Williams and Van Syoc (2007) and CAS ethanol preservation techniques). Samples in the ethanol treatment were fixed in 95% undenatured ethanol for 24 h, and then stored in 75% undenatured ethanol. Samples in the formalin treatment were fixed in 10% formalin with sodium borate added (until saturation) to prevent acidification. After one week in 10% formalin, the bivalves were transferred to 70% undenatured ethanol for long-term storage. For both techniques, the bivalves were relaxed in $MgCl_2$ solution, as described earlier, prior to preservation.

Bivalves were removed from the storage solutions after 7, 30, and 90 days and then transferred to 70% or 75% undenatured ethanol. At each time-point, 8 individuals were removed from the storage jar, measured, rinsed, pat dried, shucked, weighed (wet weight), and individually lyophilized for later analysis.

2.2. Historical study

The historical study focused on *M. senhousia* and *P. amurensis* specimens collected from two sites: South San Francisco Bay near Dumbarton Bridge (hereafter, South Bay) and San Pablo Bay in the northern reach of the Estuary (Fig. 1). Bivalves from these locations were frequently collected and preserved from 1970 to 2012. South Bay is of particular interest because mercury concentrations are generally higher in this embayment than in San Pablo and Central Bays (Conaway et al., 2007; Greenfield et al., 2013b). San Pablo and Central Bays are flushed as the Sacramento and San Joaquin Rivers flow from the Sacramento-San Joaquin River Delta to the Pacific Ocean (Conomos et al., 1985). In contrast, South Bay receives relatively little fresh water (<10% of the total), primarily from wastewater treatment plants and local tributaries, such as Guadalupe River and Coyote Creek, which drain the New Almaden mines (Conaway et al., 2008; Conaway et al., 2004).

The analyses focused on bivalve collections within open-water stations, as close together as the historical collections permitted. Samples were selected closely together to avoid variation in MeHg concentrations that could be associated with spatial differences in habitat type (Heim et al., 2007), phytoplankton biomass concentrations (Powell et al., 1989), or regional differences in mercury sources (Greenfield et al., 2013b). In South Bay, collection site distances were <0.1–2 km. In North Bay, the distances were slightly larger, ranging from 1 to 11 km. To examine small-scale spatial variability, in two cases samples were

analyzed from the same date, but at different sites, within the Dumbarton Bridge sampling area.

Once appropriate collections were identified, 4 to 15 individuals were obtained from each collection location and date, depending on the number of available specimens. For CAS samples, the collection information and preservation technique (e.g., formalin or ethanol) for each catalog number were described in the specimen database, http://researcharchive.calacademy.org/research/izg/iz_coll_db/Index.asp. Using trace metal clean techniques, the bivalve subsample was transferred to acid-cleaned Teflon vials filled with 75% ethanol for CAS samples or 70% ethanol for USGS samples.

Bivalves were measured and the tissues were individually extracted in a clean room at the University of San Francisco Mercury Laboratory. The bivalves were measured for their antero-posterior and dorso-ventral shell dimensions using a digital caliper (Grizzle et al., 2001). Then, a clean razor was used to open the shell and expose the tissues. Each bivalve was thoroughly rinsed with Milli-Q (18.2 MΩ) purified water to remove any particulates and dried with a Kimwipe (Gunther et al., 1999). Bivalves were then shucked, individually placed in acid-cleaned numbered Teflon vials, weighed to obtain wet weights, and lyophilized (Horvat and Byrne, 1992). Once lyophilization was complete, the tissues were weighed (dry weights), homogenized and set aside for future analysis.

2.3. Tissue digestion and MeHg analysis

For each individual bivalve, approximately 2 mg of tissue was weighed (to four significant figures) into 5 mL Savillex vials for a microdigestion. The method was a modification of the commonly employed alkaline digestion procedure first developed by Bloom (1992) for larger sample masses. Samples were digested by adding 0.500 mL of 25% KOH: methanol (w/v) and then heating at 65 °C for 4 h. Prior to analysis, samples were allowed to stand for three days and were diluted with 2.50 mL of methanol.

Samples were analyzed for MeHg as per the U.S. Environmental Protection Agency's (EPA) Method 1630 using ethylation, gas separation, pyrolysis, and cold vapor atomic fluorescence spectrophotometry (CVAFS) (EPA, 2002). For analysis, a thirty microliter aliquot of digestate was added to the sample vials, pH was adjusted by addition of 300 μL of acetate buffer, and samples were ethylated with 1% sodium tetraethylborate in 2% potassium hydroxide (Bloom, 1989). Detection was by CVAFS using a MERX Automated Methylmercury System (Brooks Rand Instruments). Calibration was performed using the method of standard additions (all standards, calibration verifications, and calibration blanks received 30 μL of a 1:5 dilution of the 25% KOH:methanol solution) to address any potential matrix interferences (Bloom, 1989). The instrument was calibrated daily using a 7-point calibration curve with an $r^2 > 0.99$.

The accuracy and precision of MeHg data was checked by analyzing standards and blanks including 1) analytical standards traceable to the U.S. National Institute of Standards and Technology (NIST); 2) analytical and methodological blanks; 3) a certified reference material, lobster hepatopancreas (TORT-2) from the National Research Council of Canada; 4) matrix spikes and duplicates; and 5) analytical and methodological replicates. Initial calibration and on-going precision and recovery was verified with a secondary standard (also traceable to NIST); recovery was within 67–133%. The mean method detection limit, averaged across multiple sets of analyses, was 5 ng/g dry weight for a 2 mg tissue sample. All samples were above detection limits. The mean concentration of MeHg in TORT-2 ($\bar{x} \pm SD$) during study analyses was 135 ± 22 ng/g, which was within the acceptable range (70–130%) of its certified value of 152 ± 13 ng/g. Matrix spikes and matrix spike duplicates had quantitative recoveries within 65–135%. The procedural reproducibility, calculated as the relative percent difference of replicate subsamples that were separately digested and analyzed, averaged 8%.

MeHg concentrations in the preservative were measured to rule out contamination, which can be a problem for other trace metals in museum specimens (e.g., Renaud et al., 1995). However, MeHg was not detected in the preservative, and this analyte was selected partially because it is much more difficult to contaminate for MeHg than Hg_T (Vo et al., 2011).

2.4. Stable isotope analysis

Dried, homogenized samples for ^{13}C and ^{15}N analysis were weighed (~2 mg to four significant figures), packaged into tin capsules, and sent to the University of California, Santa Cruz Stable Isotope Laboratory for analysis. Samples were analyzed with a CE Instruments NC2500 elemental analyzer interfaced to a ThermoFinningan Delta Plus XP isotope ratio mass spectrometer (IRMS). Results are expressed as delta (δ) values, which are the deviation in parts per thousand (‰), relative to international standards (PeeDee belemnite limestone for $\delta^{13}C$ and air for $\delta^{15}N$) (Peterson and Fry, 1987). Calibrated in-house standards were used to correct sample data for instrumental drift and linearity. Calibration was verified with a secondary standard, acetanilide, which was analyzed repeatedly with each run. The precision (SD of acetanilide, averaged across multiple runs) was 0.05‰ for carbon and 0.04‰ for nitrogen. Accuracy ranged from 0.02 to 0.5‰ for carbon and 0.02 to 0.09‰ for nitrogen.

2.5. Statistical analysis

Statistical analyses for the preservation study consisted of a two-way analysis of covariance (ANCOVA), with fixative type (ethanol vs. formalin) and storage time (fresh, 7 days, 30 days, and 90 days) treated as categorical independent variables, and tissue fresh weight as a covariate. A fixative type versus storage time interaction was also included. ANCOVA was chosen because it does not assume a linear or monotonic response with time. To achieve a balanced statistical design in the preservation study, all MeHg and isotope results from the fresh samples were randomly allocated to either the ethanol or formalin treatment (N ~ 10 per treatment). MeHg concentrations and isotope results in fresh samples treated with $MgCl_2$ did not differ from fresh samples without $MgCl_2$. Accordingly, it was appropriate to combine the samples into a single treatment, the “fresh” treatment.

Preservation study data were analyzed using the general linear model routine in Systat (version 13.00.05). Data were transformed when needed to achieve normally distributed residuals. When a storage time effect was determined to be significant ($p < 0.05$), pairwise differences between days were evaluated using Tukey's honestly significant difference post hoc test (Tukey's HSD). When both storage time and another variable (e.g., weight) were significant, pairwise differences were assessed by evaluating the residuals of other significant model terms. For example, residuals of a significant weight effect were evaluated for pairwise differences on a significant storage time effect. When there were significant interactions between model terms, graphical analyses and parameter estimates for model terms were used to evaluate the contribution of interaction terms.

For the historical study, Kendall's τ correlation coefficients were employed to examine trends in MeHg with time on all study samples ($N = 243$). Kendall's τ was selected because it is a non-parametric test that is robust to nonlinearity, outliers, and the presence of tie-values. The strength of association between MeHg and $\delta^{15}N$ or $\delta^{13}C$ was evaluated using Pearson's correlation coefficients.

Mixed models were performed in R (v. 3.2.1; R Core Team, 2015) to further evaluate temporal trends in MeHg, $\delta^{15}N$, and $\delta^{13}C$, while accounting for other factors, including spatial location, species, and biological variables. Data were centered and scaled to a standard range prior to statistical analysis to reduce parameter correlation. Methylmercury was log-transformed to improve residual normality. In the mixed model analyses, an individual sampling event (i.e., date) was treated as a random effect with all other parameters treated as fixed effects; this accounted for observed heterogeneity of residuals with sampling date

Table 1
Average methylmercury (MeHg) concentrations (±1 SD) in ng/g dry weight (dw), shell length, and number of individuals (N) analyzed at each time-point in the preservation study for two bivalve species. Fresh individuals (without $MgCl_2$) and fresh individuals treated with $MgCl_2$ (as a relaxant to open the valves) were processed immediately after collection. Samples were then preserved in formalin or ethanol. After 7, 30, and 90 days, a subsample of bivalves was removed from the storage solution and analyzed individually.

Preservation technique	Preservation time (days)	*M. senhousia*			*P. amurensis*		
		Mean MeHg (ng/g dw)	Mean shell length (mm)	N	Mean MeHg (ng/g dw)	Mean shell length (mm)	N
Fresh	0	73 ± 23	16.8	9	75 ± 22	12.5	9
Fresh $MgCl_2$	0	64 ± 15	15.5	10	75 ± 19	12.6	10
Formalin	7	124 ± 26	14.8	8	83 ± 20	11.9	8
Formalin	30	116 ± 31	15.4	8	94 ± 17	10.9	8
Formalin	90	159 ± 38	15.1	8	121 ± 21	9.1	4
Ethanol	7	118 ± 40	15.0	8	123 ± 21	11.6	7
Ethanol	30	147 ± 46	15.3	8	140 ± 22	11.0	8
Ethanol	90	151 ± 41	15.9	7	149 ± 36	11.3	8

when using multiple linear regression (Zuur et al., 2009). For $\delta^{13}C$, which exhibited different trends over time, a breakpoint analysis (i.e., piecewise regression) was also performed, following Crawley (2012).

The models that best predicted MeHg, $\delta^{15}N$ and $\delta^{13}C$ in the bivalve museum specimens were selected based on information theoretic criteria (AIC and BIC) and application of the likelihood ratio test for individual parameter contributions ($p < 0.05$ for each parameter addition) (Zuur et al., 2009). Amount of variability explained by the models was calculated based on the marginal and conditional r^2, described elsewhere (Johnson, 2014; Nakagawa and Schielzeth, 2013). The marginal r^2 indicates variability in the data explained by fixed model effects, and the conditional r^2 indicates variability explained by both fixed and random effects. For consistency, analysis focused on the subset of samples with MeHg, $\delta^{15}N$, $\delta^{13}C$, moisture, and C:N ratio data ($N = 156$).

3. Results

3.1. Preservation study

Methylmercury concentrations in fresh tissues treated with $MgCl_2$ did not substantially differ from concentrations in fresh tissues without $MgCl_2$ (rows 1 and 2 in Table 1). There was no significant difference between the two treatments for either *M. senhousia* (*t*-test with pooled variance, $n = 19$, $p = 0.31$) or *P. amurensis* (*t*-test with pooled variance, $n = 19$, $p = 0.97$). Accordingly, fresh tissues that had received $MgCl_2$ were pooled with those that had not in subsequent statistical analyses, and then randomly allocated to either the formalin or the ethanol treatments (as in Fig. 2).

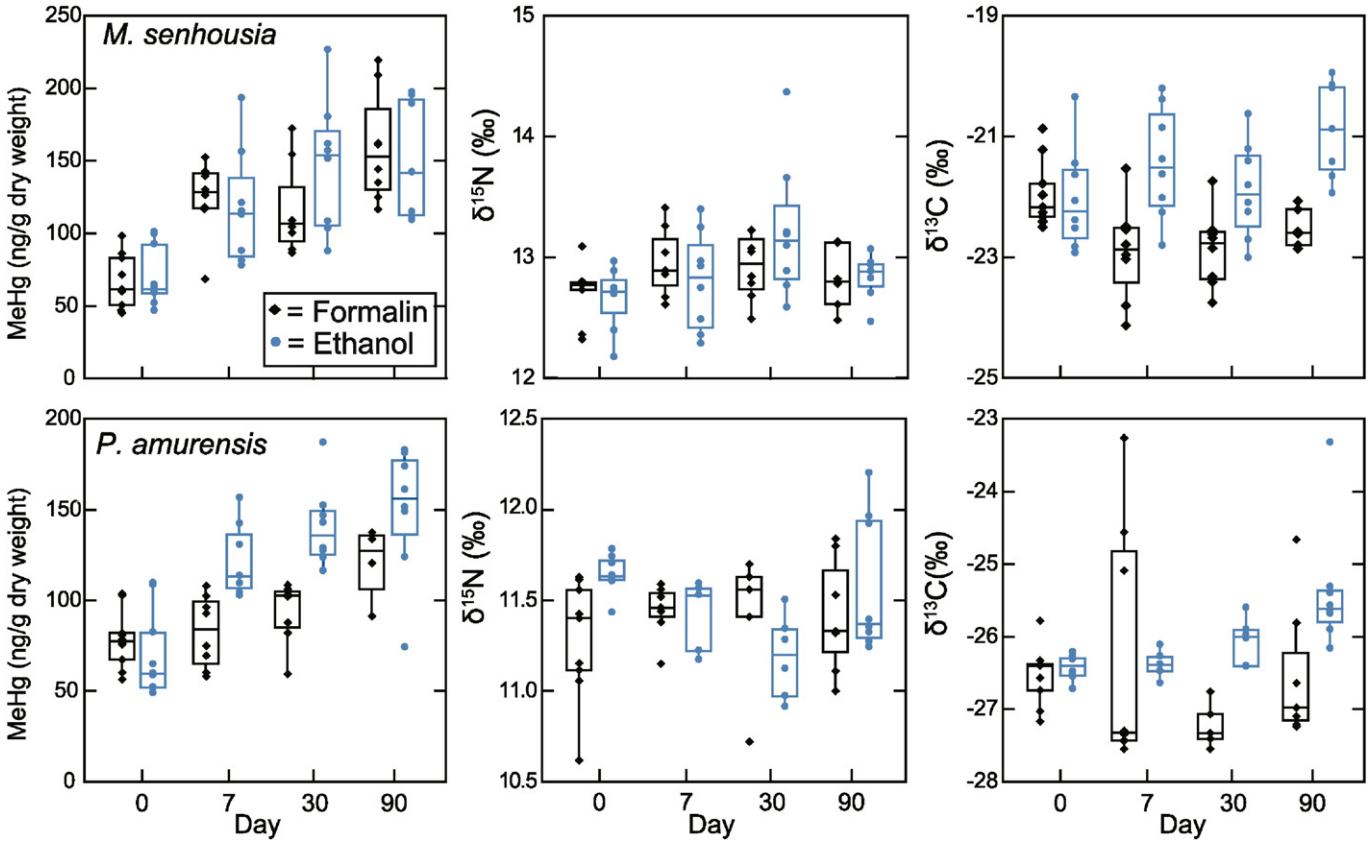

Fig. 2. Box and whisker plots showing the effects of preservation on MeHg concentrations and stable isotope signatures over time. Boxes represent the middle half of the data, horizontal lines are the medians, points show raw data, and points not connected with whiskers are outliers. Day zero shows samples that were not preserved. Top row: *M. senhousia*. Bottom row: *P. amurensis*. Note different scales in y-axes.

3.1.1. Preservation study MeHg trends

For *M. senhousia*, concentrations of MeHg in the preserved tissues increased over time and then stabilized for both the formalin and ethanol treatments (Table 1, Fig. 2). MeHg in *M. senhousia* was significantly related to the number of days in the storage solution ($r^2 = 0.59$, $p < 0.0001$, $N = 66$) but not dry mass, fixative type, or day by fixative interaction. Pairwise comparison indicated significant difference between the fresh samples (0 days) and samples that had been held for 30, 60, and 90 days. There was also a marginally significant ($p = 0.06$) increase from 7 days to 90 days. No other pairwise differences were observed between days (Tukey's HSD). In the formalin treatment, the mean MeHg concentration increased by a factor of 1.9 in the first week, starting at 67 ± 19 ng/g dry weight (dw) for fresh tissues that had been randomly allocated to the "formalin treatment" and increasing to 124 ± 26 ng/g dw (Table 1). Similarly, in the ethanol treatment, the mean MeHg concentration increased by a factor of 1.7 in the first week, starting at 69 ± 20 ng/g dw and increasing to 118 ± 40 ng/g dw (Table 1).

Methylmercury in *P. amurensis* was significantly related to storage time, fixative type, and storage time by fixative interaction terms, but not dry body mass. There was an increase in MeHg for both fixatives, but the temporal pattern differed between fixatives. For formalin, the temporal pattern was variable but suggestive of a gradual increase in MeHg over the experiment duration (Fig. 2). Concentrations of MeHg in tissues fixed in formalin did not statistically increase until 90 days. For example, the mean MeHg concentration was 78 ± 16 ng/g dw in fresh (pretreatment) *P. amurensis* tissues randomly allocated to the formalin treatment. That baseline was comparable to 83 ± 20 ng/g dw after 7 days. Finally, after 90 days, that concentration increased to 121 ± 21 ng/g dw, which was a 1.5-fold increase from the fresh tissues. In contrast, MeHg in tissues fixed in ethanol increased after 7 days and then stabilized, similar to *M. senhousia* (Fig. 2). Specifically, in the ethanol treatment, the mean MeHg concentration was 71 ± 24 ng/g dw in fresh tissues and increased to 123 ± 21 ng/g dw after just 7 days.

3.1.2. Preservation study stable isotope trends

For *M. senhousia*, $\delta^{15}N$ initially increased with addition of fixative (ethanol or formalin) but did not continue to increase over time (Fig. 2). Specifically, $\delta^{15}N$ was significantly related to storage time and dry mass (model $r^2 = 0.28$, $p < 0.01$, $N = 62$), but not fixative, or storage time by fixative interaction. Residuals from a dry mass versus $\delta^{15}N$ regression ($r^2 = 0.07$, $p = 0.04$) were analyzed for pairwise preservation time differences in $\delta^{15}N$. The only significant pairwise difference among preservation times was an increase between the fresh samples (0 days) and 30 days.

For $\delta^{13}C$ in *M. senhousia*, there was no significant effect of dry mass ($p > 0.05$), and it was removed from the model. In the final model, there was a significant ($p = 0.009$) interaction between storage time and fixative and a significant effect of fixative ($p < 0.01$). Graphical analysis indicated a clear decline after 7 days in formalin, but no further decline thereafter. In contrast, ethanol exhibited a weak and variable storage time effect with no clear time trends.

For $\delta^{15}N$ in *P. amurensis*, there was no significant ($p > 0.05$) effect of dry mass, which was removed from the model. In the final model, there was no significant ($p > 0.05$) effect of preservation time or fixative, but there was a marginally significant ($p = 0.06$) interaction between preservation time and fixative. Graphical analysis and parameter estimates for model terms indicated a decline in $\delta^{15}N$ at 30 days in the ethanol treatment that was not seen in the formalin treatment. Because this treatment showed a decline on a single day, rather than an overall trend (Fig. 2), the decline could be attributed to variability between samples.

For $\delta^{13}C$ in *P. amurensis*, there was no significant ($p > 0.05$) effect of dry mass and no significant interaction between storage time and fixative type. There was a significant ($p = 0.02$) effect of fixative type, with lower $\delta^{13}C$ concentrations found in formalin relative to ethanol (Fig. 2). There was no significant ($p > 0.05$) effect of preservation time.

3.2. Historical study

3.2.1. MeHg trends

Examining all study data across both embayments, the MeHg temporal trend varied according to bivalve species (Fig. 3). For *M. senhousia*, samples spanned the entire 42 year study time range (November 1970 through November 2012), and mean MeHg concentrations were highest in South Bay in October 1975 (218 ng/g dw) and January 1976 (180 ng/g dw). By November 2012, concentrations had declined to 57 ng/g dw, a 3.8-fold decrease from peak concentrations. In contrast, for *P. amurensis*, which was introduced into San Francisco Bay in the late 1980s, samples were available from 1988 through 2002. The first samples from 1988 were located in Grizzly Bay (the embayment to the east of the study's San Pablo Bay focus-area) and had a mean MeHg concentration of 73 ng/g dw. Subsequent samples from the study focus areas (South Bay and San Pablo Bay) averaged 62 ng/g dw in the 1990s and 81 ng/g dw in the 2000s.

Overall, there was not a significant trend when combining samples from both species (Kendall's $\tau = -0.05$, $p = 0.21$, $N = 243$). Separating by species, *M. senhousia* exhibited a significant negative trend ($\tau = -0.15$, $p = 0.004$, $N = 169$), whereas *P. amurensis* exhibited a significant positive trend ($\tau = 0.20$, $p = 0.018$, $N = 74$).

Stable isotope and C:N ratio analyses were performed for a subset of samples from Dumbarton Bridge (South Bay) and San Pablo Bay only (the study focus area; $N = 156$). For these samples, the best model to predict MeHg (log-transformed) included a random effect of date and the following fixed structure:

$$\begin{aligned}\mathrm{Ln(MeHg)} = {} & -0.53 + 0.06(\mathrm{Date}) \\ & + 1.07(\mathrm{South\,Bay}) - 0.94(\mathrm{Formalin\,Treated}) \\ & + 0.63(\textit{P.amurensis}) - 0.61(\mathrm{Date} * \mathrm{South\,Bay})\end{aligned}$$

The marginal r^2 (fixed effects only) was 0.38, and the conditional r^2 (also including random variability among dates) was 0.66 for the model. Continuous variables were centered and rescaled. All the above model terms were included based on likelihood ratio test ($p < 0.05$), AIC, and BIC. Bivalve characteristics, including length, moisture content, C:N ratio, $\delta^{15}N$, and $\delta^{13}C$, were not in the final model, indicating that they did not contribute meaningfully to understanding MeHg content in this study. Rather, the model indicates elevated MeHg in *P. amurensis* (vs. *M. senhousia*) and in samples collected from South Bay, and reduced MeHg in formalin fixed samples (versus ethanol). Furthermore, for South Bay samples, there was a decrease in MeHg for later dates; i.e., South Bay samples declined over the study duration. Graphical analysis indicated elevated MeHg tissue concentrations in the 1970s compared to other dates, for South Bay, but not San Pablo Bay (Fig. 3).

To examine small-scale spatial variability, *M. senhousia* were compared from two occasions where specimens had been collected on the same date, but at different sites within the South Bay sampling area. On January 24, 1976, *M. senhousia* specimens from two sites that were 2.7 km apart did not significantly (*t*-test with separate variance, $p > 0.05$) differ in MeHg concentrations (Fig. 3). Similarly, on May 1, 1976, *M. senhousia* from two sites that were 2.1 km apart did not significantly (*t*-test with separate variance, $p > 0.05$) differ in MeHg concentrations (Fig. 3).

3.2.2. Nitrogen isotope trends

For the 156 samples examined for $\delta^{15}N$, the best model (based on likelihood ratio test for parameter inclusion, AIC, and BIC) included a model fixed structure (on centered and rescaled continuous variables) of:

$$\begin{aligned}\delta^{15}\mathrm{N} = {} & -1.09 - 0.03(\mathrm{Date}) + 1.49(\mathrm{South\,Bay}) + 0.39(\textit{P.amurensis}) \\ & + 0.74(\mathrm{Date} * \mathrm{South\,Bay})\end{aligned}$$

The marginal r^2 was 0.83 and the conditional r^2 was 0.98, indicating that the model explained almost all observed variation in the data.

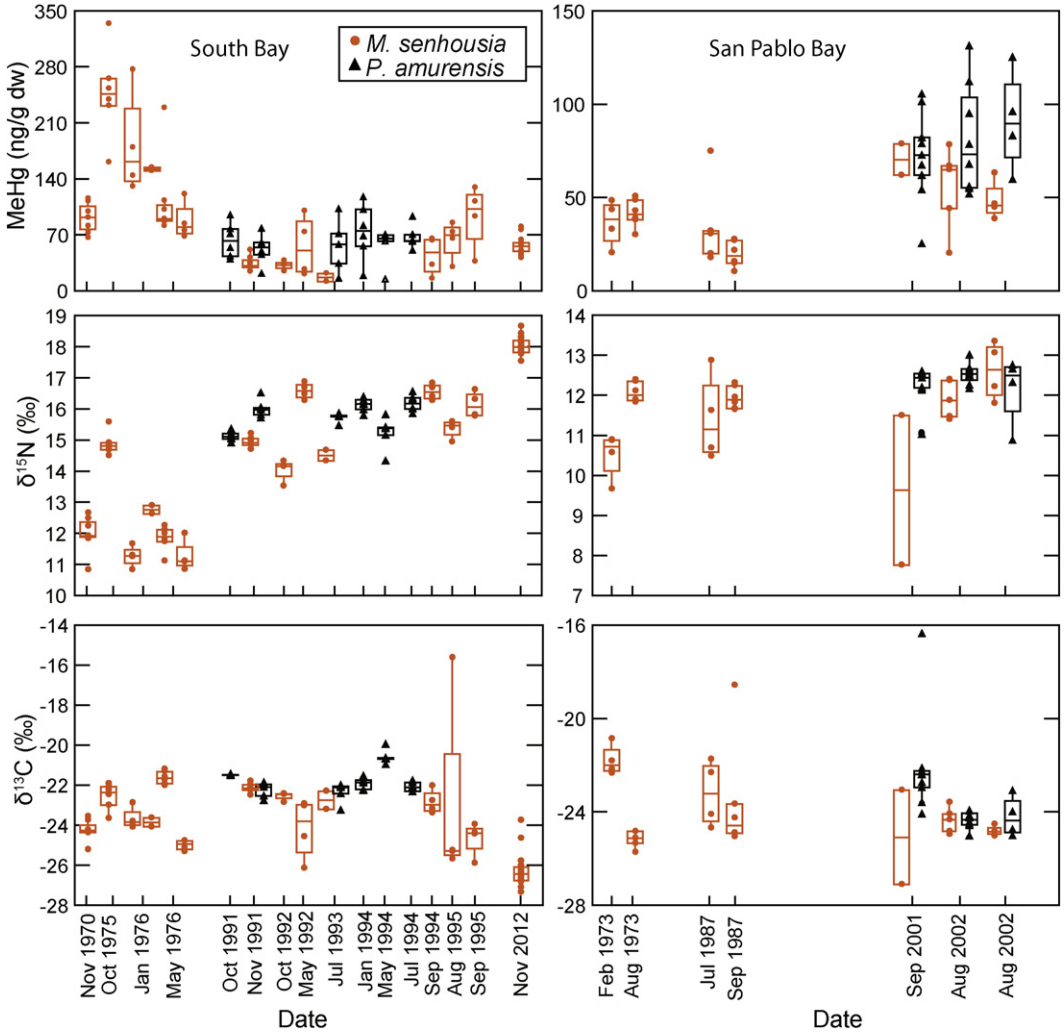

Fig. 3. Box and whisker plots of methylmercury (MeHg), $\delta^{15}N$, and $\delta^{13}C$ in museum preserved bivalves collected from San Francisco Bay between 1970 and 2012 for the subset of samples (N = 156) where stable isotope data were available. Boxes represent the middle half of the data, horizontal lines are the medians, points show raw data, and points not connected with whiskers are outliers. Left column: South Bay. Right column: San Pablo Bay. On some dates (Nov. 1991, Jul. 1993, Sep. 2001, and Aug. 2002) both species were collected from the same site. For *M. senhousia*, two boxes are plotted on Jan. 1976 and May 1976 to distinguish samples collected from different locations on the same date. For San Pablo Bay, there were two different sampling dates in Aug. 2002. Note different scales in y-axes for South versus San Pablo Bay. The x-axis is not to scale.

Other effects examined (length, $\delta^{13}C$, C:N ratio, moisture, and fixative treatment) did not meaningfully contribute to understanding of $\delta^{15}N$. Examining the model structure and parameter estimates indicates that $\delta^{15}N$ was substantially greater in South Bay (Dumbarton Bridge) than San Pablo Bay and moderately greater for *P. amurensis* than *M. senhousia*. Additionally, $\delta^{15}N$ increased strongly with date for South Bay but not San Pablo Bay. This trend is visually apparent, with South Bay median results around 12‰ in the 1970s samples, then 16‰ in the 1990s samples, and 18‰ in the 2012 samples (Fig. 3). To meet requirements of residual independence and variance homoskedasticity, the model included a random date effect (intercept term), a date-specific random effect of body length on $\delta^{15}N$ (i.e., slope term for body length), and a treatment-specific residual variance structure (ethanol versus formalin).

Given the trend in $\delta^{15}N$ and in MeHg in South Bay, there was also a weak negative association between $\delta^{15}N$ and MeHg (log-transformed) for the South Bay (Pearson's r = −0.37, N = 104), but not for San Pablo Bay (Pearson's r = 0.20, N = 52), or for both embayments combined (Pearson's r = −0.06, N = 156). The negative association between $\delta^{15}N$ and MeHg in South Bay contradicts a hypothesis of $\delta^{15}N$ indicating increased MeHg with increased trophic position.

3.2.3. Carbon isotope trends

In contrast to $\delta^{15}N$, which increased across the duration of the historical study, $\delta^{13}C$ exhibited no clear temporal trend until after 2002, when $\delta^{13}C$ appeared to decline. In particular, $\delta^{13}C$ was visibly lower (mean ± SD = −26.2 ± 0.9‰; N = 15), on November 15, 2012, the final date analyzed, than on earlier dates (−23.1 ± 1.7‰; N = 141). Consequently, both linear and quadratic negative date effects were significant predictors of $\delta^{13}C$ (N = 153), in addition to nitrogen isotope in the final fixed model structure:

$$\delta^{13}C = 0.36 - 0.55(\text{Date}) - 0.45\left(\text{Date}^2\right) + 0.28\left(\delta^{15}N\right)$$

The marginal r^2 for the model fixed effects was 0.38 and the conditional r^2, including both fixed and random effects, was 0.91. The model indicated that decline was strongest in the most recent samples (Fig. 4). There was no significant difference among species or sampling locations.

For $\delta^{13}C$, there were three positive outliers (−15.6, −16.4, and −18.6‰). These were identified as outliers based on exhibiting values 1.4–4.4‰ heavier than the remaining 153 samples (range: −27.4 to −20.0‰), relatively high C:N ratios, and graphical analysis indicating extreme values on a given sampling date (see Fig. 3). Inclusion

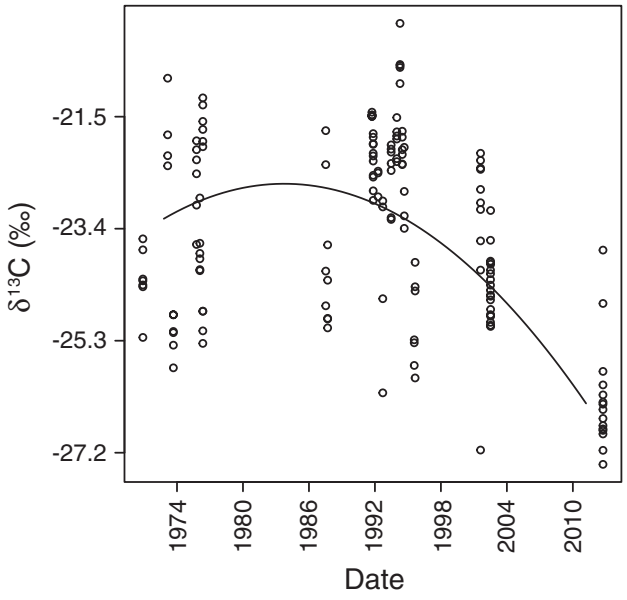

Fig. 4. The best statistical model to describe $\delta^{13}C$ included a negative quadratic date effect (marginal $r^2 = 0.38$).

versus exclusion of these outliers did not change the significant linear and quadratic date effects. For all models, the model structure required to achieve acceptable residuals also included a sample fixative effect on residual variation, as well as a random date effect.

A breakpoint analysis (i.e., piecewise regression) was performed and also indicated declining concentrations in recent years only. Based on minimizing AIC across all possible dates, a breakpoint was indicated between July 1994 and August 1995. There was no significant slope before the breakpoint (i.e., flat concentrations), and a significant decline afterwards, indicating declining $\delta^{13}C$ starting in the mid-1990s. In a mixed model with a September 1994 breakpoint, marginal and conditional r^2 were 0.43 and 0.91, similar to the quadratic model described above.

Although $\delta^{13}C$ and MeHg both exhibited temporal trends, $\delta^{13}C$ and MeHg were not significantly associated with each other for South Bay (Pearson's $r = 0.00$, $N = 104$), San Pablo Bay (Pearson's $r = -0.16$, $N = 52$), or both embayments combined (Pearson's $r = -0.02$, $N = 156$).

4. Discussion

4.1. Preservation study

4.1.1. MeHg trends

Preservation notably increased MeHg tissue concentrations, but concentrations generally stabilized after a week, with the exception of *P. amurensis* in formalin, which took 90 days to stabilize. This result was consistent with previous research showing that Hg_T in preserved fish samples increases and then stabilizes (Hill et al., 2010; Kelly et al., 1975; Levengood et al., 2013). The Hg_T increase has been attributed to loss of sample biomass due to dehydration and lipid loss; this loss concentrates mercury, which remains behind, associated with the sulfhydryl groups in proteins. Initial biomass loss generally occurs rapidly, leading to the most pronounced increases in mercury concentrations within the first 10 to 40 days (Hill et al., 2010; Kelly et al., 1975; Wetzel et al., 2005).

In this study, the initial MeHg increase was greater than that reported in prior studies of finfish but was consistent with expectations based on preservation effects on invertebrates. For the formalin treatment, MeHg increased by 86% for *M. senhousia* after 7 days and 54% for *P. amurensis* after 90 days (Table 1), a much greater increase than the 18% reported by Hill et al. (2010) for formalin-isopropanol preserved fish. The increase also seemed high compared to the increase (~30%) that would be expected from the amount of dehydration observed in fish (Kelly et al., 1975). However, Wetzel et al. (2005) observed dry mass decreases of 20–50% in invertebrates preserved in ethanol or formalin, which could result in up to two-fold MeHg concentration increases if the majority of MeHg was retained in tissue.

Neither this study nor prior studies (Hill et al., 2010; Kelly et al., 1975; Wetzel et al., 2005) indicate the relative importance of different mechanisms (lipid loss versus dehydration versus other possible mechanisms) for initial increases in MeHg concentrations upon sample preservation. In the current study, analysis of covariance did not indicate a statistical association between dry tissue mass and MeHg for either bivalve species, perhaps suggesting that loss of biomass was not important in the preservation study. However, this study did not compare the mass of each bivalve before and after it was preserved. Instead, it compared the mass of individual bivalves in the "fresh" treatment to the mass of individual bivalves in other treatments, a design that could have obscured small changes in the biomass of a single organism. The question of what caused the increase in MeHg following preservation and the time-frame for stabilization is an area where more research is needed, including direct measurements of lipid loss after preservation across multiple phyla and measurements over longer durations of years to decades (e.g., Rennie et al., 2012).

Because of the nontrivial effect from preservation (up to 1.9-fold increase in MeHg after 7 days of preservation) and the short time-frame of the preservation study, this study cannot rule out a preservation effect as a factor contributing to the historical trends (Fig. 3). However, several factors support the conclusion that the observed decline in MeHg in bivalves in the historical study is not simply a preservation artifact. First, the magnitude of the decline in the historical study (3.8-fold decrease in MeHg) is double that of the preservation effect. Second, if MeHg concentrations simply increased as samples aged, it would be hard to explain the lower MeHg in *M. senhousia* in the early 1990s compared to the mid-1990s (Fig. 3). Third, the historical study data did not support a trend of decreasing biomass over time; the samples in 1975–1976, which had some of the highest MeHg concentrations, did not have the lowest dry mass. Fourth, a continuing increase in MeHg in preserved samples over time would be inconsistent with the statistical results showing that MeHg in *P. amurensis* is lower in older samples. Fifth, this research and previous studies (e.g., Hill et al., 2010) support the conclusion that much of the MeHg increase occurs immediately upon preservation. Because all of the samples in this study were preserved, this 1.9-fold factor would not contribute to the historical trend.

Some researchers (e.g., Drevnick et al., 2007) have corrected for dehydration during preservation to compare preserved specimens to fresh ones. However, long-term trends in fish mercury concentrations have been reported without the use of a correction factor when all of the samples were preserved (Martins et al., 2006). All of the historical data in the current study were collected from samples that had been preserved for years, with the exception of the 2012 samples that had been preserved for about six months. Therefore, no correction factor was applied to the historical data in the current study.

4.1.2. Preservation study stable isotope trends

The effects of fixation on $\delta^{15}N$ depended on species but were limited overall. For *M. senhousia*, $\delta^{15}N$ values increased by about 0.3‰ after a week of fixation (ethanol or formalin), but then stabilized. For *P. amurensis*, fixation did not seem to affect $\delta^{15}N$ signatures in any systematic way. These results were consistent with literature reviews that report either no effect of preservation on $\delta^{15}N$ signatures (Rennie et al., 2012) or a very minimal change of 0.5‰ at most (Sarakinos et al., 2002). Preservation generally does not affect $\delta^{15}N$ signatures to the same extent that it affects $\delta^{13}C$ signatures (Sarakinos et al., 2002).

The study's experimental $\delta^{13}C$ data suggest that formalin fixation decreases $\delta^{13}C$ isotopic values, but ethanol fixation does not systematically affect $\delta^{13}C$ signatures. These results were in general agreement

with past studies reporting a depletion in $\delta^{13}C$ following formalin fixation (Rennie et al., 2012), but no change following ethanol fixation (Sarakinos et al., 2002). Averaged across multiple taxa, $\delta^{13}C$ declines following formalin fixation have been reported to be around 1.3‰ (Vander Zanden et al., 2003), 1.7‰ (Sarakinos et al., 2002), and 2‰ (Rennie et al., 2012). However, there is a lot of variability between species. For example, while $\delta^{13}C$ values for most taxa declined following formalin preservation, Sarakinos et al. (2002) reported an increase of 0.7‰ for the Asian clam, *Corbicula fluminea*. In the current preservation study, the effects of formalin fixation were at most a 0.5‰ decrease.

Overall, preservation study data suggest that it is appropriate to use preserved specimens to evaluate general temporal trends in bivalve $\delta^{15}N$ and $\delta^{13}C$, provided that caution is applied to small changes (i.e., <1‰). Of interest, a long-term comparison shows similar $\delta^{13}C$ and $\delta^{15}N$ signatures in specimens preserved with formalin for 1 year versus those preserved for 15 years (Rennie et al., 2012). In the current preservation study, the effects of fixation on $\delta^{15}N$ values varied with species and time. However, the largest change was a 0.3‰ enrichment in $\delta^{15}N$ in *M. senhousia* with increasing storage time (Fig. 2), which was relatively small, and in the opposite direction, compared to the ~6‰ long-term enrichment in the historical study of South Bay (Fig. 3). Similarly, the depletion in $\delta^{13}C$ (at most 0.5‰ for formalin fixed samples) with increasing storage time in the preservation study was of a smaller magnitude and opposite direction from the strongest temporal trend in the historical study, which was a 3‰ depletion in $\delta^{13}C$ in 2012 compared to prior years.

4.2. Historical study

4.2.1. MeHg trends

The study's main hypothesis was that MeHg concentrations in bivalves would decline due to a widespread reduction in mercury use prior to and during the study period. Mercury usage in the United States peaked in 1964, with consumption of 2.7 million kg per year (Conaway et al., 2007). Shortly thereafter, mercury was recognized as an environmental hazard, and production tapered off (Conaway et al., 2007). In San Francisco Bay sediments, mercury concentrations peaked in the middle of the 20th century and subsequently declined, although concentrations have not fallen to background levels (Conaway et al., 2004; Donovan et al., 2013).

Methylmercury declined over time in bivalves from South Bay, but there was no apparent time trend in San Pablo Bay. This difference between embayments was consistent with Mussel Watch data showing that trends vary by site; among 51 estuarine and coastal sites in California, analyzed across dates ranging from 1977 to 2010 (depending on site), Hg_T declined in *Mytilus* spp. at 24 sites but exhibited no trend at 27 sites (Melwani et al., 2013).

In the current study, the difference between sites likely results from embayment-specific primary mercury sources and reservoirs, which themselves exhibit differing time trends. There were two main anthropogenic sources of mercury to San Francisco Bay: elemental Hg (Hg^0) that was used during the Gold Rush in the Sierra Nevada region and mercury ore from the California Coast Range (Alpers et al., 2005). Mercury was also used in the San Francisco Bay watershed in a variety of historical applications, including as agricultural pesticides, as anti-fouling paint, and in chloralkali plants for the production of chlorine and caustic soda (Conaway et al., 2008). Mercury associated with gold mining reached North San Francisco Bay when mercury-contaminated sediments were transported downstream through the Sacramento-San Joaquin watershed (Domagalski, 2001; Donovan et al., 2013). Mercury associated with mercury mining in the Coastal Range reached South Bay when it was leached from tailings and calcine (roasted ore) and transported with sediments into the small waterways that empty into South Bay, such as Guadalupe River and Coyote Creek (Donovan et al., 2013; Thomas et al., 2002).

Mercury stable isotopes have been used to distinguish elemental mercury used in the Sierra Nevada from mercury leached from ores in the Coast Range (Gehrke et al., 2011). Stable isotope results show that the contribution of mercury from mining in the Coast Range has declined for open-water sites in South Bay, such as our field site. Around 1960, mercury from mining in the Coast Range accounted for 37% of the mercury in lower South Bay surface sediments; that number has dropped to 16% at present (Donovan et al., 2013). Elemental mercury, such as that used in the Sierra Nevada, is the now dominant source in surface sediments (Donovan et al., 2013).

The timing of the decline in the contribution of mercury from the Coast Range corresponds with the timing of the decline observed in this study. The highest concentrations of MeHg in this study were observed in *M. senhousia* in October 1975 (average 218 ng/g dw) and January 1976 (180 ng/g dw), corresponding with the closure of New Almaden in 1975. Lower MeHg concentrations observed in *M. senhousia* in later years (e.g., 57 ng/g dw in 2012) likely reflect the decline in release of mercury source material after closure of this mine (Conaway et al., 2007; Conaway et al., 2004). It is worth noting that this overall decline is significant even with the slightly lower MeHg (average 92 ng/g dw, Fig. 3) 1970 samples included; the 1970 samples were from the most westerly of the Dumbarton Bridge collection sites and were presumably furthest from the mine inputs (Fig. 1).

Bivalves in this study did not show a continued decline in MeHg concentrations in the 1990s although continued downward trends have been seen in sediments (Conaway et al., 2007). By compiling monitoring data, Conaway et al. (2007) found that mercury concentrations in sediment samples from the Dumbarton Bridge declined by 32% from 1993 to 2001. They tentatively attributed the decline in mercury in sediments to a wet period in the 1990s that lead to high flow from the Sacramento River, bringing less contaminated sediments all the way into South Bay (Conaway et al., 2007).

It would be reasonable to expect a continuing decline in mercury in bivalves, following remediation of New Almaden. After closure of the New Almaden mines in 1975, the site was opened as a county park until the California Department of Toxic Substances Control ordered its clean-up and temporary closure in 1987 (URS, 2012). Remediation occurred in two phases in 1999 and 2000 (URS, 2012). Further remediation to minimize erosion and thus release of mercury to San Francisco Bay followed after a Total Maximum Daily Load (TMDL) was developed for the Guadalupe River watershed in 2008. Unfortunately, one of the limitations of using preserved specimens is limited sample availability (Campbell and Drevnick, 2015). Lack of samples in the 2010s limits the ability to detect further declines in mercury in bivalves.

Although sample availability limited the time-frame of observations, this is the first study to report a long-term decline in mercury in biota from South San Francisco Bay. Long-term trends in MeHg in fish and wildlife have been observed in other waterbodies (e.g., Bhavsar et al., 2010; Bond et al., 2015; Campbell and Drevnick, 2015; Frederick et al., 2004; Gandhi et al., 2014; Hill et al., 2010; Vo et al., 2011). However, San Francisco Bay is somewhat unique in that it has a wide range of mercury sources, some of which were active over century timescales (e.g., historical gold and mercury mining and industrial applications) and some of which are still ongoing (e.g., atmospheric deposition) (Conaway et al., 2008; Davis et al., 2012).

Multiple sources make it difficult to observe declines, a problem that is further compounded by using biota to monitor trends. Biota mercury concentrations tend to fluctuate substantially from year to year in San Francisco Bay (Greenfield et al., 2005; Greenfield et al., 2013a; Gunther et al., 1999) and elsewhere (Claveau et al., 2015). These fluctuations can obscure trend detection. Another challenge is that concentrations in biota are frequently decoupled from those in sediments, as seen in the lack of a relationship between sediment Hg_T concentrations and fish Hg_T concentrations across Western North America (Eagles-Smith et al., 2016). Furthermore, rates of change in biota are slow and are most likely to be detected when initial concentrations are high

(Melwani et al., 2013). For example, Mussel Watch data showed no significant trend at two South Bay sites (Dumbarton Bridge and San Mateo Bridge) from 1986 to 2009 (Melwani et al., 2013). The declining trend that we observed in South Bay was due to the much higher MeHg concentrations that we were able to observe in the 1970s by using historical samples. The Mussel Watch study did report a weak declining trend in *Mytilus* spp. collected at the Emeryville shoreline (Central San Francisco Bay) from 1986 to 2009, with a calculated half-life of 54 years. Despite the different mercury form (Hg_T) and bivalve species examined, both Mussel Watch and the current study suggest limited change from the mid-1980s to the present.

The slow recovery suggests that the extensive health impacts from mercury usage in ongoing global gold mining (Gibb and O'Leary, 2014) may not be immediately curtailed when mining is stopped. The slow recovery also indicates that despite extensive ongoing restoration and management efforts, MeHg in the Bay is likely to require decades to exhibit detectable declines. There is a large existing mercury reservoir in Bay sediment and watersheds, which serves as an ongoing source of mercury to biota (Davis et al., 2012). As a result, restoration efforts and monitoring will need to be implemented on a long-term basis if discernable benefits are intended. Monitoring efforts will also need to account for ecological changes that can affect how much MeHg is transferred in the food web (Monson et al., 2011), such as the introduction of *P. amurensis* in the northern reach of the estuary in 1986. This species has shown an increase in MeHg from 1988 to 2002.

In the historical study, the statistical model indicated higher concentrations of MeHg in *P. amurensis* relative to *M. senhousia* during the time period when both species were available. Previous studies have considered both biodynamics (e.g., update and loss rates) and feeding niches to explain differing concentrations of MeHg among marine bivalve species (Pan and Wang, 2011). In this study, bivalves had similar feeding niches (as indicated by $\delta^{13}C$ signatures) so it seems likely that the differences are due to the way in which *P. amurensis* bioaccumulates MeHg and its rate of ingestion. *P. amurensis* is known to be a voracious filter feeder; its grazing rate can equal the rate of phytoplankton primary production in San Francisco Bay (Alpine and Cloern, 1992). The extremely high ingestion rate of *P. amurensis* has been cited as a factor contributing to its high selenium concentrations, which exceed concentrations in transplanted bivalves (Linville et al., 2002), lower trophic level crustaceans (Stewart et al., 2004), and the clam *Corbicula fluminea* (Lee et al., 2006). Dietary uptake may also explain the elevated MeHg in *P. amurensis*.

One implication of the differing MeHg levels between the two bivalve species is that although MeHg concentrations in *M. senhousia* have declined, if predators switched their diet to include *P. amurensis*, which became abundant in some parts of the estuary following its introduction in 1986, their overall MeHg body burden may have remained the same. Given the abundance and ecological importance of *P. amurensis* (Zeug et al., 2014), pulse-chase experiments may be warranted to compare uptake and loss rates and MeHg body burden among San Francisco Bay bivalve species.

4.2.2. Stable isotopes compared to MeHg

Methylmercury bioaccumulation occurs primarily through dietary exposure and researchers can employ multiple stable isotopes (e.g., $\delta^{13}C$ and $\delta^{15}N$) to track food sources, food web structure, and trophic position (Peterson and Fry, 1987). For example, different feeding niches (e.g., benthic versus pelagic) can be detected by $\delta^{13}C$ signatures, provided that the food sources have distinct isotopic signatures (France, 1995). Additionally, relative trophic positions can be detected with stable nitrogen isotope ratios because $\delta^{15}N$ becomes enriched between predator and prey, unlike $\delta^{13}C$ (Minagawa and Wada, 1984). This study therefore employed $\delta^{13}C$ and $\delta^{15}N$ as indicators of potential causes of mercury trends or mercury difference between species.

In this study, neither $\delta^{13}C$ nor $\delta^{15}N$ values were predictive of MeHg concentrations in the bivalves. In particular, neither were retained in mixed models to predict MeHg. Additionally, bivariate associations between the isotopes and MeHg were generally not observed. The only exception was a weak negative association between $\delta^{15}N$ and MeHg in South Bay; however, this contradicts a hypothesis of MeHg increasing with increased trophic position of individual bivalves, suggesting that MeHg is not explained by food web position. Previous studies of mercury accumulation in different bivalves species have argued that the carbon source, measured by $\delta^{13}C$, can be a predictor of MeHg concentrations, but that the trophic level, measured by $\delta^{15}N$, is similar among different bivalves species (Pan and Wang, 2011). In the current study, the lack of a relationship between MeHg and $\delta^{13}C$ or $\delta^{15}N$ suggests that the decline in MeHg concentrations (Fig. 3) was due to changes in exposure, not changes in food web structure. One possible reason for lack of associations between $\delta^{13}C$ signatures and MeHg was that, in contrast to fresh waters, which often exhibit clear two-source mixing (France, 1995), the food sources in San Francisco Bay exhibit highly variable and complex $\delta^{13}C$ baseline signatures (Cloern et al., 2002).

Higher $\delta^{15}N$ values were observed in *P. amurensis* relative to *M. senhousia*. This was somewhat surprising because previous studies of mercury accumulation have found no difference in $\delta^{15}N$ between bivalve species (Pan and Wang, 2011). *P. amurensis* has been shown to feed on zooplankton, such as copepod nauplii (Kimmerer et al., 1994) and microzooplankton (Greene et al., 2011), in addition to its main diet of phytoplankton (Canuel et al., 1995). These other carbon sources could potentially account for this clam's $\delta^{15}N$ enrichment relative to other filter feeding organisms in San Francisco Bay. Nevertheless, dietary information is lacking for *M. senhousia*, and thus, some other unknown factor could be causing a baseline shift in nitrogen isotope between the species. Future studies could conduct side-by-side comparisons of $\delta^{15}N$ and diet in the two species.

4.2.3. Stable isotope spatial and temporal trends

Substantially higher $\delta^{15}N$ values were observed in South Bay bivalves relative to San Pablo Bay, consistent with different nutrient and water sources between the embayments. San Pablo Bay is dominated by riverine inflow and nutrient sources from runoff, whereas wastewater sewage treatment plant discharges are the predominant sources of water and nutrients to South Bay (Novick and Senn, 2014; Smith and Hollibaugh, 2006). In many systems, anthropogenic nitrogen loading (e.g., from wastewater treatment plants) causes elevated $\delta^{15}N$ values (Wankel et al., 2006).

The increase in $\delta^{15}N$ values in South Bay bivalves over the study duration likely results from a shift in baseline over time, due to multiple processes. The magnitude of the increase (6‰) would represent two trophic levels and therefore is too large to be explained by an increase in trophic position for filter-feeding bivalves. Instead, the increase could result from a combination of changes in total inorganic nitrogen (TIN) loading (e.g., NO_3 and NH_4^+) and changes in algal biomass. From the 1970s to 1991, $\delta^{15}N$ values in South Bay bivalves increased from 12 to 16‰. The timing of this increase generally corresponded with an increase in dissolved inorganic nitrogen (DIN) loadings from the San Jose-Santa Clara wastewater treatment plant between the mid-1970s and the late 1980s (Cloern et al., 2006). The San Jose-Santa Clara wastewater treatment plant is the major municipal discharger into South Bay (Cloern et al., 2006).

From the 1990s to 2012, $\delta^{15}N$ values in bivalves continued to increase from around 16 to 18‰. However, increased TIN loading is unlikely to be the cause of this trend because improvements in wastewater treatment resulted in a decrease in TIN loading, beginning around 1990 and continuing to 2006, when data were last available (Cloern et al., 2007). Instead, the increase in $\delta^{15}N$ could potentially be the result of a long-term increase in algal biomass in South Bay, documented since 1999 (Cloern et al., 2007). Algal populations have increased in recent years, likely due to climate-mediated reductions in benthic bivalve populations and grazing (Cloern and Jassby, 2012). Increased phytoplankton biomass may have increased inorganic nitrogen

δ^{15}N-DIN, due to isotopic fractionation to the heavier isotope during phytoplankton uptake (Granger et al., 2004). Enrichment of the nitrate pool in ^{15}N in South Bay waters as a result of primary production was also previously observed (Wankel et al., 2006).

Similarly, the apparent shift in δ^{13}C to a lighter signature by 2012 (Fig. 4) is consistent with increased pelagic (versus benthic) carbon sources to the bivalves (Peterson and Fry, 1987), possibly resulting from the recent increase in phytoplankton biomass. However, there is considerable variability in δ^{13}C signatures (Cloern et al., 2002), including short-term variability from stormy conditions that can introduce waters depleted in ^{13}C (Fry, 1999). The recent decrease in δ^{13}C, possibly indicating a shift to a more pelagic food web pathway, should be investigated further because previous studies have demonstrated that pelagic food webs biomagnify mercury more efficiently than benthic food webs (Pickhardt et al., 2006; Stewart et al., 2008) and that algal blooms may mobilize MeHg in San Francisco Bay (Luengen and Flegal, 2009).

5. Conclusions

The sharp decline in MeHg in *M. senhousia* following closure of the New Almaden Mercury Mines, combined with mercury isotope results showing a decline in mine-derived mercury (Gehrke et al., 2011; Donovan et al., 2013), strongly suggests the importance of source control in decreasing MeHg concentrations in biota. However, MeHg patterns in bivalves can be decoupled from those in sediments, as in the 1990s when mercury concentrations in bivalves did not continue to decline despite decreases in Hg_T sediment concentrations and mine remediation. Furthermore, the overall impact of decreasing mercury loadings, especially to higher trophic levels, may be obscured by ecological changes. For example, invasion of *P. amurensis* may increase MeHg accumulation in the food chain; it has higher MeHg concentrations than *M. senhousia*. Stable carbon and nitrogen isotope analyses of preserved bivalve tissues can be useful in detecting ecological changes, including natural drivers, such as a recent increase in algal biomass (Cloern et al., 2007; Cloern and Jassby, 2012) that is of concern due to the potential for pelagic-based food webs to accumulate high MeHg. Ultimately, to understand and predict MeHg biotic trends, it is important to consider trends in loadings, MeHg production (i.e., methylation), and bioaccumulation to the food chain. Use of preserved specimens is one of many approaches that can help identify these multiple drivers to understand how management actions will influence biological responses.

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