Science of the Total Environment 738 (2020) 139803

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

# Formalin-preserved zooplankton are not reliable for historical reconstructions of methylmercury bioaccumulation

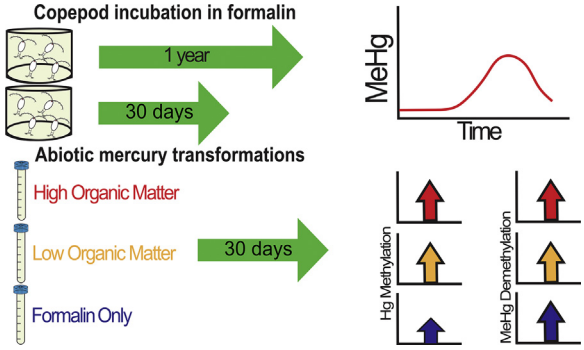
Wesley W. Huffman [a,*], Hans G. Dam [a], Robert P. Mason [a], Zofia Baumann [a,b]

[a] Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Rd, Groton, CT 06340, United States
[b] Billion Oyster Project, Governors Island, 10 South St., Slip 7, New York, NY 10004, United States

## HIGHLIGHTS

- Methylmercury in formalin-preserved copepods change over time.
- Abiotic transformations of mercury can occur in formalin solutions.
- Formalin-preserved copepods cannot reconstruct historical methylmercury levels.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 31 March 2020
Received in revised form 21 May 2020
Accepted 27 May 2020
Available online 30 May 2020

Editor: Daniel Wunderlin

*Keywords:*
Mercury
Methylmercury
Bioaccumulation
Copepods
Isotopic tracers
Historical archives

## ABSTRACT

Time-series measurements of methylmercury (MeHg) concentrations in short-lived planktic animals, such as copepods, could allow for an evaluation of mercury (Hg) inputs and transferability to organisms in marine environments. If reliable, MeHg measurements in formalin-preserved marine animals could offer insights into past environmental MeHg levels. In the present study, we examined whether the amount of MeHg changed over time in formalin-preserved copepods for two species, *Acartia tonsa*, and *Temora longicornis*. Over a 51 (*A. tonsa*) and 7 (*T. longicornis*) week incubation, we found significant changes in MeHg content in both copepods, while the timing of these changes differed between species. Furthermore, we investigated the mechanism behind these temporal changes through a separate incubation experiment of formalin spiked with two levels of organic matter (OM), and stable-isotope-enriched Hg tracers. We found that the methylation of an inorganic $^{199}$Hg tracer was significantly higher in OM-enriched solutions in comparison to a control seawater-formalin solution. Our results suggest that formalin-preserved copepods are not fit for studies of past trends due to ongoing and unpredictable abiotic transformations of Hg in chemically preserved animal tissue.



## 1. Introduction

Mercury (Hg), particularly its organic form methylmercury (MeHg), is an acute neurotoxin that efficiently bioaccumulates in aquatic biota, reaching high concentrations in apex predators which pose a risk to human health (Clayden et al., 2015; Harding et al., 2018; Lee and Fisher, 2017; Lee et al., 2016; Morel et al., 1998; Schartup et al., 2019). Mercury enters aquatic and terrestrial environments through direct atmospheric deposition, watershed inputs, or via point sources, where it can be transformed to MeHg and accumulate in biota to harmful levels (Fitzgerald et al., 2007). In response to legacy and emerging sources of

* Corresponding author.
*E-mail address:* wesley.huffman@uconn.edu (W.W. Huffman).

https://doi.org/10.1016/j.scitotenv.2020.139803
0048-9697/

Hg pollution, the 2013 Minamata Convention on Mercury was signed by over 140 countries to lower global Hg emissions and mitigate human impacts (Saito, 2019). Globally, Hg emissions peaked in the 1970s and have declined by ~20% since 1990, driven by substantial reductions in emissions from North America and Europe (Zhang et al., 2016). Coupling emissions to MeHg bioaccumulation must further consider the various processes that in concert moderate the net conversion of inorganic Hg to MeHg, and those impacting its bioaccumulation (Wang et al., 2019; Driscoll et al., 2012). While measurements of direct sources of Hg provide useful insight into the effectiveness of such treaties, changes in MeHg bioaccumulation are what ultimately influence human impacts and remain harder to quantify. Many of these processes are not well understood, and knowledge gaps result primarily from limited experimental studies (Jonsson et al., 2017; Lee and Fisher, 2016; Grieb et al., 2019).

Retroactive and current MeHg levels can be elucidated with the aid of proper sentinel organisms. In the ocean, long-lived fish have been suggested as sentinels of MeHg change over time (Drevnick et al., 2015; Evers et al., 2008; Harris et al., 2007; Lee et al., 2016; Mason et al., 2005). However, the coupling of Hg emissions and fish bioaccumulation is highly variable and inconsistent, likely due to sparse sampling over a wide size range of fish throughout multiple trophic levels (Grieb et al., 2019; Wang et al., 2019). Organisms that do not undergo extensive migration, occupy lower trophic levels, and are short-lived, would enable more meaningful inferences about shorter-term (weeks – months) feedbacks between anthropogenic Hg emissions and the pool of bioavailable MeHg that enters marine food webs (Evers et al., 2008; Mason et al., 2005). Copepods may be an ideal candidate to be used as sentinels of bioavailable MeHg as they are short-lived (e.g., 8 days to 1–2 year), occupy a low position in planktic food webs, and within their respective lifetimes tend to remain within a narrow geographic range (Mauchline, 1998). For example, the whole lake Hg isotope spike addition experiment in Canada showed that there was isotopically-labeled MeHg within the zooplankton after only 1 month of adding an inorganic Hg spike to the surface of the lake (Harris et al., 2007).

Copepods, the most abundant metazoans in the oceans, are a crucial link connecting primary producers, phytoplankton, with secondary consumers, particularly larval and juvenile fish (Mauchline, 1998). The initial transfer of MeHg from seawater to primary producers largely determines the magnitude of its bioaccumulation in apex predators (Foster et al., 2012). As copepods are primary consumers and are often the first heterotrophic link in a food web, they represent a critical step for the transfer of MeHg to upper trophic levels. Previous studies have investigated MeHg in copepods based on bulk plankton sorted through sequential filtration into size bins (Clayden et al., 2015; Gosnell et al., 2017; Hammerschmidt and Fitzgerald, 2006; Harding et al., 2018). Such samples contain a taxonomic mix, which has limited value in studies of MeHg bioaccumulation. Only a few studies have examined species or genus-level concentrations (Foster et al., 2012). Therefore, there is a need for time series measurements of MeHg concentrations in isolated taxa of copepods, which can shed light on temporal patterns in bioavailable MeHg and aid in the evaluation of the Minamata Convention (Krabbenhoft and Sunderland, 2013; Selin, 2014; Foster et al., 2012).

To assess the effectiveness of lowered human Hg emissions into the environment, knowledge of the past and current levels of Hg in aquatic ecosystems worldwide is highly desirable. Previous studies have argued for the adequacy of formalin-preserved fish to evaluate THg (total mercury) concentrations (Hill et al., 2010; Kelly et al., 1975; Levengood et al., 2013). These studies relied on the assumption that THg concentrations in fish tissues were far higher than in formalin, resulting in negligible contamination. Each of the studies demonstrated changes in fish THg concentrations between fresh and preserved samples, with equilibria in THg concentrations reached sometime between 40 days and 12 months (Hill et al., 2010; Kelly et al., 1975; Levengood et al., 2013). The observed changes in THg were attributed to fluctuations in mass during the preservation period (Hill et al., 2010; Kelly et al., 1975; Levengood et al., 2013).

The validity of chemically preserved bivalves as indicators of historical MeHg concentrations has also been explored (Luengen et al., 2016). Based on a 90-day long experiment, Luengen et al. (2016) argued that MeHg concentrations in chemically preserved bivalves were reliable indicators of MeHg conditions through three decades in San Francisco Bay. Fish and bivalves both have higher %MeHg (>95% for fish and 12–60% for bivalves) compared to copepods and similarly size binned zooplankton (2.8–24%) (Foster et al., 2012; Luengen et al., 2016; Hammerschmidt et al., 2013; Gosnell et al., 2017). Thus, MeHg concentrations may be more stable in formalin-preserved tissues that have higher %MeHg.

The present study tested whether or not copepod samples preserved in buffered formalin could be used to represent the MeHg concentrations in these organisms at the time of their capture. Formalin preservation of copepods is widespread because organism structures remain intact over long periods, allowing preservation of samples for decades or longer (Mauchline, 1998). Formalin naturally diffuses into cellular structures where it binds and stabilizes proteins, inhibiting decomposition (Thavarajah et al., 2012). Furthermore, the addition of phosphate buffer to formalin minimizes fluctuations in pH during fixation and further increases long term preservation (Thavarajah et al., 2012). The prevalence of formalin preserved copepod samples held by diverse museums, as well as marine research institutes globally, warrant an investigation into the feasibility of using these organisms for discerning past MeHg concentrations.

The aforementioned studies motivated us to experimentally determine whether chemically preserved copepods could infer formerly accumulated MeHg. We hypothesized that formalin preservation does not influence MeHg concentrations in copepods over short (days or weeks) or long (up to one year) periods. We tested this hypothesis using two copepod species, i.e., *Acartia tonsa* and *Temora longicornis*, which are ubiquitous in temperate estuarine ecosystems (Cervetto et al., 1999; Dam and Peterson, 1991; Dam et al., 1994; Kane and Prezioso, 2008). Furthermore, we conducted a set of experiments using Hg stable isotopes to test the potential for abiotic transformations of Hg within conditions similar to the preserved copepods using protein-rich organic matter. The goal of these experiments was solely to demonstrate that abiotic transformations could occur in these solutions and was motivated by findings of Luengen et al. (2016), based on an overtime-increase of MeHg concentrations in chemically preserved bivalves.

## 2. Materials and methods

### 2.1. Study species

Copepods *Acartia tonsa* and *Temora longicornis* were collected from Esker Point Beach at the shore of Long Island Sound (LIS) in Connecticut, US (41°19′N, 72°00′W) using a conical plankton net with a 200 μm mesh size. Tows were performed in August 2015 (*A. tonsa*) and April 2016 (*T. longicornis*) per their occurrence in LIS (Dam and Peterson, 1991; Dam et al., 1994). Copepods were also collected in January 2018 to measure THg and determine %MeHg (Eq. (1)), as discussed below. The derived %MeHg value from 2018 was used to determine the inorganic Hg content for *A. tonsa* and *T. longicornis* retroactively for the incubation experiments. These two species were chosen based on their size differences. *T. longicornis* is larger with average prosome length (PL) of 1.2 mm and dry mass range of 11–44 μg (Dam and Peterson, 1991) and *A. tonsa* is smaller with an average PL is 0.9 mm and dry mass range is 4–12 μg (Dam et al., 1994). Copepods were hand-picked using a transfer pipette under a dissecting microscope from the bulk plankton sample.

$$\%\mathrm{MeHg}_{\mathrm{cop}} = 100 \times \mathrm{M}_{\mathrm{MeHg,cop}} / \mathrm{M}_{\mathrm{THg,cop}} \quad (1)$$

### 2.2. Incubation of copepods in formalin

Certified phosphate-buffered formalin (10% w/v; Fisher Scientific, Toronto, Canada) was mixed with 0.2 μm-filtered seawater, reaching a final volume-based concentration of 10%. Prior to the incubations, triplicates of 50 individual copepods from each species were frozen (−20 °C) before analysis to determine their initial MeHg content. Hand-picked copepods were incubated in a seawater–formalin solution for up to one year for *A. tonsa* and 7 weeks for *T. longicornis* at room temperature. Samples were stored in 15 mL conical centrifuge tubes (Falcon tubes; Fisher Scientific, Toronto, Canada), which were filled with 10 mL of solution, sealed with parafilm, and kept under ambient light and temperature conditions in the lab. For *A. tonsa*, the incubations were terminated at 4, 8, 12, 24, and 51 weeks, and for *T. longicornis*, they were terminated at 1, 2, 4, and 7 weeks. The length of incubation periods was selected to monitor short and long-term changes in MeHg concentrations. Incubations were terminated by passing the solution that held the copepods through a 10 μm polycarbonate filter to collect the organisms, which were then stored at −20 °C before further processing.

#### 2.2.1. Mass balance of inorganic Hg in the experimental vials with copepods

To account for the sources of inorganic Hg in the experimental vials and the contribution that copepods had to the overall pool of inorganic Hg, we relied on the following equations:

$$M_{Hg(II),cop} = M_{MeHg,cop}/\%MeHg_{cop,Jan2018} \quad (2)$$

$$M_{Hg(II),v} = M_{Hg(II),cop} + M_{Hg(II),sw} + M_{Hg(II),form} \quad (3)$$

$$\%Hg(II)_{cop,v} = 100 \times M_{Hg(II),cop}/M_{Hg(II),v} \quad (4)$$

in addition to Eq. (1) as described above.

The inorganic Hg content in copepods was calculated based on the $\%MeHg_{cop,Jan2018}$, described below, and initial MeHg concentrations in copepods as described in Eq. (2), where $\%MeHg_{cop}$, and $M_{MeHg,cop}$ are respectively, the percentage and contents (pg) of MeHg in copepods within the vial. Eq. (3) describes the distribution of inorganic Hg (pg) within a single experimental vial. In this equation, $M_{Hg(II),v}$, $M_{Hg(II),cop}$, $M_{Hg(II),sw}$, and $M_{Hg(II),form}$ denote the content of inorganic Hg (pg) in the entire vial, copepods, seawater, and formalin, respectively. We assume that THg in seawater and formalin are adequate proxies of the inorganic Hg, given the extremely low %MeHg in seawater, typically on the order of 1%, and no MeHg present in formalin. The percentage of inorganic Hg associated with copepods ($\%Hg(II)_{cop,v}$) in each of the experimental vials was calculated based on Eq. (4), and the resulting data are included in Fig. 1.

### 2.3. Copepod MeHg analysis

Filters containing 50 copepods were acid digested in 7 mL of 4.5 N trace metal grade nitric acid ($HNO_3$) at 60 °C for 12 h prior to analysis following the methods described by Hammerschmidt and Fitzgerald (2005). The entire volume of the acid digest was added to 20 mL of MilliQ water. The pH of the solution was adjusted to 4.7–4.9 by adding 2 N acetate buffer and 8 N potassium hydroxide. This pH is required for sodium tetraethyl borate (NaBET-4) to effectively ethylate the MeHg present in the solution, thus forming ethylmethylmercury (EtMeHg), a volatile Hg compound that can be sampled from the headspace of the sample vial upon argon purging of the solution. EtMeHg can then be detected using cold vapor atomic fluorescence spectroscopy by a Tekran 2700 system. MeHg in copepods is reported as per vial, based on the 50 individuals per replicate.

### 2.4. THg content in seawater, formalin, and copepods

Seawater, copepods, and formalin similar to those used in the incubations were analyzed for THg. These measurements were required to further account for sources of Hg not associated with the copepods that may have influenced our experimental results. As the copepods in the time series incubations were entirely digested for the MeHg analysis, a different sample of the same species (*A. tonsa*) was used to determine both MeHg and THg content. Copepods used in this analysis were collected during a cruise by the Connecticut Department of Energy and Environmental Protection in western Long Island Sound (40° 55′N, 73° 38′W) in January 2018. Individuals were collected and picked out of the bulk sample using the same methods as described above. Copepods were acid digested at 60 °C for 12 h in 7 mL of 4.5 N $HNO_3$, then a subsample of 1 mL was resuspended in 9 mL of MilliQ water and further digested with bromine monochloride (BrCl; 0.33% v/v) at room temperature. Solutions of seawater, mixed with formalin but not additional animal tissues, were digested for 12 h at room temperature using BrCl. Hydroxylamine hydrochloride ($NH_2OH \cdot HCl$; 0.2% v/v) and lastly stannous chloride ($SnCl_2$; 0.2% v/v) were added to all digested vials holding the aqueous digestate solution immediately prior to the analysis. THg was reduced into elemental Hg ($Hg^0$) and measured by cold vapor atomic fluorescence via a Tekran 2600 system (EPA method 1631).

### 2.5. Testing for abiotic transformations of Hg(II) and MeHg

In order to investigate the potential of abiotic transformations in conditions similar to the copepod incubations, spikes of isotopically enriched $^{201}Hg(II)$ and $Me^{199}Hg$ were added to a seawater-formalin solution enriched with marine organic matter (OM). Freeze-dried and homogenized hard clam tissue at concentrations of 10 and 100 mg $L^{-1}$ was mixed with the seawater-formalin solution representing a range in OM content that encompasses those estimated for the copepod incubations (40 mg $L^{-1}$ *A. tonsa* and 140 mg $L^{-1}$ *T. longicornis*). Clam tissue was selected from a single individual sourced from a local embayment (Mumford Cove, Connecticut, US 41°32′N, 72°02′W) to represent a protein-rich OM source to simulate preserved biota samples. Concentrations of mercury in the clam tissue were measured at 0.1966 μg $kg^{-1}$ and 0.094 μg $kg^{-1}$ of THg and MeHg, respectively. The control treatment was composed of solely filtered seawater and formalin (as described above).

To test for potential abiotic methylation of Hg(II), 55 μL of the 1.18 nM solution of $^{199}Hg(II)$-enriched tracer (isotopic abundance of $^{199}Hg$: 91.95%) was added to each experimental vial (10 mL of liquid). The degree of MeHg demethylation was tested by adding 55 μL of 1.42 nM $Me^{201}Hg$ tracer (isotopic abundance of $^{201}Hg$: 98.10%) into each vial. Incubations were terminated by flash-freezing on dry ice on days 0, 4, 14, and 29. Detection of $Me^{199}Hg$ in the solution indicated an abiotic transformation of Hg(II) into MeHg, while a decline of $Me^{201}Hg$ indicated its demethylation. Three acid-digested samples containing seawater, formalin, and 100 mg OM $L^{-1}$ were spiked as in the experiment, but immediately prior to analysis. This was to account for the possibility of instantaneous methylation of the added inorganic Hg spike as an artifact of reagent addition before analysis. We refer to this treatment as the "delayed spike". The net % methylated $^{199}Hg(II)$ was calculated from a known amount of the tracer. Likewise, net % demethylated was determined from the averaged initial $Me^{201}Hg$ tracer in the delayed spike. Percent transformation (methylated or demethylated) was calculated by dividing the amount of transformed Hg from these tracers by either its initial concentration (methylation) or averaged delayed spike concentration (demethylation). Due to its high natural abundance (29.86%), $Me^{202}Hg$ was also quantified to determine ambient concentrations of MeHg within each sample (Fig. S1, Table S1). Analysis of samples from the abiotic transformation incubations made of formalin-seawater solution samples containing $^{199}Hg(II)$ and $Me^{201}Hg$ spikes, relied on Gas Chromatography - Inductively

Coupled Plasma Mass Spectrometry (GC-ICP MS; by combining a Tekran 2700 with a Perkin Elmer Elan DRC II, and using an internal standard of 0.68 nM $Me^{200}Hg$).

### 2.6. Statistical data analyses

To test for time-dependent differences in copepod MeHg, and time/OM-dependent changes in $Me^{199}Hg$ and $Me^{201}Hg$ in seawater-formalin solutions, we conducted One and Two-Way ANOVA's and Tukey HSD Post-Hoc tests (R software version 3.3.3). Data were transformed using a log + 1 transformation to achieve normality and homogeneity required for parametric statistical analysis. For $Me^{202}Hg$, data were transformed using a Tukey Ladder of Power transformation and analyzed with a Two-Way ANOVA and Tukey HSD Post-Hoc test. In the abiotic transformation experiments, values below the limit of detection for all isotopes (>5%) were removed. To account for the unbalanced replicate numbers (i.e., primarily n = 3 with occasional n = 2), we used the type II Two-Way ANOVA after determining a non-significant interaction term.

### 2.7. QA/QC

#### 2.7.1. Copepods incubations

Within the copepod incubations, MeHg concentrations in the sample vials were determined based on a calibration of MeHg standard solutions prepared from the Alfa Aesar standard traceable to a standard prepared by the National Institute of Standards and Technology (NIST). Working standards were compared with secondary standards prepared by JT Baker and were traceable to NIST to ensure accuracy. Vials containing standard additions and reagent blanks were made in the sample matrix to ensure consistency throughout the run. Standard solutions of MeHg were also tested throughout the run, at least every tenth sample, with their recoveries averaging 97 ± 6%. As the entire volume of the digest was used for the analysis of a single sample, the recovery from samples spiked with a known amount of MeHg was not possible. The limit of MeHg detection during copepod analyses was calculated at 0.016 ng $L^{-1}$, based on a threefold blank concentration. Concentrations of THg were determined based on comparison to a standard solution prepared by JT Baker and were traceable to NIST. Standard recoveries of Hg(II) were performed at least every 10 samples and averaged 126 ± 4%. The calculated limit of detection for this analysis was 0.025 ng $L^{-1}$.

#### 2.7.2. Abiotic Hg experiments

To determine MeHg concentrations in the abiotic incubations experiments, 55 μL of 0.68 nM $Me^{200}Hg$ was used as an internal standard (isotopic abundance of $^{200}Hg$: 96.4%). A solution of 4.5 $HNO_3$ digested sample was equilibrated for 1 h prior to analysis. To correct for possible mass bias during the GC-ICP MS measurement, an ambient MeHg standard of known isotopic composition was analyzed in triplicate and found to be negligible. The assumed limit of detection for isotopes within the MeHg tracers was set for 5% of the total isotope signal in all MeHg that was detected (Jonsson et al., 2017). All isotopes were detected with an average efficiency of 76 ± 21%. Isotopic signals were deconvoluted based on known Hg isotopic ratios in the tracers (Jonsson et al., 2017).

## 3. Results

### 3.1. Hg in copepods

The initial amount of MeHg in the formalin-incubation experiment was 10.05 ± 1.53 pg and 14.72 ± 2.58 pg per vial for *A. tonsa* and *T. longicornis*, respectively (mean ± SE; Fig. 1). In both cases, MeHg concentration changed significantly with time increasing to 97.30 ± 22.82 pg per vial for *A. tonsa* and 107.25 ± 6.47 pg per vial for *T. longicornis* (*One-Way ANOVA, $p < 0.001$*) with relatively similar patterns for both species (Fig. 1). In *A. tonsa*, MeHg per vial decreased slightly during the first 13 weeks, but increased sharply by 900% (week 29) and 300% (week 51). Likewise, MeHg decreased slightly during the first 4 weeks in *T. longicornis* but increased by 350% by week 4 and 600% by the end of the 7th week. MeHg and THg in *A. tonsa* collected in January 2018 from WLIS, was 5.50 ± 1.50 and 105.50 ± 2.00 pg per vial (50 individuals per sample) respectively, with a derived %MeHg of 5.3 ± 2.5%. THg content in the seawater and the formalin solution was 4.52 and 1.97 pg per vial respectively. Assuming that %MeHg in the WLIS *A. tonsa* was comparable to that of copepods used in the incubation experiments, most (> 96.4%) of the inorganic Hg in the experimental vials was accounted for by the copepods, as calculated based on Eqs. (2)–(4). Increases in MeHg content throughout the incubations track with a higher percent of methylated mercury within the entire vial, reaching as high as 59 ± 3% (Fig. 1).

### 3.2. Abiotic Hg transformations

Incubation of $^{199}Hg(II)$ and $Me^{201}Hg$ in the control (not enriched with OM) and OM-enriched formalin solutions revealed that under these experimental conditions, net methylation of $^{199}Hg(II)$ to MeHg

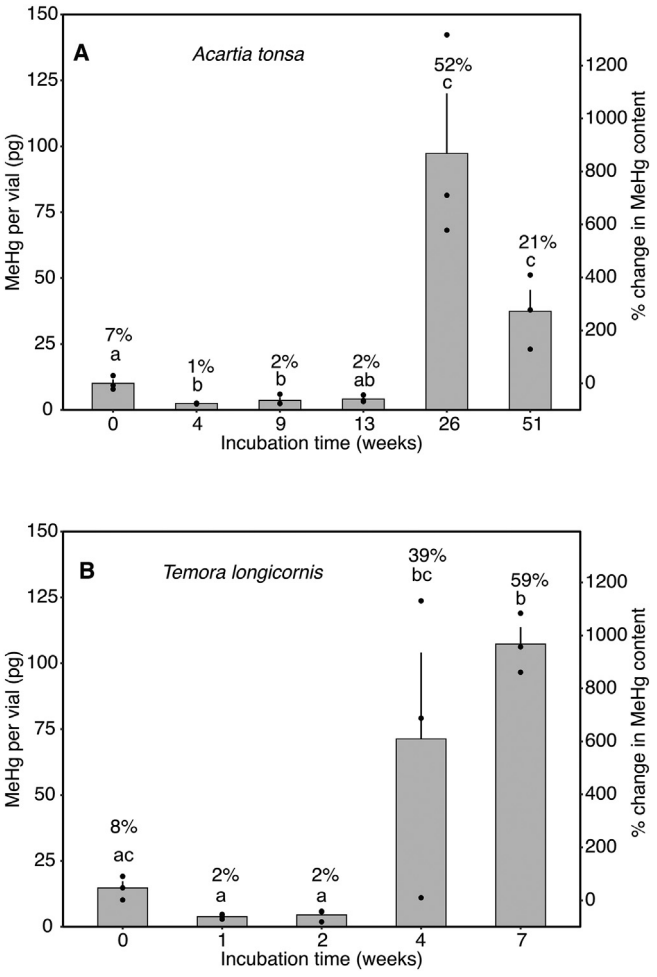



**Fig. 1.** MeHg content per vial over time in 10% buffered formalin (A) *A. tonsa* and (B) *T. longicornis*. Values are comprised of 50 individual copepods per experimental vial. The secondary axis denotes a percent change in MeHg concentration from the initial time point. Percentage values above each bar indicate % Hg as MeHg in copepods within each experimental vial. Individual replicates and mean values are depicted by points and bars, respectively, mean values ± 95% SE. Lower case letters indicate significant differences based on Tukey HSD Post-Hoc test.

could occur rapidly (Fig. 2). Within the delayed spike treatment, 1.7% of the $^{199}$Hg(II) tracer was methylated immediately after the spike addition (Fig. 2). There was significantly less methylation of $^{199}$Hg(II) in seawater-formalin solution (control) compared to the OM-enriched treatments (*Two-Way ANOVA, Tukey (HSD)* Post-Hoc, $p < 0.001$; Fig. 2).

There were no significant differences in the degree of the Me$^{201}$Hg tracer demethylation between the two OM-enriched formalin solution treatments among time points (*Two-Way ANOVA, Tukey HSD* Post-Hoc, $p = 0.31$; Fig. 2). With only a significant difference found between the control and OM-enriched treatments at 29 days (*Two-Way ANOVA, Tukey HSD* Post-Hoc, $p < 0.05$; Fig. 2). However, there was a significant difference in the amount of demethylated Me$^{201}$Hg tracer between the delayed spike (see above) and the other samples that were incubated for a longer time (*Two-Way ANOVA, Tukey HSD* Post-Hoc, $p < 0.05$; Fig. 2).

Additionally, ambient MeHg, calculated based on the Me$^{202}$Hg signal obtained from the ICP-MS analysis, also significantly increased with the level of OM (*Two-Way ANOVA, Tukey HSD* Post-Hoc, $p < 0.001$; Fig. S1), reinforcing the notion that its presence enhances net methylation over time. Percent MeHg followed a negative relationship with OM, i.e., the highest fraction of MeHg was in the control, and the lowest in the high OM-solutions (Fig. S1).

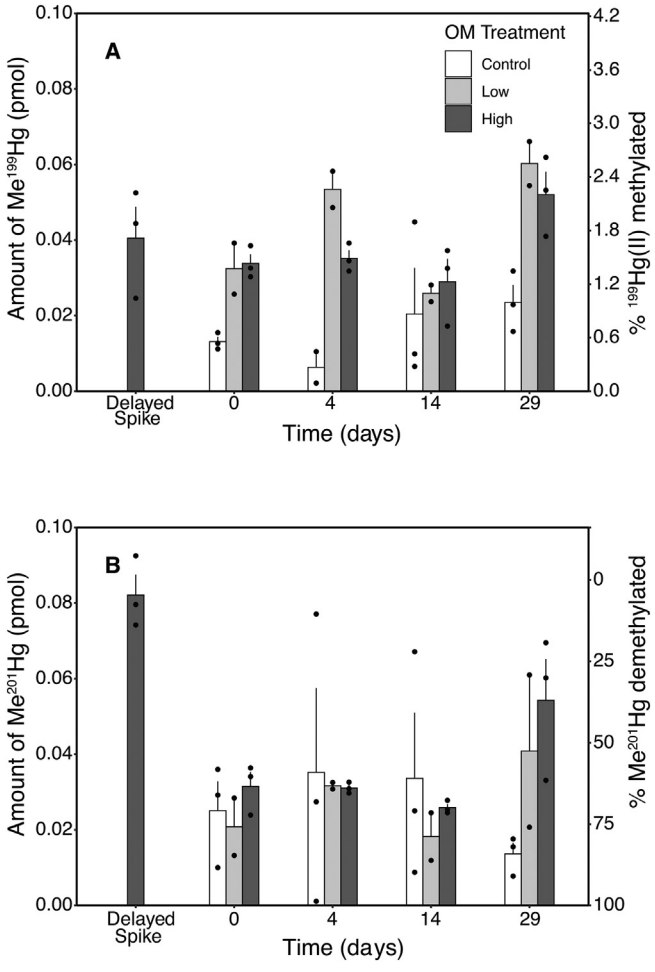



**Fig. 2.** (A) Methylation: Amount (left Y-axis) and percent (right Y-axis) of $^{199}$Hg(II) that was transformed into Me$^{199}$Hg during the up to a 29-day incubation of 10% buffered-seawater-formalin OM solutions. (B) Demethylation: Amount (left Y-axis) of Me$^{201}$Hg tracer that was detected and percent (right Y-axis) of Me$^{201}$Hg demethylated based on initial "delayed spike" concentration in the three 10% buffered-seawater-formalin OM solutions. Colors indicate OM treatments: no additional organic matter (white; "Control"), 10 mg L$^{-1}$ (gray; "Low"), and 100 mg L$^{-1}$ (black; "High"). Error bars represent ± SE on mean values. N = 3.

## 4. Discussion

In this study, the concentration of MeHg detected in formalin-preserved copepods initially declined and later increased in both species that were tested, however, the specific timing of the change was species-dependent. A steady-state concentration of MeHg in the formalin preserved copepods was not reached for either of the tested species. The changes over time indicate that both methylation and demethylation are occurring, and the factors controlling each transformation are different. Furthermore, in the incubation of the OM and formalin solution, we also detected that abiotic transformations of Hg(II) and MeHg via methylation and demethylation could occur in these solutions. However, more work is required to quantify and determine the exact mechanism behind these transformations. These results provide additional levels of confidence in our overall conclusion that long-term formalin preservation of copepods, and possibly other invertebrates that contain lower fractions of MeHg, are not suitable for measurements of MeHg.

The question of whether chemically preserved animal tissue samples can accurately document natural levels of MeHg in aquatic ecosystems has been previously discussed in the literature (Luengen et al., 2016). Our observation of the initial change in MeHg content in the preserved bivalve tissues was similarly observed by Luengen et al. (2016). These authors, however, argued that the MeHg content stabilized during the 90-day incubation period of their experiments, and therefore provide a valid proxy for the initial concentration. Based on our results, we argue, however, that the steady-state of MeHg in animal tissue, particularly in tissues with a lower %MeHg, may not be reachable during storage in chemical preservative solution as various chemical compounds continue reacting with Hg(II) and MeHg via complex pathways. Previous studies have already demonstrated the possibility of abiotic MeHg formation in the presence of high levels of inorganic Hg and OM (Fitzgerald et al., 2007; Hammerschmidt et al., 2007; Weber, 1993). Moreover, recent publications suggested a possible role of proteins in forming dimethylmercury, a supposed intermediate to monomethylmercury under experimental conditions (Jonsson et al., 2017; Kanzler et al., 2018). Buffered formalin also contains methyl alcohol as a stabilizer, which may act as a suitable methyl group donor for abiotic methylation of inorganic Hg. Therefore, in view of these findings, our results showing that these transformations can occur are not surprising and indicate a need for further investigating these processes.

The inconsistency in temporal changes of MeHg between the two copepod species remains unexplained, but likely reflects differences in the relative rates of methylation and demethylation occurring in the preservation solutions. Again, abiotic demethylation of MeHg is possible in the presence of biological organic matter (Asaduzzaman and Schreckenbach, 2011; Khan and Wang, 2010). The temporal variation in MeHg content in copepods incubated for different periods in a buffered formalin solution may have been due in part to these abiotic transformations. The additional inconsistencies between replicates could be due to the biological variability of the accumulated MeHg. The increase of MeHg occurred sooner in the incubation of the larger copepod, *T. longicornis*, than in the smaller, *A. tonsa*, suggesting the possibility that the larger pool of protein in *T. longicornis* available to react with Hg facilitated a more rapid increase of MeHg. To address the potential for rapid methylation, we showed that in animal tissue, which is rich with proteins, inorganic Hg could be methylated abiotically, however, via what mechanism remains a question for future studies (Fig. 2).

MeHg formation in copepod tissue requires a sufficient amount of available Hg. Results from our THg measurements representing the experimental vials indicate that newly formed MeHg in the copepod incubations could be derived from the inorganic Hg that was present within the copepods. As %MeHg decreased with MeHg content, THg within the vials remained constant over the incubation period. Consequently, when MeHg increased, so did %MeHg. Therefore, the

inorganic fraction of THg contained in copepod bodies could serve as a methylation substrate. Other Hg pools, such as seawater or formalin, contained negligible Hg in comparison to copepod associated Hg.

Aside from the new insights on the non-reliability of chemically preserved zooplankton for MeHg determination, the present study further demonstrates that Hg transformations can occur rapidly and do not require microbial activity, which is consistent with the results of early studies (Weber, 1993). This further motivates consideration for an abiotic route of net MeHg formation in diverse environments, including the ocean, although the limitation may be the levels of organic material which serves as the methyl donor. The possibility of abiotic formation of MeHg has been recently supported by experimental series of seawater incubations with stable isotope enriched Hg tracers (Munson et al., 2018) and builds on previous studies showing abiotic pathways of MeHg formation (Gårdfeldt et al., 2003; Hammerschmidt et al., 2007). Furthermore, the magnitude of Hg methylation in the present study is comparable with other environmental media, such as sediments and seawater (Hammerschmidt and Fitzgerald, 2004; Lehnherr et al., 2011; Monperrus et al., 2007).

## 5. Conclusions

As the climate and Hg inputs continue to change, substantial alterations across ecosystems in response to various natural and human-induced perturbations are expected. These, in turn, will affect Hg and MeHg concentrations and their fluctuations in seawater and biota. Given these changing environmental conditions and our limited knowledge of mechanisms ruling MeHg bioaccumulation at low trophic levels in marine food webs, there is a need for more research efforts in this area (Grieb et al., 2019; Wang et al., 2019). Based on the results in this study, we urge caution in the use of formalin-preserved organisms to study historic MeHg changes in aquatic biota. Instead, approaches such as freezing should be used in order to determine temporal changes in MeHg bioaccumulation in aquatic biota.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139803.

## CRediT authorship contribution statement

**Wesley W. Huffman:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Hans G. Dam:** Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Robert P. Mason:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Zofia Baumann:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The authors thank the Connecticut Department of Energy and Environmental Protection and the crew of the R.V. John Dempsey for helping to collect zooplankton in January 2018, S. Jonsson and N. Mazrui for their assistance on the ICP-MS instrument, and L. Norton for her help with identifying copepods. This research was funded by Connecticut Sea Grant Award # R/P-2 (2016-2018) and R/P-5 (2018-2020).

## References

Asaduzzaman, A., Schreckenbach, G., 2011. Degradation mechanism of methylmercury selenoamino acid complexes: a computational study. Inorg. Chem. 50, 2366–2372. https://doi.org/10.1021/ic1021406.

Cervetto, G., Gaudy, R., Pagano, M., 1999. Influence of salinity on the distribution of *Acratia tonsa* (Copepoda, Calanoida). J. Exp. Mar. Biol. Ecol. 239, 33–45. https://doi.org/10.1016/s0022-0981(99)00023-4.

Clayden, M.G., Arsenault, L.M., Kidd, K.A., O'Driscoll, N.J., Mallory, M.L., 2015. Mercury bioaccumulation and biomagnification in a small Arctic polynya ecosystem. Sci. Total Environ. 509, 206–215. https://doi.org/10.1016/j.scitotenv.2014.07.087.

Dam, H.G., Peterson, W.T., 1991. In situ feeding behavior of the copepod *Temora longicornis*: effects of seasonal changes in chlorophyll size fractions and female size. Mar. Ecol. Prog. Ser. 71, 113–123. https://doi.org/10.3354/meps071113.

Dam, H.G., Peterson, W.T., Bellantoni, D.C., 1994. Seasonal feeding and fecundity of the calanoid copepod *Acartia tonsa* in Long Island Sound: is omnivory important to egg production? Hydrobiologia 292–293, 191–199. https://doi.org/10.1007/bf00229941.

Drevnick, P.E., Lamborg, C.H., Horgan, M.J., 2015. Increase in mercury in Pacific yellowfin tuna. Environ. Toxicol. Chem. 34, 931–934. https://doi.org/10.1002/etc.2883.

Driscoll, C.T., Chen, C.Y., Hammerschmidt, C.R., Mason, R.P., Gilmour, C.C., Sunderland, E.M., Greenfield, B.K., Buckman, K.L., Lamborg, C.H., 2012. Nutrient supply and mercury dynamics in marine ecosystems: a conceptual model. Environ. Res. 119, 118–131. https://doi.org/10.1016/j.envres.2012.05.002.

Evers, D.C., Mason, R.P., Kamman, N.C., Chen, C.Y., Bogomolni, A.L., Taylor, D.L., Hammerschmidt, C.R., Jones, S.H., Burgess, N.M., Munney, K., Parsons, K.C., 2008. Integrated mercury monitoring program for temperate estuarine and marine ecosystems on the North American Atlantic Coast. Ecohealth 5, 426–441. https://doi.org/10.1007/s10393-008-0205-x.

Fitzgerald, W.F., Lamborg, C.H., Hammerschmidt, C.R., 2007. Marine biogeochemical cycling of mercury. Chem. Rev. 107, 641–662. https://doi.org/10.1021/cr050353m.

Foster, K.L., Stern, G.A., Pazerniuk, M.A., Hickie, B., Walkusz, W., Wang, F., Macdonald, R.W., 2012. Mercury biomagnification in marine zooplankton food webs in Hudson Bay. Environ. Sci. Technol. 46, 12952–12959. https://doi.org/10.1021/es303434p.

Gårdfeldt, K., Munthe, J., Strömberg, D., Lindqvist, O., 2003. A kinetic study on the abiotic methylation of divalent mercury in the aqueous phase. Sci. Total Environ. 304, 127–136. https://doi.org/10.1016/s0048-9697(02)00562-4.

Gosnell, K.J., Balcom, P.H., Tobias, C.R., Gilhooly, W.P., Mason, R.P., 2017. Spatial and temporal trophic transfer dynamics of mercury and methylmercury into zooplankton and phytoplankton of Long Island Sound. Limnol. Oceanogr. 62, 1122–1138. https://doi.org/10.1002/lno.10490.

Grieb, T.M., Fisher, N.S., Karimi, R., Levin, L., 2019. An assessment of temporal trends in mercury concentrations in fish. Ecotoxicology, 1–11 https://doi.org/10.1007/s10646-019-02112-3.

Hammerschmidt, C.R., Fitzgerald, W.F., 2004. Geochemical controls on the production and distribution of methylmercury in near-shore marine sediments. Environ. Sci. Technol. 38 (5), 1487–1495. https://doi.org/10.1021/es034528q.

Hammerschmidt, C.R., Fitzgerald, W.F., 2005. Methylmercury in mosquitoes related to atmospheric mercury deposition and contamination. Environ. Sci. Technol. 39, 3034–3039. https://doi.org/10.1021/es0485107.

Hammerschmidt, C.R., Fitzgerald, W.F., 2006. Bioaccumulation and trophic transfer of methylmercury in Long Island Sound. Arch. Environ. Contam. Toxicol. 51, 416–424. https://doi.org/10.1007/s00244-005-0265-7.

Hammerschmidt, C.R., Lamborg, C.H., Fitzgerald, W.F., 2007. Aqueous phase methylation as a potential source of methylmercury in wet deposition. Atmos. Environ. 41, 1663–1668. https://doi.org/10.1016/j.atmosenv.2006.10.032.

Hammerschmidt, C.R., Finiguerra, M.B., Weller, R.L., Fitzgerald, W.F., 2013. Methylmercury accumulation in plankton on the continental margin of the Northwest Atlantic Ocean. Environ. Sci. Technol. 47, 3671–3677. https://doi.org/10.1021/es3048619.

Harding, G., Dalziel, J., Vass, P., 2018. Bioaccumulation of methylmercury within the marine food web of the outer Bay of Fundy, Gulf of Maine. PLoS One 13, e0197220. https://doi.org/10.1371/journal.pone.0197220.

Harris, R.C., Rudd, J.W.M., Amyot, M., Babiarz, C.L., Beaty, K.G., Blanchfield, P.J., Bodaly, R.A., Branfireun, B.A., Gilmour, C.C., Graydon, J.A., Heyes, A., Hintelmann, H., Hurley, J.P., Kelly, C.A., Krabbenhoft, D.P., Lindberg, S.E., Mason, R.P., Paterson, M.J., Podemski, C.L., Robinson, A., Sandilands, K.A., Southworth, G.R., Louis, V.L.S., Tate, M.T., 2007. Whole-ecosystem study shows rapid fish-mercury response to changes in mercury deposition. Proc. Natl. Acad. Sci. 104, 16586–16591. https://doi.org/10.1073/pnas.0704186104.

Hill, J.J., Chumchal, M.M., Drenner, R.W., Pinder, J.E., Drenner, M.S., 2010. Use of preserved museum fish to evaluate historical and current mercury contamination in fish from two rivers in Oklahoma, USA. Environ. Monit. Assess. 161, 509–516. https://doi.org/10.1007/s10661-009-0764-5.

Jonsson, S., Andersson, A., Nilsson, M.B., Skyllberg, U., Lundberg, E., Schaefer, J.K., Åkerblom, S., Björn, E., 2017. Terrestrial discharges mediate trophic shifts and enhance methylmercury accumulation in estuarine biota. Sci. Adv. 3, e1601239. https://doi.org/10.1126/sciadv.1601239.

Kane, J., Prezioso, J., 2008. Distribution and multi-annual abundance trends of the copepod *Temora longicornis* in the US Northeast Shelf Ecosystem. J. Plankton Res. 30, 619–632. https://doi.org/10.1093/plankt/fbn026.

Kanzler, C.R., Lian, P., Trainer, E., Yang, X., Govind, N., Parks, J.M., Graham, A.M., 2018. Emerging investigator series: methylmercury speciation and dimethylmercury production in sulfidic solutions. Environ. Sci. Process Impacts 20, 584–594. https://doi.org/10.1039/c7em00533d.

Kelly, T., Jones, J., Smith, G., 1975. Historical changes in mercury contamination in Michigan Walleyes (*Stizostedion vitreum vitreum*). J. Fish. Board Can. 32, 1745–1754. https://doi.org/10.1139/f75-208.

Khan, M.A., Wang, F., 2010. Chemical demethylation of methylmercury by selenoamino acids. Chem. Res. Toxicol. 23, 1202–1206. https://doi.org/10.1021/tx100080s.
Krabbenhoft, D.P., Sunderland, E.M., 2013. Global change and mercury. Science 341, 1457–1458. https://doi.org/10.1126/science.1242838.
Lee, C.-S., Fisher, N.S., 2016. Methylmercury uptake by diverse marine phytoplankton. Limnol. Oceanogr. 61, 1626–1639. https://doi.org/10.1002/lno.10318.
Lee, C.-S., Fisher, N.S., 2017. Bioaccumulation of methylmercury in a marine copepod. Environ. Toxicol. Chem. 36, 1287–1293. https://doi.org/10.1002/etc.3660.
Lee, C.-S., Lutcavage, M.E., Chandler, E., Madigan, D.J., Cerrato, R.M., Fisher, N.S., 2016. Declining mercury concentrations in bluefin tuna reflect reduced emissions to the North Atlantic Ocean. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.6b04328.
Lehnherr, I., Louis, V.L.St, Hintelmann, H., Kirk, J.L., 2011. Methylation of inorganic mercury in polar marine waters. Nat. Geosci. 4 (5), 298–302. https://doi.org/10.1038/ngeo1134.
Levengood, J.M., Soucek, D.J., Taylor, C.A., Gay, D.A., 2013. Mercury in small Illinois fishes: historical perspectives and current issues. Environ. Monit. Assess. 185, 6485–6494. https://doi.org/10.1007/s10661-012-3040-z.
Luengen, A.C., Foslund, H.M., Greenfield, B.K., 2016. Decline in methylmercury in museum-preserved bivalves from San Francisco Bay, California. Sci. Total Environ. 572, 782–793. https://doi.org/10.1016/j.scitotenv.2016.07.070.
Mason, R.P., Abbott, M.L., Bodaly, R.A., Bullock Jr., R.O.J., Driscoll, C.T., Evers, D., Lindberg, S.E., Murray, M., Swain, E.B., 2005. Monitoring the response to changing mercury deposition. Environ. Sci. Technol. 39, 14A–22A. https://doi.org/10.1021/es053155l.
Mauchline, J., 1998. The biology of calanoid copepods. Adv. Mar. Biol. 33, 1–710.
Monperrus, M., Tessier, E., Amouroux, D., Leynaert, A., Huonnic, P., Donard, O.F.X., 2007. Mercury methylation, demethylation and reduction rates in coastal and marine surface waters of the Mediterranean Sea. Mar. Chem. 107 (1), 49–63. https://doi.org/10.1016/j.marchem.2007.01.018.
Morel, F.M., Kraepiel, A.M., Amyot, M., 1998. The chemical cycle and bioaccumulation of mercury. Ecol. Syst. 29, 543–566. https://doi.org/10.1146/annurev.ecolsys.29.1.543.
Munson, K.M., Lamborg, C.H., Boiteau, R.M., Saito, M.A., 2018. Dynamic mercury methylation and demethylation in oligotrophic marine water. Biogeosciences 15, 6451–6460. https://doi.org/10.5194/bg-15-6451-2018.
Rosman, K.J.R., Taylor, P.D.P., 1998. Isotopic compositions of the elements 1997. J. Phys. Chem. Ref. Data 27, 1275–1287. https://doi.org/10.1063/1.556031.
Saito, M., 2019. Minamata convention on mercury - our challenges and its future. J. Environ. Saf. 10, 95–97. https://doi.org/10.11162/daikankyo.e19cm0802.
Schartup, A.T., Thackray, C.P., Qureshi, A., Dassuncao, C., Gillespie, K., Hanke, A., Sunderland, E.M., 2019. Climate change and overfishing increase neurotoxicant in marine predators. Nature 572, 648–650. https://doi.org/10.1038/s41586-019-1468-9.
Selin, N.E., 2014. Global change and mercury cycling: challenges for implementing a global mercury treaty. Environ. Toxicol. Chem. 33, 1202–1210. https://doi.org/10.1002/etc.2374.
Thavarajah, R., Mudimbaimannar, V.K., Elizabeth, J., Rao, U.K., Ranganathan, K., 2012. Chemical and physical basics of routine formaldehyde fixation. J. Oral Maxillofac. Pathol. 16, 400–405. https://doi.org/10.4103/0973-029x.102496.
Wang, F., Outridge, P.M., Feng, X., Meng, B., Heimbürger-Boavida, L.-E., Mason, R.P., 2019. How closely do mercury trends in fish and other aquatic wildlife track those in the atmosphere? – implications for evaluating the effectiveness of the Minamata Convention. Sci. Total Environ. 674, 58–70. https://doi.org/10.1016/j.scitotenv.2019.04.101.
Weber, J.H., 1993. Review of possible paths for abiotic methylation of mercury(II) in the aquatic environment. Chemosphere 26, 2063–2077. https://doi.org/10.1016/0045-6535(93)90032-z.
Zhang, Y., Jacob, D.J., Horowitz, H.M., Chen, L., Amos, H.M., Krabbenhoft, D.P., Slemr, F., Louis, V.L., Sunderland, E.M., 2016. Observed decrease in atmospheric mercury explained by global decline in anthropogenic emissions. Proc. Natl. Acad. Sci. 113, 526–531. https://doi.org/10.1073/pnas.1516312113.