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Effects of formalin preservation on invertebrate stable isotope values over decadal time scales

Michael D. Rennie, Ted Ozersky, and David O. Evans

Abstract: Stable isotope values derived from chemically preserved organisms are a valuable resource for documenting long-term ecosystem changes. However, isotopic correction factors of preservation effects applied to samples stored for decades are frequently based on studies lasting only months, assuming that the effects of preservation stabilize within a short time frame. Very few studies test this critical assumption. We validated this assumption for formalin-preserved invertebrate tissues, finding no significant difference between mean isotopic $\delta^{13}C$ and $\delta^{15}N$ values of material stored 1–15 years across taxa. Preservation effects were evaluated for Amphipoda, Chironomidae, Dreissenidae, Ephemeroptera, Gastropoda, Isopoda, Sphaeridae, Oligochaeta, and Trichoptera. On average, freshwater benthos $\delta^{13}C$ was lower by approximately 2‰ after formalin fixation, whereas $\delta^{15}N$ values were not different from control samples. Fixation effects were similar among taxa, but were more pronounced in Gastropoda and Sphaeridae for $\delta^{13}C$ and in Trichoptera for $\delta^{15}N$. We reviewed the literature to show that preserved freshwater zooplankton $\delta^{13}C$ were slightly but significantly lower relative to control samples (–0.2‰) and higher in $\delta^{15}N$ (+0.25‰). The mean decline among marine invertebrate $\delta^{13}C$ was greater than for freshwater invertebrates after 1+ years of formalin preservation, but effects on $\delta^{15}N$ were not different between marine and freshwater invertebrates.

Key words: formaldehyde, frozen, aquatic, Lake Simcoe, preservative.

Résumé : Les valeurs d'isotopes stables dérivées d'organismes conservés chimiquement constituent une ressource utile pour la documentation de changements écosystémiques à long terme. Toutefois, les facteurs de correction isotopique des effets de la conservation appliqués à des échantillons ayant été entreposés pendant des décennies reposent souvent sur des études étalées sur quelques mois seulement, en partant du principe que les effets de la conservation se stabilisent rapidement. Très peu d'études ont cependant testé cette hypothèse clé. Nous l'avons validé en ce qui concerne les tissus d'invertébrés conservés dans le formole, n'ayant décelé aucune différence significative entre les valeurs moyennes de $\delta^{13}C$ et $\delta^{15}N$ de matériel de divers taxons entreposé sur des périodes de 1 à 15 ans. Les effets de la conservation ont été évalués pour des amphipodes, des chironomidés, des dreissenidés, des éphéméroptères, des gastéropodes, des isopodes, des sphaeriidés, des oligochètes et des trichoptères. En moyenne, le $\delta^{13}C$ du benthos d'eau douce avait diminué d'environ 2 ‰ après la fixation au formol, alors que les valeurs de $\delta^{15}N$ n'étaient pas différentes de celles des échantillons témoins. Les effets de la fixation étaient semblables pour tous les taxons, bien qu'ils aient été plus prononcés chez les gastéropodes et les sphaeriidés en ce qui concerne le $\delta^{13}C$, et chez les trichoptères en ce qui concerne le $\delta^{15}N$. Nous avons passé en revue la littérature pour démontrer que les valeurs de $\delta^{13}C$ de zooplancton d'eau douce conservé étaient légèrement mais significativement plus faibles que celles d'échantillons témoins (–0,2 ‰), alors que celles de $\delta^{15}N$ étaient plus élevées (+0,25 ‰). La diminution moyenne du $\delta^{13}C$ était plus grande pour les invertébrés marins que pour les invertébrés dulcicoles après un an ou plus de conservation dans le formol, mais les effets sur le $\delta^{15}N$ étaient semblables pour les deux types d'invertébrés.

Mots-clés : formaldéhyde, gelé, aquatique, lac Simcoe, produit de conservation.

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Introduction

Stable isotope analysis is a powerful tool for ecologists, providing insights into resource use and trophic relationships among ecosystem members where the actual linkages are difficult to establish or information is lacking or insufficient (Hecky and Hesslein 1995; Vander Zanden et al. 1999; Walters et al. 2009). Given the immense amount of time and effort required to document diets of organisms over relevant time scales to account for inherent variability in foraging, stable isotope analysis—when interpreted correctly—can serve as a practical and cost-effective means of providing temporally integrated information about the structure and energy flow within food webs. This information becomes even more powerful when combined with ecological data such as diet composition and species distributions (e.g., Vander Zanden et al. 1997; Vander Zanden and Rasmussen 1999; Rennie et al. 2009).

Recent investigations have begun to exploit stable isotope measurements of archived biological materials to reconstruct past food-web structure during periods of significant ecosystem change (Vander Zanden et al. 2003; Rennie et al. 2009; Schmidt et al. 2009). These hindcasting studies often rely on archives of state, provincial, or federal long-term monitoring programs (Rau et al. 2003; Vander Zanden et al. 2003; Maguire and Grey 2006), where formalin is frequently used to fix invertebrate samples. A key challenge for these studies is the need to account for the influence of chemical preservation methods on isotopic composition at time scales relevant to the archival tissues being investigated.

Isotope ratios in tissue samples that are chemically preserved may be subject to long-term changes that are not captured by short-term evaluation studies. Currently published evaluations of fixative effects on the isotopic values of invertebrate organisms in formalin typically do not evaluate changes of preservation greater than 1 year, but hindcasting studies have applied these corrections to material that has been stored for decades (Vander Zanden et al. 2003). Prior to the current study, evaluation studies on the effects of formalin preservation for freshwater benthic invertebrate taxa have only evaluated effects to either 6 months (Sarakinos et al. 2002) or 1 year (Syväranta et al. 2011), and for only two taxa (a genera of Trichoptera and the Asiatic clam *Corbiculus fulminea* (O.F. Müller, 1774)). Although $\delta^{15}N$ values appear to stabilize within the time frame of these studies, $\delta^{13}C$ values reported from either study do not show evidence of stabilization in preservation effects at the end of the study period. In a recent evaluation of marine benthic organisms, a 1-year preservation study reported similar trends; stability in $\delta^{15}N$, but no evidence of stability in $\delta^{13}C$ at the end of the study period (Fanelli et al. 2010). Slight but gradual changes in $\delta^{13}C$ observed over 6 months or 1 year extrapolated to a time frame of decades or centuries could lead to significant biases in the interpretation of what might otherwise appear to be ecologically relevant change. It is clear that a definitive test of the long-term effects of formalin fixation in invertebrate tissues is still lacking, and that there is a need for data on preservation effects on a wider variety of freshwater invertebrate taxa than currently exists in the literature.

The objectives this study were (*i*) to determine whether chemical preservation effects (10% buffered formalin) from short-term (e.g., 1 year) studies accurately reflect long-term (e.g., 15 years) preservation effects in a variety of benthic invertebrate tissues, and (*ii*) to examine these results within the context of the published studies that have evaluated the effects of formalin fixation on invertebrate tissues over shorter time periods.

Materials and methods

Empirical evaluation of formalin preservation on isotopic values

To evaluate the long-term effects of 10% formalin preservation on the isotopic composition of freshwater benthic organisms, we examined isotopic values ($\delta^{13}C$ and $\delta^{15}N$) of benthic invertebrates collected from Lake Simcoe, Ontario, Canada, in 1993 and 2009. Samples taken in 1993 were collected in the fall from sites in the northwest part of Lake Simcoe (44°34'N, 79°20'W) between 2 September and 10 October 1993. Benthic organisms were collected using 500 μm mesh kick-and-sweep nets in >0.5 m of water or using airlift samplers at depths of 2 m. Details on airlift sampling can be found in Barton and Hynes (1978) and Evans et al. (2011). A subset of samples taken were separated within hours of collection into broad taxonomic groups and frozen in lake water at –20 °C. Remaining samples were preserved in 10% buffered formalin. In October 2008, frozen samples collected in 1993 were thawed, sorted, and prepared for stable isotope analysis. These were classified as our "control" or unfixed reference data. In January 2009, preserved samples were sorted, rinsed with deionized water (to remove excess fixative), and prepared for isotopic analysis.

To compare long-term (15-year) fixative effects to a shorter term (1-year) evaluation period more commonly employed in preservation effect studies, we examined samples taken 13–27 August 2008 that were part of a larger survey of the Lake Simcoe benthos (Jimenez et al. 2011). From all sites surveyed, samples were sieved (500 μm mesh) and preserved in 10% buffered formalin. From a subset of these sites, additional samples were taken from 10, 20, 25, and 30 m depths, sieved, and all organisms and remaining debris were frozen in lake water at –20 °C. During the fall of 2009, frozen samples were thawed, sorted, and prepared for stable isotope analysis. Preserved samples that had previously been sorted, enumerated, and returned to their original fixative solution in 2008 were rinsed with deionized water and also prepared for isotopic analysis at this time.

To provide additional control (unpreserved) material for comparison with preserved samples over the 1-year preservation period, we repeated benthic invertebrate sampling along a single transect from the 2008 study during 16–24 August 2009 (Rennie and Evans 2012). These samples were sieved (500 μm mesh), kept in lake water on ice for less than 24 h, sorted, and immediately prepared for stable isotope analysis. To verify that these fresh samples were similar to frozen material collected in 2008 and could therefore also be utilized as control samples, we matched mean estimates of organism isotopic values by taxa and depth in both years (6 means in total, 31 observations) and estimated differences between frozen and fresh means for both carbon and nitrogen isotopes. We conducted single-sample Student's *t* tests and found that the mean differences were not significantly different from zero ($\delta^{13}C$: $t_{[5]} = -1.1$, $P = 0.3$, mean difference ± 1 SE = –0.6‰ ± 0.6‰; $\delta^{15}N$: $t_{[5]} = 0.88$, $P = 0.4$, mean differ-

ence = –0.9‰ ± 1‰). Neither carbon nor nitrogen showed any systematic pattern in differences between fresh versus frozen material with depth of sample collection (by visual inspection and linear regression, $P >> 0.05$, both carbon and nitrogen). Based on these findings, we included 2009 freshly prepared isotopic values as reference controls for 2008 preserved material that had no frozen counterparts: Oligochaeta at 10, 15, 20, and 30 m, Isopoda and Chironomidae at 15 m, and Ephemeroptera at 10 m.

Isotope analysis

Invertebrate samples used for isotopic analyses consisted of single or multiple individuals from each combination of taxa and depth, the number depending on the relative sizes of each taxa to achieve a single dry mass sample of between 0.25 and 1 mg (e.g., Amphipoda, 4–8; Chironomidae, 3–10; Dreissenidae, 1–3; Ephemeroptera, 1–2). The experimental unit for the evaluation of preservation effects was the stable isotopic values of carbon and nitrogen for each of these independently prepared samples. Where possible, replicate independent samples were prepared for each taxa collected at each depth. These samples were dried to constant mass at 60 °C (typically achieved within 18–24 h). Dried samples were ground to a powder in their drying vessels using a glass rod. Between 0.2 and 0.5 mg of material was weighed into 3.5 mm × 5 mm tin cups and analyzed for $\delta^{13}C$ and $\delta^{15}N$ at the University of Waterloo Environmental Isotope Laboratory, Waterloo, Ontario, Canada. Analyses were performed on a Delta continuous flow stable isotope mass spectrometer (Micromass) coupled to a Carlo Erba elemental analyzer (CHNS-O EA1108). Analytical error is reported to be ±0.2‰ and ±0.3‰ for carbon and nitrogen, respectively. To standardize isotope ratios and monitor data quality, international standards were employed for use with analyses of carbon (IAEA-CH6) and nitrogen (IAEA-N1, IAEA-N2), respectively. Additionally, we prepared an internal laboratory standard based on a zebra mussel tissue homogenate from mussels collected at a single site during July 2008. This standard was run during every series of isotope test samples over the course of 3 years. Analytical error (mean ± 1 SD) based on this standard were –26.5 ± 0.4 ($n = 41$) and 7.4 ± 0.4 ($n = 40$) for $\delta^{13}C$ and $\delta^{15}N$, respectively, where n is the sample size. Forty-three benthic taxa at depth were analyzed in duplicate or triplicate. The mean standard errors for these replicate determinations of $\delta^{13}C$ and $\delta^{15}N$ were 0.3‰ and 0.3‰, respectively.

Statistical analyses

To evaluate formalin (10%) preservation effects on benthic invertebrate fauna, we compared mean isotopic values of each taxa collected at each of the sampling depths that could be matched between our preserved and control (frozen) data sets. Data published elsewhere (Vander Zanden and Rasmussen 1999; Sierszen et al. 2006; Rennie et al. 2009), as well as data for Lake Simcoe specifically (M.D. Rennie and D.O. Evans, unpublished data), show that depth significantly influences benthic invertebrate isotopic values. Therefore, we evaluated preservation effects for each isotope using all unique combinations of taxa and depth on matched observations (preserved and control) using independent Student's t tests, with a Welch correction to df values (Table 1). During 1993, samples were collected nearshore at 0.5–2 m depths and comparisons were matched by taxa only (Table 2). Where sufficient data were available (Chironomidae, Oligochaeta from the 1-year preservation effect study), we evaluated the effects of preservation with the depth at which organisms were collected using linear regression.

Preservation effects on benthic taxa were further summarized by broader taxonomic definitions to provide correction factors that could be more generally applied, and to allow for a comparison of preservation effects between the 1 and 15 year data sets (Supplementary Table S1).[1] We also estimated 25th and 75th percentiles around mean preservation effects of broad taxa. We chose to represent error around means as percentiles because sample sizes were small, and therefore difficult to quantitatively assess for normality. Means and percentiles in the 1-year data set were estimated from replicate observations of taxa at different depths and from the 15-year data set across multiple subtaxa. We used a resampling technique to derive percentile estimates where replicate observations by depth or taxa were unavailable. Distributions of preserved and control treatments for each taxa were sampled with replacement, means estimated, and the difference between means (pseudoestimates of preservation effects) was calculated and saved. This process was repeated 1000 times and the 25th and 75th percentiles of those pseudoestimates were calculated. Effects of preservation after 1 and 15 years among taxa were compared with Student's t tests for both C and N isotopes. All statistical analyses were carried out using the statistical program R (R Development Core Team 2008).

Literature review

To provide context for the direction and magnitude of preservation effects observed in our study, we compared our results with those of other published evaluations describing the effects of formalin preservation on invertebrate tissues. Studies were found using a combination of relevant search terms in Web of Science (March 2012). Data on formalin-preserved benthic invertebrates and zooplankton were plotted separately, and preserved organisms from saltwater and freshwater systems were also identified in evaluations. We compared changes in preservation effects with time, as well as differences between preservation effects on organisms collected from freshwater versus marine environments.

Results

Effects of formalin preservation on freshwater invertebrate isotopic values

Following 1 year of preservation, 78% of all taxa at specified depths demonstrated significant differences between preserved and control values for $\delta^{13}C$ at a cutoff of $P < 0.1$ (61% at $P < 0.05$; Table 1), and 91% were lower relative to control samples. By contrast, only 22% of differences between preserved and control values for $\delta^{15}N$ were significant at $P < 0.1$ (13% at $P < 0.05$) and showed no clear or consistent increasing or decreasing trend following preservation. Within taxa, preservation for 1 year was variable but showed no systematic increase or decrease with site depth (data sufficient to evaluate for Chironomidae and Oligochaeta only, by visual inspection and linear regression, both $P >> 0.05$).

[1]Supplementary Table S1 is available with the article through the journal Web site (http://nrcresearchpress.com/doi/suppl/10.1139/z2012-101).

Table 1. Effects of 1 year of preservation on benthic invertebrate isotopic signatures.

Organism	Depth (m)	n_P	n_F	$\delta^{13}C_P$	$\delta^{13}C_F$	$\Delta^{13}C$	P_C	$\delta^{15}N_P$	$\delta^{15}N_F$	$\Delta^{15}N$	P_N
Amphipoda	10	3	5	–25.3	–23.8	**–1.5**	0.009	8.1	7.3	0.8	0.275
Chironomidae	10	4	7	–26.4	–21.2	**–5.2**	<0.001	8.8	7.5	1.3	0.181
	15	5	2	–26.3	–27.6	1.3	0.176	10.2	11.4	–1.2	0.123
	20	3	5	–30.4	–28.6	**–1.8**	0.020	11.6	11.2	0.4	0.305
	25	3	5	–30.9	–29.8	**–1.1**	0.010	11.8	10.6	1.2	0.123
	30	3	5	–32.7	–29.3	**–3.4**	0.002	11.3	11.3	0.0	0.964
Ephemeroptera	10	3	4	–25.3	–23.8	**–1.5**	0.048	9.0	8.9	0.1	0.890
Gastropoda	10	4	3	–22.9	–22.0	–0.9	0.286	9.6	9.0	**0.6**	0.080
Gastropoda (Planorbidae)	15	3	2	–27.6	–24.1	***–3.5***	0.061	10.2	11.0	–0.8	0.159
Gastropoda	20	3	4	–30.6	–27.0	**–3.6**	0.024	10.4	10.2	0.2	0.667
Gastropoda (Planorbidae)	25	2	1	–30.5	–27.6	–2.9	0.101	10.7	9.4	1.3	0.199
Isopoda	10	2	5	–25.4	–23.2	**–2.2**	0.011	8.4	8.5	–0.1	0.848
	15	1	2	–26.3	–26.5	0.2	0.725	8.7	10.2	***–1.5***	0.074
Oligochaeta	10	1	4	–27.7	–24.0	**–3.7**	0.008	10.0	9.9	0.1	0.566
	15	1	2	–29.8	–27.8	***–2.0***	0.097	11.3	10.9	0.4	0.236
	20	4	2	–31.2	–30.2	***–1.0***	0.094	10.1	9.4	**0.7**	0.035
	30	3	2	–31.4	–29.1	**–2.3**	0.004	10.3	11.7	**–1.4**	0.024
Sphaeridae	20	1	2	–31.1	–27.6	***-3.5***	0.076	9.3	9.6	–0.3	0.476
	25	3	3	–31.6	–27.2	**–4.4**	<0.001	10.4	10.0	***0.4***	0.074
Quagga mussel	10	2	3	–28.7	–27.2	**–1.5**	0.035	7.2	7.0	0.2	0.561
Zebra mussel	10	3	5	–29.1	–27.5	**–1.6**	0.002	7.0	7.4	–0.4	0.444
	15	3	1	–30.9	—	—	—	7.3	7.9	–0.6	0.497
	25	1	3	–32.5	–30.0	**–2.5**	0.005	7.4	7.8	–0.4	0.606

Note: The quagga mussel is *Dreissena bugensis rostriformis* (Therriault et al., 2004) and the zebra mussel is *Dreissena polymorpha* Pallas, 1771. Depth indicates site depth from which organisms were collected. *n* is sample size; Δ notation indicates changes in preserved (P) samples relative to frozen (F) control samples. *P* indicates probability of significance of difference between preserved versus control samples from Student's *t* tests; significant isotopic differences at the $P = 0.05$ level are in boldface type and at the $P = 0.1$ level are in boldface–italic type.

Table 2. Effects of 15 years of preservation on benthic invertebrate isotopic signatures.

Organism	Taxa	n_P	n_F	$\delta^{13}C_P$	$\delta^{13}C_F$	$\Delta^{13}C$	P_C	$\delta^{15}N_P$	$\delta^{15}N_F$	$\Delta^{15}N$	P_N
Amphipoda	*Gammarus*	4	5	–25.6	–23.8	***–1.8***	0.092	5.4	3.6	**1.8**	0.028
Ephemeroptera	Ephemerellidae	1	5	–24.5	–25.2	0.7	0.523	3.9	6.9	**–3.0**	0.003
	Heptageniidae	6	4	–26.1	–25.2	***–0.9***	0.052	6.6	5.3	***1.3***	0.097
Gastropoda	*Goniobasis*	6	3	–25.2	–21.6	**–3.6**	0.002	4.8	5.5	–0.7	0.409
	Physidae	5	5	–24.1	–21.2	***–2.9***	0.096	5.1	5.3	–0.2	0.722
Trichoptera	Hydropsychidae	2	4	–27.5	–28.2	0.7	0.689	5.3	8.6	**–3.3**	0.043
	Leptoceridae	2	2	–26.7	–23.6	–3.1	0.290	3.6	7.6	**–4.0**	0.034

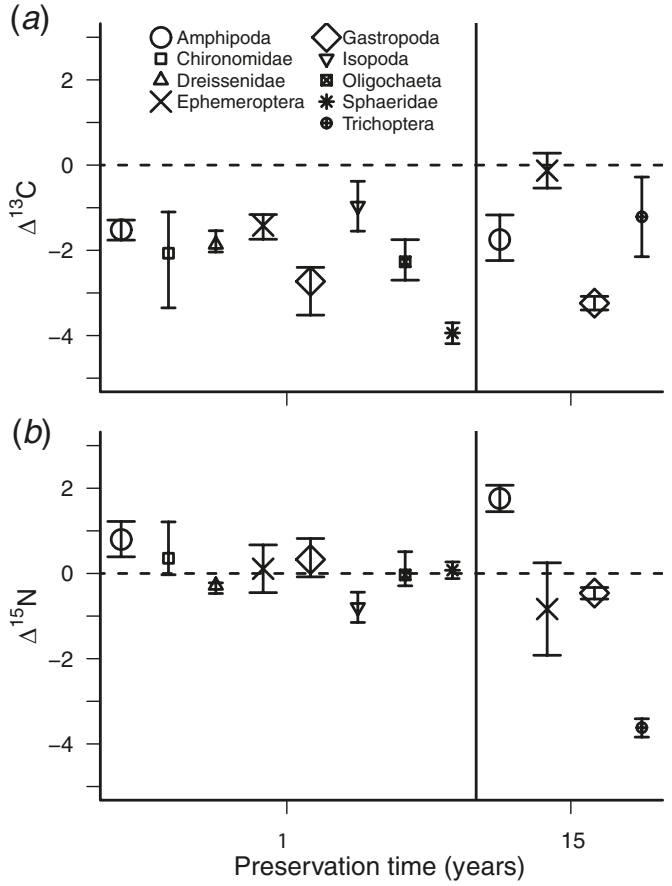
Note: *n* is sample size; Δ notation indicates changes in preserved (P) samples relative to frozen (F) control samples. *P* indicates probability of significance of difference between preserved versus control samples from Student's *t* tests; significant isotopic differences at the $P = 0.05$ level are in boldface type and at the $P = 0.1$ level are in boldface–italic type.

After 15 years of preservation, $\delta^{13}C$ values for four out of seven taxa declined significantly at the $P < 0.1$ level. Although five out of seven taxa were significantly different for $\delta^{15}N$ (Table 2), there was again no clear or consistent increase or decrease among taxa for this isotope.

When observations at depth and more detailed taxonomic levels were summarized, benthic invertebrate $\delta^{13}C$ isotope values after 1 year of preservation were significantly lower relative to control samples by –2.1‰, on average ($t_{[7]} = -6.4$, $P < 0.0004$; Fig. 1*a*), whereas $\delta^{15}N$ showed no significant change following preservation: 0.1 ‰ on average ($t_{[7]} = 0.42$, $P = 0.7$; Fig. 1*b*). Expecting a shift in the same direction as for 1 year of preservation, we evaluated preservation effects following 15 years of preservation with a one-tailed Student's *t* test. Similar to 1-year preservation results, benthic invertebrate $\delta^{13}C$ isotope values were also lower relative to control samples by –1.6‰, on average ($t_{[3]} = -2.44$, $P = 0.046$; Fig. 1*a*). Preservation effects on $\delta^{15}N$ were nonsignificant ($t_{[3]} = -0.71$, $P = 0.5$; Fig. 1*b*).

Across all freshwater benthic taxa in both time periods, we found no significant difference between 1 and 15 years of preservation on invertebrate $\delta^{13}C$ ($t_{[4.6]} = -0.71$, $P = 0.5$). Preservation effects on freshwater benthic $\delta^{13}C$ were also not different among taxa ($t_{[3.1]} = 0.77$, $P = 0.5$). We also evaluated differences between 1 and 15 years of preservation among only those taxa common to both data sets using a paired *t* test. Among these common taxa only, samples preserved for 15 years were not significantly different than samples preserved for 1 year ($t_{[2]} = -0.33$, $P = 0.8$). Nitrogen isotopic

Fig. 1. Change in carbon (*a*) and nitrogen (*b*) isotopic values resulting from formalin preservation on various benthic taxa collected from Lake Simcoe, Ontario, Canada. Vertical lines separate changes in isotopic values due to preservation after 1 year (left) and 15 years (right) of fixation. Broken horizontal lines indicate 0 (e.g., no preservation effect). Values are means and interquartile ranges (25th percentile to 75th percentile).



values were also not different between 1 and 15 years of preservation ($t_{[2]} = 0.42$, $P = 0.7$).

The decrease in $\delta^{13}C$ due to preservation following 1–15 years of formalin preservation was largely similar among taxa, but tended to be greatest among Gastropoda and Sphaeridae (Fig. 1*a*). Changes due to preservation for freshwater benthic $\delta^{15}N$ were centered around zero, but increased significantly after 15 years for Amphipoda and decreased for Trichoptera (Fig. 1*b*).

Comparison with published studies

Our literature search of formalin preservation effects on invertebrates returned 14 studies, reporting preservation effects on 22 taxa. Including the data from Lake Simcoe reported above, we were able to analyze 35 independent assessments of formalin preservation on invertebrate taxa across all studies.

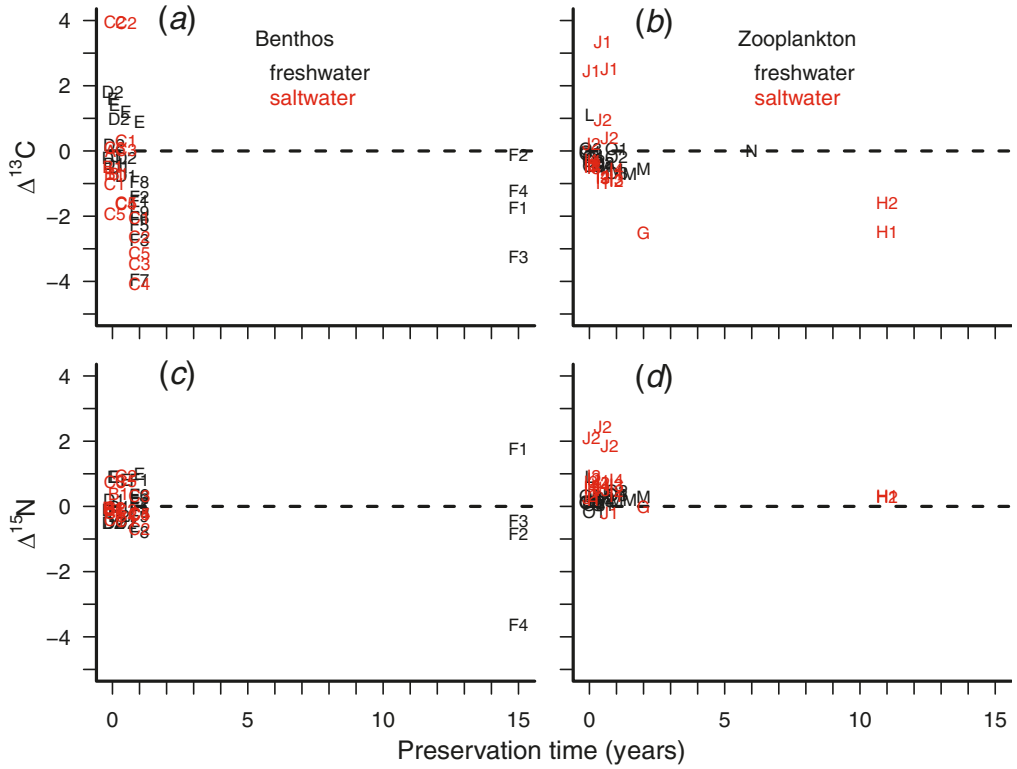
With a single exception, all taxa for which preservation effects on $\delta^{13}C$ isotope values (marine and freshwater) were evaluated for 1 year or more were lower relative to control samples (Fig. 2*a*), and significantly lower than control samples, on average (–2.1‰, $t_{[17]} = -6.9$, $P < 0.0001$). Significant drift of $\delta^{13}C$ isotope values with increasing preservation time—with no evidence of stabilization at the end of the study—was apparent among prior evaluations of both freshwater and saltwater taxa lasting 1 year or less (Fig. 2*a*). Without a comparison to samples preserved for longer time periods, it is impossible to determine from these studies whether preservation effects after 1 year represent an endpoint of effects on tissue $\delta^{13}C$ isotope values. We compared preservation effects after 1 year to 15 years across all studies and found no significant difference for $\delta^{13}C$ (1-year mean = –2.2‰, 15-year mean = –1.6‰, $t_{[4.9]} = 0.89$, $P = 0.4$). On average, over all studies included in the analysis, the effects of 1 year of preservation on freshwater benthic invertebrate $\delta^{13}C$ was –1.8‰, significantly less than the decrease observed for marine benthic $\delta^{13}C$ (–3.1‰, $t_{[11.9]} = 2.23$, $P = 0.04$; Fig. 2*a*). Unlike $\delta^{13}C$, $\delta^{15}N$ did not appear to be affected by time of preservation and was not significantly different from 0 ($t_{[41]} = -0.793$, $P = 0.9$). Results were similar when we considered only samples preserved for 1 year ($P = 0.8$). There was no significant difference between preservation effects on benthic invertebrate $\delta^{15}N$ between 1 and 15 years of fixation ($t_{[3.1]} = 0.74$, $P = 0.5$). The response of benthic invertebrate $\delta^{15}N$ to preservation was not significantly different between taxa from marine and freshwater environments ($t_{[34.0]} = -0.24$, $P = 0.8$).

We evaluated formalin preservation effects from published studies on zooplankton in a similar fashion. Formalin preservation effects on $\delta^{13}C$ in marine zooplankton appear to be much more variable compared with freshwater samples, particularly in samples preserved for less than 1 year (Fig. 2*c*). We therefore excluded marine zooplankton preserved for less than 1 year from comparisons of $\delta^{13}C$. Considering all studies and time periods, freshwater zooplankton $\delta^{13}C$ was only slightly but significantly lower relative to control samples (–0.2‰, $t_{[17]} = -2.38$, $P = 0.03$). By comparison, the decline in marine zooplankton $\delta^{13}C$ preserved for 1 or more years was more pronounced (–1.4‰, $t_{[6]} = -4.37$, $P = 0.005$). Accordingly, comparing only samples preserved for 1 year or more, marine zooplankton $\delta^{13}C$ (–1.4‰) declined to a greater extent than did freshwater zooplankton $\delta^{13}C$ following preservation (–0.4‰, $t_{[7.8]} = -2.96$, $P = 0.019$). Formalin preservation effects on marine zooplankton resulted in significantly lower $\delta^{13}C$ values at 2- to 11-year time intervals (–2.2‰) compared with only 1 year of preservation (–0.76‰; $t_{[2.3]} = -4.51$, $P = 0.03$). As with $\delta^{13}C$, marine zooplankton showed more variation in $\delta^{15}N$ values among samples preserved for less than 1 year compared with freshwater zooplankton (Fig. 2*d*) and were excluded from comparisons. Over all time periods, freshwater zooplankton $\delta^{15}N$ increased following formalin preservation ($t_{[17]} = 4.69$, $P = 0.0002$), but like $\delta^{13}C$, the difference from control samples was small (0.25‰, on average). Marine zooplankton $\delta^{15}N$ preserved for 1 year or longer also increased ($t_{[6]} = 4.08$, $P = 0.007$), but again only slightly (0.4‰). The response of zooplankton $\delta^{15}N$ to 1 or more years of fixation was not significantly different between those from marine and freshwater environments ($t_{[8.1]} = 0.89$, $P = 0.4$).

Discussion

Our study provides the first strong evidence that formalin preservation studies lasting 1–2 years likely accurately capture endpoints of preservation effects on stable isotope values of invertebrate organisms. Nitrogen isotopic values,

Fig. 2. Review of current literature (including results from Figs. 1*a*, 1*b*) regarding changes in isotopic values of carbon (*a*, *b*) and nitrogen (*c*, *d*) for both benthos (*a*, *c*) and zooplankton (*b*, *d*). Broken horizontal lines indicate 0 (e.g., no preservation effect). Results of studies from freshwater habitats are in black type, while those from marine (saltwater) habitats are in grey type (red type on the Web site). Data points represent taxa from a particular study, described in Supplementary Table S1.[1]



on average, appear to be largely unaffected by formalin preservation, with virtually all observations from the current study and the published literature falling within ±1‰ of control samples. Unlike nitrogen, carbon isotopic values following formalin preservation demonstrated directional changes (declines) up to 1 year. Prior to our study, evaluations of formalin preservation in benthic invertebrates lasted a maximum of 1 year. Of those studies, few could clearly demonstrate any evidence that preservation effects at the end of the study had stabilized. Despite this lack of evidence, correction factors from short-term studies have been applied in the published literature, and in some cases to samples stored for decades (Vander Zanden et al. 2003). The results of our study suggest that the correction factors based on studies of at least 1 year in duration can be applied to samples stored for longer time periods (e.g., decades); for both Lake Simcoe and across data from all published studies, there was no significant difference between the mean reduction in benthic invertebrate stable isotopes of carbon and nitrogen from samples preserved for 1 versus 15 years. This was also true comparing specific taxa from Lake Simcoe with observations at both 1 and 15 years. Given error around the mean estimates, preservation effects after 1 and 15 years were similar for nitrogen and carbon for Amphipoda, Ephemeroptera, and Gastropoda. Across all studies, all but one reported significant declines in formalin-preserved samples relative to control specimens after 1 year of fixation. Edwards et al. (2002) reported preservation effects on fish samples for 12–15 years, based on formalin fixation followed by ethanol preservation. The same study reported $\delta^{13}C$ fixation effects of formalin alone of approximately –2‰, similar to the mean value reported in this study for benthic invertebrates. In fish, this preservation effect appears to be reflected more quickly, without the temporal drift up to 1 year as we observed for invertebrates in this study (Edwards et al. 2002).

Our study provides isotopic correction factors of formalin preservation for a number of freshwater benthic taxa not previously reported. Prior to this study, evaluations of formalin preservation existed for the Asiatic clam (Sarakinos et al. 2002; Syväranta et al. 2011) and Trichoptera (Sarakinos et al. 2002), lasting 1 year and 6 months in duration, respectively. We report taxa-specific formalin preservation correction factors for eight additional taxa, as well as a longer term estimate of preservation effects for Trichoptera. Compared with the results from Sarakinos et al. (2002), we report comparable drift for Trichoptera $\delta^{13}C$, but far greater changes (declines) in $\delta^{15}N$ after 15 years of fixation. It is possible that some component of the variation around taxa-specific mean preservation estimates may be due to taxonomic variation (e.g., genus level, species level) below the level of resolution employed here. Although our study estimated taxa-specific correction factors for common benthic invertebrate taxa, the effects on both $\delta^{13}C$ and $\delta^{15}N$ are consistent enough across taxa, habitat (e.g., freshwater vs. saltwater), and time periods that generic correction factors reported here can likely provide a reasonable estimate of the effects of formalin preservation on benthic invertebrates.

Our review of the literature suggests that zooplankton isotopic values may respond differently in magnitude to long-term formalin preservation than benthos, depending on the source habitat. Like benthic invertebrates, freshwater zooplankton $\delta^{13}C$ was lower relative to control samples, but the magnitude was minor by comparison. Furthermore, the decline in marine zooplankton $\delta^{13}C$ due to formalin preservation was greater and the magnitude of the shift more closely reflected the decline in $\delta^{13}C$ that we report for benthic invertebrates. Unlike the largely neutral effect of preservation on benthic invertebrate $\delta^{15}N$, zooplankton $\delta^{15}N$ increased relative to control samples, but the effect was small (<0.4‰).

Samples taken from marine habitats had preservation effects of the same direction as those from freshwater habitats, but of consistently greater magnitude in both zooplankton and benthic invertebrates. Although the processes underlying this observation are unclear, it does suggest that investigators may wish to apply correction factors specific to the habitat they are investigating. Although long-term (>1 year) preservation studies for marine benthos are currently lacking, long-term studies in zooplankton appear to suggest that preservation effects after 2 years are similar to those stored for at least one decade.

We do not believe that the results of this study were influenced by using a combination of frozen and fresh material as a control for our evaluation of preservation effects on freshwater invertebrate taxa. We found no significant differences in frozen versus fresh benthic taxa taken from the same sites and depths in our study. Furthermore, a review of the literature shows that six out of eight previously published studies that evaluated the effects of freezing on isotopic values of biological tissues report no significant effect relative to control samples (Bosley and Wainright 1999; Bugoni et al. 2008; Gloutney and Hobson 1998; Kaehler and Pakhomov 2001; Sweeting et al. 2004). Though not evaluating freezing effects explicitly, Sarakinos et al. (2002) reported remarkable stability in frozen (–25 °C) invertebrate tissues from 3 days to 6 months of storage. In contrast, Barrow et al. (2008) reported a significant change in the isotopic values of turtle epidermis after 60 days due to freezing at –10 °C. However, a close examination of their Appendices A and B reveals that the significant results they report are for two individual specimens only and that the direction of the effect was not consistent (*Chelonia 2*: no significant change in $\delta^{13}C$, significant decline in $\delta^{15}N$; *Caretta 2*: significant decline in $\delta^{13}C$, no significant change in $\delta^{15}N$; we estimated *t* statistics and df values based on reported means, sample sizes, and SD values assuming unequal variance; patterns were the same with or without lipid extraction, or for a pooled analysis of results disregarding lipid extraction treatment). Feuchtmayr and Grey (2003) reported a small but significant change (1‰, lower $\delta^{13}C$, higher $\delta^{15}N$) in isotopic values associated with freezing (–10 °C). Dannheim et al. (2007) reported that bulk-frozen samples (–20 °C) of marine infauna were significantly lower in $\delta^{13}C$ and $\delta^{15}N$ relative to samples that were sorted and frozen individually. Unlike Dannheim et al. (2007) who froze the entire grab (e.g., sediments, water, and organisms), our "bulk-frozen" samples from 2008 included only the organisms collected on 500 μm screens, with only residual sediment–rocks–shells and mostly lake water. Recent work reported significant effects of freezing on the isotopic values of a bivalve (Syväranta et al. 2011), where both the direction and the magnitude of preservation effects in formalin would depend greatly on whether frozen or fresh material was used as a control. In contrast, we found no differences between fresh material and that frozen for 1 year in the bivalve *Dreissena polymorpha* (zebra mussel) (10 m depth: fresh = –27.6‰, frozen = –27.5‰, $t_{[1.24]} = 0.19$, $P = 0.9$; 25 m depth: fresh = –29.9‰, frozen = –30.2‰, $t_{[1]} = 1.5$, $P = 0.4$) or among Sphaeridae collected in our samples (25 m depth: fresh = –26.8‰, frozen = –27.4‰, $t_{[1]} = 2.3$, $P = 0.3$).

Although we were unable to control for the brand of formalin used in each of the time periods in our study (1993, 2008), we believe that this had little effect on our results. Edwards et al. (2002) reported differences as great as 14.7‰ for $\delta^{13}C$ between different brands of 10% formalin. However, variation among tissues fixed with these different preservatives was comparatively small, ranging only between 0.2‰ and 0.5‰. This is not dramatically greater than instrument error, typically reported in the range of 0.2‰–0.3‰ and much smaller than our findings of a –2‰ effect of formalin preservation.

Although the mean change in $\delta^{15}N$ values of invertebrate taxa over 15 years was, on average, not different from zero, there was considerable variability around that mean estimate, both across and within taxa. Both Trichoptera taxa evaluated were highly depleted following 15 years of preservation. Though we had no organisms from our 1 year collections with which to compare these values, the changes are far greater than those reported by Sarakinos et al. (2002) after 6 months of preservation. By comparison, Gastropoda taxa showed very little change over a 15-year time period. However, trends differed even within taxa, with Heptageniidae mayflies increasing by 1.3‰ and Ephemerellidae mayflies decreasing by –3.0‰. This variation suggests that more work on long-term taxa-specific studies of preservation effects may be required. However, until those studies are conducted, our study provides correction factors that can be applied generally and with some degree of confidence to samples that have been preserved for longer (e.g., >1 year) time periods.

Overall, this study provides the most comprehensive evaluation of long-term formalin preservation on isotopic values of invertebrates to date and reports appropriate taxa- and habitat-specific correction factors based on the current experiment, as well as reported values in the published literature. This information will be of value to researchers who wish to use stable isotopes from long-term archives of preserved samples to reveal temporal ecological change and to assist with the selection of the appropriate correction factor depending on the taxa and habitat under study.

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