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RESEARCH ARTICLE

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Effects of preservation methodology on stable isotope compositions of sea stars

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Rationale: Stable isotope analysis is used to investigate the trophic ecology of organisms and, in order to use samples from archived collections, it is important to know whether preservation methods alter the results. This study investigates the long-term effects of four preservation methods on isotopic compositions and isotopic niche parameters of sea stars.

Methods: We assessed the effects of preservation method (freezing, drying, formaldehyde, ethanol) and duration (0, 1, 3, 6, 9, 12, 24 months) on the stable isotope ratios of carbon, nitrogen and sulfur of sea star tissues. Isotopic ratios were measured using continuous-flow elemental analysis and isotope ratio mass spectrometry. We also monitored the evolution of commonly used ecological metrics (isotopic niche parameters) throughout the experiment.

Results: Clear changes in $\delta^{13}C$ values were observed for samples stored in formaldehyde and ethanol. None of the preservation methods had significant or consistent effects on $\delta^{15}N$ values. Formaldehyde preservation induced a decrease in $\delta^{34}S$ values. All these changes could be mitigated using correction factors. Isotopic niche parameters slightly changed over time when computed with $\delta^{13}C$ and $\delta^{15}N$ values, but inconsistent variations occurred when computed with $\delta^{13}C$ and $\delta^{34}S$ values.

Conclusions: Overall, these results show that preservation may affect the stable isotope ratios of sea stars. Correction factors can be used to mitigate the effects of the preservation method on stable isotope ratios. Isotopic niche parameters are overall unchanged. Consequently, in most cases, museum samples are suitable for calculation of isotopic niche parameters.

1 | INTRODUCTION

Stable isotope analysis is now a common tool for food web studies. Carbon isotopic compositions (^{13}C:^{12}C; $\delta^{13}C$ values) are generally used to determine the origin of primary sources of carbon in food webs or feeding areas[1,2] because of the differences in stable isotope composition between the different types of primary producers (phytoplankton, phytobenthos, terrestrial organic matter, etc.) and the low ^{13}C enrichment in organisms relative to their diet.[2,3] Nitrogen isotopic compositions (^{15}N:^{14}N; $\delta^{15}N$ values) are used to assess nitrogen sources and to estimate the trophic level of consumers, as organisms are generally enriched in ^{15}N relative to their diet, resulting in increasing $\delta^{15}N$ values with trophic level.[2,4] Similarly to $\delta^{13}C$ values, sulfur isotopic compositions (^{34}S:^{32}S; $\delta^{34}S$ values) are used in studies of marine food webs to refine the discrimination between primary producers, and notably of benthic and pelagic sources, because of the differences in $\delta^{34}S$ values between seawater sulfates and sediment sulfides.[5-7]

Stable isotope ratios of samples from museum collections may represent a readily accessible source of information for food web studies. They can notably help to fill gaps in our knowledge of the ecology of species coming from data-poor regions or ecosystems.

Samples from museum collections were often collected during periods when environmental conditions were different and usually more pristine than today, and thus may be used to study past trophic ecology of organisms. Unfortunately, preservative fluids are known to alter stable isotope ratios in samples.[8-10] Furthermore, the impacts of preservation methods are taxon-specific[10,11] and studies of these impacts on particular taxa are necessary.

The teleosts are the taxon for which the effects of preservation methodology on stable isotope ratios have been most studied.[8,12-16] The influence of preservation methodology on stable isotope ratios has also been investigated in various other taxa such as elasmobranchs,[17,18] chelonians,[19] birds,[20] marine mammals,[21] terrestrial mammals,[22] and even photosynthetic organisms.[8,11,23] The influence of preservation methods on stable isotope ratios has also been investigated in several invertebrate taxa such as cnidarians,[11,24] mollusks,[8,10,11,25,26] polychaetes,[10,26] sipunculids,[10] and aquatic[12,14,27] and terrestrial arthropods.[28,29] Nevertheless, the influence of preservation method on stable isotope ratios has been poorly investigated for several taxa. This is the case for echinoderms, for which the effects of preservation on stable isotope ratios have only been studied in one holothuroid species.[10]

With some exceptions,[23-25,30] most of these studies agree that freezing and drying do not alter stable isotope ratios and that preservation or fixation of organisms with formaldehyde induces a negative shift of $\delta^{13}C$ values. However, more conflicting results have been reported for the impact of ethanol on $\delta^{13}C$ values, with either no significant changes or increasing $\delta^{13}C$ values being observed.[8-10] The study of the impact of formaldehyde and ethanol on $\delta^{15}N$ values also led to conflicting results (Table 1). Contrary to $\delta^{13}C$ and $\delta^{15}N$ values, the impact of preservation on $\delta^{34}S$ values has been poorly investigated so far (but see Edwards et al[13] and Javornik et al[22]).

The increasing use of stable isotope analysis has also led to the development of new ecological metrics and models for assessing between- and/or within-group trophic diversity and variability,[32-34] source partitioning,[35] or trophic levels of organisms.[36] All these approaches take into account the isotopic variability of consumers (intragroup or intergroup comparisons) and allow us to calculate diverse ecologically meaningful isotopic metrics. However, to date only one study has investigated the impact of preservation on mixing model performance.[31] To the best of our knowledge, the influence of preservation method on isotopic niche modelling has never been tested despite these metrics being increasingly used.

Among echinoderms, sea stars (class: Asteroidea) are usually predatory organisms. With the exception of species from the Paxillosida order, which represent roughly 20% of extant species, sea stars are known to revert their stomach in order to preliminarily digest their prey externally. As a result, stomach content analyses are complicated for this taxonomic group, although some authors have been able to use this method.[37-39] Stable isotope ratios are therefore an interesting tool for investigating the trophic role of sea stars in ecosystems and the impact of preservation methods on stable isotope ratios in sea star tissues should be investigated.

In this context, the aim of the study reported here was to determine whether chemically preserved sea star samples, such as those stored in museum collections, were suitable for trophic ecology studies. To achieve this goal, we (1) experimentally assessed the modifications of stable isotope ratios in sea star tissues preserved for up to two years with different methods (freezing, drying, formaldehyde, ethanol), (2) attempted to determine if correction factors could be used to correct eventual modifications, and (3) investigated the influence of these modifications on the resulting isotopic niches and associated parameters.[34]

2 | EXPERIMENTAL

2.1 | Sampling and stable isotope analysis

Sea stars of the species *Marthasterias glacialis* ($n = 20$) were collected in the Atlantic Ocean, near the Roscoff biological station (Brittany, France), in April 2016. The sea stars were maintained alive until their transfer to the laboratory. For each sea star, the arms were separated from the central disc and internal organs were removed in each arm. The first arm of each sea star was immediately dried and homogenized into powder (T_0). The other arms were randomly assigned to each preservation method (freezing, drying, formaldehyde, ethanol) and cut into six sections, each section being randomly assigned to a time of analysis (1, 3, 6, 9, 12, 24 months; $n = 20$ samples per method and per time of analysis). Each arm section was individually either frozen at −28°C, oven dried or preserved in 3.7% formaldehyde or 99.8% ethanol. At the assigned date of analysis, with the exception of the already dried samples, the arm sections were rinsed with distilled water and dried. All samples were then ground into powder for homogenization using a mixer mill (MM301, Retsch, Haan, Germany) prior to stable isotope analyses.

Carbonates in the endoskeleton of sea stars are more ^{13}C-enriched than other tissue components.[3] Consequently, carbonates were removed from the samples by exposing subsamples to 37% hydrochloric acid vapor for 48 h.[40] The subsamples were then precisely weighed (*ca* 2.5–3 mg) in 5 mm× 4 mm tin cups with *ca* 3 mg of tungsten trioxide, and analyzed with an elemental analyzer (Vario MICRO Cube, Elementar, Hanau, Germany) coupled to a continuous-flow isotope ratio mass spectrometer (IsoPrime100, Elementar UK, Cheadle, UK). The stable isotope ratios of carbon, nitrogen and sulfur were expressed in the δ notation ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values, respectively[41]) in ‰ relative to international references (Vienna Pee Dee Belemnite for $\delta^{13}C$ values, N_2 in atmospheric air for $\delta^{15}N$ values and Canyon Diablo troilite for $\delta^{34}S$ values). Certified reference materials from the International Atomic Energy Agency (IAEA, Vienna, Austria), namely IAEA N-1 (ammonium sulfate; $\delta^{15}N = 0.4 \pm 0.2$‰), IAEA C-6 (sucrose; $\delta^{13}C = -10.8 \pm 0.5$‰) and IAEA S-1 (silver sulfide; $\delta^{34}S = -0.3$‰), were used as primary standards. Sulfanilic acid (Sigma-Aldrich, Overijse, Belgium; $\delta^{13}C = -25.6 \pm 0.4$‰, $\delta^{15}N = -0.1 \pm 0.4$‰, $\delta^{34}S = 5.9 \pm 0.5$‰, means ± SD) and one of the samples (randomly selected; $\delta^{13}C = -15.1 \pm 0.3$‰,

TABLE 1 Examples of reported shifts (mean ± SD) of preservation methods on stable isotope ratios in aquatic animals

Method	Phylum	Species	Experiment duration	$\Delta\delta^{13}C$ (‰)	$\Delta\delta^{15}N$ (‰)	Ref.
Freezing	Cnidarians	*Aurelia aurita*	6 months	Not significant	↓ −2.1	[24]
	Mollusks	*Corbicula fluminea*	12 months	↑ +2.1 ± 0.3	↑ +1.0 ± 0.3	[25]
		Octopus vulgaris	12 weeks	Not significant	Not significant	[8]
	Crustaceans	Bulk zooplankton	4 days	↓ −0.9	↑ +0.6	[30]
	Echinoderms	*Marthasterias glacialis*	24 months	Not significant	Not significant	This study
	Teleosts	*Argiosomus hololepidotus*	12 weeks	Not significant	Not significant	[8]
		Various species	1 month	Not significant	Not significant	[16]
Drying	Mollusks	*Octopus vulgaris*	12 weeks	Not significant	Not significant	[8]
	Echinoderms	*Marthasterias glacialis*	24 months	Not significant	Not significant	This study
	Teleosts	*Argiosomus hololepidotus*	12 weeks	Not significant	Not significant	[8]
		Various species	625 days	Not significant	Not significant	[31]
Formaldehyde	Polychaetes	*Chirimia biceps*	12 months	↓ −4.1	Not significant	[10]
		Magelona spp.	18 weeks	↓ −2.1	↓ −1.0	[26]
		Nephtys hystricis	12 months	↓ −3.1	Not significant	[10]
	Sipunculid	*Sipunculus norvegicus*	12 months	↓ −3.5	Not significant	[10]
	Mollusks	*Corbicula fluminea*	12 months	↑ +2.2 ± 0.3	↑ +1.0 ± 0.2	[29]
		Abra longicalus	12 months	↓ −2.1	Not significant	[10]
		Octopus vulgaris	12 weeks	↓ −0.3 ± 0.1	Not significant	[8]
		Mya arenaria	18 weeks	↓ −1.5 ± 0.8	Not significant	[26]
		Tellina fabula	18 weeks	↓ −2.7 ± 0.3	Not significant	[26]
	Crustaceans	Bulk zooplankton	4 days	↑ +1.1	↑ +0.8	[30]
	Echinoderms	*Molpadia musculus*	12 months	↑ +3.9 (6 months) ↓ −2.6 (12 months)	Not significant	[10]
		Marthasterias glacialis	24 months	↓ −0.8 ± 0.5	Not significant	This study
	Teleosts	*Argiosomus hololepidotus*	12 weeks	↓ −0.5 ± 0.1	Not significant	[8]
		Various species	625 days	↓ −1.0	Not significant	[31]
Ethanol	Cnidarians	*Aurelia aurita*	6 months	Not significant	↓ −2.4	[24]
	Polychaetes	*Chirimia biceps*	12 months	Not significant	Not significant	[10]
		Magelona spp.	18 weeks	↑ +1.4 ± 0.2	↑ +0.9 ± 0.0	[26]
		Nephtys hystricis	12 months	Not significant	Not significant	[10]
	Sipunculid	*Sipunculus norvegicus*	12 months	Not significant	↓ −1.7	[10]
	Mollusks	*Abra longicalus*	12 months	Not significant	Not significant	[10]
		Corbicula fluminea	12 months	↑ +1.3 ± 0.3	↑ +0.9 ± 0.2	[25]
		Octopus vulgaris	12 weeks	↑ +1.6 ± 0.3	Not significant	[8]
		Mya arenaria	18 weeks	↓ −1.4 ± 6.5	Not significant	[26]
		Tellina fabula	18 weeks	↑ +0.8	↑ +0.6	[26]
	Crustaceans	Bulk zooplankton	4 days	Not significant	↑ +0.8	[30]
	Echinoderms	*Molpadia musculus*	12 months	↑ +3.6	Not significant	[10]
		Marthasterias glacialis	24 months	↑ +0.6 ± 0.5	Not significant	This study
	Teleosts	*Argiosomus hololepidotus*	12 weeks	↑ +0.7 ± 0.2	Not significant	[8]
		Various species	625 days	↑ +0.7	↑ +0.4	[31]
		Various species	1 month	↑ +0.4 ± 0.4	↑ +0.6 ± 0.4	[16]

$\delta^{15}N = 12.3 \pm 0.2$‰, $\delta^{34}S = 16.9 \pm 0.8$‰) were used as secondary analytical standard and replicate, respectively. T_0 samples were analyzed four times, i.e. once per method, in order to have a balanced data design. Elemental data are expressed as a ratio between the concentrations of C and N (C/N mass ratio), measured relative to dry mass (%DM).

2.2 | Data analysis

All the data analyses were performed using R 3.3.3.[42]

Two-way repeated measures analyses of variance (ANOVA) were performed on $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values and on C/N ratios to assess the effects of preservation methods and time of preservation on those parameters. In the case of significant differences, subsequent one-way repeated measures ANOVA were performed for each preservation method to assess the effect of time of preservation on $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values and on C/N ratios. In the case of significant differences, pairwise comparisons with Bonferroni correction[43] were computed to compare $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values between T_0 and preserved samples at each time to determine when preservation alters stable isotope ratios. Normality of residuals was checked for all models using Q–Q plots and Shapiro tests. In the case of a consistent effect of preservation time, i.e. a significant change of $\delta^{13}C$, $\delta^{15}N$ or $\delta^{34}S$ values at a given time of preservation that still occurs after this time, correction factors were computed. To do so, mean differences of $\delta^{13}C$, $\delta^{15}N$ or $\delta^{34}S$ values between T_0 samples and significantly different preserved samples ($\Delta\delta^{13}C$, $\Delta\delta^{15}N$ and $\Delta\delta^{34}S$, respectively) were calculated: the correction factors are the opposite values of these calculated differences. One-way repeated measures ANOVA and subsequent *post hoc* analyses were then performed to compare the differences between corrected $\delta^{13}C$, $\delta^{15}N$ or $\delta^{34}S$ values and non-corrected $\delta^{13}C$, $\delta^{15}N$ or $\delta^{34}S$ values from previous times of analysis and T_0 samples.

For each preservation method and for each time of analysis, standard ellipses representing isotopic niches (see Figure 1 in Jackson et al[34] and Reid et al[44] for details) were computed using the $\delta^{13}C$ and $\delta^{15}N$ values, or the $\delta^{13}C$ and $\delta^{34}S$ values and the temporal evolution of the following parameters was investigated: lengths of the semi-

major (a_c) and semi-minor (b_c) axes (sample size corrected), angle (θ) of the semi-major axis with the x axis, and the eccentricity (ε) of the ellipse ($\varepsilon = 0$ means that the "ellipse" is a circle, i.e. $a_c = b_c$). Finally, sample size corrected (SEA_c) and Bayesian (based on 5.10^5 successive iterations; SEA_B) estimates of the standard ellipse area (SEA) were computed with the SIBER package.[34] For each method of preservation, the SEA_B was directly compared with the SEA_B of T_0 samples by assessing the proportion of estimated SEA computed by the SIBER package for which the SEA values of the preserved samples were higher or lower than those of the T_0 samples. If this proportion of higher or lower SEA values exceeded 95%, the SEA_B values of fresh and preserved samples were considered as being different.

3 | RESULTS

Significant influences of the preservation method ($F_{3,57} = 113.338$, $P < 0.001$) and of its interaction with the time of analysis ($F_{18,342} = 6.718$, $P < 0.001$) were observed for $\delta^{13}C$ values. Subsequent ANOVA performed in each preservation method revealed different effects of preservation on $\delta^{13}C$ values. The $\delta^{13}C$ values are strongly altered by formaldehyde preservation ($F_{6,114} = 14.360$, $P < 0.001$): the values immediately decreased at the first month of preservation and then remained stable throughout the experiment (Figure 1A). The difference in $\delta^{13}C$ values between T_0 samples and preserved samples was -0.8 ± 0.5‰. Consequently, adding 0.8‰ to the $\delta^{13}C$ values of samples preserved in formaldehyde suppressed significant differences in the $\delta^{13}C$ values between T_0 samples and preserved samples whatever the treatment time ($F_{6,114} = 0.374$, $P = 0.894$). Ethanol had a significant effect on $\delta^{13}C$ values ($F_{6,114} = 5.701$, $P < 0.001$), which increased through time until reaching an asymptote (Figure 1A). Subsequent pairwise comparisons with Bonferroni correction showed that a significant change in $\delta^{13}C$ values occurred at 9 months of preservation and was still present at 12 and 24 months of preservation (Table S1, supporting information). The difference in $\delta^{13}C$ values between T_0 samples and preserved samples after 9 months was 0.6 ± 0.5‰. Adding −0.6‰ to the $\delta^{13}C$ values of samples preserved in ethanol after 9 months suppressed significant differences in the $\delta^{13}C$ values between the T_0 samples and the preserved samples but some differences appeared between the time of analysis ($F_{6,114} = 4.532$, $P < 0.001$).

Significant influences of the preservation method ($F_{3,57} = 22.848$, $P < 0.001$) and of its interaction with the time of analysis ($F_{18,342} = 2.986$, $P < 0.001$) were observed on $\delta^{15}N$ values. Subsequent ANOVA performed for each preservation method revealed inconsistent effects of drying on $\delta^{15}N$ values ($F_{6,114} = 4.436$, $P < 0.001$; Figure 1B) as there were no significant differences in the $\delta^{15}N$ values between T_0 and other times of analysis, but some differences between the times of analysis (Table S1, supporting information). Furthermore, storage in formaldehyde ($F_{6,114} = 2.136$, $P = 0.055$) and ethanol ($F_{6,114} = 2.178$, $P = 0.050$) appeared to have a marginally significant effect on $\delta^{15}N$ values (Figure 1B).

The results of the two-way repeated measures ANOVA showed an influence of preservation method ($F_{3,57} = 87.415$, $P < 0.001$) and time of analysis ($F_{6,114} = 7.371$, $P < 0.001$) and of their interaction ($F_{18,342} = 6.617$, $P < 0.001$) on $\delta^{34}S$ values. The $\delta^{34}S$ values changed

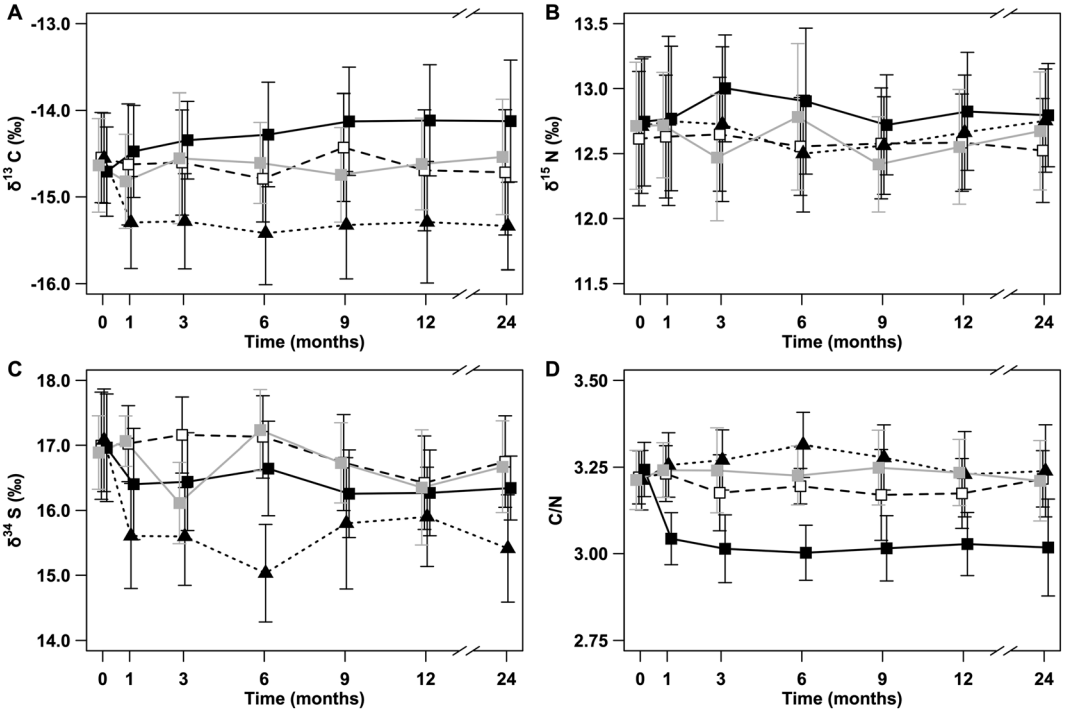

FIGURE 1 Evolution of mean ± SD of (A) $\delta^{13}C$ values, (B) $\delta^{15}N$ values, (C) $\delta^{34}S$ values, and (D) C/N ratios in *Marthasterias glacialis* tissues stored frozen (white squares and dashed lines), dried (gray squares and lines), in formaldehyde (black triangles and dotted lines) or in ethanol (black squares and solid lines) for 24 months

inconsistently in frozen samples ($F_{6,114} = 3.168$, $P = 0.007$), with samples stored for 3 and 12 months having significantly different $\delta^{34}S$ values (Figure 1C; Table S1, supporting information). Inconsistent changes of $\delta^{34}S$ values also occurred in dried samples ($F_{6,114} = 7.255$, $P < 0.001$), with significant deviance from the $\delta^{34}S$ values of T_0 samples occurring only at 3 months of preservation but not earlier or later (Figure 1C). The $\delta^{34}S$ values changed significantly in samples stored in formaldehyde ($F_{6,114} = 11.950$, $P < 0.001$): the $\delta^{34}S$ values of preserved samples were significantly lower than those of T_0 samples for all time periods, with the mean shift between T_0 and those times of analysis being −1.5 ± 1.2‰ (Figure 1C). Adding 1.5‰ to the $\delta^{34}S$ values of samples preserved in formaldehyde suppressed any significant differences between times of analysis despite the ANOVA remaining significant, but with a very low F value ($F_{6,114} = 2.327$, $P = 0.037$; Table S1, supporting information). A significant influence of ethanol preservation on $\delta^{34}S$ values was observed ($F_{6,114} = 2.659$, $P = 0.018$) but different $\delta^{34}S$ values could be seen only between T_0 samples and samples stored for 24 months in the *post hoc* analysis (Figure 1C; Table S1, supporting information). The mean shift of $\delta^{34}S$ values between T_0 samples and samples stored in ethanol for 24 months was −0.7 ± 1.0‰. Adding 0.7‰ to the $\delta^{34}S$ values of samples preserved for 24 months in ethanol suppressed the slightly significant difference in the $\delta^{34}S$ values between them and T_0 samples. However, this correction created differences between the $\delta^{34}S$ values of samples stored for 24 months and other times of analysis (Table S1, supporting information), and caused an increase of the ANOVA F value ($F_{6,114} = 4.323$, $P < 0.001$).

Significant influences of preservation method ($F_{3,57} = 162.972$, $P < 0.001$) and time of analysis ($F_{6,114} = 2.641$, $P = 0.020$) and of their interaction ($F_{18,342} = 10.209$, $P < 0.001$) were observed on C/N ratios. Subsequent ANOVA and *post hoc* analyses for each preservation method showed marginal effects of freezing on C/N ratios ($F_{6,114} = 2.252$, $P = 0.043$) and pairwise comparisons with Bonferroni correction did not detect any significant change in C/N ratios (Table S1, supporting information). Storage in formaldehyde ($F_{6,114} = 3.948$, $P = 0.001$) and ethanol ($F_{6,114} = 20.740$, $P < 0.001$, Figure 1D) induced changes of C/N ratios. For samples stored in formaldehyde, higher C/N ratios were observed at 6 months of preservation than in T_0 samples (Table S1, supporting information). For ethanol, the C/N ratios immediately decreased at the first month of preservation and then remained stable throughout the experiment (Table S1, supporting information). In this case, the difference of C/N ratios between T_0 samples and preserved samples was 0.22 ± 0.10.

When computed with $\delta^{13}C$ and $\delta^{15}N$ values, the ellipse parameters changed little and inconsistently and, as a result, they were similar at the beginning and at the end of the experiment (Figure S1, supporting information). Changes in SEA_B occurred between T_0 samples and each time of preservation for samples stored dried or in ethanol (Figure 2). For samples stored dried, the SEA_B values for samples stored for 3 months were higher than for the T_0 ones (Figure 2B). For samples stored in ethanol, the SEA_B values for samples stored during 1 month and 24 months were higher than for the T_0 ones (Figure 2D). Yet, these changes did not occur consistently for other times of preservation. The overlap between T_0

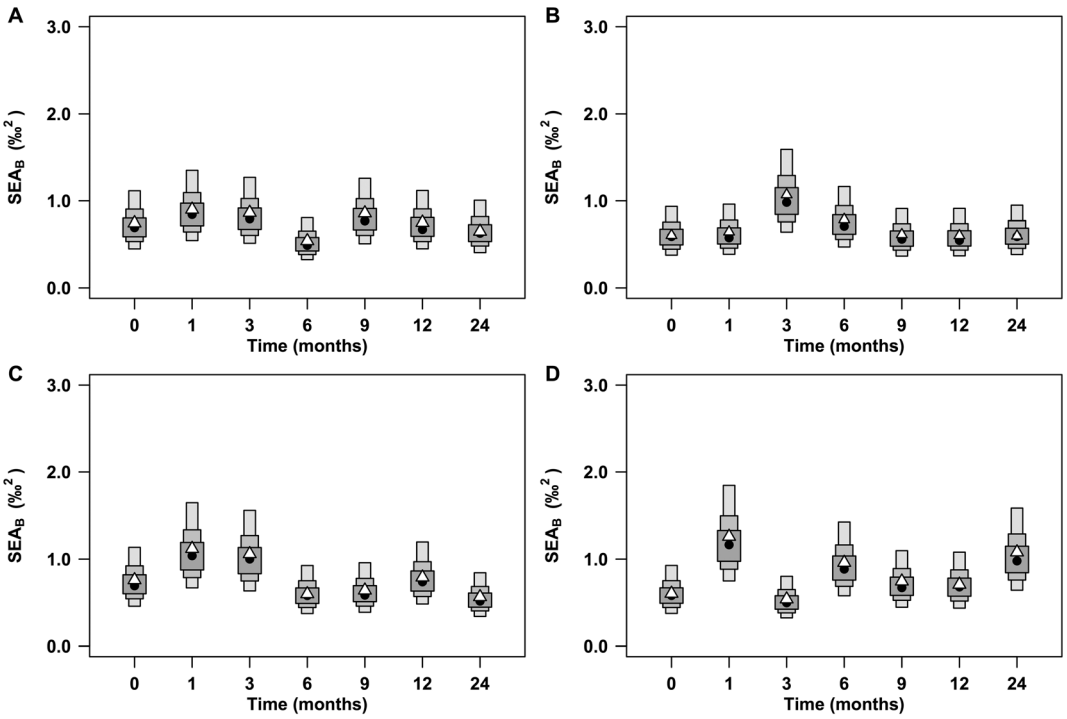

FIGURE 2 SIBER density plots depicting evolution of standard ellipse areas computed with $\delta^{13}C$ and $\delta^{15}N$ values and estimated with Bayesian analysis, as well as standard ellipse areas corrected for sample size, in *Marthasterias glacialis* tissues stored (A) frozen, (B) dried, (C) in formaldehyde, or (D) in ethanol for 24 months. Black dots are the modes. Shaded boxes represent the 50%, 75% and 95% confidence intervals, from dark to light gray. White triangles are standard ellipse areas corrected for sample size

ellipses and ellipses for other times of analysis appeared to be weak in samples stored in formaldehyde and in ethanol (Figure 3) because of the shift of mean $\delta^{13}C$ values previously observed for these two preservative fluids (Figure 1A).

When the standard ellipses were computed with $\delta^{13}C$ and $\delta^{34}S$ values, more important and more inconsistent changes in the parameters occurred (Figure S2, supporting information). Indeed, changes in the length of ellipse axes frequently exceeded 0.1‰ (Figures S2A and S2B, supporting information). In all methods, the angles of the ellipses were the opposite of the angle of ellipses from T_0 samples at least at one time of analysis, and even the angles of ellipses from T_0 samples were different between preservation methods (Figure S2C, supporting information), resulting in inverted orientation of the ellipses. Changes in the SEA_B did not occur during the experiment (Figure 4). The absence of overlap between the T_0 ellipses and ellipses for other times of analysis that appeared in samples stored in formaldehyde, as well as the weak overlap that appeared in ethanol (Figure 5), is mostly the result of the shift of the mean of both the $\delta^{13}C$ and the $\delta^{34}S$ values previously observed for these two preservative fluids (Figures 1A and 1C).

4 | DISCUSSION

Contrasting effects of preservation on $\delta^{13}C$ values in sea stars were observed. Freezing and drying had no or marginal effect on $\delta^{13}C$ values throughout time, while formaldehyde induced a rapid decrease of -0.8 ± 0.5‰ for $\delta^{13}C$ values during the first month of preservation. Those values were subsequently stable throughout the experiment. Decrease followed by stability of $\delta^{13}C$ values has been frequently observed for organisms stored in formaldehyde.[8,9,31] However, the time at which the change in $\delta^{13}C$ values occurs may differ, going from several weeks[8,9,31] to one year.[10] Furthermore, the decrease in $\delta^{13}C$ values that we observed is usually lower than the previously reported shift induced by formaldehyde. After the initial change, the $\delta^{13}C$ values seem to remain stable during longer term preservation.[27] Protein lysis[9] and/or integration of C from the preservative liquid into the samples[8,9,13] are proposed mechanisms to explain this phenomenon. Increasing C/N ratios in samples stored in formaldehyde[10,14] support this hypothesis, and higher C/N ratios were observed at 6 months of preservation in our experiment. Considering that $\delta^{13}C$ values are not further altered by formaldehyde following the initial change, we recommend using a correction factor for $\delta^{13}C$ values of sea star samples that have been in formaldehyde for more than 1 month no matter how long they have been preserved. Indeed, adding 0.8‰ to $\delta^{13}C$ values of samples stored in formaldehyde resulted in similar carbon isotopic ratios for fresh and preserved samples in our experiment. Testing of the influence of ethanol on isotopic ratios has led to conflicting results: either stable[9,10] or increasing[8-10,31] $\delta^{13}C$ values in samples were previously observed. In *Marthasterias glacialis*, a gradual increase in $\delta^{13}C$ values was observed. This increase became significant after 9 months of preservation where it went up to 0.6 ± 0.5‰. This phenomenon may be explained by the extraction of lipids by ethanol,[45] as highlighted by the decrease in C/N ratios that we observed for samples stored in ethanol. Long-term preservation in ethanol could also induce leaching of other compounds such as amino acids.[45] These results suggest that using a correction factor

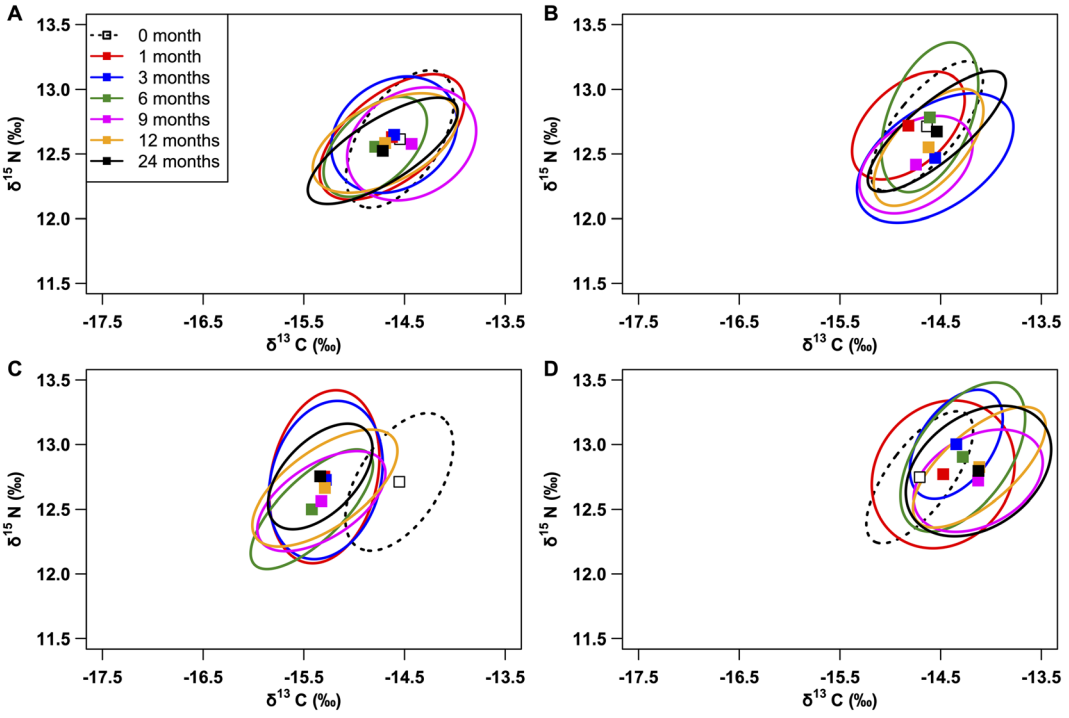

FIGURE 3 Evolution of mean stable isotope ratios and isotopic niche computed with $\delta^{13}C$ and $\delta^{15}N$ values in *Marthasterias glacialis* tissues stored (A) frozen, (B) dried, (C) in formaldehyde, or (D) in ethanol for 24 months [Color figure can be viewed at wileyonlinelibrary.com]

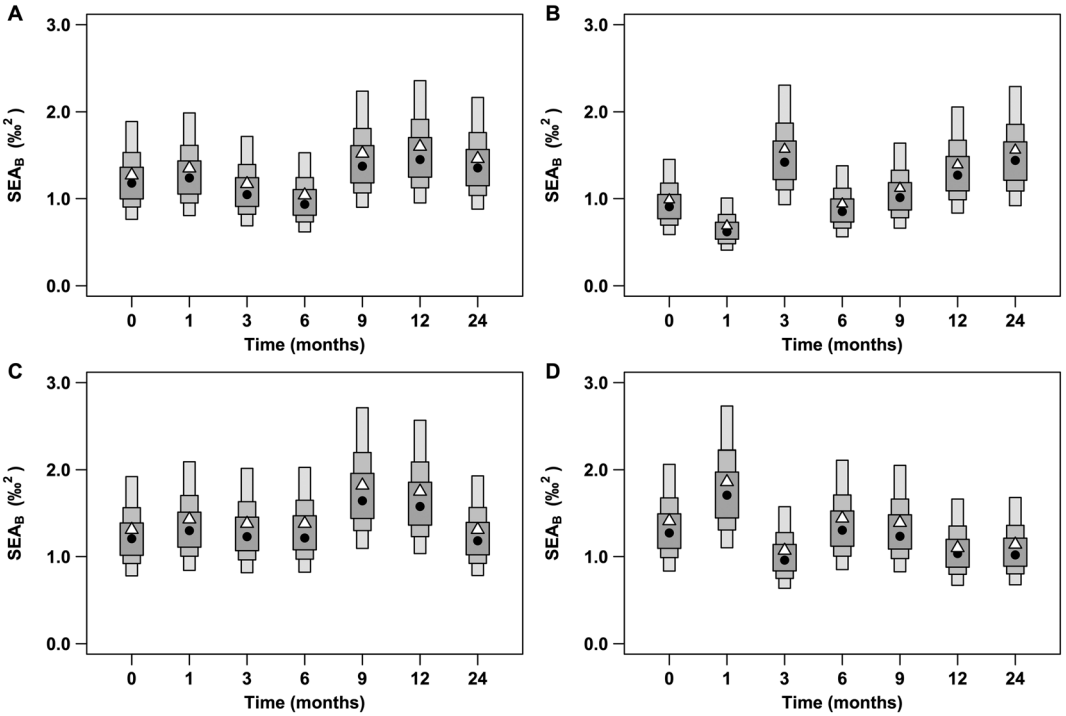

FIGURE 4 SIBER density plots depicting evolution of standard ellipse area computed with $\delta^{13}C$ and $\delta^{34}S$ values and estimated with Bayesian analysis, as well as standard ellipse areas corrected for sample size, in *Marthasterias glacialis* tissues stored (A) frozen, (B) dried, (C) in formaldehyde, or (D) in ethanol for 24 months. Black dots are the modes. Shaded boxes represent the 50%, 75% and 95% confidence intervals, from dark to light gray. White triangles are standard ellipse areas corrected for sample size

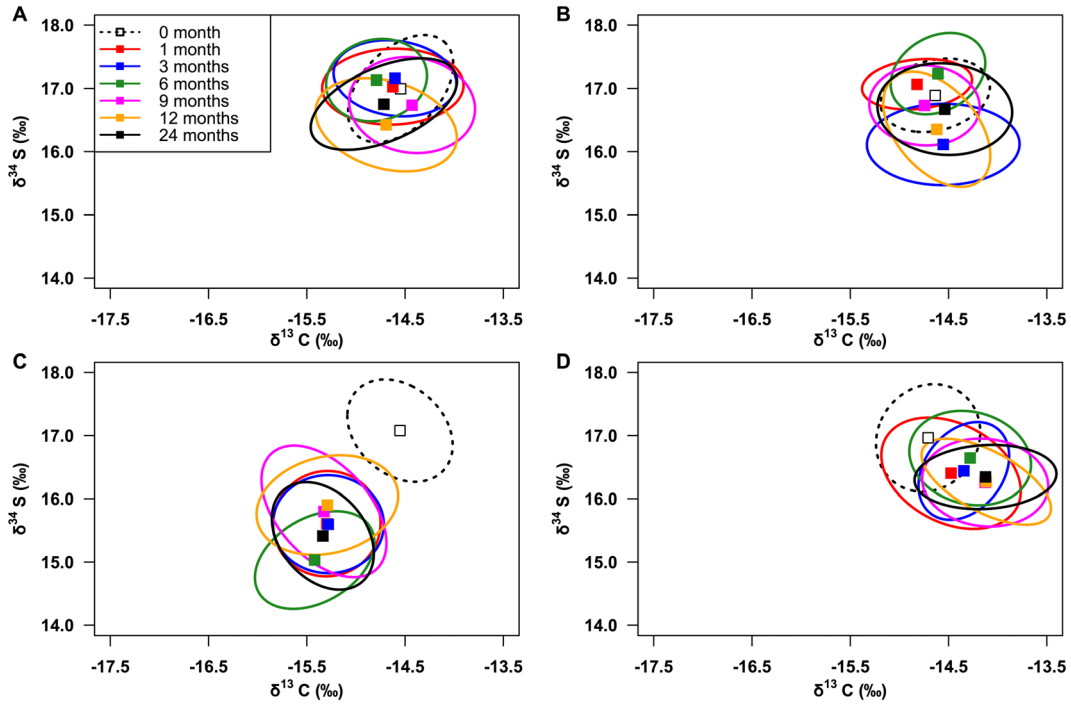

FIGURE 5 Evolution of mean stable isotope ratios and isotopic niche computed with $\delta^{13}C$ and $\delta^{34}S$ values in *Marthasterias glacialis* tissues stored (A) frozen, (B) dried, (C) in formaldehyde, or (D) in ethanol during 24 months [Color figure can be viewed at wileyonlinelibrary.com]

for $\delta^{13}C$ values of ethanol-preserved sea stars stored for more than 9 months is advised. Indeed, adding −0.6‰ to $\delta^{13}C$ values of samples stored for more than 9 months in ethanol suppressed significant differences between fresh and preserved samples in our experiment.

No major $\delta^{15}N$ changes were recorded for any of the preservation methods. Freezing or the use of formaldehyde and ethanol did not lead to any significant differences. Some differences were present in the drying experiment, but these changes were not consistent over time and occurred between times of analysis and not between $\delta^{15}N$ values of dried samples and those of T_0 samples. Seasonal variations in temperature and humidity in the storage room could contribute to this inconsistent variability in $\delta^{15}N$ values throughout the experiment. Conflicting results have been reported for the impact of formaldehyde and ethanol on $\delta^{15}N$ values,[9,10,14,27] suggesting that $\delta^{15}N$ values are generally not affected by preservation.

In this study, the $\delta^{34}S$ values of sea stars were much more variable than the two other isotopic ratios. The standard deviation for a sea star sample randomly chosen as a secondary analytical standard was 0.3‰ for $\delta^{13}C$ values, 0.2‰ for $\delta^{15}N$ values, but 0.8‰ for $\delta^{34}S$ values. This could be caused by a higher natural variability of this parameter in sea stars, but also because of a higher analytical error, as sea star tissues contain low amounts of total sulfur. Our results therefore have to be interpreted with caution. Nevertheless, they suggest that formaldehyde and possibly ethanol reduce $\delta^{34}S$ values. In formaldehyde, the $\delta^{34}S$ values of preserved samples were significantly lower than those of fresh samples after the first month of preservation. However, adding a correction factor of 1.5‰ to the $\delta^{34}S$ values of preserved samples in our experiment allowed the effects of preservation to be corrected, despite the within-treatment error being close to the average $\delta^{34}S$ value shift (−1.5 ± 1.2‰). A weaker and slower decrease in $\delta^{34}S$ values occurred in samples stored in ethanol, with the decrease being slightly significant only at 24 months of preservation. By comparison, previous studies observed different effects of preservative fluids on $\delta^{34}S$ values. Indeed, an increase in mean $\delta^{34}S$ values was observed in teleosts fixed with formaldehyde and then stored in ethanol (0.8 ± 0.5‰)[13] while no effects of ethanol preservation were observed on $\delta^{34}S$ values in bear tissues.[22] Our results suggest that using a correction factor to mitigate the effects of ethanol on $\delta^{34}S$ values is not adequate. The within-treatment error was indeed higher than the average $\delta^{34}S$ value shift (−0.7 ± 1.0‰). Moreover, although using this correction factor prevented significant differences between the $\delta^{34}S$ values of T_0 samples and those of samples stored for 24 months, it created previously non-existing significant differences between samples stored for 24 months and several other times of analysis. Furthermore, use of this correction factor seemed to increase the overall inter-treatment variability, as shown by the higher ANOVA *F* value. Considering these results, we do not advise using correction factors for the $\delta^{34}S$ values of star tissues preserved in ethanol. For samples stored frozen or dried, no significant or consistent differences in $\delta^{34}S$ values between fresh and preserved samples were observed.

The ellipse parameters computed with $\delta^{13}C$ and $\delta^{15}N$ values were slightly affected by preservation, resulting in estimation of SEA_B being inconsistently affected in samples stored dried or in ethanol while not affected by freezing and storage in formaldehyde. Consequently, preservation does not seem to be an obstacle to the study of isotopic niches computed with $\delta^{13}C$ and $\delta^{15}N$ values, and thus trophic niches of sea stars using ellipse-based methods and the lack of overlap between fresh and preserved samples are more likely to be the result of the changes in mean $\delta^{13}C$ values. By contrast, inconsistent variations in ellipse parameters occurred when computed with $\delta^{13}C$ and $\delta^{34}S$ values, because of both the preservation-induced changes in $\delta^{34}S$ values and the higher variability of this parameter. While these results need to be further tested both in sea stars and in other taxa, caution is advised when dealing with sulfur isotopic ratios of fluid-preserved samples.

5 | CONCLUSIONS

Our results show that the preservation method has to be taken into account when determining stable isotope ratios of carbon in sea stars. Freezing and drying appear to be the best preservation methods (Table 2). Freezing did not induce changes in $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values, or ellipse parameters when computed with $\delta^{13}C$ and $\delta^{15}N$ values. This result is in accordance with previous studies where freezing is generally considered as one of the best preservation methods with no effect on stable isotope ratios being observed,[8,12,23,46] although some exceptions occurred.[25,29,30] Drying appeared to have no effect on $\delta^{13}C$ values and minimal effect on $\delta^{34}S$ values but long-term drying could induce inconsistent variability of $\delta^{15}N$ values. While formaldehyde induced a sharp decrease of −0.8 ± 0.5‰ in $\delta^{13}C$ values during the first month of preservation, the $\delta^{13}C$ values remained stable once altered and it is thus possible to correct the effects of preservation with a correction factor, no

TABLE 2 Summary of the influence of preservation methods on $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values, C/N ratios and Bayesian estimation standard ellipse area (SEA_B) computed with $\delta^{13}C$ and $\delta^{15}N$ values and with $\delta^{13}C$ and $\delta^{34}S$ values in *Marthasterias glacialis* tissues preserved for 24 months

	$\Delta\delta^{13}C$ (‰)	$\Delta\delta^{15}N$ (‰)	$\Delta\delta^{34}S$ (‰)	C/N	SEA_B with $\delta^{13}C$ and $\delta^{15}N$	SEA_B with $\delta^{13}C$ and $\delta^{34}S$
Freezing	Not significant	Not significant	Inconsistent	Not significant	Not significant	Not significant
Drying	Not significant	Inconsistent	Inconsistent	Not significant	Inconsistent	Not significant
Formaldehyde	↓ −0.8 ± 0.5	Not significant	↓ −1.5 ± 1.2	Inconsistent	Not significant	Not significant
Ethanol	↑ +0.6 ± 0.5	Not significant	↓ −0.7 ± 1.0[a]	↑ 0.22 ± 0.10	Inconsistent	Not significant

[a]Significant difference only between samples at T_0 and preserved samples at 24 months.

matter how long sea stars were stored in formaldehyde. A decrease and then stability of $\delta^{13}C$ values in samples stored in formaldehyde was previously observed,[8,9,31] including at the decadal scale.[27] Furthermore, $\delta^{15}N$ values and ellipse parameters computed with $\delta^{13}C$ and $\delta^{15}N$ values did not appear to be strongly affected by preservation in formaldehyde, and the change of the position of the ellipse is the result of the changes of mean $\delta^{13}C$ values. $\delta^{34}S$ values decreased in samples stored in formaldehyde but this change can be corrected for. The results showed that $\delta^{13}C$ values are affected by storage in ethanol, probably because of lipid extraction. Considering the significant increase in $\delta^{13}C$ values observed in other taxa,[8-10] some knowledge of the lipid (or other ethanol-soluble compounds) content of samples may be beneficial before analyzing sea star samples stored in ethanol. By contrast, $\delta^{15}N$ values were not affected by ethanol preservation. Furthermore, long-term preservation in ethanol appeared to induce a decrease in $\delta^{34}S$ values. However, using a correction factor for $\delta^{34}S$ values in sea star samples stored in ethanol is not advised. Overall, the four preservation methods tested in this experiment minimally impacted stable isotope ratios or induced impacts that could be dealt with by using correction factors. Such results tend to indicate that sea star samples stored in preservative fluids and, thus, those stored by museums, may be used for trophic ecology studies using stable isotope ratios.

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DATA AVAILABILITY STATEMENT

The raw data underlying this article are freely available at the PANGAEA database (https://www.pangaea.de/) at https://doi.pangaea.de/10.1594/PANGAEA.906520.

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