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EFFECTS OF PRESERVATION METHODS ON THE $\delta^{13}C$ AND $\delta^{15}N$ SIGNATURES IN MUSCLE TISSUES OF TWO FRESHWATER FISH SPECIES

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ABSTRACT

Stable isotope is a powerful method for characterizing flows of energy through ecosystems. The power of this method, however, may be affected by preservation methods of the samples. We investigated the effects of four common preservatives (salt, formalin, and ethanol and freezing [control] and preservation duration (six and three months) on $\delta^{15}N$ and $\delta^{13}C$ values of two freshwater fish species, *Perca fluviatilis* (perch) and *Blicca bjoerkna* (silver bream). Six-month preservation caused little enrichment in $\delta^{15}N$ of both species compared to three month but had almost the same effects on $\delta^{13}C$ values of both species as in three-month preservation. All methods caused significant shifts (enrichment) in $\delta^{15}N$ of both species, and the effects in general were greater in perch (range: 0.28‰-2.19 ‰) than in bream (range: 0.31‰-1.29‰), which suggested that preservative induced shifts in $\delta^{15}N$ was species-specific. The methods caused little enrichment (ethanol-range: 0.03‰ - 0.26‰ bream and 0.30‰-0.48 ‰ perch and salt: 0.18 ‰ perch three month) and depletion (salt-range: 0.03 ‰-0.13‰ bream and 0.13‰ perch six month) in $\delta^{13}C$. Of the preservatives, however formalin had significant but consistent effects on $\delta^{13}C$ in both species (-1.27‰ and -1.25‰) for the entire preservation duration. Preservation-induced shifts in $\delta^{13}C$ were consistent in direction and magnitude for both species. The results suggested that ethanol and salt could be used without correction factor and formalin with correction factor for preservation of samples solely in $\delta^{13}C$ studies.. For the studies requiring use of carbon and nitrogen together, however, ethanol at least six month in preservation may be suitable for storing samples when considering detection of changes less than 2 ‰ is required in ecological applications.

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KEYWORDS: Formalin, ethanol, salt, preservation, stable isotopes, freshwater fish, *Perca fluviatilis*, *Blicca bjoerkna*

1. INTRODUCTION

Stable-isotopes of carbon and nitrogen have been increasingly used as a tool to evaluate the sources of energy and organic carbon in ecosystems [1-5], food web structure [5,6], trophic position [7,8] and anthropogenic impacts on ecosystems [9-11].Stable isotope methods providing valuable insights into ecosystem processes, species interactions, and community dynamics are preferred over stomach contents analysis. Stable isotopes provide information on food items assimilated and it is an easy method to determine food webs compared to stomach contents analysis. Although stable isotopes of carbon and nitrogen provide powerful information on ecosystem dynamics, inconsistencies in the use of techniques for preparing and storing samples prior to the analysis preclude the comparison of results from different investigations.

From the methodological point of view, the usual way of processing biological samples prior to stable isotope analysis is drying. However, drying immediately after collection is not always possible. Therefore, samples often need to be stored for some time prior to analysis, ideally by deep-freezing or immersion in liquid nitrogen [12]. Again, these methods of preservation are not always feasible in field situation, especially when working in remote areas or sampling a large number of species. To overcome this difficulty, researchers have tried to preserve samples using chemical solution including formalin, ethanol and salt. A number of studies have been performed in both aquatic and terrestrial organisms in order to determine how much a chemical product alters isotopic values [12-26]. But there is little apparent agreement on the significance of observed preservation effects [26]. Some studies found both significant and non-significant statistical preservation effects

[17] and Edwards et al. [19] related significant variations to the differences in species. Bosley and Wainright [16] showed that formalin, formalin/ethanol and mercuric chloride solutions produced a significant increase in $\delta^{15}N$ values and a decrease in $\delta^{13}C$ values in two marine organisms. Arrington and Winemiller [18] concluded that salt was the best way of preserving fish samples because of the fact that it changes isotopic signature little and is easily applicable in remote field conditions.

The studies on the effects of preservation on stable isotope signature in general indicate that chemical preservative changes isotopic signature of organisms. Some studies indicate that changes in isotopic signature are species-specific [17, 26, 27], whereas others point out the importance of preservation duration of animal tissues [19, 27]. Therefore, the aim of this study is to quantify time and species-specific changes in isotopic signatures of muscle tissues of two freshwater fish species, European perch (*Perca fluviatilis*) and silver bream (*Blicca bjoerkna*). These organisms are chosen because they occupy different trophic position; the perch being a carnivore and bream an omnivore. We used the same preservative (deep-freezing, formalin, ethanol and salt) used in literature for this kind of research in order to compare our results with those of others.

2. MATERIALS AND METHODS

2.1. Sampling methods and preservations

We collected 27 individuals from each species (perch and silver bream) from Suat Uğurlu Dam Lake located on the Yeşilırmak River of Turkey. Immediately after capture, fishes were euthanized and four pieces of muscle tissue (~3 g) were removed from the dorsum of each individual. Each piece of fish muscle tissue was rinsed with distilled water. A piece of muscle tissue sample was separately placed in closable plastic bag and stored at -20ºC in deep-freezer. We used -20 ºC to froze samples as control as it is used in other studies [18]. The remaining three pieces of tissue were randomly put into three 50 ml plastic bottles and bottles were filled with ethanol (70% v/v), buffered formalin (10%) or crystallized non-iodized salt. Each bottle was fully filled with given preservative ensuring that preservative was in contact with the entire surface area of the muscle sample. In order to understand the effects of preservatives separately, we did not transfer formalin prererved muscle tissue into the ethanol.

First and second set of samples consisting of 15 and 12 individuals from each species were prepared for stable isotope analysis after 3 and 6 months of preservation. The samples were soaked and then rinsed with distilled water to remove excess preservative [18]. Deep-frozen samples were rinsed with distilled water only. Salt-preserved samples were rinsed with distilled water and then soaked in distilled water for 4h [18]. Formalin and ethanol preserved samples were rinsed with distilled water and soaked in distilled water for 48 h [18]. All samples were then dried at 60 ºC for approximately 48 h in an oven. After drying, samples were ground to a fine powder with a mortar and pestle. Approximately 2 mg of powdered fish muscle were weighed into ultraclean tin capsules (Elemental Microanalysis Limited, England). Prepared samples were analyzed for percent carbon, percent nitrogen and isotope ratio (^{13}C:^{12}C and ^{15}N:^{14}N) by an isotope-ratio mass spectrometer (Carlo Erba NA1500 CHN Elemental Analyzer coupled to a Thermo Delta V Isotope Ratio Mass Spectrometer via a Thermo Conflo III Interface) at Analytical Chemistry Laboratory, University of Georgia, USA. Results of stable isotope analyses were reported as parts per thousand (‰) deviations from the international standards Pee Dee Belemnite limestone for carbon and atmospheric N_2 for nitrogen, according to the following equation.

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where X is ^{15}N or ^{13}C and R is the corresponding ratio ^{13}C:^{12}C and ^{15}N:^{14}N.

2.2. Data Analysis

Impacts of preservation method on stable isotope signatures ($\delta^{13}C$ and $\delta^{15}N$) of each species within each time period were analysed by using one way analysis of variance (ANOVA) model. Combined effects of all preservatives and preservation duration on stable isotope signatures were evaluated by using two-way ANOVA model. When there were significant differences among the means, we used Student-Newman-Keuls (SNK) test to evaluate all pair wise comparison tests after testing for data normality and homogeneity of variance. All statistical analyses were done using SAS 8.0 for Windows software. We also performed linear regression analysis between the freezing and other preservatives to evaluate the predictability of the preservatives.

3. RESULTS

3.1. The effects of preservative within each time period

We found significant differences for $\delta^{15}N$ values between frozen and preserved samples of each species for both (three and six month) preservation duration (bream: $F_{3,42}$=19.16; P<0.001(three); $F_{3,33}$=6.40; P=0.002 (six); perch: $F_{3,42}$=272.52; P=0.001 (three); $F_{3,33}$=28.03; P=0.001 (six) (Table 1). Increasing $\delta^{15}N$ values from 0.28‰ to 2.19‰ relative to frozen samples, salt had greater impacts on $\delta^{15}N$ values of both species for each preservation duration, except for six month preservation of perch sample, on which ethanol had more impact elevating the mean by 0.70‰ (Table 1). The magnitudes of salt-induced increases in $\delta^{15}N$ values of each species for each preservation duration, on the other hand, were not significant than those of formalin and ethanol with the exception for three month preservation of bream samples, on which salt had significantly greater impact (1.29 ‰) than ethanol (0.80‰) and formalin (0.71‰). In general, three month preservation of the samples with

TABLE 1 - Species-specific mean isotopic signature of nitrogen and carbon stable isotopes for each preservation duration. Diff: Differences between mean of frozen samples and other preservatives. r^2 regression coefficient between frozen samples and other preservatives. Bolded values of r^2 are significant at P<0.05. SL: Standard length of fish. The same letter indicates no differences among the mean values.

Fish species	*N*	*Time (Month)*	*Preservative*	*Mean $\delta^{15}N(\pm SD)$*	*r^2*	*Diff.*	*Mean $\delta^{13}C(\pm SD)$*	*r^2*	*Diff.*	*SL (±SD)(mm)*
Blicca bjoerkna	15	3	Freezing	13.78±0.96 (A)			(A)-29.05±0.80			
Blicca bjoerkna	15	3	Salt	15.07±0.34 (B)	**0.293**	1.29	(A)-29.09±0.64	**0.468**	-0.03	
Blicca bjoerkna	15	3	Ethanol	14.58±0.30 (C)	0.018	0.80	(B)-28.79±0.66	**0.688**	0.26	**165.63±15.59**
Blicca bjoerkna	15	3	Formalin	14.49±0.30 (C)	0.003	0.71	(C)-30.14±0.64	0.003	-1.09	
Perca fluviatilis	15	3	Freezing	12.71±0.62 (A)			(A)-27.15±0.75			
Perca fluviatilis	15	3	Salt	14.91±0.54 (B)	**0.557**	2.19	(B)-26.97±0.72	**0.966**	0.18	
Perca fluviatilis	15	3	Ethanol	14.90±0.46 (B)	**0.484**	2.19	(C)-26.67±0.67	**0.945**	0.48	**155.00±12.30**
Perca fluviatilis	15	3	Formalin	14.76±0.44 (B)	**0.489**	2.05	(D)-28.35±0.67	**0.946**	-1.20	
Blicca bjoerkna	12	6	Freezing	13.72±0.54 (A)			(A)-28.97±0.79			
Blicca bjoerkna	12	6	Salt	14.27±0.48 (B)	**0.653**	0.54	(A)-29.10±0.74	**0.925**	-0.13	
Blicca bjoerkna	12	6	Ethanol	14.07±0.46 (B)	**0.640**	0.35	(A)-28.94±0.67	**0.930**	0.03	**159.08±15.04**
Blicca bjoerkna	12	6	Formalin	14.04±0.46 (B)	0.086	0.31	(B)-30.45±0.61	**0.410**	-1.48	
Perca fluviatilis	12	6	Freezing	14.10±0.59 (A)			(A)-27.44±0.81			
Perca fluviatilis	12	6	Salt	14.38±0.71 (B)	**0.698**	0.28	(A)-27.56±0.92	**0.764**	-0.12	
Perca fluviatilis	12	6	Ethanol	14.80±0.68 (B)	**0.940**	0.70	(B)-27.14±0.84	**0.981**	0.30	**148.83±10.71**
Perca fluviatilis	12	6	Formalin	14.45±0.64 (B)	**0.875**	0.34	(C)-28.59±0.83	**0.927**	-1.14	

all three preservatives had higher impact on $\delta^{15}N$ values of each species (range 0.71‰-2.19‰) (i.e., more enriched) than six month preservation (range 0.28‰-0.70‰) (Table 1).

Relationships between $\delta^{15}N$ values of frozen and preserved samples for six month preservation were all significant for each species (P<0.05), except for formalin preserved bream samples that had weaker and insignificant association (P<0.05; r^2 =0.086) (Table 1). Except for ethanol and formalin preserved bream samples, three month preserved samples had also significant but relatively weaker (mean: r^2=0.307; range: 0.003-0.557) relationships with frozen samples for $\delta^{15}N$ values of both species (p<0.05), compared to six month (mean r^2=0.649; range 0.086-0.940). The salt-induced $\delta^{15}N$ values of both species had significant association with frozen samples for each preservation duration (mean r^2=0.550; range: 0.293-0.698) (Table 1). Compared to bream, $\delta^{15}N$ values of perch samples preserved with all preservatives had significant relationships with frozen samples for each preservation duration (P< 0.05), with r^2 ranging from 0.484 to 0.940. Of the preservatives, ethanol had the highest association with frozen samples for $\delta^{15}N$ values, which was observed only for perch for six month preservation duration (r^2=0.940) (Table 1).

In the case of $\delta^{13}C$ values, we also found significant differences between frozen samples and preserved ones of both species for each preservation duration (bream $F_{3,42}$= 61.06;P=0.001 (three); $F_{3,47}$=66.62;P=0.001(six); perch: $F_{3,42}$=6 36.61;P=0.001 (three); $F_{3,33}$=28.03; P=0.001(six) (Table 1). We observed for both species for each preservation duration that ethanol and salt, except for six month preservation of perch samples with salt, caused $\delta^{13}C$ to decrease (i.e., depletion). The magnitude of decrease (depletion) in $\delta^{13}C$ caused by salt (range:0.03 ‰ to 0.13 ‰) did not differ significantly (p>0.05) from frozen samples of both species for each preservation duration, except for six month preservation of perch samples that were elevated by 0.18 ‰. Except for six month preservation of bream samples that were enriched little (0.03‰), ethanol, on the other hand, had statistically significant impact on $\delta^{13}C$ values, elevating the mean by 0.26‰ and 0.48 ‰ for three month preservation of both species and by 0.30 ‰ for six month preservation of perch samples (Table 1). Formalin caused significant (P<0.05) and greater impacts on $\delta^{13}C$ values than ethanol and salt, decreasing (i.e. depleting) the mean values of bream and perch samples by 1.09 ‰, 1.48 ‰, 1.20 ‰ and 1.14 ‰ for three and six month preservation duration, respectively. Unlike $\delta^{15}N$ values, differences between mean $\delta^{13}C$ of frozen samples and preserved ones did not vary much between preservation durations (Table 1).

Associations between $\delta^{13}C$ values of frozen samples and preserved ones of both species were significant (p< 0.05) for each preservation duration, except for bream samples preserved with formalin for three months (Table 1). Regression coefficients obtained for $\delta^{13}C$ were higher (mean r^2 =0.746; range: 0.003-0.981) than the coefficients obtained for $\delta^{15}N$ (mean r^2 =0.478; range: 0.003-0.940)). The $\delta^{13}C$ values of ethanol-preserved samples of both species had greater association (i.e., higher regression coefficient) with the frozen samples for each preservation duration (range: r^2=0.688-0.981). Salt, on the other hand, another preservative having stronger associations with frozen samples, especially for three-month preserved perch samples (r^2=0.966) and six-month preserved bream samples (r^2=0.925). Except for three-month preserved bream samples, regression coefficients for $\delta^{13}C$ did not vary much between preservation durations (Table 1).

3.2. The effects of preservation for the entire preservation duration

Averaging for the entire preservatives, three month preserved bream samples had significantly higher mean $\delta^{15}N$ values (14.48± 0.71 SD) than those preserved for six month (14.02±0.51 SD) ($F_{1,100}$=15.23; P<0.0001) (Table 1). Contrary to bream samples, mean $\delta^{15}N$ values of perch samples preserved for three (14.32±1.06) and six month (14.43± 0.68 SD) did not exhibit significant differences ($F_{1,100}$= 0.99; P=0.323). Mean $\delta^{13}C$ values of three month (-29.26 ± 0.91 SD) and six month (-29.36±0.93 SD) preserved bream samples did not differ significantly, but did differ significantly for perch samples (-27.28±0.94 SD (three); -27.73± 1.03 SD (six) ($F_{1,100}$=8.78; P=0.004) (Table 1).

Averaging for the entire preservation duration, preservatives significantly altered $\delta^{15}N$ values of both species ($F_{3,100}$=5.59;P<0.0001 bream; $F_{3,100}$=38.89;P<0.0001 perch) (Table 2).

Salt, elevating the mean by 0.96‰, had significantly higher impact on $\delta^{15}N$ values of bream samples than ethanol (0.59 ‰) and formalin (0.51‰), which did not show significant difference from each other. Three preservatives, on the other hand, significantly altered the mean $\delta^{15}N$ values of perch samples by 1.34‰ (salt), 1.49 ‰ (ethanol) and 1.29 ‰ (formalin), which were higher than their effects on bream samples (Table 2).

In the case of $\delta^{13}C$ values averaged for the entire preservation duration, we also found significant differences between the mean values of frozen samples and preserved ones for both species ($F_{1,100}$=23.82; P<0.001 bream; $F_{3,100}$= 23.53; P<0.0001 perch) (Table 2). Formalin had significantly higher impact on $\delta^{13}C$ values of both species, depleting (more negative) the mean values by 1.27‰ (bream) and 1.25‰ (perch). The other preservatives, on the other hand, did not significantly alter the mean $\delta^{13}C$ values of frozen samples of both species. Ethanol enriched (less negative) the mean $\delta^{13}C$ values of frozen samples of both species by 0.16 ‰ (bream) and 0.40‰ (perch), whereas salt increased and decreased the mean $\delta^{13}C$ value of perch by 0.05 ‰ and 0.07‰, respectively (Table 2).

Aside from barely significant associations between the $\delta^{15}N$ values of frozen and salt preserved samples for bream ($F_{1,25}$=5.85; P=0.023; r^2=0.189) and ethanol preserved for perch samples ($F_{1,25}$=6.58; P=0.017; r^2=0.210), the relationships between $\delta^{15}N$ values of frozen and preserved samples pooled by preservation duration for both species were not significant with regression coefficients (r^2) ranging from 0.025 to 0.098. Unlike these weak and insignificant relationships for $\delta^{15}N$ values, $\delta^{13}C$ values of frozen samples for bream had stronger and significant associations with preserved ones ($F_{1,25}$=81.48; P<0.001; r^2=0.76 ethanol; $F_{1,26}$=248.15; P<0.001; r^2=0.661 salt; $F_{1,26}$=25.59; P<0.001; r^2=0.51 formalin) (Table 2). The associations for $\delta^{13}C$ values were stronger for perch ($F_{1,25}$=455.22; P<0.001; r^2=0.95, ethanol; $F_{1,25}$=331.91; P<0.001; r^2=0.93 formalin; $F_{1,25}$=130.40; P<0.001; r^2=0.83 salt) compared to bream (Table 2).

4. DISCUSSION

Preservatives (ethanol, formalin and salt) significantly altered $\delta^{13}C$ and $\delta^{15}N$ values of dorsal muscle tissues obtained from perch and silver bream. This result agreed with previous studies suggesting the magnitude of the preservation effect was medium dependent [14, 17-19, 22, 24, 26]. The mean $\delta^{15}N$ values of frozen samples were significantly lower than those of mean $\delta^{15}N$ values of other preservatives. Mean $\delta^{15}N$ values of ethanol, formalin and salt samples did not differ from each other, suggesting that all three preservatives had almost the same effects on nitrogen isotopic signatures of both species. Of the preservatives, however, salt had the greatest effects, except for six-month preserved perch samples, enriching the isotopic signature of both species from 0.28‰ to 2.19‰ with a mean value of 1.08‰ for each preservation duration and from 0.96‰ to 1.34‰ for bream and perch across preservation duration, a result agreed with the result obtained by Arrington and Winemiller [18] with a value of 0.72‰ enrichment. From a fieldwork perspective, salt is an easy method to use to preserve a range of tissues and is applicable in remote field sites; but it greatly affected $\delta^{15}N$ and resulted in greater sample variability for each and across preservation dura-

TABLE 2 - The mean values of *Blicca bjoerkna* and *Perca fluaviatilis* samples pooled by the entire preservation duration. Diff: Differences between mean of frozen samples and other preservatives. r^2 regression coefficient between frozen samples and other preservatives. SL: Standard length of fish. The same letter indicates no differences among the mean values.

Fish species	*N*	*Preservatives*	*Mean $\delta^{15}N$ ±SD*	*Diff.*	*r^2*	*Mean $\delta^{13}C$±SD*	*Diff.*	*r^2*
Blicca bjoerkna	27	Freezing	13.76±0.78(A)			(A)-29.01±0.78		
Blicca bjoerkna	27	Salt	14.71±0.57(B)	0.96	**0.189**	(A)-29.09±0.67	-0.07	**0.660**
Blicca bjoerkna	27	Ethanol	14.35±0.66(C)	0.59	0.098	(A)-28.85±0.66	0.16	**0.766**
Blicca bjoerkna	27	Formalin	14.29±0.63(C)	0.53	0.064	(B)-30.28±0.63	-1.27	**0.508**
Perca fluviatilis	27	Freezing	13.33±0.78(A)			(A)-27.28±0.78		
Perca fluviatilis	27	Salt	14.67±0.85(B)	1.34	0.025	(A)-27.23±0.85	0.05	**0.839**
Perca fluviatilis	27	Ethanol	14.86±0.73(B)	1.49	**0.210**	(A)-26.88±0.73	0.40	**0.948**
Perca fluviatilis	27	Formalin	14.62±0.76(B)	1.29	0.074	(B)-28.53±0.76	-1.25	**0.929**

tion for both species. In parallel to a previous study, our results suggested that salt is as good as freezing for preservation of fish muscle for $\delta^{13}C$, but not for preserving of fish muscle for $\delta^{15}N$.

While formalin preservations depleted $\delta^{13}C$ values of both species, ethanol enriched $\delta^{13}C$ relative to the $\delta^{13}C$ value of frozen samples. Similar results were obtained for previous studies [17, 18] and studies cited by Barrow et al. [28]. As suggested by Hobson et al. [14], this depletion in $\delta^{13}C$ is likely related to the direct incorporation of isotopically light carbon from the formalin. Formalin binds to certain biochemical constituents of the tissues and contains its own source of carbon. Therefore, it is likely that changes in isotope signatures of samples are at least dependent on isotopic composition of fixative and the amount of the fixative bound to the tissue [17]. The mean depletion caused by formalin in $\delta^{13}C$ values of both species was approximately 1.23‰, which was close to the values obtained by some previous studies [14,17]. Kaehler and Pakhomov [17] indicated that formalin should not be used as a preservative for storing samples intended for carbon isotope analysis due to the fact that depletion in $\delta^{13}C$ varied greatly between species. Formalin preservation that had greater impact on $\delta^{13}C$ values of both species could not be at least used for the reasons (i.e. high variability among species) presented by Kaehler and Pakhomov [17]. Consistency in shifts caused by formalin between species and preservation duration in our study, however, may indicate that formalin could also be used with correction factor.

Ethanol significantly increased $\delta^{15}N$ values of both species with the values ranging from 0.35 ‰ to 2.19 ‰. On the other hand, ethanol significantly enriched $\delta^{13}C$ values of the species for each preservation duration with the exception of six month preservation of bream samples, which did not significantly differ from frozen samples. The mean values of ethanol-preserved samples pooled across entire preservation duration significantly differed from the mean values of frozen samples of both species (0.16 ‰ bream and 0.40 ‰ perch). These mean values were lower than those obtained by Kaehler and Pakhomov [17] ranging from 0.7‰ to 1.5 ‰. Enrichment caused by ethanol was more likely due to fact that ethanol acted as solvent of isotopically lighter compounds which have lower carbon values such as lipids present in the samples [29]. The studies [15, 30-32] showed that extraction of isotopically lighter lipids from body tissues may enrich the whole body $\delta^{13}C$ of an organism. As Kaehler and Pakhomov [17] indicated, as ethanol is known to act as a solvent as well as a preservative, ethanol-induced enrichment in $\delta^{13}C$ may, therefore, be explained by lipid extraction. Kaehler and Pakhomov [17] advised that ethanol should not be used for storing samples due to fact that ethanol-induced enrichment varied from species and over time. Ethanol-induced changes in mean values of $\delta^{13}C$ varied from 0.03 ‰ to 0.26‰ for six and three month preserved bream samples and from 0.30 ‰ to 0.48 ‰ for six and three month preserved perch samples, respectively. Enrichments by this preservative across preservation duration for bream and perch were 0.16‰ and 0.40‰, respectively. These results suggested that mean values of ethanol preserved samples exhibit little variation among species and preservation duration.

The regression coefficients (r^2) between $\delta^{13}C$ values of frozen samples and other preservatives for both species were higher (range: 0.010-0.980, mean: 0.750±0.300 SD for each preservation duration; range:0.510-0.950 mean: 0.770± 0.170 SD across preservation duration) compared to $\delta^{15}N$ (range: 0.01-0.94, mean:0.48±0.31 SD for each preservation duration; range 0.03-0.21, mean: 0.11±0.07 SD across preservation duration). Ethanol among preservatives yielded higher coefficients (r^2) (range: 0.690-0.980 mean: 0.890± 0.130 SD for each preservation duration; 0.770 bream and 0.950 perch, across preservation duration). Meanwhile, $\delta^{13}C$ values of salt (range: r^2= 0.010-0.950, mean: 0.570±0.450 SD for each preservation duration ; r^2= 0.660 and 0.840 for bream and perch samples across preservation duration, respectively) and formalin preserved samples (range: r^2= 0.470-0.970, mean: 0.780±0.230 SD for each preservation duration and r^2=0.510 and r^2=0.930 for bream and perch across preservation duration) increased with $\delta^{13}C$ values of frozen samples. From the point of the regression analysis, all three preservatives could be used for storing samples due to fact that all three preservatives changed $\delta^{13}C$ values in predictable way. Our study, contrary to the study by Kaehler and Pakhomov [17], suggests that ethanol may be used to store samples. Although ethanol may be used as storing samples, it is flammable and difficult to transport because of the safety regulations [28]. Due to ease nature of transport, salt which also changed predictable way with frozen samples may be used as an alternative preservative medium to store sample for $\delta^{13}C$ in remote areas.

Earlier temporal preservation studies indicated that long-term preservation does not appear to alter isotopic ratios [19, 33] as after a few weeks of preservations [17] the tissue has come to equilibrium with the preservative and no addition occurs [34].We found a similar trend of temporal changes in isotopic signatures of samples. Compared to three month preservation, in general, the six month preservation for both species caused little depletion in $\delta^{15}N$ values. This stabilizing (decline in differences between freezing and other preservatives) trend was more pronounced in $\delta^{15}N$ values of both species compared to $\delta^{13}C$ values. Regression coefficients between frozen samples and other preservatives also suggested that long term preservation of samples may stabilize $\delta^{13}C$ and $\delta^{15}N$ values of both species, especially for bream.

Kaehler and Pakhomov [17], combining their data and other studies, suggested that the impacts of formalin and ethanol on $\delta^{13}C$ values are highly variable between species and that of use of correction factors may, therefore, not be possible. On the other hand, Kaehler and Pakhomov [17] found that $\delta^{15}N$ values were affected to a far lesser degree. Kaehler and Pakhomov [17] argued that nitrogen signature of preserved samples may facilitate the use of

preserved samples in trophic dynamic studies that are concerned solely with stable isotope of nitrogen. Our study, however, presented data that were not in accordance with Kaehler and Pakhomov [17] findings. Compared to nitrogen signature, carbon signatures varied far less among species and preservation duration. Even so, formalin depleted carbon signature most, but amount of depletion caused by formalin are nearly constant, i.e., it did not change much among species and preservation duration. Therefore, we suggest that preserved samples may be used in trophodynamic studies that are concerned solely with stable isotopes of carbon. The differences between the results of our study and Kaehler and Pakhomov [17] study may be due to differences in species used in the studies.

Carbon sources differ by greater than 2‰ for many ecosystems [19]. For example, the average $\delta^{13}C$ value was found to be -22‰ compared to -17‰ for marine benthic algae [35] and $C_3$and C_4 plants were also found to differ approximately 14‰ [35]. Using this information, Carabel et al. [36] reasoned that as long as carbon sources are isotopically different by more than 2‰, a shift of this magnitude in preserved specimens would not therefore confound any results. In our study, significant shift caused by preservation techniques in $\delta^{13}C$ values were less than 2‰. Preservation-induced shifts in carbon isotopic signature in our study were lower than 2‰. From this point of view, in all cases, except for formalin which made the largest variation (less than 2‰ though) it seemed that other preservatives could be used to preserve samples especially for the studies solely using carbon signature. On the other hand, diet-tissue fractionation of $\delta^{13}C$ in trophodynamic studies is assumed to be 0-1‰ per trophic level or smaller among species in a community [37]. The magnitudes of shifts caused by all preservative except for formalin, salt and ethanol were lower than 1‰ and directionally predictable; suggesting that ethanol and salt could be used as preservatives when freezing is not available at remote settings.

Some studies indicated that freezing at 20-25 °C, the range of degree commonly used to store samples for stable isotope samples [5,18,38] when fresh processing of sample especially in remote field settings is not possible, may have significant effects on $\delta^{13}C$ and $\delta^{15}N$ values of several organisms including clams [33]. Some other studies, on the other hand, indicate that freezing at 20-25°C did not significantly alter the $\delta^{13}C$ and $\delta^{15}N$ values of fish, octopus, and kelp [5, 17, 28, 39]. As it is seen, freezing at 20-25°C has varying effects on $\delta^{13}C$ and $\delta^{15}N$ values. More recently, Fanneli et al. [38] used the frozen samples of deep-sea macrofauna at 20°C as control to compare the effects of formalin and ethanol. In our study, we also used the samples frozen at 20 °C as control and believe that freezing did not alter significantly $\delta^{13}C$ and $\delta^{15}N$ values of fish samples. Considering the effect of formalin, salt and ethanol are less than 2 ‰, the effects of freezing at 20 °C, commonly used to store samples when rapid processing of the sample is not possible, may not have significant effects on $\delta^{13}C$ and $\delta^{15}N$ values.

5. CONCLUSIONS

The results presented in this study indicated that three preservation methods have significantly different effects on isotopic signatures of muscle tissues of two freshwater fish species. The data in this study presented the results contrary to other studies indicating that the effects of formalin and ethanol on carbon signature are highly variable between species and that the use of correction factors may not be possible. The data of our study indicated that salt, ethanol and formalin made almost the same effects on carbon isotopic signatures of both species for both preservation duration, which suggested that all three preservatives could be used in place of frozen samples to preserve samples in studies of using solely $\delta^{13}C$. However, largest depletion caused by formalin may indicate that formalin could be used to preserve samples of carbon isotopic signature with the use of correction factor. On contrary to $\delta^{13}C$, preservation effects on $\delta^{15}N$ are highly variable among species and preservation duration and that of use of correction factor may, therefore, not be possible to preserve samples for nitrogen isotopic signature. The fact that, however, salt and ethanol causing differences lower than one and change predictable way with frozen sample may suggest that ethanol and salt could be used as preservatives after at least storing the samples for six month when freezing is not available at remote settings for carbon and nitrogen isotopic studies requiring to use both of them. For the studies requiring use of carbon and nitrogen together, however, ethanol at least six month in preservation may be suitable for storing samples when considering detection of changes less than 2 ‰ is required in ecological applications.

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