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Influence of sample preparation on estuarine macrofauna stable isotope signatures in the context of contaminant bioaccumulation studies

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ABSTRACT

The ratios of stable isotopes of carbon and nitrogen provide important information on food sources of aquatic organisms and trophic structure of aquatic food webs. For many studies, trophic position and food source are linked to bioaccumulation and trophic transfer of contaminants from prey to predators. In these cases, it is useful to use measurements on whole organisms to make direct comparisons of contaminant bioaccumulation and food web attributes. There is a great deal of variation in methods used for stable isotope analysis, particularly in the selection of tissue type and sample preparation prior to stable isotope analysis. While there have been aquatic studies that examined methodological differences, few have focused on estuarine organisms. In this study, the effects of depuration and tissue dissection on the stable isotope enrichment of common estuarine invertebrates and fish were examined. Homogenized tissues of non-depurated whole organisms were compared to dissected muscle tissue or depurated whole organisms. A 24 h depuration did not change the mean $\delta^{15}N$ and $\delta^{13}C$ values for most species examined. Additionally, as expected, significant differences in carbon and nitrogen signatures were found when muscle tissues were compared to whole organisms. However, differences were small enough that food source as inferred by $\delta^{13}C$ or trophic level as inferred from $\delta^{15}N$ would not be inaccurately represented (differences of $<1.9‰$ for $\delta^{13}C$ and $<1.2‰$ for $\delta^{15}N$). The results of this study suggest that for these common estuarine fish and macroinvertebrates, stable isotopes ratios of samples can be analyzed without depuration in the same way as samples for contaminant analysis, but differences in tissue type must be taken into account when combining data from different sources.

1. Introduction

Stable isotope ratios of carbon and nitrogen are an important ecological tool used to characterize trophic pathways and contaminant transfer in aquatic ecosystems (Peterson and Fry, 1987; Kidd et al., 2001; Post, 2002; Campbell et al., 2005; Chen et al., 2009, and Chumchal et al., 2011). Because ^{15}N increases relative to ^{14}N from producer to consumer, $\delta^{15}N$ is used to identify the relative trophic level of organisms within a site (Minagawa and Wada, 1984 and Peterson and Fry, 1987). In aquatic systems, $\delta^{13}C$ can indicate whether an organism relies on autochthonous (benthic, pelagic, or macroalgae) or allochthonous (terrestrial inputs) materials as food (Fry and Sherr, 1984 and France, 1995). Organisms that consume primarily planktonic algae are generally depleted in $\delta^{13}C$ compared to organisms that consume primarily benthic algae or detrital material (France, 1995; Deegan and Garritt, 1997; Vander Zanden et al., 2006 and Winemiller et al., 2007).

The study of contaminants, such as methylmercury, has been linked to stable isotope measurements of carbon and nitrogen in order to relate the fate of metal contaminants to food sources and food web structure (Chen et al., 2009, 2014, 2016; Coelho et al., 2013; Fox et al., 2014; Becker et al., 2016). In these cases, contaminant samples as well as their paired stable isotope samples must be minimally handled and processed to avoid contamination. Most methodological comparison studies have focused on terrestrial invertebrates or freshwater systems, while few have examined estuarine systems (Fantle et al., 1999; Kaehler and Pakhomov, 2001; Yokoyama et al., 2005; Mateo et al., 2008). However, the reasons for decisions about what types of samples to use for these comparative studies are often not clear and method comparisons are necessary to ensure the validity of choosing a particular approach.

A variety of sample preparation methods including acid washing, lipid extraction, depuration (confinement of live organisms to allow for full gut clearance), preservation, and tissue dissection are commonly used to prepare samples for stable isotope analysis (Mateo et al., 2008). However, sample preparation prior to stable isotope analysis can potentially bias the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios and affect the interpretation of food web structure, which is important to consider in studies that use stable isotopes to investigate trophic transfer of metals (Atwell et al., 1998, Kidd et al., 1995, Stewart et al., 2004, Campbell et al., 2005 and Croteau et al., 2005) and other contaminants (Kidd et al., 1998; Kidd

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et al., 2001 and Kiriluk et al., 1995) in estuarine and freshwater aquatic food webs. In studies where trophic position and food source are linked to bioaccumulation of contaminants, it is optimal to measure contaminant concentrations and stable isotopes on the same individual samples to eliminate variation across tissues and reduce the amount of sample handling (e.g. tissue dissection) and sample contamination. However, analysis of whole organisms results in homogenizing muscle tissue with endo- or exoskeletons as well as gut contents, all of which can affect the isotope ratios (DeNiro and Epstein, 1978; Peterson and Fry, 1987; Pinnegar and Polunin, 1999; Feuchtmayr and Grey, 2003; Stenroth et al., 2006; Jaschinski et al., 2008 and Perkins et al., 2013). Some researchers have depurated organisms after collection in order to eliminate potential biases associated with gut contents (France, 1995; Griffin and Valiela, 2001; Hill and McQuaid, 2011 and Cremona et al., 2014), but none have examined whether depuration is necessary or whether isotope values differ between depurated and non-depurated organisms.

In this study, we compared methods for obtaining and preparing a broad range of estuarine species for stable isotope analysis. Specifically, we examined the effects of a 24 h depuration period or muscle tissue dissection on $\delta^{13}C$ or $\delta^{15}N$ values. The goal of this study was to determine if non-depurated and non-dissected whole organisms could be used for stable isotope analyses to allow for sample preparation in the same manner as for analysis of contaminants.

2. Material and methods

We chose species that are widespread in estuarine environments along the East Coast of the United States. Mummichog (*Fundulus heteroclitus*), striped killifish (*Fundulus majalis*), Atlantic silverside (*Menidia menidia*), sheepshead minnow (*Cyprinodon variegatus*), and sticklebacks (family Gasterosteidae) are all estuarine fish species commonly found in coastal waters of the East Coast of the United States (Conover and Ross, 1982; Abraham, 1985; Bigelow and Schroeder, 2002 and Chitty and Able, 2004). Green crabs (*Carcinus maenas*), blue mussels (*Mytilus edulis*), common periwinkle snails (*Littorina littorea*), soft shell clams (*Mya arenaria*), and Eastern oysters (*Crassostrea virginica*) are also well distributed along the coast of the North Atlantic (Buroker, 1983; Newell, 1989; Strasser et al., 1999; Klassen and Locke, 2007 and Cunningham, 2008). To test effects of depuration, we collected animals from the field: mummichogs from Drakes Island (Wells, ME) and Jefferson Patterson State Park (St. Leonard, MD), striped killifish from Jefferson Patterson State Park, sticklebacks from Bass Harbor (Tremont ME), snails from Drakes Island and the Piscataqua River (Kittery, ME), blue mussels from Drakes Island and the Piscataqua River, soft shell clams from Drakes Island and Harbor Road (Wells, ME), oysters from Drakes Island, and green crabs from Drakes Island and Harbor Road. We immediately froze a subset of the individuals of each species after capture and stored them at −20 °C while depurating another comparable subset in seawater for 24 h prior to freezing (see Table 1 for sample sizes). This allowed for comparison of $\delta^{15}N$ and $\delta^{13}C$ values in depurated versus non-depurated mummichogs, sticklebacks, silversides, striped killifish, sheepshead minnows, green crabs, blue mussels, snails, soft shell clams, and oysters.

Additionally, the $\delta^{15}N$ and $\delta^{13}C$ values of whole body, dissected muscle tissue, and the carcass (the tissue remaining after muscle tissue dissection) were compared in mummichogs from North Beach (Cape May Courthouse, NJ), sticklebacks from Bass Harbor, sheepshead minnows from Goshen Cove (Waterford, CT), silversides from Bass Harbor, and green crabs from Harbor Road. We dissected individual specimens and either claw (crab) or filet (fish) tissue was removed and analyzed separately from the remaining carcass. Lastly, whole green crabs, silversides, sticklebacks, and sheepshead minnows were analyzed for comparison (see Table 2 for sample sizes). Tissue comparisons were only made with organisms collected at the same site because stable isotope signatures are known to vary by location.

Table 1
Sample sizes of organisms used to examine the effect of depuration on $\delta^{15}N$ and $\delta^{13}C$ values.

Species	Number depurated	Number non-depurated
Mummichogs from Drakes Island	9	10
Mummichogs from Jefferson Patterson	9	9
Striped killifish from Jefferson Patterson	10	10
Sticklebacks from Bass Harbor	12	8
Snails from Drakes Island	10	10
Snails from Piscataqua River	6	6
Mussels from Drakes Island	10	10
Mussels from Piscataqua River	6	6
Clams from Drakes Island	10	10
Clams from Harbor Road	3	3
Oysters from Drakes Island	9	4
Crabs from Drakes Island	5	5
Crabs from Harbor Road	4	3

All samples were kept frozen until they were freeze-dried, ground, and homogenized. Approximately 1 mg of homogenous powder of organisms was analyzed for stable isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$) at the Stable Isotope Laboratory, Dartmouth College. We flash combusted samples at >1020 °C using a Carlo Erba elemental analyzer and the produced gases were carried by helium through a reduction column, a gas chromatography column and into a Conflo II unit, in which subsamples of the helium stream were input into a Delta Advantage isotope ratio mass spectrometer. We assessed quality control with international and in-house standards as well as sample duplicates. The uncertainty (1 σ) was 0.09 for $\delta^{13}C$ and 0.23 for $\delta^{15}N$.

We conducted all statistical analyses using the software program JMP 10.0. For the comparison of non-depurated versus depurated samples, the mean $\delta^{15}N$ and $\delta^{13}C$ for each species were calculated with outliers removed. We identified outliers by examining the distribution of data in JMP using an outlier box plot. Further, we compared plots/analyses of data with outliers removed and outliers included and the removal of outliers did not impact the results. We plotted mean and standard errors as bi-directional error bars, compared them to a one-to-one ($y = x$) line and also plotted a best-fit line. The degree to which the relationships diverged from the 1:1 line was a measure of their lack of association. We used an ANOVA to test for differences in mean $\delta^{15}N$ and $\delta^{13}C$ values among muscle, carcass, and whole body tissues. All assumptions of normality and equal variances were met after removal of outliers (one outlier in the $\delta^{13}C$ of green crabs and one outlier in the $\delta^{13}C$ of sticklebacks). When we observed a significant main effect ($\alpha = 0.05$), pairwise comparisons were made using Tukey's HSD means comparison. Because we compared muscle and carcass only for mummichogs, a *t*-test was used to examine the difference in $\delta^{15}N$ (with a square root transformation) and $\delta^{13}C$ values.

3. Results

$\delta^{15}N$ and $\delta^{13}C$ values of most of the non-depurated and depurated organisms examined were strongly related to each other. Isotopic ratios of both N and C either fell on the 1:1 line or the error bars intersected the 1:1 line, indicating that isotopic values of depurated organisms were

Table 2
Sample sizes of organisms used to determine the effect of tissue dissection on $\delta^{15}N$ and $\delta^{13}C$ values.

Species	Number of whole organism samples	Number of carcass samples	Number of muscle samples
Green crab	13	13	13
Atlantic silverside	10	11	11
Stickleback	8	10	10
Sheepshead minnow	10	10	10
Mummichog	0	10	10