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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 $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ or trophic level as inferred from $\delta^{15}\text{N}$ would not be inaccurately represented (differences of $<1.9\text{‰}$ for $\delta^{13}\text{C}$ and $<1.2\text{‰}$ for $\delta^{15}\text{N}$). 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 types must be taken into account when combining data from different sources.

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Author Contributions

A.N.C. prepared the samples, analyzed the data and prepared the manuscript; D.M.B prepared samples and assisted with writing the manuscript; K.L.B. assisted with the stable isotope results and assisted with writing the manuscript; X.F. ran the stable isotope samples and assisted with writing the manuscript; A.F. ran the stable isotope samples; C.Y.C. designed the study and assisted with writing the manuscript

Keywords

Stable isotopes; Food web; Depuration

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, $\delta^{15}\text{N}$ 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, $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ 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 $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$ 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 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 24h depuration period or muscle tissue dissection on $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ 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 $-20\text{ }^{\circ}\text{C}$ while depurating another comparable subset in seawater for 24 h prior to freezing (see Table 1 for sample sizes). This allowed for comparison of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ 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 $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ 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.

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 ($^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$) at the Stable Isotope Laboratory, Dartmouth College. We flash combusted samples at $>1020^{\circ}\text{C}$ 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 (1σ) was 0.09 for $\delta^{13}\text{C}$ and 0.23 for $\delta^{15}\text{N}$.

We conducted all statistical analyses using the software program JMP 10.0. For the comparison of non-depurated versus depurated samples, the mean $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ 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 $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ of green crabs and one outlier in the $\delta^{13}\text{C}$ 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 $\delta^{15}\text{N}$ (with a square root transformation) and $\delta^{13}\text{C}$ values.

3. Results

$\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ 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 indistinguishable from non-depurated organisms (Fig.1). Additionally, best-fit lines matched data closely for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (for $\delta^{15}\text{N}$: $r^2=0.99$ and for $\delta^{13}\text{C}$: $r^2=0.95$). However, deviations from the 1:1 line were observed in the $\delta^{15}\text{N}$ values of oysters and snails from the Piscataqua River and $\delta^{13}\text{C}$ values of oysters and mummichogs from Jefferson Patterson, MD.

Analysis of muscle tissue versus carcass or whole organism showed significant $\delta^{15}\text{N}$ enrichment of muscle tissues relative to carcass or whole-body measurements for sheepshead minnow, Atlantic silverside, and stickleback (Fig. 2). Similarly, enrichment of $\delta^{13}\text{C}$ was observed in green crabs and sheepshead minnow muscle tissues relative to whole bodies or carcasses.

4. Discussion

Methods vary for determining ecological characteristics of aquatic organisms, particularly in relation to sample preparation for use in concurrent contaminant and food web analyses, making it difficult to compare stable isotope data across studies. Therefore, studies comparing existing methods are needed to help determine the comparability of past studies and to select the most efficient approaches for new studies. Our comparison of sample preparation methods for stable isotope samples demonstrated that a 24 h depuration period did not significantly alter $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ values of common estuarine fish and invertebrates, suggesting that depuration may not be necessary for these species, and organisms can be frozen immediately after collection. In addition, the type of tissue analyzed affected the $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ values, with a significant enrichment in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in muscle tissues. However, for the studied food webs, the use of whole homogenized organisms should not substantially affect trophic-level assignment through analysis of $\delta^{15}\text{N}$ data or categorization of species into benthic or pelagic food webs from $\delta^{13}\text{C}$. We suggest that selection of tissues for stable isotope analysis should ultimately be based on the specific research questions and contaminants to be analyzed and urge against comparing different tissues.

Previous studies on freshwater and marine organisms have found conflicting results in the use of gut clearance prior to the preparation of invertebrate organisms for stable isotope analyses (Kaehler and Pakhomov, 2001 and Jardine et al., 2005). For example, Kaehler and Pakhomov (2001) found significant $\delta^{13}\text{C}$ enrichment and $\delta^{15}\text{N}$ depletion in littorinid snails when confined without food for hours and suggested sacrificing organisms immediately after collection, whereas Jardine et al. (2005) found the isotope ratios of invertebrates were related to their diet and suggest that gut clearance is unnecessary for primary consumers. Similarly, Cremona et al. (2014) found that a 12 h depuration period did not significantly alter $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ signatures in the freshwater macroinvertebrate family, Chironomidae. In the current study, a 24 h depuration period did not significantly affect the $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ values and although some organisms (e.g. oysters, mummichogs, and snails) diverged slightly from the one-to-one line, the differences between depurated and non-depurated organisms were small and interpretation of trophic level or food source would not be compromised. Our results suggest that for the majority of species tested here, depuration is not necessary prior to stable isotope analysis, and samples can be processed in the same manner as samples are for contaminant analysis.

In this study, muscle tissue was enriched in $\delta^{15}\text{N}$ and/or $\delta^{13}\text{C}$ compared to the rest of the body in most species examined (green crabs, sheepshead minnows, Atlantic silversides, and sticklebacks). Enrichment of $\delta^{15}\text{N}$ in muscle tissue has been reported by others and is often attributed to recent dietary switches or differences in turnover, assimilation rates, or amino acids incorporated (Sholto-Douglas et al., 1991, Bearhop et al., 2002, Stenroth et al., 2005, Mateo et al., 2008, Marques et al., 2011, Malpica-Cruz et al., 2013, Perkins et al. 2013, and Svensson et al., 2014). Additionally, the enrichment of $\delta^{13}\text{C}$ in muscle tissue observed in this study can be attributed to differences in tissue lipid content, since lipids are depleted in $\delta^{13}\text{C}$ compared to carbohydrates and protein (DeNiro and Epstein, 1978, Stenroth et al., 2006 and Mateo et al., 2008). However, the significant enrichment in $\delta^{13}\text{C}$ in crab claw muscle tissue compared to remaining crab was unexpected given that inorganic carbon in

crustacean exoskeletons should be enriched in $\delta^{13}\text{C}$ compared to soft tissue. Nonetheless, the findings are consistent with the belief that the carbonate content of crustacean exoskeleton may be small and have a negligible effect on carbon isotope values (Yokoyama et al., 2005 and Mateo et al., 2008). While the apparent enrichment of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in muscle tissues was statistically significant, it may not be relevant from a biological standpoint (e.g. trophic levels or dietary source would not be misinterpreted) and the dissection of muscle tissues for such small prey fish would not be necessary when assessing the trophic transfer of contaminants. It has been well established that $\delta^{15}\text{N}$ increases by about 3–5‰ per trophic level (Peterson and Fry, 1987); therefore in both the depuration and dissection analyses it is unlikely that the <1.2‰ differences in $\delta^{15}\text{N}$ would result in misclassification of trophic level. In addition, the range of $\delta^{13}\text{C}$ in marine phytoplankton and seagrasses within a single system can be large (pelagic phytoplankton range: –24‰ to –30‰, benthic algae range: –10‰ to –20‰, seagrasses range: from –3‰ to –15‰), thus a < 1.9‰ difference between tissues and <1.1‰ difference between depurated and non-depurated organisms should not result in uncertainty in classification of carbon food source (Fry and Sherr, 1984 and France, 1995). However, caution is advised when $\delta^{13}\text{C}$ values are near to or within the overlap of potential food source isotopic ratios, especially if the organism examined has a more opportunistic or generalist diet. For example, terrestrial inputs into an estuary average around –27‰ (although there are wide $\delta^{13}\text{C}$ ranges both within and between C_3 and C_4 plants), and pelagic phytoplankton range from 24‰ to –30‰. Given the fact that $\delta^{13}\text{C}$ increases at 0–1‰ per trophic level it would be possible to misclassify food source if the measured value fell within the overlap of these sources (Fry and Sherr, 1984, Peterson and Fry, 1987, Deegan and Garritt, 1997). Therefore, it is necessary when using stable isotopes to acknowledge the caveats of the method used, understand the ecology of the organism(s) being studied, and perhaps, support the interpretation of results with stomach content analysis.

In contaminant studies of lower trophic level organisms, whole organisms should be used in order to link trophic position and food source to contaminant bioaccumulation and to determine the exposure of their predators to contaminants. Additionally, lower trophic level organisms are often too small to adequately dissect out enough muscle tissue for analysis of both contaminants and stable isotopes. However, muscle tissue can often provide better estimates of what is actually assimilated than whole body samples (Yokoyama et al., 2005) and are more realistic for larger organisms. Due to concerns about human exposures to fish contaminants, muscle tissue from filet samples are often used to measure contaminant concentrations since that is the portion that humans would consume. However, whole body contaminant concentrations are of interest for wildlife exposure to contaminants because they would consume the entire fish (Vinikour et al., 1980). Since contaminant concentration can vary by tissue type, it is imperative to analyze the same sample type (i.e. all whole organisms or all muscle tissue) in order to accurately match contaminant burden and trophic structure.

A number of methods are used to prepare samples for stable isotope analysis, including acidification, lipid extraction, depuration or tissue specific dissection and each method includes its own advantages and disadvantages (Bosley and Wainwright, 1999, Feuchtmayr and Grey, 2003, Jardine et al., 2005, Sweeting et al., 2006, Mateo et al., 2008 Perkins et al.,

2013). While the use of depuration ensures that gut contents do not influence stable isotope ratios, it can be time consuming and is not universally practical for all organisms (Table 3). Likewise, the use of tissue dissection allows for a better picture of long-term assimilation, but is time-consuming, is not practical for smaller organisms, and could potentially result in sample contamination (Table 3). Our study clearly demonstrates that for the organisms examined, depuration does not appreciably change stable isotopic values, but that care should be taken with tissue dissection to ensure that enrichment in muscle tissue versus whole body analysis does not affect interpretation of results in the context of the research question. Future comparative studies should be conducted to examine other differing approaches in estuarine food webs as well as in other aquatic and terrestrial ecosystems.

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Highlights

- Depuration of estuarine taxa did not change $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values
- Different tissues differ in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values.
- Sample preparation does not change stable isotopes values.

Fig. 1.

Comparisons of mean ($\pm$ SEM) $\delta^{15}\text{N}$ (A) and $\delta^{13}\text{C}$ (B) values for non-depurated versus depurated organisms with bi-directional error bars on both axes and a best-fit line $\delta^{15}\text{N}$ ($r = 0.99$) and $\delta^{13}\text{C}$ ($r = 0.95$). Two outliers (one clam and one oyster) were removed from the $\delta^{15}\text{N}$ analyses and three outliers (one mummichog from Jefferson Patterson, MD and two sticklebacks) were removed from $\delta^{13}\text{C}$ analyses. Inclusion of outliers produced the same results (not shown here). Solid line - best fit line; dotted line - 1:1 line.

Fig. 2. Mean $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values ($\pm$ SEM) of dissected green crab (A), sheepshead minnow (B), Atlantic silverside (C), and stickleback (D) and mummichogs (E). Different letters indicated significant differences based on Tukey's post-hoc multiple comparisons ($P < 0.05$). Two outliers were removed, one green crab and one stickleback from $\delta^{13}\text{C}$ analyses. Analyses on the whole dataset (including all outliers) produced the same result (not shown here). Whole body: triangle, tissue remaining after dissection: circle, muscle tissue: diamond.

Table 1

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

Table 2

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

Comparisons of advantages and disadvantages of depuration and dissection methods and implications for future directions

Table 3

Method	Advantages	Disadvantages	Best use	Implications/future directions	References
Depuration	<ul style="list-style-type: none"> Stomach contents will not alter results Eliminates skew of an opportunistic catch Eliminates bias of prey or sediment 	<ul style="list-style-type: none"> Time-consuming Requires extra materials and space Not practical for all organisms Will not detect recent dietary shifts 	<ul style="list-style-type: none"> Small organisms Organisms with large gut:body ratio 	<ul style="list-style-type: none"> Inclusion or exclusion of gut contents should be based on whether studies are focused on individual bioaccumulation or predator-prey trophic transfer Examine differences between predators and prey Inconsistencies in earlier research, some show changes in isotopes values, our study found no significant differences 	Kaehler and Pakhomov, 2001, Feuchtmayr and Grey, 2003, Jardine et al., 2005, Winemiller et al., 2007 Hill and McQuaid, 2011
Non-depuration	<ul style="list-style-type: none"> Easy Minimal processing Directly links contaminant level in trophic transfer 	<ul style="list-style-type: none"> Potential skew of results 	<ul style="list-style-type: none"> Limited resources and time Snapshot of what the organisms is consuming 		Jardine et al., 2005, Cremona et al., 2014
Tissue dissection	<ul style="list-style-type: none"> Indicates long-term assimilation Reduces bias from exoskeletons Tissue-specific contaminant load Relevant to human exposure 	<ul style="list-style-type: none"> Contamination risk Time-consuming Not practical for all organisms Increases potential of lipid bias 	<ul style="list-style-type: none"> Tissue-specific contaminant question Large animals 	<ul style="list-style-type: none"> Whole organisms can be useful for food web questions but less so for physiological or tissue-specific questions The decision to use dissected tissue or the whole body needs to be based on the specific research question 	Hesslein et al., 1991, Yokoyama et al., 2005, Stenroth et al., 2006, Sweeting et al., 2006
Whole organism	<ul style="list-style-type: none"> Easy Minimal processing Directly track trophic transfer of contaminant(s) 	<ul style="list-style-type: none"> Tissue-specific influence on isotope ratios Not feasible for large organisms 	<ul style="list-style-type: none"> Limited time and resources Trophic transfer of contaminants 		Stenroth et al., 2006