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Origin determination of the Eastern oyster (*Crassostrea virginica*) using a combination of whole-body compound-specific isotope analysis and heavy metal analysis†

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Various samples of the Eastern oyster, *Crassostrea virginica*, were collected from five harvest bay areas in the Gulf of Mexico coastal waters of Florida (FL), Louisiana (LA) and Texas (TX). Cadmium and lead concentrations from the extracted whole-body soft tissues were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS), and bulk $\delta^{13}C$ and $\delta^{15}N$ isotope ratios and amino-acid-specific $\delta^{13}C$ values were analyzed *via* isotope ratio mass-spectrometry (IRMS). The combined data was subjected to multivariate statistical analysis to assess whether oysters could be linked to their harvest area. Results indicate that discriminant analysis using the $\delta^{13}C$ values of five amino acids—serine, glycine, valine, lysine and phenylalanine—could discriminate oysters from two adjacent harvesting in Florida with 90% success rate, using leave-one-out cross validation. The combination of trace elements and isotope ratios could also predict geographic provenance of oysters with a success rate superior to the isolated use of each technique. The combinatory approach proposed in this study is a proof-of-concept that compound specific stable isotope analysis is a potential tool for oyster fisheries managers, wildlife, and food safety enforcement officers, as well as to forensics and ecology research areas, although significantly more work would need to be completed to fully validate the approach and achieve more reliable statistical results.

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Introduction

Wildlife and food safety enforcement agencies have expressed a need for chemical or biological methods to identify the harvest areas of oysters for two reasons: (1) to protect consumers from harmful chemical and biological exposures, and (2) to protect oyster fisheries from damage caused by overfishing. Habitat protection in the U.S is important in light of examples of oyster population collapses that were caused by various habitat modifications.[1]

Estuary biomes are the nurseries for nearby marine ecosystems and therefore provide vital benefits to the health of nearshore and offshore fisheries.[2] Given the importance of these sensitive ecosystems, the ecological integrity of estuaries and harvesting of estuarine species are managed by local, state and federal regulatory agencies in partnership with various non-regulatory programs, such as the National Estuary Program (NEP).[3] Oysters are among the overseen species because of their significant biological role as filter feeders and maintainers of the nutrient balance of the estuary.[4,5] Kellogg *et al.* reported that estuaries with fully restored oyster reefs were more successful in improving the flux of nutrients like O_2, NH_4^+, nitrates and soluble reactive phosphorous in all seasons by more than an order of magnitude relative to areas that are not restored.[5]

Oyster reefs are a key component in the estuarine food web. They provide a habitat for numerous estuarine organisms and the oysters also serve as food for various species, including oyster drills (*Stramonita haemastoma, Urosalpinx cinereal*), crabs (*Menippe* sp., *Panopeus herbistii*) and fishes (*Paralichthys dena-tus*, *Pogonias cromis*, *Archosargus probatocephalus*).[6,7] Like other organisms, oysters are vulnerable to changes in their habitat and diverse factors can contribute to their overall survival. Estuaries receive freshwater from rivers, streams and creeks that can be contaminated by local waste discharges and runoff from nearby industrial, agricultural, and suburban areas. Therefore, the land use ascribed to the areas that drain into a given estuary greatly influences the chemical and biological content of the oysters that feed within its boundaries.

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Oysters can bioaccumulate the contaminants carried into the estuary, posing great public health concern especially when consumed raw or undercooked.[7] The harvest areas of commercial oysters may be closed when concerns for human health from either microbiological or chemical contamination occur. Typically, these areas are closed for short periods of time after heavy rainfall because runoff from nearby freshwater sources increases the bacteria and virus levels in the water. However, oyster harvest areas and entire estuaries can be closed for months or years due to chemical or microbial contamination. An example of a protected area is Lavaca bay, Texas, where mercury release caused severe chemical contamination and closure to oyster harvesters. After decades of work to restore the estuary, the bay was reopened to seasonal shellfish harvest in 2007.[8]

In addition to chemical contamination and common microbial contaminants like hepatitis A, noroviruses, and *Salmonella*,[9,10] one of the greatest oyster-related health concerns, especially in the Gulf of Mexico, is the consumption of raw shellfish that is contaminated with *Vibrio* sp.[7,11–13] *Vibrio vulnificus* and *V. parahaemolyticus* cause most of the deadly foodborne infections in the US,[14] and the fatality rate of infected patients can exceed 50%.[15] In 2014, 196 out of the 286 cases of domestically acquired foodborne *Vibrio* involved the consumption of oysters and 174 of those cases had involved raw oysters.[16] This disease outbreak scenario has not improved, as the incidence of foodborne vibriosis infections in 2019 reached 466 cases in the U.S., an increase of 79% compared to 2016–2018 surveillance period.[17]

The Gulf of Mexico (GOM) has been one of the largest commercial oyster fisheries in the U.S. and in 2016 supplied approximately 69% of the domestic commercial wild oysters.[18] Apalachicola bay, Florida, used to produce approximately 10% of commercial oysters harvested in the GOM,[19] but since 2012, its oyster production has been greatly reduced due to overharvesting, declining water quality and significant reductions in freshwater inflows to the Apalachicola river, which feeds into the bay.[20,21] The reduced outflows caused by severe drought conditions in the southeast U.S., combined with the increased water needs of nearby urban and suburban areas, have contributed to the increased salinity of Apalachicola bay and has had a deleterious effect on the oyster production. Additionally, a temporary increase in the number of oysters allowed for harvesting immediately after the Deepwater Horizon oil spill in 2010 has further damaged the oyster fishery.[7,20]

The Florida Fish and Wildlife Conservation Commission (FWC) has authority for bay closures to protect and manage the Apalachicola bay oyster fishery and public health. The reduced availability of oysters due to environmental conditions and fishing pressure has led to harvesting restrictions on certain oyster reefs for certain times for the year. Despite the restrictions, oystermen continue to illegally harvest from the restricted areas and, when caught, claim that their catches come from waters that are legal to harvest.[7,20] In 2020, the FWC reported more than 15 issued citations for various illegal oyster harvesting practices, including oyster removal from restricted or prohibited areas.[22] These illegal actions have long hampered the recovery of the commercial fishery and poses a threat to the restoration of the Apalachicola bay system. Given the current collapse of the oyster population, FWC has recently suspended wild oyster harvest and prohibited possession of related harvesting equipment in Apalachicola bay from August 2020 to the end of 2025.[23]

The popular Eastern oyster, *Crassostrea virginica*, is a remarkably resilient species widely dispersed in many U.S. estuaries.[24] *C. virginica* has been one of the most popular species for the commercial aquaculture business, despite its reduced availability due to overfishing, loss of habit and some parasitic diseases.[25,26] Many studies involving the controlled harvesting of this oyster are based on genetic analyses, which aim to identify differentiation patterns in the distinct populations.[27–30] The isotopic or elemental composition of this species, as a way to determine geographic provenance, is underexplored.

Inductively coupled plasma-mass spectrometry (ICP-MS) and isotope ratio mass spectrometry (IRMS) have been successfully used to determine the geographic source of a variety of samples.[31–35] However, the few studies that combined both techniques focused on the ecology of a species or ecosystem changes due to seasonal or anthropogenic factors.[36,37] In this study we demonstrate the potential of lead (Pb) and cadmium (Cd) concentrations combined with bulk and compound-specific isotope analysis (CSIA) of *C. virginica* whole-body tissues to determine harvesting locations. Cadmium and lead were chosen because they are primarily associated with anthropogenic activity and are easily detected in almost all oysters tested in North America.[38] Sources of these trace elements in the environment include fertilizers, lead acid battery production and the mining and smelting of metals. Also, lead is still permitted in aviation fuel and remains in the environment from legacy sources such as gasoline and lead–arsenic pesticides.[39,40] The ability to use chemical signatures to identify harvest areas can play an important role in several areas, including (1) the protection of consumers from the purchase of counterfeit oysters sold as "boutique" brand oysters, (2) to help regulatory agencies enforce harvesting regulations, and (3) to protect consumers from foodborne infections.

Experimental

Instrumentation

Cadmium and lead concentrations were determined by ICP-MS (PE Elan 6100 or PE Nexion, CT USA). Carbon and nitrogen bulk isotope ratio measurements ($\delta^{13}C$ and $\delta^{15}N$) were performed using a Thermo Flash HT Plus elemental analyzer (EA) coupled to a Thermo Delta V Advantage isotope ratio mass spectrometer *via* a Conflo IV interface. For the $\delta^{13}C$ CSIA, we used a Dionex ICS5000 ion chromatography system (ICS) (Dionex, Sunnyvale, CA, USA) coupled through an LC-Isolink interface to a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

Sample collection

C. virginica oysters were sampled from Apalachicola bay (Florida beds 1642 and 1662); Louisiana harvest area 28; and Copano

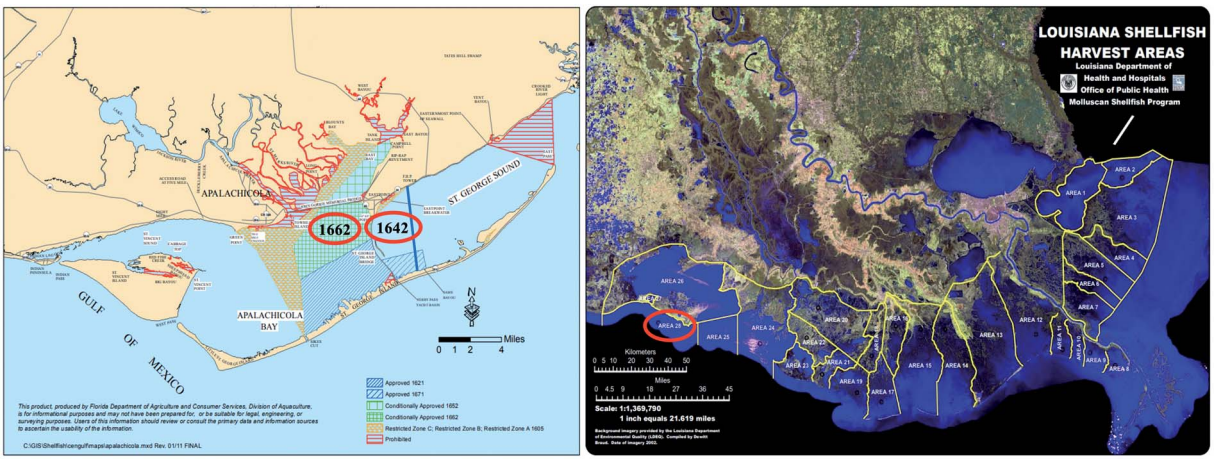


Fig. 1 Map of shellfish harvest areas in Apalachicola bay, Florida (left panel) and in Louisiana (right panel). Red circles indicate the areas included in this study: FL 1642 and FL 1662 beds (left panel) and LA area 28 (right panel). Sources: Florida Department of Agriculture and Consumer Affairs and Louisiana Department of Health and Hospitals, Office of Public Health, Molluscan Shellfish Program, respectively.

and Galveston bays, both in Texas (Fig. 1 and 2). These oysters were randomly sampled, typically within days of harvest, from wholesale and retail outlets through 2011–2013. Ideally, multivariate mathematical models should generally have 5–10 independent measures per independent variable: however, practical limitations prevented us from employing so many samples. Therefore, as a proof-of-concept, we used a limited number of samples (ten per region) in this study to assess the feasibility of combined methodologies to discriminate oyster harvest areas from both a macro and micro-geographical perspective. Ten oysters from each region were placed in plastic freezer bags, sealed, and shipped in coolers with cold packs overnight to the partner laboratory for analysis. Upon receipt, all samples were stored in a −5 °C freezer until homogenization. In the analysis laboratory, harvest areas were recorded from the sanitation tags that were included in the original inspection reports.

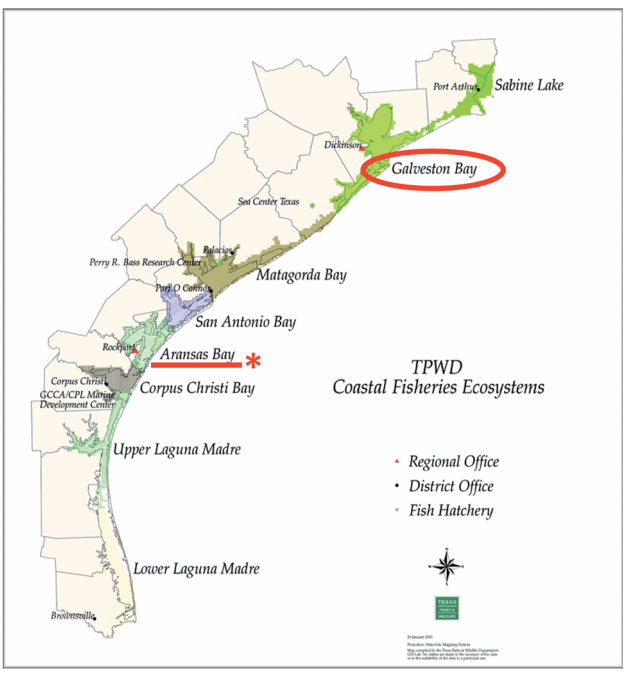


Fig. 2 Map of Texas bays including oyster harvest areas. Red circle indicates one of the areas included in this study, Galveston bay. The other area was Copano bay, which is a small unimpacted bay within the Aransas bay (marked by the asterisk symbol). Source: Texas Parks and Wildlife.

After defrosting, the oyster shells were first measured, then rinsed using metals-free water before shucking and rinsing, which helped avoid oyster tissue contamination from external debris. After shucking, the soft tissue weight was recorded, and the oysters were divided in two portions: one homogenized for the ICP-MS protocol and the other segregated for posterior stable isotope analysis. Although the immediate analysis of fresh samples is ideal, the isotope analysis of our samples was only possible a few years after their original collection. Between sampling and isotopic analysis, the samples were stored in 50 mL screw top centrifuge tubes and maintained in a −5 °C freezer. Freezing was selected as the storage method due to its minimal impact on the isotopic composition of tissues. Chemical preservation is also a method of choice for marine organisms, but the effects of chemical preservatives must be heavily considered during the isotope data analysis.[41,42]

ICP-MS analysis

Digestion and analysis were performed as previously described by Adams and Engel.[43] Briefly, approximately 0.5 g of homogenized tissue was digested in 5 mL of metals-free nitric acid. Samples were diluted to 100 mL with metals-free water, and the internal standards rhodium and lutetium were added prior to analysis of Cd and Pb using ICP-MS. The metal concentrations were determined from duplicate measurements of each sample following the method described by Mudge *et al.*[38] A laboratory reagent blank, a quality control sample, SRM 2976 Mussel tissue (NIST, Gaithersburg, MD), and a calibration check

standard (High Purity Standards, Charleston, SC) were analyzed after every ten samples. For cadmium and lead, the certified values expressed in mass fractions ± the expanded uncertainty presented by the NIST Certificate of Analysis are 0.82 ± 0.16 and 1.19 ± 0.18, respectively. The results from this ICP-MS analysis were described in the context of other metals by Mudge *et al.* in 2015.[38]

EA-IRMS and LC-IRMS

Prior to stable isotope analysis, oyster soft tissues were lyophilized in microcentrifuge tubes for approximately four hours to remove any remaining water. The dried samples were placed in 2 mL polypropylene tubes containing four 3.2 mm chrome steel beads and pulverized for 5 min at a setting of 3450 rpm in a minibead beater (Biospec Products Inc., Bartlesville, OK, USA). This process creates homogenous samples and facilitates the precise weighing in small aliquots, which leads to more reproducible results.[44]

For bulk isotope analysis, samples of approximately 0.5 mg were weighed in tin capsules and placed in the instrument mentioned above. Isotopes were measured relative to the respective reference working gases (CO_2 and N_2, Matheson, Morgantown, WV). USGS-40 ($\delta^{13}C = -26.39 \pm 0.04‰$, $\delta^{15}N = -4.52 \pm 0.06‰$) and USGS-41 ($\delta^{13}C = +37.63 \pm 0.05‰$, $\delta^{15}N = +47.57 \pm 0.11‰$) (USGS, Reston, VA, USA) were used as two-point calibration standards to express the measured isotope values in the international scale against VPDB and air, respectively.[45] Sulfanilamide was analyzed as a quality control every 12 samples to account for any drift. All samples were run in triplicate. Isotope ratios were converted to the delta or per mil (‰) scale as suggested by IRMS guidelines.[46,47]

For CSIA, approximately 2 mg of each pulverized oyster was hydrolyzed in 6 M hydrochloric acid for 24 h at 110 °C in a vacuum oven to lyse the peptidic bonds. Afterwards, each mixture was filtered using a 0.45 μm PTFE syringe filter and evaporated to dryness at 60 °C under a stream of lab air. 1 mL of deionized water was used to re-dissolve the dry residues, which were then filtered using a 0.45 μm PVDF syringe filter. Despite the destructive effect of HCl on some amino acids, the $\delta^{13}C$ values of the recovered amino acids are not considerably affected the hydrolysis conditions applied in this approach.[48–50]

Amino acid chromatographic separation was performed in a Primesep A mixed-mode column (2.1 × 250 mm, 5 μm, 100 Å) (SIELC Technologies, Prospect Heights, IL, USA). For the first 18.7 minutes, mobile phase was composed by pure deionized water (at least 17.5 MΩ), followed by a successive decrease of pH through a gradient of pure deionized water with 0.03 M sulfuric acid. The flow rate of the mobile phase was 160 μL min^{-1}. Samples of 17 standard amino acids (98–99% purity Sigma-Aldrich, St. Louis, MO, USA) were randomly analyzed throughout the sequences as a quality control for the chromatographic separation. All carbon-containing compounds eluting from the HPLC column were quantitatively oxidized to CO_2 in the LC-Isolink interface using wet chemical oxidation. This process occurred in the aqueous phase at 99.9 °C using sodium persulphate (100 g L^{-1}) and phosphoric acid (1.5 M). The resulting CO_2 was extracted from the cooled solution by a membrane exchanger, and any remaining water in the extracted CO_2 was removed in two water traps before the transfer to the IRMS system. Similar procedures using LC system for isotope analysis were previously reported.[49,51] The isotope reference materials mentioned above (USGS-40 and USGS-41) were also used for the two-point calibration curve applied to this analysis.

Data collection and statistical analysis

Isodat 3.0 was used as the data acquisition software, and IBM SPSS Statistics 25 was used in the statistical analyses. The normality of each oyster group was checked by the histograms and QQ-plots of residuals, the skewness and kurtosis of residuals and Shapiro–Wilk normality tests. General correlations and multicollinearity assessment were investigated using bivariate correlation coefficients. Any variable showing a correlation above 0.8 in the correlation matrix was removed from further analysis for high multicollinearity. Multivariate outliers were accessed *via* measurement of Mahalanobis distance. For the assumption of equal variance of the data (homoscedasticity) we checked Levene's test statistic. The more robust Welch's test was used to verify variables violating this assumption. The data was mean-centered and standardized prior to multivariate analysis using SPSS Statistics v25.

For bulk isotope ratios, multivariate analysis of variance (MANOVA) followed by Tukey's HSD post hoc test determined the mean differences between each of the investigated harvest regions. For CSIA results, we tested the multiple amino acid variables using one-way between-groups multivariate analysis of variance (MANOVA) followed by Games–Howell post hoc test and bootstrapping multiple regression analysis. The bootstrapping sampling was set up to 2000 samples and Bias Corrected and accelerated ranges (BCa) were used to estimate the 95% confidence intervals. Linear discriminant analysis (LDA) using original discriminant rules and leave-one-out cross-validation (LOOCV) was performed as an attempt to classify the oysters according to harvest area.

Statistical tests were performed on the bulk isotopic data ($\delta^{13}C$ and $\delta^{15}N$ values), $\delta^{13}C$ values of the amino acid peaks of Asx, Glx, Ser, Gly, Val, Lys, Phe, Arg and Xle, and the Pb and Cd concentrations. Ile and Leu co-eluted, so the isotope ratio value for the co-eluting pair (Xle) was used as a single variable in the model. The $\delta^{13}C$ values of aspartic acid (Asp) and glutamic acid (Glu) included a small contribution of asparagine (Asn) and glutamine (Gln), respectively, which were deaminated to their respective dicarboxylic acids during acid hydrolysis.[49,52,53] Because of the inability to fully resolve the relative contributions of Asp/Asn and Glu/Gln, we reported these amino acid peaks as Asx and Glx, respectively.

Results and discussion

Bulk $\delta^{13}C$ and $\delta^{15}N$ analysis

We tested Pb and Cd concentrations along with bulk and CSIA results of ten oyster tissue samples from each of the following

areas: FL 1642 and FL 1662 areas (Apalachicola bay); LA 28 area from Louisiana; Galveston and Copano bays, from Texas. The combined data was used to attempt the classification of samples based on their geographic origin. Bulk measurements of oyster tissues from each of the regions were normally distributed. Samples with missing data were removed from the analysis, making $n = 9$ or 10 samples per region. The mean $\delta^{13}C$ values ranged from $-24.5‰$ to $-21.9‰$, where LA 28 area gave the most depleted values and FL 1642 gave the most enriched values. For $\delta^{15}N$, values ranged from $8.8‰$ to $15.1‰$, where FL 1642 and Galveston bay were the most depleted and enriched, respectively (Fig. 3).

One-way ANOVA showed a statistically significant difference between the carbon and nitrogen bulk signatures of the harvest areas ($P < 0.05$) (Table S1†). Tukey's HSD post-hoc test (Table S2†) revealed that the $\delta^{13}C$ values of FL 1642 oysters were significantly different from the samples from LA area 28 ($P < 0.05$) and FL 1662 ($P = 0.06$), although the latter was different at the 94% confidence interval. On another hand, oysters from LA area 28 were significantly different from both Galveston and Copano bays ($P < 0.05$). Based on the $\delta^{13}C$ values, oysters from Galveston and Copano bays were not distinguishable from each other.

Regarding the nitrogen isotope ratios, all the areas were significantly different from Galveston bay ($P < 0.05$), but not different from each other. Oyster tissues from Galveston area had a very enriched ^{15}N signature ($\sim 15‰$) relative to the other bays. Galveston bay's $\delta^{15}N$ values, which exceed $10‰$, suggest that this bay receives an extra nutrient influx, most likely in the form of nitrate from some anthropogenic activity such as fertilizer runoff or animal or sewage waste. Although $\delta^{15}N$ measurements have been previously applied to indicate eutrophication of different aquatic environments,[54–57] our results endorse the beneficial use of bivalve organisms, especially the Eastern oyster *C. virginica*, in the monitoring and management

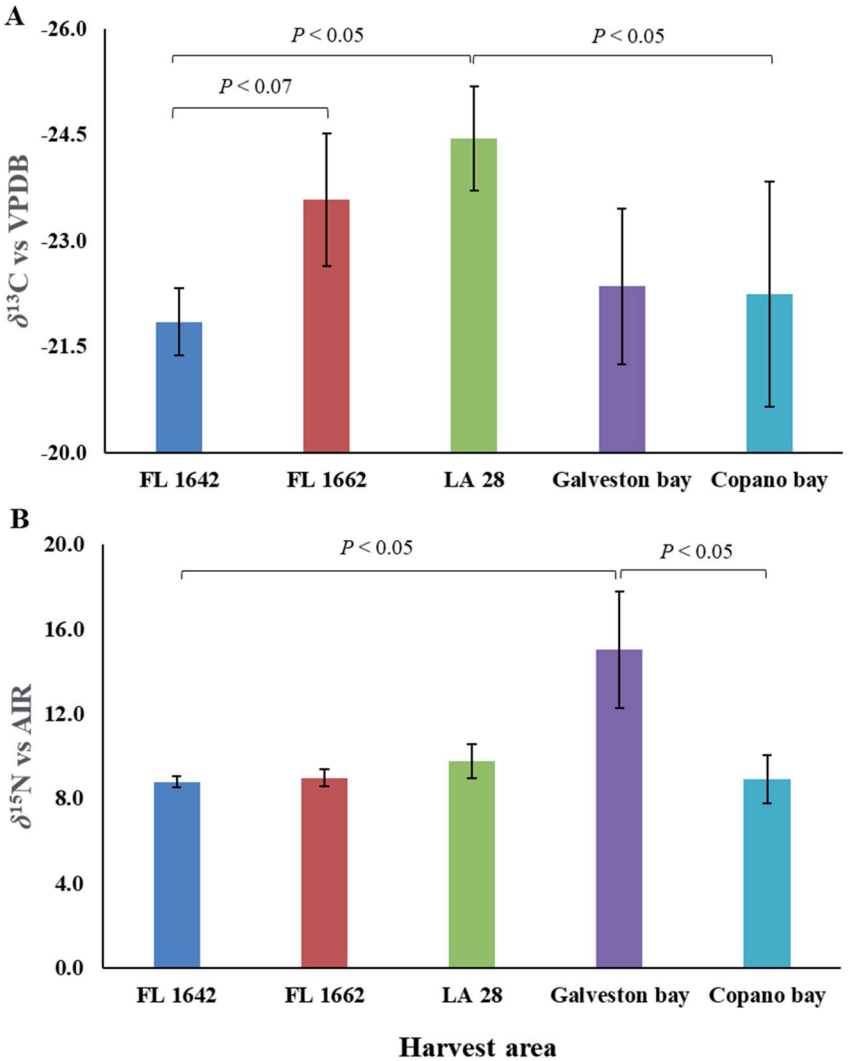


Fig. 3 (A) Bulk $\delta^{13}C$ and (B) $\delta^{15}N$ values of oyster tissue samples from five harvest areas. In (A) LA 28 was significantly different from FL 1642, Galveston and Copano bays ($P < 0.05$); FL 1642 statistically differed from FL 1662 ($P < 0.07$). In (B) Galveston bay was significantly different from all the other regions ($P < 0.05$) (B). $n = 9$ or 10 for each region. Error bars show the 95% confidence intervals of the means, assuming unequal variances.

Table 1 Linear discriminant analysis (classification) according to geographic origin using bulk $\delta^{13}C$ and $\delta^{15}N$ values and Cd concentration from whole-body oyster samples collected in different harvest areas $n = 9$ or 10 for each region

			Predicted group membership					
		Harvest area	FL 1642	FL 1662	LA 28	Galveston bay	Copano bay	Total
Original	Count	FL 1642	9	0	0	0	1	10
		FL 1662	2	7	0	0	0	9
		LA 28	0	1	9	0	0	10
		Galveston bay	0	2	0	8	0	10
		Copano bay	3	0	2	0	4	9
	%	FL 1642	90.0	0.0	0.0	0.0	10.0	100.0
		FL 1662	22.2	77.8	0.0	0.0	0.0	100.0
		LA 28	0.0	10.0	90.0	0.0	0.0	100.0
		Galveston bay	0.0	20.0	0.0	80.0	0.0	100.0
		Copano bay	33.3	22.2	0.0	0.0	44.4	100.0
Cross-validated	Count	FL 1642	9	0	0	0	1	10
		FL 1662	2	7	0	0	0	9
		LA 28	0	1	8	0	1	10
		Galveston bay	0	2	0	8	0	10
		Copano bay	3	1	3	0	2	9
	%	FL 1642	90.0	0.0	0.0	0.0	10.0	100.0
		FL 1662	22.2	77.8	0.0	0.0	0.0	100.0
		LA 28	0.0	10.0	80.0	0.0	10.0	100.0
		Galveston bay	0.0	20.0	0.0	80.0	0.0	100.0
		Copano bay	33.3	11.1	33.3	0.0	22.2	100.0

of contaminated aquatic ecosystems, including their use in some biomonitoring programs around the world.[58]

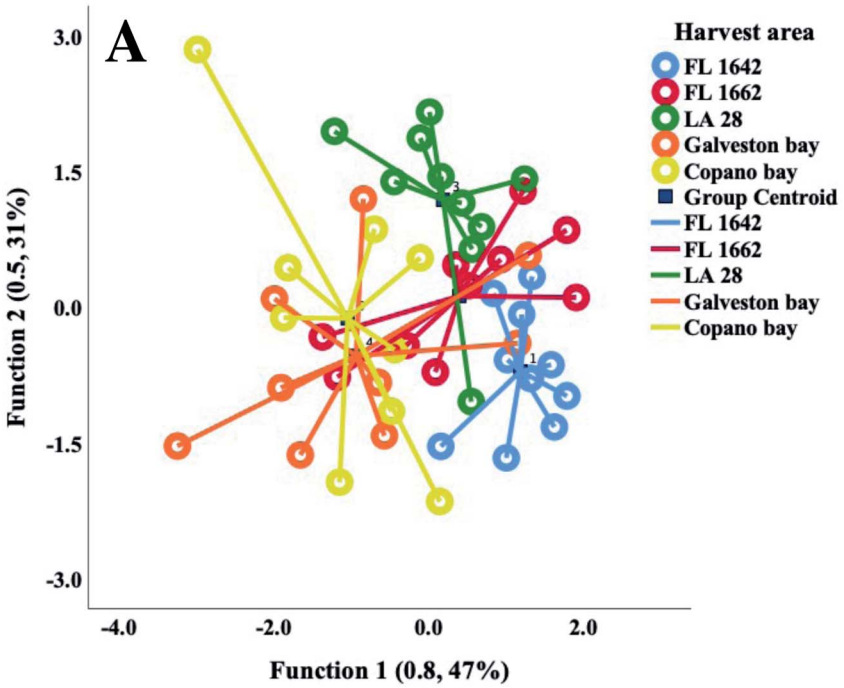
Attempts to classify oysters based on geographic region using LDA of the bulk isotope ratio values showed that 54.2% of the samples were correctly classified to their harvest area after leave-one-out cross-validation (LOOCV) (Table S3†). When using the two variables [Pb] and [Cd], correct classification only reached 32% of accuracy, which is not much better than the random assignment probability of 20%. However, the combination of bulk isotope ratios with Pb and Cd concentrations increased the success of classification to ~63% using LOOCV (data not shown). However, when using just Cd concentration along with the bulk isotope ratios, approximately 71% of the oysters were correctly classified to their geographic origin after LOOCV (Table 1). The first two canonical functions explained 92% of the total variance, which indicates that a combination of bulk isotope ratios (of ^{13}C and ^{15}N) and the concentration of Cd shows better discrimination power to assign samples to specific sources than if working with only metal concentrations or only bulk isotope ratios. Still, the classification rates using bulk isotope values are hardly sufficient for prosecutorial purposes.

$\delta^{13}C$ CSIA of amino acids

For multivariate analysis of CSIA data, the independent variable was the oysters' harvest area, and the dependent variables were the $\delta^{13}C$ values of all the amino acid peaks. $\delta^{13}C$ values were normally distributed, except for the $\delta^{13}C$ values of Asx and Glx. No serious violations were noted when testing the validity of the test assumptions. MANOVA results from the combination of the amino acids' carbon isotope ratios indicated a statistically significant difference between the difference harvest regions ($F = 2.904$, $P = 3 \times 10^{-6}$; partial eta squared = 0.408), meaning that the geographic harvest area explained approximately 41% of the variance in the linear combination of $\delta^{13}C$ values of the amino acids. After further investigation of which amino acids were strongly influencing the model, the $\delta^{13}C$ values of Ser, Gly, Val, Xle, Lys, and Phe were the most significant at the 95% confidence level ($P < 0.05$), followed by the $\delta^{13}C$ value of Glx, which was significant at the 91% confidence level ($P = 0.086$). However, we also noticed that the $\delta^{13}C$ values of Val, Xle, Lys, Phe and Arg were highly correlated among each other ($r > 0.8$), indicating multicollinearity. Multicollinearity means that one of these variables can be used to predict the behavior of the others. A high degree of multicollinearity affects the statistical power of statistical models that rely on mild correlations, like analysis of variance, regression and some multivariate analysis (such as LDA), because too much variance is shared among the correlated variables, making it difficult to access their individual contributions to the analysis outcome.[59,60] For this reason, we omitted the isotope values for Val, Xle, Lys, Phe and Arg from some of the further analysis when their inclusion would cause misinterpretation of results.

Games–Howell post hoc test on the remaining valid stable isotope variables revealed which harvest areas were statistically different from each other (Table S4†). Despite their proximity, oysters from the two adjacent Apalachicola bays (FL 1642 and 1662) showed significantly different $\delta^{13}C$ values of the amino acids Ser ($P < 0.05$) and Gly ($P = 0.067$). The other variables could not discriminate these two regions with adequate accuracy.

The $\delta^{13}C$ values of Ser were also prominent in segregating oysters from FL 1642 *vs.* Copano bay ($P < 0.05$), samples from FL



B

Classification Results[a,c]

		Region_code	Predicted Group Membership 1	2	3	4	5	Total
Original	Count	1	9	1	0	0	0	10
		2	1	7	0	1	1	10
		3	1	0	9	0	0	10
		4	2	0	1	6	0	9
		5	1	1	1	2	4	9
	%	1	90.0	10.0	.0	.0	.0	100.0
		2	10.0	70.0	.0	10.0	10.0	100.0
		3	10.0	.0	90.0	.0	.0	100.0
		4	22.2	.0	11.1	66.7	.0	100.0
		5	11.1	11.1	11.1	22.2	44.4	100.0
Cross-validated[b]	Count	1	8	2	0	0	0	10
		2	1	4	1	3	1	10
		3	1	3	5	0	1	10
		4	2	1	1	2	3	9
		5	1	1	2	2	3	9
	%	1	80.0	20.0	.0	.0	.0	100.0
		2	10.0	40.0	10.0	30.0	10.0	100.0
		3	10.0	30.0	50.0	.0	10.0	100.0
		4	22.2	11.1	11.1	22.2	33.3	100.0
		5	11.1	11.1	22.2	22.2	33.3	100.0

a. 72.9% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 45.8% of cross-validated grouped cases correctly classified.

Fig. 4 (A) Linear discriminant analysis (LDA) plot of CSIA δ^{13}C values of four amino acids (Asx, Glx, Ser and Gly), and Cd concentrations from *C. virginica* oyster samples. (B) Classification results showing the five bays could be discriminated with ~46% accuracy after LOOCV. A larger sample size would likely minimize the discrepancies between biased and unbiased classification rates, ultimately improving the classification success based on harvest region. Region code represents the harvest areas, where 1 = FL 1642; 2 = FL 1662; 3 = LA 28; 4 = Galveston bay; and 5 = Copano bay.

1642 *vs.* Galveston bay ($P = 0.070$) and LA area 28 *vs.* Copano bay ($P = 0.073$), the latter two results at the 93% confidence level. According to the δ^{13}C values of Asx, oysters from FL 1642 were significantly different compared to the ones from both Copano bay ($P < 0.05$) and Galveston bay ($P = 0.086$), at the 95% and 91% confidence levels, respectively. Additionally, the δ^{13}C values of Glx also significantly contributed to the separation between samples from FL 1662 *vs.* Copano bay ($P < 0.05$). These results demonstrate the impact that estuary conditions pose on bivalves and how oyster soft tissue's isotope analysis of amino acids can be used as a supplementary tool for source determination. When combining the Pb and Cd concentrations into the MANOVA model, Games–Howell post hoc test indicated that Cd concentrations provided statistically significant separation

between LA area 28 and both Apalachicola bays (FL 1642 and FL 1662), both with P values < 0.05. Pb concentrations did not differentiate any of the regions (Table S4†).

To confirm these results, we built a regression model based on 2000 bootstrapping samples including the same variables mentioned above and verified the BCa values (Table S5†). This parameter was used because it accurately confirms confidence intervals while correcting for bias and skewness in the distribution of bootstrap estimates.[61] The BCa intervals for the Cd concentrations and the δ^{13}C values of Ser and Asx excluded the value zero, suggesting these predictors played the strongest roles in the harvest region segregation model at the 95% confidence level.

We also investigated if the δ^{13}C values of amino acids would be helpful to the assignment of oysters to geographic harvest locations using LDA. Classification results of Asx, Glx, Ser and Gly δ^{13}C values showed that 54% of the samples were correctly classified based on the original rules of the method and only ~42% after leave-one-out cross-validation (LOOCV). After combining the isotope variables with the respective Cd concentrations for each oyster sample, the success rate improved to 73% based on original rules, and ~46% after LOOCV (Fig. 4). For the combined model, 78% of the variance was explained by the first two canonical functions. The divergence between the biased and unbiased classifications indicates instability in the model, which is usually caused by an inadequate number of samples per group. A larger sample size per region would be more appropriate to improve the classification success based on harvest source. Interestingly, the only linear correlation between metal concentrations and isotope ratios revealed by the correlation analysis was observed for Cd and the δ^{13}C values of Gly (P < 0.05). Nevertheless, the first two linear functions used to discriminate between the regions were significant based on the Wilk's lambda significance test (P < 0.05).

Despite the current classification results based on CSIA, our results from this feasibility study suggest that the combination of IRMS and ICP-MS variables is a prospective alternative to discriminate between different harvest areas of *C. virginica*. However, successful classification will depend on having a sufficient sample size and significant differences in the variance between locations and within locations. For instance, different nutritional conditions, temperatures, salinity and contaminants can all lead to different δ^{13}C values in the feedstock and fractionation of amino acids in the oysters.[62] Different micro-areas might exist within a bay, depending upon the fresh water inflows and movement of sediments,[63] making areas with similar water flow patterns and anthropogenic activities harder to differentiate.

Despite their geographic proximity, we showed the fortunate result that oysters sampled from adjacent Apalachicola bay areas could be discriminated from each other with greater than 90% confidence (LOOCV). Such small differences in fishing locations are often the hardest to prove using DNA or GPS data, especially if GPS tracking devices are temporarily suspended during illegal harvesting. For example, the mean δ^{13}C values (±95% confidence interval) for the amino acids Ser and Gly

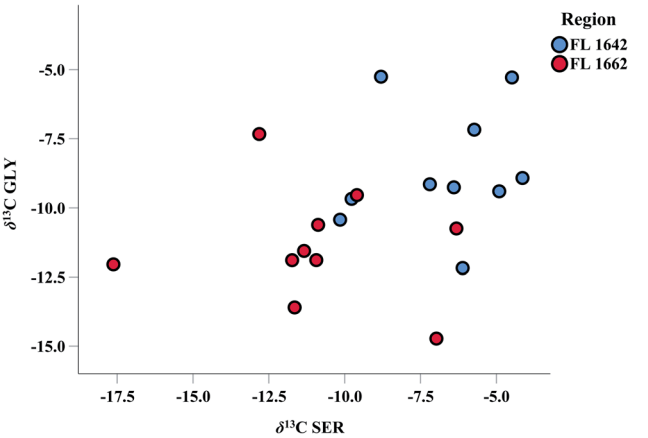
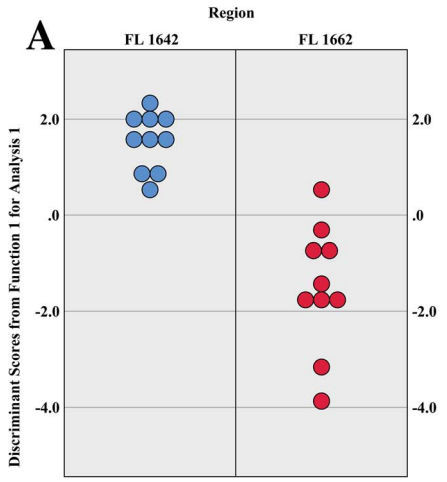


Fig. 5 Bivariate plot of δ^{13}C values of Ser and Gly, two of amino acids in oysters that were significantly different between two adjacent Apalachicola bay areas at the 95% and 93% confidence intervals, respectively.

were −6.8 ± 1.3‰ and −8.7 ± 1.4‰ in area FL 1642, respectively, whereas the mean values in area FL 1662 were −11.0 ± 1.9‰ and −11.4 ± 1.3‰, respectively. When comparing all the analyzed areas, FL 1642 showed the most enriched δ^{13}C values of Ser and FL 1662 showed the most depleted δ^{13}C values of Gly. The bivariate plot of the δ^{13}C values of Ser and Gly in Fig. 5 shows reasonable separation of the two adjoining harvesting areas using these two amino acids. An attempt to classify the Apalachicola bays solely based on these two stable isotope variables showed that 85% of the samples were correctly classified based on the original rules of the method, whereas 70% accuracy was obtained after LOOCV (data not shown).

However, MANOVA (Table S6†) using the two Florida harvesting areas as the fixed factor showed a total of five amino acids' isotope ratios, including δ^{13}C values of Ser, Gly, Val, Lys and Phe, to be significantly different at the 95% confidence interval. As shown in Fig. 6A, inclusion of these five amino acids in the discriminant analysis provided successful differentiation between the two regions and classification results of 95% and 90% for original rules and LOOCV, respectively (Fig. 6B). When using only δ^{13}C values of Ser and Gly, the final classification reached 70% accuracy (data not shown). It is important to mention that Val, Lys and Phe still presented high correlation (multicollinearity) among themselves when the two Florida regions were used as fixed factors. This parameter might adversely affect LDA because individual canonical coefficients will depict the effect of their original variables and the correlated ones. However, multicollinearity does not affect the cross-validated prediction accuracy of a model if the strength of each variable within the model is not of uttermost importance.[64] In our case, the goal of this particular analysis was to accurately predict the allocation of samples from two adjacent locations, thus making the inclusion of those variables valid. From a regulatory point of view, an investigator could use these results to help verify if the confiscated oysters were harvested in their reported area of origin, or an illegal area nearby, although



B

Classification Results[a,c]

			Predicted Group Membership		
		Region_code	FL1642	FL1662	Total
Original	Count	FL1642	10	0	10
		FL1662	1	9	10
	%	FL1642	100.0	.0	100.0
		FL1662	10.0	90.0	100.0
Cross-validated[b]	Count	FL1642	10	0	10
		FL1662	2	8	10
	%	FL1642	100.0	.0	100.0
		FL1662	20.0	80.0	100.0

a. 95.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 90.0% of cross-validated grouped cases correctly classified.

Fig. 6 (A) Contrast of oyster samples from FL 1642 and FL 1662 using LDA discriminant scores for five amino acids' isotope values. Only one sample from FL 1662 statistically overlapped with the adjacent region. (B) Table showing the linear discriminant classification results of oysters from the same two harvesting areas using δ^{13}C values of Ser, Gly, Val, Lys and Phe. 90% of the oysters were correctly assigned to their source after LOOCV.

90% successful classification does not provide unequivocal identification of the source.

A few considerations must be taken when one is using these techniques. As discussed by Mudge *et al.*,[38] shellfish elemental concentrations are highly affected by their site location, seasonal and inter-annual variability. Therefore, the proximity to waste disposal sites or changes in the oceanic currents can influence the ways in which shellfish are exposed to contaminants and nutrients. Our oyster samples included seasonal variance within each harvest area, but we hypothesize it would be possible to achieve greater discrimination between areas if seasonal variations were controlled. To strengthen the predictive power of this approach, future studies should include a larger number of samples from each area, and a sufficient number of samples should be collected from each season to enable the effects of season to be controlled as a factor of isotopic variance.

Conclusions

This feasibility study examined the ability to discriminate the harvesting areas of *C. virginica* oysters by using contaminant heavy metals like Cd and Pb and bulk or CSIA measurements. Using leave-one-out cross validation of a database of various oysters from five harvesting areas on the U.S. shore of the Gulf of Mexico, harvest areas could be correctly predicted with around 32% accuracy using the metal concentrations alone, which is not much better than the random match probability of 20%. When combining bulk δ^{13}C and δ^{15}N values with Cd concentrations, the reliability of predicting the harvesting area of oysters increased to approximately 71%. Pb concentrations did not seem to play a role in the region classification. Individually, the δ^{13}C values of amino acids Asx, Glx, Ser and Gly significantly differentiated the harvest regions at high confidence level on many occasions, even achieving separation of the Florida adjacent bays. The accuracy of harvest area predictions improved from 54% using only the δ^{13}C values for amino acid peaks of Asx, Glx, Ser and Gly, to 73% by adding Cd concentrations, based on the original rules of LDA. However, the cross-validated cases were correctly grouped with only 46% accuracy, likely due to an insufficient number of samples per region.

Surprisingly, oysters from the adjacent harvesting areas FL 1642 and FL 1662 provided five amino acids with δ^{13}C values significantly different at the 95% confidence level. Linear discriminant analysis using these five amino acids, Ser, Gly, Val, Lys and Phe, provided successful discrimination with 90% accuracy (LOOCV) for oysters from these two adjacent harvesting areas. This latter finding is valuable because these two adjacent areas are subject to different amounts of freshwater inputs, which leads to distinct exposures to anthropogenic contamination. For instance, FL 1662 is more susceptible to both microbial and chemical contamination from inland outflows because it receives extensive freshwater inputs. In times of heavy rainfall, this area is more likely to be closed by regulators due to contamination concerns. Conversely, FL 1642 is not exposed to extensive freshwater inputs, therefore it is not usually exposed to contamination from freshwater runoff. Hence, our ability to discriminate adjacent areas within Apalachicola bay has direct implications for the protection of human health.

In summary, the overall isotope analysis of bulk ^{13}C and ^{15}N measurements and CSIA of the δ^{13}C values of amino acids in the soft oyster tissues yielded noteworthy multivariate results for data analysis, ultimately providing more accurate predictions. Site differentiation would certainly improve with a larger sample size and analysis of additional trace elements, such as nickel, silver, mercury, selenium, cadmium, or zinc,[43,65–68] which were shown to be geographic markers for other marine organisms. The capability for high accuracy results and practical sample size requirements of this combinatory IRMS and ICP-

MS approach make it a potentially valuable tool for a direct application by oyster fisheries management and food safety enforcement agencies, but additional studies would be required to help validate the proposed approach.

Conflicts of interest

The authors declare no conflict of interest.

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