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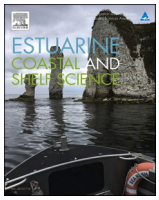

Promoting the use of non-lethal sample collection for analysing the trophic relationships of inshore flatfish populations using stable isotope analysis

Irmak Kurtul [a,b,*], Ali Serhan Tarkan [b,c,d], J. Robert Britton [b]

[a] Marine and Inland Waters Sciences and Technology Department, Faculty of Fisheries, Ege University, İzmir, Turkiye
[b] Department of Life and Environmental Sciences, Faculty of Science and Technology, Bournemouth University, Poole, Dorset, UK
[c] Department of Basic Sciences, Faculty of Fisheries, Muğla Sıtkı Koçman University, Muğla, Turkiye
[d] Department of Ecology and Vertebrate Zoology, Faculty of Biology and Environmental Protection, University of Łódź, Łódź, Poland

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ABSTRACT

Trophic studies on inshore flatfish populations usually rely on stomach content analyses and/or stable isotope analysis (SIA) of dorsal white muscle that involves the collection of samples from euthanised fishes. To promote the use of non-lethal sampling methods in inshore flatfish populations of relatively high intrinsic angling and/or ecological value, the applicability of using fin tissue and/or epidermal mucus as non-lethal alternatives to muscle in SIA studies was assessed for European flounder *Platichthys flesus*, plaice *Pleuronectes platessa* and common sole *Solea solea*. In all species, the results indicated that there were significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values of dorsal muscle versus their fin and mucus samples. These significant differences were, however, predictable by linear regression, with regression coefficients produced for converting fin and mucus SI values to the equivalent muscle SI values. The use of combined data across the species also provided regression coefficients for converting fin and mucus SI to equivalent muscle values for flatfish populations more generally. These results indicated that there are tissue alternatives to dorsal muscle that can be applied to the SIA of flatfish populations, with these tissues able to be collected using non-lethal sampling methods.

1. Introduction

The trophic ecology of commercially and/or functionally important inshore flatfishes has traditionally been completed using stomach content analyses, where insights have included prey selectivity in relation to prey availability, use of foraging habitats, and ontogenetic dietary shifts (e.g. Hinz et al., 2005; Vinagre et al., 2008). The application of stomach contents to dietary analyses is, however, usually destructive, where relatively large number numbers of individuals need sacrificing to provide statistically robust samples over time and space (Sandlund et al., 2016).

Populations of flatfishes can be vulnerable to high exploitation by inshore fishers (e.g. Cardinale et al., 2010) and they are often threatened at their range margins (e.g. Morais et al., 2011). Flatfishes are an important, seasonal species for inshore recreational anglers (Ladle and Pitts, 2013). They are increasingly used in tracking studies to measure their movements and habitat use (e.g. Mitamura et al., 2020; Baden et al., 2022). Correspondingly, ecological studies requiring the destructive sampling of relatively large numbers of juveniles and/or adults potentially represent a pressure on already stressed and/or valuable populations.

In recent decades, studies trophic and spatial ecology of fish have increasingly used stable isotope analysis (SIA; Trueman et al., 2012), where the stable isotope ratios of carbon and nitrogen (as $\delta^{13}C$ and $\delta^{15}N$) are typically applied to assess trophic interactions and food web structure (Fry, 2006; Perkins et al., 2014). The application of SIA to inshore flatfish populations has revealed, for example, the energy sources of juvenile production (Kostecki et al., 2012), spatial variation in feeding strategies (Cicala et al., 2019), and the trophodynamic linkages between river runoff and inshore fishery yields (Darnaude et al., 2004). These studies have, however, tended to use white muscle for SIA, sampled from euthanised fish that were often captured by professional fishers (e.g. Cicala et al., 2019). Although muscle can also be sampled non-lethally using a muscle plug, the use of these plugs to collect muscle samples from smaller fish can result in biased SI data due to its selective sampling of specific muscle types or regions within the fish body (Schielke and Post, 2010). As studies on the trophic ecology of flatfish populations need to at least consider using non-destructive sampling methods, there

* Corresponding author. Marine and Inland Waters Sciences and Technology Department, Faculty of Fisheries, Ege University, İzmir, Turkiye.
E-mail addresses: irmak.kurtul@ege.edu.tr (I. Kurtul), serhantarkan@gmail.com (A.S. Tarkan), rbritton@bournemouth.ac.uk (J.R. Britton).

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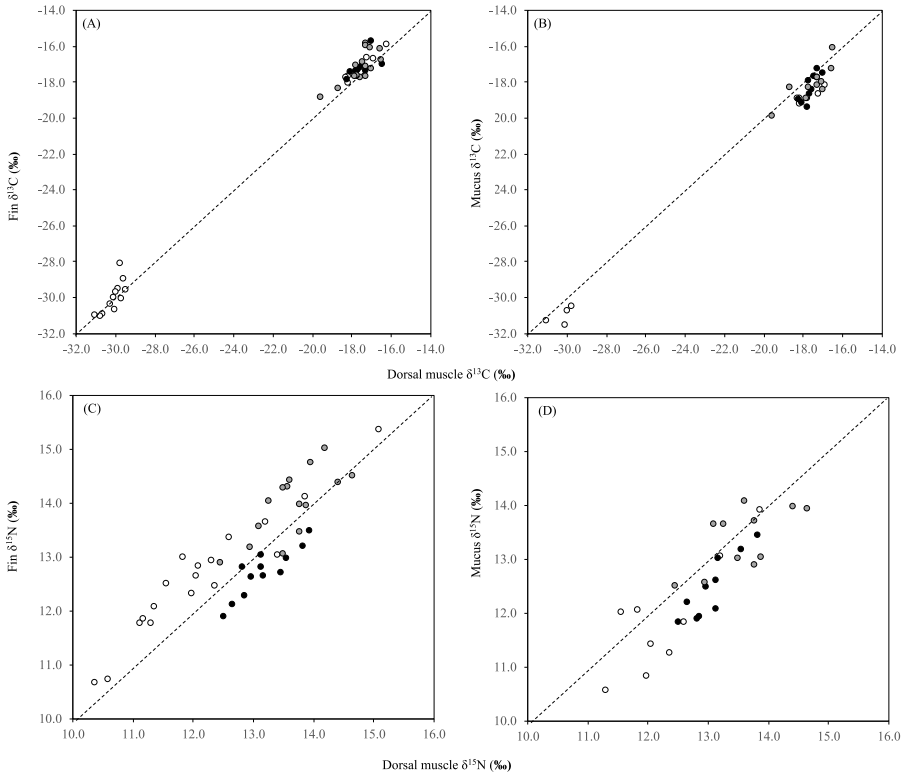

Fig. 1. Relationships between of dorsal muscle $\delta^{13}C$ with fin tissue (A) and mucus (B), and dorsal muscle $\delta^{15}N$ with fin tissue (C) and mucus (D) of European flounder (clear circle), plaice (filled circle) and common sole (grey circle).

is a need to assess the tissues that can be used as SI alternatives to muscle that can also be collected non-lethally - such as fin and mucus (e.g. Hutchinson and Trueman, 2006; Boardman et al., 2022).

While alternative tissues for SIA can provide reliable substitutes for white muscle, their values often need correcting if they are to be compared with dorsal muscle values from other studies (e.g. Maitland and Rahel, 2021; Roberts et al., 2021; Boardman et al., 2022). Compared with white muscle $\delta^{13}C$ data, fin values are often enriched, while mucus tends to be depleted (Winter et al., 2019a,b). However, there can be inter-specific differences in these relationships. For example, the differences in SI values between these tissues are minimal in European eel *Anguilla anguilla* (Boardman et al., 2022). Where there are substantial differences between the SI values of different tissues, these values are usually significantly correlated within species (Busst et al., 2015; Boardman et al., 2022).

Relationships between the SI values of different fish tissues, and their application in non-lethal sampling programmes, have mainly focused on species of the Salmonidae and Cyprinidae families (e.g. Busst et al., 2015; Church et al., 2009). Despite the regular application of SIA in ecological studies on inshore flatfish populations, there are no similar SI tissue relationships currently available. Consequently, the aim here was to test whether fin and mucus samples could be used to replace dorsal muscle samples in the SIA (as $\delta^{13}C$ and $\delta^{15}N$) of three inshore flatfish species in southern England, with development of both species-specific and general SI tissue conversion equations. We posit that the relationships between the SI values of the different tissues of these fishes will be predictable and significant, with SI values of fin tissue and mucus able to be reliably converted to equivalent dorsal muscle values for use in comparative studies.

2. Materials and methods

2.1. Study species and study area

The three flatfishes used were European flounder *Platichthys flesus*, plaice *Pleuronectes platessa* and common sole *Solea solea*, with samples collected in Dorset, southern England. Flounder were collected from three areas: lower River Frome (50°40′46″ N; 2°10′53″ W), Poole Harbour (50°41′25″ N; 1°56′22″ W) and the English Channel in Poole Bay (50°43′04″ N; 1°50′21″ W). The River Frome enters Poole Harbour approximately 8 km downstream of the sampling site, and Poole Harbour drains into Poole Bay. Plaice and sole were only present in samples from Poole Bay. Sample collection was in May and June 2022, where flounders were sampled from the River Frome using back-mounted electric fishing (Smith Root LR-24). All samples from Poole Harbour and Poole Bay were collected using rod and line angling, where all individuals were above the minimum conservation reference size (flounder and plaice: 270 mm, sole: 240 mm; Southern Inshore Fisheries Conservation Authority, 2023). Following their capture, all fishes were euthanised (anaesthetic overdose, MS-222) and frozen individually before subsequent processing in the laboratory.

2.2. Laboratory analyses

In the laboratory, fish were defrosted individually, identified to species and measured (total length, nearest mm). Samples of dorsal white muscle and dorsal fin were excised and rinsed in distilled water, with a mucus sample taken by running a cover slip along the length of the body. All samples were then transferred into individual 1.5 ml centrifuge tubes and dried at 60 °C to constant weight. The samples were then ground to powder, weighed to ~1000 μg in tin capsules, and analysed on a Thermo Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyser (CE Elantach Inc., USA), with the resultant data converted to values of $\delta^{13}C$ and $\delta^{15}N$ (as ‰). Analytical

Table 1
Results of linear regression of the relationships of dorsal muscle versus fin tissue and mucus SI data per species, and all species sampled in Poole Harbour and Poole Bay, and the associated regression coefficients of *a* and *b* for use in the equation to convert fin tissue and mucus SI data to the equivalent muscle values: $SI_{muscle} = (SI_{other} \times a) + b$, where SI_{other} = SI value of fin tissue or mucus.

	Species	Tissue	Test result	*a*	*b* (±95% CI)
$\delta^{13}C$	Flounder	Fin vs. muscle	$F_{1,16} = 2082$; $R^2 = 0.99$; $P < 0.01$	−0.96	0.97 (±0.02)
		Mucus vs. muscle	$F_{1,7} = 2552$; $R^2 = 0.99$; $P < 0.01$	1.34	1.02 (±0.02)
	Plaice	Fin vs. muscle	$F_{1,10} = 6.6$; $R^2 = 0.33$; $P = 0.03$	−7.85	0.56 (±0.22)
		Mucus vs. muscle	$F_{1,8} = 19.6$; $R^2 = 0.67$; $P < 0.01$	−10.13	0.41 (±0.09)
	Sole	Fin vs. muscle	$F_{1,13} = 19.4$; $R^2 = 0.57$; $P < 0.01$	−5.77	0.69 (±0.16)
		Mucus vs. muscle	$F_{1,9} = 17.3$; $R^2 = 0.62$; $P < 0.01$	−3.71	0.76 (±0.18)
	All	Fin vs. muscle	$F_{1,31} = 52.0$; $R^2 = 0.63$; $P < 0.01$	−5.06	0.73 (±0.21)
		Mucus vs. muscle	$F_{1,24} = 36.0$; $R^2 = 0.60$; $P < 0.01$	−5.83	0.64 (±0.22)
$\delta^{15}N$	Flounder	Fin vs. muscle	$F_{1,16} = 163.4$; $R^2 = 0.90$; $P < 0.01$	−0.53	1.00 (±0.08)
		Mucus vs. muscle	$F_{1,7} = 16.1$; $R^2 = 0.65$; $P < 0.01$	4.69	0.64 (±0.16)
	Plaice	Fin vs. muscle	$F_{1,10} = 33.8$; $R^2 = 0.75$; $P < 0.01$	2.07	0.87 (±0.15)
		Mucus vs. muscle	$F_{1,8} = 29.7$; $R^2 = 0.76$; $P < 0.01$	5.43	0.61 (±0.11)
	Sole	Fin vs. muscle	$F_{1,13} = 15.2$; $R^2 = 0.50$; $P < 0.01$	4.48	0.65 (±0.17)
		Mucus vs. muscle	$F_{1,9} = 5.9$; $R^2 = 0.33$; $P < 0.01$	4.31	0.69 (±0.28)
	All	Fin vs. muscle	$F_{1,31} = 42.3$ $R^2 = 0.58$; $P < 0.01$	4.55	0.65 (±0.21)
		Mucus vs. muscle	$F_{1,23} = 36.91$ $R^2 = 0.60$; $P < 0.01$	3.44	0.76 (±0.26)

precision of the $\delta^{13}C$ and $\delta^{15}N$ sample runs was estimated against an internal standard sample of animal (deer) material every 10 samples, with the overall standard deviation estimated at 0.08 and 0.04‰ respectively. C:N ratios were below 4.0 (mean 3.39 ± 0.04) and so did not require lipid correction (Post et al., 2007).

2.3. Statistical analyses

Species-specific conversion equations were developed to convert the stable isotope values of fin tissue and mucus samples to equivalent dorsal muscle samples. Initial analyses tested differences in muscle versus fin and mucus SI values using Wilcoxon tests (SI data were not normally distributed) or t-tests (SI data were normally distributed). Where differences in SI values between the tissues were significant, regression analyses then tested the relationships in the SI data between the tissues, where regression outputs included the regression coefficients of *a* and *b* to enable conversion of fin and mucus values (x axis) to their equivalent muscle values (y axis) for each species according to: $SI_{muscle} = (SI_{other} \times a) + b$ (Equation 1), where SI_{muscle} = equivalent value of dorsal muscle $\delta^{13}C$ and $\delta^{15}N$ and SI_{other} = actual SI value of fin or mucus.

Standard ellipse areas were then calculated for each species using $\delta^{13}C$ and $\delta^{15}N$ data from Poole Harbour and Poole Bay to evaluate the similarity in trophic (isotopic) niche positions and sizes (River Frome data were excluded due to their considerable differences in SI values to these two sites; *cf*. Results). Where these niches were similar in their position in isotopic space between the three species then it was considered that their SI data could be combined to calculate common regression coefficients for converting fin and mucus to the equivalent muscle SI data for flatfishes more generally. The standard ellipse areas (SEA) were calculated in the SIBER package in R (Jackson et al., 2011, 2012), where SEA is a bivariate measure of the distribution of individuals in isotopic space. As the ellipses enclose the core 40% of data, they represent the typical resource use of the analysed population (Jackson et al., 2011, 2012). The isotopic niches were expressed as SEA_c in SIBER (subscript '$_c$' indicates a small sample size correction was used; Jackson et al., 2012).

In the results, variations expressed around the mean were 95% confidence limits unless otherwise stated.

3. Results

Mean lengths of the fishes sampled from Poole Harbour and Poole

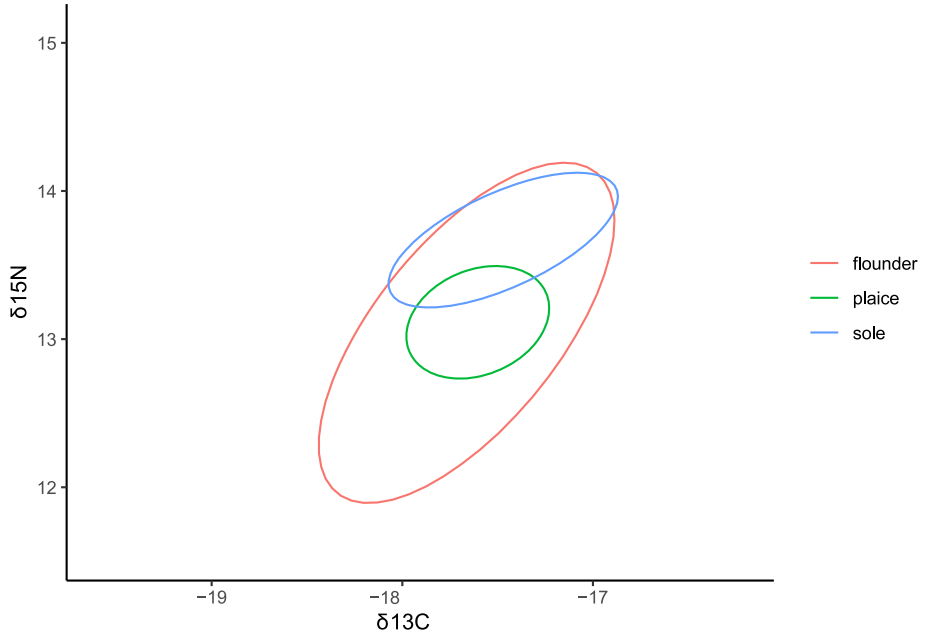

Fig. 2. Trophic niche sizes (as the isotopic niches, standard ellipse areas, SEAc) of European flounder, plaice and sole sampled from Poole Harbour and Poole Bay in May/June 2022.

Bay were flounder: 288 ± 0.7 mm (n = 6), Plaice: 302 ± 17 mm (n = 12) and sole: 274 ± 23 mm (n = 15), with the mean length of flounder sampled in the River Frome being 163 ± 37 mm (n = 12). All tissue samples were suitable for SIA of muscle and fin, but mucus data were only available from 9 flounder, 10 plaice and 11 sole (Table S1). In flounder, the samples from the River Frome were substantially depleted in $\delta^{13}C$ versus samples from Poole Harbour and Poole Bay (Frome: muscle: -30.12 ± 0.27, fin: -29.99 ± 0.51, mucus: -31.00 ± 0.48‰; Poole Harbour and Poole Bay: -17.51 ± 0.68, −17.11 ± 0.66; −18.77 ± 0.33‰ respectively; Fig. 1, Table S1). Frome flounders were also depleted in $\delta^{15}N$ versus the other samples (Frome: muscle: 11.67 ± 0.48, fin:12.09 ± 0.45, mucus: 1.58 ± 0.78‰; Poole Harbour and Poole Bay: 13.02 ± 1.06, 13.67 ± 0.80; 12.58 ± 0.78 ± 0.66; −18.77 ± 0.33‰ respectively; Fig. 1; Table S1).

There were significant differences in the muscle versus fin tissue, and muscle versus mucus SI data of all of the species (*t*-test/Wilcoxon tests; Table S2). Consequently, linear regression was applied to the $\delta^{13}C$ and $\delta^{15}N$ data of each species and all tissues to produce the regression coefficients of *a* and *b* for application in Equation 1 (Table 1). For the SI data collected in Poole Harbour and Poole Bay only, the trophic niches (as the isotopic niche through standard ellipse areas, calculated from muscle data) of plaice and sole sat almost entirely within the niche of flounder (Fig. 2). Thus, their data were combined here to determine standard regression coefficients for flatfishes more generally (Table 1). Across all species, fin SI data had general patterns of enrichment versus muscle, while values of mucus SI were depleted (Fig. 1; Table 1).

4. Discussion

The $\delta^{13}C$ and $\delta^{15}N$ data of these three flatfish species revealed significant differences between muscle and fin tissue (generally enriched), and muscle and mucus (generally depleted), as per our prediction. These SI differences between the tissue combinations were consistent, enabling the calculation of regression coefficients for converting fin tissue and mucus SI data to their equivalent dorsal muscle values. Thus, the sampling of fin tissue and mucus from these species provide reliable and non-lethal SI tissue alternatives to dorsal muscle in these three species, as is the case in many freshwater fishes, such as Northern pike *Esox lucius* (Winter et al., 2019a) and common carp *Cyprinus carpio* (Winter et al., 2019b, 2021). Moreover, the use of combined data from the three flatfish populations sampled from Poole Harbour and Poole Bay provided regression coefficients that could be suitable for applying to flatfish SI studies generally.

Dietary studies on marine fishes have generally relied on destructive sampling, with it acknowledged that stomach content analyses have provided considerable insights into the trophic relationships of focal species over time and space, such as cod *Gadus morhua* (Townhill et al., 2021). In inshore areas, however, there is increasing evidence of the socio-economic importance of recreational angling to local economies (Roberts et al., 2017), where catch and release practises are adopted by many anglers (Ferter et al., 2013). Thus, the intrinsic values to anglers of larger individual fish in populations of many inshore fishes (such as flatfishes and sport fishes such as sea bass *Dicentrarchus labrax*) can be high (Ladle and Pitts, 2013). Accordingly, we argue that the use of lethal sampling for dietary analyses in these populations should be avoided where possible. Moreover, movement studies based on tracking methodologies are increasingly using flatfishes as the focal species (e.g. Mitamura et al., 2020; Baden et al., 2022). Should these studies wish to couple movement data with trophic (SI) data then there will be a need for applying non-destructive sampling methods, as suggested here. For flatfishes, our results indicate that fin tissue and mucus samples are both suitable as these non-lethal SIA alternative tissues. As mucus tends to have a more rapid turnover rate than the other tissues then its application to studies assessing short-term dietary shifts is most appropriate. Fin tissue is then more suitable in studies where longer-term dietary perspectives are required (Winter and Britton, 2021). The application of mucus and fin tissue samples to trophic studies based in SI approaches of these flatfishes can thus move researchers away from using destructive sampling for valuable species and enable approaches that can generate data on a wider range of ecological and behavioural metrics to answer more complex research questions. Notwithstanding these insights and recommendations, the approach used in the study here was based on the sampling of inshore areas and a lower river reach in southern England only. Although there is confidence that the detected SI relationships between the tissues of the fishes will be valid across the range of these species, and not just in the vicinity of the sampling areas, this remains an assumption given it was not tested here.

Plaice and sole were only present in samples from Poole Harbour and Poole Bay, whereas flounder samples were available from across a salinity gradient from freshwater through to inshore areas. Within the inshore samples, the isotopic niches of plaice and sole were all in the same isotopic space as the inshore flounders, indicating these sympatric flatfishes were using relatively similar dietary resources (although their prey resources were not quantified any further). In these inshore samples, the number of flounders captured was relatively low versus the other fishes. Flounder sample numbers were thus complemented by fish captured from the non-tidal reaches of the River Frome. Juvenile flounders regularly encountered in the lower reaches of rivers, with these riverine habitats acting as important nursery areas (Kerstan, 1991). In the lower River Frome, a previous study based on stomach content analyses indicated that the diets of these riverine flounders were based entirely on freshwater prey (i.e. the individuals were not moving further downstream into estuarine or inshore areas to forage), with the use of the river advantageous in enabling faster growth rates versus flounders sampled from nearby inshore habitats (Beaumont and Mann, 1984). Indeed, the SI data from the riverine flounders here had a very strong freshwater signal (Nolan et al., 2019), which were strongly differentiated from SI values of all the species sampled from Poole Harbour and Poole Bay.

In summary, we suggest that there are strong arguments for increasingly adopting non-destructive sampling methods for inshore populations of fishes that have intrinsic angling and/or ecological value. For inshore flatfishes, we suggest that dietary studies can adopt SIA as its method of choice for analysing trophic relationships between species, where fin tissue and mucus provide tissues that can be collected non-invasively and non-destructively.

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CRediT authorship contribution statement

Irmak Kurtul: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Ali Serhan Tarkan:** Writing – original draft, Formal analysis. **J. Robert Britton:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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