Final Report

Brilliant Marine Research Idea 2025

This report presents the results of research conducted under the Brilliant Marine Research Idea grant, supported by The Sea as a Good Cause (VLIZ philanthropy).

1. General information

Title of the idea	Time travelling: reconstruction of historical dietary changes in fish through stable isotope analysis of otoliths
Name PhD student	Tuan Anh Bui
Name supervisor	Promotor: Prof. Dr. Marleen De Troch (Ghent University) Co-promotors: Dr. Jochen Depestele (Flanders Research Institute for Agriculture, Fisheries and Food, ILVO); Prof. Dr. Jan Jaap Poos (Wageningen University & Research)
Flemish University or University College	Ghent University

2. Brilliant Marine Research Idea – Report about the activities

Abstract

Over recent decades, there has been significant variation in the growth rates of common sole (*Solea solea*) populations in the Northeast Atlantic. Whether, and to what extent, this variation is related to dietary changes within the population remains unclear due to a lack of long-term dietary data. In this study, we aim to reconstruct historical dietary changes in sole using stable isotope analysis of otoliths, or fish ear stones, which act as a natural archive of an individual fish's lifetime. A total of 97 otoliths from two periods representing high growth (2004–2007) and low growth (2019–2022) in the Irish Sea were selected for analysis. All otoliths were obtained from age-2 fish within a size range of 250–280 mm, minimizing dietary variation associated with ontogeny, as larger and older sole tend to have more diverse diets. In addition, 39 age-2 otoliths from the 2022 cohort, spanning a range of body sizes from 198 to 331 mm, were selected to examine variation in isotopic composition associated with body size. Prior to analysis, protocols for measuring $\delta^{13}C$ and $\delta^{15}N$ of the organic fraction were developed and tested using five otoliths. The results of these analyses, along with the challenges and opportunities associated with otolith isotope analysis, will be presented in this report.

Intro

Over recent decades, there has been significant variation in the growth rates of common sole (Solea solea) populations in the Northeast Atlantic[1]. Part of this variation can be attributed to variation in annual temperature as we found that at higher temperature growth rate of juvenile fish increases but growth rate of adult fish decreases[1]. Another important

[1] Bui, T.A., et al., Otolith increments in common sole (Solea solea) reveal fish growth plasticity to temperature. Estuarine, Coastal and Shelf Science, 2025. 312: p. 109041.

factor that can contribute to the observed growth changes is dietary changes. Food availability and quality are very important to fish growth[2]. The biomass and composition of benthic prey items of sole may have changed due to climate change[3] and fishing activities, which disturbed the seabed[4]. However, whether, and to what extent, this variation is related to dietary changes within the population remains unclear due to a lack of long-term dietary data.

Otoliths, or fish ear stones, are calcified structures within the inner ear of fish that are deposited periodically in the fish's lifetime. The deposition rates of otoliths are affected by the environment and physiology of a fish, making them an effective natural archive of fish life history[5]. Stable isotope analysis of historical otoliths can thus provide insights into the temporal changes in fish diet[6]. Stable isotope analysis is based on the principle "you are what you eat", in which the stable isotope ratios of prey items are incorporated into the consumer's body tissues[7]. Specifically, values of ^{13}C and ^{15}N ratios can be used to infer the range and trophic level of prey items. Assessment of diets using stable isotope analysis can overcome the limitations of the traditional stomach contents analysis, such as reducing bias towards species that are digested more slowly, provide insights over a longer times scales (instead of a few hour before the fish is caught), and avoid misidentification of prey taxa[4].

A major challenge of otolith stable isotope analysis for diet studies is the low concentration of organic matter in otolith (<1-10% by mass)[6,8]. Conventional approach developed by Grønkjær et al. (2013)[6] requires about 65-100 mg of otolith sample to reliably identify ^{13}C and ^{15}N. A more recent protocol, applying oxidation and bacterial conversion processes, developed by Lueders-Dumont et al. (2018)[9] allows to reliably identify ^{15}N using otolith samples of as small as 3-4 mg. In this study, we aim to (1) develop a protocol to identify simultaneously ^{13}C and ^{15}N in sole otoliths by applying oxidation and bacterial conversion processes similar to the protocol developed by Lueders-Dumont et al. (2018), and (2) investigate the variation in isotopic composition of otoliths across time, focusing on two periods representing high and low growth, and across ontogeny.

Material & Methods

Otolith selection

This study focused on the recent 20 years in the Irish Sea population where sole growth has been declining significantly (Figure 1). For each otolith pair collected in this period, one otolith was processed for age determination and one otolith was kept intact as a backup. These intact otoliths enable stable isotope analysis without the risk of contamination from

[2] Wolf, N., et al., Assessing the relationship between diet and size-at-age in Pacific halibut (Hippoglossus stenolepis) using δ13C and δ15N analysis. Canadian Journal of Fisheries and Aquatic Sciences, 2019. 76(12): p. 2326-2331.
[3] Kröncke, I., et al., Changes in North Sea macrofauna communities and species distribution between 1986 and 2000. Estuarine, Coastal and Shelf Science, 2011. 94(1): p. 1-15.
[4] Hinz, H., et al., Stable isotopes reveal the effect of trawl fisheries on the diet of commercially exploited species. Scientific Reports, 2017. 7(1): p. 6334.
[5] Campana, S. E., and Thorrold, S. R. 2001. Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? Canadian Journal of Fisheries and Aquatic Sciences, 58: 30-38.
[6] Grønkjær, P., Pedersen, J.B., Ankjærø, T.T., Kjeldsen, H., Heinemeier, J., Steingrund, P., Nielsen, J.M. and Christensen, J.T., Stable N and C isotopes in the organic matrix of fish otoliths: validation of a new approach for studying spatial and temporal changes in the trophic structure of aquatic ecosystems. Canadian Journal of Fisheries and Aquatic Sciences, 2013. 70(2), pp.143-146.
[7] Hobson, K.A., Tracing origins and migration of wildlife using stable isotopes: a review. Oecologia, 1999. 120(3): p. 314-326.
[8] Sirot, C., Grønkjær, P., Pedersen, J.B., Panfili, J., Zetina-Rejon, M., Tripp-Valdez, A., Ramos-Miranda, J., Flores-Hernandez, D., Sosa-Lopez, A. and Darnaude, A.M., Using otolith organic matter to detect diet shifts in Bardiella chrysoura, during a period of environmental changes. *Marine Ecology Progress Series*, 2017. *575*, pp.137-152.
[9] Lueders-Dumont, J.A., Wang, X.T., Jensen, O.P., Sigman, D.M. and Ward, B.B., Nitrogen isotopic analysis of carbonate-bound organic matter in modern and fossil fish otoliths. *Geochimica et Cosmochimica Acta*, 2018. *224*, pp.200-222.

burning treatment that was applied to otoliths collected in the earlier period.

Sole otoliths often weigh around 5-20 mg, and one whole otolith can provide sufficient sample for stable isotope analysis using the protocol from Lueders-Dumont et al. (2018)[9]. However, the analysis cannot be conducted using micromilled samples from otolith rings as initially proposed due to insufficient sample mass. Therefore, the analysis was done using whole otoliths.

For the analysis of otolith across time, age-2 fish within the size range of 250-280 mm were selected. This size range minimised the variation of diet across ontogeny as larger and older sole have a more diverse diet[10]. While the selection of age-1 fish would be preferable, the sample size was insufficient. Overall, 97 otoliths were selected in two periods representing high growth (2004-2007) and low growth (2019-2022).

For the analysis of otolith across ontogeny, 39 age-2 otoliths from the 2022 cohort, spanning a range of body sizes from 198 to 331 mm, were selected to examine variation in organic ^{13}C and ^{15}N associated with body size.

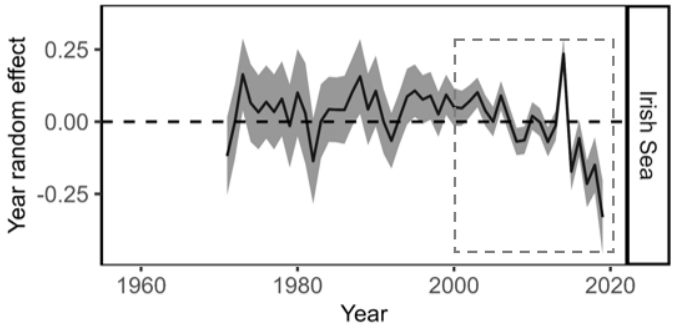

Figure 1. Time series of sole growth reconstructed from otolith data in the Irish Sea population. Lines above and below the horizontal dotted line indicate good and poor growth years, respectively.

Stable isotope analysis of otolith

The protocol for stable isotope analysis of otoliths for both organic carbon (^{13}C) and nitrogen (^{15}N) was developed based on previous protocols developed by Grønkjær et al. (2013)[6] and Lueders-Dumont et al. (2018)[9]. A short description of the protocol is as follows:

- Otolith cleaning
    - Otoliths were soaked in 10mL NaOCl 15% overnight in 15mL polypropylene centrifuge tubes on a continuous shaker (100 times/min)
    - Soaked otoliths was rinsed 3 times with milliQ water and then transferred to a pre-combusted 12 mL glass vial for over-night dry at 60 °C
    - Dried otoliths were weighed into 4 mL pre-combusted glass vials and stored at room temperature
- Organic carbon analysis
    - Cleaned otoliths were treated with acid to remove the inorganic carbon. For this 0.5 mL 0.1 M HCl was first added, after 24h at 4°C 2.2mL 0.5M HCl was added and kept at 4°C until total demineralization after which an additional 24 h was waited before further treatment.
    - Demineralized samples were centrifuged 40 min at 3000 g and supernatant was transferred on a 10kDa centrifuge filter. HCl is removed by centrifugation (2500 g) and rinsed 2 times with 4mL miliQ, until only 150 to 500 µL remained on the filter.

[10] Rijnsdorp, A.D. and Vingerhoed, B.V., Feeding of plaice Pleuronectes platessa L. and sole Solea solea (L.) in relation to the effects of bottom trawling. Journal of Sea Research, 2001. 45(3-4), pp.219-229.

- Filtered samples were transferred to vial. 1mL of acidic persulfate solution (40 $g \cdot L^{-1}$ $K_2S_2O_8$ + 2 $mL \cdot L^{-1}$ 85% H_3PO_4) was added for oxidation. The vials were flushed with He and heated at 100°C for 60 minutes.
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- Organic nitrogen analysis
  - Samples after organic carbon analysis were neutralized with $CaCO_3$ to remove excess acid. 3 mL Milli-Q water was added.
  - Neutralized samples were transferred for bacterial conversion.

After testing the protocol using 5 test otoliths (apart from the sampled 136 otoliths for the final analyses), several challenges of simultaneous analysis of organic ^{13}C and ^{15}N occurred. First, the $K_2S_2O_8$ - H_3PO_4 solution used for oxidation made the samples prone to contamination of NH_3 presenting in the laboratory. In addition, the large amount of Ca^{2+} required for neutralization, together with the presence of PO_4^{3-}, made the samples very viscous and difficult to transfer to the anoxic bacterial culture. An alternative approach was to split the filtered sample prior to oxidation for separate analyses of ^{13}C and ^{15}N. However, due to limited mass of sole otoliths in this study and the high blank experienced during the test phase, this approach was not possible. Ultimately, we decided to conduct only the analysis for ^{13}C.

Results/Conclusions

Variation of organic ^{13}C across time

$\delta^{13}C$ values of otolith across time ranged between -28.41 ‰ to -16.85 ‰ v.s. VPDB. The mean of $\delta^{13}C$ of otoliths in the period of high and low growth were -21.94 ± 2.77 ‰ and -22.97 ± 2.79 ‰, respectively. There was no statistically significant difference in mean $\delta^{13}C$ between the two periods. This result suggests no difference in the diet composition of age-2 fish within the 250–280 mm size range across periods. Although this finding is unexpected given the marked differences in growth rate and size at age 2 between periods, it may be explained by the fact that the diet of fish within the 250–280 mm size range is dominated by Annelida, with limited contributions from other prey[9]. As a result, potential dietary shifts between periods may be difficult to detect. An additional analysis focusing on older and larger fish, whose diet composition is more diverse[9], would be a useful follow-up to further investigate potential diet shifts associated with growth changes. However, conducting this analysis on sole is challenging because it requires micromilled samples from specific growth increments, which may yield insufficient otolith mass and organic matter content for isotopic analysis.

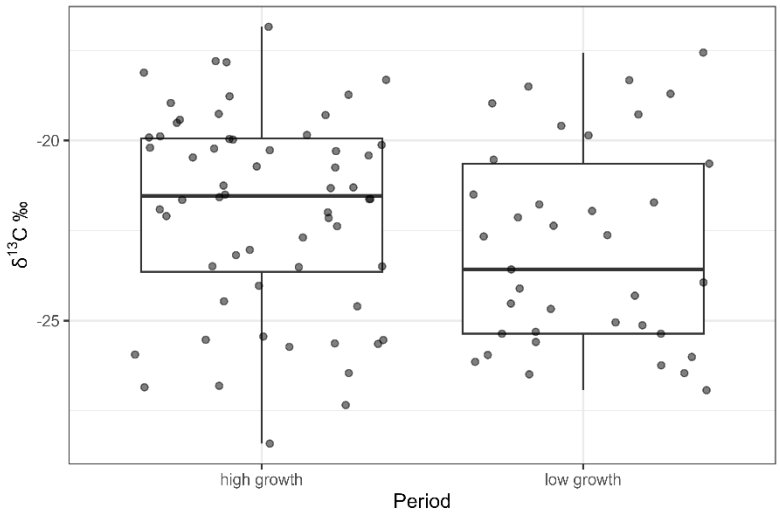


Figure 2. Variation in $\delta^{13}C$ across time.

Variation of organic ^{13}C across body sizes

The mean organic carbon content in otolith was 0.65 µg/mg, which is consistent with the results of previous studies on the otoliths of other fish species[6,8]. $\delta^{13}C$ values of otolith across fish sizes ranged between -23.11 ‰ to -12.14 ‰. $\delta^{13}C$ values were positively correlated with fish size (R = 0.51, p-value < 0.05), i.e. larger fish having a less negative $\delta^{13}C$ values (Figure 3). This positive correlation was also observed in the analysis using muscle tissues of sole under 500 g in the Atlantic shelf seas[11] and seems to represent the ecological difference in diet across fish size. A study in the North Sea showed that the diet of small sole under 250 mm is dominated by Annelida, while the contribution of Crustacea, Bivalvia, Echinodermata, and other preys increases as fish size increases[10]. Many species of Annelida are deposit feeders that consume organic matter settled in the sediment. Sediments in coastal areas, where sole inhabit, often contain terrestrial organic matter, which is more depleted in ^{13}C (i.e., lower $\delta^{13}C$ values[12]). In contrast, Crustacea and Bivalvia are often filter feeders that consume food from more pelagic sources and therefore tend to have more enriched in ^{13}C (i.e., higher $\delta^{13}C$ values).

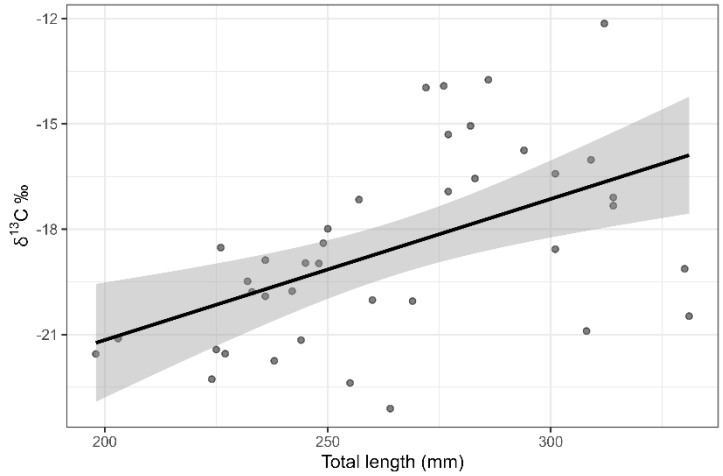


Figure 3. Positive correlation between $\delta^{13}C$ and fish length.

[11] Jennings, S. and Cogan, S.M., Nitrogen and carbon stable isotope variation in northeast Atlantic fishes and squids: Ecological Archives E096-226. Ecology, 2015. 96(9), pp.2568-2568.

[12] Peterson, B. J., & Fry, B., Stable isotopes in ecosystem studies. *Annual review of ecology and systematics*, 1987. 293-320.

Challenges and opportunities of isotope analysis of organic matter in otolith

Challenges:

- Simultaneous analysis of organic carbon (C) and nitrogen (N) in small sole otoliths remains technically challenging. Further methodological development and protocol optimization are required.
- Separate analyses of C and N are feasible; however, they require otoliths with sufficient mass, which may limit applicability for small individuals or micromilled samples.

Opportunities:

- Stable isotope analysis of archived otoliths provides an opportunity to reconstruct historical diet information over time. This approach adds a valuable dimension to the existing analytical toolbox and enables further exploitation of the scientific potential of the thousands of otoliths stored in archival collections.
- Although additional methodological refinement is needed, the ability to simultaneously analyze organic C and N from the same otolith sample is promising. This advancement would maximize the information obtained from limited material and improve our capacity to investigate long-term trophic and ecological changes.

3. Overview of the expenditures

Describe in detail how the requested fund was spent within the implementation period (1 March 2025 and 28 February 2026). Be as specific as possible.

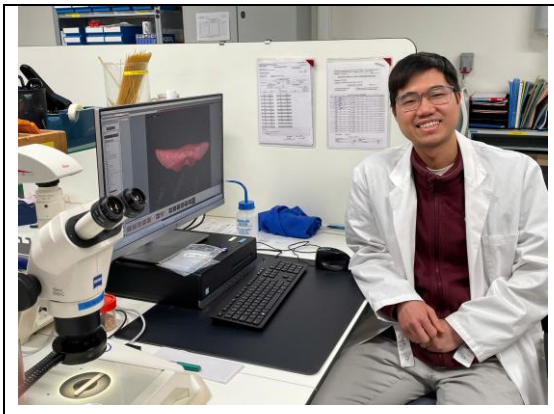
The total BMRI prize amounted to €4842. In accordance with UGent regulations, 10% overhead was deducted, resulting in a net available research budget of €4357,8. The full net amount of €4357,8 was spent within the implementation period and used exclusively for project-related laboratory analyses and essential research consumables. The majority of the budget (€3915; SAP 5106086408) was allocated to stable isotope analyses performed by ISOFYS. The remaining funds were used for laboratory consumables and materials required for sample preparation and biochemical analyses (SAP documents 5106016560, 5106021701, 5106098412, 5106098416). All expenses were incurred in 2025, recorded in the institutional SAP system, and directly aligned with the approved project objectives. All further details in the table below and the attached excel file.

The difference between the amounts on the invoice and the amounts reported here stems from the differentiated VAT-regime of UGent

ID	Description	Supplier	Amount (€)	Notes	SAP documents	Date
1	Stable isotope analyses	ISOFYS	3,915.00	Lab analyses	5106086408	24/09/2025
2	2.5LT Sodium hypochlorite, 13% active chlorine	Fisher Scientific	39.50	Chemicals	5106016560	03/03/2025
3	Corning(r) falcon(r) centrifuge tubes, 1	Merck Life Science	222.85	Lab materials	5106021701	16/03/2025
4	Ripa buffer, 100ml	Fisher Scientific	132.97	Chemicals	5106098412	27/10/2025
5	Microcentrifuge tube, 1.5 ml, polyprop&	Merck Life Science	63.08	Lab materials	5106098416	27/10/2025
	Total		4,373.40			

4. Pictures

A set of five pictures (low resolution in this document). The five high resolution pictures should be delivered to VLIZ by email to karen.rappe@vliz.be.



Picture 1. Tuan Anh and the image of a sliced sole otolith

Picture 2. The otolith archive at ILVO

Picture 3. Selected otoliths for the analysis

Picture 4. Otolith cleaning

Picture 5. Otolith cleaning