



Statolith-Based Squid Stock Analysis of European  
*Illex coindetii* and *Loligo forbesii* to Support  
Sustainable Fisheries Management

CUMULATIVE DISSERTATION

for the attainment of the academic degree  
Doctor of Natural Sciences (Dr. rer. nat.)

at the Institute of Biosciences  
Faculty of Mathematics and Natural Sciences  
University of Rostock

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born on 19.08.1989 in Goslar

Rostock, 2025

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Year of submission: 2025

Year of defense: 2026



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## Abstract

Although cephalopods are commercially used in Europe, they are currently not covered by the European Common Fisheries Policy (CFP), and are therefore not subject to fisheries management, resulting in unregulated fishing in Europe. To avoid overfishing and promote sustainable fisheries, it is essential to identify and differentiate so-called fishery stocks. In this study, I focused on the squids *Illex coindetii* and *Loligo forbesii*, two of the most economically important cephalopod species in the North East Atlantic and Mediterranean Sea. To distinguish stocks from different fishing areas, shape analysis and microchemical analysis of statoliths combined with age determination were employed. The results revealed distinct stocks and cohorts, and suggested potential migration patterns both between stocks (shared feeding grounds) and throughout ontogeny (shared spawning or nursery grounds). In addition, spawning areas and seasonal patterns of *L. forbesii* were determined. These findings contribute to the identification and distinction of *Illex coindetii* and *Loligo forbesii* stocks and thus provide an important foundation for future sustainable fisheries management of the two studied species in Europe. Furthermore, this study highlights current gaps in the assessment and management of cephalopods in the North East Atlantic and Mediterranean Sea and illustrates the opportunities and limitations of assessing European cephalopod stocks.

### KEY WORDS:

broadtail shortfin squid, veined squid, Loliginidae, Ommastrephidae, Myopsida, Oegopsida, statolith shape analysis, statolith microchemistry analysis, stock assessment, stock structure, MSFD

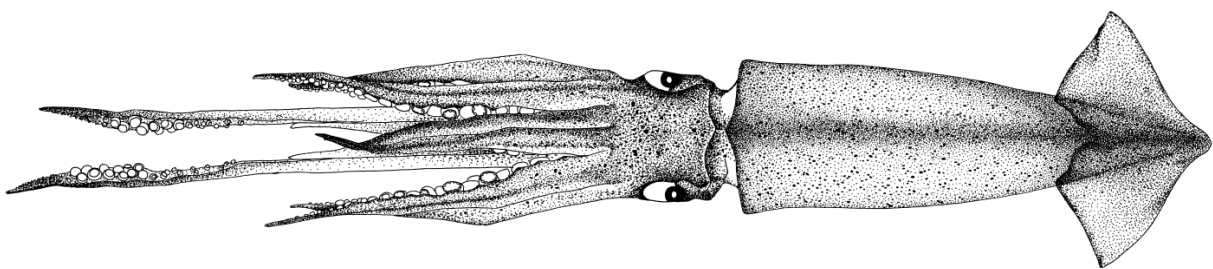


Figure 1: Habitus illustration of *Illex coindetii* in dorsal view



## Zusammenfassung

Obwohl Kopffüßer in Europa kommerziell genutzt werden, sind sie derzeit nicht in die Gemeinsame Fischereipolitik (GFP) der Europäischen Union integriert und unterliegen daher keinem Fischereimanagement, welches in einer unregulierten Befischung in Europa resultiert. Um Überfischung zu vermeiden und eine nachhaltige Fischerei zu ermöglichen, ist es notwendig, sogenannte Fischereibestände zu identifizieren und voneinander abzugrenzen. In der vorliegenden Studie konzentrierte ich mich auf die Kalmare *Illex coindetii* und *Loligo forbesii*, zwei der wirtschaftlich bedeutendsten Tintenfischarten im Nordostatlantik und Mittelmeer. Zur Unterscheidung von Beständen aus verschiedenen Fanggebieten wurden die Umrissanalyse sowie die mikrochemische Analyse der Statolithen in Kombination mit Altersbestimmung verwendet. Die Ergebnisse zeigten abgegrenzte Bestände und Kohorten und deuteten auf potentielle Migrationsmuster sowohl zwischen Beständen (gemeinsame Nahrungsgründe) als auch im Laufe der Ontogenese (gemeinsame Laich- oder Aufzuchtgründe) hin. Ergänzend dazu konnten Schlupfgebiete und saisonale Muster von *L. forbesii* ermittelt werden. Diese Erkenntnisse tragen zur Identifizierung und Trennung von *Illex coindetii* und *Loligo forbesii* Beständen bei und bilden somit eine wesentliche Grundlage für ein zukünftiges, nachhaltiges Fischereimanagement der beiden untersuchten Arten in Europa. Darüber hinaus weist die Studie auf bestehende Lücken in der Bewertung und dem Management von Kopffüßern im Nordostatlantik und dem Mittelmeer hin und zeigt sowohl die aktuellen Möglichkeiten als auch die Grenzen der Bestandsbewertung europäischer Kopffüßer auf.

### SCHLÜSSELWÖRTER:

Breitschwanz-Kurzflossenkalmar, Nordischer Kalmar, Loliginidae, Ommastrephidae, Myopsida, Oegopsida, Statolithen Umrissanalyse, Statolithen Mikrochemieanalyse, Bestandsstruktur, MSRL



## Acknowledgements

I am deeply thankful to the Begabtenförderungswerk Evangelisches Studienwerkt Villigst e.V., which funded this PhD through its scholarship program. In addition to financial security, their support also provided access to an inspiring community and opportunities for personal and academic growth.

I would also like to express my sincere thanks to Prof Dr Inna Sokolova and Dr Daniel Oesterwind. This work would not have been possible without their excellent supervision.

Although I wrote my thesis outside of the University of Rostock, the supervision by Prof Dr Inna Sokolova, as head of the Department of Marine Biology, was invaluable. She always had an open ear - or rather an open door - for all non-subject-specific questions, was patient, responded quickly, and was always happy to hear about good news, the progress of my work, and the written manuscripts. A special thanks goes to Dr Daniel Oesterwind, whose support was instrumental even before the official start of the thesis. As my co-supervisor, he was the perfect contact person for all subject-specific questions throughout my PhD. His role as chair of the WGCEPH of ICES also allowed me to connect with the European cephalopod research community, establish valuable contacts, and receive further support for my research.

I also want to thank the Thünen Institute of Baltic Sea Fisheries for the opportunity to work there as a guest scientist, with my appreciation to the head of the institute, Dr Christopher Zimmermann, who responded quickly to any question I had. I am especially grateful to my dear colleagues Carl Bukowski, Christina Henseler and Maraja Riechers for their support, inspiring discussions, shared meal breaks, and the many memorable moments at the institute.

My gratitude also extends to the Paleoclimate Research Group headed by Klaus Peter Jochum at the Max Planck Institute in Mainz. The microchemical analysis of my samples would not have been possible without the expert support of Brigitte Stoll and Ulrike Weis.

Moreover, I want to thank all co-authors for their support, collaboration and the collected samples. Speaking of which, I also want to express my gratitude to the squids that gave their lives for science – they not only provided the data this thesis is based on but also served as a valuable source of protein for my dear colleagues.

Finally, I would like to thank my family and my friends, most of whom are unfamiliar with academic life, yet have accompanied me on the path to my PhD.





## List of abbreviations

|           |                                                                 |
|-----------|-----------------------------------------------------------------|
| AuWiSS    | Autumn-Winter Spawning Stock                                    |
| CFP       | Common Fisheries Policy                                         |
| DML       | Dorsal mantle length                                            |
| GFP       | Gemeinsame Fischerei Politik                                    |
| IAMS      | Irish Anglerfish and Megrin Survey                              |
| IBTS      | International Bottom Trawl Survey                               |
| ICES      | International Council for the Exploration of the Sea            |
| LA-ICP-MS | Laser Ablation – Inductively Coupled Plasma – Mass Spectrometry |
| MEDITS    | Mediterranean International Bottom Trawl Survey                 |
| MSFD      | Marine Strategy Framework Directive                             |
| MSRL      | Meeresstrategie Rahmenrichtlinie                                |
| MSY       | Maximum sustainable yield                                       |
| PC1       | Principal component one                                         |
| PC2       | Principal component two                                         |
| PCA       | Principal component analysis                                    |
| Q1        | Quarter 1 – January to March                                    |
| Q3        | Quarter 3 – July to September                                   |
| Q4        | Quarter 4 – October to December                                 |
| SCOWCGFS  | Scottish West Coast Groundfish Survey                           |
| SpSS      | Spring Spawning Stock                                           |
| SpSuSS    | Spring-Summer Spawning Stock                                    |
| SpSuSS    | Spring-Summer Spawning Stock                                    |
| SuSS      | Summer Spawning Stock                                           |
| SuSS Q1   | Summer Spawning Stock captured in Q1                            |
| SuSS Q3   | Summer Spawning Stock captured in Q3                            |
| WGCEPH    | Working Group on Cephalopod Fisheries and Life History          |
| WiSpSS    | Winter-Spring Spawning Stock                                    |
| WiSS      | Winter Spawning Stock                                           |



## Part I      Summary of the doctoral study





# 1 Introduction

## 1.1 Relevance of the Thesis

Climate change and food-web changes have caused a worldwide distribution shift and a change in abundance of cephalopod species (Chen et al., 2006; Guerreiro et al., 2023). Both increases and decreases in abundance are reported, reflecting a variety of underlying causes. Climate-induced changes in abiotic factors and habitat suitability can extend, shift or decrease the distribution area via migration, especially in highly mobile species like squids (Schickele et al., 2021; Guerreiro et al., 2023). Furthermore, cephalopods can profit from changes in the food web, e.g., due to overfishing of predatory finfish stocks. With absent key competitors, highly adaptable cephalopods can quickly occupy ecological niche spaces. Additionally, the lack of main predators reduces predation pressure, further benefiting cephalopod populations (Caddy and Rodhouse, 1998; Boyle and Rodhouse, 2005). Contrarily, fishing pressure on cephalopods from both indirect (bycatch) and direct (targeted) fisheries can lead to an overexploitation (Rodhouse et al., 2014), especially without catch limits. Currently, most cephalopods are not subject to fisheries management, resulting in unregulated fishing in Europe. To avoid overfishing and promote sustainable fisheries, it is essential to identify and differentiate so-called fishery 'stocks'. Fish 'stocks' are defined as more or less isolated, self-sustaining units of living resource populations (FAO Fishery Glossary, n.d.). In fisheries, this definition is combined with economic considerations to characterise those stocks that are significant for fisheries exploitation. This study highlights current gaps in the assessment and management of cephalopods in the North East Atlantic and Mediterranean Sea and illustrates the opportunities and limitations for an assessment of European cephalopods.

Intense fishing activity could lead to stock collapse in cephalopods, particularly given their semelparous life cycle, where a single reproductive event is followed by death (Vaupel et al., 2013). To avoid an overexploitation and guarantee sustainable fisheries, it is necessary to define, assess and manage cephalopod stocks which have gained increasing commercial interest in fisheries (Pierce and Portela, 2014). Although the European Marine Strategy Framework Directive (MSFD) has mandated an assessment for the Good Environmental Status (GES) since 2012, cephalopods have so far received limited attention due to incomplete data as well as a lack of appropriate indicators (**Manuscript I**). Furthermore, the European Common Fisheries Policy (EU-CFP) aims at an assessment for sustainable fisheries, which requires even



more data than an assessment within the MSFD. Ten key challenges are evident for the management of cephalopod fisheries: (i) rapid growth rates, (ii) individual variability in growth rates, (iii) high productivity, (iv) ecological opportunism, (v) short lifespan, (vi) complex life cycle phenology, and (vii) fluctuations in population abundance. Additionally, management are hindered by (viii) difficulties in species identification, (ix) challenges in accurate age determination, and (x) the absence of clearly defined populations or stocks (ICES, 2023). These conditions make a traditional finfish stock assessment difficult or impossible to transfer to cephalopods.

However, existing data on cephalopods already provide a foundation for assessment. Fisheries landings data as well as various surveys, like the International Bottom Trawl Survey (IBTS) in the North East Atlantic or the Mediterranean International Trawl Survey (MEDITS) in the Mediterranean Sea, while not focused on cephalopods, allow a limited assessment of many cephalopod species in Europe (**Manuscript I**; ICES, 2023). Nevertheless, stock identification as a key component of fisheries stock assessment entails various difficulties. For example, the swimming capacity of various cephalopod species suggests an ‘intermediate’ level of population exchange (Payne et al., 2013), which seems to correspond to an intermediate level of genetic differentiation in cephalopod populations (Wolfram et al., 2006). Hence, populations might share spawning grounds but migrate into distinct areas outside this region, which results in biological differences (Begg et al., 1999; Arvanitidis et al., 2002; Moreno et al., 2002). For this reason, genetic microsatellite analysis appears to reveal differences only at a larger geographical scale (Göpel et al., 2022). Moreover, conventional age-based models are often impractical for identifying and assessing cephalopod stocks (Arkhipkin et al., 2021), requiring alternative methods to meet the unique challenges posed by these short-lived species.

One such alternative approach is morphological analysis of hard structures, such as statoliths or beaks. It has shown promise in distinguishing stocks, albeit with some limitations (Fang et al., 2014). Various studies used statolith shape to develop regional-scale markers for an ‘ecological’ stock identification based on the shape alone or in combination with microchemical analyses of the statolith or genetic surveys (Green et al., 2015; Fang et al., 2018; Sheerin et al., 2022; Wang et al., 2024). Statoliths, as calcified structures, contain valuable ecological information in their microstructure and chemical composition (Zumholz,



2005) and can serve as archives of life cycle information (Lipiński, 2001). Morphological Fourier shape analysis of otoliths is a valuable tool for fish stock discrimination, as numerous studies have shown differences in otolith shapes among stocks of the same fish species (Campana and Casselman, 1993; Smoliński et al., 2020). Fourier shape analysis also shows promise for analysis of cephalopod statoliths, as their shapes are species-specific, with biologically-controlled crystallisation involving various activation and inhibition factors (Lipiński, 2001). Further, laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) is an effective tool for analysing the microchemistry of both fish otoliths and cephalopod statoliths, providing insights into individual life histories, migratory behaviour, and habitat use (Zumholz et al., 2007). Variations in elemental compositions, detected by LA-ICP-MS, can reflect environmental conditions (e.g., water chemistry, temperature, and salinity) as well as ontogenetic differences (Fowler et al., 1995; Thorrold et al., 1997).

The current study focuses on the stock identification and differentiation of two of Europe's most commercially important species, *Illex coindetii* and *Loligo forbesii*, through analyses of the statolith shape (**Manuscript II**) and microchemistry (**Manuscript III**) analysis using Fourier analysis and LA-ICP-MS, respectively, in combination with biological data. In addition to stock structure and distribution, identification of the potential spawning grounds and seasons of *L. forbesii* (**Manuscript IV**) represent a crucial element in the management of these stocks.



## 1.2 Focus and Key Contributions

The increasing commercial importance of cephalopods, their unpredictable abundance fluctuations, as well as the risk of overfishing highlight the urgent need for stock assessment and fisheries management (ICES, 2023). Protective measures, such as temporal and spatial fishing restrictions, can help prevent the exploitation of reproducing individuals and should account for regional differences in spawning seasons. This thesis aims to provide scientific data for such protective measures. More specifically, the objective of this thesis was to differentiate populations of *Illex coindetii* and *Loligo forbesii* in the North East Atlantic and Mediterranean into distinct stocks, providing a basis for sustainable fisheries management.

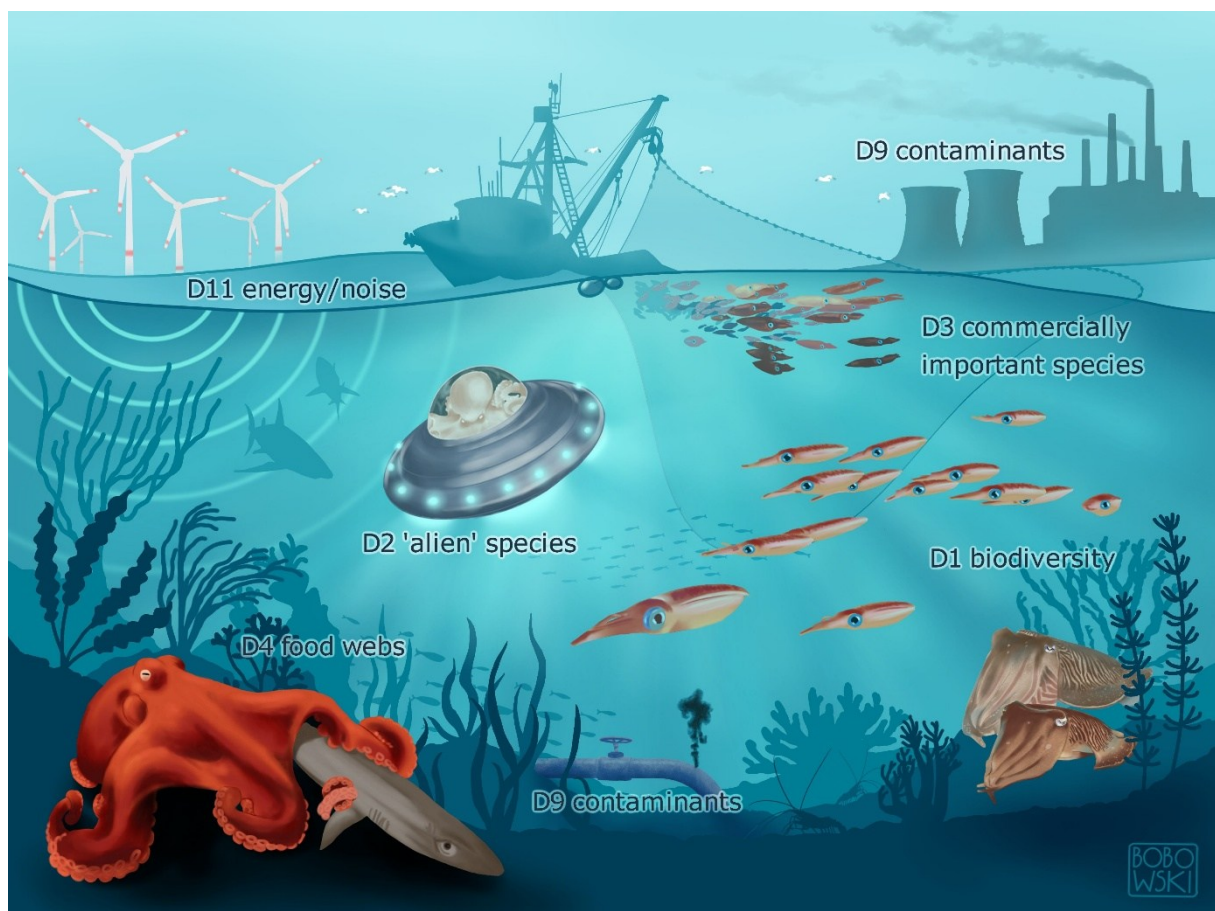


Figure 2: Illustration of the different MSFD cephalopod related Descriptors. This illustration was also used as graphical abstract for Manuscript I (Bobowski et al. 2023)

**Manuscript I** summarizes potential indicators for meeting the criteria of each cephalopod-related Descriptor within the framework of the European Marine Strategy Framework Directive (MSFD), highlighting those that can already be addressed using existing survey data and analytical methods. It also introduces the individual Descriptors (D1 – D11) and identifies those under which cephalopods should be included (Figure 2). Furthermore, the





manuscript examines the current state of cephalopod inclusion in the MSFD, detailing which member states are obligated to report on cephalopods, which have already done so in the 2012 and 2018 reporting cycles, and the reason some states provide for the exclusion of cephalopods within their reporting. In addition, it provides an overview of the MSFD's primary goal of protecting and preserving the marine environment and its natural resources.

**Manuscript II** and **III** are focused on the stock discrimination of European *I. coindetii* and *L. forbesii*, using three statolith based approaches: (i) shape analysis (**Manuscript II**), (ii) microchemical analysis (**Manuscript III**) and (iii) age determination (also in **Manuscript III**). For comparability and data integration, all three methods were conducted on the same statolith samples. In addition to distinguishing between different areas, **Manuscript II** examined the influence of sex and maturity stage on statolith shape. Meanwhile, **Manuscript III** incorporated age determination to identify hatching periods of the analysed individuals. The combination of methods revealed distinct stocks across the studied areas for both species. Additionally, **Manuscript IV** focused on spawning areas and seasons of *Loligo* spp. within the Lusitanian Zoogeographical Province over a period of more than 30 years.

In summary, the current status of cephalopods within the MSFD (**Manuscript I**), the identified stocks of *I. coindetii* and *L. forbesii* across the sampled areas in the North East Atlantic and Mediterranean Sea (**Manuscript II & III**), along with the identification of spawning areas and seasons of *L. forbesii* (**Manuscript IV**), provide a solid foundation for a sustainable fisheries management of these two species. Additionally, the study highlights both the limitations of individual methods and how their combination can offer a more comprehensive overall view.



### 1.3 Scientific approval

The results of this study were presented in international and national conferences and seminars as well as at an internal institute colloquium:

1. ICES WGCEPH 2021. WebEx, June 2021
  - **Presentation - Bobowski, B.T.C.** – Assessment status of cephalopods in Europe
2. CIAC (Cephalopod International Advisory Council) 2022. Sesimbra, Portugal, April 2022
  - **Poster - Bobowski, B.T.C., Power, A.M., Pierce, G.J., Moreno, A., Iriondo, A., Valeiras, J., Sokolova, I., Oesterwind, D., 2022.** The missing assessment of European cephalopods – Future integration of cephalopod species under Descriptor 1 and 3 within the MSFD.  
<https://doi.org/10.13140/RG.2.2.29016.16641>
  - **Presentation - Pierce, G.J., Abad, E., Ainsworth, G.B., Allcock, A.L., **Bobowski, B.T.C.** et al., 2022.** Management for sustainable cephalopod fisheries in Europe: review and recommendations
3. ICES WGCEPH 2022. Tenerife, Spain, June 2022 – online participation
  - **Poster - Bobowski, B.T.C.** – Cephalopods within the MSFD
4. Lange Nacht der Wissenschaft – Rostock University. Rostock, Germany, May 2023
  - **Presentation - Bobowski, B.T.C.** - Tintenfische – Alles andere als Fisch
5. ICES WGCEPH 2024. Lowestoft, England, June 2024 – online participation
  - **Presentation - Bobowski, B.T.C.** - Squid stock discrimination by statolith shape analysis and microchemistry
6. Thünen Institute of Baltic Sea fisheries colloquium. Rostock, Germany, February 2024
  - **Presentation - Bobowski, B.T.C., Oesterwind, D.** - Bestandsidentifikationsmethoden von Kopffüßern

The material of this study was published in four original publications across three different peer- reviewed scientific journals:

1. **Bobowski, B.T.C.**, Power, A.M., Pierce, G.J., Moreno, A., Iriondo, A., Valeiras, J., Sokolova, I.M., Oesterwind, D., 2023. Cephalopods, a gap in the European Marine Strategy Framework Directive and their future integration. *Mar. Biol.* 170, 26.  
<https://doi.org/10.1007/s00227-022-04148-2>
2. **Bobowski, B.T.C.**, Power, A.M., Burns, F., Carbonara, P., Cuccu, D., Donnaloia, M., Follesa, M.C., Moreno, A., Sokolova, I.M., Valls, M., Oesterwind, D., 2024. Stock discrimination of two European squids (*Illex coindetii*, *Loligo forbesii*) by statolith shape analysis. *Fish. Manag. Ecol.* e12689. <https://doi.org/10.1111/fme.12689>



3. **Bobowski, B.T.C.**, Power, A.M., Burns, F., Carbonara, P., Cuccu, D., Follesa, M.C., Jochum, K.P., Moreno, A., Sokolova, I.M., Valls, M., Oesterwind, D., 2025. Classification of European *Illex coindetii* and *Loligo forbesii* Squid Stocks by Trace Element Analysis of Statoliths. *Fish. Manag. Ecol.* <https://doi.org/10.1111/fme.70016>
4. Laptikhovsky, V., Moreno, A., Oesterwind, D., Perales Raya, C., Pierce, G.J., Robin, J.-P., Sobrino, I., Valls, M., Villanueva, R., Allen, A., Abad, E., Bello, G., **Bobowski, B.T.C.**, Fernández-Álvarez, F.Á., Cabanellas-Reboredo, M., González, A.F., Hernández-Urcera, J., Krstulović Šifner, S., Lefkaditou, E., MacLeod, E., Valeiras Mota, J., Onsoy, B., Pereira, J., Salman, A., Seixas, S., Power, A.M. 2025. The reproductive ecology of *Loligo* squids (Cephalopoda: Myopsida) in Lusitanian Zoogeographical Province. *Reg. Stud. Mar. Sci.* 91. <https://doi.org/10.1016/j.rsma.2025.104506>

Moreover, the ICES WGCEPH 2022 Meeting output was published in a Technical Report:

1. ICES, 2023. Working Group on Cephalopod Fisheries and Life History (WGCEPH; Outputs from 2022 meeting). ICES Scientific Reports. 5:01. 163 pp. <https://doi.org/10.17895/ices.pub.21976718>





## 2 Material and Methods

### 2.1 Analytical Approaches Using Squid Statoliths

Three different methods, based on the same individual statolith, were used to identify distinct stocks of the squids *Illex coindetii* and *Loligo forbesii* in Europe. These methods include (i) statolith shape analysis (**Manuscript II**), based on the statolith contour; (ii) statolith microchemistry analysis (**Manuscript III**), which examines various elements measured at the nucleus (representing early-life stages) and the outermost edge (representing the later life stages immediately preceding the capture); and (iii) age determination (**Manuscript III**), based on counted increments (growth rings), where one increment presumably corresponds to one day of life (Figure 3).

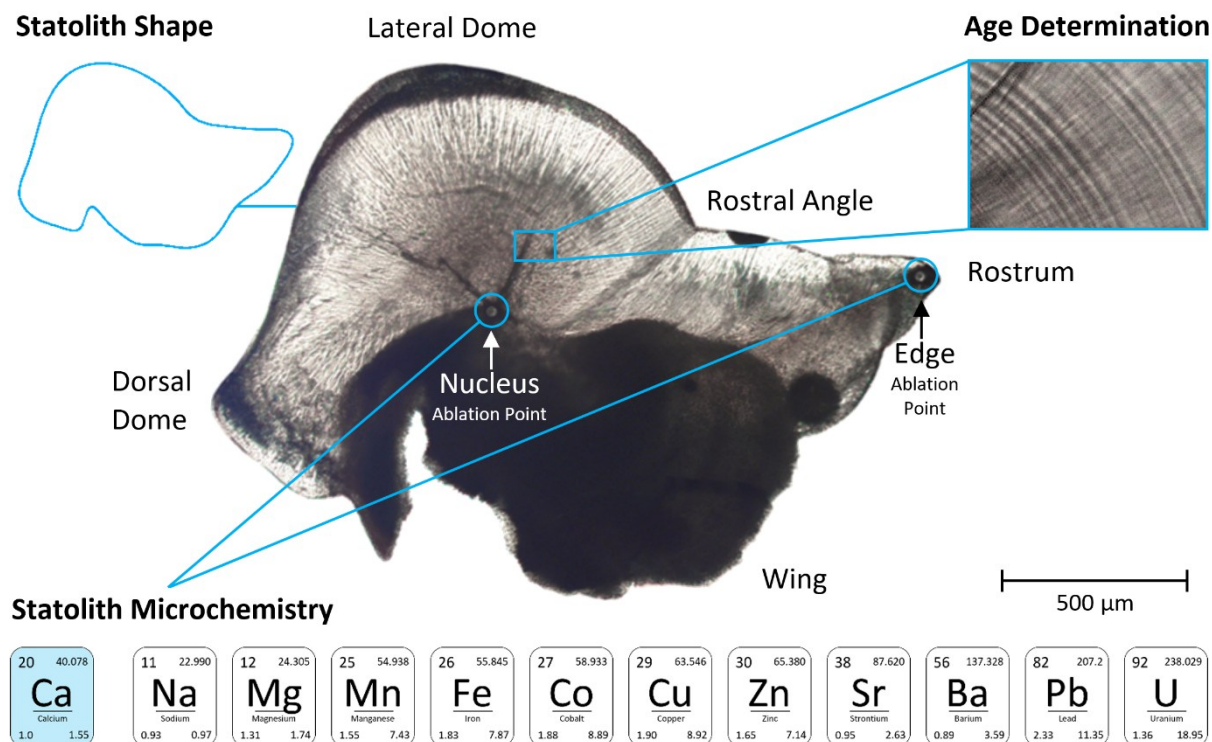


Figure 3: Polished right-side statolith of a mature North Sea *Loligo forbesii* (centre) with key morphological features labelled. Surrounding the statolith are the methods used for stock identification in this study: Statolith Shape – mean statolith shape of North Sea *L. forbesii*; Age Determination - visible increments (representing days of life); Statolith Microchemistry – considered elements measured during microchemical analysis at the nucleus and edge ablation points. Element values were considered in relation to Ca.



## 2.2 Sampling and preparation

Samples of *I. coindetii* and *L. forbesii* were either collected by me (North Sea) or by the corresponding co-authors within a one-year timeframe (Q1 2021 - Q1 2022), in the North East Atlantic and Mediterranean Sea. The samples from the following eight areas were analysed: Balearic Sea, Celtic Sea, North Sea, Sea East of Sardinia, Sea West of Sardinia, Sea West of Scotland, South Adriatic Sea and Western Ionian Sea (**Manuscript II & III**). The collection was part of four different surveys, namely: the International Bottom Trawl Survey (IBTS), Mediterranean International Bottom Trawl Survey (MEDITS), Irish Anglerfish and Megrim Survey (IAMS), and Scottish West Coast Groundfish Survey (SCOWCGFS) (Reid et al., 2007; ICES, 2012, 2017, 2020a). The sampled areas were visualised using QGIS 3.40 (QGIS.org, 2024; Figure 4).

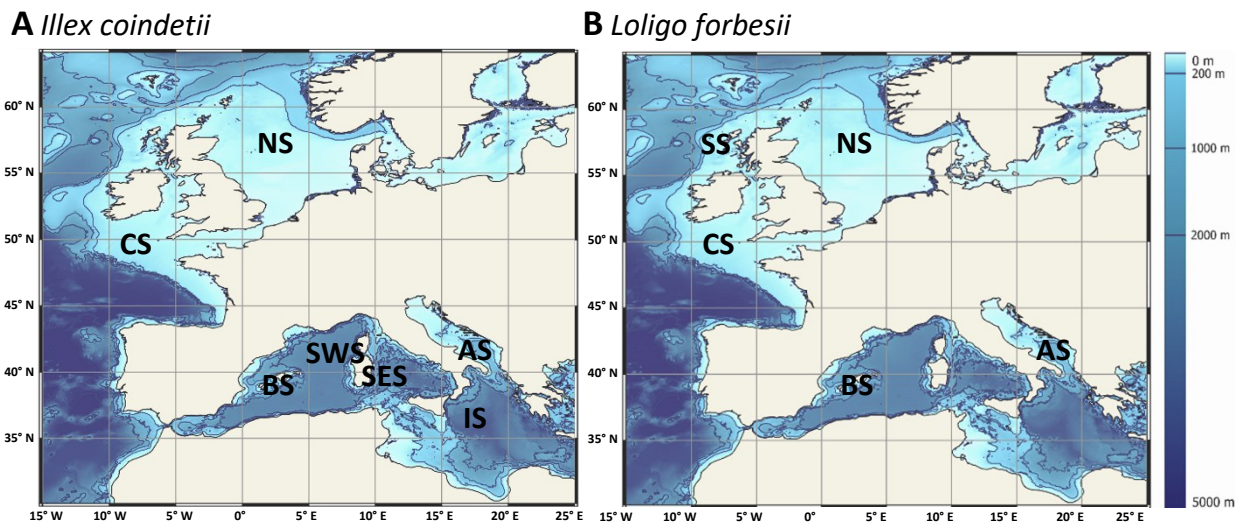


Figure 4: Sampling areas of *Illex coindetii* (A) and *Loligo forbesii* (B) in the North Sea (NS), Sea West of Scotland (SS), Celtic Sea (CS), Balearic Sea (BS), Sea West of Sardinia (SWS), Sea East of Sardinia (SES), Western Ionian Sea (IS), South Adriatic Sea (AS) in the North East Atlantic and Mediterranean Sea during Q1 2021- Q1 2022. Bathymetric map of GEBCO 2024 Grids (GEBCO Compilation Group, 2024), land polygons are based on data from Natural Earth (Natural Earth, 2024).

The biological data of each individual (dorsal mantle length (DML), weight, sex and maturity stage) were recorded for each individual, and statoliths were prepared for analysis (**Manuscript II**), with a significant portion of the samples provided by the corresponding co-authors. For the detailed statolith preparation for shape analysis, age determination and microchemical analysis, conducted by me, see **Manuscript II** and **Manuscript III**, respectively. A total of 482 right-side statoliths were analysed in the studies. Due to the exclusion of some statoliths in the individual analysis for various reasons (breakage, over-grinding, no measured values), the respective numbers were slightly lower than the total sample size (Table 2). To ensure consistent naming throughout the thesis and across both species, sample groups are



referred to base on their inferred spawning season, rather than solely on area or quarter of caption. The following table (Table 1) provides the correspondence between the original, capture-based sample identifiers (used in **Manuscript II**) and the spawning season-based labels (introduced in **Manuscript III**), which are used consistently in all subsequent figures and tables.

Table 1: Sample group labels used throughout the thesis, including original capture information and inferred spawning seasons.

| Species                | Sample Label           | Original Area ID     | Quarter of Capture | Spawning Season | Abbreviation |
|------------------------|------------------------|----------------------|--------------------|-----------------|--------------|
| <i>Illex coindetii</i> | Balearic WiSS          | Balearic Sea         | Q3                 | Winter          | WiSS         |
|                        | Celtic SpSuSS          | Celtic Sea           | Q1                 | Spring, Summer  | SpSuSS       |
|                        | North Sea SuSS Q1      | North Sea Q1         | Q1                 | Summer          | SuSS         |
|                        | North Sea SuSS Q3      | North Sea Q3         | Q3                 | Summer          | SuSS         |
|                        | Eastern Sardinian WiSS | Sea East of Sardinia | Q3                 | Winter          | WiSS         |
|                        | Western Sardinian WiSS | Sea West of Sardinia | Q3                 | Winter          | WiSS         |
|                        | South Adriatic WiSpSS  | South Adriatic Sea   | Q3                 | Winter, Spring  | WiSpSS       |
|                        | Western Ionian WiSS    | Western Ionian Sea   | Q3                 | Winter          | WiSS         |
| <i>Loligo forbesii</i> | Balearic WiSS          | Balearic Sea         | Q3                 | Winter          | WiSS         |
|                        | Celtic SuSS            | Celtic Sea           | Q1                 | Summer          | SuSS         |
|                        | North Sea SpSuSS Q1    | North Sea Q1         | Q1                 | Spring, Summer  | SpSuSS       |
|                        | North Sea AuWiSS Q3    | North Sea Q3         | Q3                 | Autumn, Winter  | AuWiSS       |
|                        | Western Scotland SpSS  | Sea West of Scotland | Q4                 | Spring          | SpSS         |
|                        | South Adriatic AuWiSS  | South Adriatic Sea   | Q3                 | Autumn, Winter  | AuWiSS       |



Table 2: Numbers of analysed right-side statoliths of *Illex coindetii* and *Loligo forbesii* in the respective method of analysis per spawning stock. The numbers refer to the same total sample size, with minor differences due to exclusions specific to each method. North Sea individuals captured in Q1 and Q3 are combined in shape analysis.

| Species                | Spawning Stock         | Shape analysis | Microchemical analysis | Age determination | Total sample size |
|------------------------|------------------------|----------------|------------------------|-------------------|-------------------|
| <i>Illex coindetii</i> | Balearic WiSS          | 13             | 10                     | 12                | 13                |
|                        | Celtic SpSuSS          | 42             | 40                     | 37                | 44                |
|                        | North Sea SuSS Q1      | 50             | 31                     | 36                | 37                |
|                        | North Sea SuSS Q3      |                | 22                     | 21                | 23                |
|                        | Eastern Sardinian WiSS | 12             | 15                     | 10                | 16                |
|                        | Western Sardinian WiSS | 23             | 28                     | 22                | 29                |
|                        | South Adriatic WiSpSS  | 25             | 32                     | 24                | 32                |
|                        | Western Ionian WiSS    | 22             | 26                     | 19                | 29                |
| <i>Loligo forbesii</i> | Balearic WiSS          | 33             | 31                     | 32                | 33                |
|                        | Celtic SuSS            | 25             | 24                     | 24                | 26                |
|                        | North Sea SpSuSS Q1    | 128            | 54                     | 57                | 59                |
|                        | North Sea AuWiSS Q3    |                | 65                     | 67                | 71                |
|                        | Western Scotland SpSS  | 49             | 50                     | 46                | 50                |
|                        | South Adriatic AuWiSS  | 19             | 20                     | 19                | 20                |

### 2.3 Analytical methods

Stanolith shapes (contour) of *I. coindetii* and *L. forbesii* were analysed by Fourier Shape analysis using the software SHAPE (ver.1.3 by Iwata and Ukai, 2002). Microchemistry analysis of the statolith nucleus and outermost edge of the same species were conducted with a Laser Ablation – Inductively Coupled Plasma - Mass Spectrometry (LA-ICP-MS). For the detailed methods see **Manuscript II** and **Manuscript III**, respectively.

### 2.4 Data analysis

Extracted statolith shapes and measured elemental values were visualized with principal component analysis (PCA) to represent differences in the statoliths of each sampled area. Additionally, a variation between groups (defined as areas in **Manuscript II** and spawning stocks in **Manuscript III**) and the correlation between observed and predicted classes were analysed based on the PCAs using the statistical programming language R. For details on used packages in R for each analysis see **Manuscript II** and **Manuscript III**, respectively.





## 2.5 Egg masses

Nearly 700 observations of egg masses and egg mass groups along the European and West African shore were collected from 1992 to 2023. The observations were reported with decreasing frequency by recreational divers, commercial and research fishing activities, attached to artificial substrates or observed by ROV (Remotely Operated Vehicle) – see **Manuscript IV** for detailed information – with a significant part of the data from recreational divers compiled by me. Collected data included individual egg masses and group of egg masses classified as ‘spawning sites’, identified of the species *L. vulgaris*, *L. forbesii* or unclassified *Loligo* species.





### 3 Results

#### 3.1 Stock identification based on Statolith Shape and Statolith Element Differences

Based on the statolith shape and the microchemistry analysis of the statolith nucleus and edge, both *I. coindetii* and *L. forbesii* can be divided into geographical-distinct groups within the studied areas. Individuals of both species from the North Sea (black colour) exhibited the most pronounced statolith key-morphological characteristics (Figure 5 *Illex coindetii* & *Loligo forbesii* A.2). In contrast, individuals from the Balearic Winter Spawning Stock (WiSS - dark blue colour) showed less pronounced statolith shape characteristics in both species. These results are also reflected in Principal Component Analysis (PCA); although most data points (95% confidence intervals) showed strongly overlapping components, individuals from the North Sea and the Balearic showed the least overlap in both species with those from other areas with regard to the statolith shape (Figure 5 *Illex coindetii* & *Loligo forbesii* A.1).

Age determination revealed only one spawning season for North Sea *I. coindetii* (**Manuscript III**). The microchemical analysis results reflect these findings, as North Sea *I. coindetii* individuals showed a high overlap and also the lowest elemental variability for both quarters of the year (grey & black colour) at both the nucleus and edge measurement points (Figure 5 *Illex coindetii* B.1, C.1). The highest element variability was driven by Cu, Pb and Zn and was shown by the Western Ionian WiSS of *I. coindetii* (purple colour) at the nucleus (Figure 5 *Illex coindetii* B.1, B.2) and in both, Eastern and Western Sardinian WiSS (red & orange colour) at the statolith edge (Figure 5 *Illex coindetii* C.1, C.2). Although *L. forbesii* individuals from the Western Scotland Spring Spawning Stock (SpSS - green colour) showed a high overlap in the nucleus microchemical PCA with both spawning stocks from the North Sea (grey & black colour) (Figure 5 *Loligo forbesii* B.1), the edge measurement point revealed a strong influence of Ba, Cu, Pb and Zn only in individuals from the Western Scotland SpSS (Figure 5 *Loligo forbesii* C.1 & C.2).

**Figure 5:** *Illex coindetii* (left page) and *Loligo forbesii* (right page)

from the North East Atlantic and Northern Mediterranean Sea during 2021–2022.

**A.1, A.2 - Statolith Shape:** Sample PCA (A.1) and mean statolith shapes (A.2), grouped by inferred spawning stock

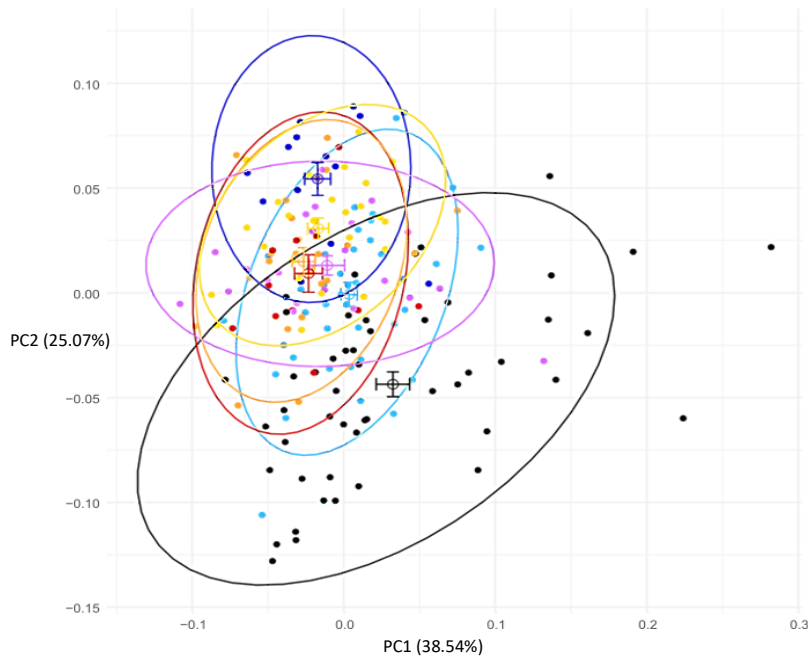
**B.1, B.2 - Statolith Nucleus Microchemistry:** Sample (B.1) and variable (B.2) PCA biplot of statolith nucleus microchemistry

**C.1, C.2 - Statolith Edge Microchemistry:** Sample (B.1) and variable (B.2) PCA biplot of statolith edge microchemistry

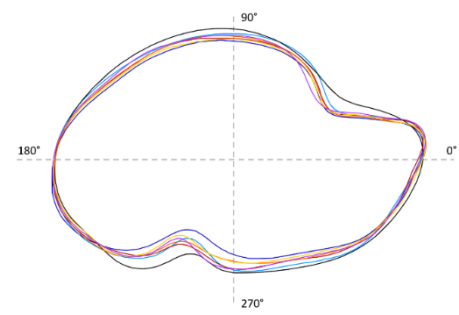
Spawning stocks (SS) are color-coded as follows: Balearic SS (dark blue), Celtic SS (light blue), North Sea SS (grey & black), Eastern Sardinian SS (red), Western Sardinian SS (orange), Western Scotland SS (green), South Adriatic SS (yellow), Western Ionian SS (purple). The specific spawning stocks represented in each subplot are indicated in the respective legends. Open circles represent the mean ( $\pm$  standard error), and ellipses encompass 95% of samples per spawning stock.

# *Illex coindetii*

## A.1 Statolith Shape



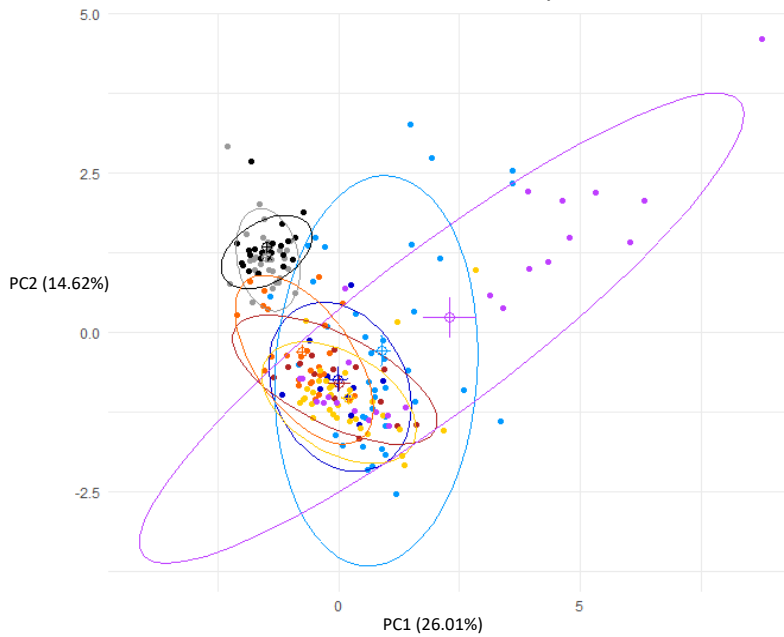
## A.2



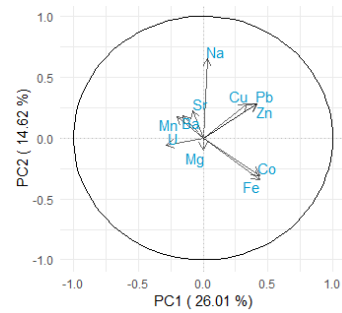
### Spawning stocks

- Balearic WiSS (n=13)
- Celtic SpSuSS (n=42)
- North Sea SuSS (n=50)
- Eastern Sardinian WiSS (n=12)
- Western Sardinian WiSS (n=23)
- South Adriatic WiSpSS (n=25)
- Western Ionian WiSS (n=22)

## B.1 Statolith Nucleus Microchemistry



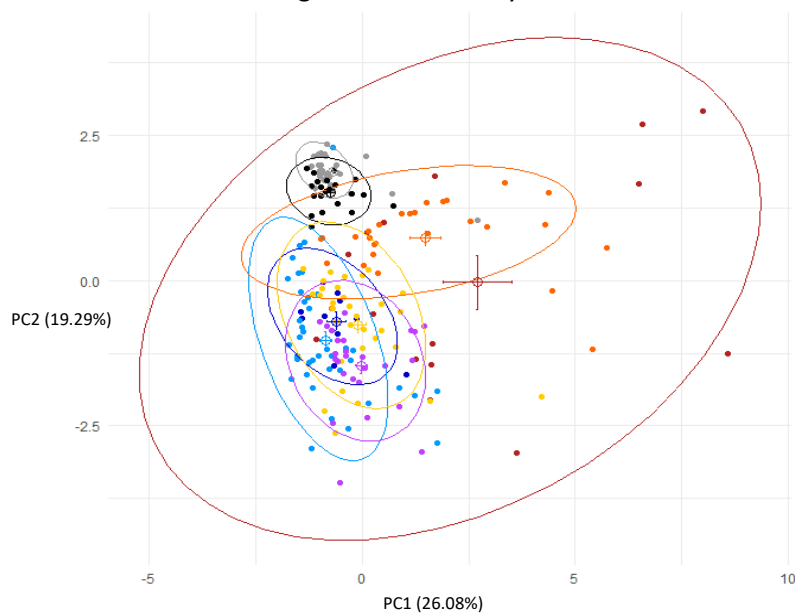
## B.2



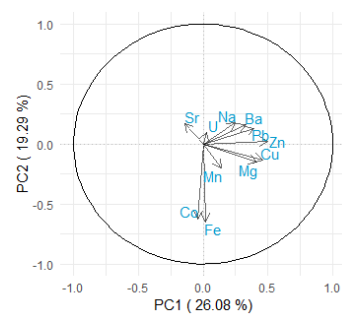
### Spawning stocks

- Balearic WiSS (n=10)
- Celtic SpSuSS (n=40)
- North Sea SuSS captured in Q1 (n=31)
- North Sea SuSS captured in Q3 (n=22)
- Eastern Sardinian WiSS (n=15)
- Western Sardinian WiSS (n=26)
- South Adriatic WiSpSS (n=32)
- Western Ionian WiSS (n=25)

## C.1 Statolith Edge Microchemistry



## C.2

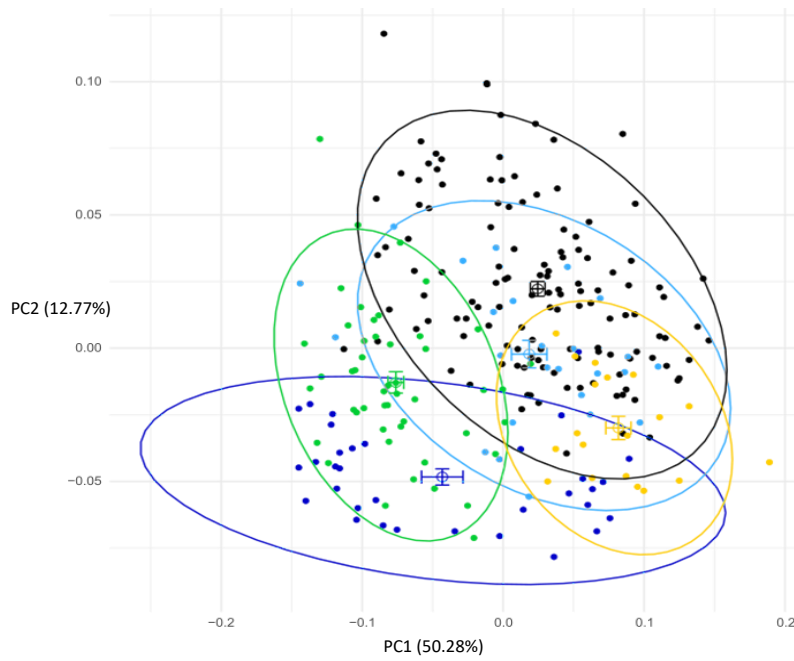


### Spawning stocks

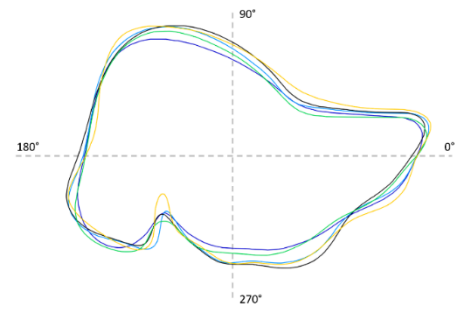
- Balearic WiSS (n=10)
- Celtic SpSuSS (n=40)
- North Sea SuSS captured in Q1 (n=31)
- North Sea SuSS captured in Q3 (n=22)
- Eastern Sardinian WiSS (n=15)
- Western Sardinian WiSS (n=28)
- South Adriatic WiSpSS (n=32)
- Western Ionian WiSS (n=26)

# *Loligo forbesii*

## A.1 Statolith Shape



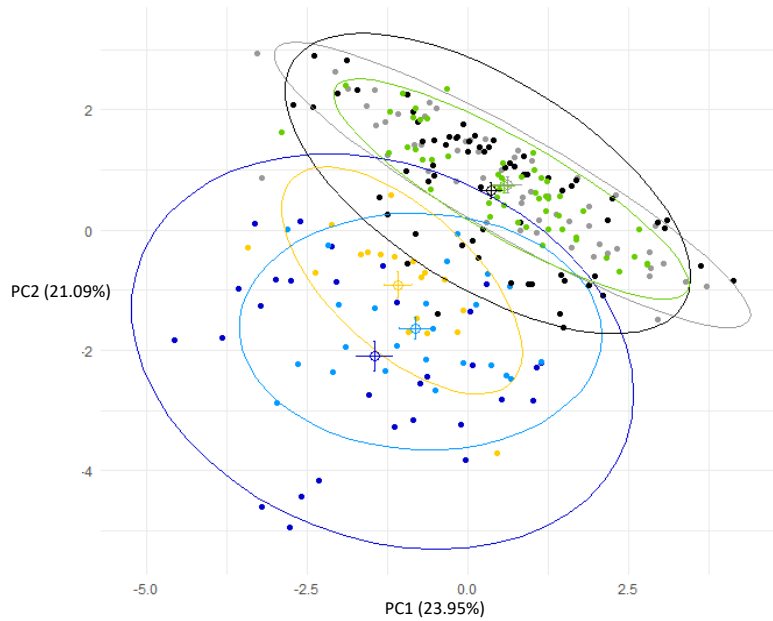
## A.2



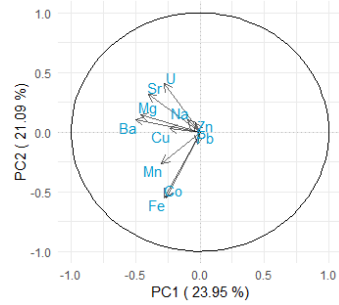
### Spawning stocks

- Balearic WiSS (n=33)
- Celtic SuSS (n=25)
- North Sea SpSuSS & AuWiSS (n=128)
- Western Scotland SpSS (n=49)
- South Adriatic AuWiSS (n=19)

## B.1 Statolith Nucleus Microchemistry



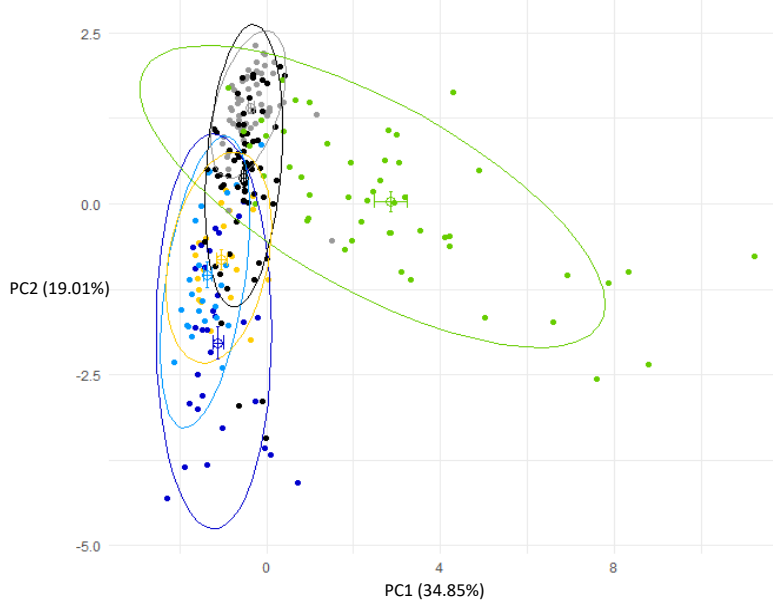
## B.2



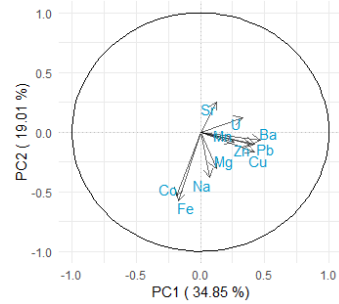
### Spawning stocks

- Balearic WiSS (n=31)
- Celtic SuSS (n=24)
- North Sea SpSuSS captured in Q1 (n=54)
- North Sea AuWiSS captured in Q3 (n=65)
- Western Scotland SpSS (n=50)
- South Adriatic AuWiSS (n=18)

## C.1 Statolith Edge Microchemistry



## C.2



### Spawning stocks

- Balearic WiSS (n=30)
- Celtic SuSS (n=24)
- North Sea SpSuSS captured in Q1 (n=54)
- North Sea AuWiSS captured in Q3 (n=65)
- Western Scotland SpSS (n=49)
- South Adriatic AuWiSS (n=20)



PCA graphs for both species (Figure 5 *Illex coindetii* & *Loligo forbesii* A.1, B.1, C.1), show distinct clustering patterns and are explained by different percentages of PC1, PC2 and PC<sub>sum</sub> in shape and microchemistry analysis (Table 3). The highest percentage values are given by shape PCA for both species, whereas the lowest are given by microchemical PCA for the nucleus measurements (both analyses having been conducted on the same individuals).

Table 3: Principal component analysis results (PC1, PC2 and PC<sub>sum</sub> [PC1 and PC2 combined]) in percentage per method (shape & microchemistry analysis) per species

| Species             | Method                  |         | PC1 (%) | PC2 (%) | PC <sub>sum</sub> (%) |
|---------------------|-------------------------|---------|---------|---------|-----------------------|
| <i>I. coindetii</i> | Shape analysis          |         | 38.54   | 25.07   | 63.61                 |
|                     | Microchemistry analysis | nucleus | 26.01   | 14.62   | 40.63                 |
|                     |                         | edge    | 26.08   | 19.29   | 45.37                 |
| <i>L. forbesii</i>  | Shape analysis          |         | 50.28   | 12.77   | 63.05                 |
|                     | Microchemistry analysis | nucleus | 23.95   | 21.09   | 45.04                 |
|                     |                         | edge    | 34.85   | 19.01   | 53.86                 |

In the pairwise comparisons of statolith shape, *I. coindetii* showed differences between the Balearic WiSS, Celtic SpSuSS, and North Sea Summer Spawning Stock (SuSS) and most other area-specific spawning stocks, whereas Mediterranean spawning stocks, except for the Balearic WiSS, showed no differences among each other (**Manuscript II**, Table 2). By comparison, *L. forbesii* exhibited differences in statolith shape between all area-specific spawning stocks, except between North Sea (SpSuSS & AuWiSS) and Celtic SuSS. Considering the elemental composition, differences were observed between most area-specific spawning stocks for *I. coindetii* and *L. forbesii* in pairwise comparison (**Manuscript III**, Table 2). However, no significant differences were found in elemental values at the nucleus measurement point for either species between the Balearic WiSS and other Mediterranean spawning stocks, nor between Balearic WiSS and Celtic spawning stocks. Similarly, no differences were observed in *I. coindetii* between the Eastern Sardinian WiSS and other Mediterranean spawning stocks. Additionally, both species showed no differences in the elemental composition of the nucleus between North Sea individuals captured in Q1 and Q3. At the statolith edge, no differences were evident between Eastern and Western Sardinian spawning stocks in *I. coindetii*.



### 3.2 Area Classification

The classification success rates of observed and predicted area-specific spawning stocks, based on statolith shape and elemental ratios, were highly accurate in the North East Atlantic for both species (>80%) across both analysis methods and measurement points. However, *L. forbesii* Celtic SuSS was an exception, with a lower classification success rate in shape analysis (44%) and microchemical analysis (>60%) (Table 4). In the Mediterranean, most individuals were classified more accurately using shape analysis compared to microchemistry. For detailed numbers of correctly classified individuals as well as misclassified individuals per area specific spawning stock, see **Manuscript II**, Table 3 and **Manuscript III**, Table 3.

Table 4: Classification success rates per method in percentage per spawning stock (SS); Balearic SS, Celtic SS, North Sea SS, Eastern Sardinian SS, Western Sardinian SS, Western Scotland SS, South Adriatic SS, Western Ionian SS. North Sea spawning stocks captured in Q1 and Q3 are combined in Shape Analysis. A second value in parenthesis in microchemical analysis stands for the classification success rate for North Sea (captured in Q1 & Q3) combined.

| Species                | Spawning Stock         | Classification success rate [%] |                                 |                              |
|------------------------|------------------------|---------------------------------|---------------------------------|------------------------------|
|                        |                        | Shape Analysis                  | Microchemistry Nucleus Analysis | Microchemistry Edge Analysis |
| <i>Illex coindetii</i> | Balearic WiSS          | 53.85                           | 0.00                            | 0.00                         |
|                        | Celtic SpSuSS          | 80.95                           | 72.50                           | 80.00                        |
|                        | North Sea SuSS Q1      | 88.00                           | 100.00 (100.00)                 | 90.32 (93.55)                |
|                        | North Sea SuSS Q3      |                                 | 0.00 (100.00)                   | 31.82 (100.00)               |
|                        | Eastern Sardinian WiSS | 33.33                           | 0.00                            | 33.33                        |
|                        | Western Sardinian WiSS | 56.52                           | 69.23                           | 85.14                        |
|                        | South Adriatic WiSpSS  | 72.00                           | 59.38                           | 43.75                        |
|                        | Western Ionian WiSS    | 54.55                           | 40.00                           | 61.54                        |
| <i>Loligo forbesii</i> | Balearic WiSS          | 87.88                           | 64.52                           | 48.39                        |
|                        | Celtic SuSS            | 44.00                           | 62.50                           | 66.67                        |
|                        | North Sea SpSuSS Q1    | 87.50                           | 35.19 (62.96)                   | 83.33 (96.30)                |
|                        | North Sea AuWiSS Q3    |                                 | 63.08 (90.77)                   | 63.08 (87.69)                |
|                        | Western Scotland SpSS  | 81.63                           | 28.00                           | 79.59                        |
|                        | South Adriatic AuWiSS  | 89.47                           | 50.00                           | 20.00                        |



### 3.3 Seasonal egg mass occurrence and hatching period of *Loligo*

*Loligo* egg masses were found in offshore areas at depths ranging from 3 m to 556 m across all European Mediterranean Seas, including the Balearic, Ligurian, Tyrrhenian, Sicilian, Ionian, Adriatic, Aegean and Levantine Sea. Throughout the year, most recorded egg masses were identified as *L. vulgaris*, with rare records of *L. forbesii* in the Western and Eastern Mediterranean Sea (**Manuscript IV**). This reflects the low abundance of *L. forbesii* in the Mediterranean Sea and is consistent with the smaller sample size of this species in statolith shape and microchemical analysis in Mediterranean areas. Most of the egg mass records were documented between November to July. For detailed information on the recording periods and detection methods used, see **Manuscript IV**. The majority of the analysed Mediterranean *L. forbesii* individuals for shape and microchemistry analysis, counted statolith increments revealed a hatching period in winter (Q1) 2021 (**Manuscript III**).





## 4 Discussion

This thesis aimed to identify and differentiate populations of *I. coindetii* and *L. forbesii* in the North East Atlantic and Mediterranean into distinct stocks, providing a basis for sustainable fisheries management. The results revealed distinct stocks, cohorts and potential migration patterns both between stocks (shared feeding grounds) and throughout ontogeny (shared spawning or nursery grounds). In addition, identified spawning grounds and seasons can inform protective measures, and therewith prevent the exploitation of reproducing individuals by accounting for regional differences in spawning seasons. These findings provide an important foundation for future sustainable fisheries management of the two studied species in Europe. In the following, the findings are discussed with a particular focus on comparing the results from **Manuscript II** and **III**.

Statoliths, as calcified structures growing over time, develop their crystalline structure through nucleation influenced by environmental factors (Lipiński, 2001), with their shape and size changing during ontogeny (Morris and Aldrich, 1984; Thomas and Moltschaniwskyj, 1999). The principal component analysis (PCA) of statoliths shape and microchemistry of the same individuals, yielded differing levels of explained variance. While localized measurement points (microchemical analysis – **Manuscript III**) reflect element values over short time periods (a few weeks each), statolith shape (**Manuscript II**) integrates biotic and abiotic environmental influences across the entire lifespan of an individual. Thus, statolith shape can serve as a marker of the individual's main residence area, leading to higher PC1 and PC2 values. Influenced by ontogeny, habitat, and species-specific traits, statolith shape varies in response to environmental factors. During early life stage development, temperature and salinity of surrounding water bodies already affect statolith shape (Chung and Lu, 2005). Furthermore, statolith morphology can be classified into demersal and pelagic types, reflecting the squid's ecological niche (Arkhipkin and Bizikov, 2000). However, the shape varies not only between species from different families, but also among closely related species (Pineda et al., 1998; Fang and Chen, 2017) and even within the same species (Green et al., 2015; Sheerin et al., 2022, **Manuscript II**). By encompassing all influences and variations in the system (e.g., seasonal fluctuations, long-term trends, external factors), shape analysis allows principal components to capture more dominant patterns and explain a larger proportion of the variance. In contrast, elemental values measured at the statolith nucleus, which represents



early life stages, show the lowest explained variance. This may be due to individuals from different areas experiencing more similar environmental conditions during this stages, as specific environmental parameters (temperature, light intensity, salinity, pH and specific ion concentrations) are required for hatching and development of squids (O'Dor et al., 1982; D'aniello et al., 1989; Paulij et al., 1990; Barón, 2002; Sen, 2004). Additionally, early life stages include the egg phase, during which the semipermeable nature of cephalopod eggs may influence elemental incorporation. These gelatinous egg cases can act as a barrier against environmental stressors (Amida et al., 2019) and provide physical and chemical protection (Cornet et al., 2015), potentially leading to more uniform elemental compositions in statoliths across different geographical areas. However, statoliths of some squid species appear to be hollow during embryogenesis, as reported for *L. vulgaris* (Rosa et al., 2013). If this also applies to the statoliths of *I. coindetii* and *L. forbesii*, the presumed post-embryonic onset of elemental incorporation would reflect similar environmental conditions experienced throughout early life stages following hatching. Consequently, principal component differences in explained variance between shape and microchemical analysis can be attributed to their temporal and structural statolith contexts. However, although PCA values were lower in microchemical analysis, variation was also evident (at both the statolith nucleus and edge measurement point) in *I. coindetii* and *L. forbesii* among geographically and seasonally separated groups, which were interpreted as separate stocks.

Until 2010, it was assumed that the North East Atlantic harboured only one *I. coindetii* stock (Pierce et al., 2010). However, Oesterwind et al. (2020) identified a new spawning stock in the North Sea based on survey data and age determination. Supporting this conclusion, the age determination of North Sea individuals captured in Q1 and Q3 (**Manuscript III**) revealed only one spawning season (summer) in this area. Moreover, observed gelatinous egg spheres in the Norwegian Trench, genetically identified as *I. coindetii* (Ringvold et al., 2021), provide evidence of a North Sea spawning stock. Corroborating this, both methods (Statolith Shape and Microchemistry - **Manuscript II & III**) showed a high classification success rate based on statolith differences for the *I. coindetii* North Sea Summer Spawning Stock (SuSS) and Celtic Spring-Summer Spawning Stock (SpSuSS), suggesting that the North East Atlantic now harbours at least two distinct *I. coindetii* stocks.



In the Mediterranean, gelatinous egg spheres, likely belonging to *I. coindetii*, were observed in the Balearic Sea, Golfe du Lion, Ligurian Sea, Tyrrhenian Sea, Sicilian Sea and central Adriatic Sea (Ringvold et al., 2021). These free-floating gelatinous egg masses are suspended in the mesopelagic zone suitable for embryonic development conditions, and drift passively (O'Dor and Balch, 1985). This suggests that the observed locations are not necessarily *I. coindetii* hatching areas but might be adjacent to these areas. If spawning areas are near observed egg masses, analysed *I. coindetii* individuals from different Mediterranean areas appear to have local spawning areas nearby. However, the misclassification between Mediterranean spawning stocks using both statolith shape and microchemistry revealed a high similarity between individuals. Overall, shape analysis results indicate substantial exchange of *I. coindetii* individuals and the absence of independent stocks in the Mediterranean region, especially around Italy (Sardinian spawning stocks, Western Ionian Winter Spawning Stock (WiSS), South Adriatic Winter-Spring Spawning Stock (WiSpSS)). Although microchemical analysis yielded similar results, certain additional patterns were captured. The highest classification success rate based on microchemical analysis in the Mediterranean was observed in individuals from the Western Sardinian WiSS (**Manuscript III**). Combined with the misclassification of individuals from the Eastern Sardinian WiSS as belonging to the western side, particularly in early life stages, suggests Sardinian waters may host a distinct stock. This stock seems to be more separated from the Ionian and Adriatic spawning stocks, possibly due to biogeographic barriers like the Calabrian peninsula between the Western and Eastern Mediterranean basins (Serra et al., 2007). However, surface connectivity reveals strong links between the Ionian Sea and the Strait of Sicily, as well as between the Eastern Ionian Sea and Adriatic Sea (Celentano et al., 2020). These connections correspond with the misclassifications observed in the microchemical analysis of individuals from sampled Italian Sea spawning stocks, indicating that the Sardinian 'sub-stock' is not entirely distinct, but rather part of an intermediate exchange between the remaining spawning stocks. These findings support the description of Mediterranean *I. coindetii* by Sánchez et al. (1998), who characterized it as exhibiting a 'middle course' situation, with multiple cohorts, micro-cohorts, or heavily overlapping cohorts. Moreover, geographically distinct spawning stocks, such as the Balearic WiSS and the Western Ionian WiSS or South Adriatic WiSpSS, as well as the Mediterranean spawning stocks and the Celtic SpSuSS, exhibited some misclassification among each other. Although, *I. coindetii* is a highly mobile



species, with adults capable of reaching critical swimming speeds of 70-90 cm/s (*I. illecebrosus*; Webber and O'Dor, 1985), a consistent exchange of all life stages between these biogeographic separated areas seems unlikely. Instead, the observed misclassification is more likely driven by similarities in incorporated elemental values, influenced by comparable water conditions or consumed prey. However, as statoliths function as life history 'black boxes' (Arkhipkin, 2005), their full complexity is not yet fully understood and additional factors may contribute to these misclassifications. Considering geomorphological features and habitat characteristics (Quetglas et al., 2012), it is reasonable to assume that the Balearic Sea hosts a distinct stock of *I. coindetii*, which is not only separated from the Celtic WiSS but also from those in Italian Sea areas.

As early as 1974, migration of *L. forbesii* through the English Channel was described (Holme, 1974). Observed statolith shape similarities (**Manuscript II**) between individuals from the Celtic SuSS and the North Sea suggest that some individuals originating from the Celtic SuSS may temporarily reside in the North Sea. Since statolith shape develops and changes over an individual's entire lifetime (Thomas and Moltschaniwskyj, 1999), the environmental influences (both biotic and abiotic) must have persisted for a significant period. This indicates that statoliths of misclassified Celtic SuSS individuals were possibly shaped during a foraging phase in the North Sea. A suggested immigration into the North Sea likely to exploit feeding grounds, and emigration back into the Celtic Sea for mating and spawning based on the observed statolith shape similarities aligns with the described migration pathway by Holme (1974). The water conditions of the English Channel are highly influenced by water inflow from the North Sea (Lumby, 1925). According to this, the partial misclassification of Celtic SuSS individuals as North Sea Autumn-Winter Spawning Stock (AuWiSS) based on elemental values of the statolith edge (**Manuscript III**) suggests either a return into the Celtic Sea shortly before capture, or exposure to similar water conditions, likely located in the English Channel, during these life stages. Based on the statolith shape findings, compared with the occurrence of different size classes at least two temporally separated stocks within the North Sea were suggested (**Manuscript II**). On one hand individuals hatched in the Celtic Sea and on the other hand North Sea spawners as well as presumed Scottish individuals. Although, individuals from the Western Scotland Spring Spawning Stock (SpSS) were correctly classified by statolith shape (**Manuscript II**), they revealed the lowest classification success rate (28%) based on elemental values (**Manuscript III**) of the statolith nucleus, frequently misclassified as North Sea spawning



stocks. However, statolith edge elemental values revealed a much higher classification success rate (80-96%) for both area-specific spawning stocks, suggesting that the *L. forbesii* Western Scotland SpSS and North Sea spawning stocks experienced similar environmental conditions during early life stages. Based on these results it seems likely that *L. forbesii* Western Scotland SpSS and North Sea spawning stocks share a common hatching area at northern Scotland, where egg masses have been reported from January to June (Laptikhovsky et al., 2022), and diverge into two distinct areas in later life stages.

Genetic analysis (microsatellite and mitochondrial DNA studies) indicate an overall genetic homogeneity of *L. forbesii* across the Mediterranean Sea (Göpel et al., 2022; Sheerin et al., 2022). However, the high classification accuracy (>87%) and significant differences in statolith shape (**Manuscript II**) of *L. forbesii* Balearic WiSS and South Adriatic AuWiSS suggests the presence of distinct stocks in these areas. As *L. forbesii* appears to be much less common than *L. vulgaris* in the Mediterranean (Guerra et al., 1994; ICES, 2023), most unidentified egg masses in **Manuscript IV** likely belong to *L. vulgaris*. However, if some of these unidentified *Loligo* egg masses belong to *L. forbesii*, the hatch season determined in **Manuscript III** (January – March) indicates specific spawning areas, namely the Balearic, Tyrrhenian, Western Ionian, and Adriatic Seas. These results suggest different spawning areas for analysed *L. forbesii* individuals from the Balearic WiSS and South Adriatic AuWiSS, albeit some individuals were misclassified between these spawning stocks based on elemental values of the statoliths deposited at early life stages (50-65% correct classification) or during the period preceding capture (20-48% correct classification) (**Manuscript III**). Although *L. forbesii* can reach maximum speeds of 0.8 m/s, it typically hovers off-bottom and may use ‘soaring’ to conserve energy in currents (O’Dor et al., 1995). Given these movement patterns, long distance migration between geographically distinct areas – especially across biogeographic barriers – seems unlikely. Notably, misclassification was not only observed between Balearic WiSS and South Adriatic AuWiSS but was even more frequent between the former two spawning stocks and the Celtic SuSS and North Sea AuWiSS. Given low likelihood of long-distance migration among these areas, these data suggest that adult *L. forbesii* of these spawning stocks were experiencing similar environmental conditions, which could influence the elemental composition of their statoliths. However, as already described, the precise influence of environmental conditions on statoliths are not fully understood and requires further investigation.



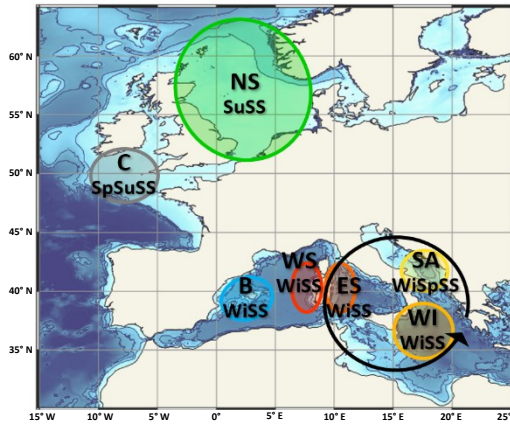


## 5 Conclusion

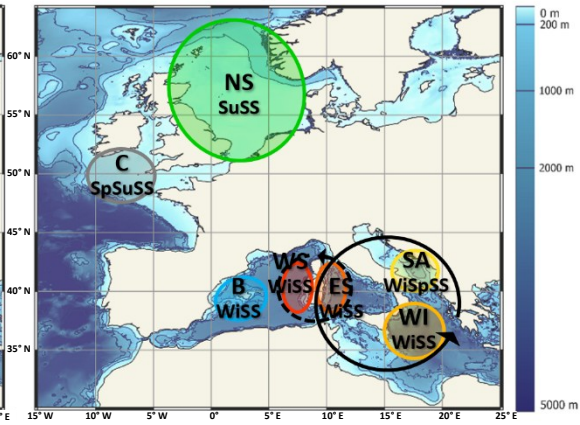
The findings based on statolith shape analysis and microchemical analysis provide valuable insights into stock discrimination for European *I. coindetii* and *L. forbesii*. Despite some variation in results between the two methods with respect to the amount of variation explained by the PCA, pairwise comparisons, and classification matrices, most individuals from both species exhibited similar patterns across the studied areas in Europe. Based on the results from **Manuscript II** and **III**, Figure 6A (*I. coindetii*) and Figure 6B (*L. forbesii*) illustrate the spawning stocks and their geographic distribution, as well as their interconnections.

### *Illex coindetii*

#### A.1 Shape analysis

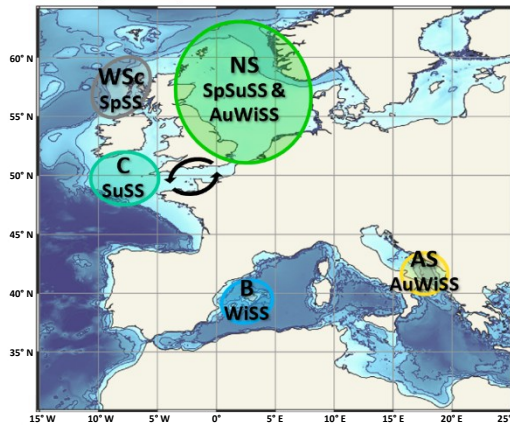


#### A.2 Microchemical analysis



### *Loligo forbesii*

#### B.1 Shape analysis



#### B.2 Microchemical analysis

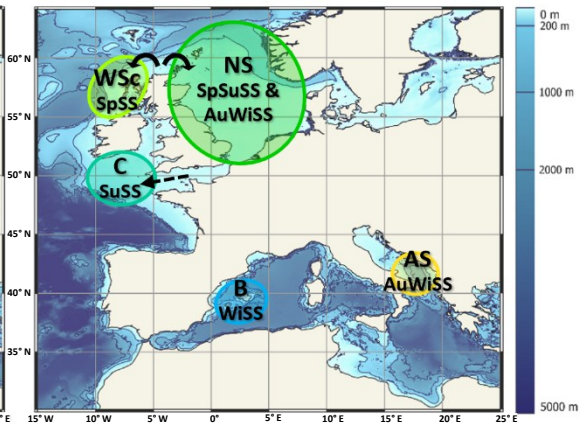


Figure 6: Geographic distribution and connectivity of *Illex coindetii* (A) and *Loligo forbesii* (B) spawning stocks in the North East Atlantic and Mediterranean Sea based on statolith shape (1) and statolith microchemical analysis (2). Stocks are labelled according to geographic region and spawning season as spawning stocks (SS): North Sea Autumn-Winter SS (NS AuWiSS), Spring-Summer SS (NS SpSuSS), and Summer SS (NS SuSS); Western Scotland Spring SS (WSc SpSS); Celtic Spring-Summer SS (C SpSuSS), and Summer SS (C SuSS); Balearic Winter SS (B WiSS); Western Sardinian Winter SS (WS WiSS); Eastern Sardinian Winter SS (ES WiSS); Western Ionian Winter SS (WI WiSS); South Adriatic Autumn-Winter SS (SA AuWiSS), and Winter-Spring SS (SA WiSpSS). Similar colour schemes and directional arrows indicate potential interconnections between stocks. Bathymetric map of GEBCO 2024 Grids (GEBCO Compilation Group, 2024), land polygons are based on data from Natural Earth (Natural Earth, 2024).



Both analytical methods identified similar distinct stocks throughout the sampled areas. While microchemical analysis provided a more detailed view of stock structure in certain life stages, shape analysis indicated temporal residence in a neighbouring fishing area. To achieve a more comprehensive stock discrimination – including migration patterns, shared hatching areas, seasonally distinct stocks, and sub stocks – a combination of both methods along with biological data such as age determination, is recommended. Furthermore, effective fisheries management should consider stock delimitation, temporal or permanent mixing, and potential stock splitting to ensure sustainability.

The results of both analytical methods (**Manuscript II & III**) represent an important first step toward stock assessment within the CFP. In addition to stock structure and distribution, the identification of spawning grounds and seasons (**Manuscript IV**) is crucial for effective stock management. Spawning grounds are vital for maintaining reproduction and ensuring sustainable stock levels. Protecting spawning grounds allows management to implement tailored measures, such as protected areas, fisheries regulations and comprehensive environmental actions, to ensure a long-term preservation of these species.

For sustainable management, innovative depletion models (i.e., models used to estimate population abundance), incorporating seasonal data, are recommended, along with high-frequency data collection to enable separate assessment of individual cohorts (Arkhipkin et al., 2021). However, current European fisheries surveys are not focused on cephalopods and performed either annually (MEDITS) (Bertrand et al., 2002) or conducted in different quarters of the year (IBTS) (ICES, 2020b). Given the existing data limitations in many cephalopod fisheries, developing alternative assessment methods or increasing the frequency of relevant surveys is essential for more accurate stock assessments and management.



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## Part II Manuscripts



## I. Cephalopods, a gap in the European Marine Strategy Framework Directive and their future integration

## Contribution letter Manuscript I

### Cephalopods, a gap in the European Marine Strategy Framework Directive and their future integration

Bobowski, B.T.C., Power, A.M., Pierce, G.J., Moreno, A., Iriondo, A., Valeiras, J.,  
Sokolova, I.M., Oesterwind, D.

This manuscript has been published in Marine Biology:

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The major contributions to this (review) manuscript were done by me. I was responsible for the design and the main literature review. I developed the framework of the review, including key issues and proposed possible solutions to address them. I was responsible for the preparation and writing of the manuscript drafts as well as the revised versions from the co-authors, the Journal reviewers and the editor. The graphical abstract, which were requested by the journal for the manuscript, was also prepared by me.

Based on the CRediT taxonomy (Brand et al., 2015), my contributions to this manuscript were as follows:

- Conceptualization
- Investigation
- Writing – Original Draft
- Writing – Review and Editing
- Visualization

#### Signatures of consent



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Brand, A., Allen, L., Altman, M., Hlava, M., Scott, J., 2015. Beyond authorship: Attribution, contribution, collaboration, and credit. Learn. Publ. 28, 151-155. <https://doi.org/10.1087/20150211>





# Cephalopods, a gap in the European Marine Strategy Framework Directive and their future integration

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Received: 25 May 2022 / Accepted: 17 November 2022  
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## Abstract

With the aim of maintaining or obtaining good environmental status in the Northeast Atlantic and northern Mediterranean Sea, the European Marine Strategy Framework Directive (EU-MSFD) came into force in 2008. All EU Member States (MS) have the task of assessing the current state of their adjacent waters to guarantee sustainable use of marine resources and healthy ecosystems for the current and future generations. Although the MSFD has been implemented for over a decade, there are still gaps in the assessment of various ecologically and commercially important marine species, such as cephalopods. Here we describe the status of cephalopods, a key component of marine ecosystems and a valuable fishing resource, within the MSFD. To do so, we review how MS deal with this group in their reporting and identify and explain the gaps in the cephalopod assessment. We describe the main challenges including the limited data and the rarity of dedicated surveys on cephalopods. However, we argue that cephalopods can be partially integrated into the EU-MSFD assessment, illustrating the current opportunities and future possibilities of their integration into the MSFD, mainly using Descriptors 1–4.

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Responsible Editor: Ulrich Sommer.

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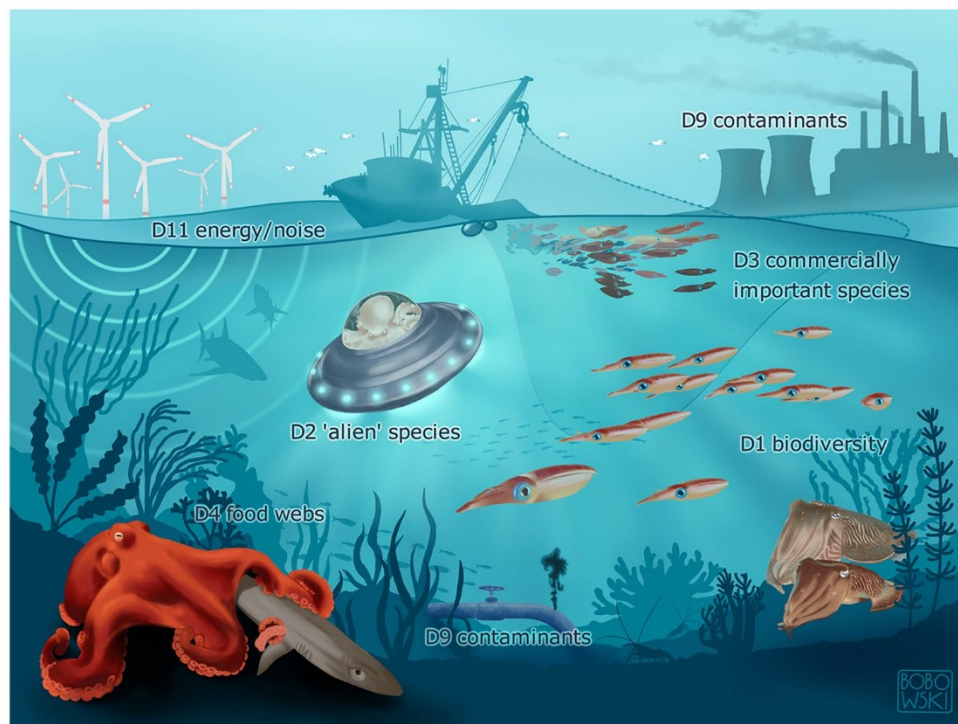
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## Graphical abstract



**Keywords** European waters · Good environmental status · Indicators · Squids · Stock assessment · MSFD · Descriptor

## Introduction

Global change involves a variety of different human pressures including climate warming (rising sea temperatures), ocean acidification, invasive species and resource use such as fishing, sand mining and energy generation, all of which affect marine ecosystems worldwide (Oesterwind et al. 2016). To manage the different anthropogenic pressures in European Union seas, diverse directives have been implemented, including the European Marine Strategy Framework Directive (EU-MSFD).

The EU-MSFD aims to protect and preserve the marine environment and its natural resources. It came into force in 2008 and was implemented in 2010 to form the environmental pillar of the future maritime policy of the European Union (EU Directive 2008/56/EC). With the intention of introducing an ecosystem-based management of biodiversity, habitats and resources, this directive focuses on obtaining or maintaining good health of marine ecosystems and the sustainable use of the marine environment by current and upcoming generations (Morato et al. 2016). The overall goal was to maintain or achieve this ‘Good Environmental Status’ (GES) by 2020, something which has not so far been achieved for most indicators and areas.

The EU-MSFD process started in 2012 and involves a 6-year cycle with the following three main phases: (i) reporting the current environmental status (performed in 2012 and 2018), (ii) setting up monitoring programmes to assess progress in achieving GES (conducted in 2014) and (iii) setting up programmes of measures to deliver the objectives of the MS (performed in 2016) and reporting on implementation progress (performed in 2018) (COM/2020/259). As the second cycle started in 2018, the GES assessment in support of the updated assessment guidance is due in 2024. The description of GES is based on the initial assessment from 2012 and refers to the following 11 qualitative descriptors: D1—biodiversity, D2—non-indigenous species, D3—commercial fish and shellfish, D4—food webs, D5—eutrophication, D6—sea-floor integrity, D7—hydrography, D8—contaminants in environment, D9—contaminants in seafood, D10—marine litter, D11—energy/noise (EU Directive 2008/56/EC). A number of criteria and indicators have been developed for both biodiversity-related and pressure descriptors. However, these criteria and indicators are constantly being supplemented and revised so that their number and nature change continually. In 2010, the Commission Decision 2010/477/EU named 29 associated criteria and 56 indicators for the different descriptors. Seven years later, the



number of criteria had increased to 42 (Commission Decision 2017/848/EU).

Even if the MSFD considers all European marine waters, the assessments (and monitoring and measures) can be subdivided into subareas by the MS (EU Directive 2008/56/EC). Until now the requirements of the MSFD have not yet been met following a holistic approach and various publications illuminate the weak points on different aspects of the MSFD (e.g. Santos and Pierce 2015; Probst et al. 2016; Borja et al. 2021; Tsiamis et al. 2021). Furthermore, some large and/or important groups of species are still not (or insufficiently) considered, including keystone groups or species, which are, in this case, species which have an important impact on the ecosystem or are important commercial resources.

One of these key groups is the Cephalopoda. Most cephalopods are opportunistic predators (e.g. Lipinski 1992) with a fast growth rate and a short lifespan. Their semelparous lifecycle mainly ends after 1–2 years and only a few species live longer (Mangold and Boletzky 1988). Their short lifespan enables them to quickly adapt to changing environments and they might also benefit from overfishing of commercial finfish stocks (Caddy and Rodhouse 1998; Balguerías et al. 2000; Hunsicker et al. 2010), due to decreasing predation pressure, less food competition and likely due to ‘free’ ecological niches, that can quickly be occupied by cephalopods as opportunists and r-strategists. Many cephalopods display typical r-selected features such as a high production to biomass ratio (Clarke 1996b; Smale 1996) and a high number of eggs (Cuccu et al. 2013; Laptikhovsky 2000). However, the life-cycles of some cephalopods also show K-selected features such as a relatively low number of eggs (e.g. in the octopus *Eledone cirrhosa*; Regueira et al. 2013), parental egg or brood care (e.g. in octopuses and some deep-sea squids like *Gonatus onyx* and *Bathyteuthis berryi*; Mangold and Boletzky 1973; Seibel et al. 2000; Bush et al. 2012) as well as a large body size (e.g. in the squid *Dosidicus gigas*; Argüelles et al. 2008). Furthermore, because cephalopods have a strong influence on other trophic levels (TLs) and, therefore, play a structuring role in their food webs, they can be defined as keystone species according to Paine (1995). The importance of a species in its community or ‘Keystoneness’ can be ranked by an index (Hurlbert 1997; Coll et al. 2013). For example, cephalopods are the group with the third highest ‘Keystoneness Index’ in the Azores, after pelagic sharks and toothed whales (Morato et al. 2016), and have a relatively high ‘Keystoneness’ in other marine waters (Coll et al. 2013; Smith et al. 2014), which emphasizes their importance in the ecosystem and consequently the need to include cephalopods into the MSFD. Various studies show significant changes in the occurrence and abundance of cephalopods from year to year (in addition to within-year changes associated with the life cycle)

(Doubleday et al. 2016), while others illustrate impressive range expansions which affect regional biodiversity (D1) (Chen et al. 2006; Zeidberg and Robinson 2007; van der Kooij et al. 2016; Moura et al. 2020; Oosterwind and Schaber 2020; Oosterwind et al. 2022).

However, not all range expansions are natural. Many established non-indigenous marine species (NIS) are registered in the European Union, comprising 824 taxa including five cephalopod species (Tsiamis et al. 2018). The bioregions most heavily affected by introduced species in general include the Mediterranean Sea and the Northeast Atlantic (Hewitt and Campbell 2010), where the local community structure, biodiversity and ecosystem functions are influenced by the direct and indirect impacts caused by these species (Thomsen et al. 2015). Besides negative effects such as the reduction in native biodiversity and ecosystem shifts, there might also be benefits from NIS, such as the replacement of lost ecological functions and the strengthening of biogenic complexity (Kleitou et al. 2021). A recent study describes 13 non-indigenous cephalopod species for the Mediterranean Sea, which entered the region over the last few decades, most likely by the following three different routes: the natural pathway Strait of Gibraltar, the human-constructed pathway Suez Canal or through other anthropogenic pathways like shipping (Bello et al. 2020). Consequently, cephalopods should also be considered in the NIS assessment within the MSFD (D2).

Fisheries landings of cephalopods are increasing in parts of the world (Arkhipkin et al. 2015; Doubleday et al. 2016), but in some species/areas, there is an opposite trend in cephalopod catches (Ospina-Alvarez et al. 2021), likely due to unsustainable fisheries (Katağan et al. 2015). For example, the catch biomass of Octopoda in the Aegean Sea showed a strong decrease between 2001 and 2013. Katağan et al. (2015) attributed this to unsuitable policies such as non-optimal closed seasons in cephalopod fisheries, along with weak controls on cephalopod fisheries. In European waters, catching cephalopods in large-scale fisheries is largely unregulated, but not all fluctuations have been attributed to fishing, as there have also been significant decreases associated with changing environmental conditions (e.g. Sobrino et al. 2020). However, the combination of high fishing pressure and the semelparous life cycle of cephalopods, with only a single spawning event before they die, make them a potentially vulnerable resource for both targeted and bycatch fisheries. Around 17 of nearly 70 cephalopod species inhabiting European marine waters are commercially relevant (Jereb et al. 2015; Katağan et al. 2015). Cephalopods are fished in many areas of the EU, where their commercial landings show both increasing (Caddy and Rodhouse 1998; Arkhipkin et al. 2015) and decreasing trends, depending on the area and species fished (Ospina-Alvarez et al. 2021). These cephalopod species should be considered in the assessment

under D3 (status of commercial fish and shellfish) and are also potentially relevant for D9 (contaminants in seafood) as they are able to accumulate contaminants in their tissues (Bustamente et al. 2002).

In food webs, cephalopods exert top-down pressure on lower TLs but also have a bottom-up function due to their role as major prey for some marine mammals and predatory fishes, depending on their life stage and size. For example, cephalopods are a major food source for sperm whales, dolphins, seals and some elasmobranchs (Clarke 1996a, 1996b; Katağan et al. 2015). They also play an important role in the diet of some teleosts (Bello 1991; Daly et al. 2001; Battaglia et al. 2013). Changes in fish and/or cephalopod abundances have already caused a community shift towards squid-dominated food webs in some places (Caddy and Rodhouse 1998; Balguerías et al. 2000; Hunsicker et al. 2010; Doubleday et al. 2016). Logically, cephalopods play an important key role in marine coastal and open sea food webs and should therefore be integrated in MSFD D4 (food webs).

In summary, one should expect that, in accordance to the MSFD, information on cephalopods is reported under D1, D2, D3, D4 and D9. Of course, other Descriptors including eutrophication and noise might also have a possible impact on the state of cephalopods, though the relationship between pressure and state is still unclear. However, some studies on acoustic exposure in cephalopods (Guerra et al. 2011; André et al. 2011; Solé et al. 2013a, b, 2017, 2022) do already exist. Nevertheless, we will review the current integration of cephalopods within the MSFD focusing on D1–D4 and D9 but will also deal with D11 (energy/noise). The aim of this

review is to illustrate the gaps and to provide some preliminary suggestions on how to deal with this keystone group in future MSFD reporting.

## State-of-the-art: cephalopods within the MSFD

Until now, only 23 EU–MS were obliged to complete MSFD reporting due to their geographical location and access to marine waters. Furthermore, in addition to MS without a coastline, those with a lack of cephalopods in their marine areas (e.g. Baltic and Black Sea) are not committed to reporting on cephalopods. This leaves 16 EU-MS for which it could be assumed that they would include cephalopods in their reporting. However, our results show that only a few even mentioned cephalopods in their MSFD reporting (Table 1). The non-reporting of cephalopods in the MSFD usually occurs without any justification (Dupont et al. 2014a, b, c, d, e).

Within the first reporting cycle (2012), the Netherlands reported that only an insignificant number of cephalopods occurs in their national waters (Dupont et al. 2014f). Germany stated that no methods for the analysis and evaluation of the cephalopod species group have currently been developed (Dupont et al. 2014g) and the UK justified the reporting gap citing insufficient information and data on cephalopods in their waters and stated that further development may be required (Dupont et al. 2014h).

**Table 1** Report of cephalopods in the first (2012) and second (2018) reporting cycle within corresponding descriptors of the MSFD as well as the given reason by MS for their non-reporting

| Member States | First MSFD reporting cycle 2012                  | Second MSFD reporting cycle 2018                           |
|---------------|--------------------------------------------------|------------------------------------------------------------|
| Belgium       | Non-reporting without justification              | D9                                                         |
| Croatia       | –                                                | Lack of monitoring, data and methodological standards      |
| Cyprus        | Non-reporting without justification              | No indicators developed                                    |
| Denmark       | Non-reporting without justification              | Not relevant                                               |
| France        | D9                                               | –                                                          |
| Germany       | No methods for analysis and evaluation developed | D1: abundance trends                                       |
| Greece        | –                                                | –                                                          |
| Ireland       | Non-reporting without justification              | Lack of biological information and data collecting systems |
| Italy         | D9                                               | D1: D1C2, D1C3                                             |
| Malta         | –                                                | D1: D1C1, D1C2, D1C3, D1C4                                 |
| Netherlands   | Insignificant population                         | Not relevant                                               |
| Portugal      | D1: D1C2, D1C3<br>D3: D3C1, D3C3                 | D1: D1C2, D1C3<br>D3: D3C1, D3C3                           |
| Slovenia      | –                                                | D1: D1C1, D1C2, D1C3                                       |
| Spain         | D9                                               | D1, D9                                                     |
| Sweden        | Most likely not relevant for the North Sea area  | Not relevant                                               |
| UK            | Insufficient information and data                | –                                                          |



For the initial assessment of cephalopods under D3, Portugal described the stock development using secondary indicators (for an explanation see section ‘Descriptor 3’) for D3C1 and D3C3 (Dupont et al. 2014i) and included cephalopod species in D1, indicator 1.6 (pelagic habitat conditions) and the states of two functional groups (Cochrane et al. 2010) were assessed: *Coastal/shelf cephalopods* and *deep-sea cephalopods*. Although no verdict regarding the GES of cephalopods was made, it was assumed to be achieved in Portuguese waters. Spain included cephalopods under D9 and assessed the heavy metal concentration of seven species from the Spanish Atlantic waters (Dupont et al. 2014j) and three species from the western Mediterranean Sea. All of them had concentrations below the respective reference values for Cd, Hg and Pb (Dupont et al. 2014j). France and Italy also included cephalopods in D9. The contamination by hazardous substances in cephalopods for the French Mediterranean region was described as moderate (Dupont et al. 2014d), whereas Dupont et al. (2014k) provided no information on such levels in cephalopods in Italian waters. The remaining countries ignored cephalopods in their reports for the contaminants in seafood Descriptor, so that it seems that countries where more cephalopods are eaten, take this descriptor more seriously.

Within the second reporting cycle in 2018, the integration of cephalopods had only changed slightly and around half of the 16 MS still ignored this species group. However, most MS which have not carried out a cephalopod assessment have justified their non-reporting. Denmark, Netherlands and Sweden designated cephalopods in their waters as ‘not relevant’ for assessment (Banfi et al. 2021a, b, c). Croatia justified its non-reporting by the lack of monitoring, available data and methodological standards, while Cyprus argued that developed indicators are missing (Banfi et al. 2021d, e). Ireland excused the non-reporting by the lack of biological information and data collection systems (Anonymous 2020). Germany illustrated the trends in abundance within D1, but without any evaluation (BMU 2018). Italy, Malta, Slovenia and Spain considered cephalopods under D1 in relation to different criteria (see Table 1) (Banfi et al. 2021f, g). The contamination of cephalopods (D9) was assessed in the second cycle (2018) by two MS. Spain focused on heavy metals (Banfi et al. 2021g), whereas Belgium focused on PAH and dioxins (Banfi et al. 2021h). Portugal included cephalopods within D1 and D3 in the second reporting cycle and used criteria D1C2, D1C3 (for more information about criteria see Descriptor sections), D3C1 and D3C3, following Commission Decision 2017/848/EU (about criteria and methodological standards on good environmental status of marine waters and specifications and standardized methods for monitoring and assessment) for their status reporting. Poor GES for biodiversity of coastal/shelf cephalopods was reported,

mainly due to the low biomass of *Alloteuthis* spp. in recent years but increasing biomass trends for three cephalopod species were also reported by Portugal in D3 (Ministerio do Mar 2020). Thus, to our best knowledge, out of 16 MS, only Portugal described the status of cephalopods in D3. Meanwhile, the International Council for the Exploration of the Sea (ICES) already advises the inclusion of five cephalopod species (*Illex coindetii*, *Illex illecebrosus*, *Loligo vulgaris*, *Octopus vulgaris* and *Sepia officinalis*) in the reference list of D3 species (ICES 2020a) (although *I. illecebrosus* can be ignored in European waters as it most likely does not occur (Oosterwind et al. 2020)).

Where a reason was provided by MS to exclude cephalopods within their reporting, most MS argue that the lack of data is the primary reason (COM/2020/259). However, many standardized fisheries research surveys report cephalopods, particularly the commercially important species. While such data are insufficient to determine absolute abundance of cephalopods, for various reasons, these data allow comparisons of species distribution, relative abundance, size distribution, age distribution, and size at maturity (by sex) of different cephalopod species, as well as comparisons between years and seasons (Pierce et al. 1998; Krstulović Šifner et al. 2011; Oosterwind et al. 2015; van der Kooij et al. 2016). Furthermore, commercial landings data also exist for many regions, although these are often at higher taxonomic level and therefore would often be insufficient for a species-specific assessment in many cases. However, even if these studies might not provide species-specific datasets over decades, they allow the description of the current status and, in some areas, can indicate changes in MSFD-relevant aspects like species distribution and abundance, reproduction and phenology (e.g. Pierce et al. 2005; Oosterwind et al. 2020, 2022).

As an example, the ICES Working Group on Cephalopod Fisheries and Life History (WGCEPH) analyses the landings data and fishery research survey data (in those species that are commercially important for D3) by regions, species groups (at different taxonomic levels) and fishing effort, to describe the trend in indices of biomass and/or abundance. These exercises demonstrate the potential of using existing data, but also highlight the data gaps (e.g. ICES 2020b). In the following sections, we discuss the current and potential future possibilities to integrate cephalopods into the MSFD using the existing and publicly available data, along with any limitations and knowledge gaps. As an overview we have listed descriptors that can already be addressed in Table 2, including those indicators which can already be used to assess status (marked in bold) and additional potential indicators for cephalopod assessment.

**Table 2** Potential indicators for the assessment of cephalopod stocks within the EU-MSFD; in bold: can serve current criteria of described Descriptors; in unbold: additional potential indicators

| Descriptor    | Criteria          | Indicator                                                      | Method                                                                                           | References                          |
|---------------|-------------------|----------------------------------------------------------------|--------------------------------------------------------------------------------------------------|-------------------------------------|
| <b>D1</b>     | <b>D1C1</b>       | <b>Mortality rate per species from incidental by-catch</b>     | –                                                                                                | –                                   |
| <b>D1, D3</b> | <b>D1C2</b>       | <b>Population abundance</b>                                    | <b>CPUE (i t<sup>-1</sup>)</b>                                                                   | Palma-Pedraza et al. (2020)         |
| <b>D1, D3</b> | <b>D1C3, D3C3</b> | <b>Demographic characteristics (size and age distribution)</b> | <b>Measurement series and age determination</b>                                                  | Oesterwind et al. (2020)            |
| <b>D1, D3</b> | <b>D1C4</b>       | <b>Distributional range and pattern</b>                        | $\Delta_{\text{distribution}} \left( \frac{b}{N} - \frac{a}{N} \right) \times 100$               | OSPAR (2016)                        |
| <b>D1, D3</b> | <b>D3C2</b>       | <b>Spawning stock biomass</b>                                  | <b>CPUE<sub>mature</sub> (Kg t<sup>-1</sup>)</b>                                                 | Borja et al. (2021)                 |
| D1, D3        |                   | Nursery stock biomass                                          | CPUE <sub>immature</sub> (Kg t <sup>-1</sup> )                                                   | –                                   |
| D1, D3        |                   | Nursery grounds                                                | Modelling                                                                                        | Colloca et al. (2015)               |
| D1, D3        |                   | Biomass                                                        | CPUE (Kg t <sup>-1</sup> )                                                                       | Quincoces et al. (2011)             |
| D1, D3        |                   | Biomass of small cephalopods                                   | CPUE (Kg t <sup>-1</sup> )                                                                       | ICES (2014a, 2018)                  |
| D1, D3        |                   | Small squid abundance                                          | CPUE (i t <sup>-1</sup> )                                                                        | Modified after Pierce et al. (2016) |
| D1, D3, D4    |                   | Guild level biomass (and production)                           | CPUE (Kg t <sup>-1</sup> )<br>(CPUE year <sup>-1</sup> (Kg t <sup>-1</sup> year <sup>-1</sup> )) | Modified after Whitfield (2016)     |
| <b>D2</b>     | <b>D2C1</b>       | <b>Detection of non-indigenous species (NIS)</b>               | $\sum \text{newNISintroductions}$                                                                | OSPAR (2018)                        |
| <b>D2</b>     | <b>D2C2</b>       | <b>Abundance of NIS</b>                                        | $\sum \text{NIS}_{t-1} - \sum \text{NIS}_t$                                                      | OSPAR (2018)                        |
| <b>D2</b>     | <b>D2C2</b>       | <b>Spread of NIS</b>                                           | $\left( \frac{M_s}{M} \right)_{t-1} - \left( \frac{M_s}{M} \right)_t$                            | OSPAR (2018)                        |
| <b>D3</b>     | <b>D3C1</b>       | <b>Harvest rate (fishing mortality)</b>                        | $Y_{iy}/B$                                                                                       | Piet et al. (2010)                  |
| <b>D4</b>     | <b>D4C1</b>       | <b>Trophic level of catch</b>                                  | <b>Stable isotopes <sup>15</sup>N</b>                                                            | Jennings and van der Molen (2015)   |
| <b>D4</b>     | <b>D4C4</b>       | <b>Key predator productivity (production per unit biomass)</b> | <b>(CPUE year<sup>-1</sup> (Kg t<sup>-1</sup> year<sup>-1</sup>))</b>                            | Modified after Rogers et al. (2010) |
| D4            |                   | Trophic level of surveyed species                              | $TL_i = 1 + \sum_j (TL_j * DC_{ij})$                                                             | Joshi et al. 2016                   |
| D4            |                   | Mean trophic level                                             | $TL_y = \frac{\sum_i (TL_i * Y_{iy})}{\sum_i Y_{iy}}$                                            | Joshi et al. (2016)                 |
| <b>D9</b>     | <b>D9C1</b>       | <b>Level and number of contaminants</b>                        | <b>Tissue analyses</b>                                                                           | Maggi et al. (2014)                 |

*a* number of spatial units (e.g. sub-areas, grid cells) occupied during reference period *a*, *b* number of units occupied in a subsequent period *b*, *B* biomass, *N* total number of spatial units, *CPUE* catch per unit effort, *Kg* weight (kg), *t* assessment period, *M<sub>s</sub>* species positive monitoring locations, *M* total monitoring locations, *Y* fisheries catch, *i* number of individuals, *TL<sub>i</sub>* trophic level of species (*i*), *TL<sub>j</sub>* trophic level of prey (*j*), *DC<sub>ij</sub>* contribution of prey (*j*) in the diet of species (*i*), *Y<sub>iy</sub>* catch of the species or group (*i*) in year (*y*)

## Descriptor 1 (biodiversity)

According to Commission Decision 2010/477/EU, the objective under D1 is as follows: "Habitat quality and occurrence, and species distribution and abundance, are consistent with prevailing physiographic, geographic and climatic conditions."

Different indicators or methods to assess fish species might serve as a useful starting point for potential application to cephalopods. Among them are trophodynamic, climatic (Fortibuoni et al. 2017), pressure and state indicators that can be used for assessing GES (Lynam and Mackinson 2015). In the latest MSFD assessment, D1 contained five relevant criteria for cephalopods, the first four of which relate directly to the individual species (Commission Decision 2017/848/EU). These include species mortality rate (D1C1) (Table 2), which is mainly relevant for species considered at risk due to bycatch and discarding; species population abundance (D1C2) (Table 2), which is important

for non- or less commercial species with a relevant ecological role in the ecosystem and not assessed in D3; species demographic characteristics (D1D3) (Table 2); and species distributional range and pattern (D1C4) (Table 2). The last criterion (D1C5) relates to the habitat, which has to meet all the necessary requirements for the life stages of a species. These indicators are intended for use to assess the status of cephalopods directly and as a constituent part of marine communities.

While mortality data are very rare for cephalopods and fishing mortality is, in most cases, documented at higher taxonomic level (in so far as it is meaningful for species which usually die when 1–2 years old regardless of whether they have been fished), data about species population abundance (D1C2), species demographic characteristics (D1D3), and species distributional range and pattern (D1C4) are, in many cases, provided by fishery research cruises and can be used at least for some species in some areas. Arkhipkin et al. (2021) describes the challenges for stock assessment of



short-lived species, and exploratory stock assessments of the main European cephalopods have been carried out under the auspices of ICES WGCEPH based on the use of natural mortality (M) and fishing mortality (F), which can be estimated, for example, using a surplus production model in continuous time (SPiCT) (Larivain 2020). In addition, several assessments of various stocks of octopuses have been carried out in the Mediterranean, most recently using production models or related methods (Froese et al. 2018; Geraci et al. 2021; Tsikliras et al. 2021). The only routine stock assessment for a cephalopod takes place in the Western Asturias small-scale fishery for *O. vulgaris*, where it is required under the terms of Marine Stewardship Council (MSC) certification and is based on the depletion model described by Roa-Ureta et al. (2021).

Indices of relative species population abundance provide important information and are collated by the ICES WGCEPH (e.g. ICES 2020b) based on a data call to MS, and has already been used in various publications (e.g. van der Kooij et al. 2016; Oosterwind et al. 2022). Due to the short life cycle, and other reasons, most species show strong interannual fluctuations which require special attention during the analysis. Fishery research surveys also provide some information about species demographic characteristics, as, in many cases, length data of the survey catches are provided. However, due to the high plasticity of cephalopod life cycles and the occurrence of micro-cohorts (Guerra and Rocha 1994; Oosterwind et al. 2010), because within any given year hatching and recruitment may occur in several discrete 'pulses' over an extended period of time or there may even be separate summer and winter breeding groups, interpretation of length data should be performed with caution.

Occurrence and distribution depend on natural factors such as habitat preferences, temperature, salinity and food availability, as well as seasonality of the life cycle, which applies to most species of commercial interest in Europe. Due to the high sensitivity of cephalopods to environmental conditions, a change in their distribution could indicate either significant natural change or man-made pressures, and these two are not easily disentangled. Understanding spatial distributions is an important aspect of developing management and conservation strategies (Planque et al. 2011). However, cephalopods are highly mobile species so, for most species, their geographic distribution can vary from year to year (Pierce et al. 1994). Another reason for distribution changes (in this case within years) in cephalopods is ontogenetic migrations during their life cycle. Such migrations occur when the range of a species is divided into spawning/nursery and feeding grounds (Nesis 1985). In a 6-step approach, Colloca et al. (2015) identified annual nursery areas for *E. cirrhosa* and *I. coindetii* by modeling the spatial distribution of recruits with the purpose of reconstructing

the distribution of 'density hot-spots', regarded as more productive areas in the Mediterranean Sea. Within the Northeast Atlantic spawning, nursery and feeding grounds for *Loligo vulgaris* and *Loligo forbesii* were published recently (Laptikhovsky et al. 2022). Nursery grounds of coastal species in the European Mediterranean Sea are consistently protected from trawling fisheries (Colloca et al. 2015) due to a ban on use of this gear for three nautical miles of coastline or waters < 50 m depth (Council Regulation (EC) No 1967/2006), which is in contrast to the less consistent (or non-existent) protection of nursery areas for species with offshore recruitment. Last but not least, many knowledge gaps exist regarding habitat requirements in cephalopods, although studies are beginning to be performed to fill this gap (recently e.g. Barrett et al. 2021; Laptikhovsky et al. 2022). All-in-all, cephalopods can be integrated in D1, at least for some of the criteria, as described above.

## Descriptor 2 (non-indigenous species)

To maintain or obtain GES within D2 requires "non-indigenous species introduced by human activities are at levels that do not adversely alter the ecosystems" (Commission Decision 2010/477/EU). In D2, one primary and two secondary criteria are relevant for cephalopods. According to Commission Decision 2017/848/EU, the primary criterion considers the number of NIS introduced into the area by human activity (D2C1) (Table 2). The secondary criteria consider the abundance and distribution of these species (D2C2) (Table 2), and the species and habitat affected by NIS (D2C3).

OSPAR (2018) listed three parameters (indicators) for the assessment of non-indigenous or cryptogenic species. These include the 'new introductions' parameter for the number of new introductions within the assessment area and assessment period (t), the 'community abundance' parameter showing the relative change in the total number of NIS between different assessment periods and the 'dispersal' parameter for the relative change in the proportion of monitoring locations (M) within which the species is found (OSPAR 2018). The list of new NIS requires understanding of the invasion process, as the species relevant to MSFD monitoring and future assessment are those related to human-mediated transmission. The status of NIS can be regularly monitored by existing fishing research cruise surveys, in which the sampling protocols have been improving in the last decade to assure good cephalopod identification. Where ship-based surveys cannot be executed, e.g. in Marine Protected Areas, and within hotspots or likely entry points for NIS, the use of scientific divers (Deidun et al. 2019) or other survey technologies will be helpful for some species.

In addition to survey-based indicators, eDNA (Obst et al. 2020) and Citizen Science [e.g. Environmental NGO iSea project “Is it Alien to you? Share it!!!” (iSea 2022)] can be cost-effective tools to supplement routine monitoring, by filling knowledge gaps on the distribution and dynamics of marine bioinvasions, hence contributing to the assessment of GES within D2 of the MSFD (Giovos et al. 2019). Although none of the MS with non-indigenous cephalopods in their marine regions included them in their assessment, it seems that cephalopods could already be integrated in D2.

### Descriptor 3 (commercial fish and shellfish)

The aim of D3 is that “populations of all commercially exploited fish and shellfish are within safe biological limits, exhibiting a population age and size distribution that is indicative of a healthy stock” (Commission Decision 2010/477/EU). The Commission Decision 2017/848/EU names three primary criteria for D3; fishing mortality rate (D3C1), spawning stock biomass (D3C2) and age and size distribution (D3C3) of commercial species (Table 2), which are usually derived from the analytical stock assessments required under the European Common Fishery Policy (CFP). The first two criteria aim to achieve maximum sustainable yield (MSY) of the commercially fished stocks. Thus, fishing mortality should be at or below the level (D3C1) and spawning stock biomass should at or above a level (D3C2) which can achieve MSY (Commission Decision 2017/848/EU). However, not all commercially exploited fish stocks in EU waters (and only one cephalopod stock) are routinely subject to full analytical assessments and, hence, for the non-assessed stocks, time series for fishing mortality (F) and spawning stock biomass (SSB) are generally not available, and the age structure is usually unknown. In addition, bycatch data for commercially exploited species are often still lacking. Another issue is that typical analytical assessment methods are developed for long-lived finfish stocks and many are not appropriate for cephalopod species, which possess 1- or 2-year life cycle (Pierce and Guerra 1994; Payne et al. 2006; Arkhipkin et al. 2021). Obviously, the European CFP is (or would be if cephalopods were included) faced with a special challenge in order to develop sustainable cephalopod fisheries management (Arkhipkin et al. 2021). While some authors point towards the advantages of using depletion methods to assess cephalopod stocks (e.g. Arkhipkin et al. 2021), other have favoured a simpler approach based on production models (e.g. Froese et al. 2018). The former requires intensive monitoring and (ideally) real-time assessment while the latter make the questionable assumption of a fixed carrying capacity—but have the advantage of rapidly generating simple indicators (e.g.  $F/F_{MSY}$ ,  $B/B_{MSY}$ ). As an alternative to analytical assessment and its indicators,

the European Commission suggested the use of so-called ‘secondary indicators’ which can serve at least for some fish stocks as reliable indicators (Probst and Oesterwind 2014). These include the ‘Catch Per Unit Effort’ (CPUE) indices (Table 2), based in numbers or biomass, which are assumed to be proportional to stock size (King 2007), and can be based on data from commercial fisheries or fisheries-independent scientific surveys, though the latter are considered more reliable for estimating stock abundance (Harley et al. 2001). However, Probst and Oesterwind (2014) note that the quality of such indicators depends on the stock in question, as well as on the chosen metric. In the case of cephalopods, it might be that the patchy spatial distribution in some species (Pierce et al. 1998) and the difficulty of ensuring that surveys coincide with the same part of life cycle decrease the quality of the indicators. Nevertheless, the CPUE can also be modified to estimate recruitment, or SSB (Table 2). While the ICES WGCEPH already use the CPUE to describe general trends for different species in different regions (ICES 2020b), size-modified CPUE to assess spawning stock, for example, may require further refinement for cephalopods because, in many cases, the relationship between length and maturity stage is highly variable in cephalopod species and depends on sampling location, particularly latitude, and sampling season (Jackson and Yeatman 1996; Oesterwind et al. 2018), and because recruits and spawners may be present only in certain seasons.

Another secondary index to reflect fishing mortality is the Harvest Ratio (HR) (Table 2), which is the ratio between the biomass of commercial catches (or landings) and abundance indices (Piet et al. 2010). A two-stage biomass model has been developed to calculate HR for cephalopods in the English Channel (Gras et al. 2014; Alemany et al. 2017), but without a routine assessment in this area. To estimate the HR, reliable documentation of commercial landings is needed but this is somewhat lacking in cephalopod species because many species are fished as bycatch and landings are often reported at higher taxonomic level. Better sampling of the cephalopods landings by MS could address this issue.

Various length-based indicators exist to assess the demography of cephalopod stocks but, as mentioned in D1, high plasticity, a short life cycle and the possible existence of micro-cohorts might render the demography criterion useless. Furthermore, size-dependent indicators for D3 in fish are based on the concept that overfishing tends to result in the loss of the larger and older animals. This might make sense in long-lived animals but it is not as relevant in fished species with a short lifespan, non-overlapping generations (in many cases) and high plasticity where length depends not only on the life cycle stage but is also driven by environmental factors like oxygen and temperature (Pecl and Jackson 2008; Rosa et al. 2012; Rogov et al. 2019). A potentially useful length-based indicator for control purposes is the



‘small squid indicator’ (Table 2) because a catch with many small squids could be a sign of fishing in a recruitment or nursery area, which could result in overfishing and endanger the stock (Pierce et al. 2016). The seasonal variability of cephalopod sizes, however, can pose a challenge to using this indicator for GES assessment. Nevertheless, as Laptikhovsky et al. (2022) mentioned, as quarterly research cruises are timed consistently across the years, any massive changes in the sizes of the sampled population at a particular time of year might indicate that more investigations are necessary and could also point to climate-associated shifts. In addition to the assessment of commercially important species (D3), this size indicator is just as important for any non-commercial species in D1. In summary, the integration of cephalopods within D3 is partly possible for the main commercial species. However, the primary indicators mentioned above seem problematic for cephalopods due to the short life cycle and the general absence of routine assessment, and it might be better to develop a separate indicator approach for this group instead.

#### Descriptor 4 (food webs)

The focus in D4 is that “all elements of the marine food webs, to the extent that they are known, occur at normal abundance and diversity and levels capable of ensuring the long-term abundance of the species and the retention of their full reproductive capacity” (Commission Decision 2010/477/EU). According to Commission Decision 2017/848/EU, four criteria are related to D4, which are divided into two primary and two secondary criteria. The primary criteria are trophic guild diversity (D4C1) (Table 2) and abundance balance (D4C2). These two are complemented by the secondary criteria, size distribution of individuals (D4C3) and productivity (D4C4) (Table 2). As with D2, these indicators are related to ecological communities in marine ecosystems and cephalopods should not be neglected.

As indicators of community structure, trophic levels (TLs) are important for monitoring ecosystem changes (Joshi et al. 2016). Besides calibration, parameterization and validation of food web models, the estimation of TL is also used to calculate metrics and indicators of food web structure and human impact (Jennings and van der Molen 2015). A distribution change in a cephalopod species, for example, may lead to a change in its role as predator and prey and thus its trophic relationship in the food web (Lishchenko et al. 2021). Shannon et al. (2014) made a comparison between indicators based on TL, whereby landings-based TL indicators (e.g. TL of the landed catch) show the pressure on the system, whereas survey-based TL indicators show (potentially) unbiased state changes of the

surveyed community. In addition, model-based TL indicators (based on Ecopath with Ecosim models) can facilitate the inclusion of poorly sampled species in ecosystem assessments (e.g. Gasalla et al. 2010; Coll et al. 2013). However, it should be noted that model-based indicators, which are based on equilibrium measures, have some difficulties with interannual variability in cephalopod biomass. As Christensen and Walters (2004) describe, assuming equilibrium for a reference year can lead to overly optimistic estimates of sustainable fishing mortality.

Biomass and productivity are related to the structural properties of food webs and can thus serve as proxies for ecosystem functioning (Zador et al. 2016). ICES (2014b) and Tam et al. (2017) have stated that the guild level biomass indicator is commonly used for fish, but should include guilds across all TLs. With this, both the food web condition and the relative stability of its main components could be assessed. Some predators or groups of species play a more important role in food webs than others and can thus be used as energy flow indicators for the whole system. The major predator–prey processes are measured using the productivity of these key predators (Rogers et al. 2010). To assess cephalopods using these indicators, high-quality and regularly updated fisheries landings and survey data time series from regional seas are required. Furthermore, information on regional and ontogenetically specific prey composition is something which is still lacking in many species and needs to be improved in future. Overall, cephalopods can partly be integrated in D4 on the basis of currently available data.

#### Descriptor 9 (contaminants in seafood) and Descriptor 11 (energy/noise)

According to Commission Decision 2010/477/EU, the aim for D9 is that “contaminants in fish and other seafood for human consumption do not exceed levels established by Community legislation or other relevant standards”. For D11, the following applies: “Introduction of energy, including underwater noise, is at levels that do not adversely affect the marine environment” (Commission Decision 2010/477/EU). One primary criterion is listed by Commission Decision 2017/484/EU for D9, which is contaminant levels in seafood (D9C1) (Table 2), whereas D11 has two primary criteria related to anthropogenic impacts on marine animals, concerning impulsive sound source levels (D11C1) and levels of continuous low-frequency sound (D11C2) (Commission Decision 2017/848/EU).

Unlike the ecologically and environmentally focused D1–D4, contaminants in seafood (D9) and introduction of energy and noise (D11) have a clear human focus and refer directly to the extent of human impact on the environment

**Table 3** Summary of Ommastrephidae trends based on landings in tonnes. The historical mean is the entire landings data series (2000–2021) (modified from ICES 2023)

| Region                              | Historical<br>(mean tonnes) | 2016–2018<br>(mean tonnes) | 2019–2021<br>(mean tonnes) | Trends                      |                                |
|-------------------------------------|-----------------------------|----------------------------|----------------------------|-----------------------------|--------------------------------|
|                                     |                             |                            |                            | 2019–2021 vs.<br>historical | 2019–2021<br>vs. 2016–<br>2018 |
| North Sea                           | 9                           | 35                         | 29                         | Positive                    | Negative                       |
| Celtic Seas                         | 271                         | 420                        | 557                        | Positive                    | Positive                       |
| English Channel                     | 144                         | 495                        | 315                        | Positive                    | Negative                       |
| Bay of Biscay                       | 1325                        | 1198                       | 1549                       | Positive                    | Positive                       |
| Western Iberia and<br>Gulf of Cadiz | 1079                        | 604                        | 1126                       | Positive                    | Positive                       |

(O'Higgins 2016). Nevertheless, it is possible to use cephalopods as indicators for heavy metal pollution in the oceans. As Pierce et al. (2016) already describe, they could be used as an early warning of high environmental levels due to their accumulation of cadmium and PCBs in their tissues.

The impact of noise as underwater pollution and its impact on the surrounding environment are unclear in most cases. Therefore, information on the effects on most cephalopods is also lacking, although there are some documented effects on squid behavior (Fewtrell and McCauley 2012), on squid and cuttlefish statocyst development (Solé et al. 2013a, b, 2017, 2022) and on (giant) squid mortality (Guerra and González 2006; Guerra et al. 2011). In addition, the topographic conditions of an area, for example, great depths and steep slopes, or a large number of islands and islets play a major role in this assessment because they influence noise transmission in complex ways (Paramana et al. 2021). However, André et al. (2011) and Solé et al. (2013a, b, 2017, 2022) exposed several cephalopod species to low-frequency sounds (which is the main type of noise generated by coastal industry, marine traffic, geophysical surveys, offshore wind energy, etc.) and this resulted in permanent and substantial alterations of sensory hair cells of the statocysts of the individuals.

## Case study

To illustrate how existing data from the ICES-DATRAS-database and EU Data Collection Framework (DCF) could be used to integrate cephalopods into the MSFD, we present some examples, with graphic representations and tables from which conclusions can be drawn for a first cephalopod assessment. The time analyses and associated trend analyses in our case study are not intended as a recommendation and can be adapted or modified. In addition, due to the Covid-pandemic in Europe, some cruises have not taken place or have been shortened, so that the data quality within the past 2 years seems to be limited, but this should increase again in further years.

## Mortality rate (D1C1), fishing mortality (D3C1)

Landings data collected within the DCF provide an indication of fishing pressure on commercially important cephalopods. Landings tend to be highly variable across ICES management units.

A summary of the Northeast Atlantic fishing mortality trends in Ommastrephidae indicated by landings shows an increasing fishing mortality in all areas, when recent landings are compared with historical landings, and this also happens in most areas when compared with the recent period (Table 3). Interpreting the reasons for these trends is more difficult and abundances as well as fishing effort should be considered.

The summary trends in Loliginidae based on the landings show an increase in the North Sea, Western Iberia and Gulf of Cadiz and Azores, while landings decreased in Celtic Seas, English Channel and Bay of Biscay (Table 4). However, the Azores *Loligo* stock is considered to be reproductively isolated and independent of the European Shelf populations, while the latter population shows high genetic gene flow and connectivity amongst the constituent shelf regions (Brierley et al. 1995; Shaw et al. 1999; Göpel et al. 2022; Sheerin et al. 2022).

## Population abundance (D1C2, D3C2)

The data of the standardized fishery surveys (available at DATRAS) can address abundance indicators. Except for the North Sea CPUE trend of *Todaropsis eblanae*, CPUEs in ommastrephids show an increasing trend within the recent years in the North Sea, Celtic Seas and Bay of Biscay, while CPUE values at the Gulf of Cadiz decreased for *I. coin-detii* and *T. eblanae* (Table 5). However, as it is very typical that cephalopod CPUE fluctuates across years, especially for ommastrephid squids, ideally further analysis should be performed to determine whether the declining CPUE is part of its natural fluctuation, or not. Ommastrephid squids are

**Table 4** Summary of Loliginidae trends based on landings in tonnes. The historical mean is the entire landings data series (2000–2021) (modified from ICES 2023)

| Region                              | Historical<br>(mean tonnes) | 2016–2018<br>(mean tonnes) | 2019–2021<br>(mean tonnes) | Trends                      |                                |
|-------------------------------------|-----------------------------|----------------------------|----------------------------|-----------------------------|--------------------------------|
|                                     |                             |                            |                            | 2019–2021 vs.<br>historical | 2019–2021<br>vs. 2016–<br>2018 |
| North Sea                           | 1816                        | 2226                       | 2560                       | Positive                    | Positive                       |
| Celtic Seas                         | 1423                        | 1794                       | 1418                       | Negative                    | Negative                       |
| English Channel                     | 3518                        | 4710                       | 2880                       | Negative                    | Negative                       |
| Bay of Biscay                       | 1755                        | 1635                       | 1188                       | Negative                    | Negative                       |
| Western Iberia and<br>Gulf of Cadiz | 848                         | 703                        | 1047                       | Positive                    | Positive                       |
| Azores                              | 397                         | 252                        | 671                        | Positive                    | Positive                       |

**Table 5** Summary of trends in Ommastrephidae based on existing ICES-DATRAS surveys (modified from ICES 2023)

| Region                | Survey      | Recent trends (2019–2021 vs. 2016–2018) |                   |                      |
|-----------------------|-------------|-----------------------------------------|-------------------|----------------------|
|                       |             | <i>I. coindetii</i>                     | <i>T. eblanae</i> | <i>T. sagittatus</i> |
| North Sea             | NS IBTS Q1  | Positive                                | Negative          | Positive             |
| Celtic Seas           | ES-PorcGFS  | Positive                                | Positive          | Positive             |
|                       | UK-SWBEAM   | NA                                      | NA                | NA                   |
| Bay of Biscay         | FR-EV HOE   | Positive                                | Positive          | Positive             |
| North-Western Iberian | ES-IBTS Q4  | NA                                      | NA                | NA                   |
|                       | PT-IBTS Q4  | NA                                      | NA                | NA                   |
| Gulf of Cadiz         | SP-GCGFS Q1 | Negative                                | Negative          | NA                   |
|                       | SP-GCGFS Q4 | Negative                                | Negative          | NA                   |

known to show short-lived outbreaks, as seen in Norway, Scotland, and at Rockall Bank in recent decades. Even for very well-documented stocks like those of *Illex argentinus* and *Todarodes pacificus*, separating fishing and environmental influences on abundance is difficult (Sundet 1985; Hastie et al. 1994; Rodhouse et al. 2014).

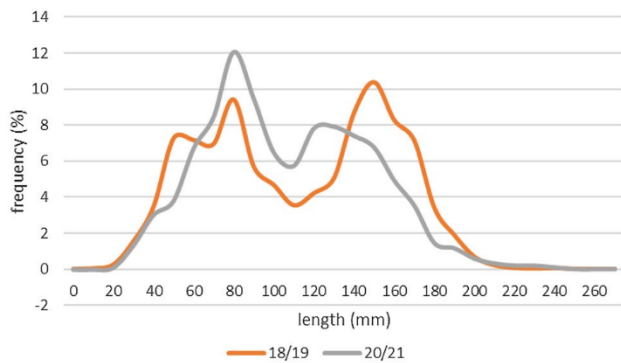
Within the southern areas (Gulf of Cadiz, North-Western Iberia), all available data show an increasing trend for *L. forbesii*, *L. vulgaris* and *Alloteuthis* spp., while in the

northern area, loliginids do not show a consistent trend in biomass among surveyed areas (Table 6). CPUE values for *L. forbesii* increased in the North Sea, while in the Celtic Seas both increasing and decreasing trends are observed, depending on the sub-area. A negative trend is also documented for *Alloteuthis* spp. in the Celtic Seas and North Sea in winter, while the CPUE of this taxon in the North Sea increased in summer. In the North Sea, CPUE trends of *L. vulgaris* are neutral or negative depending on the quarter.

**Table 6** Summary of Loliginidae trends based on standardized ICES coordinated surveys (modified from ICES 2023)

| Region                | Survey      | Recent trends (2019–2021 vs. 2016–2018) |                    |                         |
|-----------------------|-------------|-----------------------------------------|--------------------|-------------------------|
|                       |             | <i>L. forbesii</i>                      | <i>L. vulgaris</i> | <i>Alloteuthis</i> spp. |
| North Sea             | GER-IBTS Q3 | Positive                                | Neutral            | Positive                |
|                       | GER-IBTS Q1 | Positive                                | Negative           | Negative                |
| Celtic Seas           | UK-NWGFS    | NA                                      | NA                 | NA                      |
|                       | UK-SWBEAM   | NA                                      | NA                 | NA                      |
|                       | ES-PorcGFS  | Negative                                | NA                 | NA                      |
| Bay of Biscay         | IR-GFS      | Positive                                | NA                 | Negative                |
|                       | FR-EV HOE   | Negative                                | Negative           | Negative                |
| North-Western Iberian | ES-IBTS Q4  | Positive                                | Positive           | NA                      |
|                       | PT-IBTS Q4  | NA                                      | NA                 | NA                      |
| Gulf of Cadiz         | PT-IBTS Q4  | NA                                      | NA                 | NA                      |
|                       | SP-GCGFS Q1 | Positive                                | Positive           | Positive                |
|                       | SP-GCGFS Q4 | Positive                                | Positive           | NA                      |





**Fig. 1** Size distribution of *I. coindetii* from ICES IBTS surveys in the North Sea from Q1 and Q3 in 2018 & 2019 and 2020 & 2021 (ICES DATRAS download 09/2022)

An exclusively decreasing trend for all considered loliginid species is shown by the Bay of Biscay data.

### Species demographic characteristics (D1C3)

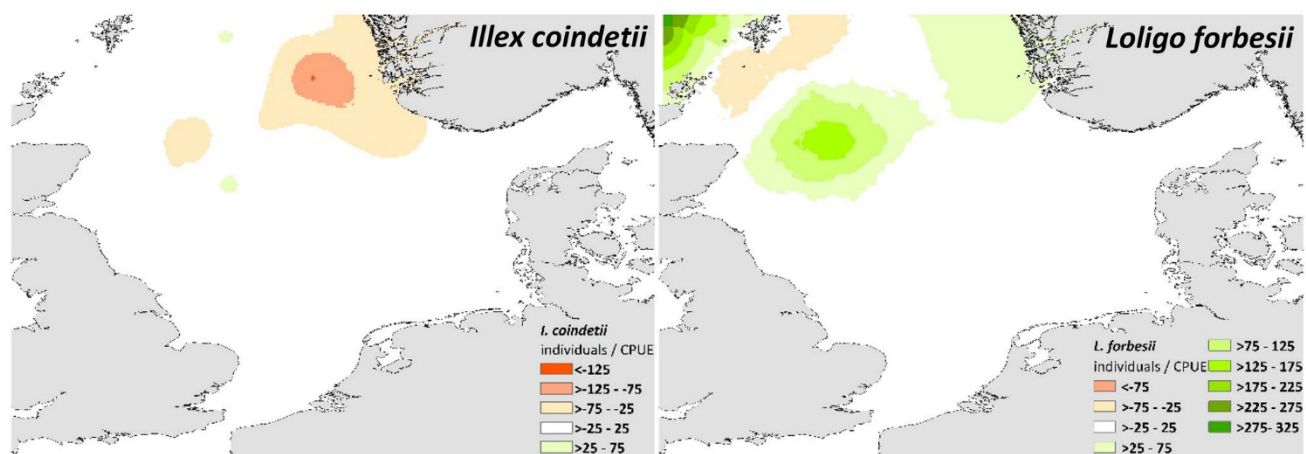
Although the frequency distribution of lengths of *I. coindetii* individuals in 2018/2019 fluctuated more than those in 2020/2021, they showed a similar trend (Fig. 1). Both show the highest frequency of individuals with dorsal mantle length of approx. 80 mm, with a decrease in frequency of subsequent size classes, followed by a renewed increase in larger length classes. An irregular drop in this length distribution, compared to previous years, could provide information on fishing pressure on a particular life stage of cephalopods, or, since survey timings tend to be invariable from year to year, could indicate a seasonal shift in abundance of the sampled demographic stages.

### Distributional range and pattern (D1C4)

The same survey data set from DATRAS can provide some information about the geographic distribution and any changes in the distributional pattern on a regional scale. As shown in Fig. 2, a heatmap for the differences in CPUE of *I. coindetii* and *L. forbesii* in the North Sea can allow potential differences in distribution to be visualized and compared over a time period, always assuming that seasonal data collection remains the same. This may be used to tease apart natural variability from longer-term climate-associated impacts on both the seasonal timing of distribution and annual changes in spatial distribution.

### Discussion

Our results show that not all MSFD criteria are currently suitable for cephalopods due to missing data and the very specific biology (fast and flexible lifecycles) in this group. Many cephalopods are key components in ecosystems (Coll et al. 2013) as well as being important commercial species, yet regular assessment comparable with commercial fish stocks does not exist in Europe (with the exception of the western Asturias octopus fishery, where assessment is required as part of the justification for MSC certification (Roa-Ureta et al. 2021)). Other countries around the world prove that an ecosystem approach to assessment and management, at least of commercially important cephalopod species, is quite possible (e.g. Arkhipkin et al. 2021). Therefore, we recommend including cephalopods within the MSFD instead of giving them little attention. Marchal et al. (2016) compares EU fisheries management with the innovative fisheries management of non-EU countries such as Australia,



**Fig. 2** Differences (Q1 2017–Q1 2019 versus Q1 2020–Q1 2022) in CPUE of *I. coindetii* (left) and *L. forbesii* (right) from ICES IBTS surveys in the North Sea (ICES DATRAS download 09/2022)

New Zealand and Iceland, whose approach is characterized by standard tools such as licensed commercial fishing vessels, assessment of key stocks and catch limitations through input controls. For instance, fisheries in the United States are regulated by law through the Magnuson–Stevens fishery conservation and management Act. The Act requires individual states to comply with various standards for fishing effort and management (NOAA 2007). Thus, each species, or species complex (e.g. all octopus species are grouped into a single assemblage) has its annual catch limit, which applies even to non-commercial species. For example, the overfishing limit (OFL, which corresponds to the stock's MSY) of *Enteroctopus dofleini* in the Gulf of Alaska is calculated by multiplying the best available estimate of octopus biomass by the best estimate of their natural mortality (Ormseth and Conners 2018). However, the MSY concept is based on equilibrium points, which can be inappropriate for cephalopods due to the particular highly dynamic life cycle of semelparous populations, where the growth rate and their yield is not constant (Roa-Ureta et al. 2021). One way to assess the seasonal mortality of short-lived species is to use a surplus production model in continuous time (SPiCT), which has already been tested for an assessment of cuttlefish (*Sepia officinalis*) in the English Channel, though these assessments are expected to show large uncertainty in model outputs due to larger inter-annual fluctuations, as well as possible non-equilibrium between stock production and environmental carrying capacity, and they may require long time series with seasonal inputs (Larivain 2020). Ultimately, environmental covariates which predict recruitment success may enable assessments based on stock-production to be strengthened for cephalopods.

Most EU-MS justify their lack of reporting by citing insufficient data availability or non-harmonized assessment methods. This is partly valid, and in many regions, the available datasets might be insufficient to perform an optimal assessment, especially as reliable data at species level is problematic (Lishchenko et al. 2021). However, an evaluation at taxonomic family level for some criteria may be sufficient, for example to describe substantial changes in the cephalopod fauna in European waters, as presented in various published studies (e.g. Chen et al. 2006). MS have conducted standardized surveys in the Northeast Atlantic, such as the standardized International Bottom Trawl Survey (IBTS) and the Beam Trawl Survey (BTS), for almost 30 years (ICES 2009, 2012a). Similarly, in the Mediterranean Sea, the standardized International Bottom Trawl Survey (MEDITS) has been performed over a long time period. Consequently, a large amount of data already exist and data collection is ongoing in many areas, including long-term data series for many cephalopod species, particularly those of commercial importance. In addition, fisheries landings data are available and can

be used to evaluate the level of fishing pressure, although taxonomic identification problems are even more obvious in these data. However, even if the quality of available datasets has to improve in future, survey and landings data sources are already used by ICES WGCEPH to describe the trends in landings, biomass and abundances, as shown in case studies presented in the present study. In addition to the development of particular metrics over time, such analyses can also be used to provide a certain degree of information about the status and trends of commercially exploited cephalopod groups. It should also be noted that, like many highly mobile marine organisms, due to their natural migratory behavior, cephalopods are not bounded inside the marine areas of a particular MS and, therefore, subregional monitoring programs are necessary for GES assessments. Indeed, Shephard et al. (2015) proposed that ICES working groups could help integrate the assessment of GES at a subregional level. We argue that, a non-harmonized assessment provides more information than no assessment, and will serve as initial baseline, which can be optimized in future. In particular, against the background of an increased interest in fisheries diversification to non-quota species, there is little fisheries management (with the exception of local management in small-scale cephalopod fisheries, e.g. for *O. vulgaris*) and almost no stock assessment in cephalopods, hence the need to include this species group in the MSFD framework is all the more appropriate.

For easier integration of cephalopods into an assessment within the MSFD, we have listed some indicators in Table 2. In addition to the indicators highlighted as serving existing criteria in the descriptors, additional 'potential' indicators are also listed, which might be used in future for a more comprehensive assessment of cephalopods within this framework (although these probably require further evaluation first). Moreover, there are some inconsistencies or difficulties within the indicators themselves, not all of which are due to the particular biology of cephalopods. The detection, assessment of abundance and distribution of non-indigenous or cryptogenic cephalopod species is important due to their potentially strong influences on native biodiversity. Indicators are available and immediately applicable (Table 2), but which species are included in the category NIS requires clarification. NIS (synonyms: alien, exotic, non-native, allochthonous) are species, subspecies or lower taxa which have been introduced intentionally or accidentally in new areas (past or present) by different anthropogenic vectors or natural means (Olenin et al. 2010). The European Commission itself named NIS as species introduced by human activities (Commission Decision 2017/848/EU). However, the exact meaning of anthropogenically influenced NIS could be interpreted in different ways. All cephalopod species are more or less powerful swimmers and highly mobile. Various squids



undertake diurnal vertical migrations which result in temporal and spatial fluctuations in their distribution and contribute significantly to the transfer of energy, resources and nutrients between deep water and shallow water systems. Therefore, deep-sea studies should include the assessment of cephalopods as well (Kazanidis et al. 2020). Horizontal movements are also common, due to long feeding and spawning migrations, which can likewise lead species into new areas. In addition, squid and some octopus species have planktonic paralarvae which may travel with ocean currents. Migrations are common and can be extensive in ommastrephids, to a lesser extent in loliginids, and only exist to some extent in cuttlefish. *I. coindetii* in the North Sea, for example, apparently represents a new spawning stock, which migrated into this area within the past 10 years (Oesterwind et al. 2020). Thus, this species entered the North Sea through natural pathways but was probably only able to colonize this new area due to global warming and the associated rise in temperature in the North Sea, which is influenced by human activity. Whether this is considered a natural or human-mediated range shift is somewhat ambiguous under present definitions.

In relation to fishing mortality for D3 we could illustrate that some data exist but those data are at a higher taxonomic level and, therefore, give only a rough idea about fishing mortality within the group. The unknown fishery status of certain commercially exploited species, particularly in some areas, as well as the unknown spatial structure of commercially fished stock units generally, needs to be addressed (Lishchenko et al. 2021) in future. Also, analysis of suggested descriptors by taxonomic family is clearly inappropriate in some cases, e.g. our analysis shows that commercially targeted species within a taxon show contrasting trends in fishing mortality and CPUE abundance relative to historical means (e.g. Tables 3, 4). Hence, in this case, assessing the group obscures contrasting trends within the group members.

Finally, achieving the major targets of the EU-MSFD requires knowledge of the food web and prediction of the trophic consequences of major changes to it (Heiskanen et al. 2016; Lynam and Mackinson 2015; Hastie et al. 2009). Different datasets can present different information in this regard. For example, survey-based TL indicators represent unbiased state changes at the level of the surveyed community while catch-based TL indicators show the pressure on the system (Heiskanen et al. 2016). Various food web models exist but cephalopods are not included in many cases, most likely due to knowledge gaps in knowledge of prey composition (Gasalla et al. 2010) and total cephalopod biomasses. However, Mackinson and Daskalov's (2007) North Sea ecosystem model, for example, lists cephalopod biomass, productivity, catchability and the role of cephalopods as prey for apex predators. Given that some of the current D4

indicators and criteria are borrowed from other descriptors, there should be no problem assessing cephalopods under this Descriptor.

As a first approach, it seems reasonable to calculate the existing criteria and to fill the indicator gaps with adapted fish indicators. While the calculation of various indicators is possible, they clearly need a sensible interpretation because they have not been adapted to the specific biology of cephalopods. The typical annual fluctuations in abundance and biomass, the sensitivity of the life cycle stages to environmental factors and the short life cycle of cephalopods complicate the interpretation of any changes. In a worst case, the substantial biological differences between cephalopods and finfish make some of the fish indicators unhelpful (Arkhipkin et al. 2021). Most importantly, annual or short-lived species show a seasonal cycle in terms of distribution, abundance and size composition. The seasonal variability, against a background of strong environmental forcing, must, therefore, be considered for any assessment with use of the corresponding indicators. Due to this high variability, it is difficult to determine or set baselines/threshold values/reference points. Possible strategies to overcome these difficulties are to identify a threshold range that encompasses the normal natural variations. Such an environmentally dependent threshold can be determined, for example, by modeling the threshold as a function of the relevant environmental parameters and recalculating the threshold, as needed, to assess GES. Since cephalopod populations are subject to natural fluctuations, it is advisable to focus on trends rather than status. If there is a sustained decreasing trend over a period of time, this is likely a sign of a negative impact. Responding to short-term declines, while they may be environmental, might be advisable in order to reduce fishing pressure on stocks with low biomasses in particular assessment areas.

In conclusion, there is a need for indicators to be adapted and additional indicators developed to evaluate the status of European cephalopods inside the MSFD. Certainly, better definitions of GES are needed and thresholds for GES are not yet available, when it comes to cephalopods. Furthermore, the species identification of cephalopods has already improved in some surveys but is still insufficient in some areas and for some families, and has to improve especially, in commercial landings (see Lishchenko et al. 2021) to fill the data gaps. The lack of routine assessment of almost all cephalopod stocks in European waters should also be addressed, for both MSFD assessment and fishery management (Lishchenko et al. 2021). Despite all of this, we advocate that, based on our case studies, a first integration of cephalopods into the MSFD framework is already possible and would provide useful preliminary information on their status.

## Conclusions and outlook

To ensure GES in Europe within the MSFD, cephalopods must be considered in the assessment, as it is the case for fish, marine mammals and marine birds. Besides CPUE-based indicators, other methods can already be applied using the existing data collected from commercial fisheries and research surveys incorporating cephalopods. The indicators, which can already be implemented in the framework of MSFD, are listed in bold in Table 2. For a more comprehensive cephalopod assessment in future, further potential indicators are listed (non-bold in Table 2). The total catch, total biomass and population abundance of cephalopods, for example, can be measured within the framework of ICES Trawl surveys (IBTS, BTS and MED-ITS) on the basis of CPUE (catch per tow, catch per time or catch per area) using standardized fishing gear. However, absolute abundance estimates depend on an appropriate survey design (e.g. locations, timing) and the fishing gear (Pierce et al. 1998), which is always size-selective, to a certain extent, as the entire size range of a species cannot be caught with one net size. Another consideration is that these are finfish surveys and cephalopods are only fished as bycatch. Nevertheless, the data collected in these surveys over decades, along with newly collected data in future, possibly involving tweaked data collection frameworks, can and should be used for an assessment using relative trend analyses of commercially fished cephalopod species. Furthermore, indicators can be developed on the basis of these data (including threshold values, e.g. following trends analysis) and implemented in the cephalopod assessment within the MSFD. Using current and long-term data, it should then be possible to define or determine GES of the corresponding cephalopod species (or, at the very minimum, for higher taxa, even if this has large disadvantages). Arkhipkin et al. (2021) have already discussed indicators for the assessment of cephalopods for management purpose in target fisheries and have shown the inutility and faulty advice arising from some methods. For instance, serious problems have been shown with using MSY to manage *Octopus* fisheries (Roa-Ureta et al. 2021). Nevertheless, it is important to start with some kind of assessment, for example, using the indicators and methods we have listed here. In addition to the adaptation of the potential indicators, which are similar to ICES fisheries assessment category three (ICES 2012b), where stocks are assessed on the basis of surveys or other trend indices (ICES 2015), more data are needed to increase the quality of the indicators. This includes, for example, the monitoring design, quality of species identification, stock discrimination (Lishchenko et al. 2021; Göpel et al. 2022; Sheerin et al. 2022), and survey timing in relation to the life cycle.

Further enhancement of cephalopod assessment methods is also required, along with improved understanding of their trophic importance and ‘Keystoneness’ within ecosystem models (e.g. Gasalla et al. 2010; Coll et al. 2013).

**Acknowledgements** BTCB thanks the German Foundation ‘Evangelisches Studienwerk Villigst’ for funding the doctorate in the context of which this review was written. In this context, she would also like to thank Dr. Stefan Forster from the marine biology department at the University of Rostock, who made the application for the scholarship possible. We also thank ICES WGCEPH, where elements and contents of this review were discussed. Finally, we thank the anonymous reviewers for their comments that improved the manuscript.

**Author contributions** BTCB and DO contributed to the study conception the design and wrote the first draft. AMP, AI, AM developed landings and survey tables, DO analysed length and geographic distribution for the case study. All authors reviewed and revised the first draft and gave final approval for submission. Finally, all authors contributed to the revision of the manuscript.

**Funding** Open Access funding enabled and organized by Projekt DEAL. This project was funded by the Begabtenförderungswerk Evangelisches Studienwerk Villigst e.V.

**Data availability** The datasets used for the case study are available in ICES DATRAS Database ([https://datras.ices.dk/Data\\_products/Download/Download\\_Data\\_public.aspx](https://datras.ices.dk/Data_products/Download/Download_Data_public.aspx)) or by the corresponding author on reasonable request.

## Declarations

**Conflict of interest** There is no conflict of interest.

**Ethics approval** A corresponding declaration that all applicable international, national and/or institutional guidelines for the sampling, care and experimental use of organisms for the study have been followed and all necessary permissions have been obtained is accordingly not necessary.

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## II. Stock discrimination of two European squids (*Illex coindetii*, *Loligo forbesii*) by statolith shape analysis

## Contribution letter Manuscript II

### Stock discrimination of two European squids (*Illex coindetii*, *Loligo forbesii*) by statolith shape analysis

Bobowski, B.T.C., Power, A.M., Burns, F., Carbonara, P., Cuccu, D., Donnalioia, M.,  
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This manuscript has been published in Fisheries Management and Ecology:

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The major contributions to this manuscript were done by me. I was responsible for the study design and methods. I was involved in the sample collection (North Sea), while samples from other studied areas were collected by the respective co-authors. The main part of the laboratory procession was performed by me, as well as the statistical analysis. I was responsible for the preparation and writing of the manuscript drafts as well as the revised versions from the co-authors, Journal reviewers and the editor.

Based on the CRediT taxonomy (Brand et al., 2015), my contributions to this manuscript were as follows:

- Conceptualization
- Methodology
- Validation
- Formal Analysis
- Investigation
- Data Curation
- Writing – Original Draft
- Writing – Review and Editing
- Visualization

#### Signatures of consent



Bianca Bobowski

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Brand, A., Allen, L., Altman, M., Hlava, M., Scott, J., 2015. Beyond authorship: Attribution, contribution, collaboration, and credit. Learn. Publ. 28, 151-155. <https://doi.org/10.1087/20150211>

## ARTICLE

# Stock discrimination of two European squids (*Illex coindetii*, *Loligo forbesii*) by statolith shape analysis

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## Funding information

Evangelisches Studienwerk Villigst; European Union through the European Maritime and Fisheries Fund (EMFF)

## Abstract

We evaluated the feasibility of classifying ommastrephid and loliginid squid species by statolith shape analysis into fisheries management units or stocks. Statoliths of *Illex coindetii* (Verany, 1839) and *Loligo forbesii* (Steenstrup, 1856) were studied from multiple areas of the North East Atlantic and Northern Mediterranean Sea during 2021–2022. *I. coindetii* and *L. forbesii* individuals were categorized into multiple stocks across the areas studied. Stocks migrating between fishing areas as well as multiple stocks in the same area, as identified for *L. forbesii* in this study, need to be considered for sustainable fisheries management.

## KEYWORDS

broadtail shortfin squid, Loliginidae, Myopsida, Oegopsida, Ommastrephidae, statolith shape analysis, stock structure, veined squid

## 1 | INTRODUCTION

Cephalopods are increasingly important in commercial fisheries due to changes (extending or expanding) in their global distribution (Chen et al., 2006; Kooij et al., 2016; Oesterwind et al., 2022) and because finfish populations are declining worldwide (Sabolić

et al., 2021). The landed biomass of squid, cuttlefish and octopus has steadily increased (Doubleday et al., 2016), from 2.7 million tonnes (Mt) in 1993 to a record high of 4.9 Mt in 2014 (FAO, 1998, 2022). Since 1980, East Asia and South America (Peru and Argentina) are main contributors to these landings (Ospina-Alvarez et al., 2022).

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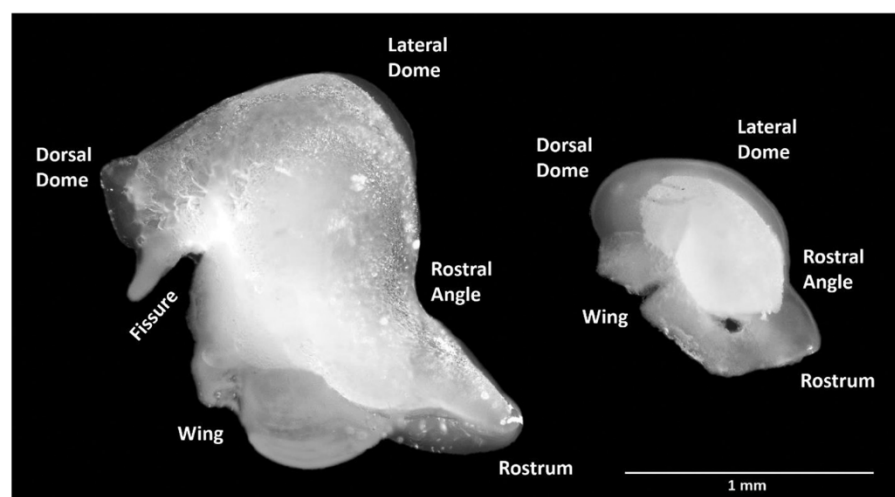
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The veined squid *Loligo forbesii* is important in European fisheries and one of the most commonly caught myopsid squid in the North East Atlantic fishery (Pierce et al., 2013). Although Scotland and France target squid fishing (Marchal & Vermard, 2023; Young et al., 2006), *L. forbesii* is usually caught as bycatch in trawl nets that primarily target demersal fish species (Pierce et al., 2013). European Atlantic annual landings of loliginid squid species (which include *L. forbesii*, *L. vulgaris*, *Alloteuthis media*, *A. subulata*) ranged 8000–12,000 tonnes in the last 5 years, with highest yields from the North Sea and English Channel (ICES, 2023). The broadtail shortfin squid, *Illex coindetii*, is another commercially important species found throughout the Atlantic Ocean that is among the most important commercial species of its genus (Martínez et al., 2002). In Europe, this species is predominantly caught as bycatch in demersal and pelagic trawl fisheries (Sánchez et al., 1998) with landings of 5000–7000 tonnes per year (since 2018), mostly by Spanish Fisheries (ICES, 2023). For effective fisheries management and assessment, understanding population dynamics and defining management units (stocks) is crucial (Rodhouse et al., 2014). Fish stocks encompass the living resources within a population used for fishing, typically implying a self-sustaining population isolated from other stocks of the same species (FAO Fishery Glossary, n.d.). To support effective management, statolith shape analysis is a promising method for defining different stocks of a species, including squids (Fang et al., 2014; Green et al., 2015; Sheerin et al., 2022).

Like other hard structures (beak, gladius or cuttlebone), statoliths can provide valuable information for species identification (Guo et al., 2021) and delineation of stocks (Green et al., 2015; Sheerin et al., 2022). Statoliths are paired calcified structures (Jackson, 1994) composed of aragonite crystals and organic matter (Liu et al., 2016). Several substances in a mineral structure inhibit, or favour, primary or secondary nucleation and control crystalline structure formation. These regulatory factors, in combination with the limited available space, lead to a certain mineral structure (Bettencourt & Guerra, 2000). Moreover, statolith size can vary among different cephalopod species and may be

influenced by their locomotion patterns. In slow-moving cephalopods, larger statoliths can be advantageous for detecting and responding to slow movements. Larger statoliths provide a greater inertial mass within the statocyst that potentially increases sensitivity to changes in acceleration and movement (Budelmann, 1988). Ommastrephid species, including *I. coindetii*, commonly known as flying squids or arrow squids, are highly active and agile swimmers (Muramatsu et al., 2013). In contrast, loliginid squids are slower swimmers (O'Dor et al., 1995). Consequently, the behaviour of *I. coindetii* is associated with significantly smaller statoliths than those of *L. forbesii* (Figure 1). Furthermore, bathymetry is assumed to affect behaviour and thereby influence statolith development and shape (Ching et al., 2022; Fang et al., 2018). Statolith growth, shape, and microstructure are also affected by temperature (Chemshirova et al., 2023; Moreno et al., 2012). In particular, the four main areas of statolith shape (dorsal dome, lateral dome, rostrum and wing; Figure 1) have distinguishing characteristics that can be used to differentiate among species within the same family (Fang & Chen, 2017). Furthermore, statolith shape can differentiate individuals from different stocks of the same species. For instance, a combination of genetic analysis and statolith shape analysis for *L. forbesii* revealed variations in statolith shape among individuals from different areas of the Irish Shelf (Sheerin et al., 2022), while microsatellites of *L. forbesii* from the North East Atlantic and Mediterranean Sea differed at a larger geographical scale (Göpel et al., 2022). Hence, genetic traits alone may not consistently differentiate stocks, but rather define isolated breeding groups. Populations may share spawning grounds (or partially exchange breeding individuals), but exist as distinct entities outside the spawning season, and consequently differ biologically, such as growth and maturity, and different seasonality patterns (Arvanitidis et al., 2002; Moreno et al., 2002). Therefore, a comprehensive understanding of the “stock concept” requires additional approaches (Begg et al., 1999) in some cases.

For sustainable use of fishery resources, management based on previously defined stocks is necessary. For example, along



**FIGURE 1** Photomicrograph of right-side statoliths from mature Q1 2021 North Sea *Loligo forbesii* (left) and Q3 2021 North Sea *Illex coindetii* (right) individuals with labelled key morphological features.





the northwest coast of Africa, the common octopus (*Octopus vulgaris*) is heavily exploited, with its three stocks already considered overfished (FAO, 2012). Management measures implemented to reduce fishing pressure on this species include fishing licences, closed seasons, minimum landing size limits and prohibitions on trawling in waters shallower than 20 m (FAO, 2012; Fernández-Rueda & García-Flórez, 2007). However, octopuses are benthic species that migrate little, so their stock definition should be more straightforward than squid, which are known for migratory behaviour over medium to long distances, which might contribute to inadequate stock discrimination by genetic analysis (Göpel et al., 2022). Cephalopod management measures implemented by non-EU countries, Australia, New Zealand and Iceland, include licenced commercial fishing vessels, assessment of key stocks, catch restrictions and input controls (Marchal et al., 2016). The Magnuson-Stevens Fishery Conservation and Management Act of the United States also requires states to meet standards for fishing effort and management (NOAA, 2007). However, this management is either based on stock definition by species distribution, including timing and occurrence of larval and juvenile stages (for *I. illecebrosus*; Dawe & Hendrickson, 1998), or genetic analysis of *L. pealeii* (Shaw et al., 2012). For European cephalopods, no such management measures have been implemented (Bobowski et al., 2023), and cephalopod stocks are not currently differentiated based on biological characteristics for fisheries management. Therefore, stock differentiation is needed for commercially used cephalopod species (Jereb et al., 2015; Lishchenko et al., 2021) to ensure a sustainable fishery.

We evaluated the potential of statolith shape analysis to delineate loliginid and ommastrephid squid stocks across their distribution in European waters. We hypothesized that shape analysis would provide a more detailed view of spatial and temporal discrimination of stocks than genetic analysis. Our findings would provide relevant information for fisheries management and contribute to the development of more sustainable fishing of European squids.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection and preparation

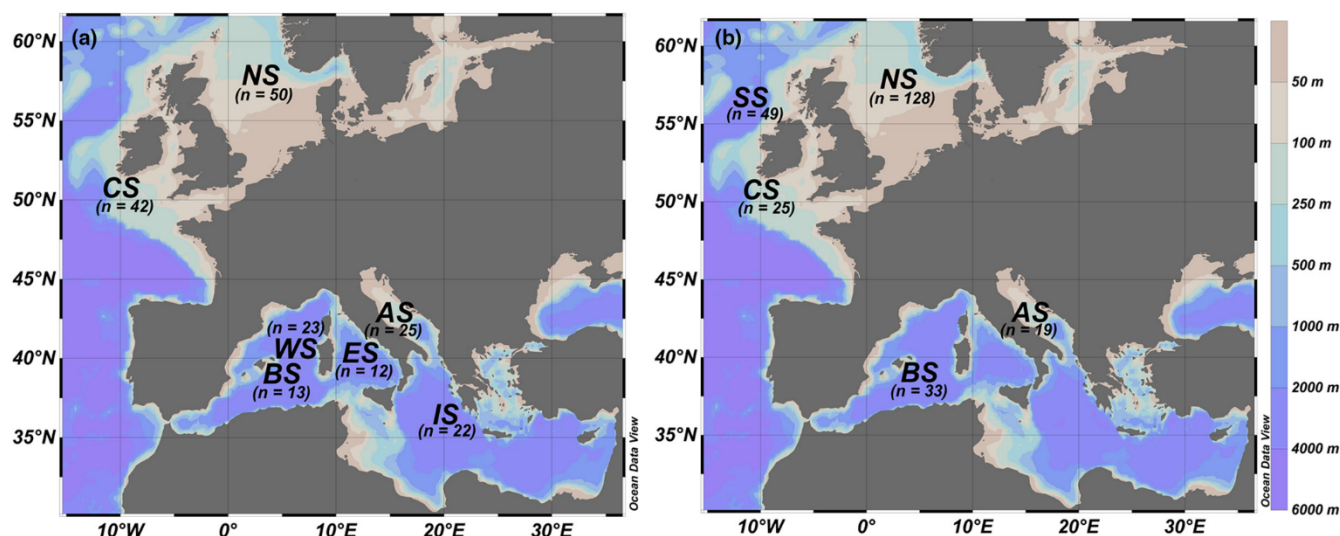
Squid samples were collected during ICES (International Council for the Exploration of the Sea) co-ordinated International Bottom Trawl Surveys (IBTS) and Mediterranean International Bottom Trawl Surveys (MEDITS), following standardized protocols (ICES, 2012, 2017). Samples were also collected during the Irish Anglerfish and Megrim Survey (IAMS) using standardized protocols (Reid et al., 2007) and the Scottish West Coast Groundfish Survey (SCOWCGFS) (ICES, 2020a). Samples were collected within a one-year period, but in different quarters (Q) encompassing eight areas in the North East Atlantic and Northern Mediterranean Sea, including North Sea, Sea West of Scotland, Celtic Sea, Balearic Sea, Sea

West of Sardinia, Sea East of Sardinia, Western Ionian Sea and South Adriatic Sea (Figure 2). Mediterranean samples were collected in Q3 2021, whereas North East Atlantic samples were collected in different year quarters (North Sea: Q1, Q3 2021; Sea West of Scotland: Q4 2021, Celtic Sea: Q1 2022).

Dorsal mantle length (DML, mm), total weight (W, 0.01 g), sex and maturity stage (ICES, 2022) of *I. coindetii* and *L. forbesii* were measured or classified, and statoliths were extracted from the statocyst. Statoliths were cleaned with distilled water, affixed to a microscope slide using a thermoplastic adhesive (Crystalbond™) and photographed under reflected light with an Axiocam 105 colour camera attached to an Olympus BX60 microscope and microscopy software Zen 2 blue edition (ver.2.0.0.0 by Carl Zeiss Microscopy GmbH, 2011). Images of dust particles and fibres were removed from photographs to ensure that statolith outlines were suitable for Fourier shape analysis. To avoid ontogenetic bias due to development of the statolith wing (similar to *I. illecebrosus*, see Morris & Aldrich, 1984), only statoliths from adult *I. coindetii* (Maturity stage 2a-3b (Table 1)) were included. Broken statoliths were excluded. Of 441 total right-side statoliths, 187 statoliths were from *I. coindetii* and 254 statoliths were from *L. forbesii*. Due to the availability of two different seasonal data sets (Q1 and Q3) for each species from the North Sea (Table 1), seasonal differences were tested for this area.

### 2.2 | Statistical analyses

Statolith shape (contour) of *I. coindetii* and *L. forbesii* was extracted from a bitmap image, described with Elliptic Fourier Descriptors (EFDs) and principal component analysis (PCA) of EFDs, using SHAPE (ver.1.3 by Iwata & Ukai, 2002) software. Statolith shapes were normalized based on the first harmonic ellipse that corresponds to the first Fourier approximation to the contour information with a standardized size and orientation of the contour in accordance with the size and alignment of the major axis of the ellipse (Iwata & Ukai, 2002). A scatterplot was used to represent statolith shape differences for each sampled area (Q1 and Q3 North Sea samples combined) in the plane of first two principal components (PC1 and PC2) using the ggplot2 package in RStudio (ver.4.2.2 by Posit PBC, 2022). Partitioning of variation among groups, based on principal components, was assessed through permutational multivariate analysis of variance (PERMANOVA) using the vegan package in RStudio. A Bonferroni correction was implemented on *p*-values of multiple comparisons to reduce Type I errors. If multivariate spatial dispersions of variances were not homogenous, an analysis of similarities (ANOSIM) was used instead of PERMANOVA. To test for correlation between observed and predicted classes, PCAs were classified and analysed in Statistica (ver.14.0.1.25 by TIBCO, 2020). Maps of individuals in areas were created in Ocean Data View version 5.6.5 (Schlitzer, 2023). Additionally, an MDS plot was created in Primer v6.1.13 to visualize proximity of maturity stage 1 of *L. forbesii* in different areas.



**FIGURE 2** Sampling areas of *Illex coindetii* (a) and *Loligo forbesii* (b), with number of individuals analysed (*n*), in the North Sea (NS), Sea West of Scotland (SS), Celtic Sea (CS), Balearic Sea (BS), Sea West of Sardinia (WS), Sea East of Sardinia (ES), Western Ionian Sea (IS), South Adriatic Sea (AS) in the North East Atlantic and Northern Mediterranean Sea during 2021–2022.

**TABLE 1** Biological characteristics of *Illex coindetii* and *Loligo forbesii* and quarters sampled for the North Sea in the North East Atlantic and Northern Mediterranean Sea during 2021–2022.

| Species             | Sex           | DML (mm) |      |                 | Weight (g) |      |                 | Individuals ( <i>n</i> ) | Maturity stage |
|---------------------|---------------|----------|------|-----------------|------------|------|-----------------|--------------------------|----------------|
|                     |               | Min.     | Max. | Mean $\pm$ SD   | Min.       | Max. | Mean $\pm$ SD   |                          |                |
| <i>I. coindetii</i> | All males     | 79       | 197  | 144 $\pm$ 24.85 | 13         | 232  | 104 $\pm$ 45.94 | 85                       |                |
|                     | All females   | 92       | 285  | 169 $\pm$ 33.37 | 16         | 549  | 132 $\pm$ 72.21 | 102                      |                |
|                     | Q1 NS         | 79       | 158  | 122 $\pm$ 23.37 | 13         | 158  | 46 $\pm$ 24.39  | 27                       | 2a, 2b         |
|                     | Q3 NS         | 108      | 224  | 162 $\pm$ 30.77 | 33         | 331  | 143 $\pm$ 67.45 | 23                       | 3a, 3b         |
| <i>L. forbesii</i>  | All juveniles | 78       | 225  | 152 $\pm$ 79.04 | 24         | 320  | 164 $\pm$ 152.3 | 4                        |                |
|                     | All males     | 72       | 485  | 195 $\pm$ 85.14 | 16         | 1820 | 256 $\pm$ 308.6 | 135                      |                |
|                     | All females   | 74       | 365  | 184 $\pm$ 55.42 | 20         | 1057 | 223 $\pm$ 174.3 | 115                      |                |
|                     | Q1 NS         | 107      | 356  | 214 $\pm$ 69.37 | 44         | 704  | 261 $\pm$ 166.7 | 58                       | 2a, 2b, 3a     |
|                     | Q3 NS         | 87       | 298  | 165 $\pm$ 49.81 | 25         | 595  | 164 $\pm$ 129.9 | 68                       | 2a, 2b, 3a     |

Abbreviations: DML, dorsal mantle length; NS, North Sea; Q1, Sampling period Quarter 1 (25.01.2021–26.02.2021); Q3, Sampling period Quarter 3 (19.07.2021–17.08.2021).

### 3 | RESULTS

#### 3.1 | Biological data

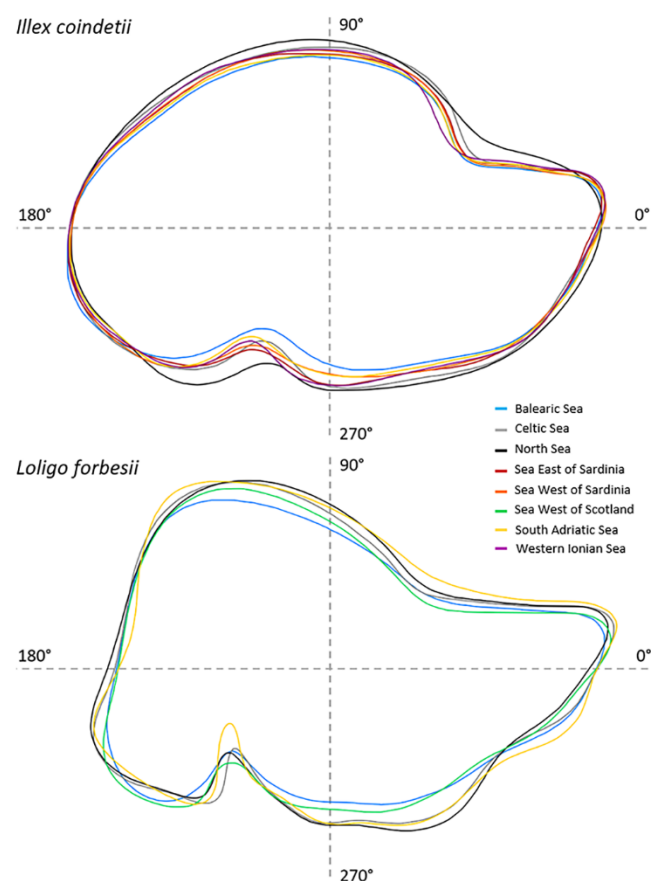
Mean dorsal mantle length (DML) of *I. coindetii* was shorter for males than females (Mann–Whitney *U*-test,  $p \leq 0.001$ , Table 1). Mean DML of mature *L. forbesii* males did not differ from mature females (Mann–Whitney *U*-test,  $p = 0.825$ ). For *I. coindetii* from the North Sea, mean DML was shorter (*t*-test,  $p \leq 0.001$ ), mean wet weight was lighter (Mann–Whitney *U*-test,  $p \leq 0.001$ ), and mean maturity stage was lower (Mann–Whitney *U*-test,  $p \leq 0.001$ ) in Q1 (including developing and maturing individuals) than in Q3 (including mature and spent individuals). Conversely, for *L. forbesii* from the North Sea, mean DML was longer (*t*-test,  $p \leq 0.001$ ), mean wet weight was heavier (Mann–Whitney *U*-test,  $p \leq 0.001$ ), and mean maturity stage was higher

(Mann–Whitney *U*-test,  $p \leq 0.001$ ) in Q1. However, both quarters included the same maturity categories: developing, maturing and mature individuals (Table 1).

#### 3.2 | Statolith shape differences

For *I. coindetii*, mean statolith shape from the North Sea (Q1 and Q3 combined) differed more than from other regions (Mediterranean areas: Q3 2021, CS: Q1 2022). Compared to other areas, statoliths were more rounded, with a prominent wing and wide rostral angle (Figure 3). The first two principal components explained 63.61% (PC1: 38.54%; PC2: 25.07%) of statolith shape variation. Statolith shape grouped together in geographical distance-dependent groups (Figure 4). To achieve homogenous multivariate dispersion





**FIGURE 3** Mean statolith shapes of *Illex coindetii* and *Loligo forbesii* (all maturity stages and sexes) from the Balearic Sea (blue), Celtic Sea (grey), North Sea (Q1 & Q3 combined; black), Sea East of Sardinia (red), Sea West of Sardinia (orange), Sea West of Scotland (green), South Adriatic Sea (yellow), Western Ionian Sea (purple) of the North East Atlantic and Northern Mediterranean Sea during 2021–2022. Intersection of coordinate lines corresponds to the statolith centroid.

in space among areas, North Sea samples were excluded from PERMANOVA analysis (homogeneity when North Sea excluded:  $p=0.4175$ ,  $F_{5,131}=1.005$ ). Excluding the North Sea, statolith shape differed among all areas and the Balearic Sea, except South Adriatic Sea, and between the Celtic Sea and all areas, except the Sea East of Sardinia. Furthermore, Sardinian Seas and South Adriatic Sea did not differ in statolith shape; similarly, statolith shape from the North Sea did not differ from those from the Sea East of Sardinia (Table 2).

For *L. forbesii*, mean statolith shape from the North Sea (Q1 and Q3 combined) had a more pronounced dorsal dome and wing structure than from other areas (Mediterranean areas: Q3 2021, SS: Q4 2021, CS: Q1 2022) and were less pronounced from the Balearic Sea (Figure 3). PC1 (50.28%) and PC2 (12.77%) accounted for 63.05% of statolith shape variation (Figure 4). Statolith shape of individuals from North Sea and Celtic Sea was similar, whereas statolith shape of individuals from Scottish waters differed from these regions. Statolith shape of individuals from the Balearic Islands (part of the Mediterranean) was closer to that of Scottish individuals

and differed from those in the Southern Adriatic Sea. Due to non-homogenous multivariate dispersion in space of individual areas, a one-way ANOSIM was used for *L. forbesii* (Table 2). Statolith shape differed among all areas, except between the North Sea and Celtic Sea (Table S1).

### 3.3 | Area classification

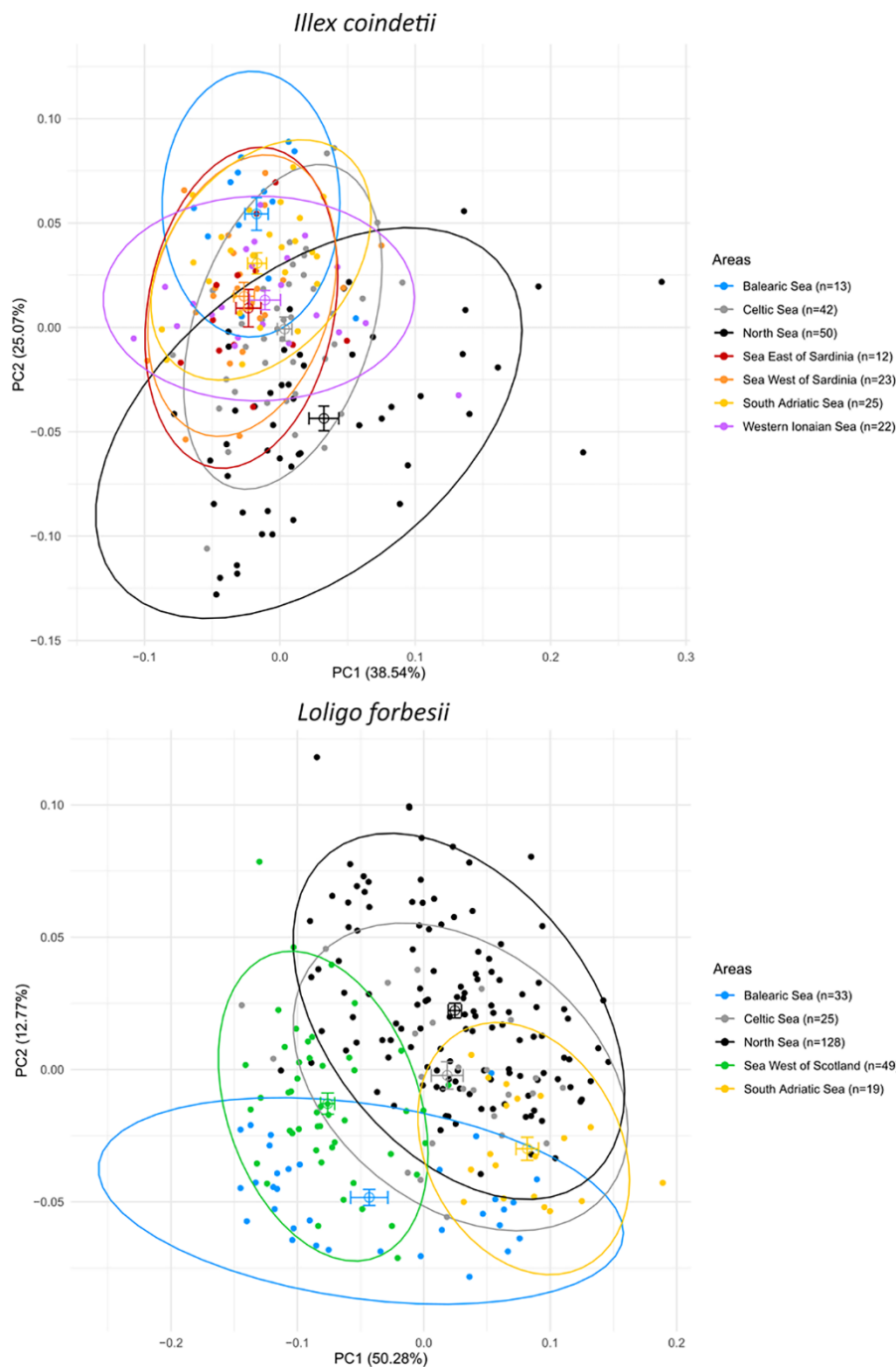
Classification of *I. coindetii* to sample areas based on statolith shape was highly accurate, with >80% of specimens from the North East Atlantic and >50% overall from the Mediterranean correctly classified (Table 3). Within the Mediterranean, however, only ~33% of individuals from the Sea East of Sardinia were correctly classified. Classification of *L. forbesii* to sample areas based on statolith shape was also highly accurate, with 80%–90% of specimens classified to correct areas (Table 3), although only 44% from the Celtic Sea were correctly classified.

Within the North Sea, statolith shape did not differ significantly between FAO areas (<https://www.fao.org/fishery/en/area/27/en>) in both *I. coindetii* ( $F_{1,48}=0.2108$ ,  $p=0.64852$ ) and *L. forbesii* ( $F_{2,125}=0.5193$ ,  $p=0.5962$ ) (Table S8). However, statolith shape differed significantly between samples from Q1 and Q3 for both species (*I. coindetii* ANOSIM:  $p=0.006$ ; *L. forbesii* PERMANOVA;  $p=0.006$ ).

## 4 | DISCUSSION

We found that statolith shape variation of *I. coindetii* and *L. forbesii* distinguished among geographically and seasonally separated groups within the North East Atlantic and the Mediterranean, which we interpreted as stocks, in contrast to stock identification studies based on genetic variation (ICES, 2020b) that only revealed differences at a larger geographical scale for *L. forbesii* in European waters due to high migration rate (Göpel et al., 2022; Sheerin et al., 2022). Genetic stock discrimination may be limited for highly mobile species with large gene flow, whereas the present and other studies illustrate the use of statolith shape analysis to distinguish stocks of squid species on a smaller geographic scale (shape markers may indicate ecologically separate groups of species with similar migration patterns; Fang & Chen, 2017; Green et al., 2015; Guo et al., 2021; Sheerin et al., 2022).

High accuracy of correct area classification for North Sea and Celtic Sea and differences in statolith shape between areas, in the present study, suggests the presence of at least two distinct *I. coindetii* stocks in the North East Atlantic. Previously, single adult individuals without any evidence of reproduction occurred in the North Sea, which were thought to have migrated into the North Sea from adjacent waters (Oesterwind et al., 2020). The current reproductive success in the North Sea (first observed in 2016) probably results from rising temperatures and prevailing currents (Barrett et al., 2021; Oesterwind et al., 2022) and has led to an independent stock. The



**FIGURE 4** Principal components one and two of *Illex coindetii* and *Loligo forbesii* statolith shape from the Balearic Sea (blue), Celtic Sea (grey), North Sea (Q1 & Q3 combined; black), Sea East of Sardinia (red), Sea West of Sardinia (orange), Sea West of Scotland (green), South Adriatic Sea (yellow), and Western Ionian Sea (purple) of the North East Atlantic and Northern Mediterranean Sea during 2021–2022. Open circles represent the mean ( $\pm$  standard error) and ellipses encompass 95% of specimens from each area.

North Sea appears to be the northernmost breeding area, with main spawning grounds in the Central and Northern North Sea (Barrett et al., 2021), while floating egg masses have been observed in the Norwegian Channel (Ringvold et al., 2021). The bathymetry and hydrology of the North Sea, as a shelf sea with an average depth of 90m and a relatively narrow connection to the Atlantic Ocean (ESaTDOR, 2013), could prevent emigration from this area by passive drifting egg spheres and hatchlings of *I. coindetii*. Despite our observed differences in *I. coindetii* statolith shape between two sampling periods within the North Sea, this species has only one spawning season in the area (Oesterwind et al., 2020) and statolith shape did not differ among maturity stages (Q1: 2a, 2b; Q3: 3a, 3b) (Table S4).

Therefore, we assume only one stock is present in this region. Factors responsible for statolith shape differences between sampling quarters need to be studied in the future. Based on genetic homogeneity, Pierce et al. (2010) concluded that only one stock of *I. coindetii* occurs throughout the North East Atlantic, which could be interpreted as a single breeding stock with ecologically distinct sub-units (e.g., due to separate migration patterns). However, as mentioned above, a new stock of *I. coindetii* reproduced since at least 2016 in the North Sea (Barrett et al., 2021; Oesterwind et al., 2020). Hence, the North Sea stock was likely established since the study by Pierce et al. (2010), primarily due to the previously limited number of *I. coindetii* individuals found in this region (Oesterwind et al., 2010, 2015).



**TABLE 2** Pairwise comparisons of *Illex coindetii* and *Loligo forbesii* statolith shape from the Balearic Sea, Celtic Sea, North Sea (Q1 & Q3 combined), Sea East of Sardinia, Sea West of Sardinia, Sea West of Scotland, South Adriatic Sea, and Western Ionian Sea in the North East Atlantic and Northern Mediterranean Sea during 2021–2022 using permutation MANOVAs, respectively one-way ANOSIM on Euclidian distance matrix.

|                                  |                   | Balearic Sea | Celtic Sea | Sea east of Sardinia | Sea west of Sardinia | South Adriatic Sea   |                    |
|----------------------------------|-------------------|--------------|------------|----------------------|----------------------|----------------------|--------------------|
| <i>Illex coindetii</i><br>MANOVA | Celtic Sea        | 0.001        | –          | –                    | –                    | –                    |                    |
|                                  | S. E. o. Sardinia | 0.033        | 0.601      | –                    | –                    | –                    |                    |
|                                  | S. W. o. Sardinia | 0.049        | 0.010      | 1.000                | –                    | –                    |                    |
|                                  | S. Adriatic Sea   | 0.845        | 0.001      | 1.000                | 1.000                | –                    |                    |
|                                  | W. Ionian Sea     | 0.025        | 0.042      | 1.000                | 0.735                | 1.000                |                    |
|                                  |                   | Balearic Sea | Celtic Sea | North Sea            | Sea East of Sardinia | Sea West of Sardinia | South Adriatic Sea |
| <i>Illex coindetii</i><br>ANOSIM | Celtic Sea        | 0.001        | –          | –                    | –                    | –                    | –                  |
|                                  | North Sea         | 0.004        | 0.001      | –                    | –                    | –                    | –                  |
|                                  | S. E. o. Sardinia | 0.005        | 0.272      | 0.287                | –                    | –                    | –                  |
|                                  | S. W. o. Sardinia | 0.027        | 0.021      | 0.011                | 0.434                | –                    | –                  |
|                                  | S. Adriatic Sea   | 0.238        | 0.001      | 0.001                | 0.224                | 0.178                | –                  |
|                                  | W. Ionian Sea     | 0.009        | 0.003      | 0.003                | 0.600                | 0.240                | 0.118              |
|                                  |                   | Balearic Sea | Celtic Sea | North Sea            | Sea West of Scotland |                      |                    |
| <i>Loligo forbesii</i><br>ANOSIM | Celtic Sea        | 0.001        | –          | –                    | –                    |                      |                    |
|                                  | North Sea         | 0.001        | 0.654      | –                    | –                    |                      |                    |
|                                  | S. W. o. Scotland | 0.001        | 0.001      | 0.001                | –                    |                      |                    |
|                                  | S. Adriatic Sea   | 0.001        | 0.001      | 0.027                | 0.001                |                      |                    |

Note: Significant comparisons ( $p < 0.05$ , adjusted with Bonferroni method) are shown in bold.

The Mediterranean *I. coindetii* population structure is described as a middle-course situation, involving a combination of C-type (multiple cohorts or microcohorts) and D-type (multiple, heavily overlapping cohorts) population structures (Sánchez et al., 1998). This conclusion corresponds with our findings, as the stock interpretation by statolith shape analysis in this area was more complex than in the North East Atlantic. The complexity was because of a high similarity of statoliths from southern Italy (Sea East of Sardinia, Western Ionian Sea, South Adriatic Sea) and lower classification accuracy, which collectively suggested a substantial exchange of individuals belonging to the species *I. coindetii* and the absence of distinct stocks in this part of the Mediterranean Sea. Furthermore, statolith shape from the Balearic Sea differed from all other areas, except the Southern Adriatic Sea, while the Celtic Sea and North Sea did not differ from the Sea East of Sardinia. We expected to find different stocks in geographically distant or topographically separated areas, such as the Balearic Sea and the Adriatic Sea (assuming different stocks in these areas). Unlike the Fourier method, which is based on cosine and sine functions, wavelets are well-suited for approximating data with sharp discontinuities (Graps, 1995; Libungan & Pálsson, 2015), and therefore future analysis using wavelets might detect differences between these regions. This shows the limitation of (Fourier) shape analysis and a need of further studies on determinants of statolith shape. However, we assume that the Mediterranean Sea harbours a separate *I. coindetii* stock in the Balearic Sea area, probably related to geomorphology and habitat characteristics (Quetglas et al., 2012), which would benefit from further research.

In *L. forbesii*, we found that statolith shape differed between the Balearic Sea and Adriatic Sea, whereas microsatellite analysis only revealed small differences between individuals from these two areas (Göpel et al., 2022). In general, due to a high rate of correct classification and differences in statolith shape of *L. forbesii* among most areas, we conclude that these regions harboured separate stocks of this species, at least at the times of year sampled. *L. forbesii* has varying spawning seasons within the North East Atlantic, encompassing Scottish waters, Celtic Sea, English Channel, Faroe Bank and North Sea (Gaard, 1987; Holme, 1974; Laptikhovsky et al., 2022; Oesterwind et al., 2022; Pierce et al., 1994, 2005). However, we found relatively high misclassification of *L. forbesii* samples from the Celtic Sea, and that statolith shape only failed to differ significantly between samples from the Celtic Sea and the North Sea, which suggests connectivity between the North Sea and Celtic Sea, a confirmed spawning area for *L. forbesii* (Laptikhovsky et al., 2022) connected by migration (Holme, 1974). Juvenile *L. forbesii*, hatched near the western end of the English Channel, migrate eastward to foraging areas in the eastern Channel and southern North Sea, and migrate back to the Celtic spawning areas in autumn (Holme, 1974). The timing of eastward migration within the English Channel likely depends on water temperature and can vary up to 150 days (Sims et al., 2001). Consequently, we conclude that statolith shape develops during foraging in the North Sea. Alternatively, lower classification accuracy in the Celtic Sea in our study may have been caused by small sample sizes from this area. In addition to the exchange between



TABLE 3 Classification matrix for *Illex coindetii* and *Loligo forbesii* from the Balearic Sea, Celtic Sea, North Sea (Q1 & Q3 combined), Sea East of Sardinia, Sea West of Sardinia, Sea West of Scotland, South Adriatic Sea, and Western Ionian Sea in the North East Atlantic and Northern Mediterranean Sea during 2021–2022.

| <i>Illex coindetii</i> | Observed area     | Predicted area |            |           |                    |                    |                 |               | Classification success rate (%) |
|------------------------|-------------------|----------------|------------|-----------|--------------------|--------------------|-----------------|---------------|---------------------------------|
|                        |                   | Balearic Sea   | Celtic Sea | North Sea | Sea E. of Sardinia | Sea W. of Sardinia | S. Adriatic Sea | W. Ionian Sea |                                 |
|                        | Balearic Sea      | 7              | 2          | 0         | 1                  | 0                  | 1               | 2             | 53.85                           |
|                        | Celtic Sea        | 0              | 34         | 5         | 1                  | 1                  | 0               | 1             | 80.95                           |
|                        | North Sea         | 0              | 2          | 44        | 0                  | 0                  | 2               | 2             | 88.00                           |
|                        | S. E. o. Sardinia | 0              | 1          | 2         | 4                  | 1                  | 0               | 4             | 33.33                           |
|                        | S. W. o. Sardinia | 2              | 6          | 1         | 0                  | 13                 | 0               | 1             | 56.52                           |
|                        | S. Adriatic Sea   | 1              | 3          | 0         | 0                  | 2                  | 18              | 1             | 72.00                           |
|                        | W. Ionian Sea     | 0              | 3          | 1         | 0                  | 0                  | 6               | 12            | 54.55                           |

| <i>Loligo forbesii</i> | Observed area     | Predicted area |            |           |                    |                 |  |  | Classification success rate (%) |
|------------------------|-------------------|----------------|------------|-----------|--------------------|-----------------|--|--|---------------------------------|
|                        |                   | Balearic Sea   | Celtic Sea | North Sea | Sea W. of Scotland | S. Adriatic Sea |  |  |                                 |
|                        | Balearic Sea      | 29             | 0          | 0         | 1                  | 3               |  |  | 87.88                           |
|                        | Celtic Sea        | 5              | 11         | 8         | 0                  | 1               |  |  | 44.00                           |
|                        | North Sea         | 1              | 7          | 112       | 6                  | 2               |  |  | 87.50                           |
|                        | S. W. o. Scotland | 1              | 3          | 3         | 40                 | 2               |  |  | 81.63                           |
|                        | S. Adriatic Sea   | 0              | 0          | 2         | 0                  | 17              |  |  | 89.47                           |

Note: Predicted versus observed area classes in individual numbers and correct classification are also provided as percentage. Correct classified samples are shown in bold.



North Sea/Celtic Sea, immigration from northern and western Scottish waters into the North Sea cannot be excluded, as indicated by migration patterns (Viana et al., 2009) and temporal patterns of egg mass, paralarvae, and mature female movements (Laptikhovsky et al., 2022). However, *L. forbesii* spawning also occurs in warmer months in the northern North Sea, so a “native” *L. forbesii* stock also occurs in the area (Laptikhovsky et al., 2022).

The occurrence of different *L. forbesii* stocks within the North Sea is also supported by different size classes and breeding populations. Pierce et al. (2005) described two different breeding populations in the North Sea, while Kristensen (1959) identified two size groups and speculated about disparate spawning areas – one in the English Channel and another in the North. However, Laptikhovsky et al. (2022) were not able to identify any evidence of spawning activity of *L. forbesii* in the English Channel so that it seems that Kristensen's (1959) former assumed southern spawning area is the Celtic Sea nowadays. Again, the combination of significant differences between the statolith shape of Q1 and Q3 North Sea individuals and the occurrence of different size classes infers that the North Sea harbours at least two temporally separated stocks of *L. forbesii*, namely individuals hatched in the Celtic Sea and North Sea spawners and very likely Scottish individuals (but the latter were not identified in this study). Furthermore, the slightly higher misclassification within the North Sea in Q1 (Tables S9 and S10) indicates that the stock mixture in Q1 is higher compared to Q3. It is not yet possible to quantify the mix of individuals spawning in the (northern) North Sea and those of Celtic and (presumably) Scottish west/north coast origin which use the North Sea as a foraging area.

Although *L. forbesii*, like *I. coindetii*, differed in statolith shape between sampling periods in the North Sea, *L. forbesii* size distribution and potential immigration from Scottish and Celtic waters clearly demonstrates that shape analysis itself provides only coarse stock identification that is not singularly interpretable without a multi-method approach (Sheerin et al., 2022). Rather, we propose that a combination of shape analysis and biological data (e.g., maturity, size and age) is sufficient for stock discrimination. The many factors that influence statolith shape are not completely understood or identified, which complicates interpretation of the results of shape analysis (Arkhipkin, 2005; Sheerin et al., 2022). For instance, some squid species are sexually dimorphic, including *I. coindetii* and *L. forbesii* (Jereb & Roper, 2010). Furthermore, some squids migrate separately in sex-specific schools (Kubodera et al., 1983; Sauer et al., 1992). Neither species are known to migrate separately by sex like other species (e.g., *Todarodes sagittatus*, Jereb et al., 2015; *Doryteuthis gahi*, Arkhipkin & Middleton, 2002), but sexually mature female *I. coindetii* were significantly larger than males, whereas male and female length in *L. forbesii* were similar in our study. Because of these biological differences, we tested for sex-specific differences in statolith shape (Tables S2 and S3). However, results indicated that statolith shape did not differ significantly between males and females.

We did find that statolith shape of *L. forbesii* differed with maturity stage (size or age) in Balearic and Scottish waters, which was

consistent with other research showing maturity stage (a proxy for age or size) of squid influencing statolith shape (Wang et al., 2021). Several factors may contribute to shape differences within a species (Arkhipkin & Bizikov, 2000). For example, we found that statoliths of earlier maturity stages (young small squid) differed in shape from later maturity stages (older larger squid), because statoliths from squid of earlier maturity stages were more similar than statoliths from squid of later maturity stages (Tables S6 and S7 and Figure S1). Potentially, statolith shape characteristics need time to develop (Sheerin et al., 2022). Furthermore, diet of *L. forbesii* from the Balearic Sea varied with body size and season, with larger squid migrating into offshore regions, potentially indicating that statolith shape differences between maturity stages can be affected by habitat type or different prey species (Valls et al., 2015). However, even if these differences were less pronounced than shape variations among regions, we recommend to compare only similar life stages for future stock identification by statolith shape analysis.

## 5 | CONCLUSION

Our findings indicate that statolith shape analysis is useful for stock discrimination on a large geographical scale. While extending the applicability of this method to stock discrimination of ommastrephid squids (*I. coindetii*) with less differentiated statolith forms, we also showed the limitation of this method and recommend combining statolith shape analysis with biological or genetic analysis for further stock discrimination. We found the seasonal occurrence of at least two different stocks of *L. forbesii* in the North Sea, i.e., Celtic Sea and North Sea spawners, and likely also some immigration from Scottish waters. Thus, our results confirm the presence of multiple stocks in the North Sea, which was previously inferred by the presence of different size classes (Gaard, 1987; Holme, 1974; Laptikhovsky et al., 2022; Oesterwind et al., 2022; Pierce et al., 1994, 2005). These results illustrate the advantage of including length distribution in fisheries management. Mixing of stocks from other areas through immigration and emigration (Celtic Sea and presumably Scottish waters) should be considered in sustainable fisheries management, after further investigation. A multi-method approach can be useful for cephalopod stock separation due to the extreme environmental responsiveness in life history traits within this taxon (Sheerin et al., 2022). An improved knowledge of the factors that influence statolith shape would improve our understanding of migration of stocks and thus connectivity of stocks from different areas.

## ACKNOWLEDGEMENTS

BTCB thanks Dr Anne Sell and the technical assistants Samira Peter, Anne Georgi, Britta Rotzoll, Cornelia Albrecht and Thomas Hogh for their support and supervision in the laboratory. She also thanks Fedor Lishchenko for the introduction of SHAPE and Dr Christina Henseler as well as Dr Juan Santos for helping with RStudio queries. Furthermore, thanks to the crew and scientific officers of the RV *Dana* and RV *Celtic Explorer* who supplied samples, especially Susan



Maersk Lusseau and Dr Eoghan Kelly, as well as Lisa Hendrickson for some literature recommendations. Last but not least we thank two anonymous reviewers and Michael J Hansen for their comments which have improved the manuscript. Open Access funding enabled and organized by Projekt DEAL.

## FUNDING INFORMATION

This project was funded by the Begabtenförderungswerk Evangelisches Studienwerk Villigst e.V. Open-access funding enabled and organized by Projekt DEAL. The MEDITS surveys are co-funded by the European Union through the European Maritime and Fisheries Fund (EMFF) within the National Programme of collection, management and use of data in the fisheries sector and support for scientific advice regarding the Common Fisheries Policy.

## CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ETHICS STATEMENT

There is no approval necessary.

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## SUPPORTING INFORMATION

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**How to cite this article:** Bobowski, B.T.C., Power, A.M., Burns, F., Carbonara, P., Cuccu, D., Donnaloia, M. et al. (2024) Stock discrimination of two European squids (*Illex coindetii*, *Loligo forbesii*) by statolith shape analysis. *Fisheries Management and Ecology*, 31, e12689. Available from: <https://doi.org/10.1111/fme.12689>



### III. Classification of European *Illex coindetii* and *Loligo forbesii* Squid Stocks by Trace Element Analysis of Statoliths

## Contribution letter Manuscript III

### Classification of European *Illex coindetii* and *Loligo forbesii* Squid Stocks by Trace Element Analysis of Statoliths

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This manuscript has been published in Fisheries Management and Ecology:

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The major contributions to this manuscript were done by me. I was responsible for the study design and methods. I was involved in the sample collection (North Sea), while samples from other studied areas were collected by the respective co-authors. The main part of the laboratory procession was performed by me, as well as the statistical analysis. I was responsible for the preparation and writing of the manuscript drafts as well as the revised versions from the co-authors, Journal reviewers and the Editor.

Based on the CRediT taxonomy (Brand et al., 2015), my contributions to this manuscript were as follows:

- Conceptualization
- Methodology
- Validation
- Formal Analysis
- Investigation
- Data Curation
- Writing – Original Draft
- Writing – Review and Editing
- Visualization

#### Signatures of consent



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Brand, A., Allen, L., Altman, M., Hlava, M., Scott, J., 2015. Beyond authorship: Attribution, contribution, collaboration, and credit. Learn. Publ. 28, 151-155. <https://doi.org/10.1087/2015021>

## ARTICLE OPEN ACCESS

# Classification of European *Illex coindetii* and *Loligo forbesii* Squid Stocks by Trace Element Analysis of Statoliths

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**Received:** 7 October 2024 | **Revised:** 11 August 2025 | **Accepted:** 21 August 2025

**Funding:** This project was funded by Evangelisches Studienwerk Villigst (Protestant Academic Foundation). The MEDITS surveys are co-funded by the European Union through the European Maritime and Fisheries Fund (EMFF) within the National Programme of collection, management and use of data in the fisheries sector and support for scientific advice regarding the Common Fisheries Policy. Open Access funding was enabled and organized by Projekt DEAL.

**Keywords:** Loliginidae | Myopsida | Oegopsida | Ommastrephidae | statolith microchemistry analysis | stock structure

## ABSTRACT

We investigated whether statolith microchemistry analysis could classify ommastrephid and loliginid squid species into fishery stocks. Statolith nucleus (early life stage) and edge (life stage at time before catch) of *Illex coindetii* (Verany, 1839) and *Loligo forbesii* (Steenstrup, 1856) from various areas of the North East Atlantic Ocean and Mediterranean Sea during 2021–2022 were analysed using laser ablation ICP-MS to measure values of 11 trace elements (Na, Mg, Mn, Fe Co, Cu, Zn, Sr., Ba, Pb and U) next to calcium. Four stocks were categorised for *I. coindetii* and five stocks for *L. forbesii* amongst sample areas. Common hatching areas and migration into distinct fishing regions were also identified. Our results provide substantial information to sustainably manage fisheries for both species in European waters by providing stock definitions, spawning periods and migration patterns.

## 1 | Introduction

Cephalopods have gained importance in European commercial fisheries due to abundance shifts and range expansions (Chen et al. 2006; van der Kooij et al. 2016; Oesterwind et al. 2022), whilst European finfish populations and fishery harvests have declined (Sabolić et al. 2021). Due to their rapid growth, high productive rates and adaptability, cephalopods are likely able to exploit ecological opportunities arising from the overfishing of commercial finfish stocks (Mangold and Boletzky 1988; Caddy and Rodhouse 1998). These features, along with their high

market price, make cephalopods valuable harvest targets (Vaz-Pires et al. 2004). However, little is known about the stock structure of commercially important and highly mobile squid species in Europe (Pierce et al. 2025).

Two squid species that migrate extensively are the broadtail shortfin squid *Illex coindetii* and the veined squid *Loligo forbesii*. *I. coindetii*, an oceanic and neritic species, is widely distributed in the Atlantic and Mediterranean and plays an essential role in oceanic ecosystems by acting as an ‘ecosystem accelerator’ that converts food into energy-rich biomass which sustains higher

<sup>†</sup>Klaus Peter Jochum, deceased 2024.

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trophic levels (González and Guerra 2013). Genetic analyses of *I. coindetii* from the Iberian Atlantic and the central Mediterranean revealed a homogenous population structure, albeit with moderate temporal and geographical variation (Martínez et al. 2005). However, statolith shape analysis revealed multiple stocks for *I. coindetii* in the North East Atlantic Ocean and Mediterranean Sea (Bobowski et al. 2024). *L. forbesii*, a demersal squid species that typically migrates ontogenetically, with juveniles moving offshore to feed and adults moving inshore to spawn, occurs primarily over the continental shelf (Pierce et al. 2013). However, around the Balearic Islands, adults have also been observed moving offshore to feed (Valls et al. 2015). Despite a lack of evidence for distinct stocks in European waters from morphometric and genetic analyses, isolated populations occur around the Azores and Rockall Bank (Shaw et al. 1999; Göpel et al. 2022; Sheerin et al. 2022). Statolith shape analyses, in contrast, indicated the presence of at least three stocks in the North East Atlantic Ocean and two stocks in the Mediterranean Sea (Bobowski et al. 2024).

Although squid species are currently not managed in Europe (Bobowski et al. 2023; Pierce et al. 2025), both species are important in European fisheries (Martínez et al. 2002; Pierce et al. 2013), especially as bycatch in trawls (Sánchez et al. 1998; Pierce et al. 2013). Furthermore, cephalopod fishing in Europe, especially for cuttlefish and squid, is increasing in the North East Atlantic Ocean. For example, Danish cephalopod landings from the northern North Sea and Skagerrak increased from 50 t in 2011–2014 to over 430 t in 2020 (Vinther 2021). Although cuttlefish and octopus are main contributors to cephalopod landings in Europe, landings of longfin squids (*L. forbesii*, *L. vulgaris*, *Alloteuthis* spp.) increased after 2017, with peak landings in 2022 (ICES 2023). In parallel, landings of short-finned squids (*I. coindetii*, *Todarodes sagittatus* and *Todaropsis eblanae*) increased significantly in 2021, with peak landings in 2022 (ICES 2023). Understanding population dynamics and defining management units or stocks (FAO Fishery Glossary, n.d.) is crucial for sustainable fishery assessment and management (Rodhouse et al. 2014).

Statolith microchemistry can be a valuable tool for defining stocks of cephalopod species and for identifying migration pathways or hatching grounds, to support their sustainable management (Zumholz, Hansteen, et al. 2007; Liu et al. 2015, 2016; Avigliano et al. 2020; Han et al. 2022). Statoliths are one of the few hard structures in cephalopods that consist mainly of aragonite, a polymorph of calcium carbonate (Dilly 1976). In addition to their use for age estimation based on statolith increments consisting of pairs of light (aragonite) and dark rings (organic material) that are laid down over time (usually daily) (Hanlon et al. 1989; Jackson 1994; Perez et al. 2006), statoliths can be used for estimating growth rates (Jackson and Choat 1992). Another important application of statoliths is stock discrimination based on statolith shape (Sheerin et al. 2022; Bobowski et al. 2024) or microchemistry analysis of trace elements in statoliths (Green et al. 2015). During cephalopod ontogeny, trace elements are absorbed into statoliths from surrounding water and ingested food that give rise to distinct elemental compositions caused by different habitats (water masses) occupied by individual squid, which can be used as a natural marker to distinguish cephalopod stocks (Avigliano et al. 2020). Besides stock

identification, statolith element profiles can provide information on natal origin (Liu et al. 2015) or migration patterns of individuals (Liu et al. 2016; Han et al. 2022). Most trace elements are incorporated in squid statoliths during darkness when squids are at the sea surface, so sea surface temperature may have a more significant impact on the uptake of elements than deep water temperature (Liu et al. 2016).

With a concentration of ~400,000 ppm (Zumholz, Hansteen, et al. 2007; Han et al. 2022), calcium is the main component in cephalopod statoliths (Ikeda et al. 1998; Liu et al. 2013). Therefore, concentrations of key trace elements in statoliths are commonly expressed as a ratio in relation to calcium, including elemental ratios correlated with temperature, such as Barium/Calcium, Iodine/Calcium, Strontium/Calcium, Uranium/Calcium or salinity, such as Iodine/Calcium, Magnesium/Calcium, of surrounding water (Ikeda et al. 1998; Zumholz, Hansteen, et al. 2007; Liu et al. 2011). These ratios can be used as indicators of population migration and habitat (Han et al. 2022). Considering the complex relationship between statolith elemental composition and element availability in the environment, between elements and squid physiology, and the fact that no microchemical statolith studies of *I. coindetii* and *L. forbesii* have been conducted in Europe, as many of the relevant elements as possible should be examined.

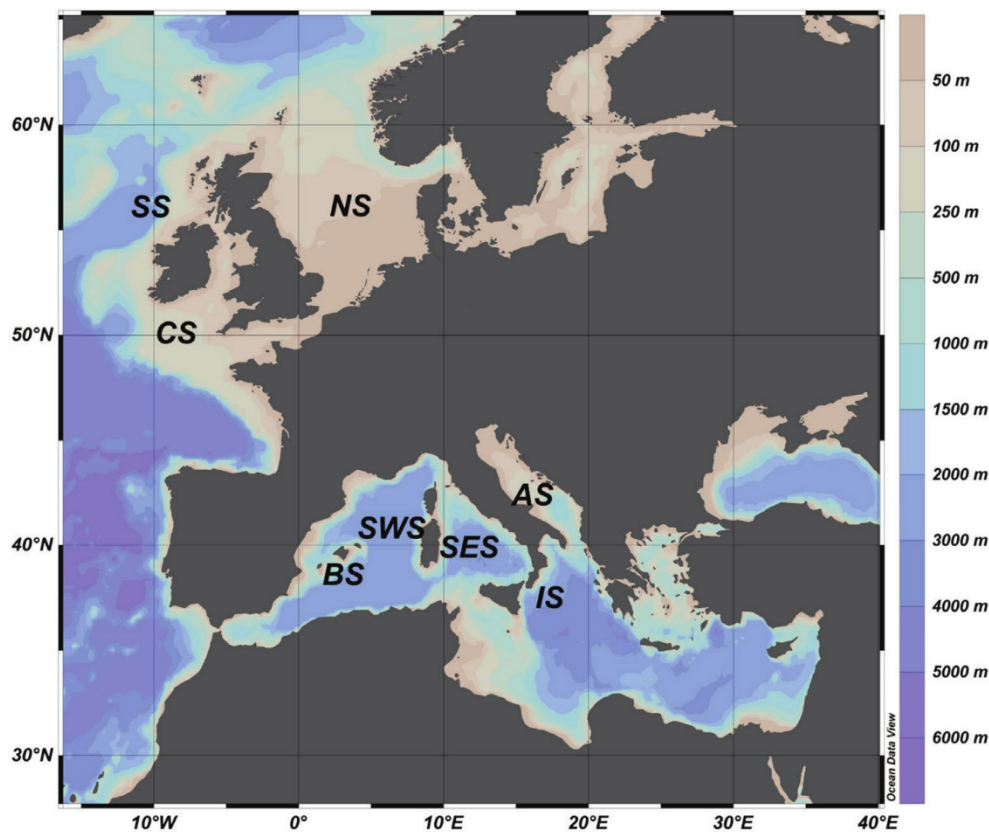
Our objective was to determine if different stocks of *I. coindetii* and *L. forbesii* occur in the North East Atlantic Ocean and Mediterranean Sea, based on the elemental composition of statoliths. We also sought to determine which elements contributed most to stock differentiation. Two different spots on each statolith, the nucleus and the edge, were chemically analysed to provide complementary insights into stock structure: spawning origin (nucleus) and environmental history close to the capture date (edge). Age was also estimated to ascertain hatching periods and detect temporal overlap amongst stocks with respect to cohorts in the study area. We also determined if similar elemental ratios were present at different times (yearly quarters). Our findings provide a potential basis for developing sustainable fisheries management plans and actions for squids in European waters of the North East Atlantic Ocean and Mediterranean Sea.

## 2 | Material and Methods

Squid were collected using established protocols as part of ICES (International Council for the Exploration of the Sea) coordinated International Bottom Trawl Surveys (IBTS) and Mediterranean International Bottom Trawl Surveys (MEDITS) (ICES 2017, 2012). Additional squid were collected using standardised protocols as part of the Irish Anglerfish and Megrim Survey (IAMS) (Reid et al. 2007) and Scottish West Coast Groundfish Survey (SCOWCGFS) (ICES 2020). Squid were collected from the North East Atlantic Ocean in the North Sea in Q1 and Q3, 2021, Sea West of Scotland in Q4, 2021 and Celtic Sea in Q1, 2022. Squid were collected from the Mediterranean Sea in the Balearic Sea, Sea East of Sardinia, Sea West of Sardinia, South Adriatic Sea and Western Ionian Sea in Q3 of 2021 (Figure 1 and Table 1).

Dorsal mantle length (DML, mm) and total weight (W, 0.01 g) were measured, sex and maturity stage (ICES 2022) of *I.*





**FIGURE 1** | Sampling areas for *Illex coindetii* and *Loligo forbesii* in the North Sea (NS), Sea West of Scotland (SS), Celtic Sea (CS), Balearic Sea (BS), Sea West of Sardinia (SWS), Sea East of Sardinia (SES), Western Ionian Sea (IS), South Adriatic Sea (AS) in the North East Atlantic Ocean and Mediterranean Sea during 2021–2022.

*coindetii* and *L. forbesii* were classified, and statoliths were extracted from the statocyst. Statoliths previously used for shape analysis (Bobowski et al. 2024) were cleaned with distilled water, affixed to a microscopic glass slide using a thermoplastic adhesive (Crystalbond), polished to the core with micro-mesh polishing paper (6000, 8000 and 12,000 grid) and rinsed with distilled water.

For age estimation, polished statoliths were photographed under transmitted light with an AxioCam 105 colour camera attached to an Olympus BX60 microscope and microscopy software Zen 2 blue edition (ver.2.0.0.0 by Carl Zeiss Microscopy GmbH, 2011). To minimise distortion and blurring caused by diffraction of the statolith that would cause miscounting of increments, a series of depth-focussed images was captured from the nucleus to the edge of the statolith. Increments were counted from the nucleus to the edge (dorsal dome) for *I. coindetii* and from the natal ring to the edge (dorsal dome) for *L. forbesii*. To estimate the precision of age estimates, increments on each statolith were counted twice by the same trained person. Only those age estimates with a standard deviation of less than 10 increments (days) were used for age estimation ( $n=181$  *I. coindetii*; and  $n=245$  *L. forbesii*). Based on the interpretation of statolith growth increments, we assumed that material at the nucleus corresponds to early life stages and material at the edge represents environmental conditions during the final weeks before capture.

Elemental composition of 453 polished statolith nuclei and edges (Figure 2) was quantified by laser ablation-inductively coupled

plasma-mass spectrometry (LA-ICP-MS), using a New Wave Research UP213-LA paired with a Thermo Fisher Scientific ELEMENT2 ICP-MS. To ensure that measuring points were positioned as correctly as possible, microscopy images of polished statoliths taken by the same person were used as reference material. Statolith regions were ablated with a  $40\mu\text{m}$  spot size at 70% power, a pulse repetition rate of 10 Hz and  $\sim 14\text{J cm}^{-2}$  energy density over  $\sim 40\text{s}$ . Values were recorded every 0.836 s. A blank value was calculated from the first 16 measurements, and the average value for each corresponding measurement point was computed from the remaining 31 measurements. Reference materials for calibration included a synthetic silicate glass NIST612 (National Institute of Standards and Technology) and a calcium carbonate pressed powder pellet MACS-3 (United States Geological Survey) (Jochum et al. 2011). Both reference materials were measured in triplicate at the beginning and end of each laser session, with up to 14 samples between, and a dwell duration of 24 s.

Raw data were expressed as ratios to calcium ( $^{43}\text{Ca}$ ). Raw concentration (ppm) was first corrected using relative sensitivity factors (RSFs) based on reference materials and then divided by calcium concentration in statoliths to express element-to-calcium ratios. Values below detection limits were excluded. Elements included sodium ( $^{23}\text{Na}$ ), magnesium ( $^{25}\text{Mg}$ ), manganese ( $^{55}\text{Mn}$ ), iron ( $^{57}\text{Fe}$ ), cobalt ( $^{59}\text{Co}$ ), copper ( $^{65}\text{Cu}$ ), zinc ( $^{66}\text{Zn}$ ), strontium ( $^{88}\text{Sr}$ ), barium ( $^{137}\text{Ba}$ ), lead ( $^{208}\text{Pb}$ ) and uranium ( $^{238}\text{U}$ ). Crystalbond adhesive was also measured to ensure elements in the adhesive did not interfere with sample measurements.



**TABLE 1** | Dorsal mantle length (DML), weight, age and sex distribution (male = M, female = F, juvenile = J), capture period and hatch period of *Illex coindetii* and *Loligo forbesii* (*n*) sampled in the Balearic Sea, Celtic Sea, North Sea, Sea East of Sardinia, Sea West of Scotland, South Adriatic Sea and Western Ionian Sea in the North East Atlantic and Mediterranean Sea during 2021–2022 (Q1 = January–March, Q2 = April–June, Q3 = July–September, Q4 = October–December; AuWiSS = Autumn–Winter spawning stock, SpSS = Spring spawning stock, SpSuSS = Spring–Summer spawning stock, SuSS = Summer spawning stock, WiSS = Winter spawning stock, WiSpSS: Winter–Spring spawning stock).

| Species                | Spawning stock         | Sex    | DML (mm)    |              | Weight (g) |              | n <sub>DML+weight</sub> | Age (days) |             | n <sub>age</sub> | Capture | Hatch      |
|------------------------|------------------------|--------|-------------|--------------|------------|--------------|-------------------------|------------|-------------|------------------|---------|------------|
|                        |                        |        | Min-Max     | Mean ±SD     | Min-Max    | Mean ±SD     |                         | Min-Max    | Mean ±SD    |                  |         |            |
| <i>Illex coindetii</i> | Balearic WiSS          | M      | 106–143     | 130 ± 14.36  | 58–104     | 86 ± 18.55   | 5                       | 177–203    | 191 ± 12.38 | 4                | Q3 2021 | Q1 2021    |
|                        |                        | F      | 141–190     | 170 ± 16.61  | 106–184    | 135 ± 26.06  | 8                       | 159–194    | 176 ± 14.02 | 8                |         |            |
|                        | Celtic SpSuSS          | M      | 108–185     | 165 ± 16.19  | 28–200     | 125 ± 40.81  | 24                      | 190–241    | 216 ± 13.86 | 20               | Q1 2022 | Q2/Q3 2021 |
|                        |                        | F      | 160–285     | 206 ± 27.57  | 73–549     | 191 ± 103.95 | 20                      | 185–262    | 225 ± 26.39 | 17               |         |            |
|                        | North Sea SuSS Q1      | M      | 79–157      | 114 ± 24.88  | 13–88      | 40 ± 25.66   | 19                      | 128–167    | 148 ± 12.19 | 19               | Q1 2021 | Q3 2020    |
|                        |                        | F      | 90–158      | 116 ± 22.65  | 15–92      | 37 ± 23.55   | 18                      | 116–165    | 147 ± 10.95 | 17               |         |            |
|                        | North Sea SuSS Q3      | M      | 120–197     | 158 ± 21.74  | 92–232     | 143 ± 46.11  | 13                      | 229–319    | 271 ± 28.26 | 11               | Q3 2021 | Q3 2020    |
|                        |                        | F      | 108–224     | 167 ± 40.39  | 33–331     | 143 ± 91.03  | 10                      | 216–317    | 256 ± 36.81 | 10               |         |            |
|                        | Eastern Sardinian WiSS | M      | 142–152     | 147 ± 4.16   | 115–129    | 120 ± 6.29   | 4                       | 195–195    | 195 ± 0     | 2                | Q3 2021 | Q1 2021    |
|                        |                        | F      | 140–209     | 176 ± 17.45  | 77–186     | 139 ± 31.69  | 12                      | 182–213    | 192 ± 9.41  | 8                |         |            |
| <i>Loligo forbesii</i> | Western Sardinian WiSS | M      | 124–154     | 140 ± 8.13   | 67–158     | 114 ± 29.2   | 16                      | 179–216    | 194 ± 13.67 | 12               | Q3 2021 | Q1 2021    |
|                        |                        | F      | 124–208     | 179 ± 24.78  | 49–222     | 139 ± 51.3   | 13                      | 182–205    | 190 ± 7.77  | 10               |         |            |
|                        | South Adriatic WiSpSS  | M      | 95–145      | 127 ± 13.87  | 26–123     | 86 ± 27.61   | 12                      | 137–186    | 168 ± 16.52 | 9                | Q3 2021 | Q1/Q2 2021 |
|                        |                        | F      | 115–185     | 152 ± 16.85  | 43–186     | 107 ± 36.47  | 19                      | 131–196    | 168 ± 18.23 | 15               |         |            |
|                        | Western Ionian WiSS    | M      | 100–125     | 113 ± 7.99   | 44–81      | 61 ± 14.95   | 8                       | 181–196    | 188 ± 7.51  | 3                | Q3 2021 | Q1 2021    |
|                        |                        | F      | 105–190     | 147 ± 17.5   | 31–193     | 106 ± 33.79  | 21                      | 164–201    | 185 ± 10.39 | 16               |         |            |
|                        | Balearic WiSS          | M      | 87–485      | 178 ± 131.57 | 32–1820    | 309 ± 519.38 | 14                      | 166–218    | 184 ± 15.02 | 14               | Q3 2021 | Q1 2021    |
|                        |                        | F      | 74–365      | 181 ± 98.08  | 20–1057    | 264 ± 299.22 | 14                      | 161–207    | 184 ± 11.67 | 14               |         |            |
|                        | Celtic SuSS            | J      | 78–225      | 152 ± 79.04  | 24–320     | 164 ± 152.32 | 4                       | 174–193    | 183 ± 7.78  | 4                |         |            |
|                        |                        | M      | 72–250      | 150 ± 38.02  | 16–338     | 104 ± 67.98  | 19                      | 157–204    | 180 ± 13.71 | 18               | Q1 2022 | Q3 2021    |
|                        | F                      | 82–182 | 142 ± 32.63 | 23–167       | 98 ± 50.03 | 7            | 145–190                 | 171 ± 16   | 6           |                  |         |            |

(Continues)

TABLE 1 | (Continued)

| Species | Spawning stock | Sex | DML (mm) |                 |  | Weight (g) |                  |  | $n_{\text{DML+weight}}$ | Age (days) |                 |  | $n_{\text{age}}$ | Capture | Hatch               |
|---------|----------------|-----|----------|-----------------|--|------------|------------------|--|-------------------------|------------|-----------------|--|------------------|---------|---------------------|
|         |                |     | Min-Max  | Mean $\pm$ SD   |  | Min-Max    | Mean $\pm$ SD    |  |                         | Min-Max    | Mean $\pm$ SD   |  |                  |         |                     |
|         | North Sea      | M   | 116–356  | 233 $\pm$ 83.63 |  | 61–704     | 291 $\pm$ 202.48 |  | 31                      | 162–246    | 198 $\pm$ 23.03 |  | 31               | Q1 2021 | Q2/Q3 2020          |
|         | SpSuSS Q1      | F   | 107–272  | 193 $\pm$ 40.01 |  | 44–525     | 227 $\pm$ 106.36 |  | 27                      | 162–311    | 193 $\pm$ 31.81 |  | 26               |         |                     |
|         | North Sea      | M   | 87–298   | 173 $\pm$ 56.0  |  | 25–595     | 189 $\pm$ 150.06 |  | 45                      | 148–244    | 180 $\pm$ 22.56 |  | 44               | Q3 2021 | Q4 2020/<br>Q1 2021 |
|         | AuWiSS Q3      | F   | 95–199   | 148 $\pm$ 29.02 |  | 38–206     | 114 $\pm$ 49.52  |  | 23                      | 151–230    | 174 $\pm$ 18.44 |  | 23               |         |                     |
|         | Western        | M   | 144–424  | 231 $\pm$ 93.73 |  | 99–1533    | 418 $\pm$ 503.15 |  | 17                      | 160–222    | 180 $\pm$ 18.57 |  | 16               | Q4 2021 | Q2 2021             |
|         | Scotland SpSS  | F   | 126–323  | 203 $\pm$ 56.23 |  | 79–885     | 277 $\pm$ 209.91 |  | 33                      | 156–202    | 179 $\pm$ 12.08 |  | 30               |         |                     |
|         | South Adriatic | M   | 175–405  | 274 $\pm$ 92.49 |  | 185–1170   | 575 $\pm$ 411.51 |  | 6                       | 233–268    | 247 $\pm$ 13.62 |  | 6                | Q3 2021 | Q4 2020/<br>Q1 2021 |
|         | AuWiSS         | F   | 180–215  | 201 $\pm$ 11.34 |  | 196–329    | 278 $\pm$ 41.06  |  | 13                      | 204–284    | 234 $\pm$ 25.22 |  | 13               |         |                     |

A principal component analysis (PCA) was performed in R.4.2.2 (R Core Team 2022) using the prcomp() function from the base stats package. Missing values were imputed using the mice package (v3.14.0; van Buuren and Groothuis-Oudshoorn 2011). The first two principal components (PC1 and PC2) were used to describe spatial patterns of elemental ratios. Scatterplots of PC2 against PC1, with 95% confidence ellipses for grouped PCA scores, were plotted using the ggplot2 package (v3.3.6; Wickham 2016). Group means and standard errors were plotted as error bars to visualise central tendency and variation across sampling areas. Due to inhomogeneity of multivariate spatial variances, partitioning of variation amongst groups based on principal components was assessed by analysis of similarity (ANOSIM) using the vegan package (v2.5–7; Oksanen et al. 2020). To reduce Type I error, a Bonferroni correction was applied to  $p$ -values of multiple comparisons. For the correlation between observed and predicted classes, PCAs were classified using caret (v6.0–86; Kuhn 2020) and e1071 packages (v1.7–9; Meyer et al. 2021). To test for significant differences in element ratios between nucleus and edge measurements in each area,  $t$ -tests for normally distributed data or Mann–Whitney U tests for non-normally distributed data were computed using SigmaPlot 13 (Systat Software Inc. 2014).

### 3 | Results

#### 3.1 | Biological Data

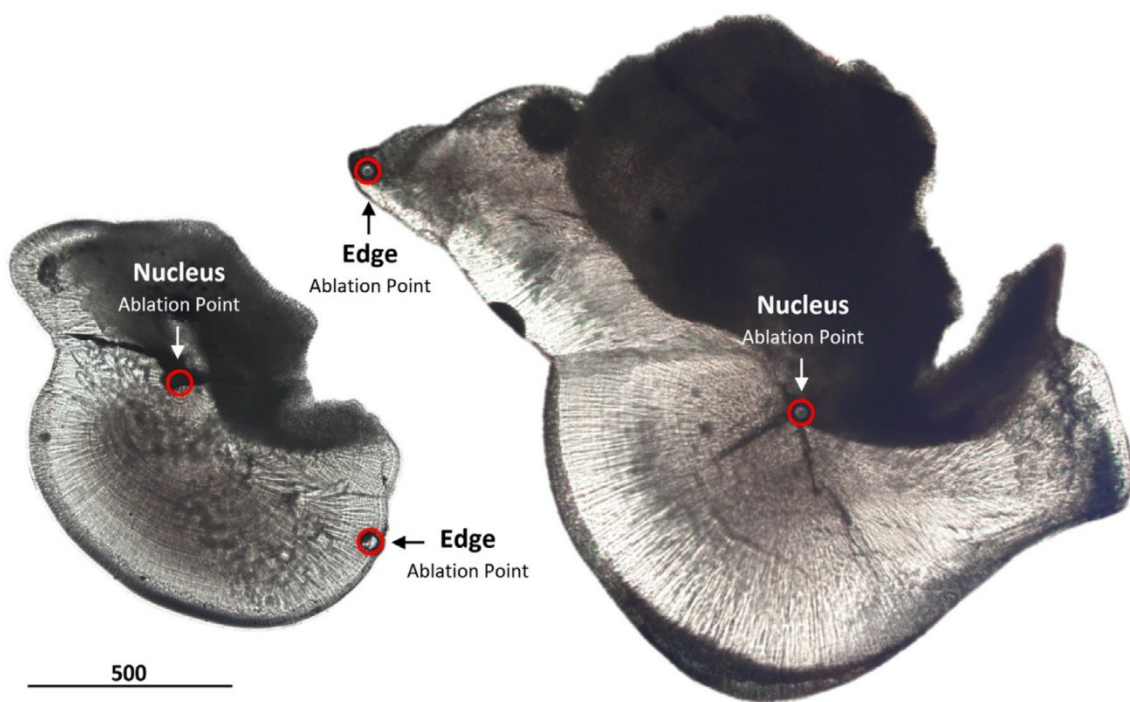
Biological data were collected from 475 squid, including 222 *I. coindetii* and 253 *L. forbesii* (Table 1). Fewer squid were analysed for statolith microchemistry, including 206 *I. coindetii* and 247 *L. forbesii*, and even fewer were analysed for age estimation (181 *I. coindetii*, 245 *L. forbesii*; Table 1).

All Mediterranean *I. coindetii* captured in Q3 of 2021 hatched in winter 2021, and four individuals from the South Adriatic Sea hatched in spring 2021 (April) (Table 1). *L. forbesii* from the Mediterranean also hatched in winter 2021, with some South Adriatic individuals hatched in autumn 2020. *I. coindetii* from the North Sea captured in Q1 and Q3 of 2021 hatched in summer of 2020, whereas *L. forbesii* from the North Sea differed in hatching periods between those caught in Q1 and Q3 of 2021. *L. forbesii* from the Sea West of Scotland hatched in spring 2021 and overlapped with spring–summer spawners in the North Sea that hatched in Q2 and Q3 of 2020.

For both *I. coindetii* and *L. forbesii*, microchemistry of the statolith nucleus deposited near the time of hatching and statolith edge deposited near the time of capture were divided into distinct groups that reflected different spawning and mating groups. However, groups differed between measurements at the nucleus and edge. For nucleus and edge measurements of both species, Co and Fe compositions explained greater variance in PC1 and PC2 than other elements, except Na at the *I. coindetii* nucleus (Figures 3B,D and 4B,D), for which a small angle of difference amongst elements indicated high similarity.

For microchemistry of *I. coindetii* at the statolith nucleus, the first two principal components explained 41% (PC1=26%; PC2=15%) of element variation, whereas at the statolith edge,





**FIGURE 2** | Ablation point locations on a polished right-sided *Illex coindetii* (left) and *Loligo forbesii* (right) statolith from the North Sea. The nucleus represents the early life stage, and the edge represents the period of capture in developing, maturing or matured squid.

the first two principal components explained 45% (PC1=26%; PC2=19%) of element variation (Figure 3). For the Western Ionian winter spawning stock, element variation was highest at the statolith nucleus and highly influenced by Cu, Pb and Zn (Figure 3A,B). For Eastern Sardinian and Western Sardinian winter spawning stocks, element variation was highest at the statolith edge (Figure 3C,D). Element concentrations did not differ significantly between Balearic and each of the Eastern Sardinian winter spawning, South Adriatic winter-spring spawning, Ionian winter spawning and Celtic spring-summer spawning stocks, either at the nucleus or the edge (Table 2). Nucleus element concentrations did not differ significantly: (i) amongst Balearic winter spawning and Western Sardinian winter spawning stocks, (ii) between North Sea summer spawning groups (captured in Q1 and Q3) and (iii) between Eastern and each of Western Sardinian winter spawning, Adriatic winter spawning and Ionian winter spawning stocks. Element concentrations at the statolith edge did not differ significantly between Eastern and Western Sardinian winter spawning stocks.

For microchemistry of *L. forbesii* at the nucleus, the first two principal components explained 45% of element variation (PC1=24%; PC2=21%), with North Sea spring-summer, autumn-winter and Western Scotland spring spawning stocks strongly overlapping (Figure 4). Element concentrations overlapped for Celtic Sea summer spawning, Adriatic autumn-winter spawning and Balearic winter spawning stocks, which were more strongly influenced by Co and Fe than those of the North Sea and Scottish Sea stocks (Figure 4A,B). For element concentrations at the statolith edge, the first two principal components explained 54% of element variation (PC1=35%, PC2=19%). At the statolith edge, element profiles were strongly influenced by Co and Fe for Balearic winter spawning, Celtic summer spawning and South Adriatic autumn-winter spawning stocks

and by Cu, Pb, Zn and Ba for the Western Scotland spawning stock (Figure 4C,D). Elemental composition at the statolith edge differed amongst sampled areas. In contrast, element concentrations at the statolith nucleus did not differ significantly: (i) amongst Balearic winter spawning and each of Celtic summer spawning and Adriatic autumn spawning stocks and (ii) between North Sea spring-summer spawning and North Sea autumn-winter spawning stocks (Table 2).

For *I. coindetii*, classification accuracy based on element ratios at the statolith nucleus was highest for the North Sea stock sampled in Q1 (100%), followed by the Celtic Sea stock (72%; Table 3). For Mediterranean Sea stocks, classification accuracy was less successful for Western Sardinian winter spawning, South Adriatic winter-spring and Western Ionian winter spawning (40%–69%), and stocks were completely misclassified for Balearic and Eastern Sardinian winter spawning stocks (0%). North Sea summer spawning squid captured in Q3 were completely misclassified based on element ratios at the statolith nucleus, but were assigned to the correct stock, albeit in a different quarter (NS Q1). Based on element composition at the statolith edge, classification was successful for squid from the North East Atlantic and Western Sardinian winter spawning stocks (80%–100%). Squid from other spawning stocks in the Mediterranean Sea were less correctly classified (33%–61%) or were completely misclassified to the Balearic winter spawning stock. Based on element ratios at the statolith nucleus and edge, Mediterranean Sea squid were mostly misclassified, with some misclassified to the Celtic Sea.

For *L. forbesii*, spawning stock classification for stocks in the North East Atlantic Ocean was higher based on element composition at the statolith edge, whereas those from the Mediterranean Sea were more correctly classified based on element composition

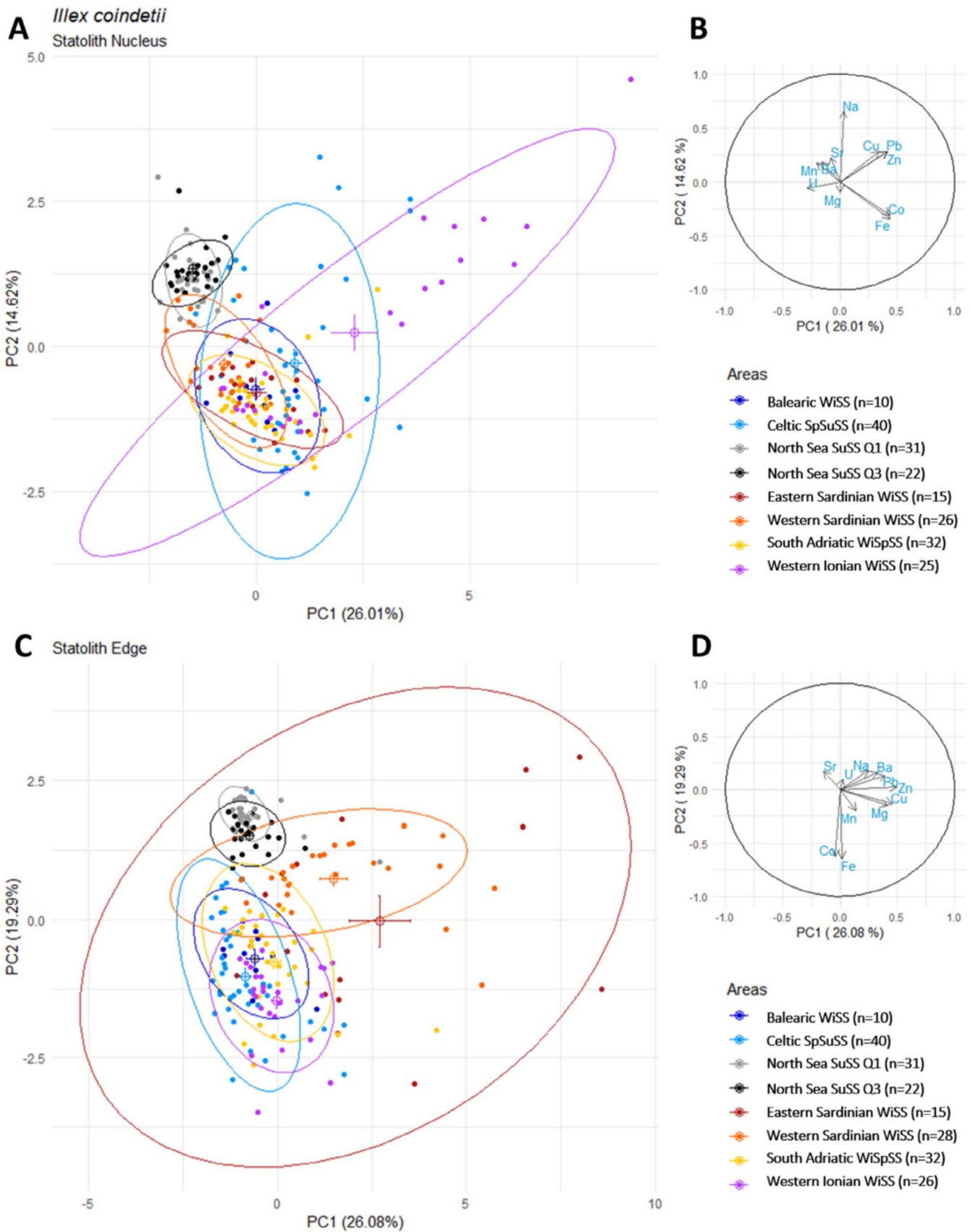


FIGURE 3 | Legend on next page.



**FIGURE 3** | Sample (A, C) and variable (B, D) biplots of the first two principal components (PC1, PC2) for statolith microchemistry of *Illex coindetii* spawning stocks sampled in the Balearic Sea (WiSS = dark blue), Celtic Sea (SpSuSS = light blue), North Sea (SuSS Q1 = grey, SuSS Q3 = black), Eastern Sardinian Sea (WiSS = red), Western Sardinian Sea (WiSS = orange), South Adriatic Sea (WiSpSS = yellow) and Western Ionian Sea (WiSS = purple) in the North East Atlantic Ocean and Mediterranean Sea during 2021–2022 (A, B = statolith nucleus, C, D = statolith edge; colours in A and C correspond to different spawning stocks: SuSS = Summer spawning stock, WiSS = Winter spawning stock, SpSuSS = Spring–Summer spawning stock, WiSpSS = Winter–Spring spawning stock). Open circles depict the mean ( $\pm$  standard error) and ellipses depict 95% of samples from each area.

at the nucleus (Table 3). Classification accuracy based on element concentrations at the statolith nucleus was higher for the North Sea autumn–winter spawning stock (> 90%) than for Balearic winter spawning, Celtic summer spawning, North Sea spring–summer spawning and South Adriatic autumn–winter spawning stock (50%–65%). Classification accuracy was lowest for the Western Scotland spring spawning stock (28%), with misclassified squid all classified to the North Sea. Based on element classification at the statolith edge, classification accuracy was higher for North Sea and Sea West of Scotland spawning stock (80%–96%) than for the Celtic summer spawning stock (67%) that were often classified to the North Sea autumn–winter spawning stock. Classification accuracy was low for Balearic winter spawning and South Adriatic autumn–winter spawning stocks (20%–48%).

Except for a few locations, element ratios of Sr, U and Zn differed between statolith nucleus and edge for both *I. coindetii* and *L. forbesii* (Table 4). For most sampling areas, Ba and Cu element ratios differed between statolith nucleus and edge for *I. coindetii*, whereas Na, Mg and Pb differed for *L. forbesii*. In contrast, whilst element ratios of Co and Fe discriminate sampling locations in the PCA, they did not differ much between the nucleus and edge of statoliths for both species, except *L. forbesii* from the Western Scotland spring spawning stock. For *I. coindetii* from the Western Ionian winter spawning stock, no element ratios differed significantly between statolith nucleus and edge.

#### 4 | Discussion

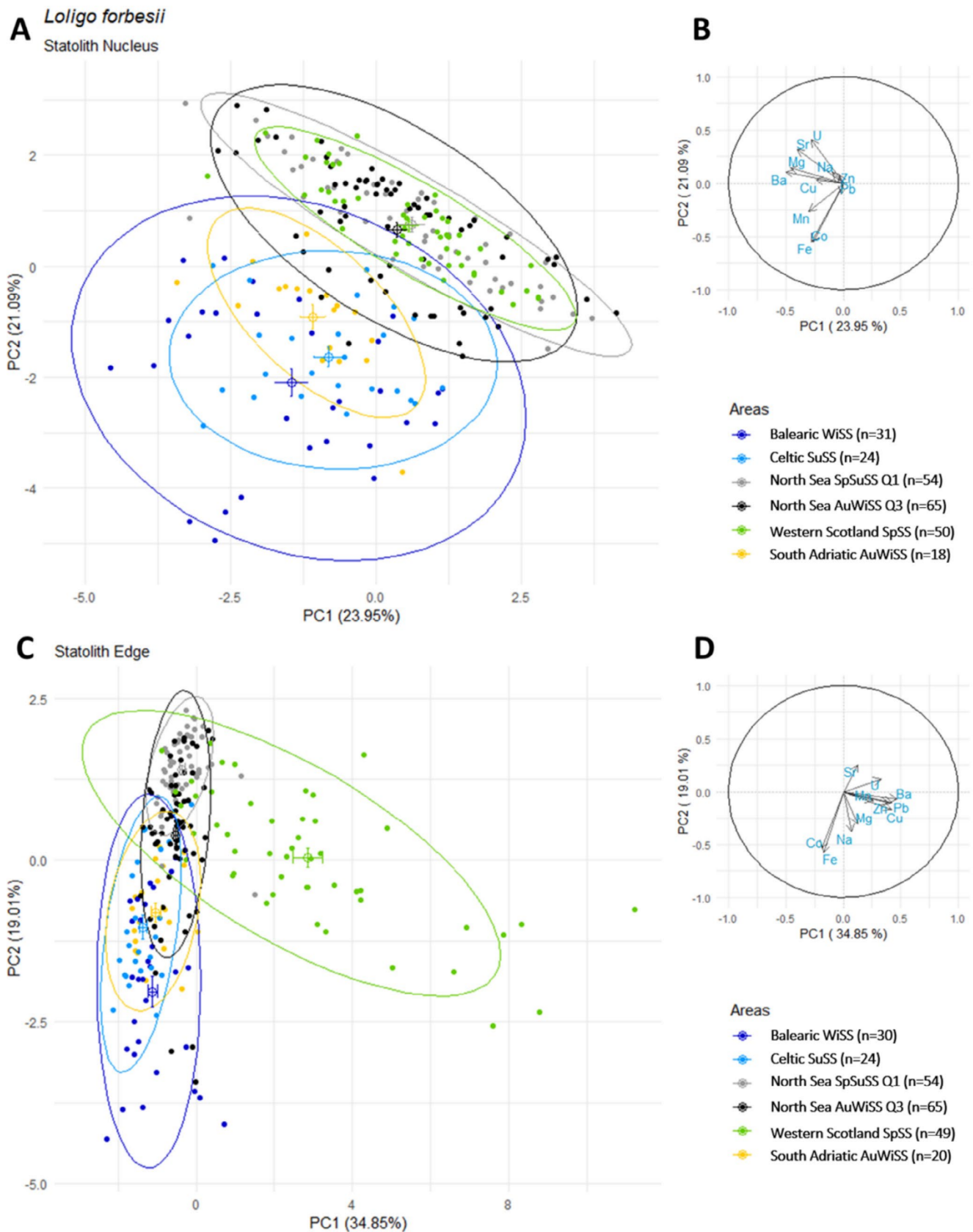
Microchemical differences in statolith element ratios we observed have been previously linked to environmental factors, such as temperature, salinity or habitat use (Zumholz, Klügel, et al. 2007; Liu et al. 2016). For example, in combination with sea surface temperature, fluctuations in U/Ca ratios can indicate ontogenetic migration routes (Zumholz, Klügel, et al. 2007). Sr/Ca and Ba/Ca ratios can provide additional insights (Liu et al. 2016), with Ba/Ca correlated with water depth (Arkhipkin et al. 2004; Jones et al. 2018). Moreover, the Sr/Ca ratio as a temperature indicator seems more suitable to identify horizontal migration patterns (e.g., onshore–offshore) in loliginid species like *L. forbesii* than in oceanic ommastrephids like *I. coindetii*, whose horizontal movement is possibly masked by extensive diel–vertical migrations (Arkhipkin et al. 2004). However, elements like Fe and Zn, along with Sr, are related to temperature in the ommastrephid squid *Todarodes pacificus* (Ikeda et al. 1996). Whilst Mg/Ca is often used to indicate salinity variations due to its positive correlation with salinity in coral skeletons (Mitsuguchi et al. 1996), this correlation is not evident in cephalopods. Instead, Mg concentrations in cephalopod statoliths are linked to the deposition of organic matter

and progressively decrease from the statolith nucleus to the edge as squid grow (Arkhipkin et al. 2004; Zumholz, Klügel, et al. 2007; Fan et al. 2023). Furthermore, I/Ca is positively correlated with temperature and negatively correlated with salinity in the common cuttlefish *Sepia officinalis* (Zumholz, Hansteen, et al. 2007). Non-biodegradable accumulating trace metals in statoliths, such as, Cd, Cr, Cu, Pb, Hg and Zn (Sharifuzzaman et al. 2016), can provide insight into residence at specific locations during ontogeny.

The ablation spot (40  $\mu$ m) in our study corresponded to 25–30 daily increments in *I. coindetii* statoliths and 10–15 daily increments in *L. forbesii* statoliths, so elemental composition at the statolith nucleus represented early life stages, and the edge represented a few weeks before capture. Based on differences in element ratios at the nucleus and edge of statoliths, *I. coindetii* and *L. forbesii* were separated into seasonally and geographically distinct groups within the areas of the North East Atlantic Ocean and Mediterranean Sea that we interpret as stocks. Similar approaches using statolith element ratios have been successfully applied to discriminate squid stocks in other species (Fang et al. 2014; Green et al. 2015; Avigliano et al. 2020). Consistent with previous findings that statolith microchemistry reflects natal origin (Warner et al. 2009; Liu et al. 2015) differences in nucleus profiles here likely indicate hatching in different regions, whereas similar profiles suggest a shared origin. Differences in both nucleus and edge microchemistry, by contrast, suggest the presence of different stocks, consistent with previous studies demonstrating that variation in statolith element composition can delineate stock structure in squid (Fang et al. 2014; Avigliano et al. 2020). Alternatively, deposition of elements is influenced by abiotic factors and diet (Zumholz 2005), so squid from different areas that were temporally and spatially separated, but experienced similar abiotic conditions, could also have similar elemental ratios in their statoliths, thereby making their classification into distinct groups less accurate for some regions.

Combining our results with those from statolith shape analysis of the same individuals (Bobowski et al. 2024) allowed for the identification of potential cohorts of *I. coindetii* around southern Italy and possible common hatching or feeding grounds of both species that provided more detailed insight into European stock structures. For *I. coindetii*, microchemical analysis indicated that three distinct stocks and one mixed stock were found in Italian waters, thereby suggesting temporary ontogenetic separation, with overall connectivity, consistent with the ‘middle course’ situation proposed for the Mediterranean (Sánchez et al. 1998). This observation is consistent with the previously reported homogenous population structure (Martínez et al. 2005), highlighting that ecological separation does not imply genetic differentiation. Our analysis of *L. forbesii* revealed five distinct





**FIGURE 4** | Legend on next page.

stocks, with some connectivity amongst North East Atlantic stocks during different ontogenetic stages, contradicts a homogenous population structure inferred from genetic analysis (Göpel et al. 2022).

Our results are consistent with earlier studies that also found no evidence for temporally separated stocks of *I. coindetii* in the North Sea based on biological data (DML, maturity stage, hatching period) and statolith shape (Oesterwind et al. 2020;

**FIGURE 4** | Sample (A, C) and variable (B, D) biplots of the first two principal components (PC1, PC2) for statolith microchemistry of *Loligo forbesii* sampled in the Balearic Sea (WiSS = dark blue), Celtic Sea (SuSS = light blue), North Sea (SpSuSS Q1 = grey; AuWiSS Q3 = black), Western Scotland (SpSS = green) and South Adriatic Sea (AuWiSS = yellow) in the North East Atlantic Ocean and Mediterranean Sea during 2021–2022 (A, B = statolith nucleus, C, D = statolith edge; colours in A and C correspond to different spawning stocks: AuWiSS = Autumn–Winter spawning stock, SpSS = Spring spawning stock, SpSuSS = Spring–Summer spawning stock, SuSS = Summer spawning stock, WiSS = Winter spawning stock). Open circles depict the mean ( $\pm$  standard error) and ellipses depict 95% of samples from each area.

Bobowski et al. 2024). Our findings, combined with observations of egg-spheres in the Norwegian Channel (Ringvold et al. 2021), support an assumption that *I. coindetii* in the North Sea complete their whole life cycle in the area as an independent North Sea stock (Oesterwind et al. 2010, 2015, 2020; Barrett et al. 2021). Based on element composition at the statolith nucleus and the edge, a proportion of the Celtic spring–summer spawning stock was assigned to the Mediterranean stock. Considering that water masses can have similar abiotic factors despite spatial and temporal separation, and the Celtic Sea and Mediterranean Sea areas around Italy are geographically distant areas, we assume that misclassified individuals inhabited environments with similar water conditions, which illustrates a limitation of our approach. Based on these considerations, and differences in statolith microchemistry (this study) and shape (Bobowski et al. 2024), we conclude that *I. coindetii* squid from the Celtic Sea belong to an Atlantic stock that originated from a different hatching area that was separated from stocks in the North Sea and Mediterranean Sea.

Stock identification by microchemistry analyses is more complex for *I. coindetii* in the Mediterranean Sea due to high misclassification and similarity amongst areas, similar to a statolith shape analysis (Bobowski et al. 2024). The Mediterranean *I. coindetii* population is complex, with multiple cohorts, micro-cohorts or heavily overlapping cohorts (Sánchez et al. 1998). *I. coindetii* does not attach its eggs to hard substrates, but lays floating gelatinous egg-spheres (Jereb and Roper 2010) that would facilitate exchange amongst areas. Because all squid we analysed were of similar age and part of the same spawning group, we conclude that squid from the Mediterranean Sea were exposed to seasonally similar water parameters during early life. Misclassified squid from the Mediterranean Sea were primarily classified for the Sea west of Sardinia and the south Adriatic Sea based on microelement composition of the statolith nucleus, whereas misclassification was lower based on statolith shape and squid did not differ amongst areas surrounding Italy (Bobowski et al. 2024). In contrast to microchemistry, statolith shape formation is a biologically regulated process that occurs over a longer period that involves proton and ion transporter control of endolymph composition and organic matrix and either promotes or inhibits crystal nucleation (Bettencourt and Guerra 2000). Consequently, shape analysis can indicate main areas of residence but provides no information about hatching areas or migration routes. Overall, our results indicated that squid around Italy might share a hatching region that splits into multiple cohorts thereafter, whilst also using similar feeding grounds.

Statoliths of *I. coindetii* from both Sardinian Sea areas were highest in concentrations of Cu, Pb and Zn at the edge, which suggests exposure to these trace metal elements near capture locations.

The harbour at Cagliari (Sardinia, Italy) has an oil terminal with significant concentrations of trace metals in surface sediments throughout the area (Schintu et al. 2016). Additionally, soils and vegetation of the mining areas in southwest Sardinia have exceptionally high levels of Ca, Pb and Zn (Leita et al. 1989), which might enter surrounding seas through runoff that accumulates in statoliths of squid in this area. Accumulation might occur through direct deposition from water or through prey exposed to these metals. Early life stages of squid from the Western Ionian Sea had the highest value of Cu, Pb and Zn in their statoliths, so we assume that ommastrephid hatchlings incorporated such trace metals in their statoliths from surrounding water. These elements were not present or were lower in concentrations in early life stages of squid from East and West Sardinian Sea, so we infer that maturing and mature individuals from waters around Sardinia might have hatched outside the area and migrated into Sardinian Seas. Furthermore, all elements we measured did not differ between life stages of squid from the western Ionian Sea. Diets of *I. coindetii* change between early and late life stages (Sánchez et al. 1998; Schäfer et al. 2024), so we infer that both early and late life stages of these squid occupied the same area, or at least in regions with similar water parameters. However, some squid from the western Ionian Sea were misclassified to the Adriatic Sea in both life stages, which also supports an assumption that squid migrated amongst areas in southern Italy, similar to statolith shape analysis of the same samples of squid (Bobowski et al. 2024).

Mature female *L. forbesii* in the North East Atlantic are mainly found in the northern and central North Sea, northern and western Scotland and Ireland, and egg clusters attached to hard substrates have been found at coastal areas in northern Scotland and the Moray Firth (Laptikhovsky et al. 2022). A relatively high number of squid we sampled from the North Sea and the Western Scotland spring spawning stock were misclassified based on measurements at the statolith nucleus and edge, with higher misclassification of early life stages. High misclassification might indicate that paralarval stages were exposed to similar water conditions, perhaps due to a common spawning and hatching area. Whilst a potential migration was described from the West of Scotland into the North Sea (Waluda and Pierce 1998), routine migration was not evident between the two areas (Viana et al. 2009), so squid likely dispersed from a more northern region of Scotland. Nonetheless, these groups of squid likely have distinct feeding grounds because misclassification of individuals from the North Sea and Sea West of Scotland was lower based on element composition at the statolith edge as well as statolith shape comparisons (Bobowski et al. 2024). Moreover, early life stages did not differ significantly in statolith microchemistry between North Sea spring–summer and autumn–winter spawning stocks, which suggests that water conditions in areas inhabited by early life stages were relatively similar amongst different periods.

**TABLE 2** | Pairwise comparisons of statolith elemental composition of *Illex coindetii* and *Loligo forbesii* between spawning stocks in the Balearic Sea, Celtic Sea, North Sea, Sea East of Sardinia, Sea West of Sardinia, Sea West of Scotland, South Adriatic Sea and Western Ionian Sea in the North East Atlantic Ocean and Mediterranean Sea during 2021 to 2022 (AuWiSS = Autumn–Winter spawning stock, SpSS = Spring spawning stock, SpSuSS = Spring–Summer spawning stock, SuSS = Summer spawning stock, WiSS = Winter spawning stock, WiSpSS: Winter–Spring spawning stock). Significant differences are in **bold** ( $p < 0.05$ , adjusted with Bonferroni method).

| <i>Illex coindetii</i> |                        | Balearic WiSS | Celtic SpSuSS | North Sea SuSS Q1   | North Sea SuSS Q3   | Eastern Sardinian WiSS | Western Sardinian WiSS | South Adriatic WiSpSS |
|------------------------|------------------------|---------------|---------------|---------------------|---------------------|------------------------|------------------------|-----------------------|
| Nucleus                | Celtic SpSuSS          | 0.602         | —             | —                   | —                   | —                      | —                      | —                     |
|                        | North Sea SuSS Q1      | <b>0.003</b>  | <b>0.003</b>  | —                   | —                   | —                      | —                      | —                     |
|                        | North Sea SuSS Q3      | <b>0.003</b>  | <b>0.003</b>  | 1.000               | —                   | —                      | —                      | —                     |
|                        | Eastern Sardinian WiSS | 1.000         | <b>0.020</b>  | <b>0.003</b>        | <b>0.003</b>        | —                      | —                      | —                     |
|                        | Western Sardinian WiSS | 0.134         | <b>0.003</b>  | <b>0.003</b>        | <b>0.003</b>        | 0.392                  | —                      | —                     |
|                        | South Adriatic WiSpSS  | 1.000         | <b>0.003</b>  | <b>0.003</b>        | <b>0.003</b>        | 1.000                  | <b>0.003</b>           | —                     |
|                        | Western Ionian WiSS    | 1.000         | <b>0.003</b>  | <b>0.003</b>        | <b>0.003</b>        | 0.224                  | <b>0.003</b>           | <b>0.008</b>          |
| Edge                   | Celtic Sea SpSuSS      | 0.666         | —             | —                   | —                   | —                      | —                      | —                     |
|                        | North Sea SuSS Q1      | <b>0.003</b>  | <b>0.003</b>  | —                   | —                   | —                      | —                      | —                     |
|                        | North Sea SuSS Q3      | <b>0.003</b>  | <b>0.003</b>  | <b>0.003</b>        | —                   | —                      | —                      | —                     |
|                        | Eastern Sardinian WiSS | 0.210         | <b>0.003</b>  | <b>0.003</b>        | <b>0.003</b>        | —                      | —                      | —                     |
|                        | Western Sardinian WiSS | <b>0.011</b>  | <b>0.003</b>  | <b>0.003</b>        | <b>0.003</b>        | 1.000                  | —                      | —                     |
|                        | South Adriatic WiSpSS  | 1.000         | <b>0.003</b>  | <b>0.003</b>        | <b>0.003</b>        | <b>0.007</b>           | <b>0.003</b>           | —                     |
|                        | Western Ionian WiSS    | 0.258         | <b>0.003</b>  | <b>0.003</b>        | <b>0.003</b>        | <b>0.003</b>           | <b>0.003</b>           | <b>0.003</b>          |
| <i>Loligo forbesii</i> |                        | Balearic WiSS | Celtic SuSS   | North Sea SpSuSS Q1 | North Sea AuWiSS Q3 | Western Scotland SpSS  |                        |                       |
| Nucleus                | Celtic SuSS            | 0.065         | —             | —                   | —                   | —                      | —                      | —                     |
|                        | North Sea SpSuSS Q1    | <b>0.002</b>  | <b>0.002</b>  | —                   | —                   | —                      | —                      | —                     |
|                        | North Sea AuWiSS Q3    | <b>0.002</b>  | <b>0.002</b>  | 0.558               | —                   | —                      | —                      | —                     |
|                        | Western Scotland SpSS  | <b>0.002</b>  | <b>0.002</b>  | <b>0.002</b>        | <b>0.002</b>        | —                      | —                      | —                     |
|                        | South Adriatic AuWiSS  | 0.372         | <b>0.002</b>  | <b>0.002</b>        | <b>0.002</b>        | <b>0.002</b>           | <b>0.002</b>           | <b>0.002</b>          |
| Edge                   | Celtic Sea SuSS        | <b>0.002</b>  | —             | —                   | —                   | —                      | —                      | —                     |
|                        | North Sea SpSuSS Q1    | <b>0.002</b>  | <b>0.002</b>  | —                   | —                   | —                      | —                      | —                     |
|                        | North Sea AuWiSS Q3    | <b>0.002</b>  | <b>0.002</b>  | <b>0.002</b>        | —                   | —                      | —                      | —                     |
|                        | Western Scotland SpSS  | <b>0.002</b>  | <b>0.002</b>  | <b>0.002</b>        | <b>0.002</b>        | —                      | —                      | —                     |
|                        | South Adriatic AuWiSS  | <b>0.006</b>  | <b>0.002</b>  | <b>0.002</b>        | <b>0.002</b>        | <b>0.002</b>           | <b>0.002</b>           | <b>0.002</b>          |



| Predicted area |                                |               |               |                   |                   |                        |                        |                       |                     |                                 |
|----------------|--------------------------------|---------------|---------------|-------------------|-------------------|------------------------|------------------------|-----------------------|---------------------|---------------------------------|
| Observed area  | <i>Illex coindetii</i> NUCLEUS | Balearic WiSS | Celtic SpSuSS | North Sea SuSS Q1 | North Sea SuSS Q3 | Eastern Sardinian WiSS | Western Sardinian WiSS | South Adriatic WiSpSS | Western Ionian WiSS | Classification success rate (%) |
|                |                                | 0             | 4             | 0                 | 0                 | 0                      | 2                      | 4                     | 0                   | 0.00                            |
|                |                                | 0             | 29            | 3                 | 0                 | 0                      | 3                      | 3                     | 2                   | 72.50                           |
|                |                                | 0             | 0             | 31                | 0                 | 0                      | 0                      | 0                     | 0                   | 100.00 (100.00)                 |
|                |                                | 0             | 0             | 22                | 0                 | 0                      | 0                      | 0                     | 0                   | 0.00 (100.00)                   |
|                |                                | 0             | 5             | 0                 | 0                 | 0                      | 8                      | 2                     | 0                   | 0.00                            |
|                |                                | 0             | 1             | 3                 | 0                 | 0                      | 18                     | 4                     | 0                   | 69.23                           |
|                |                                | 0             | 9             | 0                 | 0                 | 0                      | 3                      | 19                    | 1                   | 59.38                           |
|                |                                | 0             | 6             | 0                 | 0                 | 0                      | 1                      | 8                     | 10                  | 40.00                           |
| Predicted area |                                |               |               |                   |                   |                        |                        |                       |                     |                                 |
| Observed area  | <i>Illex coindetii</i> EDGE    | Balearic WiSS | Celtic SpSuSS | North Sea SuSS Q1 | North Sea SuSS Q3 | Eastern Sardinian WiSS | Western Sardinian WiSS | South Adriatic WiSpSS | Western Ionian WiSS | Classification success rate (%) |
|                |                                | 0             | 3             | 0                 | 0                 | 0                      | 0                      | 4                     | 3                   | 0.00                            |
|                |                                | 0             | 32            | 1                 | 0                 | 0                      | 0                      | 1                     | 6                   | 80.00                           |
|                |                                | 0             | 0             | 28                | 1                 | 0                      | 2                      | 0                     | 0                   | 90.32 (93.55)                   |
|                |                                | 0             | 0             | 15                | 7                 | 0                      | 0                      | 0                     | 0                   | 31.82 (100.00)                  |
|                |                                | 0             | 2             | 0                 | 0                 | 5                      | 3                      | 1                     | 4                   | 33.33                           |
|                |                                | 0             | 3             | 0                 | 0                 | 0                      | 23                     | 2                     | 0                   | 85.14                           |
|                |                                | 0             | 11            | 0                 | 0                 | 1                      | 0                      | 14                    | 6                   | 43.75                           |
|                |                                | 0             | 7             | 0                 | 0                 | 0                      | 0                      | 3                     | 16                  | 61.54                           |



TABLE 3 | (Continued)

|                                |                    | Predicted area   |                |                     |                     |                             |                             | Classification<br>success rate (%) |
|--------------------------------|--------------------|------------------|----------------|---------------------|---------------------|-----------------------------|-----------------------------|------------------------------------|
|                                |                    | Balearic<br>WiSS | Celtic<br>SuSS | North Sea<br>SpSuSS | North Sea<br>AuWiSS | Western<br>Scotland<br>SpSS | South<br>Adriatic<br>AuWiSS |                                    |
| <i>Loligo forbesii</i> NUCLEUS |                    |                  |                |                     |                     |                             |                             |                                    |
| Observed area                  | Balearic WiSS      | 20               | 5              | 0                   | 2                   | 0                           | 4                           | 64.52                              |
|                                | Celtic SuSS        | 5                | 15             | 0                   | 3                   | 0                           | 1                           | 62.50                              |
|                                | North Sea SpSuSS   | 0                | 0              | 19                  | 25                  | 10                          | 0                           | 35.19 (62.96)                      |
|                                | North Sea AuWiSS   | 0                | 1              | 18                  | 41                  | 3                           | 2                           | 63.08 (90.77)                      |
|                                | W. Scotland SpSS   | 0                | 0              | 12                  | 24                  | 14                          | 0                           | 28.00                              |
|                                | S. Adriatic AuWiSS | 3                | 5              | 0                   | 1                   | 0                           | 9                           | 50.00                              |
| <i>Loligo forbesii</i> EDGE    |                    |                  |                |                     |                     |                             |                             |                                    |
| Observed area                  | Balearic WiSS      | 15               | 9              | 0                   | 2                   | 0                           | 4                           | 48.39                              |
|                                | Celtic SuSS        | 1                | 16             | 0                   | 6                   | 0                           | 1                           | 66.67                              |
|                                | North Sea SpSuSS   | 0                | 0              | 45                  | 7                   | 2                           | 0                           | 83.33 (96.30)                      |
|                                | North Sea AuWiSS   | 4                | 0              | 16                  | 41                  | 0                           | 4                           | 63.08 (87.69)                      |
|                                | W. Scotland SpSS   | 0                | 0              | 5                   | 5                   | 39                          | 0                           | 79.59                              |
|                                | S. Adriatic AuWiSS | 1                | 8              | 0                   | 7                   | 0                           | 4                           | 20.00                              |

**TABLE 4** | Differences in element concentration between statolith nucleus and edge (*t*-test or Mann–Whitney *U*-test) of *Illex coindetii* and *Loligo forbesii* from Balearic Sea, Celtic Sea, North Sea, Sea East of Sardinia, Sea West of Sardinia, Sea West of Scotland, South Adriatic Sea and Western Ionian Sea in the North East Atlantic Ocean and Mediterranean Sea during 2021 to 2022 (SpSS=Spring spawning stock, SuSS=Summer spawning stock, WiSS=Winter spawning stock, AuWiSS=Autumn–Winter spawning stock, SpSuSS=Spring–Summer spawning stock, WiSpSS=Winter–Spring spawning stock). Significant differences ( $p < 0.05$ ) are in **bold**.

|                            |                  | Balearic<br>WiSS | Celtic<br>SpSuSS | North Sea<br>SuSS Q1   | North Sea<br>SuSS Q3   | Eastern<br>Sardinian<br>WiSS | Western<br>Sardinian<br>WiSS | South<br>Adriatic<br>WiSpSS | Western<br>Ionian<br>WiSS |
|----------------------------|------------------|------------------|------------------|------------------------|------------------------|------------------------------|------------------------------|-----------------------------|---------------------------|
| <i>Illex<br/>coindetii</i> | Na               | 0.116            | 0.292            | <b>0.047</b>           | <b>0.005</b>           | <b>&lt;0.001</b>             | <b>&lt;0.001</b>             | <b>&lt;0.001</b>            | 0.223                     |
|                            | Mg               | <b>0.009</b>     | <b>&lt;0.001</b> | <b>&lt;0.001</b>       | <b>&lt;0.001</b>       | 0.648                        | 0.061                        | <b>&lt;0.001</b>            | 0.224                     |
|                            | Mn               | 0.216            | 0.307            | <b>&lt;0.001</b>       | 0.054                  | 0.973                        | 0.448                        | <b>0.039</b>                | 0.110                     |
|                            | Fe               | 0.648            | 0.851            | 0.601                  | 0.846                  | 0.818                        | 0.568                        | 0.587                       | 0.992                     |
|                            | Co               | 0.515            | 0.864            | 0.530                  | 0.231                  | 0.320                        | 0.815                        | 0.712                       | 0.604                     |
|                            | Cu               | <b>0.013</b>     | <b>&lt;0.001</b> | <b>&lt;0.001</b>       | <b>&lt;0.001</b>       | <b>&lt;0.001</b>             | <b>&lt;0.001</b>             | <b>&lt;0.001</b>            | 0.618                     |
|                            | Zn               | <b>0.006</b>     | <b>0.006</b>     | <b>0.022</b>           | <b>0.021</b>           | <b>&lt;0.001</b>             | <b>&lt;0.001</b>             | <b>&lt;0.001</b>            | 0.807                     |
|                            | Sr               | <b>&lt;0.001</b> | <b>&lt;0.001</b> | 0.139                  | <b>&lt;0.001</b>       | <b>0.007</b>                 | <b>&lt;0.001</b>             | <b>&lt;0.001</b>            | 0.658                     |
|                            | Ba               | <b>0.046</b>     | <b>&lt;0.001</b> | <b>0.001</b>           | <b>&lt;0.001</b>       | <b>0.002</b>                 | <b>0.001</b>                 | <b>0.033</b>                | 0.164                     |
|                            | Pb               | <b>0.010</b>     | <b>&lt;0.001</b> | 0.265                  | 0.245                  | <b>&lt;0.001</b>             | <b>&lt;0.001</b>             | <b>&lt;0.001</b>            | 0.957                     |
| U                          | <b>&lt;0.001</b> | <b>&lt;0.001</b> | <b>&lt;0.001</b> | <b>0.001</b>           | 0.073                  | <b>&lt;0.001</b>             | <b>&lt;0.001</b>             | 0.456                       |                           |
|                            |                  | Balearic<br>WiSS | Celtic SuSS      | North Sea<br>SpSuSS Q1 | North Sea<br>AuWiSS Q3 | Western<br>Scotland<br>SpSS  | South<br>Adriatic<br>WiSS    |                             |                           |
| <i>Loligo<br/>forbesii</i> | Na               | <b>0.003</b>     | <b>0.014</b>     | <b>0.004</b>           | <b>0.011</b>           | <b>&lt;0.001</b>             | <b>0.406</b>                 |                             |                           |
|                            | Mg               | <b>&lt;0.001</b> | <b>0.001</b>     | <b>&lt;0.001</b>       | <b>&lt;0.001</b>       | <b>&lt;0.001</b>             | <b>&lt;0.001</b>             |                             |                           |
|                            | Mn               | <b>&lt;0.001</b> | <b>0.012</b>     | <b>&lt;0.001</b>       | <b>&lt;0.001</b>       | 0.718                        | 0.391                        |                             |                           |
|                            | Fe               | 0.960            | 0.714            | 0.647                  | 0.989                  | <b>&lt;0.001</b>             | 0.808                        |                             |                           |
|                            | Co               | 0.805            | 0.342            | 0.953                  | 0.964                  | 0.909                        | 0.547                        |                             |                           |
|                            | Cu               | <b>&lt;0.001</b> | <b>0.011</b>     | 0.152                  | 0.574                  | <b>&lt;0.001</b>             | <b>0.011</b>                 |                             |                           |
|                            | Zn               | <b>0.001</b>     | <b>&lt;0.001</b> | <b>0.017</b>           | 0.236                  | <b>&lt;0.001</b>             | <b>0.005</b>                 |                             |                           |
|                            | Sr               | <b>0.005</b>     | <b>&lt;0.001</b> | <b>&lt;0.001</b>       | 0.412                  | <b>&lt;0.001</b>             | <b>0.013</b>                 |                             |                           |
|                            | Ba               | <b>&lt;0.001</b> | 0.671            | <b>&lt;0.001</b>       | <b>&lt;0.001</b>       | <b>&lt;0.001</b>             | 0.179                        |                             |                           |
|                            | Pb               | <b>0.004</b>     | <b>&lt;0.001</b> | <b>0.002</b>           | <b>0.006</b>           | <b>&lt;0.001</b>             | <b>0.035</b>                 |                             |                           |
| U                          | <b>0.011</b>     | <b>&lt;0.001</b> | <b>&lt;0.001</b> | <b>&lt;0.001</b>       | <b>0.027</b>           | 0.221                        |                              |                             |                           |

*L. forbesii* from the Sea West of Scotland had the highest concentrations of Cu, Pb and Zn at the edge of the statolith, although the source of these elements was not clear, which suggests the use of feeding grounds separated from the hatching area that potentially included prey that had already accumulated these metals in tissues. *L. forbesii* hatches in the western English Channel and Celtic Sea (Laptikhovsky et al. 2022), before migrating through the English Channel into the southern North Sea and returning to the Celtic Sea to spawn (Holme 1974). Alternatively, migration into the North Sea could also be from northern Ireland or the Sea West of Scotland into the northern North Sea (Waluda and Pierce 1998). A relatively high number of squid from the Celtic spring–summer spawning stock (25%) were misclassified to the North Sea autumn–winter spawning

stock, based on element composition at the statolith edge. These squid did not likely migrate from the North Sea back into the Celtic Sea, because the shortest migration route through the English Channel spans over 560 km (López Solano et al. 2022), which is impossible for *L. forbesii* to cover in 2 weeks (statolith edge ablation point) at the average swimming speed of 3.0 km/day for *L. reynaudii* (Sauer et al. 2000). Furthermore, Celtic Sea squid that were misclassified based on statolith microchemistry to the North Sea autumn–winter spawning stock were not the same as those misclassified to the North Sea based on statolith shape analysis (Bobowski et al. 2024). Consequently, these squid more likely stayed in a region with water parameters similar to the North Sea where squid hatched in autumn–winter, perhaps separated from correctly classified squid that were primarily

found in a considerably more southern area than misclassified squid in the Celtic Sea. However, seasonal or annual differences in element incorporation could affect classification.

Microelement composition at the edge of *L. forbesii* statoliths suggested that squid caught in the southern Adriatic Sea differed from those caught in the Balearic Sea, with two separate stocks and hatching areas, similar to results of statolith shape analysis (Bobowski et al. 2024). In contrast, the similar microelement composition of the statolith nucleus we found supported the possibility of a common stock, like an earlier genetic analysis that showed a linkage between the two regions (Göpel et al. 2022). However, squid from the two areas may be separate stocks, with paralarval stages that may have been exposed to similar abiotic conditions after hatching in winter, which aligns with the November to July period when most *Loligo* spp. eggs are typically found in the Mediterranean (Laptikhovsky et al. 2025). Additionally, squid from the Adriatic winter spawning stock were the least correctly classified (20%), based on the elemental composition at the statolith edge, with 75% misclassified to the Celtic summer spawning and North Sea autumn-winter spawning stocks combined. Squid from the Balearic winter spawning stock were also misclassified (37%) for the same North East Atlantic areas based on element composition of the statolith edge. The Adriatic Sea is relatively narrow, bordered by land on three sides and influenced by the river Po, which may have influenced inputs of elements to cause high variability in nearshore seawater composition. For instance, harbours and estuaries in coastal areas of the southern Adriatic are critical hotspots for Zn, Cu, Mn and Ni (Accornero et al. 2004). Moreover, the Adriatic is an upwelling area (Vilibić and Orlić 2002) and this hydrographic element could further concentrate microelements mentioned above. Elevated concentrations of Cu and Zn at the statolith edge of *L. forbesii* from the Adriatic Sea reflect this pollution. However, squid from the North Sea had lower values of these trace metals in their statolith edge, whilst squid from the Adriatic winter spawning stock ranged widely. This suggests that misclassification between these two spawning stocks was caused by squid with low trace metal concentrations in the Adriatic Sea or by similar element concentrations as in squid from the North Sea. Misclassification of squid from both Mediterranean areas was lower in early life stages, but they were misclassified as Celtic summer spawning or North Sea autumn-winter spawning stocks. A small number of squid from Celtic summer spawning and North Sea autumn-winter spawning stocks were also misclassified to the Balearic and Adriatic winter spawning stocks based on microchemical analysis. Misclassification of squid between areas in the North East Atlantic Ocean and Mediterranean Sea based on statolith nucleus microelements may have been linked to depth differences of eggs because similar water parameters could exist at different depths in these areas. However, because only squid from areas in the Mediterranean Sea and North Sea did not differ at early life stages, the effect of depth was likely negligible. Furthermore, misclassification of elemental composition was higher at the statolith edge than at the nucleus, so early life stages may reside at more specific locations with less seawater element variability (possibly caused by pollution) than maturing and mature individuals. Last, misclassification of *L. forbesii* between North East Atlantic and Mediterranean may have been caused by the tendency of cephalopods to accumulate certain environmental microelements (e.g., Zn, Cu, Fe and Sr.; Lourenço et al. 2009). In

polluted environments, such accumulation could further obscure differences amongst areas. Overall, results of our study were similar to results of shape analysis of the same individuals (Bobowski et al. 2024), with higher or similar correct classification to most areas for both species in the life stage before capture (i.e., statolith edge).

## 5 | Conclusions

Our study clearly showed the potential and limitations of statolith microchemistry for stock discrimination. Stock discrimination in squid is highly complex, and a single approach is likely insufficient to comprehensively discriminate all stocks. Microchemistry of the statolith edge reflects the influence of water parameters or diet close to the time of capture, but provides no information about primary habitat prior to the time of capture. Nevertheless, in combination with elemental ratios at the statolith nucleus and age estimates, statolith edge microchemistry provided a good indication of whether individuals from the same capture area shared the same hatching area. However, to accurately determine the primary habitat of squid, shape analysis is preferable to microchemical analysis based solely on nucleus and edge measurement. Of all elements we measured, Co and Fe best separated capture areas. However, when all elements were considered, both species were incorrectly classified to some areas, especially the squid in the Mediterranean Sea. Therefore, we recommend that for a correct and detailed stock differentiation, including hatching area, migration pathway and the main residence area, microchemistry analysis of transect measurements from nucleus to edge be combined with shape analysis and age estimation. Additionally, based on high concentrations of statolith trace metals in some life stages and areas, we assume that microchemistry could be used as an indicator for trace metal pollution in marine areas or prey organisms.

## Acknowledgements

B.T.C.B. thanks Dr. Anne Sell and the technical assistant Samira Peter for their support in the laboratory as well as Brigitte Stoll and Ulrike Weis for their supervision and support with the microchemistry analysis. She would also like to thank Marilena Donnalioia for providing individuals from the Adriatic Sea as well as their associated biological data. Furthermore, thanks to the crew and scientific officers of the *RV Dana* and *RV Celtic Explorer* who supplied samples, especially Susan Maersk Lusseau and Dr. Eoghan Kelly. Last but not least, we thank the anonymous reviewers and Michael J Hansen for their comments, which have improved the manuscript. Open Access funding enabled and organized by Projekt DEAL.

## Ethics Statement

The authors have nothing to report.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.



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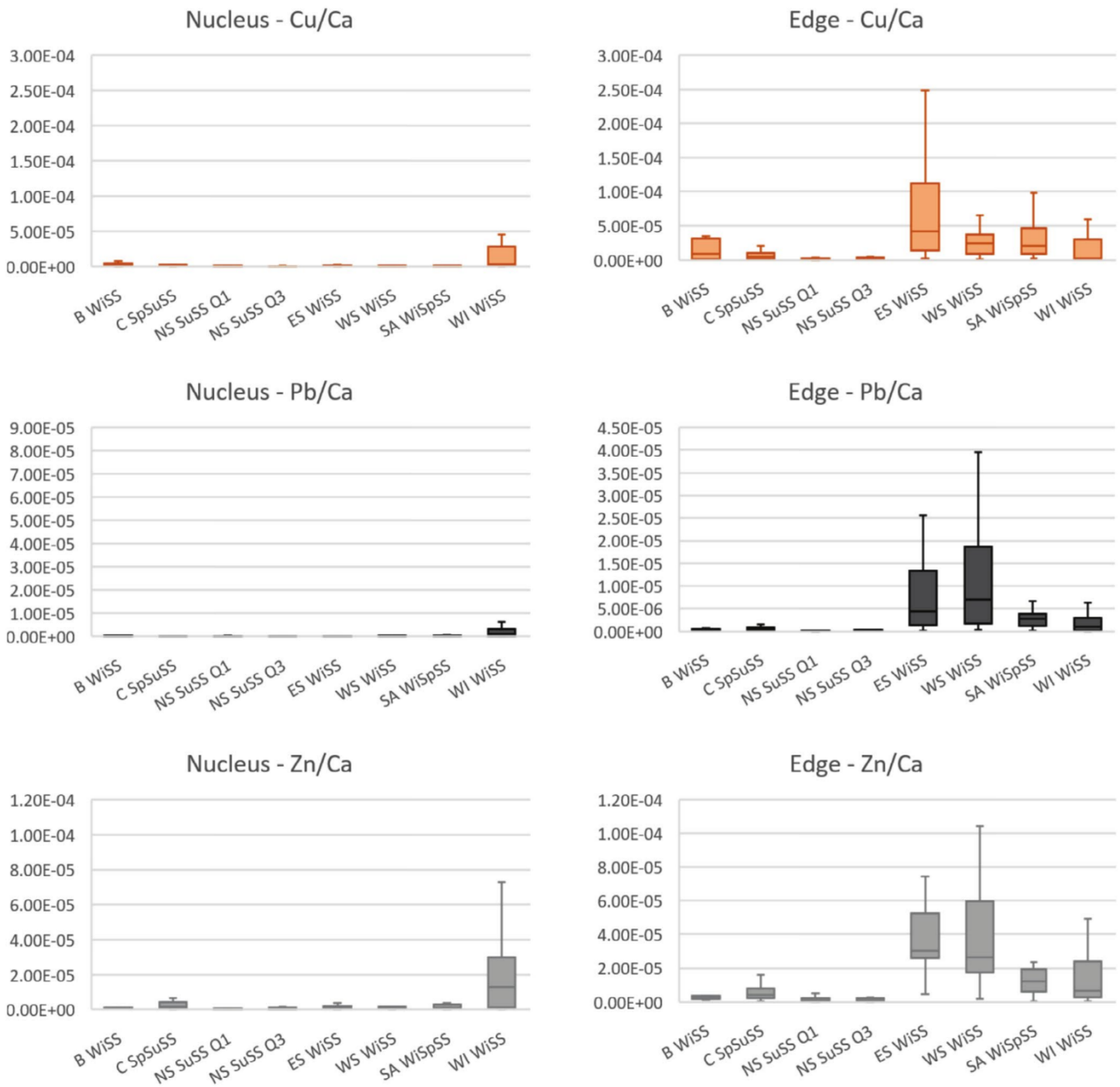
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Appendix A

Figures A1 and A2

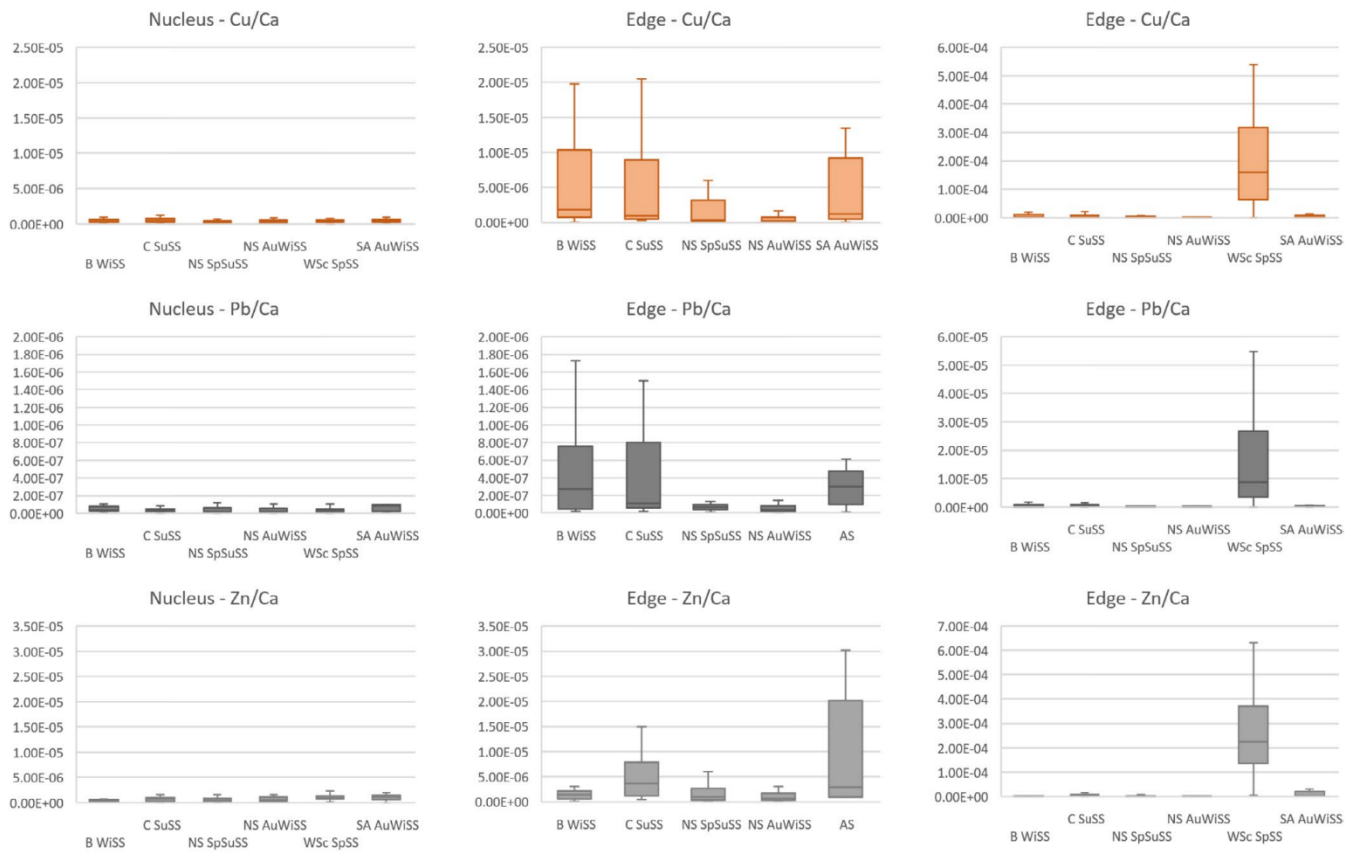
*Illex coindetii*



**FIGURE A1** | Concentration of copper (Cu), lead (Pb) and zinc (Zn) at the nucleus and edge of statoliths from *Illex coindetii* sampled in the Balearic Sea (B WiSS), Celtic Sea (C SpSuSS), North Sea (NS SuSS Q1 = captured in Q1; NS SuSS Q3 = captured in Q3), Sea East of Sardinia (ES WiSS), Sea West of Sardinia (WS WiSS), South Adriatic Sea (AS WiSpSS) and Western Ionian Sea (WI WiSS) in the North East Atlantic Ocean and Mediterranean Sea during 2021–2022 (AuWiSS=Autumn–Winter spawning stock, SpSS=Spring spawning stock, SpSuSS=Spring–Summer spawning stock, SuSS=Summer spawning stock, WiSS=Winter spawning stock, WiSpSS=Winter–Spring spawning stock). Data distributions with quartiles; box for Q1 to Q3 whiskers extend to minimum and maximum values within 1.5 times the interquartile range.



# *Loligo forbesii*



**FIGURE A2** | Concentration of copper (Cu), lead (Pb) and zinc (Zn) at the nucleus and edge of statoliths from *Loligo forbesii* sampled in the Balearic Sea (B WiSS), Celtic Sea (C SuSS), North Sea (NS SpSuSS=captured in Q1, NS AuWiSS=captured in Q3), Sea West of Scotland (WSc SpSS), South Adriatic Sea (SA AuWiSS) in the North East Atlantic Ocean and Mediterranean Sea during 2021–2022 (AuWiSS = Autumn–Winter spawning stock, SpSS = Spring spawning stock, SpSuSS = Spring–Summer spawning stock, SuSS = Summer spawning stock, WiSS = Winter spawning stock). Data distributions with quartiles; box for Q1 to Q3 whiskers extend to minimum and maximum values within 1.5 times the interquartile range. Two visualisations for microchemical edge data: right column = all sampled areas, and middle column = excluding West Scotland Spring spawning stock, where extreme values were recorded.



#### IV. The reproductive ecology of *Loligo* squids (Cephalopoda: Myopsida) in Lusitanian Zoogeographical Province

## Contribution letter Manuscript IV

### The reproductive ecology of *Loligo* squids (Cephalopoda: Myopsida) in Lusitanian Zoogeographical Province

Laptikhovsky, V., Power, A.M., Moreno, A., Oesterwind, D., Perales Raya, C., Pierce, G.J.,  
Robin, J.P., Sobrino, I., Valls Mir, M., Villanueva, R., Allen, A., Abad, E., Bello, G., Bobowski,  
B.T.C., Fernández-Álvarez, F.Á., Cabanellas Reboledo, M., Gonzalez, A., Hernández Urcera, J.  
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Seixas, S.

This manuscript has been published in Regional Studies in Marine Science:

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The final published version is available at [DOI: 10.1016/j.rsma.2025.104506]

The version included in these PhD submission copies is the Accepted Manuscript (Preprint).

I was responsible for a significant part of the research, including the collection and documentation of data on relevant egg sightings through various Citizen Science sources with the egg location coordinates. I prepared these data, summarized them with additional data from other co-authors, and provided them to the first author. I reviewed the drafts of the manuscript and provided constructive feedback and suggestions for improvement.

Based on the CRediT taxonomy (Brand et al., 2015), my contributions to this manuscript were as follows:

- Investigation
- Data Curation
- Writing – Review and Editing

## Signatures of consent



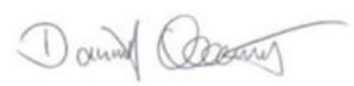
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Brand, A., Allen, L., Altman, M., Hlava, M., Scott, J., 2015. Beyond authorship: Attribution, contribution, collaboration, and credit. Learn. Publ. 28, 151-155. <https://doi.org/10.1087/20150211>

# Regional Studies in Marine Science

## The reproductive ecology of *Loligo* squids (Cephalopoda: Myopsida) in Lusitanian Zoogeographical Province --Manuscript Draft--

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| Manuscript Number: | RSMA-D-25-01286                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| Article Type:      | Research paper                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Keywords:          | <i>Loligo vulgaris</i> ; <i>Loligo forbesii</i> ; climate; reproduction; citizen science                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| Abstract:          | <p>Summary Analysis of temporal and spatial distribution of 686 egg masses and egg mass groups recorded by recreational divers and posted in the various public media, scientific survey data collected during research programs and publications in peer-reviewed literature demonstrated that the reproduction of <i>Loligo</i> squids occurs throughout the Lusitanian zoogeographical province all year round with varying seasonal peaks. This may be due to high phenotypic plasticity, with the life cycle adapting to local conditions, or the existence of small discrete stock units related to individual water features. In warmer and relatively productive waters the spawning peak is more extended, and percentage of mature females in catches is lower. In the Mediterranean the peak of occurrence of both mature females and spawned eggs gradually shifts to earlier dates from the west to the east, which is in line with respective changes of the earlier peak of productivity. There is little or no gap between peak of occurrence of mature females and peak of the egg mass records. Spawning grounds of <i>L. vulgaris</i> expand much deeper than thought before and in the Mediterranean area they extend from 3 m to 550 m. Egg masses are reported by recreational scuba divers from deeper locations in the central Mediterranean than in other areas. <i>Loligo forbesii</i> egg masses are found between 170 and 720 m.</p> |

# The reproductive ecology of *Loligo* squids (Cephalopoda: Myopsida) in Lusitanian Zoogeographical Province

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## Summary:

Analysis of temporal and spatial distribution of 686 egg masses and egg mass groups recorded by recreational divers and posted in the various public media, scientific survey data collected during research programs and publications in peer-reviewed literature demonstrated that the reproduction of *Loligo* squids occurs throughout the Lusitanian zoogeographical province all year round with varying seasonal peaks. This may be due to high phenotypic plasticity, with the life cycle adapting to local conditions, or the existence of small discrete stock units related to individual water features. In warmer and relatively productive waters the spawning peak is more extended, and percentage of mature females in catches is lower. In the Mediterranean the peak of occurrence of both mature females and spawned eggs gradually shifts to earlier dates from the west to the east, which is in line with respective changes of the earlier peak of productivity. There is little or no gap between peak of occurrence of mature females and peak of the egg mass records. Spawning grounds of *L. vulgaris* expand much deeper than thought before and in the Mediterranean area they extend from 3 m to 550 m. Egg masses are reported by recreational scuba divers from deeper locations in the central Mediterranean than in other areas. *Loligo forbesii* egg masses are found between 170 and 720 m.

**Key words:** *Loligo vulgaris*, *Loligo forbesii*, climate, reproduction, citizen science

## INTRODUCTION

The Lusitanian Zoological Province (LZP) encompasses the East Atlantic warm temperate marine waters from the Bay of Biscay in the north to southern Morocco including the Mediterranean Sea and the archipelagos of the Azores, Madeira and the Canary Islands (Almada et al. 2013). The commercial squid fauna of this region is dominated by two species of the family Loliginidae Lesueur, 1821: the European squid *Loligo vulgaris* Lamarck, 1798 and the veined (northern) squid *Loligo forbesii* Steenstrup, 1856. Both squids are highly commercially relevant species (ICES 2023). The ecology of *L. vulgaris* and *L. forbesii* is well known and comprehensively summarised in a range of publications (e.g. Mangold-Wirz 1963; Moreno et al. 2002, 2013, Pierce et al. 2013, 2015). Both species are nektonic and neritic and inhabit shelf waters being most abundant between 10–150 m (*L. vulgaris*) and 150–400 m (*L. forbesii*) with the maximum recorded depths of 550 m and 730 m respectively. The shallowest records of *L. forbesii* in the Mediterranean occur at depths of 70–80 m, although in cold northern waters it might occur at depths as shallow as 10–20 m (Jereb et al. 2015). Both species lay finger-like egg capsules in clusters attached to the sea floor (Laptikhovsky et al. 2021). *L. vulgaris* is known to spawn at depths of 2–120 m, mostly < 50 m whereas *L. forbesii* reproduce in slightly deeper waters, usually 10–150 m (North Sea) but occasionally at 300–500 m with depths exceeding 700 m in the eastern Mediterranean (Pierce et al. 2015).

In the LZP, *L. vulgaris* is the most common shallow-water species and consists of several genetic components e.g. Western Saharan, Atlantic, Western Mediterranean, Eastern Mediterranean (Moreno et al. 2013). At present, studies on the genetic structure of *L. vulgaris* across its full distribution range are scarce. A local study in the Adriatic found high gene flow and panmixia within that area (Garoia et al. 2004), while a preliminary study based on three microsatellites suggest strong differentiation between populations of the Western Mediterranean, Eastern Mediterranean and Atlantic *L. vulgaris* (ICES 2005). *Loligo forbesii* is rare throughout LZP and its range has been gradually shifting northward over recent decades (Chen et al. 2006, Oesterwind et al. 2022). The species is characterised by a complex population structure (Shaw et al. 1999; Göpel et al. 2022) and, based on statolith shape analysis, it is very likely that several stocks exist (Sheerin et al. 2022; Bobowski et al. 2024). Herein we consider the “population” as a biological unit whereas “stock” is a fisheries management unit; both words often mean the same in

practice, but a population might consist of several genetic or ecological stocks (e.g. alternative seasonal cohorts in *Doryteuthis gahi* – Roa-Ureta and Arkhipkin, 2007). Interestingly, Shaw et al. (1999) suggested that the Azorean population of *L. forbesii* is so molecularly divergent that a different subspecies could be recognized. Recent reports of *L. forbesii* occurrence and even high abundances in the Red Sea (Gabr and Riad, 2008; Riad, 2020, 2022) are not confirmed as, historically, this species is not considered to inhabit that area (Jereb and Roper 2010).

This study combines information published in peer-reviewed journals with data from public media supplemented by some unpublished materials provided by the authors. The environmental data have been downloaded from publicly available sources. The study aimed to explore seasonal and spatial variability of spawning grounds of both species based on the occurrence of egg masses, as recorded by recreational divers and in research surveys, together with literature information throughout the area from the Canary Islands to the shores of Greece and Turkey. The combination of this *ad hoc* Citizen Science information with data on spatial and seasonal occurrence of spawning females and on seasonal variability of water temperature and productivity, will help to define specific areas of seasonal spawning if such areas exist. Furthermore, both squids typically live for around one year and spawn only once in their short life. Therefore, unusually high egg masses mortality during a particular year might have harsher consequences for cephalopods than for fish reproducing during many years. Thus, improved knowledge of the seasonal and spatial distribution of loliginid spawning will provide an opportunity for protection of the most important spawning grounds supporting the sustainable use of these commercially important resources (Laptikhovsky et al. 2021).

## MATERIALS AND METHODS

### 1. Literature data on spawning seasonality

Monthly female maturity data (i.e. “% mature individuals”) for *L. vulgaris* and *L. forbesii* were obtained for a range of different areas across the LZP from published studies. Where possible, female maturity data were obtained directly from the authors or from tables in the published sources. Where these data were not available (as was often the case), data were instead extracted from graphic representations using WebPlotDigitizer v4.6 (distributed under the GNU Affero General Public License Version 3). In instances where monthly data were available for more than one year, a monthly average was calculated. Months with apparent zeros were assumed to be months with missing data unless the absence of mature females was stated explicitly in the text. Details of data sources, years sampled, and relevant maturity scale information are shown in Table 1, along with relevant scientific publications where more details can be obtained. Females were considered to be mature when having ovulated eggs in the oviduct, which falls into different stages of the various maturity scales (all details are provided in Tables 1 and 2).

**Table 1 Metadata on data sources and literature used to digitise data on % mature females in *Loligo vulgaris***

| Location          | Area              | Authors    | Years sampled        | Sample size (females) | Maturity scale used    | Maturity stage plotted | Notes/ Reference         |
|-------------------|-------------------|------------|----------------------|-----------------------|------------------------|------------------------|--------------------------|
| Northern Atlantic | N France          | This study | Nov. 1997- June 1999 | 801                   | Boyle and Ngoile, 1993 | IV + V                 | Moreno et al. 2002       |
|                   | Galicia, Spain    | This study | Apr. 1991- June 1992 | 218                   | Boyle and Ngoile, 1993 | IV + V                 | Guerra and Rocha, 1994   |
|                   | W Portugal        | This study | 1990-2002            | 9630                  | Boyle and Ngoile 1993  | IV + V                 | Moreno et al. 1994; 2002 |
|                   | Algarve, Portugal | This study | Jan. 1991- June 1992 | 487                   | Ngoile, 1987           | IV + V                 | Coelho et al. 1994       |

|                                             |                            |                                   |                      |                                            |                        |                                                                      |                                                                                                                                       |
|---------------------------------------------|----------------------------|-----------------------------------|----------------------|--------------------------------------------|------------------------|----------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|
|                                             | Gulf of Cadiz, Spain       | Vila et al., 2010                 | Apr. 2005- Dec. 2007 | n=1967 (both sexes, # females unspecified) | Lipinski, 1979         | IV + V                                                               |                                                                                                                                       |
| <b>African Atlantic &amp; Mediterranean</b> | Balearic Islands           | Valls (unpublished)               | 2009-2021            | 1153                                       | ICES 2010              | III + IV                                                             | ICES, 2010                                                                                                                            |
|                                             | Catalan Sea                | Mangold-Wirz 1963                 | 1954-1960            | N/A                                        | Mangold-Wirz 1963      | 'Mature' i.e. females with eggs that are are ripe / ready to be laid | Egg size was a key characteristic in maturity estimation                                                                              |
|                                             | Croatia (Central Adriatic) | Krstulović Šifner and Vrgoč, 2004 | Jan. 1998- Aug. 2000 | 666                                        | Lipinski 1979          | III                                                                  | 4-stage scale used (stage 0 - III)                                                                                                    |
|                                             | Greek Seas                 | Moreno et al. 2002                | Jul. 1997- June 1999 | 880 (both sexes, # females unspecified)    | Boyle and Ngoile, 1993 | 'Mature'                                                             | Stage(s) not specified                                                                                                                |
|                                             | Agadir, Morocco            | Baddy 1989                        | May 1985- May 1986   | 760 females                                | Hixon 1980             | IV                                                                   | 5-stage scale (stage V is spent). The table which the data came from probably only includes mature 'stage IV' (no 'maturing' females) |
|                                             | Western Sahara 1           | Srairi et al. 2023                | Mar 2000- Oct 2014   | 11,062                                     | unknown                | III                                                                  | 3-stage scale, 'mature' females shown. 'Maturing' are omitted                                                                         |
|                                             | Western Sahara 2           | Perales-Raya et al. 1999          | May 1993- April 1994 | 660 females                                | Lipinski 1979          | IV + V                                                               |                                                                                                                                       |
|                                             |                            |                                   | <b>TOTAL</b>         | <b>n &gt;25,437</b>                        |                        |                                                                      |                                                                                                                                       |

Metadata on maturity of *L. forbesii* are scarce due to the relatively low occurrence of this species in the study area and where data are available, these are generally based on small sample sizes (Table 2).

**Table 2 Metadata on data sources and literature used to digitise data on % mature females in *Loligo forbesii***

| Location        | Area           | Authors    | Years sampled          | Sample size (females) | Maturity scale used   | Maturity stage plotted | Notes/ Reference         |
|-----------------|----------------|------------|------------------------|-----------------------|-----------------------|------------------------|--------------------------|
| <b>Atlantic</b> | N France       | This study | 1992-1995              | 466                   | Ngoile 1987           | IV + V                 |                          |
|                 | Galicia, Spain | This study | Feb. 1991 - June 1992  | 103                   | Boyle and Ngoile 1993 | IV + V                 | Guerra & Rocha, 1994     |
|                 | Azores         | This study | Nov. 1990 – March 1992 | 358                   | Ngoile 1987           | IV + V                 | Porteiro & Martins, 1994 |



|                      |                          |                          |                                                    |               |               |                              |                     |
|----------------------|--------------------------|--------------------------|----------------------------------------------------|---------------|---------------|------------------------------|---------------------|
|                      | W Portugal               | This study               | 1990-2002                                          | 902           | Ngoile 1987   | IV + V                       | Moreno et al., 1994 |
| <b>Mediterranean</b> | Sardinia                 | Agus et al., 2018        | 2013-2015                                          | 96            | Lipinski 1979 | 'Mature' (on seasonal basis) |                     |
|                      | Straits of Sicily        | Ragonese and Jereb, 1986 | Aug. 1985-Mar.1986 (Aug-Sep., Nov-Dec., Feb.-Mar.) | 75            | 3 stage scale | N/A                          |                     |
|                      | Balearic Islands         | M.Valls (unpublished)    | 2009                                               | 356           | ICES 2010     | III + IV                     | ICES 2010           |
|                      | Cyclades Plateau, Greece | This study               | 1997-1999                                          | 61            | Ngoile 1987   | IV + V                       |                     |
|                      |                          |                          | <b>TOTAL</b>                                       | <b>n=2417</b> |               |                              |                     |

## 2. Egg masses

Data on observations of a total of 686 egg masses and egg mass groups were collected from 1992 to 2023 from different sources (351 reported by recreational divers, 126 captured during fishing activities of commercial and research vessels, 208 egg masses and group of masses found attached to artificial substrates, and 1 observed by ROV). Information was either personally provided to this study (n=314) or collected from public media (n=361), where it had been posted by recreational divers and citizen scientists (e.g. numerous web pages on Facebook, individual accounts on Instagram, iNaturalist <https://www.inaturalist.org/>, RedProMar <https://redpromar.org/> ) or taken from peer-reviewed publications where exact coordinates were provided (n=11 – Salman and Laptikhovsky, 2002; Pham et al. 2009; Orsi Relini et al. 2009; Carreira et al. 2011; Agus 2015). The dataset included 330 individual egg masses and groups of egg masses of *L. vulgaris*, 13 of *L. forbesii* and 343 classified as “unidentified *Loligo*”. These records are hereafter referred to “spawning sites” regardless of whether one or several egg masses were reported. The dataset covered European and West African shores. No data were available from North African waters of the Mediterranean.

The data we mined from public media (e.g. Facebook and Instagram) are publicly available. We followed recent recommendations for responsible use of social media data in research (Monkman et al. 2018; Di Minin et al. 2021; Sbragaglia et al. 2022), considering data privacy concerns and ensuring compliance with the European Union's (EU) General Data Protection Regulation (GDPR).

## 3. Environmental data

Monthly estimates from 2000 to 2020 of concentration of Chlorophyll A and sea surface temperatures were downloaded from the Global Ocean Physics Reanalysis of the E.U Copernicus Marine Service Information (<https://doi.org/10.48670/moi-00019>, <https://doi.org/10.48670/moi-00021>). These were converted to mean monthly estimates using R (R Core team 2021; Hijmans 2022), and then mapped using ArcGIS (ESRI, 2011).

## RESULTS

## 1. Seasonal occurrence of mature individuals

### 1.1. *Loligo vulgaris*

A substantial amount of data was available in the LZP for *L. vulgaris* (more than 20 000 females, Table 1; Supplementary Tables S1-S3). Comparison of seasonal distribution and intensity of the spawning peaks across the studied region considering also the numbers of studied females (hence data robustness) revealed several types of temporal distribution of the reproductive effort with the following main patterns (Figs. 1,2):

1. All year round spawning with one or several relatively weak seasonal peaks, whose appearance potentially might depend on the sampling pattern – Atlantic waters from off West Africa to the Algarve and Gulf of Cadiz and Balearic Islands. Mature females represent ~40-70% of females during the peak of reproduction.
2. Most reproductive activity occurs in the cold season (December – April) with one obvious peak, when mature females represent ~70-90% of females – from Western Portugal to Northern France and most of the Mediterranean basin

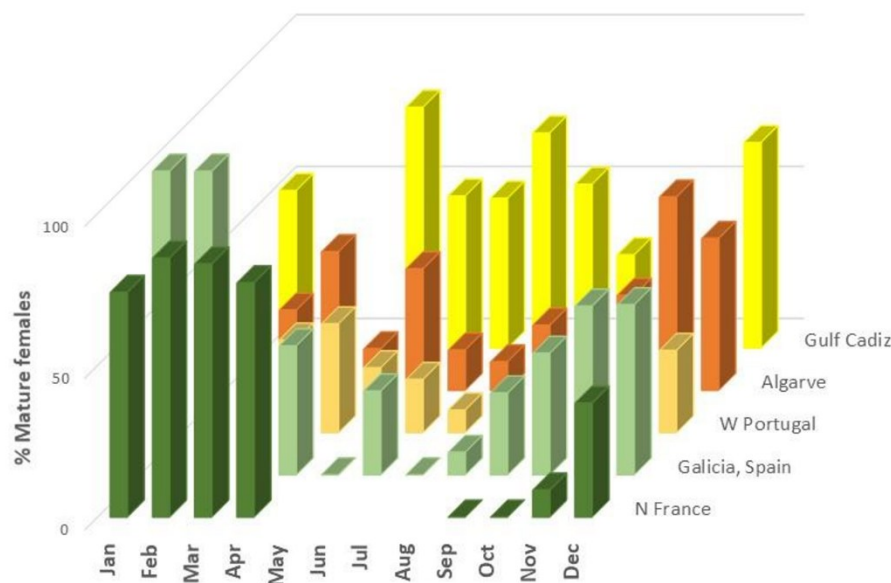


Figure 1. Percentage of mature females of *L. vulgaris* in the northern part of the LZP. Months with no data are left blank and true zeros are recorded. Metadata supporting this figure can be viewed in Table 1 and Supplementary Table S1.

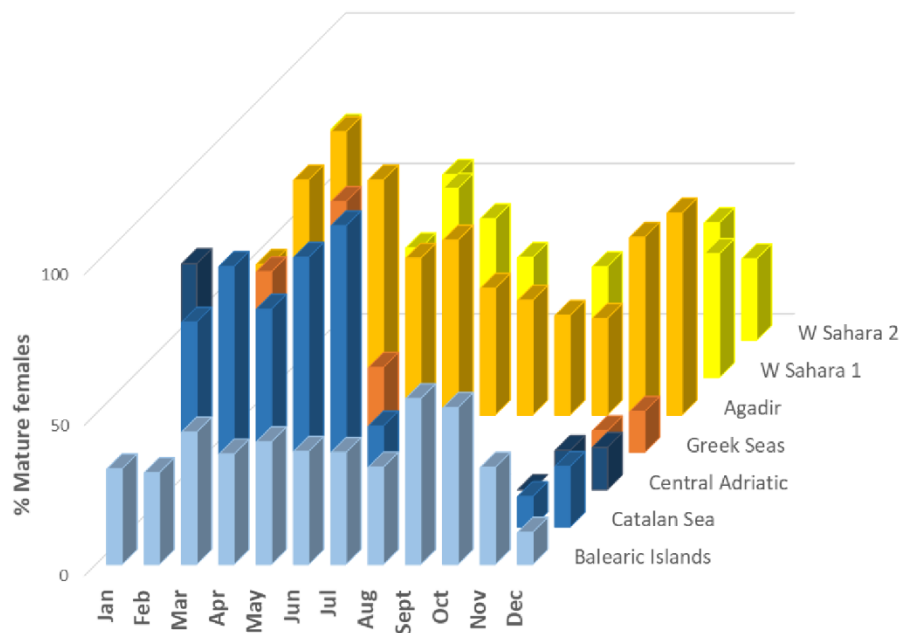


Figure 2. Percentage of mature females of *L. vulgaris* in the southern part of the LZP and the Mediterranean. Months with no data are left blank and true zeros are recorded. Metadata supporting this figure can be viewed in Table 1 and Supplementary Table S2.

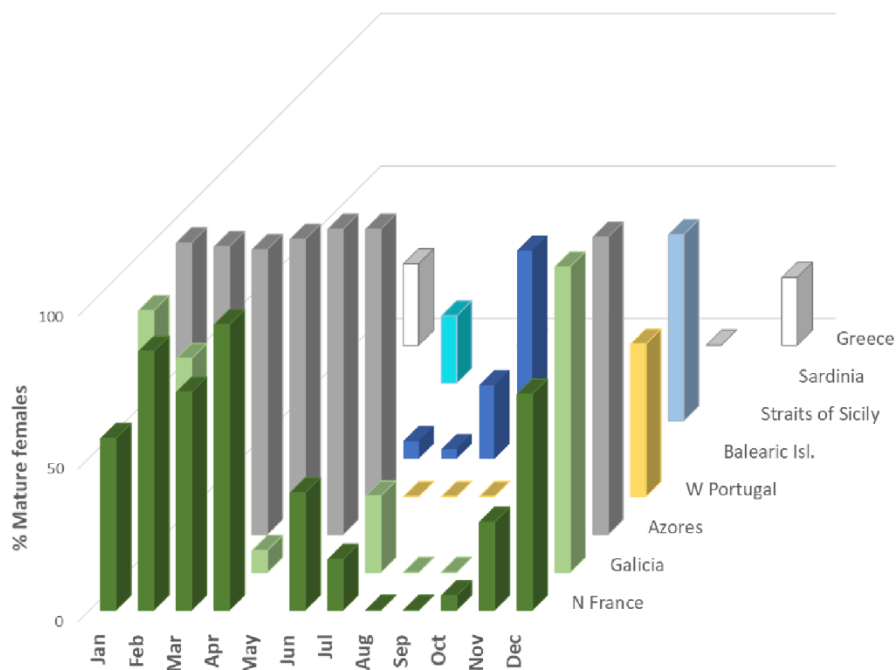


Figure 3. Percentage of mature females of *L. forbesii* in the southern part of the species range. Months with no data are left blank and true zeros are recorded. Metadata supporting this figure can be viewed in Table 2 and Supplementary Table S3.

Data on the spawning of *Loligo forbesii* in the study area are very limited (mature females n=2061, Table 2); however, the observed patterns were quite similar to those of *L. vulgaris* (Figure 3). Around Balearic Islands mature females were found all year round indicating all year-round spawning. The relative occurrence of spawning females across the area was higher in from November to June-July. The spawning peak was clearly evident in colder northern waters but not so obvious in the Mediterranean also because the species is so rare there that spotting any pattern is difficult (Fig. 3).

## **2. Seasonal egg mass occurrence**

The egg masses of squid genus *Loligo* were found throughout offshore waters of southern Europe in all component seas of the Mediterranean (Balearic, Ligurian, Tyrrhenian, Adriatic, Ionian, Sea of Sicily, Aegean and Levantine) (Figure 4). The bulk of identified eggs belonged to *L. vulgaris* with records of *L. forbesii* eggs being extremely rare in both the western and eastern Mediterranean, reflecting the general relatively low abundance of this species in the area.

Egg masses were recorded year- round in the Mediterranean, from the Balearic to the Ionian Sea, although the majority were found between November and July. Nevertheless, during autumn and early winter (November to February), egg masses were mostly reported from the western Mediterranean, whereas later in the season (March - September), they were seemingly equally common in both western and eastern Mediterranean.

Egg masses of *L. vulgaris* were found between 3 m (l'Ametlla de Mar, NE Spain, June 2004, scuba diving) and 556 m depth (NE Spain, December to April 2019-2021, bottom trawl). Divers found egg masses between 3 and 67 m, mean 21.3 m depth (depth records n=139). All egg masses that were identified by professional scientists to the species level, from both diving and catches of research and fishing vessels (depth range 5-556 m, mostly 15-100 m) belonged to *L. vulgaris*.



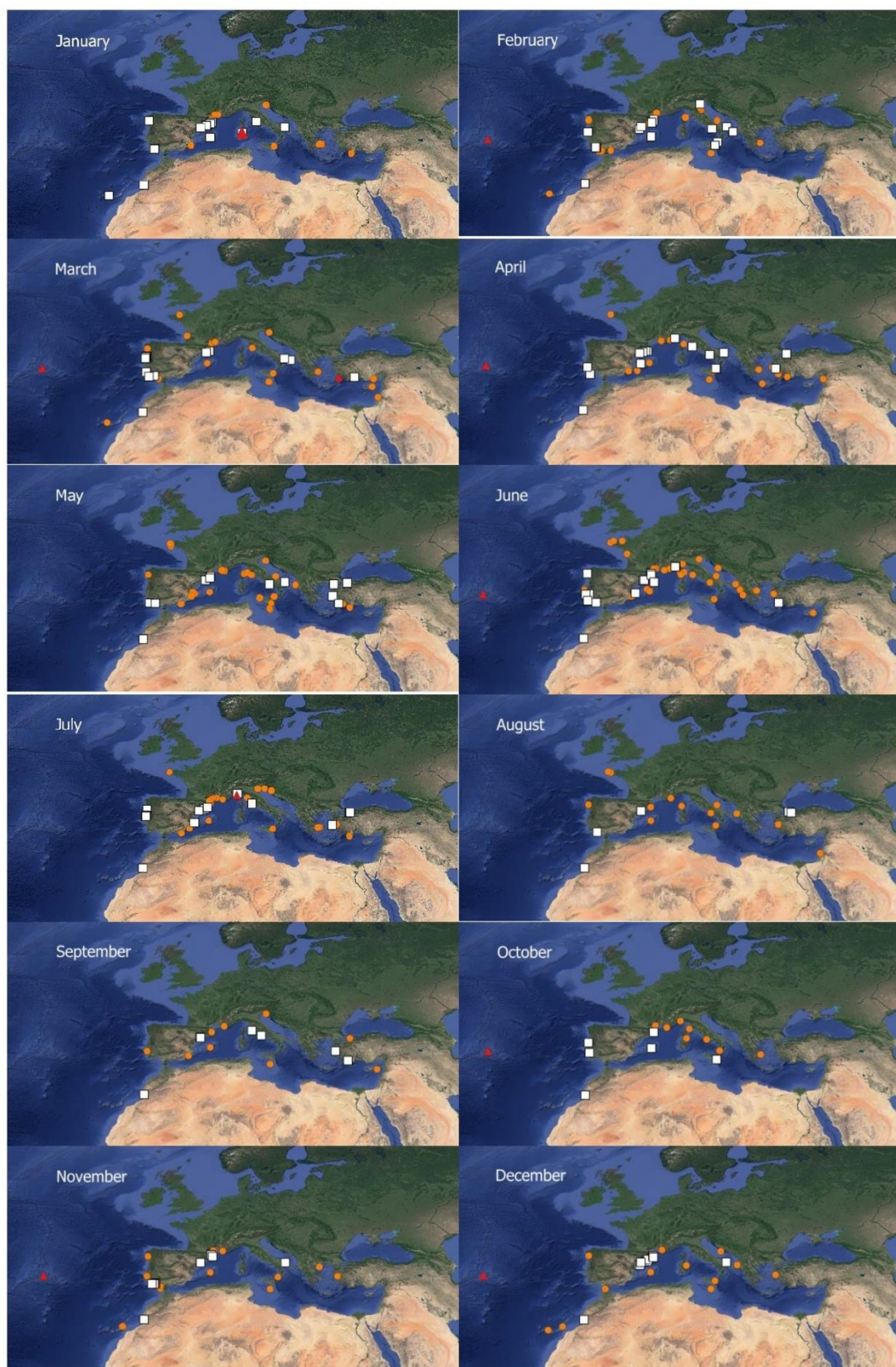


Figure 4. Distribution of egg masses from January to December (white squares – *L. vulgaris*, orange circles – *Loligo sp.*, red triangles – *L. forbesii*).

When only SCUBA-recorded egg masses are taken into consideration the peak of occurrence of egg masses gradually shifted to earlier dates from west to east. In the European Atlantic from Morocco to Galicia the maximum occurrence of spawning sites as recorded by SCUBA divers were observed in June. In the western part of the Mediterranean Sea (Spain, France) it peaked in May - June, in the central part (Italy, Malta, Slovenia, Croatia, Albania) - in April – June, the maximum in May and in the eastern part (Greece, Turkey, Cyprus, Israel) - in April – June also, but the maximum in April (Fig. 5).

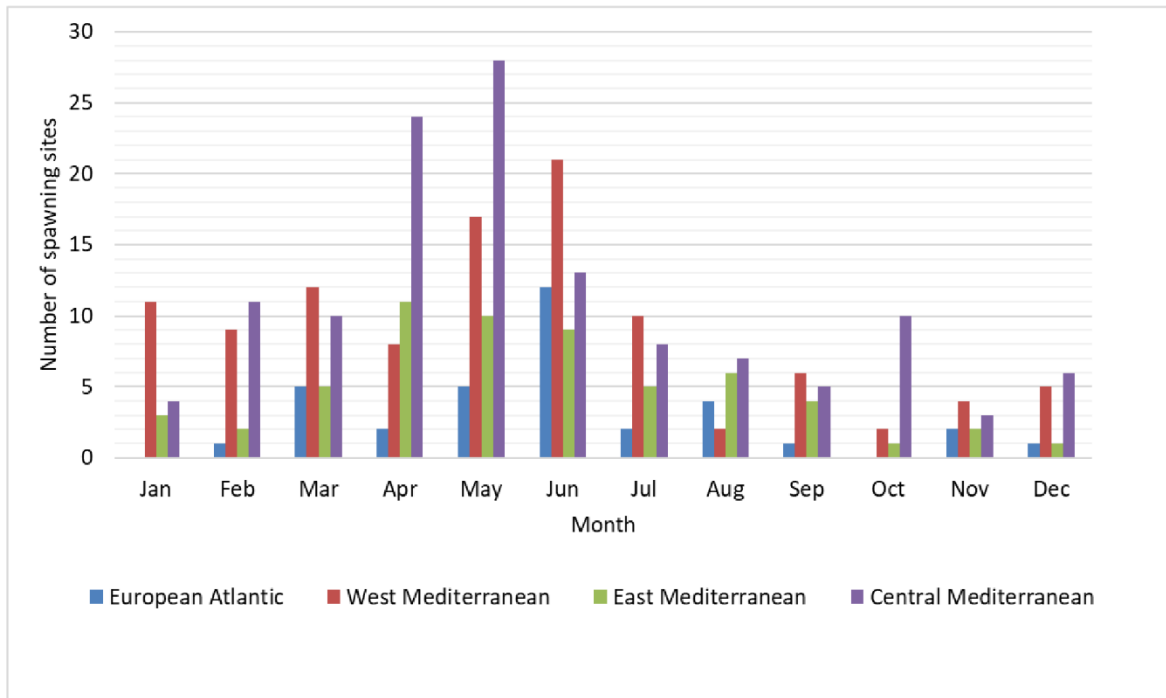


Figure 5. Seasonal occurrence of *L. vulgaris* egg masses in the Mediterranean Sea: West Mediterranean (Spain, France) Central Mediterranean (Italy, Malta, Slovenia, Croatia, Albania) East Mediterranean (Greece, Turkey, Cyprus, Israel) and European Atlantic (west Spain, Portugal).

In all areas the depth range of 11-20 m yielded more SCUBA reports on egg masses than others (Fig. 6). However, in the central Mediterranean relatively high number of egg masses were reported by divers also from depths of 30 to 50 m, the deepest dive being to 67 m. Some 38% of these “deep” egg masses were identified as *L. vulgaris*, the rest reported as unidentified *Loligo* sp.

The seasonal changes in egg deposition do not show any clear pattern with egg masses being found generally shallower in winter and summer than in autumn and spring (Fig. 7).

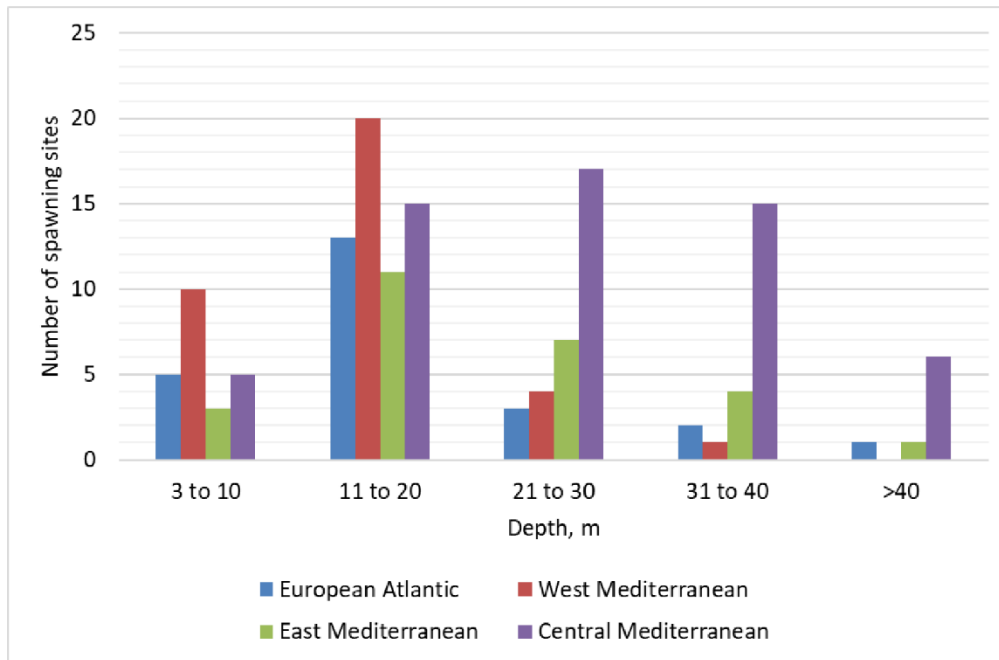


Figure 6. Depth of deposition of egg masses of *Loligo* sp. in the in the different parts of the Mediterranean and European Atlantic as recorded by SCUBA divers.

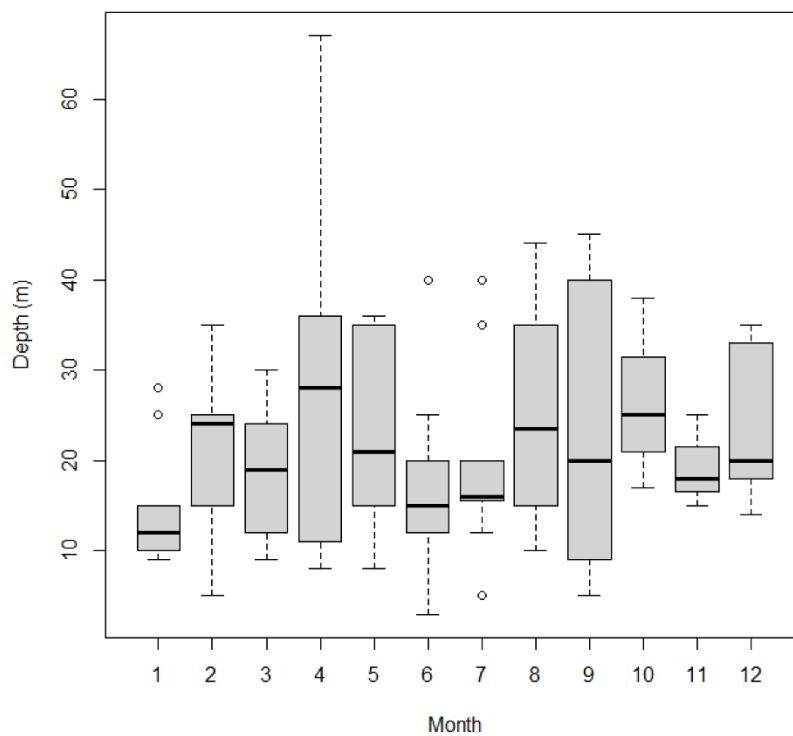


Figure 7. Seasonal changes in depth of deposition of egg masses of *Loligo* sp. as recorded by SCUBA divers (all areas combined). Box representing quartile 1 to quartile 3 of the data, whiskers extending to the minimum and maximum values within 1.5 times the interquartile range, clear circles representing outliers.



### 3. Environmental situation

In the LZP, surface temperatures fluctuate throughout the year, consistently being warmer on the east and south than in the north and west. During spring, the eastern areas warm up earlier than the western areas, which reach similar temperatures about a month later (Fig. 8). The warmest temperatures on both the east and the west occur between July and September.

Chlorophyll *a* concentrations were generally lower in southern waters compared to northern waters of the Mediterranean Basin and they decreased from west to east, with the lowest productivity observed in the southeastern Mediterranean (Fig. 9). Over the course of the year, higher chlorophyll *a* concentrations occur in winter (January – March) following a rapid decline with very low concentrations from April to November, and slow increase starting in December. The northernmost part of the Adriatic Sea maintains relatively high Chlorophyll *a* concentrations all year round.

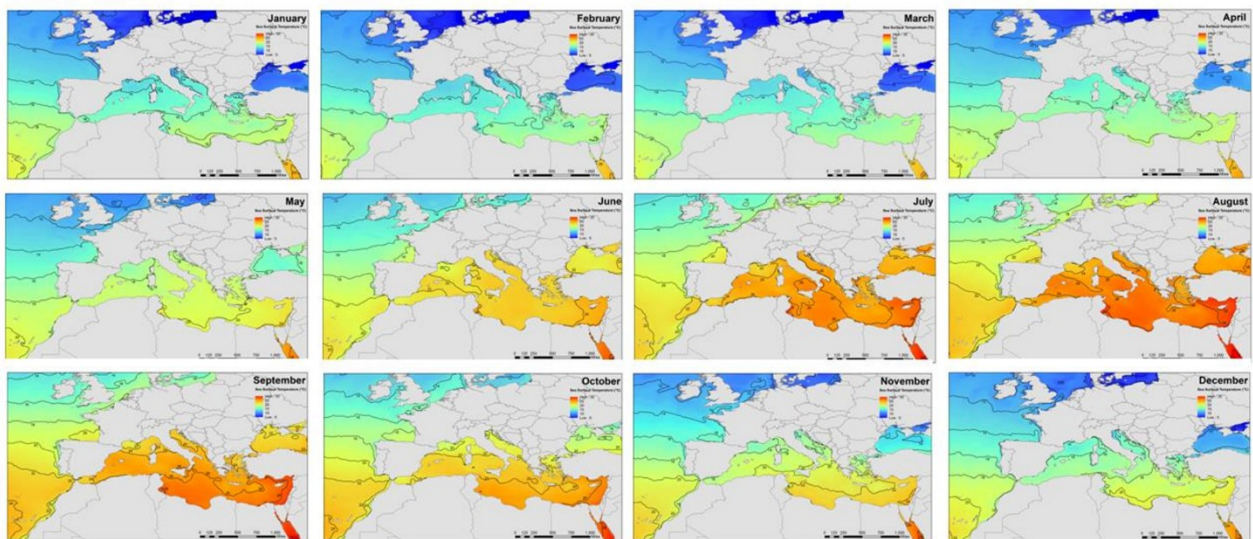


Figure 8. Monthly distribution of the surface temperatures (°C) in the LZP (July-December 2000-2020). From the Global Ocean Physics Reanalysis of the EU Copernicus Marine Service

In the Atlantic areas, chlorophyll *a* levels and their seasonal patterns are closely linked to upwelling events and significantly influenced by nutrient inputs from rivers in coastal areas. Along the northwest Iberian Peninsula to Western Sahara, chlorophyll *a* concentrations on the western coast remain relatively high throughout the year. In contrast, the Gulf of Biscay have moderate chlorophyll *a* levels from November to March, with notably lower levels during the other months, except in certain locations along the western coast of France. Similarly, in southern Portugal and the Gulf of Cadiz, chlorophyll *a* levels are moderate from November to May and decline during the remainder of the year.



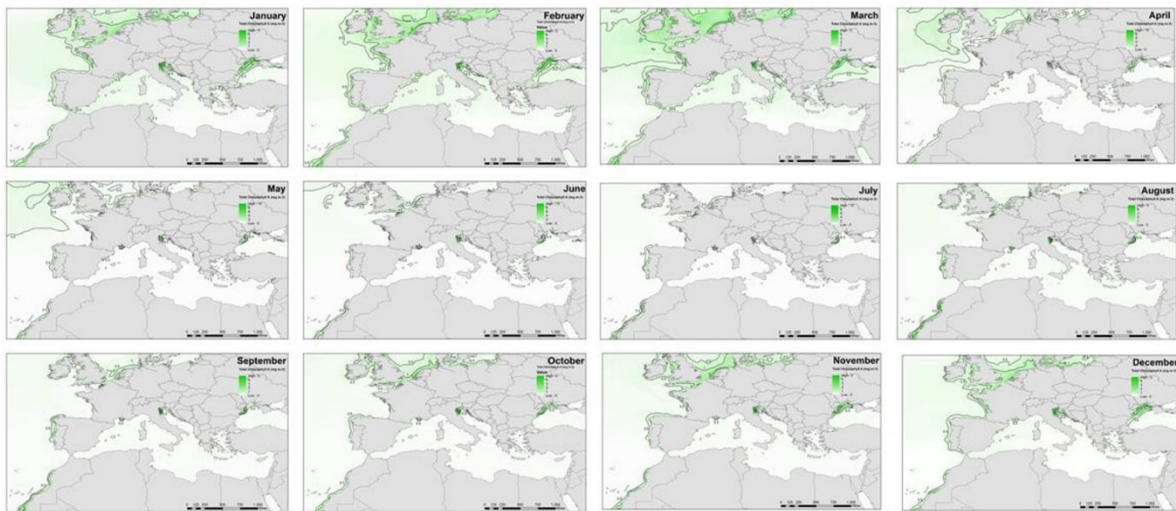


Figure 9. Monthly distribution of Chlorophyll A in the LZP, 2000-2020. E From the Global Ocean Physics Reanalysis of the EU Copernicus Marine Service

## DISCUSSION

Our study provides new insights into the spawning seasonality and intensity of two long-finned (loliginid) squid species in the LZP. Spawning seasonality and intensity are two sides of the same reproductive pattern mirroring each other in annual species like coastal squids of the family Loliginidae. The shorter spawning period - the higher percentage of mature females would be observed in these months

Temperature might be a key factor determining the duration of spawning periods. A higher proportion of the female *L. vulgaris* was mature throughout the year in Atlantic waters off Africa and the Mediterranean compared to more northern waters. This variation could be related to differences in water temperature regimes between areas. For instance, Moreno et al. (2002) showed that average surface water temperatures in N France were 4 °C lower (~13.5 °C), than in NW Portugal (~17.5 °C), and 9 °C lower than the Western Sahara (~22 °C) during the study period.

In addition, average monthly sea surface temperature fluctuations between the different months were greater in N France (8 °C) compared to NW Portugal (6°C) and the Western Sahara (4°C) Warmer temperatures in the southern part of the range, combined with the high productivity of upwelling areas off Northwest Africa and the Iberian Peninsula (the Canary/Iberia Current Upwelling System), may allow for extended spawning periods, though with relatively low numbers of mature females in the population. Prolonged periods of high proportions of mature females were linked to freshly-laid egg masses' observations during the same period; e.g. in Agadir, Morocco, where freshly-laid egg masses (white rather than brown in colour) were collected in all months (Baddy 1989). Because, eggs of *L. vulgaris* develop normally only between 12 and 24°C (Sen, 2005) , at the northern limit of the species' range (Netherlands), *L. vulgaris* seems to be able to reproduce only within a narrow warm-water window in late spring-summer (Tinbergen and Verwey 1945; Oosterwind et al. 2010), though developing egg masses may be found there during most of the year (Laptikhovsky et al. 2021).

Two different spawning patterns of *L. vulgaris* can be seen in the LZP, and appear to be closely related to the regional environmental variability. In the highly productive areas of permanent upwelling off the NW African coast and adjacent areas of the southwest Iberian Peninsula, spawning peaks are weakly defined, with the monthly proportion of mature females during the reproductive period representing ~40-70% of all females. This may arise due to consistently high productivity, which supports the survival of hatchlings throughout the year. As *L. vulgaris* paralarvae are considered generalist predators (García-Mayoral et al.

2020), their flexible prey preferences may play a role in increasing their survival under varying oceanographic conditions. For example, hatching of *L. vulgaris* off Northwest Africa occurs all year round, with an extended seven months' "peak" between January and July (Perales-Raya et al., 1999). The maximum occurrence of mature females in catches predictably occurs some two months earlier, somewhere between November and May, mostly between November and January (Fig. 2, Baddy 1989; Perales-Raya et al 1999).

The spawning peaks are much more pronounced in both colder areas of the north-western Iberian Peninsula and in less productive waters of the Mediterranean basin, where the percentage of mature females during these periods is ~70-90%. However, in these areas there is also a clear non-breeding season. Off the western Iberian Peninsula, the upwelling is seasonal and occurs between May (June) to September (October) (Relvas et al. 2007; Tenore et al. 2008), whereas the maximum relative abundance of mature females in commercial landings is recorded between November and February (Figure 1; Guerra & Rocha, 1994), well before the expected peak of productivity. But considering embryonic development of 30-50 days at water temperatures of 12-15°C (Boletzky and Hanlon, 1983), if we assume that the bulk of spawning itself occurs somewhere between March–April, this would come a month after the peak of mature female occurrence and a month or two before the peak of plankton abundance.

The seas comprising the relatively oligotrophic Mediterranean Basin represent a combination of complicated oceanographic circulation and unidirectional longitudinal changes in temperature, salinity and productivity: environmental factors that potentially might impact spawning seasonality and intensity. Among them, the northern and some coastal parts of the Adriatic Sea represent a separate case of high productivity, extended peak of occurrence of mature females (January - May) with their relative low abundance of ~50-75% (Krstulović Šifner and Vrgoč, 2004; Supplementary Table S2) a pattern somewhat like that of Moroccan and Iberian upwelling areas of the Atlantic coasts.

Across the rest of the Mediterranean basin, the temperature increases, and productivity decreases from west to the east in any season. The productivity peak begins and finishes later in the western part of the Mediterranean than in the eastern part, is about 1/3 longer and has much higher amplitude (Salgado-Hernanz et al. 2019). Predictably, as the west Mediterranean is a more productive area with a longer duration of the main phytoplankton growing season, the period of high relative abundance of mature females is longer than in the Eastern Mediterranean (Fig. 2) and it is also where the greater number of egg masses of *L. vulgaris* were observed though it might be related also with intensity of recreational diving.

The maximum relative abundance of mature females (90-100%) was observed later, peaking in May - June, in the Catalan Sea (Mangold-Wirz, 1963), as compared to April or even earlier in Greek Seas (Moreno et al. 2002). This may be because the peak of productivity occurs in the Western Mediterranean two-three months later than in the eastern part of the sea, although maturity data for the two areas were collected a few decades apart so this interpretation should be treated with caution. Eggs of *L. vulgaris* were found to die before hatching at 26°C (Sen 2005) but, as some egg masses are still found in the Aegean Sea between June and October, high temperatures do not prevent spawning in this species during the heat of summer in eastern areas (Figure 4). These egg masses were reported from depth of 5-40 m at temperatures 16-22°C although we cannot not exclude the possibility that some of them would die due to being occasionally subjected to lethal temperatures. However, spawning females are rare in Greek waters between July and September (Fig. 2, Moreno et al., 2002).

Even if the information obtained by SCUBA divers is somewhat restricted in depth, seasonally biased and limited by site accessibility, the above-mentioned shift of the beginning of the spawning to earlier dates in an easterly direction following the shift of peak of productivity within the Mediterranean Sea is consistent with observations of spawning sites as reported by Citizen Science (Figure 5). The maximum number of

reports from the west Mediterranean appeared in June, from the central Mediterranean in May, and from the east Mediterranean in April, mirroring shift in peak of productivity on one hand, and shifting the peak in the Eastern Mediterranean further away from the possibility to be subjected to lethal temperatures in the late summer.

In the literature, eggs of *L. forbesii* were recorded at much deeper: 25-373 m, mostly > 100 m around Azores (Porteiro and Martins, 1992; Pham et al. 2009; Carreira et al. 2011), ~170 m at Sardinia (Agus 2015), ~600 m in the Ligurian Sea (Orsi-Relini et al. 2009) and ~720 m in the East Mediterranean (Salman and Laptikhovsky, 2002). Therefore, in our further considerations we assume that all unidentified egg masses recorded in the Mediterranean by divers (typical depth range 3-30 m) belonged to this species as *L. forbesii* is not known to occur there shallower than 40-80 m and these squids are most abundant between 200 and 400 m depth (Agus 2015; Jereb et al. 2015).

As egg masses of *L. vulgaris* were found in each sea of the Mediterranean basin and in nearly every month, it rules out a possibility that some large geographic areas off European shores may simply represent foraging grounds of this species with squid reproduction taking place elsewhere, thus exhibiting some long-distance rather than inshore-offshore migration. It means that within revealed Western Mediterranean and Eastern Mediterranean genetic components (Moreno et al. 2013) there might be some small discrete stock units related to individual water features including the complicated surface circulation patterns of Atlantic water with a meandering and eddying flow in the general eastward direction from Gibraltar to Bosphorus (Millot and Taupier-Letage 2005). Existence of such a population was recently confirmed for the West Iberian Peninsula (García-Mayoral et al., 2024) so their existence is possible in the Mediterranean also. Though these stocks might be genetically identical due to larval transport, they still would represent a separate management unit. However, it is still a problem to explore.

Another important result of this study is that *L. vulgaris* spawning grounds extend much deeper than previously known (to ~550 m with intermediate records at 200-370 m), thus providing additional opportunity for transport of hatchlings by subsurface and deep-water circulation currents and also causing an ontogenetic migration of juveniles to forage in shallow waters as it has been described in loliginid squids. In the western Mediterranean, K. Mangold-Wirz (1963) noted that spent female *L. vulgaris* were seen in July, (although mature specimens were still obtained from this time in deeper waters), as well as in August. Juvenile *L. vulgaris* appear in coastal waters from August onwards, with a wave of 'very young animals' moving from depths of 100 m towards the coast in the second half of October (Mangold-Wirz 1963). Some inshore / offshore movement of individuals of different cohorts is seen in the late months of the year (Oct – Dec), which are probably the same individuals associated with large numbers of mature animals seen in Jan/Feb (Mangold-Wirz 1963). In the Balearic Islands, the largest individuals shifted their diet to bottom-associated prey during spring (Valls et al. 2015), probably related to movement to the spawning grounds as observed in *Loligo opalescens* (Karpov and Cailliet 1979).

Distribution of egg mass recorded by SCUBA divers by depth was very similar between the European Atlantic, West Mediterranean and East Mediterranean, with a majority of records coming from the depth range 11-21 m. Eggs were found to be deposited deeper in the Central Mediterranean (around Italy) peaking in reports from depth range 11-40 m mostly 21-30 m. If we exclude the possibility that Italian and Maltese recreational divers go deeper than those from other parts of the Mediterranean, there should be some environmental explanation (e.g. coastal bathymetry) or biological reasons (e.g. lower competition with *L. forbesii*), which is not obvious at this stage of research.

Our knowledge of maturity and reproduction of *L. forbesii* in the LZP is related mostly to Portuguese mainland and Galician waters and those off the Azores (Porteiro and Martins, 1992; Porteiro et al. 1994; Moreno et al. 1994; Guerra and Rocha, 1994; Pham et al. 2009; Smith et al. 2011) and information remains

very scarce for the Mediterranean. The species reproduces there in the cold season, in deep waters, with egg masses occasionally found between November and March in east, central and west Mediterranean (Boletzky 1987; Ragonese and Jereb, 1986; Salman and Laptikhovsky, 2002). Its current distribution is thought to have shifted northward throughout the range due to climate changes. In the 1960s it was reasonably common off Morocco (Ch.M. Nigmatullin, pers. comm) but never was found there in the 1990s despite intensive multiannual research activity of RVs “AtlantNIRO”, “Atlantida” and “Fridtjof Nansen” (V. Laptikhovsky, H. Moustafidh, pers. comm) and there was a simultaneous dramatic decrease in landings west of the Iberian Peninsula from the early 1990s onwards (Chen et al. 2006) although long-term time series from MEDITS survey show an increasing trend in abundance along the Iberian-Lions and Strait of Sicily bioregions (Quetglas et al. 2019). The deep seas of the Mediterranean might represent a potential for expansion of this species range as its eggs were found there deeper than 700 m (Salman and Laptikhovsky 2002).

Genetic analysis as well as statolith shape analysis suggest a complex stock structure of *L. forbesii* in the Mediterranean (Göpel et al. 2022, Bobowski et al. 2024). Age determination supports our study results and suggests a hatching period of *L. forbesii* from the Balearic between January and March and from the South Adriatic Sea from October to March (Bobowski et al. submitted).

*Loligo forbesii* (including mature animals) was reported as common on Mediterranean shores of Egypt and even the Red Sea, occurring in both areas together with Indo-Pacific *Uroteuthis (Photololigo) duvaucelii* (Gabr and Riad 2008; Riad and Al Werfaly 2014; Riad 2020, 2022). The authors identified the species on the basis of external morphological features (e.g. number of teeth in club and arm suckers, relative size of club suckers, relative fin length, relative hectocotylus length) rather than obvious presence of a pair of photophores on the ventral surface of the ink sac in *Uroteuthis* and absence of those in *L. forbesii* – a feature that was never mentioned in their diagnosis. Neither did the diagnosis mention the characteristic “stripes” on the body of *L. forbesii* (Riad 2020, 2022). Certainly, *L. forbesii* could be present in some numbers and likely reproduce somewhere off Egyptian shores, but we await further clarification and confirmation of *L. forbesii* presence in the Red Sea and *U. duvaucelii* presence in the southeast Mediterranean, which would challenge our current understanding of distribution of these species (Jereb and Roper 2010; Bello et al. 2020). Therefore, we did not consider the data on spawning seasonality in these areas. The records of *L. forbesii* in the Indian Ocean are unconfirmed (Jereb and Roper 2010) so its high abundance off Egypt should be treated as unconfirmed. Notably the spawning season of alleged *L. forbesii* in Egyptian waters (spring – summer) is more consistent with *U. duvaucelii* with its all year-round reproduction peaking in spring to autumn in the Red Sea (Gewida et al. 2021) and is very dissimilar from the known pattern of *L. forbesii* reproduction throughout the species’ range, including in the Mediterranean, which thus provides additional grounds for doubts.

## ACKNOWLEDGEMENTS

Female maturity data were collected during the Eurosquid Projects (EEC research programmes FAIR, contract no. MA 1.146 and FAIR-CT96-1520). We thank Lucia Coelho, Pedro Andrade, Pilar Sanchez, Angel Guerra, Helen Martins, Drosos Koutsoubas, Christos Arvanitidis and Filipe Porteiro who collected some of the Eurosquid datasets.

FÁF-Á was supported by a Beatriu de Pinós fellowship from Secretaria d’Universitats i Recerca del Departament de Recerca i Universitats of the Generalitat de Catalunya (Ref. BP2021 00035) and the Spanish government through the “Severo Ochoa Center of Excellence” accreditation (CEX2019-000928-S).



Samples from the Balearic Islands were collected within the Medits surveys and the National Programme of collection, management and use of data in the fisheries sector and support for scientific advice regarding the Common Fisheries Policy, co-funded by the IEO and the EU through the European Maritime and Fisheries Fund (EMFF).

Our team sincerely thanks all recreational divers that posted their records on squid egg masses to the public media either as a part of the different Citizen Science programs aimed on monitoring of biodiversity and collection of biological information as well as result of their own initiative.

Authors thank Dr. Rui Vieira (Cefas) for valuable comments.

The work was undertaken as part of activity of the ICES The Working Group on Cephalopod Fisheries and Life History (WGCEPH) and authors sincerely thanks all members involved into discussion of results during meetings.

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## SUPPLEMENTARY MATERIALS

**Supplementary Table S1** Percentage female maturity in *Loligo vulgaris* per area in northern locations. Data are from this study (raw spreadsheets) or digitised from literature (indicated by a reference in each case). Grey cells denote missing datapoints. Metadata for this table can be found in Table 1 of the main text.  
\*Indicates monthly percentages based on fewer than n=10 (where known).

|     | N France | Galicia,<br>Spain | W<br>Portugal | Algarve | Gulf Cadiz<br>(Vila et al.<br>2010) |
|-----|----------|-------------------|---------------|---------|-------------------------------------|
| Jan | 74       | 100               | 25            | 30      | 52                                  |
| Feb | 85       | 100*              | 34            | 26      |                                     |
| Mar | 83       | 13*               | 30            | 46      |                                     |
| Apr | 77       | 42                | 36            | 14      | 79                                  |
| May |          |                   | 21            | 40      | 50                                  |
| Jun |          | 28                | 17            | 13      | 49                                  |
| Jul |          | 0*                | 7             | 9       | 71                                  |
| Aug |          | 7                 | 6             | 21      | 54                                  |
| Sep | 0*       | 27                | 13            | 10      | 31                                  |
| Oct | 0        | 40                | 9             | 31      |                                     |
| Nov | 9        | 56                | 12            | 64      | 32                                  |
| Dec | 38       | 56                | 27            | 50      | 68                                  |

**Supplementary Table S2** Percentage female maturity in *Loligo vulgaris* per area in southern locations. Data are from this study (spreadsheets) or digitised from literature (indicated by a reference in each case). Grey cells denote missing datapoints. Metadata for this table can be found in Table 1 of the main text.

|      | Balearic Islands<br>(Valls,<br>unpublished) | Catalan Sea<br>(Mangold-<br>Wirz 1963) | Central<br>Adriatic<br>(Krstulović<br>Šifner &<br>Vrgoč, 2004) | Greek Sea:<br>(Moreno et al<br>2002) | Agadir<br>(Baddy 1989) | W Sahara 1<br>(Srairi et al.<br>2023) | W Sahara 2<br>(Perales-Raya<br>et al. 1999) |
|------|---------------------------------------------|----------------------------------------|----------------------------------------------------------------|--------------------------------------|------------------------|---------------------------------------|---------------------------------------------|
| Jan  | 32                                          |                                        | 75                                                             | 9                                    | 50                     |                                       | 70                                          |
| Feb  | 30                                          | 68                                     | 71                                                             | 60                                   | 78                     | 38                                    |                                             |
| Mar  | 44                                          | 87                                     | 50                                                             | 29                                   | 94                     | 44                                    |                                             |
| Apr  | 37                                          | 72                                     | 54                                                             | 83                                   | 78                     | 43                                    | 55                                          |
| May  | 41                                          | 90                                     | 75                                                             | 28                                   | 52                     | 63                                    | 28                                          |
| Jun  | 38                                          | 100                                    | 9                                                              |                                      | 58                     | 53                                    | 0                                           |
| Jul  | 37                                          | 34                                     | 17                                                             |                                      | 42                     | 40                                    |                                             |
| Aug  | 32                                          | 15                                     | 21                                                             |                                      | 38                     |                                       |                                             |
| Sept | 55                                          | 1                                      | 0                                                              |                                      | 33                     | 37                                    | 5                                           |
| Oct  | 52                                          | 3                                      | 0                                                              | 0                                    | 32                     | 30                                    |                                             |
| Nov  | 32                                          | 10                                     | 13                                                             | 7                                    | 59                     | 40                                    | 39                                          |
| Dec  | 11                                          | 20                                     | 14                                                             | 14                                   | 67                     | 41                                    | 27                                          |

**Supplementary Table S3** Percentage female maturity in *Loligo forbesii* per area. Data are from this study (spreadsheets) or digitised from literature (indicated by a reference in each case). Grey cells denote missing datapoints; \*Indicates monthly percentages based on fewer than n=10. Metadata for this table can be found in Table 2 of the main text.

|     | N France | Galicia | Azores | W Portugal | Balearic Isl | Straits of Sicily (Ragonese & Jereb 1986) | Sardinia (Agus et al. 2018) | Greece |
|-----|----------|---------|--------|------------|--------------|-------------------------------------------|-----------------------------|--------|
| Jan | 56       | 86      | 96     | 12         | 0*           |                                           |                             | 0*     |
| Feb | 85       | 70      | 94     | 20         | 10           | 21                                        |                             | 27     |
| Mar | 71       | 30      | 93     | 18         | 0            |                                           |                             | 0      |
| Apr | 94       | 7       | 97     | 0          | 8            |                                           | 22                          |        |
| May |          | 20*     | 100    | 2          | 6            |                                           |                             | 0      |
| Jun | 38       | 0*      | 100*   | 0*         | 3            |                                           |                             |        |
| Jul | 17       | 25*     |        | 0          | 24           |                                           |                             |        |
| Aug | 0        | 0*      |        | 0          | 68           | 0                                         |                             |        |
| Sep | 0        | 0       | 0*     | 0*         | 53           |                                           |                             |        |
| Oct | 5        | 0*      | 25     | 0          | 23           |                                           |                             | 0*     |
| Nov | 29       | 17      | 50     | 27         | 32           | 61                                        |                             |        |
| Dec | 71       | 100*    | 97     | 50*        |              |                                           |                             | 22     |





## Artificial Intelligence Statement

For clarity of the text and linguistic optimization, DeepL and ChatGPT were used. These technologies helped refine the expression and enhance comprehension. After using these tools; I reviewed and edited the content as needed and take full responsibility for the content of the thesis.

## Declaration of Authorship

I hereby certify that the submitted thesis is entirely my original work unless stated otherwise. Information or work that is derived from other publications or authors is referenced accordingly. I am aware of the University's regulations regarding plagiarism.



Bianca Bobowski