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Mismanagement and poor transparency in the European processed seafood supply revealed by DNA metabarcoding

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ABSTRACT

In the global processed seafood industry, disparate actors play different roles along the supply chain, creating multiple opportunities for mistakes, malpractice, and fraud. As a consequence, consumers may be exposed to non-authentic products, which hinder informed purchasing decisions and broader efforts to improve trade transparency and sustainability. Here, we characterised the taxonomic composition of 62 processed seafood products in Italian, British and Albanian retailers, purposefully obtained from different supply routes, using multiple DNA metabarcoding markers. By combining molecular results with metadata reported on labels, we revealed patterns of mislabelling in 24 products (39%) across sampling regions, denoting lack of transparency of processed seafood products based on resources sourced from either Europe or globally. We show that the accuracy of label claims and the mis-represented and underestimated levels of traded biodiversity are largely determined by the management of raw material by global processors. Our study shows that DNA metabarcoding is a powerful and novel authentication tool that is mature for application at different stages of the seafood supply chain to protect consumers and improve the sustainable management of fish stocks.

1. Introduction

The seafood industry handles nearly 180 million tonnes of goods annually to meet the unprecedented demand for fish products around the world (FAO, 2024). To cater to consumers, especially in developed countries, producers, processors and retailers work synergistically to supply, transform and deliver processed "convenience seafood" to supermarket chains (Barska, 2018; Mottola et al., 2022). Globally, at the point of sale, high quality standards are expected by regulatory frameworks and the consumers themselves. Recently, the quality aspects of seafood products have expanded to include not only sensory and safety concerns, but also traceability, authenticity, and sustainability (FAO, 2018). Indeed, in May 2020, the European Commission presented the fight against fraud along the supply chain as one of the main objectives of the 'From Farm to Fork' strategy at the core of the European Union's Green Deal (Schebesta et al., 2020). Particularly for mixed processed seafood (e.g. fish cakes, fish fingers, surimi, etc.), the length of the supply chain and the lack of morphological characteristics of the product on sale mean that there are more opportunities for fraud, such as the inclusion of species sourced from illegal, unreported, and unregulated fishing (IUU), or subject to specific management programmes (Fox et al., 2018). Also, the substitution of prized species for others of lower value is the most reported type of seafood fraud, which can deliver financial gain for the fraudulent operator, and is often a ploy to meet local and global market demands for dwindling popular species (Calosso et al., 2020; Luque & Donlan, 2019). At the same time, inaccurate labelling of mixed seafood exposes consumers to the risk of unknowingly consuming products (e.g., molluscs, crustaceans, dairy, pork, or eggs) that may be undesirable for health or personal dietary choices (Piredda et al., 2022). Labels are the tool through which consumers can make informed choices, so they must be filled in with accurate information by Food Business Operators (FBOs) purveying the products (Giusti et al., 2023; Paolacci et al., 2021). Meanwhile, consumer awareness of fishing pressure on ecosystems is moving towards the choice of eco-labels such as

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the Marine Stewardship Council (MSC), the leader in this sector, which has based its chain of custody management principles on maintaining target stocks at sustainable levels, and their ecosystems in a healthy status (Jardim & Currey, 2023).

Several analytical techniques have been developed to verify food authenticity, including protein-based methods (e.g., HPLC, ELISA, NMR) and DNA analysis (e.g., RAPD, LAMP, ddPCR, HRM) (Asensio et al., 2008; Cai et al., 2017; Medina et al., 2019). However, DNA specificity, sensitivity, and thermal stability have progressively made DNA-based approaches more popular and effective for species identification especially when the species integrity is lost, or food is subjected to high technological treatments (Griffiths et al., 2014). Among them, DNA metabarcoding, based on parallel sequencing of diagnostic target fragments (i.e., 'barcodes'), is the most suitable for mixed products since it allows the simultaneous in-depth characterisation of all species present in a sample, offering throughput unmatched by traditional DNA barcoding methods or quantitative PCR (qPCR) (Clark, 2015; Staats et al., 2016, Franco et al., 2021).

In this study, we utilised a dual-marker DNA metabarcoding strategy to comprehensively characterise the taxonomic composition of pre-packaged processed seafood products in three European countries. This approach included one marker specifically designed to target fish species and another that is broadly applicable to eukaryotes, which enabled the analysis of globally sourced species processed through diverse supply routes.

2. Material and methods

2.1. Sampling

Processed pre-packed point-of-sale seafood products (i.e., breaded, burgers, surimi-based, dumplings, and "fish steaks") were collected in three European countries spanning the range of current regulatory statuses: Italy (EU member), UK (recently exited from the EU), Albania (aiming to soon become an EU member). Sampling was carried out in the main supermarket chains of the Large-Scale Distribution (retailers) of these three countries. Specifically, we visited six different retailers in the UK, five in Italy and three in Albania, to cover the diversity of products sold by different brands (FBOs). To cast further light onto supply chain mechanisms, we also obtained samples from an Italian supplier, ahead of their preparation for retail; and we also sampled a range of diverse processed products from two Asian retailers in the UK, aiming to increase the biological diversity of products and supply routes (Fig. 1).To group samples by product type, we pooled under the term "steaks" all those samples that came as frozen blocks of fish and appeared to mimic a unique piece of muscle, while we used the term "breaded" to simply refer to all products coated with breadcrumbs or battered crust. All samples were stored at −20 °C until DNA extraction.

Visual inspection of the labels was carried out for each purchased product to record data such as the commercial name used and the scientific name, if declared, as well as the list of ingredients, the declared presence of traces of other animal species, the name of the FBO and the unique approval number on the identification mark. Moreover, we recorded whether or not the products were certified sustainable by the MSC.

2.2. Laboratory procedures

Three mock mixtures were prepared blending different proportions of tissue from three fish species purchased whole and unlikely to be included in the mainstream supermarket products. These species were: silver pomfret (*Pampus argenteus*), Bengal corvina (*Daysciaena albida*) and false trevally (*Lactarius lactarius*) blended respectively in these following proportions: 70 %:15 %:15 % (Sample 39), 25 %:5%:70 % (Sample 40) and 33.3 %:33.3 %:33.3 % (Sample 41). Those mixtures were included to support our traffic light criterion for mixed market products and to determine a conservative operational threshold below which a certain percentage of reads could be disregarded as "traces" for the purpose of designating whether a sample was mislabelled.

Since several packaged products contained multiple pieces (e.g., fish fingers), we endeavoured to randomly excise several pieces from each sample using a sterile scalpel and placed approximately 200 mg of each product in an 1.5 ml eppendorf tube for DNA extraction.

Genomic DNA extraction and purification of the Italian and Albanian samples was performed using the DNeasy Blood and Tissue Kit (Qiagen) as reported by Piredda et al., 2022. The DNA of products sampled in England and of the three mock mixtures was isolated following the MuDNA extraction protocol (Sellers et al., 2018). Negative extraction controls (no added tissue) were included in both cases to verify the purity of the extraction reagents. DNA concentration and purity were evaluated by a Qubit Flex™ 4.0 fluorometer with a Qubit™ dsDNA HS Assay Kit (Invitrogen).

Two different primer sets were chosen to increase the confidence with the species identification and to compare their performance in terms of discrimination power at species level. One set of PCR amplifications were performed using the mlCOIintF 5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′ and jgHCO2198 5′- TAIACYTCIGGRT GICCRAARAAYCA-3′ primers, designed to target a fragment of 313 bp of the subunit I of the Cytochrome *C* Oxidase (COI) in all eukaryotes (Geller et al., 2013; Leray et al., 2013). The other primer pair used was the Teleo02 (F: 5′-AAACTCGTGCCAGCCACC-3′, R: 5′-GGGTATCTAATCCCAGTTTG-3′) that amplifies ~ 167 bp of the 12S rRNA mitochondrial gene (12S) as described by Taberlet et al. (2018) and primarily targets teleost fishes. Both primer pairs were uniquely tagged for each sample with 8 bp sequences to ensure the parsing of sample reads during demultiplexing. The PCR amplification was performed for 72 samples (62 products, 7 negatives, and 3 mocks) in triplicate, and in a final reaction volume of 20 μl containing 10 μl MyFi™ Mix (Meridian Bioscience), 0.16 μl of Bovine Serum Albumin (Thermo Scientific), 5.84 μl of UltraPure™ Distilled Water (Invitrogen), 1 μl of the forward and reverse primer (10 μM, Eurofins), and 2 μl of the extracted DNA. The thermocycling programme for the COI primer followed the initial DNA denaturation at 94 °C for 10 min, a total of 35 cycles of 1 min at 94 °C, 1 min at 45 °C, 1 min at 72 °C, and a final elongation at 72 °C for 5 min. For the 12S primer the following amplification conditions were applied: initial DNA denaturation for 10 min at 95 °C, 35 cycles of 30 s at 95 °C, 45 s at 60 °C and 30 s at 72 °C, and a final step of 72 °C for 5 min. PCR triplicates were then pooled and checked through gel electrophoresis on a 2 % agarose gel stained with SYBRsafe (Invitrogen). The PCR product clean-up was performed using an equal volume of 45 μl of the Mag-Bind® TotalPure NGS magnetic beads (Omega Bio-tek Inc) for both primer sets. DNA quantification of all samples was used to normalize and pool samples in equimolar concentration for the two libraries' preparation. After pooling, an additional magnetic beads clean-up was performed to concentrate pooled samples in a final volume of 50 μl. The Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape (Agilent Technologies) showed the presence of a single peak of the expected size for both libraries. End repair, adapter ligation and library PCR amplification were carried out using the NEXTFLEX® Rapid DNA-Seq Kit 2.0 for Illumina® platforms (PerkinElmer) according to the manufacturer's protocol. The two libraries were quantified by a quantitative PCR (qPCR) on a Rotor-Gene Q (Qiagen) with the NEBNext® Library Quant Kit for Illumina® (New England Biolabs) and were equimolarly pooled to obtain a final concentration of 9 pM with 10 % PhiX control. Sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v2 (500 cycles).

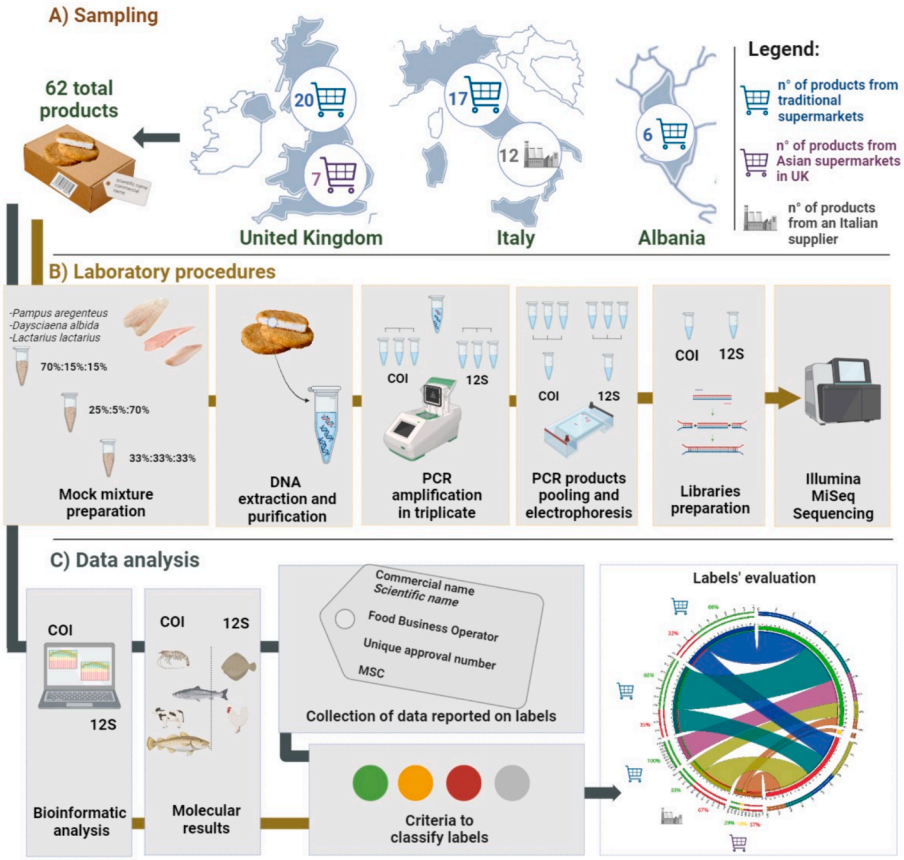

Fig. 1. Infographic showing the main steps of the study, from sampling, laboratory procedures and data analysis to the final cross-check against the established criteria to assess the accuracy of the labels.

2.3. Bioinformatics

Raw data were analysed following the OBITOOLS (Boyer et al., 2016) pipeline. First, the FASTQC program was used to assess the quality of reads and exclusively the 12S raw reads were trimmed (i.e., 200 bp for forward and 180 bp for reverse sequences) to remove low-quality ends using OBICUT. Then, pair-end reads were merged through ILLUMINAPAIREDEND and retained only those with a quality score > 40. NGSFILTER allowed the demultiplexing, based on the unique tags, with a maximum mismatch threshold of 1 bp. OBIGREP was used to filter reads based on their expected lengths (i.e., 300 – 325 bp for the COI and 109 – 229 bp for the 12S), and remove reads containing ambiguous bases "N". Sequences were dereplicated through OBIUNIQUE and chimeras were removed using UCHIME (Edgar et al., 2011). The clustering into Molecular Operational Taxonomic Units (MOTUs) was performed with SWARM (Mahé et al., 2014), setting custom threshold of d = 9 for COI and d = 3 for 12S. Finally, taxonomic assignment was performed using ECOTAG against the EMBL database (release version r143) for both the COI and 12S, generated by in silico PCR (ecoPCR). MOTUs with a percentage identity between 95 % and 98 % were manually checked on NCBI to resolve ambiguous assignments. Negative controls were used to identify the presence of contaminating MOTUs with the DECONTAM package in R, setting a threshold of 0.2 in both cases. Contaminant reads, such as *Homo sapiens* and *Pangasianodon hypophthalmus* (used as a positive control in other sequencing projects) were removed. Finally, based on the results obtained in the three mock mixtures, we set a threshold of 1 % of the total percentage abundance of reads in each sample to discard all lower percentage values as representative of contamination.

2.4. Mislabelling criteria

Since commercial designations of seafood species vary greatly both across and within countries, we compared the ingredients provided for each product to the official list of commercial designation of the country where the product was purchased. In particular, we referred to the Commercial designations of fish of the United Kingdom (updated to 03 February 2020) (https://www.gov.uk/government/publications/commercial-designations-of-fish-united-kingdom), the Annex I of the Decree of the Italian Ministry of Agriculture, Food Sovereignty and Forests (MASAF) of September 22nd, 2017 (https://www.politicheagricole.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/11953), and the Appendix 3 of the Albanian Regulation No. 407 dated May, 8th 2013 (https://www.mod.gov.al/images/akteligjore/qnod/09_VKM_%20407_08052013.pdf). If a common name was declared on the label, the relevant species name was obtained searching FishBase (Froese, 2023), while if a scientific name was provided, it was contrasted directly with the molecular results. Using matches and mismatches between label information and DNA-based identification, we classified the examined products into the following categories: (i) "green" (correctly labelled product): when the proportion of reads of the declared species was at least twice as large as the second most abundant species and constitutes the majority of the bulk; (ii) "amber" (misleading product): when the proportion of the declared species was higher than any other species, but not necessarily amounting to the majority of the bulk; (iii) "red" (mislabelled product): when the declared species was either absent or not the most abundant in the mix; (iv) "grey" (undetermined product): when the declared species couldn't be genetically identified with certainty.

These categories were established after combining the "traffic light"

information between the two genetic markers employed, as there were instances where one marker was unable to discriminate between two closely related species ("grey"), but the other could instead tell them apart (see Results for details).

2.5. Data analysis

The experimental workflow illustrated in Fig. 1 was created online on Biorender.com. Label metadata, such as the unique approval number and the country where the product brand is registered, were combined with the sampling location of each product to reconstruct trade flows between and within countries of establishments and sale. Linear regressions between biomass and the Hellinger-transformed number of reads, generated with the R package vegan (Oksanen, 2018), were calculated for the mock mixtures using ggplot2 (Wickham, 2011) and ggpmisc (Aphalo, 2016). The number of reads of each taxon within the total number of reads in each sample were converted in proportions that were used to represent the product composition, for category and gene marker applied, generating barplots through the R package ggplot2. Visualizations of labelling results, in accordance with the labelling classification criteria, were created using the Circos online tool (Krzywinski et al., 2009). The Pearson's chi-squared test was calculated within the R environment (R Core Team, 2023) (version 4.2.3) and used to test for pair-wise differences in labelling accuracy between Italian and UK supermarkets, between them and the Italian supplier, as well as between MSC-certified and non-certified products. For the purposes of this study, documentation on the suppliers' MSC certification schemes was provided and used to assess the management of the MSC-certified species within the same company.

3. Results

3.1. Label visual inspection

The sampling expansion reflected the range of diversity and availability of processed seafood in countries of sampling. We examined 62 pre-packed seafood products obtained from Italian, Albanian, and British (UK) markets (Fig. 1), purchased from 22 distinct FBOs, distributed as follows: 10 headquartered in the UK, 10 in Italy, 1 in Spain and 1 in the Netherlands. We identified 30 different unique approval numbers, each linked to the last point of food handling, across Europe, Asia, and Africa. One sample (S20) lacked a unique approval number, but reported China as country of production, therefore a code was also assigned to that product in China. Most of the samples from the UK were supplied by British establishments, with the exception of three products from Germany and one from Poland. In the Italian supermarkets, eight products were sourced from processing plants in Italy, with the other nine coming from Spain, France, Germany, and Poland. On the other hand, none of the products purchased in Albania came from a local establishment, but were sourced from Spain, the UK, Germany, and Namibia. Twelve products sourced from an Italian supplier (a-Italy) all came from a factory in the Netherlands. All the Asian-style cuisine products came from production sites in India, China, Thailand, and Malaysia, apart from one, and were all sold by FBOs in the UK and one in the Netherlands (Fig. 2). A total of 23 out of the 62 products held MSC certification (Supplementary Table 1–3). Overall, 51 of 62 products claimed to be made exclusively of a single species (Supplementary Tables 1–3).

3.2. Primer performance and taxonomic characterization

A successful COI amplification was obtained for all samples, except for one (5 reads in sample S2). The total number of reads obtained with the COI primer pair was 5,077,768, which came down to 4,775,115 after

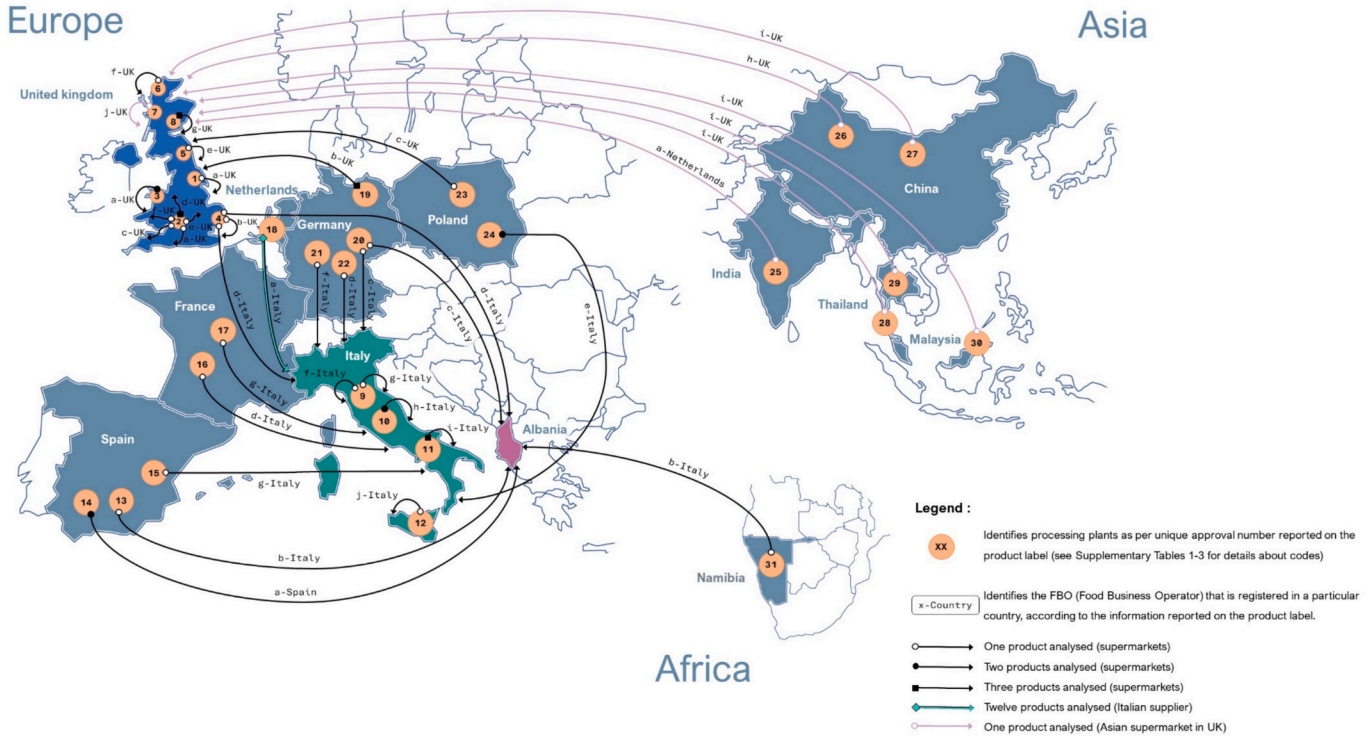

Fig. 2. The roadmap mapping the information provided on the labels about the unique approval number of the establishment from which the products originated and the brand selling the products, based on the country in which they are registered (Supplementary Tables 1–3). Their position in the country map does not represent the real geographical coordinates.

applying all filters (Supplementary Material), resulting in a mean read number per sample (61 products + 3 mocks) of ~ 75 k. The COI dataset consisted of 53 total taxa (49 at species level, three at genus level and one at family level), belonging to three kingdoms (Metazoa, Fungi and Stramenopiles), five phyla (Arthropoda, Chordata, Nematoda, Ascomycota and Ochrophyta), eight classes (Arachnida, Actinopterigyii, Chromadorea, Insecta, Mammalia, Malacostraca, Saccharomycetes and Chrysophyceae), 19 orders, and 30 families.

The COI molecular identifications highlighted that in the 44 breaded products (e.g., fish cakes and fish fingers) the main component was Alaska pollock (*Gadus chalcogrammus*), reaching over > 98 % of the reads in 14 of the samples, and between 44 % and 63 % in 11 other samples (Fig. 3; Supplementary Tables 1–3). Atlantic cod (*G. morhua*) contributed ~ 40 % of reads in eight samples; European plaice (*Pleuronectes platessa*) accounted for at least the 75 % of reads in six samples; haddock (*Melanogrammus aeglefinus*) amounted to ~ 78 % of reads in three samples (S14, S15, S26); Pacific hake (*Merluccius productus)* reached at least the 90 % of reads in three other samples (S54, S59 and S70); saithe (*Pollachius virens*) represented ~ 60 % of reads in one sample (S62), and Atlantic salmon (*Salmo salar*) comprised the entirety of sample S25. The main species in the five burgers, accounting for at least the 89 % of the reads, were Alaska Pollock (S54 and S60), Atlantic salmon (S57 and S72), and Pacific hake (S58). In the four "steaks", Alaska Pollock, hakes and *Macruronus* sp. (S48) were predominant, amounting to at least 80 % in each sample. In one of the shrimp-based dumplings (S20), 100 % of the reads belonged to Louisiana crawfish (*Procrambarus clarkii*) and in the other one (S21) the whiteleg shrimp (*Penaeus vannamei*) represented over 80 % of reads. The six surimi-based products were constituted by a broad range of species from fisheries across the world, with the predominant species being: herring (*Clupea harengus*) with > 93 % of reads in S22, Pacific hake with 84 % of reads in S42, cutlassfishes (*Trichiurus japonicus*) with 82 % in S19, and threadfin breams (*Nemipterus* sp.) with over 59 % of reads in S18 (Fig. 3; Supplementary Tables 1–3).

The 12S amplification was successful for all samples. The total number of reads obtained with this primer pair was 5,643,616, which was reduced to 4,880,925 after filtering (with mean read number per sample of ~ 75 k) (Supplementary Material). The cleaned 12S dataset consisted of 50 taxa (38 at species level, nine at genus level, and two at family level). The 12S dataset exclusively included the phylum Chordata, three classes (Actinopterigyii, Mammalia and Aves), 19 orders and 28 families. Haddock (*Melanogrammus aeglefinus*) and whiting (*Merlangius merlangus*) could not be distinguished by this marker and were recorded as 'Gadidae'. For the MOTU assigned to the Genus *Gadus* it was not possible to unambiguously assign a species-level identification.

The 12S molecular identifications highlighted that the genus *Gadus* sp. was the main component in the 44 breaded products, reaching > 93 % of the reads in 26 samples (Fig. 3; Supplementary Tables 1–3). European plaice accounted for at least ~ 50 % of reads in six samples. Also, reads of Gadidae (*Melanogrammus aeglefinus* and *Merlangius merlangus*) reached an average 61 % of reads in three samples. Hakes (*Merluccius* sp.) reached at least the 91 % of reads in five samples. Salmon (*Salmo* sp.) constituted sample S25 in its entirety. Composition in the five burgers also mirrored the findings from the COI data set, with at least 75 % of the reads of *Gadus* sp. in S55 and S60, *Salmo* sp. in S57 and S72, and *Merluccius* sp. in S58. The four "steaks" were entirely composed of *Merluccius* sp. (S47 and S71), *Gadus* sp. (S1), and hoki (*Macruronus novaezelandiae*) in S48. In one shrimp-based dumpling *Sus scrofa* reached 100 % of the reads (S21), while in the other one *Gadus* sp. amounted to 78 % of the reads (S20), in contrast to the COI results. In the six surimi-based products, in the midst of the vast number of species detected, the results confirmed the dominance of *Clupea harengus* in S22, *Nemipterus* sp. in S18, *Merluccius* sp. in S42, and *Trichiurus* sp. in S19, with only S23 showing discrepancy with COI, as *Sardinella* sp. dominated with 66 % of reads (Fig. 3; Supplementary Tables 1–3).

To add support to our criteria for the interpretation of proportional read counts, we used mock mixtures of three known fish species. These included silver pomfret (*Pampus argenteus*), Bengal corvina (*Daysciaena albida*) and false trevally (*Lactarius lactarius*), which were mixed in varying proportions. Results obtained from the three mock mixtures, from both primer sets, confirmed the presence of the three fish species included, with similar abilities of both markers to resolve the known compositions (Supplementary Fig. 1). Overall, we observed that both primers returned proportional read counts that fit well with the input

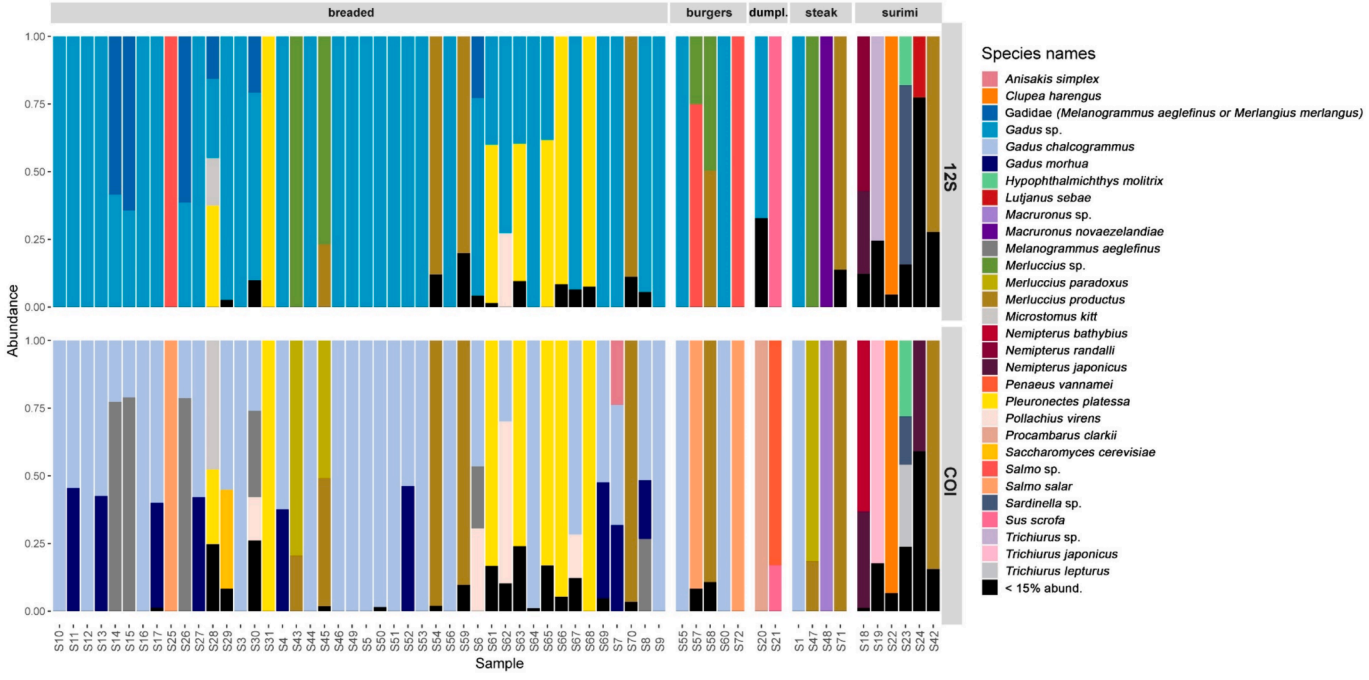

Fig. 3. Barplots showing the proportion of reads of each taxon detected by the 12S and COI primer pairs separately, grouped according to the type of product (i.e., breaded, burgers, dumplings, steaks and surimi).

proportions of tissues, with 12S (designed for teleosts) reflecting the actual proportions with greater accuracy. Notably, in S41 (where input proportions were equal across the three species), no species resulted dominant in the read counts for neither marker. To corroborate the choice of excluding taxa with < 1 % reads, we never found anything other than the three input species with read counts beyond the 1 % threshold.

3.3. Mislabelling assessment

To determine labelling accuracy, we compared molecular identifications of each marker with the commercial and/or scientific names reported on the labels. The COI dataset allowed accurate assessment for 60 out of 62 products' labels (Fig. 4A), while with 12S it was not possible to unequivocally determine label conformity in 40 samples, in large part due to DNA sequence ambiguity within the genus *Gadus* (Fig. 4B). In all those cases, COI information was instrumental to resolve the ambiguity. Mislabelled or "red" samples occurred when the declared species was either absent or not the most abundant in the product, while correctly labelled products or "green" samples needed to meet a conservative abundance (see Methods). In 21 of 61 cases there was an agreement of both markers with the traffic light criterion. In all other cases, whenever one marker failed to resolve taxonomy, the other one was able to settle identification to the species level. Overall, 36/61 (59 %) labels were "green", 24/61 (39.3 %) were "red", and one (1.6 %) was "amber" (the declared species was higher than any other species, but not the majority of the bulk). Three products were labelled with a generic name (e.g. "whitefish" and "surimi") to which no specific taxon could be attributed. Consequently, these three products were deemed "green", regardless of the identifications obtained. All six product labels form the Albanian supermarkets were "green" (100 %). Of the 17 product labels from the Italian supermarkets, 11 were "green" (65 %) and six "red" (35 %), among the 19 samples purchased from UK supermarkets, 13 labels were "green" (68 %) and six "red" (32 %). In contrast, among the 12 products' labels from the Italian supplier, four were "green" (33 %) and eight "red" (67 %). Products sampled at Asian markets located in UK included two "green" (29 %), four "red" (57 %), and one "amber" (14 %) labels (Fig. 4C).

The rate of mislabelling did not differ significantly between Italian and UK supermarkets ($\chi^2 = 0.056$, p-value = 0.81), but the mislabelling rate between the Italian supplier and both Italian and UK supermarkets was significantly different ($\chi^2 = 3.833$, p-value = 0.05).

Of the 20 supermarket products that were MSC certified, 15 (75 %) were "green", and five (25 %) were deemed "red". By contrast, of the 29 non-MSC certified products, 17 (58.6 %) were "green", and 12 (41.4 %) "red" or "amber" ($\chi^2 = 1.402$, p-value = 0.24); however, two out of three MSC-certified products handled by the Italian supplier were deemed mislabelled (Supplementary Table 3b). All the species found in MSC-certified products were within the MSC programme of their suppliers, except for *Microstomus kitt*, *Melanogrammus aeglefinus* and *Sander lucioperca*, which were all found in products from the same supplier.

4. Discussion

The advent of massively parallel sequencing is revolutionising the field of food research and monitoring (Mottola et al., 2023; Noh et al., 2021; Özkök et al., 2023). Here we show that the biodiversity underpinning widely traded processed seafood is greater than what is usually declared on labels, frequently even in products that are retailed as being of "single-species". Comparisons among countries, and between levels along the supply chain, can help form a better understanding of the mechanisms that regulate the provision and use of aquatic species in processed foods and can identify a roadmap for improving practice towards more informed consumers, a responsible trade, and sustainable

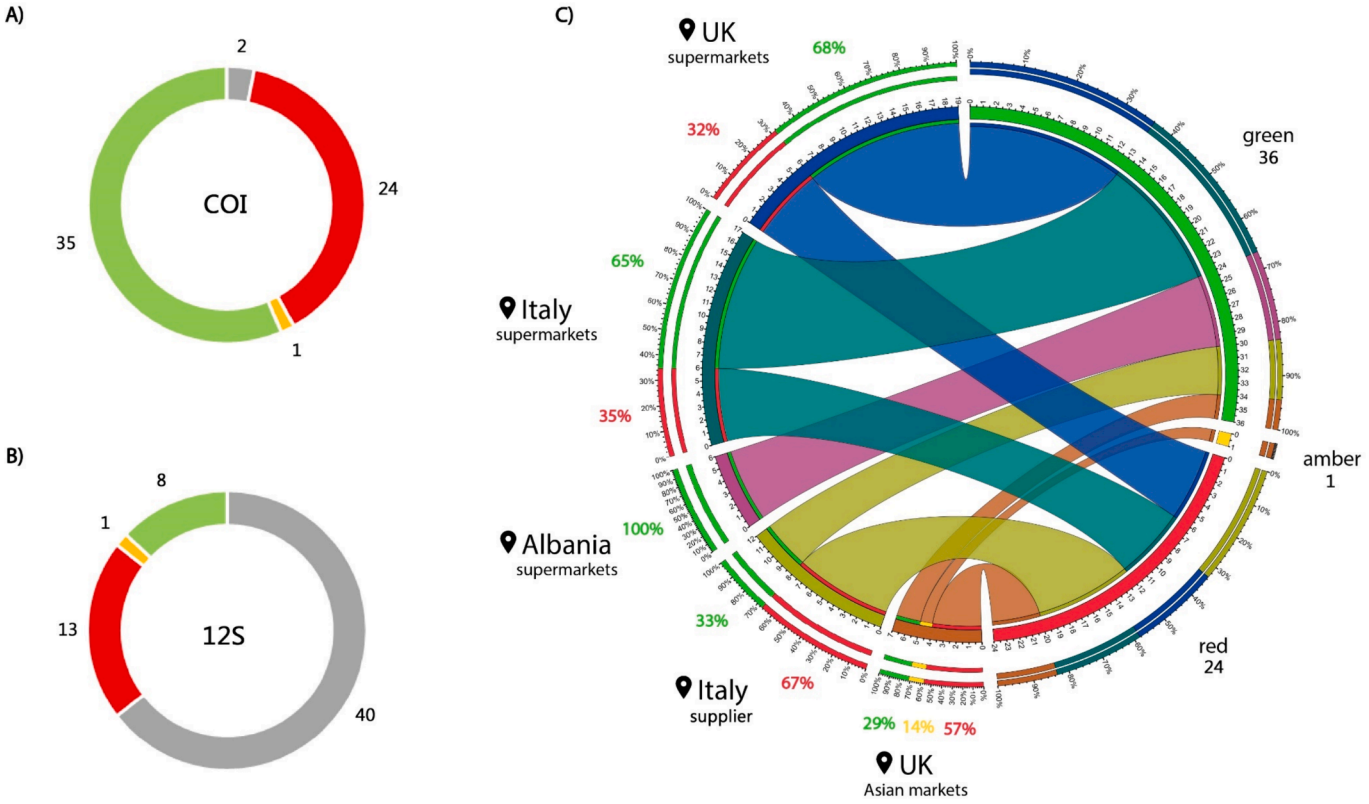

Fig. 4. Circos plots A) and B) number of "green" (correct), "red" (mislabel), "amber" (mislead) and "grey" (undetermined) labels highlighted following our criteria by the COI and 12S primer pairs. C) corresponding abundance of correct, misleading and mislabelled products for each sampling location, after collapsing the attributions.

fish production.

4.1. Molecular identification

The DNA metabarcoding approach has proven to be a powerful tool for ingredient authentication in processed seafood products, primarily owing to its ability to resolve species identity in complex mixture (Giusti et al., 2023; Kappel et al., 2017; Klapper et al., 2023; Piredda et al., 2022; Toxqui Rodríguez et al., 2023). However, to date, there is no standardised workflow for food authentication of processed, potentially mixed, products, especially in terms of the choice of primers to generate taxonomic profiles (Lorusso et al., 2024). In this study, the inclusion of two different primer pairs (one, 12S, focused on fishes, the other, COI, broadly encompassing all animals Geller et al., 2013; Leray et al., 2013; Taberlet et al., 2018), provided a comprehensive assessment of species composition, and helped to verify their reciprocal performance in multispecies seafood. As part of our test, the use of mock mixtures comprising known proportions of three species, showed that the starting biomass of each species was positively correlated with the transformed number of sequence reads for both primers, consistent with previous evidence (Supplementary Fig. 1) (Evans et al., 2016; Laporte et al., 2021) and supporting a semi-quantitative interpretation of our results. Several factors are known to affect the proportion of reads generated from complex matrices (i.e., primer affinity, PCR conditions or inhibitors, as well as competition among templates) (Deagle et al., 2019; Klapper et al., 2023), which means that we opted for a conservative approach when assessing label claim compliance (see "traffic light" criteria in the Methods). Most of the sequences in the two marker datasets belonged to the same taxa, at least at the genus level: *Gadus* sp., *Merluccius* sp. and *Pleuronectes platessa* were the most common taxa in both datasets, accounting for – alongside haddock and salmon – over 80 % of the total number of reads. The two primers performed in a complementary way, not only due to their different ability to discriminate species. Indeed, COI was able to efficiently discriminate *G. chalcogrammus* and *G. morhua* and reach species level in *M. aeglefinus* and *Salmo salar*, whereas 12S proved to be crucial in discriminating the genus *Macruronus* (Fig. 3). Moreover, only COI was able to detect crustaceans, necessary to assess the use of the declared species in shrimp-based dumplings (i.e., *Procrambarus clarkii* and *Penaeus vannamei*) or burgers (i.e., *Solenocera crassicornis* and *Parapenaeopsis hardwickii*). Additionally, 34 and 24 low-abundance species exclusively detected in the COI and 12S datasets respectively, contributed to the overall characterisation of the products.

The authenticity of food products does not only involve the accuracy of the information on the label concerning the main raw material used, but also other aspects such as the presence of allergens or uncommon species. For instance, even if the COI molecular result for S20 (dumpling) confirmed the use of the crustacean species declared on the label, those from 12S sequencing revealed the presence of terrestrial species, i.e., pig (10 %) and chicken (~1%), which were not declared either in the list of ingredients or as possible traces. The presence of fish species (e.g., *Gadus* sp., Gadidae and *Gadiculus argenteus*) was also detected, possibly due to the presence of "seafood flavoured sauce", reported in the list of ingredients. Irrespective of product compliance in terms of the declared crustacean species, the UK Food Labelling Regulation also requires the declaration of presence of fish and eggs (chicken) as allergens. Again, the 12S primer detected chicken (~2%) in sample S63 (breaded), with the possible presence of egg traces being correctly declared on the label, in accordance with Regulation (EU) No. 1169/2011 (Mottola et al., 2022). Sample S59 (breaded) contained cow DNA, as detected by both primers, which may be linked to the presence of cheese, declared in the list of ingredients. The additional power of DNA metabarcoding is revealed through the detection of uncommon species for these products. For example, the detection of freshwater species, such as zander (*Sander lucioperca*), not declared on the label and typically uncommon in processed seafood products, rises concern about the intentional sourcing and use of species of unknown origin (Nikolić et al., 2023; Selig et al., 2022). Specifically, the supplier in question publicly advertises their trade in zander products, which may accidentally lead to traces of this species being incorporated in different end products. Other useful findings from the metabarcoding approach include the detection of potential pathogens (Poms et al., 2004). For instance, sample S7 (breaded) included the DNA of *Anisakis simplex*, a parasitic nematode that can infests fish fleshes, and can be harmful for human ingestion causing allergic reactions as urticaria, angioedema and/or anaphylactic shocks, sometimes even when consuming cooked or frozen products (Packi et al., 2023; Polimeno et al., 2021). In addition, in sample S24 (surimi) the undeclared presence of *Lutjanus sebae* was detected, which may contain toxins that are not destroyed by cooking and cause ciguatera poisoning (Abraham et al., 2012; Hamilton et al., 2002). Sample S19 (surimi) showed the presence of *Ochromonas danica*, a freshwater phytoflagellate microalga that produces chlorosulpholipids, marine toxins responsible for human poisoning from seafood consumption (Nilewski et al., 2009). This calls for the need to improve the quality control of the raw materials before including them in added-value preparation.

Also, it is interesting to note the presence of the beetle *Sitophilus oryzae*, in sample S69 (breaded), and *Acarus siro*, in sample S67 (breaded), both common pests of raw materials in the food industry, therefore probably present in the wheat flour used for breadcrumbs (Bell, 2014). The reads belonging to yeast, *Saccharomyces cerevisiae*, in some samples detected by the COI marker may also be due to the declared use of breadcrumbs. However, samples S28, S29 and S30 (breaded) – all from the same processing plant ("18", Fig. 2) showed an unexpectedly high presence (8.17 % – 36.48 %), which could represent an intentional addition of brewer's yeast as a flavour enhancer in the fish mixture (Ferreira et al., 2010).

Our set threshold of 1 % for contamination was already used by the UK Food Safety Authority and by the Department for Environment Food and Rural Affairs (Defra), and it is also in accordance with other metabarcoding studies for food authentication (Giusti et al., 2023). Details on the, at times, curious findings from these trace reads can be found in the Supplementary Material.

4.2. Supply flows and mislabelling drivers

Although processed pre-packed seafood product labels do not currently require information on commercial and scientific names (Paolacci et al., 2021), several product labels did actually report some species names: eight out of the 15 (53 %) processed products sold in Italy, six out of the 20 (30 %) in UK, but none in Albania. Also, six out of the seven (86 %) products from Asian markets voluntarily indicated a scientific name, at least at the genus level, for the main species used. This is probably a marketing strategy of the FBOs or a way to anticipate what might become a much-needed future labelling requirement also for highly processed seafood products.

Overall, UK supermarket products primarily claimed Alaska pollock, cod, haddock and salmon. In Italy and Albania, the same types of products claimed Alaska pollock, South-Pacific hake, Cape hake, North-Pacific hake, hoki, cod and salmon (Supplementary Fig. 2). Thus, a pattern of preference of hakes in Mediterranean markets was observed in contrast with the UK market, probably due to consumers' historical familiarity with certain regional products (EUMOFA, 2023; Penca et al., 2021). These claims were all largely corroborated by the molecular results and mirrored a similar pattern in surimi products, where different sets of species are used as raw materials to produce similar products. Surimi prepared in Europe – such as sample S42, processed in Spain and sold in Albania – tends to be based on cold temperate species, such as hakes and anchovies, which contrasts with the species used for the preparation of surimi from Asian countries, which tend to be of tropical origin, as also documented in other studies (An et al., 2020; Zhang et al., 2024). It is also worth noting that in some of the products from Asian

countries, species were found that do not currently feature in the official list of the UK commercial designations of fish (Supplementary Table S4). Their absence within the list may stem from a variety of reasons, including their danger for consumption (such as in the case of pufferfishes) or simply because they are not known as a commercial target. This has a twofold implication for government action, as the genetic discovery of these species in seafood could either prompt more frequent updates of these approved lists or escalate prosecution for illegally marketed organisms.

By placing label metadata in a geographically explicit context, it is possible to visualise trade flows and the otherwise under-appreciated supplier-retailer relationships that may help explain mislabelling patterns (Fig. 2). Most of the UK products are supplied by UK-based processors (eight out of 10 processors being British), while Italy mostly relied on international supply (only four out of 12 providers being based in Italy), with Albania being entirely reliant on external suppliers. Such differences likely reflect the legacy of different levels of establishment of industrial fishing and processing in the various countries (Wilcox, 2012). Interestingly, however, such different supply or retail mechanisms do not seem to affect the overall extent of mislabelling in the products, which was essentially the same in Italy and the UK (Fig. 4). This seems to indicate that major *trans*-national suppliers of processed seafood depend on the level of good practice enacted in individual processing plants, echoing similar inference obtained from the supply of unprocessed seafood (Miller et al., 2012).

In this study we could also peer into the dynamics between a supplier/distributor and the products it obtains from their manufacturing hubs. Analysis of the samples provided by the Italian supplier revealed that in 11 of the 12 products declared to be based on Alaska pollock, the DNA of several other species was also detected, including saithe (S29, S30, S62, S65 and S67), haddock (S28 and S30), lemon sole (S28 and S66), European plaice (S28, S61, S63, S65, S66 and S68), zander (S30 and S63), and Atlantic cod (S29, S30, S61, S62, S63, S64, S65 and S67), sometimes in substantial proportions (Fig. 3; Supplementary Tables 1–3). All products come from a factory in the Netherlands (Fig. 2), which appears to include these additional species in the processing of the products for reasons that require further investigation. Such biocomplexity associated with the supply chain is unlikely to be unravelled without a DNA testing programme.

By far the most recurrent case of mislabelling is the existence of cod products that contain substantial amounts of Alaska pollock, in both the UK and Italy, which may be explained by the latter's greater availability, and lower price compared to Cod (Pardo et al., 2016) (Supplementary Fig. 2). Consistently, even products deemed "green", were shown to contain minority amounts of DNA sequences from species not declared on the labels and, even if these are approximations of the real proportions, there are no doubts that other species are simultaneously present in these products (Klapper et al., 2023). This is also the case of one of the "steak" products where the unexpected presence of *M. productus*, along with the declared *M. paradoxus,* may be due to its inclusion as entire fillets or parts of them. Samples S14, S15 and S26, all labelled as haddock (*M. aeglefinus*), still contained a significant percentage of Alaska pollock. These products originate, respectively, from processors "2", "19", and "8", which also provide traditional Alaska pollock products (such as S3, S9, S10 and S16 from establishment "2", or S5 from "19"), or where Alaska pollock was identified alongside the declared species (cod) but not explicitly stated (e.g., S27 from "8"). The addition of readily available cheaper species to bulk up haddock and cod products creates the conditions for financial gain (Carvalho et al., 2017), as well as when in two products (S59 and S70) from the same processing plant and brand, the declared Alaska pollock was substituted with *M. productus*, as found in frozen unprocessed products (Blanco-Fernandez et al., 2021). These large scale gadoid species substitution patterns may not just necessarily result from deliberate financial incentives, but also from the need to secure a steady and secure supply of raw whitefish. Since the regulations do not mandate the reporting of the exact species in this type of products, such practices would be acceptable, if the items on retail were not making inaccurate marketing claims. Indeed, products labelled as made of "whitefish", from the same processor, simultaneously contained *G. chalcogrammus*, *P. virens* and *M. aeglefinus* (S6) and *G. chalcogrammus*, *M. aeglefinus* and *G. morhua* (S8). Yet, as mentioned above, neither the retailers nor the large distribution companies would be in the position to ascertain the true composition of these products, without conducting DNA metabarcoding checks.

These intricate dynamics along the processing and supply chain have some bearing on sustainability schemes. We found that all the undeclared species in MSC-certified products sampled in supermarkets were included in the MSC certification programme. Therefore, these products could still be considered environmentally friendly, overall. On the other hand, the three MSC certified products from the Dutch-Italian supply flow, as discussed above, contained lemon sole, haddock, and zander, none of which is within the MSC scope. This raises concerns around some certified companies' commitment to comply with the MSC programme, as well as for the ability of MSC to control such compliance. Since the MSC already showed willingness to monitor labelling accuracy across the scheme by carrying out DNA barcoding tests on unprocessed fillet (Barendse et al., 2019), a case could be made for the expansion of testing to include metabarcoding analysis of processed mixed seafood.

5. Conclusions

Our findings highlighted the highly diverse nature of pre-packed processed fish products, even where a single species is declared. Such diversity can currently only be characterised in detail through DNA metabarcoding. By combining data on species composition and label information, we could uncover some of the mechanisms and stages of the global supply chain that are vulnerable to unintentional errors and/or deliberate malpractice. Raw material management in production plants plays a key role in determining species composition, while the long distance that exists between raw material and consumers enhances exposure to mislabelling. Our results showed that current practices expose consumers to a significant amount of poorly traceable and mislabelled products, hindering progress towards a truly sustainable seafood supply. Without more frequent and systematic DNA-based monitoring, it remains difficult to fully understand mechanisms and motives (Fox et al., 2018), as non-compliance at one of the nodes along the chain can irreversibly compromise the authenticity of the product all the way down the supply (Luque & Donlan, 2019). As a natural progression from the transformative impact that DNA barcoding had on seafood market operations globally (Mariani et al., 2015), we argue that the now established metabarcoding approach can play a major role in monitoring authenticity in complex production sectors across the agri-food arena. To further boost such a transformation, the use of low-cost, real-time and long reads sequencers, such as nanopore technology, could accelerate progress in statutory product identification in the seafood industry (Ho et al., 2020; Shum et al., 2024), although dedicated studies need to be carried out to verify its robustness (e.g., error rate) compared to traditional sequencing methods. These changes may entail voluntary monitoring within the industry and eco-labelling organisations, as well as the implementation of government-led testing programmes, which would improve the safety of products, the rights of consumers and brands, and the sustainable management of fish stocks.

CRediT authorship contribution statement

Lucilia Lorusso: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Peter Shum:** Writing – review & editing, Software, Methodology, Data curation. **Roberta Piredda:** Writing – review & editing, Data curation, Conceptualization. **Anna Mottola:** Writing – review & editing, Data curation, Conceptualization. **Giulia Maiello:** Writing – review & editing, Visualization, Software, Methodology.

Emma L. Cartledge: Writing – review & editing, Methodology. **Erika F. Neave:** Writing – review & editing, Software. **Angela Di Pinto:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Stefano Mariani:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

The fastq raw data files are available on zenodo.org under the following doi's: https://doi.org/10.5281/zenodo.13335561.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114901.

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