



# Improving the knowledge basis for advice on North Sea horse mackerel

Developing new methods to get insight on stock boundaries and

Authors: Thomas Brunel, Edward D. Farrell, Michiel Kotterman, Christiaan Kwadijk, Ruben Verkempynck, Chun Chen and David Miller

Wageningen University & Research Report C092/16

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Publication date: 29th of September, 2016

Wageningen Marine Research  
IJmuiden, 29 September 2016

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Brunel, T., Farrell, E.D., Kotterman, M., Kwadijk, C., Verkempynck, R., Chen, C and Miller, D. 2016. *Improving the knowledge basis for advice on North Sea horse mackerel. Developing new methods to get insight on stock boundaries and abundance*. Wageningen, IMARES Wageningen UR (University & Research centre), Wageningen Marine Research report C092/16 57 pp.; 10 tab.; 70. ref.

Keywords: horse mackerel, stock structure, genetics, gas chromatography, survey index.

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# Contents

<b>Summary</b>	<b>5</b>
<b>1 Introduction</b>	<b>7</b>
1.1 The North Sea horse mackerel	7
1.1.1 Biology and life cycle	7
1.1.2 Current perception of horse mackerel stock identity and stock boundaries in the North East Atlantic	8
1.1.3 North Sea horse mackerel fishery	10
1.1.4 Stock assessment and abundance indices	11
1.2 Aim of the project and organisation of the work	12
1.3 Assignment and alteration of the original plan	13
<b>2 Strategy for Sample collection</b>	<b>14</b>
2.1 Spatial and temporal requirements	14
2.2 Samples collected and selection of samples for genetics and GCXGC-MS	14
2.3 Characteristics of the samples selected and selection of the subsample for genetic and GCXGC-MS analyses	16
2.3.1 Sample available	16
2.3.2 Selection of fish for further analyses	16
<b>3 Genetics study</b>	<b>18</b>
3.1 Introduction	18
3.2 Materials and Methods	18
3.2.1 Marker discovery and primer design	18
3.2.2 Amplicon generation and sequencing	19
3.2.3 Statistical analyses	19
3.3 Results	20
3.3.1 Marker discovery and primer design	20
3.3.2 Amplicon generation and sequencing	20
3.3.3 Statistical analyses	22
3.4 Conclusions	23
<b>4 Chemical fingerprint approach to stock discrimination</b>	<b>24</b>
4.1 GCxGC-MS principle	24
4.2 Methods	24
4.2.1 Lipid content analysis	24
4.2.2 GCxGC-MS Analysis	24
4.3 Statistical analyses of the results	25
4.3.1 Post processing of the results	25
4.3.2 Row results	25
4.3.3 Presence/absence test	28
4.3.4 Classification tree	29
4.3.5 Multivariable statistical methods.	31
4.4 Discussion	34
4.4.1 Differences between early and late summer chemical fingerprints	34
4.4.2 Limitation of the statistical analyses	35
4.4.3 Differences between North Sea horse mackerel and western horse mackerel	35

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4.4.4	Towards an operational method to discriminant geographical origin?	35
<b>5</b>	<b>Survey index</b>	<b>38</b>
5.1	Goal	38
5.2	Methods	38
5.2.1	log Gaussian Cox process model based on number per haul	38
5.2.2	Delta log-normal method based on CPUE per haul	38
5.3	Survey data and data pre-processing	39
5.3.1	NS-IBTS in Q3	39
5.3.2	French CGFS in Q4	39
5.4	Results	40
5.5	Discussion	45
<b>6</b>	<b>General conclusions and recommendations for future work</b>	<b>46</b>
<b>7</b>	<b>Quality Assurance</b>	<b>48</b>
	<b>References</b>	<b>49</b>
	<b>Justification</b>	<b>52</b>
<b>Annex 1</b>	<b>Guidelines for Sampling horse mackerel for chemical pollutants</b>	<b>53</b>
	Guideline for sample collection	53
	Guideline for sample preparation	53
<b>Annex 2</b>	<b>Guidelines for Sampling horse mackerel for genetic analyses</b>	<b>55</b>

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# Summary

The North Sea horse mackerel stock is currently classified by ICES as a data poor stock, for which the catch advice is based on the trend in an abundance index. The development of an analytical stock assessment, necessary to give more accurate advice, is hampered by a number of limitations on the input data, among which the most important are the poor quality of catch-at-age data and the absence of a targeted survey for North Sea horse mackerel. The aim of this project was to study possibilities to improve the data quality used for an analytical stock assessment model.

The management boundary between the western and North Sea stocks in the English Channel (corresponding to the separation between areas VIIe, western Channel and VIId, eastern Channel) does not correspond to a real biological boundary, as mixing of the two stocks is known to occur in area VIId in autumn and winter. The catches taken in VIId, officially considered as being North Sea horse mackerel and representing around 80% of the catches from this stock, therefore also contain horse mackerel from the western stock, in an unknown proportion. This is likely to deteriorate the cohort signal in the catch at age matrix, hampering the development of an age-structured assessment model for the North Sea stock. Developing methods to separate catches from the western stock from catches from the North Sea stock in area VIId are therefore necessary to improve the quality of the catch information for the North Sea stock. Within this project, two pilot studies, based on genetics and chemical fingerprint, are conducted to investigate new methods to determine stock structure and to develop techniques to identify the stock origin of the catches taken in the eastern English Channel.

Horse mackerel samples were collected from the spawning stocks (June and July 2015) and post-spawning stocks (August-September 2015) in the western area (Celtic Sea and northwestern Ireland) and in the southern North Sea by commercial fishing vessels and research vessels. Only the samples corresponding to the spawning stocks were used for the genetic analyses, while samples from both periods were used for the chemical analyses.

The first step in the development of a method of genetic stock identification for discriminating North Sea and Western Horse mackerel was the identification of microsatellite markers. Using "next generation sequencing", the entire genome of 2 two specimens (one from the Western and one from North Sea) was sequenced, and a total of 100 potentially polymorphic microsatellites were identified. The second step consisted in amplifying (making a large number of copies) and sequencing these markers for 96 fish (half from the western stock and half from the North Sea stock) in order to look for differences in allele composition between the two stocks. Among the 100 potential markers, about 50 were found not to be polymorphic (and hence not usable to look for differences between stocks), and about 25% were confounded by a high level of error in the sequencing (typically, foreign sequences, e.g. ape, inserted in the microsatellite sequence). Of the remaining microsatellites, only 17 were found suitable to be used as markers. Statistical analyses showed a fair amount of genetic differentiation between the North Sea and the western fish, of a similar magnitude as observed between significantly different stocks for other marine fish species. However, the difference here was not significant, mainly due to low number of markers and individuals included in the statistical analyses. Although inconclusive with respect to the degree of stock structure, this study has identified a large number of markers which could potentially be used in future studies. To improve the statistical power of the analysis, future projects should intent to collect a larger number of samples, and include samples covering a larger geographical area. Especially, stocks located at the opposite end of the distribution range of the species (e.g. Mauritania) should be incorporated in order to assess the scale of the maximum possible genetic differentiation between 2 horse mackerel stocks.

The chemical fingerprint analysis was carried out using two-dimensional gas chromatography (GCxGC-MS). Common applications of this analysis technique aim at looking at the presence, or measuring the

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concentration, of a priori defined chemical compounds. Here, the aim was to establish a full chemical fingerprint of the horse mackerel samples from both origins. Contrasting results were observed between first round of analyses performed on the early summer samples (10 individuals from each stock) and the second round of analyses performed on the late summer samples (20 individuals from each stock). The first round of analyses detected 131 compounds but many of them were found only in one or few individuals. For the second round of analyses, 96 compounds were detected, and the majority of them were found in many fish. For both rounds of analyses, the general pattern for most of the compounds was that concentrations in North Sea individuals were higher than in western individuals. As the actual nature of the compounds was not identified, the chemical fingerprints of the two rounds of analyses could not be compared, and it was not possible to assess whether the chemical fingerprint for a given area changed between the early and the late summer. For each round of analyses, a small number of compounds were identified as a potential marker of the stock origin based on presence absence analysis and on classification methods (classification tree). Using these specific compounds to predict stock origin resulted in a relatively low misclassification rate (5-7.5%). However this predictive power was not estimated using cross-validation (i.e. on an independent data set) and it is therefore likely to be overestimated. In addition, because these simple classification methods are based on the value of only 1 or 2 compounds in a limited number of individuals, their reproducibility may be questioned. As a matter of fact, the compounds that were found exclusively in the North Sea horse mackerel in the late summer sample were actually found also in the western fish in the early summer sample, which suggests that they are not truthful markers. Multivariate analysis methods, comparing the concentration of a larger number of compounds and therefore less subject to chance, were also implemented, but they had a higher misclassification rate (15-35%), mostly due to the lack of contrast in the data (i.e. the main signal in the data was higher concentrations in the North Sea than in the west, but with a large overlap between the two areas). The results from this pilot study suggest that the chemical finger print approach is a potential tool to determine stock origin, with a moderate risk of misclassification. However, more insight on the sources of variation of compound concentrations (seasonal changes, influence of sex, length, age, reproducibility of the results from year to year) is required before this method can be further developed.

Finally, work was carried out to develop survey indices for the North Sea horse mackerel. The only available index (developed in previous projects carried out at Wageningen Marine Research) is derived from the Q3 North Sea IBTS survey. This survey however covers an area where only 20% of the catches from this stock are taken in the recent years. The French CGFS (Channel Ground Fish Survey) covers the area VIId in Q4 and is carried out with a similar gear and protocol as the North Sea IBTS. These surveys are not taking place at the same time of the year. Because fish probably migrates from the North Sea to the Channel between the two surveys, no attempt was made to combine the data from the two surveys to compute a single index. Instead, they were treated as two independent surveys. Two analytical methods to derive an abundance index from the raw CPUE data were investigated: a simple method, a delta log-normal model, and a more sophisticated method intending to incorporate autocorrelation (temporal and spatial) in the data, a log-Gaussian cox process model. The fit of log-Gaussian cox process model for both surveys indicated that, unlike other species (e.g. mackerel, cod), autocorrelation in the horse mackerel data was weak, and the estimated abundance indices had a large uncertainty. The simpler approach, delta log-normal model, resulted in a less uncertain abundance index. The trends in the IBTS and CGFS indices were very similar, indicating a decreasing stock from the early 1990s until around 2005, and a stock stable at low levels since then. The inclusion of this CGFS index, covering a key geographical area for this stock and supporting the perception of stock trend from the IBTS index, improves the basis for the provision of advice on the catch limits for the North Sea. The delta log-normal indices from the IBTS and the CGFS surveys have been used by ICES since WGWIDE 2015 to give catch advice.

Additional work is being carried out to develop an abundance index from commercial catch rates. However this work is not finalized and is not part of this report.

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# 1 Introduction

## 1.1 The North Sea horse mackerel

### 1.1.1 Biology and life cycle

Horse mackerel (*Trachurus trachurus*) is a species of jack mackerel from the family of the *Carangidae*. This species can be found in a depth range of 0 – 1050 m but usually resides in waters of 100 – 200 m depth. It is distributed from Norway to Western Africa and the Mediterranean Sea. Adult individuals can form large schools in coastal areas with sandy substrate where they feed on smaller fish, crustaceans, and cephalopods (Froese and Pauly, 2015). Horse mackerel in the northeast Atlantic is mainly caught with pelagic trawls and purse seines, often close to the sea floor (Abaunza et al., 2003). Van Marlen (2000) found that horse mackerel schools often mix with mackerel schools.

Maturity ogives of horse mackerel in literature are generally related to length rather than to age. The reason is obvious: it is much easier to measure length than to estimate age. On the other hand, when age is used, different criteria for interpretation among authors increase the uncertainty of establishing maturity by age-groups (Abaunza et al., 2003). Specifically horse mackerel shows great plasticity in growth. This means that it is possible for individuals of only a few years old to reach sizes that are attained by other individuals only many years old. Thus, it is difficult to establish a reliable age-length key. A review article on the growth and reproduction of horse mackerel (Abaunza et al., 2003) found a range of 18 - 24 cm of fish length for length at first maturity in North Sea and English Channel. One study (Sahrhage, 1970) found that this length range would correspond to one year old horse mackerel.

Horse mackerel have distinct areas for spawning, feeding, and over-wintering. Migration is probably driven by water temperature and availability of (prey) food. In autumn, when water temperature is below 10°C, the North Sea horse mackerel stock retreats from feeding areas in the Norwegian Sea and the North Sea, and migrate to the over-wintering areas further south, predominantly in the English Channel (Macer, 1974). However they do not reside in water with a temperature lower than 8°C (Polonsky, 1965). In winter, horse mackerel form dense schools in deeper waters, while in spring their distribution becomes far more dispersed (Polonsky, 1965). They migrate northwards with increasing water temperature during spring (Chukskin and Nazarov, 1989). The North Sea stock is found at the southern Dutch and English coasts in April (Meek, 1916), and reaches the western coast of Denmark and southern Norwegian coast by August.

Spawning takes place during May and June and tails off until August or September (Macer, 1974). There is little spawning in the English Channel, despite the abundance of horse mackerel in the western English Channel in winter. A study on horse mackerel reproductive biology (Macer, 1974) has shown that there is a major spawning area off the Dutch coast in the southern Bight of the North Sea (Figure 1.1). Fat and energy content of adult horse mackerel is lowest during and after spawning in late spring and summertime, and highest in autumn (Sahrhage, 1970). In the North Sea, after summer, body energy content of horse mackerel rises rapidly, but feeding ceases as soon as water temperatures drop below 10°C by winter. At 8-9°C, horse mackerel stop feeding and leave the area to over-winter in relatively warmer waters. In spring, before spawning, only limited fat reserves are left in the gut and muscle tissue (Leloup and Gillis, 1964).



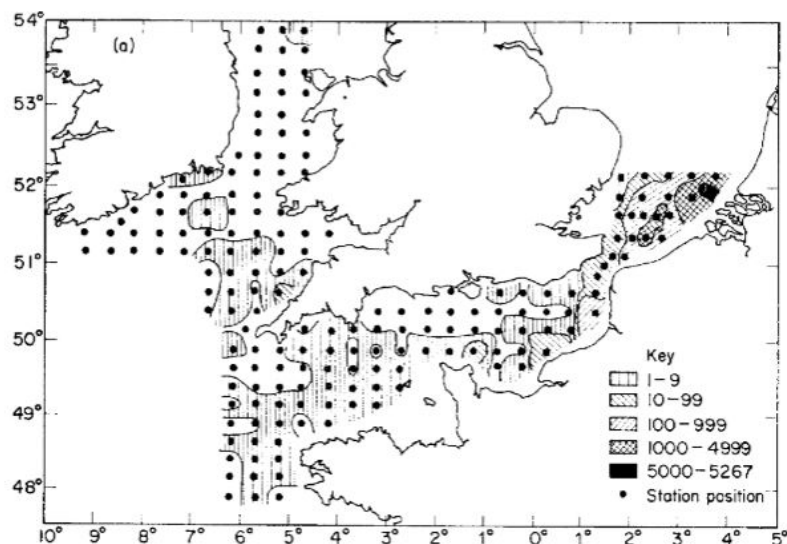


Figure 1.1: distribution of horse mackerel egg density (numbers/m<sup>2</sup> below surface) in May-June 1967 (from Macer, 1974).

Horse mackerel is a fairly long-lived species, reaching a maximum age of well over 30 years (Eltink and Kuitert, 1989). This means that an occasional strong year-class can lead to high abundance of horse mackerel. In 1982, an extraordinary strong year-class created a substantial fishery in the northern areas, which continued for more than a decade (Abaunza et al., 2003). As a result horse mackerel became an important commercial species and is now one of the three most important pelagic species in the European fish industry.

### 1.1.2 Current perception of horse mackerel stock identity and stock boundaries in the North East Atlantic

ICES has long considered horse mackerel (*Trachurus trachurus*) in the northeast Atlantic to consist of three stocks (Figure 1.2A). The southern stock was defined as that found in the Atlantic waters of the Iberian Peninsula (Division IXa), the North Sea stock in the eastern English Channel and southern North Sea area (Divisions IIIa, IVb,c, and VIId), and the western stock on the northeast continental shelf of Europe, stretching from the Bay of Biscay in the south to Norway in the north (Subarea VIII and Divisions IIa, IVa, Vb, VIa, and VIIa-c, e-k). This separation of horse mackerel was based on a variety of factors including the temporal and spatial distribution of the fishery, the observed egg and larval distributions, information from acoustic and trawl surveys and from parasite infestation rates (see ICES, 2015). A tagging programme was established in 1994 (ICES, 1995) and further studies using allozyme differentiation and morphometric characteristics, were conducted in 1997 (ICES, 1998). Tagging studies failed to recover any tagged fish, and neither the genetic or morphometric studies provided a basis for changing the stock separation as previously defined.

Further refinements of the definitions of stock units were based on the results from the EU-funded HOMSIR project (2000-2003), using a multidisciplinary approach that included various genetic approaches (allozymes, mtDNA and microsatellites), the use of parasites as biological tags, body morphometrics, otolith shape analysis, and the comparative study of life history traits (growth, reproduction and distribution) (Abaunza et al., 2008). The resulting stock structure was broadly similar to that previously considered by ICES (Figure 1.2B). However, it was observed that the population structure in the western European coasts could be more complicated and that more research was needed to clarify the migration patterns within the Northeast Atlantic Ocean. This was especially relevant to the mixing areas between the North Sea stock and the Western stock (Northern North Sea and English Channel). The sampling in this region was relatively sparse whereas the

southern regions had significantly better coverage (Figure 2.2A). The genetic components of the project failed to resolve stock structure largely due to the low number (four microsatellites) and low power of the genetic markers employed (Kasapidis and Magoulas, 2008).

The results of the HOMSIR project have been debated in terms of whether they legitimised a change in management boundaries. There was indeed only one sampling location in the area where the North Sea stock was assumed to be distributed, located in area IVb. The southern North Sea and the entire English channel were not represented in the samples (areas IVc, VIId and VIIe). It has been argued that mixing of both stocks (Western and North Sea) occurs in VIId and that catches there in fact are not solely of the North Sea stock. Indeed, while their spawning and feeding areas seem to be separated (Eltink, 1992), there are also indications that the distribution of the western and North Sea stocks overlap partly during over-wintering in the English Channel (Macer, 1977). There appears to be no solid basis for a separation of the North Sea and Western stock at the limit between area VIId and VIIe.

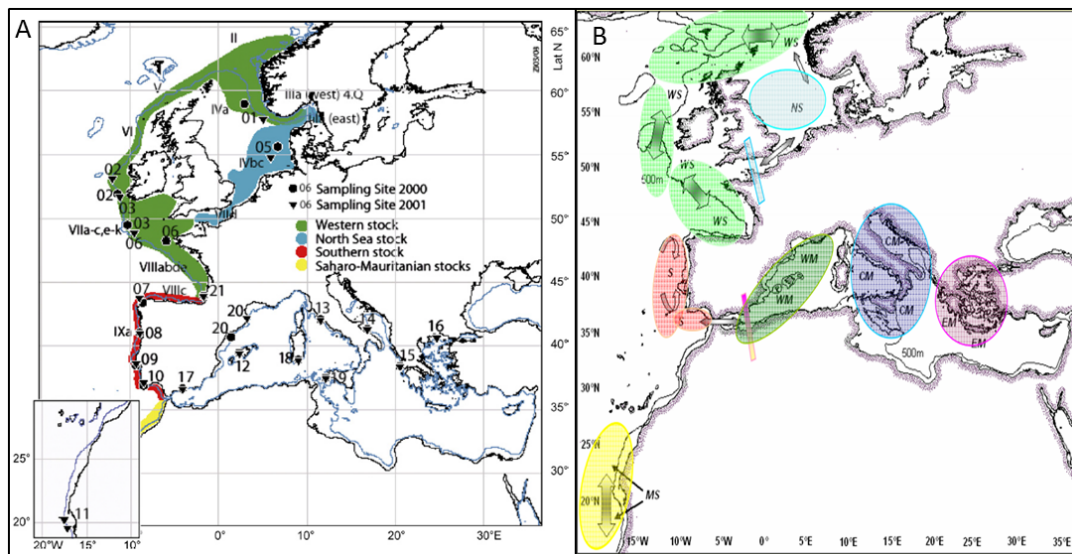


Figure 1.2. (A) The suggested stocks of horse mackerel prior to the HOMSIR project. The sampling sites in the HOMSIR project in 2000 (circles) and 2001 (triangles). (B) Proposed horse mackerel stocks according to the results of the HOMSIR project. The arrows indicate possible migratory movements. WS: western stock; NS: North Sea stock; S: southern stock; MS: Saharo-Mauritanian stock; WM: western Mediterranean stock; CM: central Mediterranean stock; EM: eastern Mediterranean stock. From Abaunza et al. (2008).

A recent preliminary study on western and North Sea horse mackerel employed 12 microsatellites (4 from horse mackerel, *Trachurus trachurus* and 8 from Chilean jack mackerel, *Trachurus murphyi*) to screen a small number of samples from both putative stocks (Figure 2.2B). The results indicated significant population structure within the samples from the western stock and no significant structure between samples collected west of Ireland and those collected in the central North Sea (Mariani, 2012). However there were a number of issues related to the genetic markers employed being non species-specific and also the samples screened not being from spawning stocks.

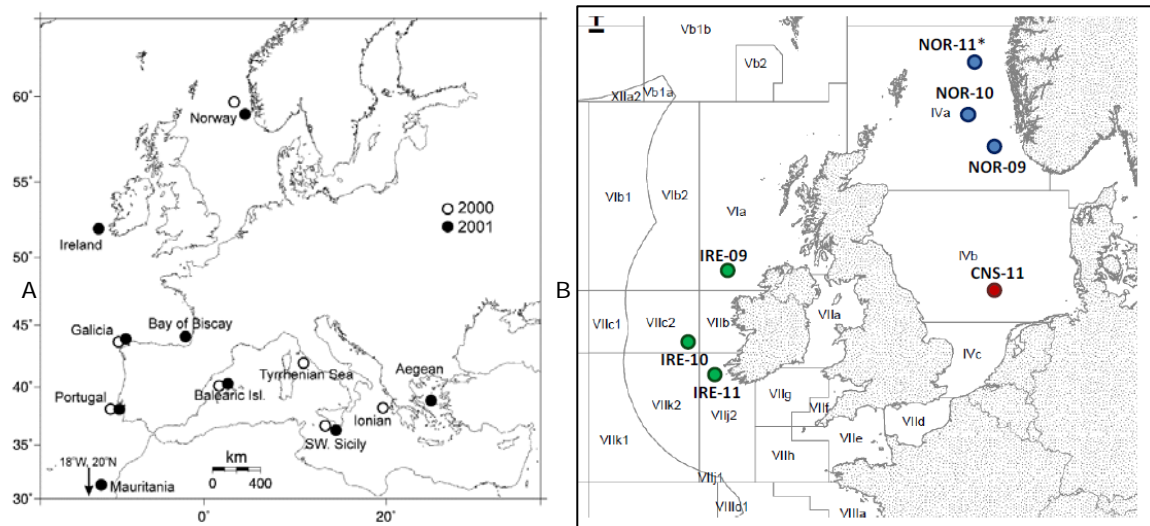


Figure 2.2. (A) The genetic samples collected and analysed in the Kasapidis & Magoulas (2008) study which was part of HOMSIR. (B) The genetic samples collected and analysed in the Mariani (2012) pilot study.

The degree of separateness of the western and North Sea stocks is also uncertain. It is known that the western stock spawns west of Ireland and that the North Sea stock has a separate spawning ground in the North Sea. However it is unclear if these grounds are used interchangeably. Unlike herring, horse mackerel are not known to be faithful to their original spawning grounds. Therefore, without strong evidence to the contrary, it cannot be assumed that the two stocks are indeed separate. Treating these stocks as separate, if indeed they are not, can lead to inaccurate stock assessments, and therefore potentially inappropriate management decisions. Further research is needed to clarify the level of differentiation between the North Sea and Western stocks and also to define the boundary areas, if any, between them.

### 1.1.3 North Sea horse mackerel fishery

Catches of horse mackerel in divisions IVb, IVc and VIId and divisions IVa and IIIa during the first half of the year are considered to correspond to the North Sea stock.

Historically, in the 1970s and 1980s, the horse mackerel fishery in the North Sea has developed as an industrial fishery for reduction into fish oil and fishmeal operated by Danish vessels. Catches were mainly taken in ICES area IVbc and VIId in quarter 4. Horse mackerel was then discarded in the human consumption fisheries because of its low value. The effort of the Danish industrial fleet sharply decreased in the 1990s, owing to an increase in fuel cost combined with a decrease in fishing opportunities and a drop in price of industrial fish. In the early 2000s the Danish pelagic fleet underwent a restructuring triggered by the introduction of individual quota management, and the current fleet now represents less than 20% of what it was in the 1980. Owing to the predominance of the Danish fishery in the North Sea horse mackerel fishery in the 1970s and 1980s, Denmark currently holds about half of the North Sea horse mackerel TAC.

Concurrently to the decrease of industrial fleet, a directed human consumption fishery, mostly operated by the Dutch owned pelagic freezer trawlers, developed in the late 1990, mainly due to the development of new markets outside Europe. Quota trading between Denmark and the Netherlands contributed to the increase of fishing opportunities for the freezer trawler fleet, but since the Danish quota management system only allows for exchange of a limited amount of quota, most of the Danish quota remained unfished, resulting in a underutilisation of about 50% of the North Sea horse mackerel TAC in the recent years. With the substantial decrease in the TAC in 2014, the rate of utilisation of the TAC has increased and the TAC was fully taken in 2015 (ICES, 2015).

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The catches were relatively low during the period 1970-1980s with an average of 18 000 tonnes (ICES, 2015). The catches increased in the late 1990s to reach in 48 425 tonnes in 2000, mostly taken by the Netherlands, and in a much smaller proportion Denmark and Germany. Between 2000 and 2010, the catches varied between 23 379 and 48 425 tonnes and subsequently decreased since 2007. In 2014 the catch reached the lowest value since the early 1990s (13 388 tonnes), mostly taken by the Netherlands and the UK.

The geographic location of the catches changed markedly over the years (ICES, 2015): the catches were mostly centred over the area IVbc, or even occasionally IIIa in the 1980s and until the mid-1990s, the proportion of catches taken in VIId increased to 50% during the 1990s and early 2000s. Catches in VIId further increased and represented around 80% of the North Sea horse mackerel catches over the recent years, but this value shows large interannual variations.

#### 1.1.4 Stock assessment and abundance indices

##### Earlier work

The ICES working group on Mackerel Horse Mackerel Sardine and Anchovy (WGMHSA) used the mean catch rates in the Q3 IBTS as a relative abundance index. Over the years 2003 to 2007 WGMHSA ran exploratory assessments (ICES, 2007) based on catch at age data and this simple IBTS index. Due to the low quality of data, the method deviated from other assessment methods in that the number of parameters was small, which was made possible by the introduction of a number of assumptions.

- 1) The selection ogive is given by one logistic curve.
- 2) The selection parameters are assumed to remain constant within pre-selected sequences of years.

The assessment output, especially the scale of the stock size were shown to be highly dependent on the weighting given to the survey index compared to the catch data. In addition, the model was considered too experimental to be used for a formal assessment. The results, were also affected by errors in data allocation and stock identification and were considered inconclusive (ICES 2007)

With the introduction of the ICES Data limited stock approach (ICES 2012a and ICES 2012b), the North Sea horse mackerel was classified as a category 5 stock, for which trends in the landings was the only information on which to base any advice. In 2013, the IBTS index was incorporated in the advice, but since no uncertainty estimate was associated to this index, the basis for advice remained the same and the stock remained in category 5.

##### Recent assessment and management plan proposal

In 2014, the Dutch pelagic industry and Wageningen Marine Research developed a proposal for a management plan for the North Sea horse mackerel stock, which would ensure sustainable management of the stock, as well as identify how the knowledge base to underpin scientific advice could be improved (Miller and Coers, 2014).

To support the development of this plan, a stock assessment model was developed (JaxAs, as new implementation of the model formerly used at WGMHSA). Given the many uncertainties in the input parameters for this model (e.g. selection pattern, natural mortality, maturity at age, analytical method used to compute a survey index), the management plan simulations were not based on a single "best" model, but took a multi-model approach.

The proposal was presented at the Pelagic advisory committee. The plan specified a rationale for establishing TACs through a Harvest Control Rule (HCR). After a special request to ICES from the Dutch ministry of Economic affairs this proposal was reviewed and its compliance with ICES precautionary criteria was assessed during an expert workshop (WKHOMMP).

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## Current Basis for advice

The work from Coers and Miller (ICES, 2014a) showed that the uncertainty on North Sea horse mackerel catch data and biological information remained too high to develop an acceptable analytical assessment. However based on this work, the ICES advice in 2014 was based on the trend in the IBTS index calculated using the zero inflated GLM method developed by these authors. The stock was classified as a category 3, for which the trend in the survey is considered representative of the trend in the stock.

In 2015 an additional survey index was provided (the French CGFS survey in Division VIIId) as a result of the work carried out in the early stage of this project. This additional survey covers the main fishing area for this stock, but as it is carried out in October, there is a risk that part of the catches of horse mackerel belong to the western stock. Both survey indices have a high variability, but show a similar trend. The stock was classified again as a category 5.

## 1.2 Aim of the project and organisation of the work

Despite long term efforts to improve the scientific perception of the state of the North Sea horse mackerel, this stock is still classified by ICES as a data poor stock. The recent work from Coers and Miller (ICES, 2014a) developed several alternative assessment configurations for this stock, but none has been yet accepted by ICES. Some of the main obstacles to the elaboration of a valid assessment method where the lack of clear stock boundaries, and the scarcity of abundance or biomass indices.

The North Sea horse mackerel and the adjacent Western horse mackerel are both present in the eastern English channel (ICES subdivision VIIId) during winter. Catches taken in this area are however reported as North Sea horse mackerel. ICES concluded that it is important to be able to identify catches as originating from that stock with some certainty. It has been shown that failing to take account of complexities in population structures (i.e. with mixing of populations) poses a major problem for management (e.g. Hintzen *et al.*, 2014). Considering the potential of mixing between Western and North Sea horse mackerel occurring in Divisions VIIId+e (the English Channel), better insight in the origin of catches from that area is important, if not crucial, for improvement of the quality of future scientific advice and thus management of the North Sea horse mackerel stock, and to a similar but lesser degree for the larger Western horse mackerel stock.

In order to improve the allocation of catches taken in area VIIId to either stock, a better understanding of the distribution of the two stocks, the existence of a geographical boundary or of an area of mixing are required.

Another obstacle to the elaboration of a robust stock assessment method is the lack of reliable abundance indices. The quarter 3 North Sea IBTS survey which was used in the collaborative project in 2014 to develop indices for this stock is a bottom trawl survey targeting ground fish (gadoids), but also catching pelagic species (e.g. horse mackerel). The use of the IBTS survey was based on the assumption that it covers the North Sea, where the population is thought to be in Quarter 3, ignoring that it does not cover Division VIIId where the majority of the fishery occurs (in Quarter 1 and 4). To develop a better understanding on the trends in this stock, it is important to explore additional sources of information in quarters 1 and 4 which may provide indicators of stock abundance.

**The general aim of the project is to improve the knowledge-basis for the management of North Sea horse mackerel, taking account of and building on the results and recommendations from the collaborative project. Specific actions focus on:**

- 1) Generating better information on stock boundaries and mixing. To achieve this goal the project investigated the potentiality of two apply modern analytical stock identification methods (chemical analyses, genetics) to distinguish horse mackerel origin.**
- 2) Improve the abundance index by incorporating additional survey data**

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## 1.3 Assignment and alteration of the original plan

The original assignment, as defined in the Wageningen Marine Research Tender document 15.43.063, comprises 5 work packages (WPs). The bulk of the work for WP 1 and 3 was supposed to be carried out by students, hosted at the Redersvereniging voor de Zeevisserij (RVZ). The role of Wageningen Marine Research was to provide scientific supervision. Unfortunately, no student could be found to carry out these tasks during the course of the project. In agreement with the client (RVZ), the budget ascribed to WP1 and 3 was reallocated to WP 2 to carry out GCXGC-MS analyses for a larger number of samples. The WP3 was carried out by the University College Dublin, as originally planned.

### **WP1 Analysis of catch composition information collected on pelagic trawlers**

Task 1.1: talks with QMs; description of available information localizing information

Task 1.2: data entry and first rough analysis (also for WP3)

Task 1.3: final analysis

Task 1.4: presentation of results to the sector

### **WP2 GCxGC-MS sample collection and analysis**

Task 2.1: definition of a sampling scheme

Task 2.2: samples collection

Task 2.3: chemical analyses

Task 2.4: statistical interpretation of the results

Task 2.5: final results and reporting

### **WP 3 Genetic sample collection and analysis**

Task 3.1: definition of a sampling scheme

Task 3.2: samples collection

Task 3.3: chemical analyses

Task 3.4: statistical interpretation of the results

Task 3.5: final results and reporting

### **WP4 Commercial fisheries search-time index for horse mackerel**

Task 4.1: talks with QMs; description of available information (also for WP1)

Task 4.2: literature review of search rate indicators

Task 4.3: data entry and first rough analysis (also for WP1)

Task 4.4: final analysis and reporting

### **WP5 Combining the French en English groundfish survey series in VIId with the IBTS survey**

Task 5.1: connect with IFREMER and CEFAS to get the data

Task 5.2: analysis of potential usability of IFREMER and CEFAS surveys for horse mackerel. If positive:

Task 5.3: combined analysis of the North Sea IBTS and the eastern channel surveys.

Task 5.4: final analysis and reporting.

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## 2 Strategy for Sample collection

### 2.1 Spatial and temporal requirements

For both techniques investigated in this projects, based on genetics and on GCXGC-MS, the aim to is find markers (microsatellite or chemical compounds) which differ depending on the geographical origin of the fish, and could be used to assess the proportion of western and North Sea horse mackerel in the catches taken in the eastern Channel. For this approach, it is essential that the fish samples taken to identify potential markers can be ascribed with certainty to each of the two putative stocks. In the case of horse mackerel, the ideal time to collect samples of individuals from the Western stock and from the North Sea stock is spawning time, as at that time both stocks are separated.

The Western stock spawns mainly from May to July (ICES, 2014b). Egg surveys data shows that there is still important spawning activity in July from the Celtic Sea to the West of Scotland, with a maximum intensity in the west of Ireland (ICES, 2014b). The North Sea stock spawns at the same period from May to June, mostly off the Eastern coast of the North Sea from Belgium to Denmark (Eltink, 1991; Macer, 1974).

Unlike genetic markers, which are permanent, the concentrations of chemical compounds are related to the physiology of the fish, and may change throughout the biological life cycle. Processes affecting the accumulation or the mobilisation of fat reverses in the fish (e.g. to produce gametes) may result in changes in compound concentrations. The horse mackerel samples taken to establish the chemical signature of a given geographical origin should therefore not be taken too long before the period of mixing in the channel (October-November).

In the case of the GCXGC-MS analyses, there are therefore two contradictory constraints for the collection of the samples to identify markers of the stock origin: 1) ensuring the stock origin of the samples by taking them at spawning time, vs. 2) avoiding changes in compound concentrations by taking samples close to the period of potential mixing in the Eastern channel.

**The collection of samples was therefore planned as follows:**

- **For the genetic analyses, the priority is to collect fish during spawning time (no later than July) on the spawning ground.**
- **For the GCXGC-MS analyses, fish samples will be collected at the end of summer to reduce the risk of changes in compounds concentration, while still having a low risk that a fish from a given spawning origin could be caught in the other geographical area**

### 2.2 Samples collected and selection of samples for genetics and GCXGC-MS

#### Samples collected

A first horse mackerel sample originating from the Bay of Biscay was set aside in March by a freezer trawler and recovered at the start of the project (figure 2.1). The project started mid-June 2015, and in order to get samples during the spawning season, collection started immediately. Following the justification exposed in the section above, the samples were collected during two main periods: the early summer (end of June / July) corresponding to the end of the spawning season, and late summer (end of August / September) corresponding to the period just prior to migration towards the eastern

Channel. For both periods, the samples from the western horse mackerel were obtained from research surveys: the Boar fish Acoustic Survey (BFAS) in July and the Irish Ground Fish Survey in September. The samples from the North Sea were collected by the Dutch demersal (flyshooters) fleet, both in early and late summer.

The unprocessed fish for the samples collected from the freezer trawler and those from the Irish surveys were frozen immediately after the catch. The horse mackerel samples from the demersal fleet were taken from hauls at the end of the fishing trips and kept in ice and were frozen after landing. Frozen samples were transported either to Wageningen Marine Research or to the UCD where they were stored at -20°C.

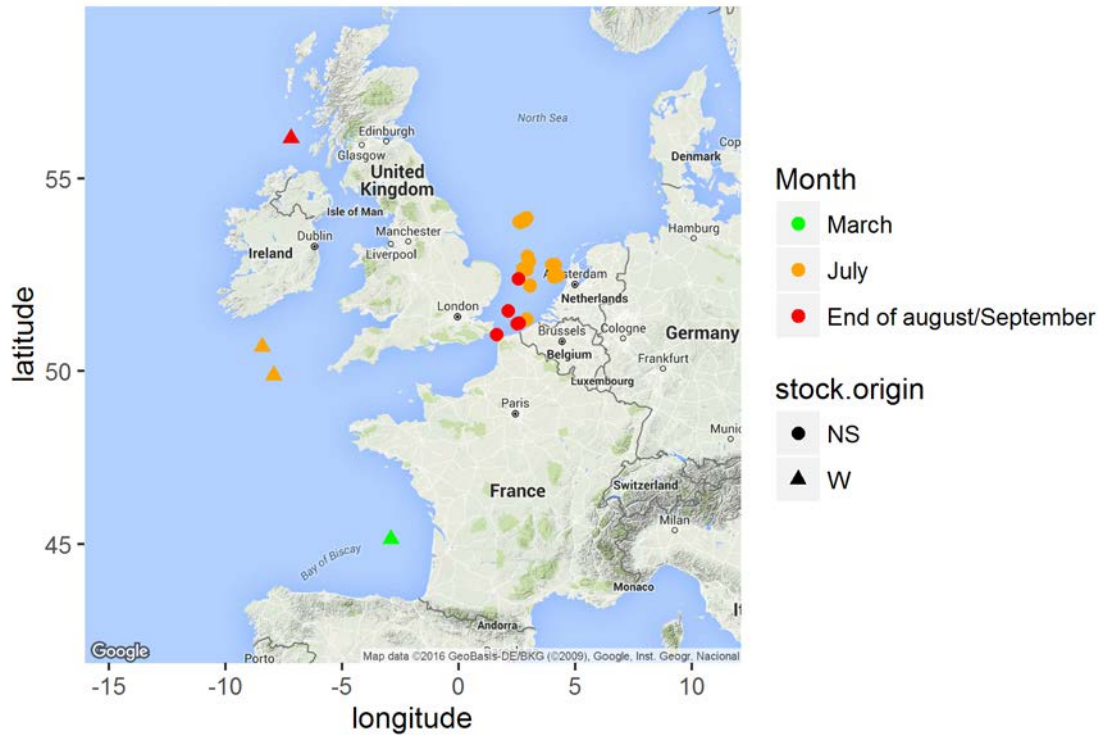


Figure 2.1: geographical origin of the horse mackerel samples collected

### Samples preparation

As explained above, only the samples taken in early and late summer were used for further analyses. The March sample was not considered suitable to establish a baseline for the western horse mackerel as it was taken before spawning time.

After thawing, individual fish were measured and weighted, their sex and maturity status were estimated, and otoliths were taken. Tissue samples were cut following protocols for the genetic and GCXGC-MS analyses. These protocols are given in annex of this report.

Samples from the western horse mackerel were processed by University College Dublin and tissue samples for GCXGC-MS were then sent to the Netherlands (and the opposite for the North Sea samples)



## 2.3 Characteristics of the samples selected and selection of the subsample for genetic and GCXGC-MS analyses

### 2.3.1 Sample available

The horse mackerel sampled from the North Sea were in general of smaller size than the samples from the western areas (figure 2.2), especially for the late summer samples for which half of the fish was smaller than 20 cm. Overall, the sex ratio was close to 1:1.

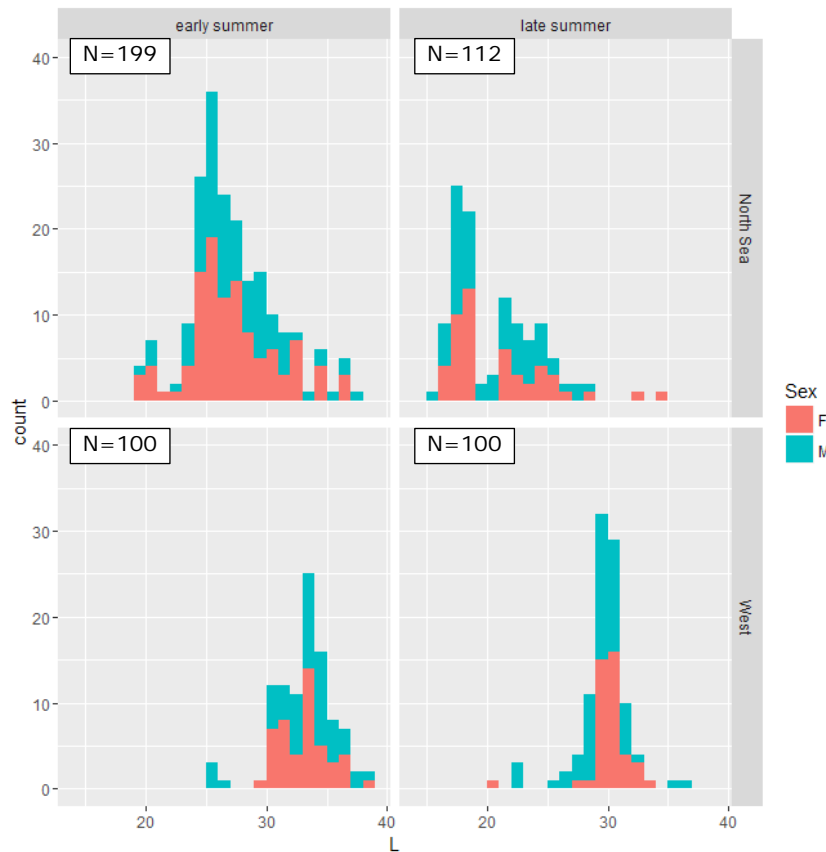


Figure 2.2: length and sex distribution of the horse mackerel in the 4 summer samples

### 2.3.2 Selection of fish for further analyses

#### GCXGC-MS analyses

The concentration of chemical compounds in fish depends on geographical origin, but also on other factors. Compounds that accumulate throughout the life are likely found in higher concentration in older individuals. The sex of the fish is also likely to affect the concentration of chemical compounds, as the level of energy requirement for sexual maturation may differ between males and females. For instance, reproductive females lose fat when producing and releasing oocytes (Abaunza et al., 2003). Therefore, in order to avoid multiplying the factors potentially influencing the outcome of the GCXGC-MS analyses, the fish selected for the GCXGC-MS analyses were only males from a restricted length range.

A first round of GCXGC-MS analyses was carried out on fish from the early summer samples, despite the fact that late summer samples were considered to be more suitable for this analysis. This decision was made after receiving information that no horse mackerel sample was set aside for this project

during the IGFS. Therefore 10 fish from the early summer samples from each geographical origin, of a length ranging from 30 to 35 cm were analysed (figure 2.3).

Later in the course of the project, horse mackerel samples from the IGFS were incidentally found at the Marine Institute (Galway, Ireland) in charge of this survey. Resource from WP 1 and WP4 were also reallocated to this specific WP, allowing for running more chemical analyses. Therefore it was decided to invest the rest of the resources in analysing the late summer samples. At total of 20 fish from each geographical origin were used in this second round of analyses. Given the lack of overlap in the length distribution between the western and North Sea fish in the late summer, fish were selected from a larger length range, from 21 to 31 cm.

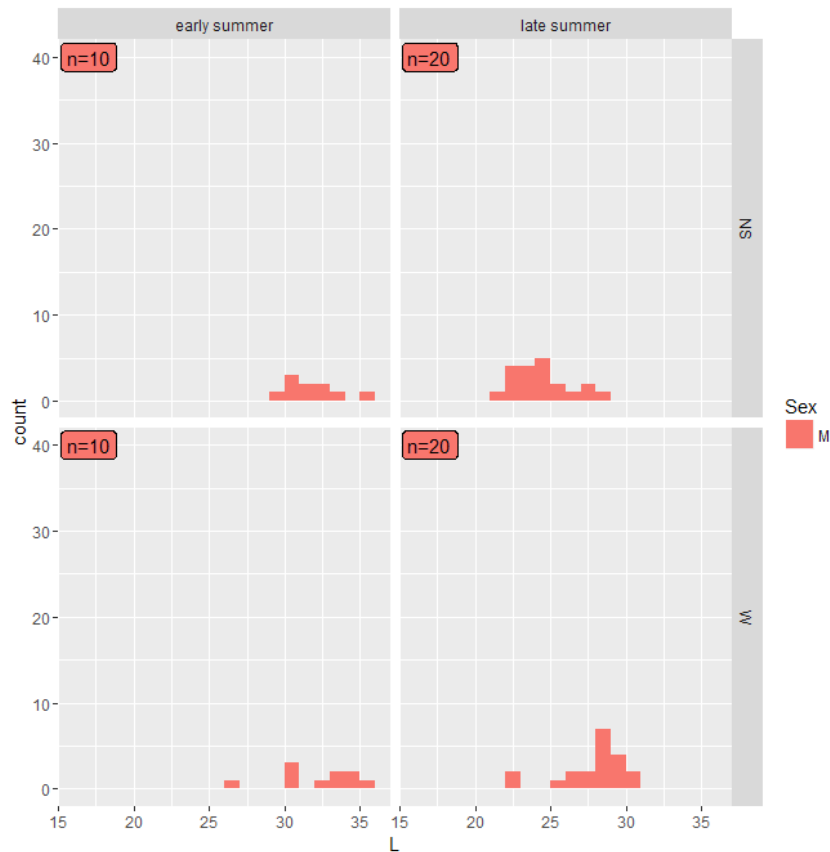


Figure 2.3: length and sex distribution of the horse mackerel selected for the GCXGC-MS analyses

### Genetic analyses

For the genetic analyses, 100 fish were randomly sampled from the early summer sample in the North Sea, and the entire early summer sample from the western area was used.

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## 3 Genetics study

### 3.1 Introduction

The aim of this pilot study was to develop a method of genetic stock identification for discriminating North Sea and Western Horse mackerel. Next Generation Sequencing (NGS) and Genotyping by Sequencing (GBS) based approaches, which were developed during UCD projects on cod (*Gadus morhua*), boarfish (*Capros aper*) and V1a herring (*Clupea harengus*) are used for marker development and screening of spawning samples (Carlsson *et al.*, 2013; Farrell *et al.*, in prep, 2015a, 2015b; Vartia *et al.*, 2014, 2016). This pilot study has two primary deliverables (1) development and validation of at least 24 polymorphic microsatellites markers and (2) screening of spawning fish collected in 2015 from the Western and North Sea stocks to establish a genetic baseline of the spawning stocks and test the presence of population structure

### 3.2 Materials and Methods

#### 3.2.1 Marker discovery and primer design

Two specimens, one from the Western and one from North Sea samples were selected for microsatellite discovery. Total genomic DNA (gDNA) was extracted from 10 mg of tissue from each fish using a modified chloroform/isoamyl alcohol protocol. Isolated gDNA (50µl at 50 ng/µl) was sent to the Duke Center for Genomic and Computational Biology (GCB, Duke University, USA) for library preparation and Illumina 300PE MiSeq shotgun sequencing.

Raw sequence data was downloaded from Illumina BaseSpace and initial quality control was performed using FastQC (Babraham Bioinformatics, 2016.). Overlapping R1 and R2 PE reads were assembled and exported in FASTA format with PANDAseq paired-end assembler for Illumina sequences (Masella *et al.*, 2012). Assembled reads were screened for microsatellites according to the protocol in Farrell *et al.* (in prep.).

Locus-specific forward and reverse primers were designed for putatively polymorphic microsatellite loci with the Primer3 application (Rozen and Skaletsky, 2000) in Geneious® 7.0 with optimal primer length set at 20bp, optimal Tm at 60°C and product size range at 120-180bp. Primers were designed to bind in conserved flanking regions to minimise the possibility of null alleles. Primers were cross-referenced with the original sequence data set to identify primers that annealed to multiple regions, which if detected were excluded. The forward and reverse locus-specific primers were adapted, to facilitate combinatorial barcoding of amplicons, by adding either an M13-R or CAG universal tail to the 5' end as described in Vartia *et al.* (2016) and Farrell *et al.* (in prep.). The modified primers were tested for the formation of secondary structures (hairpins, primer dimers and hetero dimers) with the IDT OligoAnalyzer Tool 3.1 (<http://eu.idtdna.com/calc/analyzer>) and were ordered as 100 µM stock solution (IDT, Leuven, Belgium). Multiplex panels were generated in MultiPLX 2.1 using the low grouping stringency setting and the maximum number of primers per group set at 20 (Kapinski *et al.*, 2005). Primers were diluted to 10 µM working solution and combined according to the MultiPLX 2.1 output to form five 0.25 µM multiplexes. A set of ninety-six 11bp combinatorial barcodes suitable for amplicon sequencing on the Illumina MiSeq platform were designed based on the 12-bp Golay-barcode from Caporaso *et al.* (2012), following Vartia *et al.* (2016).

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### 3.2.2 Amplicon generation and sequencing

Total genomic DNA (gDNA) was extracted from 10 mg of tissue from 96 fish from each putative stock using Chelex® 100 protocol with proteinase K. Microsatellite amplification and barcoding reactions were carried out using a two-step nested PCR as described in Farrell et al. (in prep.). Each plate of amplicons was pooled into a single sample by pipetting 9µl from each well into a new 1.5 ml Eppendorf tube. The concentration of the purified pooled amplicon samples was measured on a Qubit® Fluorometer (Invitrogen, ThermoFisher Scientific) using the Qubit® dsDNA HS Assay Kit (Invitrogen, ThermoFisher Scientific). Pooled amplicon samples were standardised and combined as described in Farrell et al. (in prep.) and sent to Duke Center for Genomic and Computational Biology (GCB, Duke University, USA) for library preparation and amplicon sequencing. Raw FASTQ sequence data was downloaded from Illumina BaseSpace. Reads were sorted and grouped using a modified python script (Vartia et al., 2016).

### 3.2.3 Statistical analyses

Two levels of genotyping success were used to explore the data and generate two datasets. Individuals and loci that had less than 60% and less than 70% genotyping success were excluded from the datasets in order not to bias the analysis. The software MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004) was used, under default settings, to identify possible genotyping errors, including stuttering, large allele drop-outs and null alleles. Deviations from Hardy–Weinberg equilibrium (HWE) and deficiency of heterozygotes were tested with Genepop 4.2 – default settings (Rousset, 2008). Microsatellite Analyzer 4.05 was used, under default settings, to assess the number of alleles, allelic richness, allele size ranges, expected and observed heterozygosities and global and pairwise  $F_{ST}$  estimates (Dieringer & Schlötterer, 2003). In all cases with multiple tests, significance levels were adjusted using the sequential Bonferroni technique (Rice 1989).

POWSIM v4.1 (Ryman & Palm, 2006) was used to estimate whether the number of loci and their allele frequencies provided sufficient statistical power to detect significant genetic differentiation. Simulations were run using default parameter values for dememorizations (1000), batches (100) and iterations per batch (1000), and a range of different values of  $F_{ST}$  (0.0005 – 0.048) were tested by varying the number of generations of drift ( $t$ ) while keeping the effective population size ( $N_e$ ) constant at 1000. The statistical power was estimated after 1000 replicates as the proportion of statistically significant test ( $p < 0.05$ ). The probability of obtaining false positives when the true  $F_{ST} = 0$  was also obtained at generation  $t = 0$  as a measure of  $\alpha$  error rate.

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## 3.3 Results

### 3.3.1 Marker discovery and primer design

Shotgun sequencing of two specimens yielded 22,328,005 reads from a possible 25,000,000, from which 5,041 microsatellite containing sequences were extracted. One hundred and nineteen contigs containing putatively polymorphic microsatellite loci with a read depth ranging from 18 to 156 were selected following visual analysis. These 119 loci were further reduced to 96 microsatellite loci following screening for redundancy in Geneious and secondary structure testing with the IDT OligoAnalyzer Tool 3.1. An additional four loci from the original HOMSIR project (Kasapidis & Magoulas, 2008) were redesigned in order to reduce their amplicon lengths so that they were suitable for MiSeq sequencing. The corresponding 100 primer pairs were divided into 5 multiplex panels, each comprising 20 primer pairs, in MultiPLX 2.1.

### 3.3.2 Amplicon generation and sequencing

Amplicon sequencing of the single library yielded 20,886,472 raw reads, 2,914,627 of which were correctly assigned to individual fish and locus when allowing for 0 errors in the barcode and primer regions and 3,276,292 when allowing for 1 barcode and 0 primer errors. Of the 100 putative polymorphic loci analysed, 34 were polymorphic of which 17 produced scorable amplicons and could be genotyped and 17 were confounded by sequence error and low read depth (Table 3.1). Forty-three loci were not polymorphic and 23 were confounded by a high level of error and it could not be determined if they were polymorphic or not. This conversion from sequence yield to useable genetic data is significantly lower than what we have previously observed in any other study using this approach. Communication with Duke Sequencing Facility is on-going to clarify sample quality, handling and sequence processing to uncover the source of low technical quality of these sequence data.

Table 3.1. Quality assessment of microsatellite loci. Those highlighted in green were genotyped and those in bold were from the HOM SIR project. Dataset 1 loci are denoted by <sup>a</sup> and Dataset 2 loci by <sup>b</sup>.

Marker	Variable	Scored	Reason	Marker	Variable	Scored	Reason
HOM111 <sup>ab</sup>	Polymorphic	Yes	NA	HOM417	Unknown	No	No amp
HOM113 <sup>ab</sup>	Polymorphic	Yes	NA	HOM511	Unknown	No	Error
HOM202 <sup>ab</sup>	Polymorphic	Yes	NA	HOM512	Unknown	No	Error
HOM203 <sup>ab</sup>	Polymorphic	Yes	NA	HOM513	Unknown	No	Error
HOM211 <sup>ab</sup>	Polymorphic	Yes	NA	HOM515	Unknown	No	Low reads
HOM213 <sup>ab</sup>	Polymorphic	Yes	NA	HOM519	Unknown	No	Error
HOM306 <sup>ab</sup>	Polymorphic	Yes	NA	HOM520	Unknown	No	Low reads
HOM315 <sup>ab</sup>	Polymorphic	Yes	NA	HOM103	Not polymorphic	No	NA
HOM316 <sup>a</sup>	Polymorphic	Yes	NA	HOM105	Not polymorphic	No	NA
HOM317	Polymorphic	Yes	NA	HOM108	Not polymorphic	No	NA
<b>HOM319<sup>ab</sup></b>	Polymorphic	Yes	NA	HOM116	Not polymorphic	No	NA
HOM412 <sup>ab</sup>	Polymorphic	Yes	NA	HOM117	Not polymorphic	No	NA
<b>HOM414<sup>ab</sup></b>	Polymorphic	Yes	NA	HOM119	Not polymorphic	No	NA
<b>HOM502</b>	Polymorphic	Yes	NA	HOM204	Not polymorphic	No	NA
HOM510	Polymorphic	Yes	NA	HOM206	Not polymorphic	No	NA
HOM516 <sup>ab</sup>	Polymorphic	Yes	NA	HOM207	Not polymorphic	No	NA
HOM517 <sup>ab</sup>	Polymorphic	Yes	NA	HOM208	Not polymorphic	No	NA
HOM102	Polymorphic	No	Error	HOM210	Not polymorphic	No	NA
HOM106	Polymorphic	No	Error	HOM212	Not polymorphic	No	Stutter
HOM109	Polymorphic	No	Error	HOM215	Not polymorphic	No	NA
HOM110	Polymorphic	No	Low	HOM216	Not polymorphic	No	NA
HOM112	Polymorphic	No	Error	HOM217	Not polymorphic	No	Stutter
HOM114	Polymorphic	No	Error	HOM219	Not polymorphic	No	NA
HOM115	Polymorphic	No	Error	HOM220	Not polymorphic	No	NA
HOM201	Polymorphic	No	Error	HOM301	Not polymorphic	No	NA
HOM205	Polymorphic	No	Error	HOM302	Not polymorphic	No	NA
HOM209	Polymorphic	No	Error	HOM305	Not polymorphic	No	NA
HOM308	Polymorphic	No	Error	HOM307	Not polymorphic	No	NA
HOM408	Polymorphic	No	Error	HOM309	Not polymorphic	No	NA
HOM409	Polymorphic	No	Low	HOM311	Not polymorphic	No	NA
<b>HOM415</b>	Polymorphic	No	Low	HOM312	Not polymorphic	No	NA
HOM418	Polymorphic	No	Error	HOM313	Not polymorphic	No	NA
HOM419	Polymorphic	No	Low	HOM314	Not polymorphic	No	NA
HOM501	Polymorphic	No	Low	HOM401	Not polymorphic	No	NA
HOM101	Unknown	No	Error	HOM403	Not polymorphic	No	NA
HOM104	Unknown	No	Error	HOM404	Not polymorphic	No	NA
HOM107	Unknown	No	Error	HOM406	Not polymorphic	No	NA
HOM118	Unknown	No	No amp	HOM407	Not polymorphic	No	NA
HOM120	Unknown	No	Error	HOM410	Not polymorphic	No	NA
HOM214	Unknown	No	Error	HOM413	Not polymorphic	No	NA
HOM218	Unknown	No	Error	HOM420	Not polymorphic	No	NA
HOM303	Unknown	No	Error	HOM503	Not polymorphic	No	NA
HOM304	Unknown	No	Error	HOM504	Not polymorphic	No	NA
HOM310	Unknown	No	Foreign	HOM505	Not polymorphic	No	NA
HOM318	Unknown	No	Error	HOM506	Not polymorphic	No	NA
HOM320	Unknown	No	Error	HOM507	Not polymorphic	No	NA
HOM402	Unknown	No	Error	HOM508	Not polymorphic	No	NA
HOM405	Unknown	No	Error	HOM509	Not polymorphic	No	NA
HOM411	Unknown	No	Error	HOM514	Not polymorphic	No	NA
HOM416	Unknown	No	Error	HOM518	Not polymorphic	No	NA

### 3.3.3 Statistical analyses

After quality control checks 2 datasets with different levels of genotyping success were used to explore the data. Dataset 1 was less stringent and contained only individuals that were genotyped at greater than 60% of loci and loci that were genotyped in greater than 60% of individuals. The resulting dataset comprised 102 individuals (61 Western and 41 North Sea) at 14 loci (Table 3.1). Dataset 2 was more stringent and contained individuals that were genotyped at greater than 70% of loci and loci that were genotyped in greater than 70% of individuals. The resulting dataset comprised 68 individuals (41 Western and 27 North Sea) at 13 loci (Table 3.1). MICRO-CHECKER analyses of both datasets indicated the potential presence of null alleles at a number of loci (Table 3.2).

Table 3.2: Results of MICRO-CHECKER analyses for Null alleles.

Dataset	1		2	
	Western	North Sea	Western	North Sea
HOM111	no	no	No	No
HOM113	no	no	No	No
HOM202	yes	yes	yes	Yes
HOM203	yes	yes	yes	Yes
HOM211	no	no	no	No
HOM213	no	yes	no	Yes
HOM306	no	yes	no	No
HOM315	no	no	no	No
HOM316	yes	yes	NA	NA
HOM319	yes	no	yes	No
HOM412	no	no	no	No
HOM414	no	no	no	No
HOM516	no	no	no	No
HOM517	no	no	no	No

The loci HOM111, HOM113 and HOM203 displayed significant deviations from Hardy-Weinberg equilibrium in both samples in both Dataset 1 and 2. HOM203 also displayed significant heterozygote deficiency in both samples in both Dataset 1 and 2. However given the small effect on number of loci and individuals available they were retained in the analyses. The global multi-locus  $F_{ST}$  for dataset 1 and dataset 2 were not significant (Table 3.3).

Table 3.3: Global multi-locus  $F_{ST}$  for the Western and North Sea horse mackerel samples

Dataset	$F_{ST}$ over all loci	95% CI	$P$
1	0.0021	-0.00437 - 0.00853	0.248
2	0.0030	-0.00696 - 0.01311	0.234

POWSIM analyses of dataset 1 and dataset 2 indicated that there insufficient power to detect significant population structure at the level observed (Tables 3.3 and 3.4).

Table 3.4. POWSIM analyses results for datasets 1 and 2. The number of generations of drift ( $t$ ) was varied while keeping the effective population size ( $N_e$ ) constant at 1000.

Dataset	Expected $F_{ST}$	Chi <sup>2</sup>	Fisher's test	$t$
1	0.0005	0.075	0.093	1
	0.0010	0.116	0.142	2
	0.0025	0.316	0.331	5
	0.0050	0.734	0.702	10
	0.0100	0.987	0.978	20
	0.0488	1.000	1.000	100
2	0.0005	0.052	0.055	1
	0.0010	0.070	0.070	2
	0.0025	0.171	0.184	5
	0.0050	0.403	0.416	10
	0.0100	0.857	0.816	20
	0.0488	1.000	1.000	100

No further statistical analyses were conducted due to the lack of significant population structure and the limited power in the current datasets.

### 3.4 Conclusions

The current pilot study successfully identified a large number of novel microsatellite markers in Horse mackerel. These markers will facilitate high throughput stock structure identification for future studies of the species. Initial data analyses were confounded by the low yield of the second sequencing run and as such the discrimination power between the western and North Sea sample was low (compare with Farrell et al., in prep). This resulted in the pilot study being unable to conclusively and unequivocally separate the two stocks. However, the level of population structure observed albeit not statistically significant ( $F_{ST} = 0.002 - 0.003$ ) is on par with what has been observed in other marine fish species that show significant stock structure.

This study highlights the potential of the genetic stock identification in horse mackerel. In order to improve the statistical power and increase the geographical scope of the analysis we suggest widening the sample area to include the southern stock and outlier stocks (e.g. Mauritania and Mediterranean samples) and increasing the number of samples per stock to ensure that a comprehensive baseline of spawning individuals with sufficient statistical power is developed for each stock. To this end, sampling is currently being conducted on the 2016 Mackerel Egg Survey (MEGS) and through Industry collaboration (PFA).



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# 4 Chemical fingerprint approach to stock discrimination

## 4.1 GCxGC-MS principle

Gas chromatography (GC) is a type of chromatography for separating and analyzing compounds that can be vaporized without decomposition. Separating compounds is carried out between a gas mobile phase and a stationary phase. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. GCxGC is a technique that allows for extra discrimination compared to normal GC by using a setup with 2 columns with different characteristics. GCxGC is used in the petrochemical industry to characterize oils and can also be used to locate the source of a spill (Bayona et al., 2015) or differentiate between natural and industrial sources for pollution (Rowland et al 2012). When applying this technique in a non-selective way the results of the analysis can be used as a kind of chemical fingerprint. With the help of this technique it is possible to tell if organisms originate from the same location based upon chemicals markers they may contain.

## 4.2 Methods

### 4.2.1 Lipid content analysis

The total lipid analysis is performed by an adapted method (Bligh and Dyer method), based on a cold chloroform-methanol extraction. The method, described in Wageningen Marine Research Standard Operating Procedure (SOP) "biota, determination of lipid according to Bligh and Dyer; gravimetric determination" is part of the scope of the Accreditation Board under laboratory number L097, method nr 1.

### 4.2.2 GCxGC-MS Analysis

Samples are homogenised with a blender after which a subsample is taken and dried using sodium sulphate. The dried sample is then loaded into an Accelerated Solvent Extraction (ASE) cel (Dionex) together with 25 grams of florisil (VWR) and 1 ml of internal standard solution (80 ng/ml PCB112 and PCB207) is added. Cells are subsequently extracted with a mixture of pentane/dichloromethane (85/15, Promochem) using an ASE350 (Dionex). The extracts were then concentrated to 1 ml using a rotavap (Heidolph) and transferred to a vial for analysis by GCxGC-MS.

5 µl of sample was injected on a Shimadzu GCMS2010 (GC) coupled to a GCMS-QP2010 Ultra (MS) detector (Shimadzu, the Netherlands) using Large Volume Injection (LVI). Analysis was performed in GCxGC mode using a Zoex ZX2 modulator (Shimadzu, the Netherlands) with a modulation of 6 s. The 1<sup>st</sup> dimension column was a 30m x 0.25 mm i.d. HT8 with a film thickness of 0.25 µm. The 2<sup>nd</sup> dimension column was a 2.3 m x 0.25 mm i.d. BPX-50 column with a film thickness of 0.15 µm. Samples were injected at 75 °C. After 85% of the solvent had evaporated the injector was brought up to 290 °C. The following temperature program was used for the analysis: Start at 75 °C and hold for 2 min. Heat up with 23.75 °C/min to 170 ° and then heat with 2.5 °C/min to 280 °. At the end of the program the column was heated to 300 ° and held for 10 min. detection was performed in electron impact (EI) mode with source and interface temperatures of 200 and 300 °C respectively. Mass between 150 - 500 were recorded between 10.1 and 50 minutes with Event time set to 0.03 sec. Chromatograms were processed using the GCImage software package (Shimadzu, the Netherlands).

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Chromatograms are processed by doing a blob detection to find relevant peaks and comparing these to a template. The template is created from the first sample by selecting all relevant peaks and added these to the templates using unique names for each peak based upon the sample name. Peaks found in later samples are also added to the template with a unique name. After blob detection and comparing with the template a blob table is saved and used for further processing.

## 4.3 Statistical analyses of the results

### 4.3.1 Post processing of the results

The raw output from the GCXGC-MS was processed before it could be used to look for differences between geographical origin:

- Inter-sample standardisation: the concentration of a given compound is measured by the value of the peak corresponding to this compound on the chromatogram. In order to standardise the outcome of the GCXGC-MS between different samples, a compound is injected with a known concentration together with the fish sample. All the peak values for all compounds were therefore first divided by the peak value of this internal standard, for all fish analysed.
- Removing compounds added during sample preparation: A GCXGC-MS analyse is also run on a blank sample, containing no fish but only the products used for the preparation of the samples before analysis. In order to "remove" all the compounds added during the preparation of the samples, the peak values of the compounds observed in the blank sample were subtracted from the output of the GCXGC-MS analyses on the fish samples.
- Identification of unreliable compound measurements: given the uncertainty in the measurement of compound concentrations, some compounds added only during the preparation of the sample may still have a non-null concentration even after subtracting the blank value. In order to take this error margin into account, all measurements of compound for which the peak value in the sample is less than twice the peak value in the blank were classified as potentially unreliable compound measurements.
- Elimination of unreliable compounds: all compounds having a null concentration in all samples (after subtraction of the blank) were removed from the data. In addition, compounds having unreliable measurement in most of the samples were also removed from the data base. The threshold used for this was a minimum of 50% of valid measurements among samples of at least one stock origin were required to keep the compound in the database.
- Correcting for the different in fat content: many of the compounds measured in the horse mackerel are liposoluble. Therefore their concentration in the muscle tissue sample analysed should be proportional to the lipid content of this sample. In order to standardise for the differences in fat content among samples, concentration of all compounds in a sample were divided by the corresponding lipid content measurement.

### 4.3.2 Row results

#### Lipid content analyses

The figure 4.1 shows the lipid concentration measured in the muscle tissue samples. It should be noted that lipid content in the muscle is not representative of the overall fish lipid content, as most of the fat is stored in adipose tissues, not muscle, located in specific parts of the body.

There was a large variability in the fat content, even between fish of a same origin and period. The North Sea early summer sample however showed less inter-individual variability and had a markedly lower lipid content than any other sample. In the late summer, North Sea fish had a higher lipid content than the western fish.

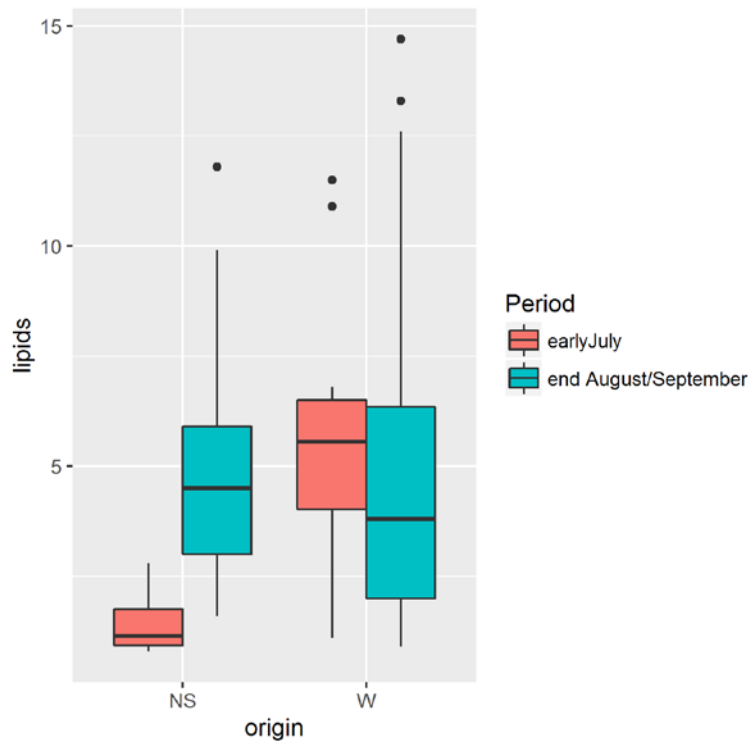


Figure 4.1: distribution of the fat content measurements in the muscle tissue among samples for the North Sea and western samples from early and late summer.

#### Chemical compounds

The figure 4.2 shows the distribution on the compound concentrations by geographical origin for the early and late summer samples. The analyses of the early and late summer samples were carried out half a year apart (November 2015 and May 2016 respectively). Within this period of time, the properties of the chromatograph (e.g. the length column) may have been modified and the labelling of the compounds are not directly comparable between the two round of analyses. To compare the results from the early and late summer samples, identifying the compound corresponding to each peak would have been necessary. This has to be done individually for each compound, which given the large number of compounds found (around 100 for each round of analyses) was not undertaken during this project. Therefore there is no basis for comparison of the results between the early and late summer compounds.

Marked differences can nevertheless be noted between the two rounds of analyses:

- A larger number of compounds were detected in the early summer samples (136 vs. 91)
- Differences in compounds concentration between geographical origin are more marked in the early summer sample (less overlap in the bar plots)
- Many compounds in early summer are found only in a small number of fish. Figure 4.3 shows that for about half of the compounds, the proportion of non-null measurement is 50% or lower. On the opposite, most of the compounds found for the late summer samples are present in at least 75% of the fish analysed.

One common observation can be made however: for both the early and late summer samples, most of the compounds are on average in higher concentrations in the North Sea fish than in the western fish (83% and 91 % of the compounds respectively).

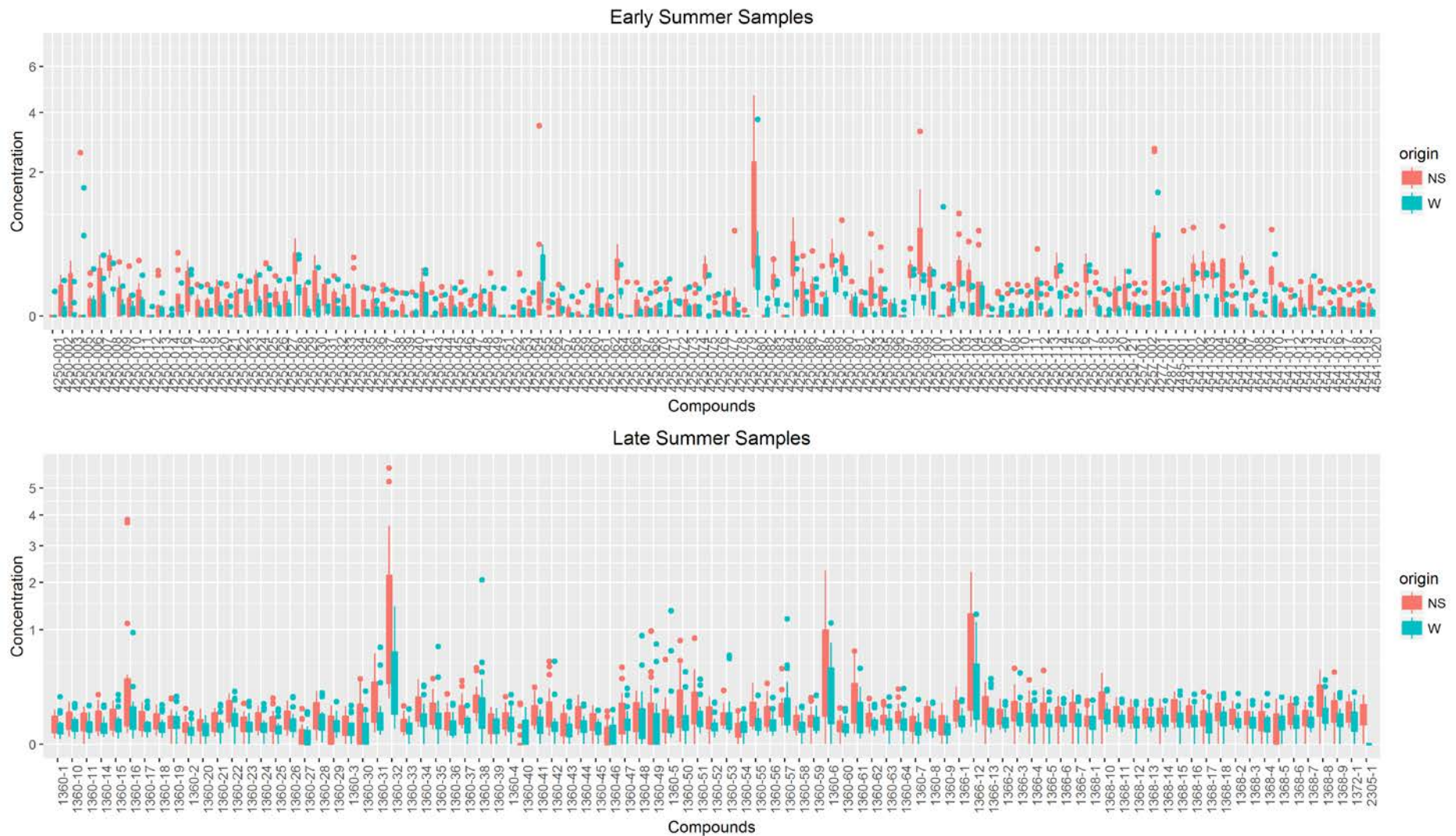


Figure 4.2: distribution of the compound concentrations (amplitude of the peak on the chromatogram) among the western and the North Sea samples for the early (10 individuals per geographical origin) and late summer samples (20 individuals per geographical origin).

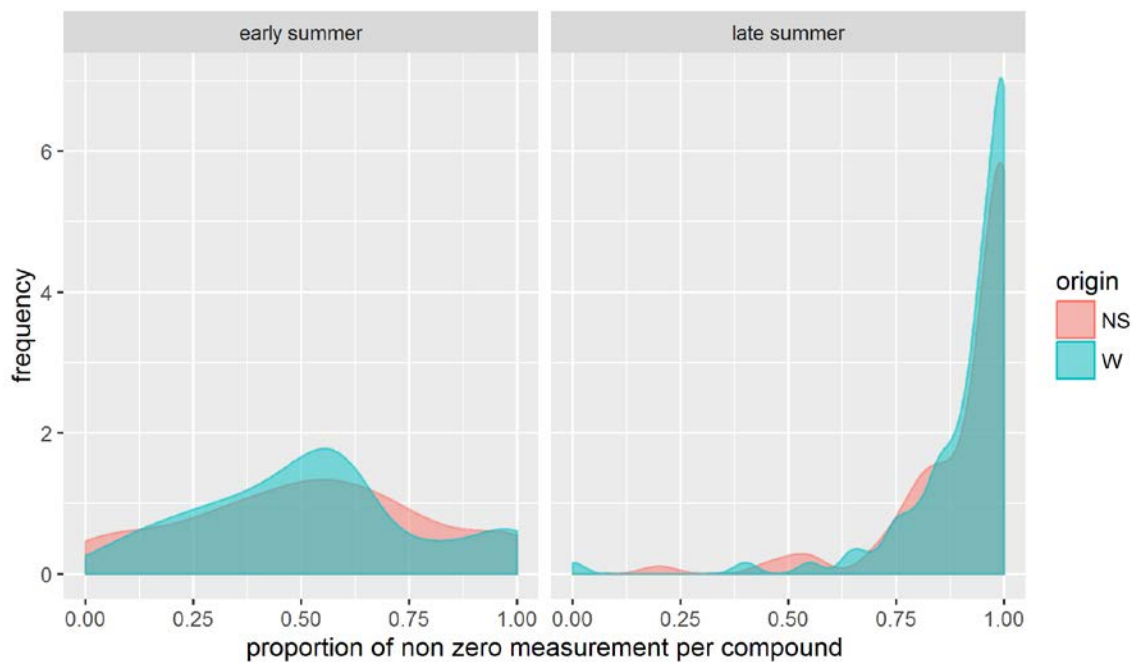


Figure 4.3: distribution of the proportion of non-zero measurement per compounds in the early and late summer samples.

#### 4.3.3 Presence/absence test

The ideal situation would be to identify compounds specific to a geographical origin. In such case, the presence/absence of that(these) compound(s) in a fish would be sufficient to identify its geographical origin.

In an attempt to identify such compounds, the frequency of occurrence (non-null concentration) of each compound per geographical origin was calculated. The table 4.1 shows the compounds for which the frequency of occurrence was the most different between the western and North Sea horse mackerel.

None of the compound was systematically present in the samples of one geographical origin and absence in the sample from the other geographical origin. The largest difference was observed for compound labelled "2305-1" in the late summer analyses, which was never found in the western fish, while being found in 85% of the North Sea fish. Using presence/absence of this compounds as a classification criteria would result in a misclassification risk of 7.5% (percentage of individuals which are assigned to the wrong stock). For the other compounds the difference in the rate of occurrence between west and North Sea fish is smaller, leading to a larger risk of misclassification.

Two compounds, labelled 4541-004 and 4250-093 were found consistently in the North Sea early summer samples, but were not systematically absent from the western samples. Combining the presence/absence information of these two compounds improves the accuracy of the classification (table 4.2). If a fish for which one or both of these 2 compounds is absent is classified as western fish, the rate of misclassification is only 5%. Incorporating the component with the third largest difference in frequency in occurrence for the early summer samples (4541-014) however increases the misclassification rate to 20%.

A further investigation of the GCxGC-MS results showed that the compound 2305-1 in the late summer analyses corresponded to a different molecule than any of the two compounds, 4541-004 and 4250-093, of the early summer sample.

Table 4.1: performance of potential markers based on presence absence criteria

compound	period	Frequency of occurrence		Misclassification rate
		NS	W	
2305-1	Late summer	85%	0%	7.5%
4541-004	Early summer	100%	30%	15%
4250-093	Early summer	100%	40%	20%
4541-014	Early summer	70%	20%	25%

Table 4.2: performance of potential combinations of markers based on presence absence criteria

compound	Frequency of absence		Misclassification rate
	NS	W	
4541-004 or 4250-093	0%	90%	5%
4541-004 or 4250-093 or 4541-014	30%	90%	20%

#### 4.3.4 Classification tree

Classification trees (Breiman et al., 1984) are machine-learning methods for constructing prediction models from data. The models are obtained by recursively partitioning the data space and fitting a simple prediction model within each partition. As a result, the partitioning can be represented graphically as a decision tree. Classification trees are designed for dependent variables that take a finite number of unordered values (here western or North Sea origin), with prediction error measured in terms of misclassification cost.

Contrary to most statistical methods, classification trees do not require normally distributed data, are robust to the presence of outliers, and can also combine numerical and categorical predictors (which was not the case here).

In the present case, the tree is built by finding the cut-off value for one of the compounds which results in the best separation of the western and North Sea samples. The data set is then divided in two parts, two first branches of the tree, based on whether the concentration is above or below the cut-off value identified. For each new branch of the tree, the same procedure is repeated until the samples from the west and from the North Sea are perfectly separated. However, a tree with too many branches would correspond to an over-fitted model: it is able to explain all the variability in the data, but its usefulness for prediction is low. For this reason, the full tree is usually "pruned" (i.e. reducing the size of the tree) in order to improve its prediction power. This is done using cross-validation (i.e. part of the data is used to build the tree, and the other part is used to assess its predictive power). The size of the final tree is therefore the result of a trade-off between cost (loss of prediction power) and complexity (i.e. explanatory power). The R package rpart used here to build the classification tree determines the optimal tree size using cross validation

Classification trees have been used in studies similar to the present horse mackerel study. Iverson et al. (1997) identified differences acid compositions according to the geographical origin of harbour seal in Alaska using classification trees and established the link with the fatty acid composition of their preys. Loewen et al. (2015) used a similar classification method to describe the stock structure of Dolly Varden char in Northern Canadian based on trace elements and strontium isotopes in the otolith.

A classification tree was built based on the compound concentrations for both the early summer and late summer samples (figure 4.4 et 4.5). For both samples, the classification tree identified a single compound in each case, which can be used to separate western individuals from North Sea individuals with a prediction error of 5% and 7.5% respectively. The compounds identified in the classification

trees are the same which were identified in the presence absence analysis. The rate of misclassification is reduced for the earlier summer samples when the classification is based on a value of 0.06057 for compound 4541-004 instead of simply the presence/absence of this compound.

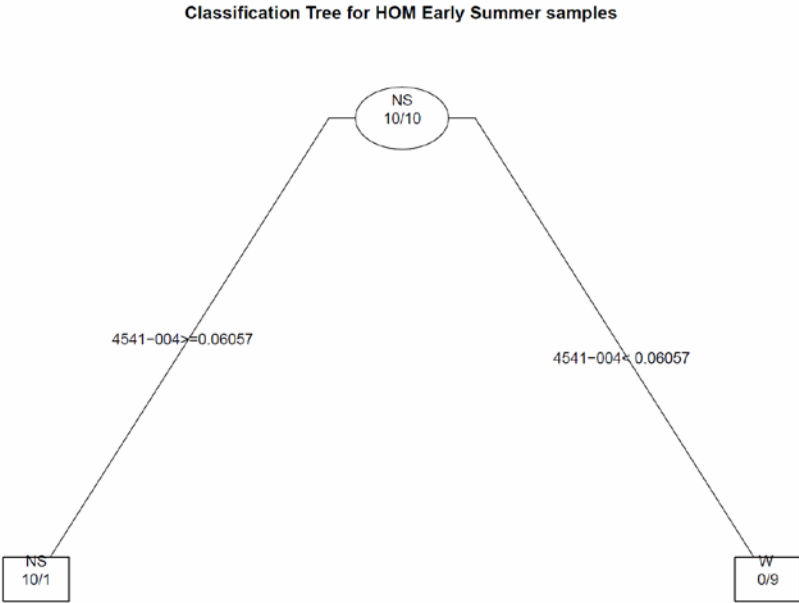


Figure 4.4: classification tree for the prediction of the geographical origin of horse mackerel based on concentrations of chemical compounds in the early summer samples.

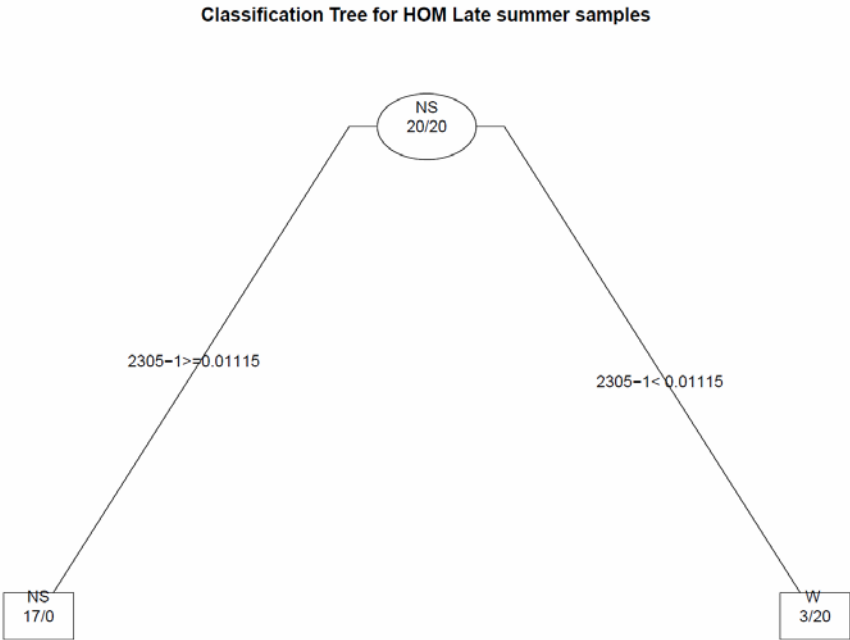


Figure 4.5: classification tree for the prediction of the geographical origin of horse mackerel based on concentrations of chemical compounds in the late summer samples.

#### 4.3.5 Multivariable statistical methods.

##### Condition of application and variable selection

The two methods presented below, principal component analysis (PCA) and discriminant analysis (DA), have strict conditions of application. In discriminant analysis, there is a number of conditions that need to be fulfilled, and their validity dictates the usefulness of the method for the data (Zuur et al. 2009). These conditions are:

- 1) The observations can be divided a priori into at least two groups. Each observation can only be in one group.
- 2) At least 2 observations per group, but ideally more than 5 times as many observations per group as number of variables.
- 3) The number of observations in the smallest group is larger than the number of variables.
- 4) Continuous variables, no categorical
- 5) No collinearity between variables (remove variables correlated with  $r > 0.8$ ).
- 6) Similar Within group variances for a given variable (but differences in variances of different variables allowed).
- 7) As a consequence of 6) variables having a constant value in one group (e.g. 0) are not allowed.
- 8) Similar covariance matrices between groups
- 9) Variables must be (approximately) normally distributed within each group.
- 10) Independent observations

The data used readily comply with condition (1, 4 and 10). In order to fulfil with the rest of the conditions, the variables (compounds) to be used in a DA were selected in a stepwise process:

- 1) Compounds frequently absent from the measurements were excluded (condition 6,7 and 9). The criteria used was that compound had to have at least 50% of non-null measurement to be kept
- 2) A normality test (Shapiro–Wilk test) was performed for each variable with both groups (condition 9). All variables with a for which the null hypothesis that the variable is normally distribution is rejected with a risk of 1% or lower ( $p.value \leq 0.01$ ) in at least one of the group were considered too different from normality and removed. This does not strictly mean that all remaining variable are normally distributed, but this results of the elimination of the variable departing the most from normality. DA, especially the quadratic DA, is relatively tolerant to the violation of condition 9.
- 3) Collinear variables were removed, using a threshold of  $r = 0.8$  (condition 5).
- 4) Compute difference between the covariance matrices for the North Se and the western samples, and remove iteratively the compounds for which the difference is too large until a maximum difference of 25% is observed.

The table 4.3 shows the number of compounds remaining after each step of this selection process. After the stepwise elimination of compounds condition 2 appears to be violated, for the early summer sample, as the number of observation (10 fish per group) would in principle allow to include only 2 compounds in the DA.

*Table 4.3: reduction of the number of compounds to comply with the conditions of application of the discriminant analysis*

Sample	Original dataset	After step 1	After step 2	After step 3	After step 4
Early summer	136	69	36	14	8
Late summer	91	87	33	10	8



## Principal component analysis

The PCA is typically used to synthesize the information of a multivariable data set into a small number of meaningful variables (the principal components) which can then be used to find trends and groupings in the data. The principal components are obtained by linear combination of the original variables, with the aim of maximising the variance explained by each principal component. A PCA was run for both samples, based on the variables remaining after the step 3 of the selection process described above.

In both case, the first PC explained a more than 40% of the variance (Figure 4.6) and the PCAs indicated that many of the variables are correlated (all arrows pointing to the right, indicating correlation with the first PC). There is more contrast for the early summer sample, for which the variance explained by the second PC is higher, and for which some compounds (e.g. 4250-055) correlate specifically to this second PC.

A general pattern in the scoring of the individual observation on the first two PC is that individuals from the west tend to have negative values on the first PC, while individuals from the North Sea have positive values. However, there is still a large overlap (especially for the late summer samples) between the individuals of the two geographical origin.

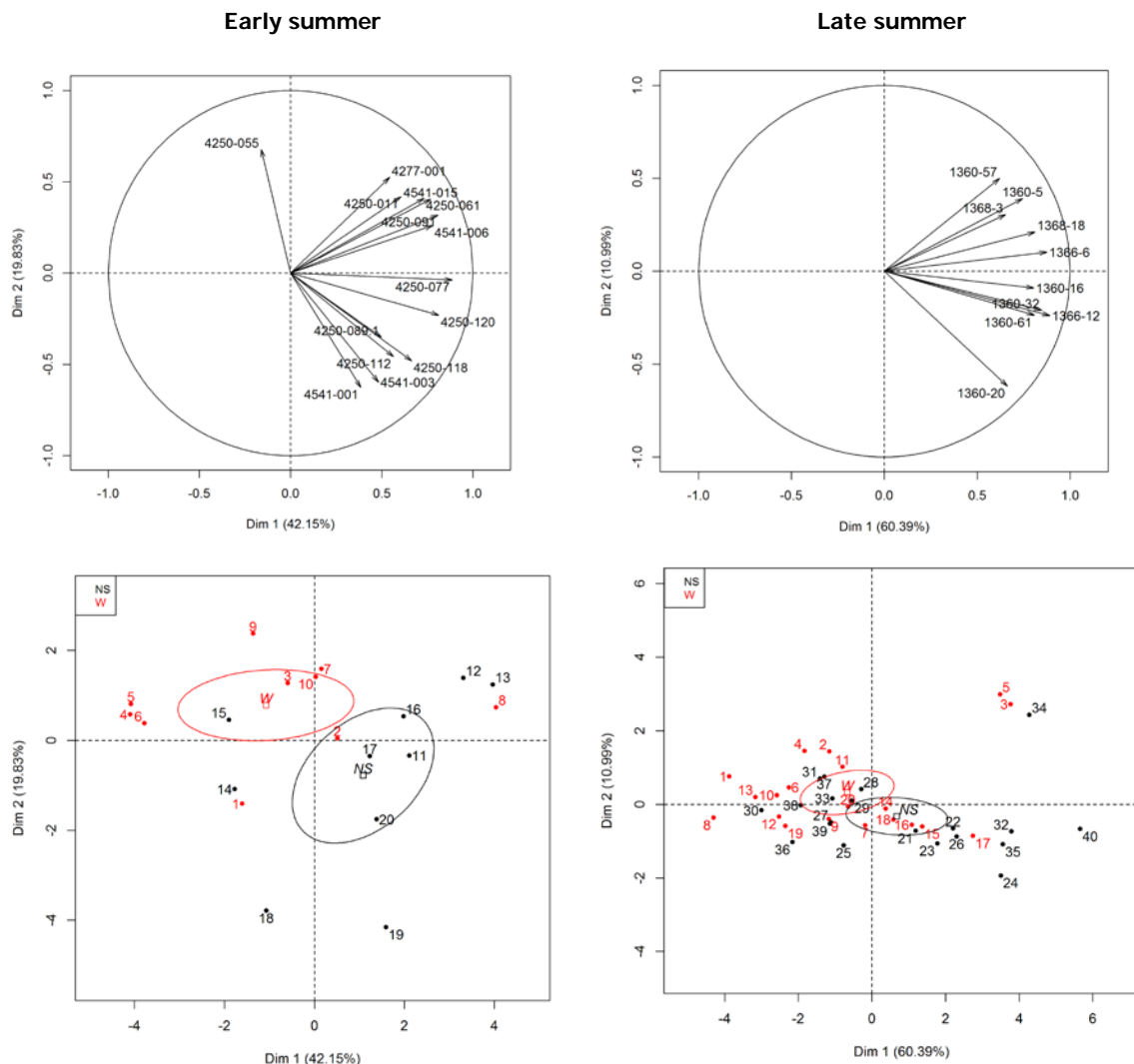


Figure 4.6 principal component analysis based on the concentration of chemical compounds in the early (left panel graphs) and late summer samples (right panel graphs). The correlation circle (above) shows the correlation of each original variable (compounds) on the first 2 principal component (percentage of variance explain given in the axis label). The projection of the individuals (below) shows how the groups are segregated by the PCA.

## Discriminant analysis

The discriminant analysis (DA) is a multivariable statistical analysis similar to a principal component analysis. In a DA, discriminant functions (the DA equivalent of the principal components) are estimated by linear combination of the original variables with the aim of achieving a maximal segregation of the observations according to a categorical grouping variable. In the present case, the concentration of the compounds are the original variables, with each fish being one observation, and the geographic origin is the grouping variable. Discriminant analysis, in its different variants, is a commonly used statistical approach to separate individuals from different population based on multivariate measurements from individuals (Cardin et al, 2013). For instance, Pangle et al. (2010) analysed otolith elemental composition of yellow perch (*Perca flavescens*) using quadratic discriminant analysis to their natal geographical origin. Jonsdottir et al (2006) used the same technique to identify geographical origin of Icelandic cod on the spawning grounds based on otolith shape characteristics.

A linear discriminant analyses was performed for each of the periods using the R function `lda()`. The DA produced only one discriminant function in each case. The figure 4.7 shows the projection of the individuals on the discriminant function. In both cases, the discriminant function separates the North Sea individuals, which have mainly negative values, from the western individuals (positive values). The segregation is however not complete, as the distribution of the individuals on the discriminant function overlap in both cases. Using this discriminant function to predict the geographical origin of the individuals (west for positive values, North Sea for negative values), the rate of misclassification (table 4.4) is of 15% for the analysis on the early summer sample and of 35% for the analysis of the late summer samples.

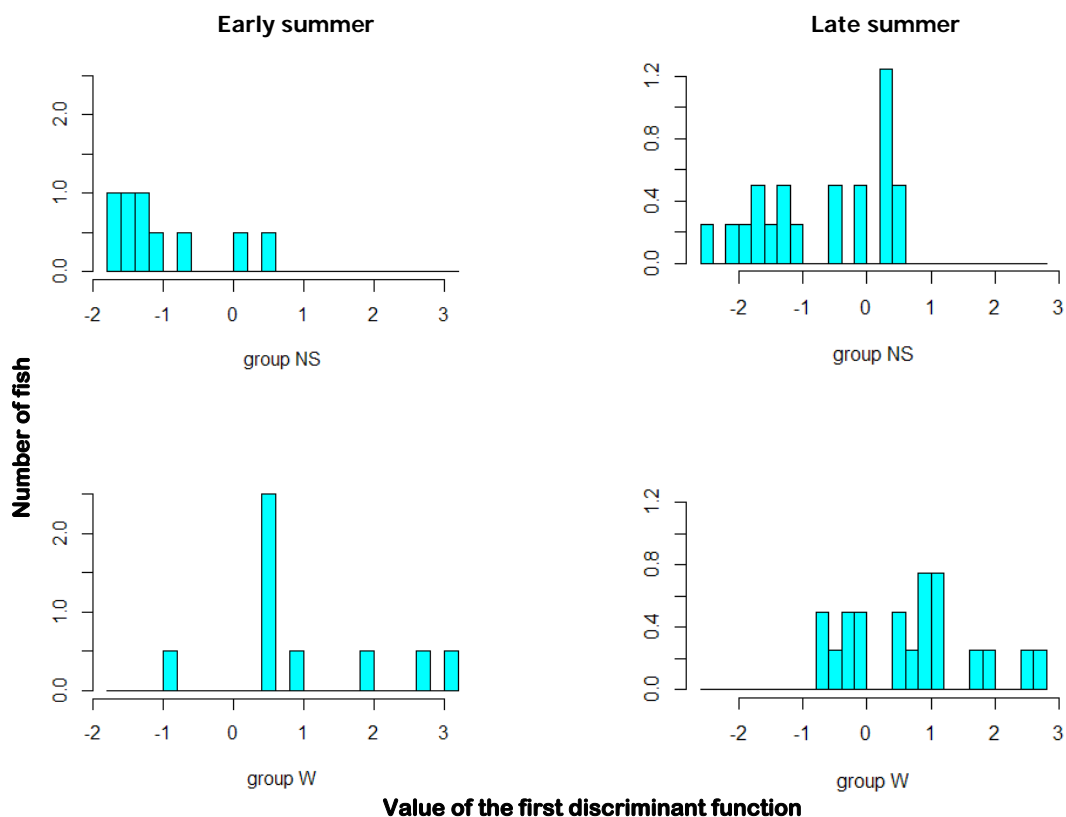


Figure 4.7: projection of the individuals (fish) on the first discriminant function for the discriminant analysis run for the early and for the late summer samples.

Table 4.4: classification accuracy of the DA

	Early summer		Late summer	
	Stock origin		Stock origin	
Classified as	North Sea	West	North Sea	West
North Sea	8	1	13	7
West	2	9	7	13
Misclassification rate	15%		35%	

## 4.4 Discussion

### 4.4.1 Differences between early and late summer chemical fingerprints

The difference in the compound profiles between the early summer and the late summer samples were striking, and found for fish from both geographical origins: many of the compounds identified in the early summer samples were found only in a minority of the fish, while most of the compounds identified in late summer were found in most of the fish. We can formulate different hypotheses to explain such differences:

- Most of the compounds detected in late summer could have accumulated in the fish during summer. This would imply that most of the compounds detected have a short residence time. These compounds could be man-made compounds with low residence time, accumulated during summer (due to diet and contamination at the feeding grounds) and/or natural compounds produced at certain time/location with a limited life-span (degraded over time, either in the water or in the fish) and/or a limited residence time in the fish. However, without having identified the nature of the compounds detected for both sampling period, it is not possible to verify this hypothesis.
- Chemical profile differences between early and late summer may be explained by differences in the protocol applied for the GCXGC-MS analyses. Indeed, the first round of GCXGC-MS analyses were done using a same amount to tissue for all fish and the lipid concentration measurements were done at the same time. The results of the first round of analyses showing a large variability in the lipid content among individuals, it was decided for the second round of analyses, to first perform the lipid measurements on a larger sample of fish, and then select fish with no extreme lipid content values to perform the GCXGC-MS analyses. In addition, the amount of tissue used per individual was taken inversely proportional to the fat content so that the amount of fat per sample was roughly constant. This was done to avoid that compounds present in the fat would not be found if their concentration was below the detection threshold. The many null measurements in the early summer samples could be explained by samples with a low fat content for which a too small amount of tissue was used for the GCXGC-MS analyses, resulting in the non-detection of compounds. However, while the fat content in the North Sea fish from early summer samples was found to be consistently low, this was not the case for the western fish. A confounding parameter is that the lipid content was analysed in the fillets. Other lipid storage compartments (abdominal cavity for example) are also relevant to the total amount of lipids in a fish important. If lipid levels in the fillets are not strictly related to lipid levels in the whole fish, this may interfere with the analysis of the contaminants in the fillet.

While it is difficult to clearly identify the cause of difference in chemical profiles, the protocol used for the analysis of the late summer samples was appropriate, and there is no reason to question the validity of the outcome of the GCXGC-MS analyses. In addition, this sample is closer in time to the

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period of the year where mixing is believed to take place in area VIId, and represents therefore a better basis for developing a method to distinguish western fish from North Sea fish.

#### 4.4.2 Limitation of the statistical analyses

The number of fish analysed is too low to conduct proper statistical analyses. In such circumstances, there is a good chance that the results cannot be generalized (i.e. model is valid only for the data used for fitting), and future classifications based on such analysis will be inaccurate. A rule of thumb for the discriminant analysis is that in each group the number of observations should be at least 5 times the number of compounds (Zuur et al, 2009). Therefore, with 10 and 20 individuals per group (for the early and late summer samples respectively), only 2 and 4 compounds should have been used in the discriminant analysis. This should not be a limitation if this method gets implemented operationally, as the number of fish analyzed would be much higher.

The predicting power of the classification methods developed here (e.g. the misclassification rate) was evaluated on the same data used for fitting the models. For an appropriate evaluation of the predictive power, independent data should be used (Tabachnick and Fidell, 1996; Green and Carroll, 1978). Any future sample should be large enough (typically larger than 100) to randomly divide the observations into two subsets. The first is used for the setup the classification model (training set), and the second is for testing the reliability of the results (test set). Hence, the results can probably not be generalized (i.e. model is valid only for the data used for fitting).

#### 4.4.3 Differences between North Sea horse mackerel and western horse mackerel

The two multifactorial analyses (PCA and DA) indicate the same: there are differences in concentration of compounds included in these analyses (after removing those not complying with the conditions of application). In fact, even when the number of variables is reduced to eliminate strongly correlated variables, the PCA still shows that the main structure in the data is the correlation of the different compounds. This reflects that in general concentrations are higher in the North Sea than in the West. However, the differences between the two geographical origins are not strong enough for good separation of the two groups, especially in the late summer samples. Even in discriminant analysis, in which the discriminant functions are constructed with the aim of maximising the segregation between the two groups, there is substantial overlap of the projection of the individuals from the west and from the North Sea on the first discriminant function.

The classification methods using presence/absence, or threshold values (classification trees) of a limited number (1 or 2) of specific compounds achieved a higher classification success than the multivariate techniques, trying to combine the information of a large number of compounds. However, since these simpler classification methods are based on the value of only 1 or 2 compounds in a limited number of individuals, their reproducibility may be questioned. One issue to consider is the potential lack of representativeness of the late summer sample from the western areas: all individuals come from a single trawl haul, and are therefore likely to be similar. For instance, the absence of compound 2305-1 in late summer samples from the western area might be a specificity of this unique sample. The North Sea samples were collected from a larger number of trawl haul, covering a larger geographical area (figure 2.1) and can therefore be considered representative of the North Sea stock.

#### 4.4.4 Towards an operational method to discriminant geographical origin?

##### Validation

The North Sea, as well as other waters with intensive human activities as industries and shipping, is known to be more polluted. Levels of certain chemical compounds in the environment, including living organisms, can therefore be higher in the North Sea than in the area western of Ireland (Pierce et al,

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2008 ; Knickmeyer and Steinshart, 1989). The fact that the differences observed in the results presented here are often small may have a range of different explanations:

- 1) Lack of imprinting because the geographical gradient in the contaminant concentration in the environment is too weak.
- 2) Lack of imprinting because of extensive fish migration (fish do not reside in a given area long enough for it to be reflected in compound concentrations).
- 3) Lack of imprinting for biological reasons (e.g. the fact of being a pelagic, the type of prey it feeds on)
- 4) Imprinting occurs, but late summer samples (for which there is less difference in concentration) are a mix of the two populations whereas the early summer sample is not (spawning time).

The validity of each of these explanations can be tested for. Other studies using chemical fingerprints (e.g. fatty acids profiles, or otolith micro elements) often combine analyses of fish, with analyses of either the environment (water) or their prey (Iverson et al., 1997; Pangle et al., 2000). This provides a validation that the differences observed in fish are also observed in their biotic or abiotic environment.

In the present case, a validation based on water or prey samples would be costly. However, a validation based on catches of more sedentary fish species would be relatively easy and cheap. Species which undertake only small scale migration and for which different stocks have been identified could be used:

- If for these non-migratory fish there is little difference between western areas and the North Sea this suggests that the geographical gradient in contaminant concentration is too weak for these compounds to be used as markers of stock origin (point 1 above).
- If for these non-migratory fish large differences are found between western areas and the North Sea this would mean that (i) that the lack of difference is due to some specifics of the horse mackerel (point 2 above) or (ii) that fish migrate between more and less contaminated areas (point 3 above). This latter explanation would suggest that there is actually no strong separation between North Sea and western horse mackerel.

#### Understanding sources of temporal variability

Some stock origin classification methods are based on patterns which are observed consistently over the years. For instance, Jonsdottir et al. (2006) studied the temporal stability of otolith shape in cod around Iceland at seven spawning locations in two consecutive years. Otolith shape differences were greater among locations than among years within a location. This means that stock origin can be established based on one year of sampling and for subsequent years the reference values for each stock origin do not need to be re-established. Other features may be less stable in time. Interannual variability in otolith micro-elemental signatures of perch in lake Erie was so high that natal location of larvae from one year could not reliably be classified in another year (Pangle et al., 2010).

The question of temporal stability of observed patterns for reference samples (in our case, the summer samples) is also relevant for shorter time scales. For instance, if the compounds showing differences between the North Sea and western fish have a short residence time, then the differences observed in the late summer samples may disappear after a few weeks when stocks are mixing in autumn/winter. On the other hand, if compounds distinguishing the 2 stocks are of human origin and accumulate in fish, there is little risk that their concentrations vary substantially between summer samples and autumn/winter samples. The identification of the compounds that separate the two stocks identified in this study would give some insight on this issue.

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In short, in order to assess the magnitude of temporal variations in compounds composition, it would be necessary to repeat the work carried out in the present project and to identify the nature of the compounds detected.

#### Applicability of the method

If this approach is to be applied operationally to separate catches from the North Sea stock from catches from the western stock, individuals need to be selected (males from a specific length range) in order to avoid any source of variability other than their geographical origin. Otherwise, other potential sources of variability, such as length, age or sex, are likely to influence compounds concentration.

The GCXGC-MS approach could be developed using a larger number of samples collected during a single year to provide a reference value for the mixing rate of North Sea and western fish in the VIId catches. This would provide useful information for stock management. For instance, a high proportion of western fish in the VIId catches would be a call for reconsidering the current practice of allocating all VIId catches to the NS stocks.

In addition, such a one-time large sampling exercise could be used to validate empirical knowledge from the fishing industry. Pelagic fishermen and especially quality managers of the fishing companies have identified two different types of horse mackerel, which can be distinguished visually only (figure 4.8). The so-called "dark" horse mackerel is characterised by its dark colour, a lower fat content and a lean body. The "blond" horse mackerel has a clearer skin colour, is rounder of shape and has a higher fat content. Dark horse mackerel are not found in the North Sea. Blond horse mackerel are not found in the western areas (Celtic Sea or west Ireland), but are found in the North Sea, and the Southern Bay of Biscay. Both types are found in the English channel and the fishermen believe that the blond horse mackerel caught there originates from the North Sea, while the dark horse mackerel comes from the western stock. Fishermen believe that the differences between the two types are due to differences in habitat (as they usually catch dark horse mackerel deeper than blond one) and food.

The GCXGC-MS approach could be used to investigate if the two types of horse mackerel in the VIId catches would correspond to two different stock origins, as it is believed by the fishermen. As the fishing industry keeps records of the type of fish along with their catch statistics, it would be possible to build up catch records for each horse mackerel type, which could also be linked to length frequency data collected by the industry. Therefore, if a validation of the fishermen's knowledge could be done using the chemical fingerprint approach, the data necessary for splitting the catch-at-age data from VIId between the two stocks could be potentially routinely collected by the fishing fleet itself.



*Figure 4.8: the two types of horse mackerel (top: dark horse mackerel, bottom: blond horse mackerel) identified by the pelagic industry (foto: courtesy of the pelagic freezer trawler association).*

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# 5 Survey index

## 5.1 Goal

At the start of this project, the advice for the North Sea horse mackerel was based on the trend in an abundance index derived from the NS IBTS Q3 computed using a zero-inflated GLM model (ICES, 2014a). The aim of this section was to investigate two methods, commonly used to derive abundance indices from survey CPUE data: a delta log normal model, and a geostatistical log Gaussian Cox process model. In addition, data from another survey, the French CGFS, covering the Channel area was used to derive a second index for this stock.

## 5.2 Methods

### 5.2.1 log Gaussian Cox process model based on number per haul

A geostatistical log Gaussian Cox (LGC) process model incorporating spatial-temporal correlation has been applied in several studies to describe catch rates over space and time (Jansen *et al.* 2012, 2014; Nielsen *et al.* 2014). The response of the model is the catch in number, in our case, number of horse mackerel  $\geq 20$  cm per haul. A spatial grid is established (e.g.  $10 \times 10$  km cell size). The combined time and spatial random variables are then assumed to follow a log Gaussian (or log-normal) distribution that determines the mean of the catch, which follows a Poisson distribution. Such LGC model has been shown to be appropriate for count data from catches that are correlated, overdispersed and contains a high proportion of zeros (Kristensen *et al.* 2013). The spatial-temporal correlations are expressed by a decorrelation distance (H) and decorrelation time (T), the distance in space and time where the correlations have decayed to  $1/e$  (explaining ca. 14% of the variance) (Jansen *et al.* 2014). The detailed structure of the LGC model is described in Kristensen *et al.* 2013.

In our study, we started with a full model, with the expected number of fishes in sample  $i$  at position  $x$  in year  $y$ :

$$E[\log(\lambda_i)] = \eta_{\text{space}}(x) + \eta_{\text{space} \times \text{time}}(x, y) + \mu(y) + \eta_{\text{nugget}}(i) + \mu(\text{Duration}) \quad (1)$$

where  $\lambda_i$  is the number of horse mackerel  $\geq 20$  cm at sample  $i$ ;  $\eta_{\text{space}}(x)$  is a mean 0 Gaussian stochastic process with covariance matrix  $\exp(-|\Delta x|/H)$ ;  $\eta_{\text{space} \times \text{time}}(x, y)$  is a mean 0 Gaussian stochastic process with covariance matrix  $\exp(-|\Delta x|/H) \times \exp(-|\Delta y|/T)$ ;  $\mu(y)$  indicates the effect of year;  $\mu(\log(\text{Duration}))$  indicates the effect of haul duration.  $\eta_{\text{nugget}}(i)$  is a mean 0 Gaussian distribution with variance  $\sigma_N^2$ , indicating the local variance between hauls within a spatial-temporal cell.

We expect that by describing the spatial-temporal correlations in the model, we would obtain a more independent and accurate estimate of the year effect. The estimated year effect, i.e. an estimated value per year, would be used as the annual indices.

### 5.2.2 Delta log-normal method based on CPUE per haul

The CPUE data distribution is highly skewed and contains a high proportion of zeros. Such data can be modeled by a delta-lognormal distribution: Consider a non-negative random variable  $Y$  (in our case CPUE) for which  $\Pr(Y>0)=p$ . We define  $X=\{\ln Y \mid Y>0\}$ , and assume that  $X \sim N(\mu_X, \sigma_X^2)$ .  $Y$  is said to have a delta-lognormal distribution. The expected value of  $Y$  is then given as (Aitchison 1955):

$$\mu_Y = p \exp\left(\mu_X + \frac{\sigma_X^2}{2}\right) \quad (2)$$

To summarize, the expected annual index values are the product of 1) the proportion of positive (non-zero) hauls and 2) the exponentiated sum of mean and variance of the CPUE from the positive hauls.

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The DLN method has been widely used in fisheries research (Pennington 1983,1991; Smith 1988,1990; Lo et al. 1992; Fletcher 2008).

Assuming the number of positive hauls is  $m$  and the total number of hauls is  $n$ , a minimum variance unbiased estimator (MVUE) for  $\mu_Y$  was given by Aitchison 1955 as:

$$\hat{\mu}_Y = p \exp(\bar{x}) G_m\left(\frac{s_x^2}{2}\right) \quad (3)$$

where  $p = m / n$ ,  $\bar{x}$  and  $s_x^2$  are the sample mean and variance of  $X$ , and

$$G_m(t) = 1 + \frac{(m-1)}{m} t + \sum_{i=2}^{\infty} \frac{(m-1)^{2i-1}}{m^i(m+1)(m+3)\dots(m+2i-3)} \frac{t^i}{i!}.$$

We refer this estimator as Aitchison's estimator and use this in our calculation.

To estimate the variance or confidence interval of  $\mu_Y$ , Fletcher 2008 summarized three parametric methods: 1) Pennington estimator (based on eq. 3) with symmetric confidence intervals; 2) A modification of Cox's method for the lognormal ( $\ln\mu_Y$  and back-transformation based); 3) Profile likelihood method. Based on simulations, profile likelihood turns out to provide the best confidence interval while Pennington estimator gives the worst performance. Despite the parametric confidence interval estimates, we applied a non-parametric bootstrapping method in this study, allowing less constraints on the distribution assumptions. Treating the sample as a pseudo-population, a simulated sample is randomly drawn and the estimated  $\hat{\mu}_Y$  is calculated according to eq. 3. Such process is then repeated for 5000 times. Subsequently, the 2.5 and 97.5 percentile of the 5000 simulation results are the 95% confidence interval of the estimated  $\hat{\mu}_Y$ .

## 5.3 Survey data and data pre-processing

### 5.3.1 NS-IBTS in Q3

Many pelagic species are frequently found close to the bottom during daytime (which is when the IBTS survey operates) and migrate upwards predominantly during the night. They are susceptible to semi-pelagic fishing gear and to bottom trawls (Barange *et al.*, 1998). Eaton *et al.* (1983) argued that horse mackerel of 2 years and older are pre-dominantly demersal in habit. Based on a comparison of IBTS data from 4 quarters in the period 1991–1996, Ruckert *et al.* (2002) showed that horse mackerel catches in the IBTS were most abundant in the third quarter of the year. Therefore, in the absence of a targeted survey for this stock, the IBTS data quarter 3 is considered a reasonable alternative.

IBTS data from quarter 3 in the period 1998-2014 were obtained from DATRAS (downloaded from DATRAS in July 2015) and analysed. Commercial catch data show that 2-year old fish and older make up 96% of the landings, which roughly coincides with fish of  $\geq 20$  cm (see Figure 4.4.3 in WGWISE report, ICES, 2014b). The number of fishes per haul of  $\geq 20$  cm (roughly corresponding to age 2 and older) were therefore used to estimate the annual stock indices.

To create indices, a subset of ICES rectangles was selected. Rectangles that were not covered by the survey more than once during the period 1991–2012 were excluded from the index area. In 2012, WGWISE expressed concern that the previously selected index area did not sufficiently cover the distribution area of the stock, especially in years that the stock would be relatively more abundant and spread out more. Ruckert *et al.* (2002) also identified a larger distribution area of the North Sea stock. Based on the above, 61 rectangles were identified to be included in the index area (ICES, 2014a).

### 5.3.2 French CGFS in Q4

In order to improve data basis for the North Sea horse mackerel assessment, alternative survey indices have been explored. Previous indices used had only cover the North Sea distribution of the stock, while the majority of catches in recent years have come from the eastern English Channel (VIId). We evaluated the potential contribution of the French Channel Groundfish Survey in VIId (CGFS) in Quarter 4. The CGFS is carried out since 1990 and has frequent captures of horse mackerel.



The survey data was downloaded from IFREMER on 28 August 2015 after contacting the relevant survey coordinator (Franck Coppin). Only the horse mackerel catches by length per half hour tow were used. We also included hauls without horse mackerel catches (number=0). Similar to the NS-IBTS data, The number of fishes per haul of  $\geq 20$  cm were used to estimate annual stock indices.

Although CGFS in quarter 4 is conducted in a different quarter to the North Sea IBTS, the observed seasonal migration patterns of horse mackerel indicate that fishes move into the channel following quarter 3. The two survey cannot be considered as giving a snapshot view of the stock, as part of the stock is moving between Q3 and Q4 and is probably sampled by each survey. Therefore, the calculation of the indices, using both LGC model or DLN method, was conducted separately on each survey.

## 5.4 Results

The number of hauls and number of rectangles covered by NS-IBTS (1998-2014) and french CGFS (1990-2014) data are presented in Table 5.1. NS-IBTS contains on average 107 hauls in 58 selected rectangles per year, while CGFS contains 79 hauls in 15 rectangles per year. For the IBTS, the years 2000 and 2002 were excluded, while for CGFS all years were included.

The spatial grid of  $10 \times 10$  km cell size in LGC model are illustrated in Figure 5.1. Due to the high-dimensionality, small sample size and high correlation among the covariates, the full LGC can not be assessed. Therefore, we applied a trial-and-error process to find the collection of assessable covariates. The final workable LGC model has the form of

$$E[\log(\lambda_i)] = \eta_{\text{space}}(x) + \mu(y) + \eta_{\text{nugget}}(i) \quad (4)$$

With similar haul duration in both surveys, the effect of haul duration is negligible and the model in Eq. 4 is valid. The estimated model parameters are presented in Table 5.2. The estimated spatial decorrelation distance is small but consistent, 9.4 km in the North Sea and 10.5 km in the English Channel. In other words, at about 10 km distance, the correlation of the numbers at catch has already decayed to  $1/e$ . Such small spatial correlation is also illustrated by the modelled catch rate distributions of horse mackerel Figure 5.2. Given such little contribution of spatial correlation in the model, the estimated year effect (annual indices) and 95% confidence intervals for NS-IBTS and French- CGFS are plotted in Figure 5.3 and Figure 5.4, respectively.

The LGC model output implies that there is little spatial correlation in the two surveys. As a result, the more straightforward DLN method could be a preferred option. The estimated mean CPUE (annual indices) and 95% confidence intervals for NS-IBTS and French CGFS are plotted in Figure 5.3 and Figure 5.4, respectively.

Table 5.1. Number of hauls and number of rectangles covered by the NS-IBTS (1998-2014) and french CGFS (1990-2014) data.

Year	NS-IBTS NO. Hauls	NS-IBTS NO. rectangle	CGFS NO. Hauls	CGFS NO. rectangle
1990	NA	NA	73	15
1991	NA	NA	79	15
1992	NA	NA	59	13
1993	NA	NA	62	14
1994	NA	NA	84	14
1995	NA	NA	85	16
1996	NA	NA	60	13
1997	NA	NA	84	15
1998	101	58	77	16

1999	110	57	94	16
2000	131	61	95	16
2001	121	59	102	16
2002	109	58	88	15
2003	102	56	73	16
2004	108	58	80	15
2005	119	59	94	15
2006	118	60	93	16
2007	103	56	83	15
2008	95	53	78	13
2009	95	55	63	13
20010	101	57	72	15
2011	105	58	86	14
2012	109	60	68	14
2013	100	59	58	11
2014	92	57	73	12

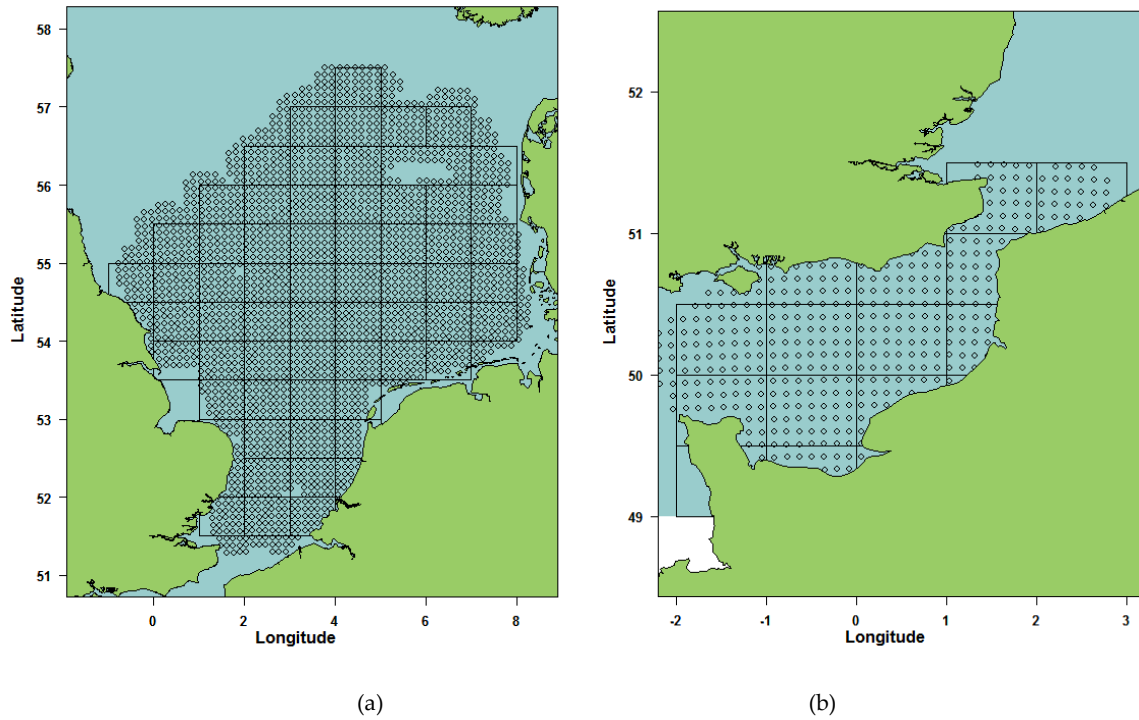


Figure 5.1. The spatial grid of  $10 \times 10$  km cell size used in the LGC model for (a) NS-IBTS and (b) French-CGFS.

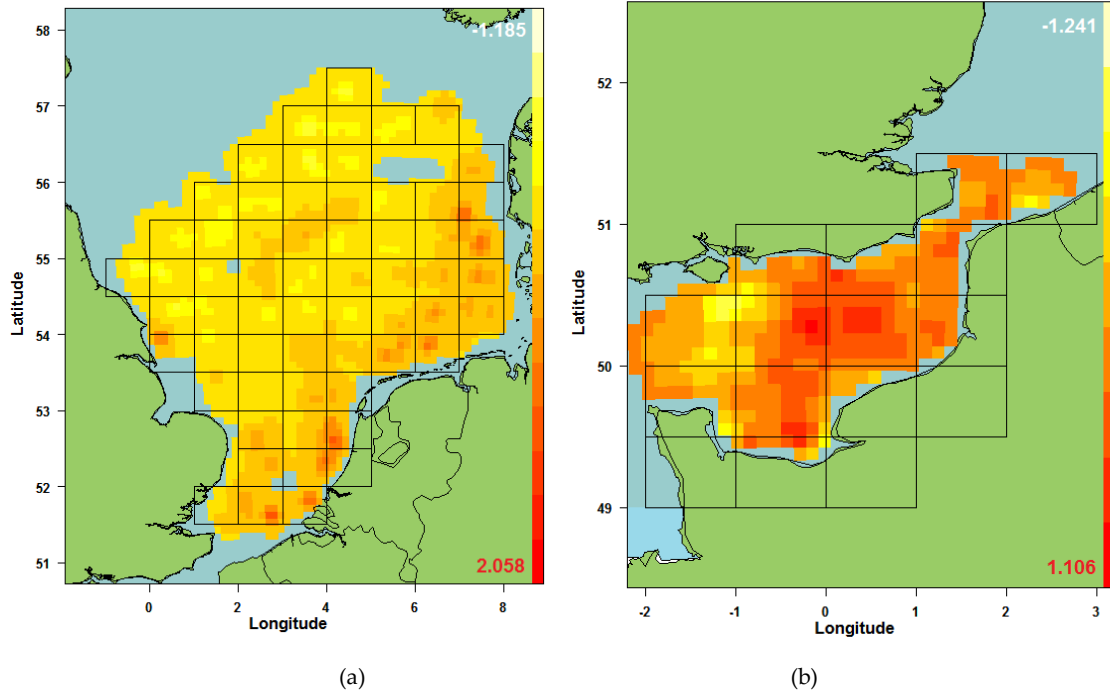
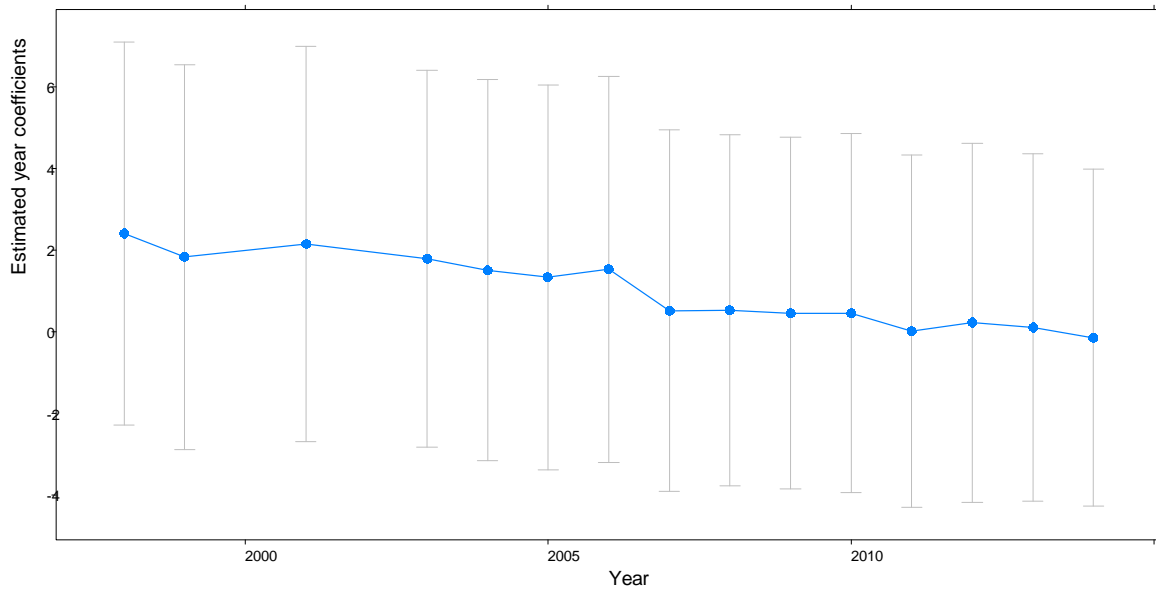


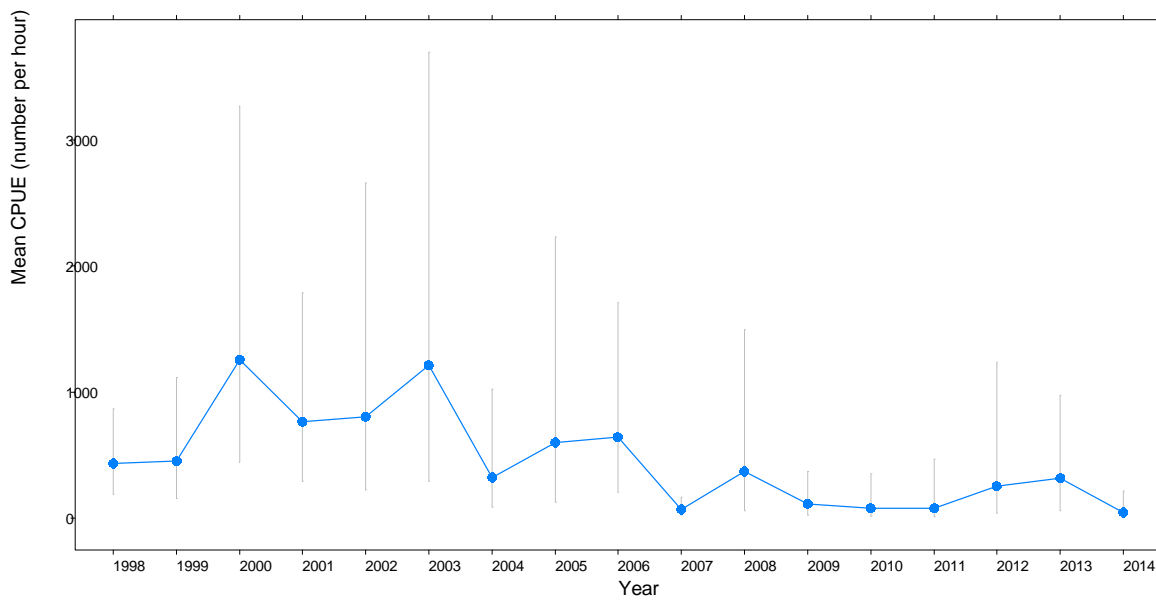
Figure 5.2. LGC process Modelled catch rate distributions of horse mackerel for (a) NS-IBTS and (b) French-CGFS.

Table 5.2. LGC model parameter estimates and standard errors.

Model	Symbol	Description	Estimate	SE
NS-IBTS	$H$	Spatial decorrelation distance (km)	9.4	2.15
1998-2014	$\sigma_{is}^2$	Spatial variance parameter (intercept surface)	0.2	0.02
	$\sigma_N^2$	Variance of the nugget effect	0.4	0.04
French-CGFS	$H$	Spatial decorrelation distance (km)	10.5	1.21
1990-2014	$\sigma_{is}^2$	Spatial variance parameter (intercept surface)	0.3	0.03
	$\sigma_N^2$	Variance of the nugget effect	0.3	0.01

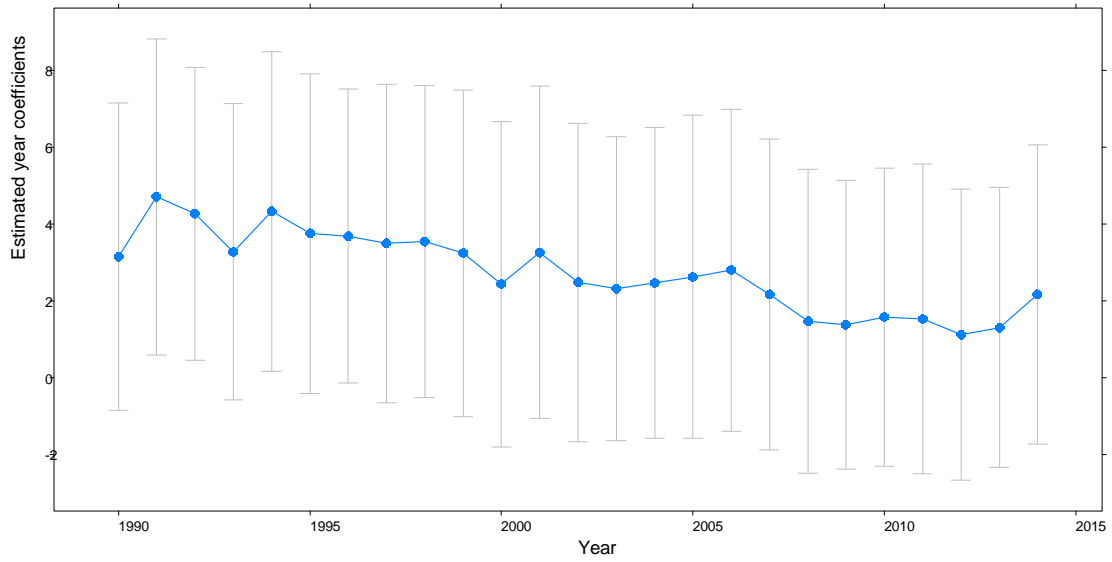


(a)

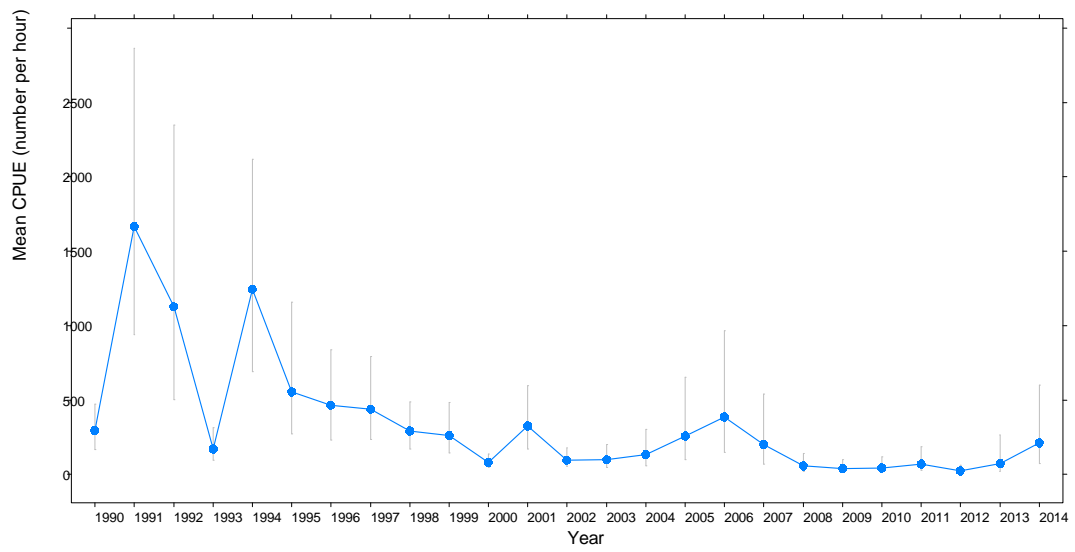


(b)

Figure 5.3. Estimated annual indices and 95% confidence interval from NS-IBTS data using (a) LGC model and (b) DLN method



(a)



(b)

Figure 5.4. Estimated annual indices and 95% confidence interval from French-CGFS data using (a) LGC model and (b) DLN method

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## 5.5 Discussion

The estimated spatial decorrelation distance (around 10 km) is very small based on our selected data, smaller than the spatial grid used for the design of both surveys (ICES rectangles of 0.5° lat x 1° long). Without considering the underlying biology, a recent study of LGC model on age 0 mackerel (Jansen *et al.* 2014) indicates a 255 km spatial decorrelation distance, 25 times larger than our estimate. That study also includes much larger area of north sea (27273 hauls) as compared to our study (1819 hauls in IBTS and 1963 hauls in CGFS). If the actual spatial cluster of  $\geq 2$  year horse mackerel are large, a larger area of data need to be included. An extreme case of such is that if we have only included data within a cluster.

The estimated annual indices from both LGC and DLN provide similar annual trends (compare Figure 5.3a vs. 5.3b, 5.4a vs.5.4b), although the absolute values differ. However, LGC method gives very large confidence intervals, the cause of such large uncertainty needs to be further investigated. Such similar trend also implies that the spatial correlation is low so that it does not make a significant change to the annual trends estimated without taking the correlation into account.

The DLN method gives resonably good CPUE estimate. The confidence intervals become small since 2007 for NS-IBTS and since 1996 for French-CGFS. In a similar period, the estimated CPUE are low. More work needs to be done to incorporate these estimate for stock assessment. For instance, to compare and explain the two survey indices consistency or inconsistency (compare Figure 5.3a vs. 5.4a, 5.3b vs.5.4b).

From 2015 onwards, the vessel R/V Gwen Drez CGFS is reformed and can no longer be used. Instead, CGFS will be carried out on another scientific vessel R/V Thalassa. To sure a consistent measurement over time series, an inter-calibration method between the two vessels has been studied (Auber *et al.* 2015). Thus, the annual indices calculation from 2015 needs to adapt such calibration.

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## 6 General conclusions and recommendations for future work

### Genetics

The genetic analyses suggest a fair amount of differentiation between North Sea and western horse mackerel, which would support the current perception of the North Sea horse mackerel being a separate stock from the western horse mackerel. However, mostly due to technical problems occurring during the sequencing (being the responsibility of a laboratory sub-contracted by UCD for this task), only a sub-set of the samples could be included in the statistical analyses, using only 17 out of the 34 potential markers identified. This dramatically decreased the power of the analyses, which therefore turned out to be inconclusive.

The work carried out during this project has however identified a large number of potentially useful markers. To improve the statistical power of the analysis, future projects should intent to collect a larger number of samples, and include samples covering a larger geographical area. Especially, stocks located at the opposite border of the distribution range of the species (e.g. Mauritania) should be incorporated in order to assess the scale of the maximum possible genetic differentiation between 2 horse mackerel stocks.

The quality of the DNA seems to have been lower for the North Sea samples (kept in ice and frozen at landing) than for the western samples (frozen immediately). The quality of the genetic material in the samples could be improved in future studies by using directly on the vessel micro-tubes especially designed to take genetics samples. The lid of such tubes is made to cut a standard amount of tissue sample and which can be store immediately in the fixating solution contained in the tube.

### Chemical fingerprint

The chemical fingerprint analysis showed generally higher compounds concentration in North Sea horse mackerel than in western horse mackerel, but the differences were, for the majority of the compounds, not large enough to allow for a reliable discrimination of stock origin. Only one compound in each round of analysis was found to be a potential marker. Given the small number of fish analysed, and the fact that all fish from the western area come from a single trawl haul, the possibility that these specific compounds were present in the most North Sea fish and absent from all western fish only due to chance cannot be ruled out. In addition, the fact that the compound identified as a specific maker of the North Sea horse mackerel in the late summer sample was actually also found in the western fish in the early summer sample suggests that this compound is not a truthful marker.

Due to the high cost of the GCxGC-MS analyses, only a limited number of fish was analysed during this project, and whether or not this approach could be useful to separate horse mackerel of different geographical origin remains unclear. The result of this project raised many questions regarding their reproducibility from year to year, the representativeness of the western horse mackerel sample, the surprising fat content results and the impact thereof on the GCxGC-MS results, the stability over time of compound concentration, the influence of fish age, length and sex on compound concentration. Answering these questions would require a larger scale study, in which sample are taken both at spawning time and at mixing time in multiple locations.

The absence of marked difference in compound concentrations could also be due to a lack of environmental gradient in the compound concentrations. Repeating this analysis on a sedentary fish, in which compound concentrations should reflect the concentration in the environment, would provide a validation for this approach. Weak differences between North Sea and western area in sedentary fish would indicate an environmental signal too weak for developing the chemical fingerprint approach.

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### Abundance indices

The additional survey information for horse mackerel in the Channel (French Groundfish Survey) has already been analysed during WGWIDE 2015 and this project concluded that the information is valuable and that no further efforts are needed to combine the information with the IBTS survey in the North Sea, since these survey are not carried out at the same time of the year. The results indicate also that the simple delta log-normal approach currently used at WGWIDE provides acceptable abundance indices, and that there is no need, given the weak autocorrelation in the data, to implement the more complex log-Gaussian Cox process model.



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# 7 Quality Assurance

Wageningen Marine Research utilises an ISO 9001:2008 certified quality management system (certificate number: 187378-2015-AQ-NLD-RvA). This certificate is valid until 15 September 2018. The organisation has been certified since 27 February 2001. The certification was issued by DNV Certification B.V.

Furthermore, the chemical laboratory at IJmuiden has NEN-EN-ISO/IEC 17025:2005 accreditation for test laboratories with number L097. This accreditation is valid until 1<sup>th</sup> of April 2017 and was first issued on 27 March 1997. Accreditation was granted by the Council for Accreditation. The chemical laboratory at IJmuiden has thus demonstrated its ability to provide valid results according a technically competent manner and to work according to the ISO 17025 standard. The scope (L097) of de accredited analytical methods can be found at the website of the Council for Accreditation ([www.rva.nl](http://www.rva.nl)).

On the basis of this accreditation, the quality characteristic Q is awarded to the results of those components which are incorporated in the scope, provided they comply with all quality requirements. The quality characteristic Q is stated in the tables with the results. If, the quality characteristic Q is not mentioned, the reason why is explained.

The quality of the test methods is ensured in various ways. The accuracy of the analysis is regularly assessed by participation in inter-laboratory performance studies including those organized by QUASIMEME. If no inter-laboratory study is available, a second-level control is performed. In addition, a first-level control is performed for each series of measurements.

In addition to the line controls the following general quality controls are carried out:

- Blank research.
- Recovery.
- Internal standard
- Injection standard.
- Sensitivity.

The above controls are described in Wageningen Marine Research working instruction ISW 2.10.2.105. If desired, information regarding the performance characteristics of the analytical methods is available at the chemical laboratory at IJmuiden.

If the quality cannot be guaranteed, appropriate measures are taken.

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# Justification

Report C092/16

Project Number: 4311100007

The scientific quality of this report has been peer reviewed by a colleague scientist and a member of the Management Team of Wageningen Marine Research.

Approved: Jan Jaap Poos  
Senior researcher

Signature:



Date: 30<sup>th</sup> of September 2016

Approved: Jakob Asjes  
Manager integratie

Signature:



Date: 30<sup>th</sup> of September 2016

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# Annex 1 Guidelines for Sampling horse mackerel for chemical pollutants

## Guideline for sample collection

If organisms to be used in the same sampling are collected on different days / from different ships, individual fishes will be frozen until all the fish to be included are available for preparing the samples.

- a) Individuals of the selected target species should be rinsed in ambient water to remove any foreign material from the external surface, handled using clean nitrile gloves, and placed in clean holding containers (plastic bags, livewell, buckets, etc.) to prevent contamination.
- b) Specimens from each species should be packed in wrapped double plastic bags, labeled in the outer bag and immediately stored in refrigerated conditions (preferably ice) to transport to the laboratory.
- c) Transport of samples to the laboratory should take place in less than 24 hours from the time of sample collection. If this is not possible, freezing of the samples is required.
- d) Contamination of the sample during collection and transport must be avoided: possible sources of contamination (smoking, exhaust engines, dust).

## Guideline for sample preparation

Once horse mackerel arrives to the laboratory samples need to be prepared following different stages

- a) If not performed earlier, individual weight, total length and sex should be registered and each specimen should have identification (to be used throughout the whole experiment)
- b) Of those specimen selected to be used for samples otoliths are taken out

With respect to the filleting, please note:

***Prepare the filets of the same part of the fish each time***

*As as little as 10 grams is enough for analysis, there is no need to cut a relative larger filets from a smaller fish (with the risk of cutting into the abdominal cavity)!!*

- c) ***Largest risks of contamination occur during sample preparation (cutting of the filet)*** and storage of the samples, thus to diminish such risks: always work under clean conditions (e.g. knives and boards), do not sample muscle from abdominal cavity in fish, carefully separate muscle tissue from spines, skin, scales and viscera to avoid disruption
- d) Preparation boards previously rinsed with detergents should be properly rinsed with water, as this can be a source of contamination
- e) Working with clean materials (without detergent and disinfectants!) in a "clean room" (filtered air) is ideal but not always practical
- f) Wipe knife and board briefly after each fish. When the abdominal cavity has been cut, the knife should be cleaned more thorough (the liver can be major source of contamination to the filet)
- g) Place each filet in (glass) container as soon as possible and close with lid\*
- h) Storage material, mainly due to the long contact times with the sample, can also be a source of significant contaminations, according to the contaminant (see table below for tips)
- i) Storage temperatures: a minimum of -20 °C is required for proper storage of fresh samples
- j) As packaging and storage can affect the quality of the sample (with respect to contaminants) all filets of horse mackerel will be, if possible, identically stored and packaged

\* **Please note:** As many lids contain either teflon or a synthetic based liner, a hard plastic lid is advised. In case of any doubt, the use of aluminum foil as liner works very well

*Table to indicate the most likely sources of contamination of the samples (filets)*

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Contaminant	Potential contamination source	Proper material to be used	Material that cannot be used
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PAHs*	Fumes engine, sediments	combustion <b>smoking!!</b>	Aluminum wrap or glass container	
BFRs	Various including dust	materials,	Aluminum wrap or glass container	Various synthetics
Endocrine disruptors	Depending on type, soaps, detergents, house dust, liver, sediment		Aluminum wrap or glass container	
PPCPs (Pharmaceuticals)	Depending on type, soaps, detergents, house dust, liver, sediment		Aluminum wrap or glass container	
PFOS	packaging materials, Teflon liners, cutting boards, dust, liver		Polypropylene or glass	<b>Teflon liners</b> , HDPE
PBDEs	dust, sediments, liver		Aluminum wrap or glass container	
Natural metabolites	Algae, seeweeds, sediments, biofilms		Aluminum wrap or glass container	

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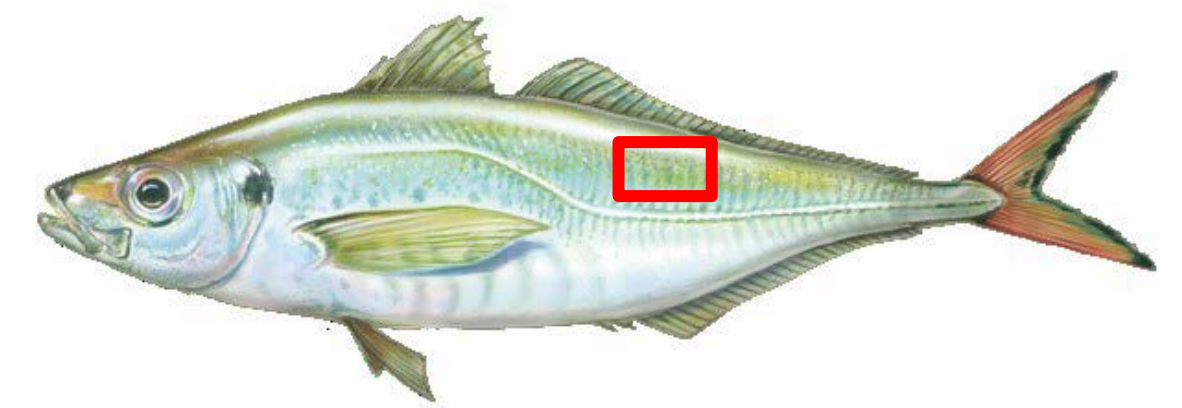
\*cross-contamination, as PAHs should not be found in fish filets, but can influence the chemical analysis.

**In conclusion;** prevent the use of synthetics as much as possible (other than pure LDPE plastic bags), avoid contact of filets with abdominal cavity, dust, sediment, smoke and vegetable materials during preparation of the filets and store in an appropriate container.

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## Annex 2 Guidelines for Sampling horse mackerel for genetic analyses



The sample kit contains 100 ml screw cap micro-tubes each filled with molecular grade ethanol. Each tube is individually labelled with a unique sample code. A sample data sheet is also provided. Please follow the following protocol to ensure the best quality samples are taken.

1. Take 100 randomly selected spawning horse mackerel from a single haul if possible.
2. Record the survey name, date, haul number, and catch position in the data sheet.
3. Select a new sample tube from the box.
4. Measure the total length, weight, sex and maturity of the fish
5. Record on data sheet beside the relevant sample no.
6. Use a clean scissors to cut a 1 cm<sup>2</sup> piece of white muscle tissue from the fish in the area indicated on the image above. Try to avoid including skin and scales with the sample.
7. Place the tissue into the sample tube and secure the lid tightly.
8. Ensure that the sample is not too big and is completely surrounded by ethanol in the sample tube.
9. Store the box of sample tubes upright in a fridge (4°C) or freezer (-20°C).





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