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PCR-RFLP analyses of formalin-fixed fish eggs for the mapping of spawning areas in the Eastern Channel and Southern North Sea

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ABSTRACT

A better knowledge on spawning grounds of principal commercial fishes in the North Sea is necessary for their monitoring. Fish egg taxonomic identification based on morphological characters cannot always be carried out effectively. This is particularly the case for the eggs of a number of species of the Gadidae (i.e. cod, haddock and whiting, and three rockling species) which have the same range of size and for which molecular-based techniques can help to improve the accuracy of taxonomic identification of eggs, and hence the reliability of studies on spawning areas. In this study, formalin fixation, egg conservation and DNA extraction were optimized in order to develop a specific PCR-RFLP method. Based on the sequence of the cytochrome *b* gene of reference specimens, a PCR-RFLP method was developed to distinguish eggs of cod, haddock and whiting on the one hand, and between eggs of three species of rocklings on the other. Among the 404 eggs tested, 80% of positive amplification was obtained for rocklings eggs and 86.36 % for Gadidae eggs. Eggs of *Enchelyopus cimbrius, Ciliata mustela* and *Ciliata septentrionalis,* and *Gadus morhua, Merlangius merlangus* and *Melanogrammus aeglefinus* were distinguished with this method. Based on morphological characters, the percentage of misidentification was less for cod than for whiting.

Key words: fish eggs, PCR-RFLP, cytochrome *b* gene, geostatistical analyses, interpolated map.

INTRODUCTION

The North Sea supports one of the world's most active fisheries. Overfishing, especially by beam trawling and other demersal fishing methods, has had strong effects on the structure of both fish populations and benthic communities and on trophic interactions (Jennings and Kaiser, 1998; Bergman *et al.*, 1996). The extensive fishing activity, especially in the south, results in many areas being trawled several times each year. High fishing pressure has caused the loss of mature and breeding fish populations. Stocks of many commercial fish species like herring (*Clupea harengus*), cod (*Gadus morhua*) (Beaugrand *et al*., 2003), mackerel (*Scomber scombrus*), and plaice (*Pleuronectes platessa)* (ICES 2005) have been fished beyond safe limits, and some stocks have been permanently depleted since the 1960s (ICES, 2004). As a result, the exploitation of most major commercial species stocks in the North Sea is mainly based on the available first year classes of the population. In this context, the influence of recruitment fluctuations on the state of stocks has increased and recruitment success is becoming a fundamental parameter for fisheries management. Spawning grounds are then regarded as sensitive habitats, both in terms of ecosystem functioning and fishery activities.

The egg stage is a critical phase of the fish life cycle, subjected to high mortality due to predation and environmental conditions. Therefore it is essential to study and protect spawning grounds to improve the management of stocks and to maintain appropriate population levels of fish species in the North Sea.

Pelagic eggs of fish are spherical, transparent and highly aggregated in time and space (Checkley *et al.*, 1997). They float in the uppr water column and are usually located just below the surface (Fritsch, 2005). Fish eggs are passive particles and their vertical distribution is determined by a few parameters such as egg density, egg diameter, wind and tidally induced turbulence and vertical hydrographic structures (Petitgas *et al.*, 2006). Many fish have a winter spawning period. Fish eggs are mainly identified by visual methods under the binocular microscope using morphological criteria such as egg size. However, many eggs, like those of cod and haddock (*Melanogrammus aeglefinus*) have the same range of size and therefore the visual identification of early stages is very difficult (Taylor *et al.*, 2002).

Biochemical techniques can be used to identify fish eggs such as isoelectric focusing (IEF) (Mork *et al*., 1983). IEF combined with histochemical staining of isozymes has been used to identify Gadidae and Pleuronectidae eggs by Heffernan *et al*. (2004), but 6% of the eggs were not identifiable by comparison with the known standards and approximately 36% of all samples analysed were unidentifiable because they expressed neither LDH nor SOD isozymes. Immunological probes based on antigen-antibody reactions have allowed identification of zebrafish eggs (*Danio rerio,* Del

Giacco *et al.*, 2000) or malabar grouper (*Epinephelus malabaricus)* egg (Chiou *et al.*, 2004), but these techniques are lengthy procedures because they require, for each studied fish species to previously purify an antigen, to induce specific antibody production, and finally to develop and validate the method. Molecular techniques seem to be the most useful tools for identification of marine organisms, applied with species-specific genetic markers (for a complete review see e.g. Teletchea, 2009 and Puyet and Bautista, 2010), they allow identification of all life-history stages from eggs to adults (Karaiskou *et al.*, 2007).

The main problem regarding genetic identification of eggs is the preservatives used for fixation and conservation of samples. Many preservatives may be used, but eggs are usually fixed and preserved in formaldehyde to be visually identified. Formalin-preserved eggs of different species have been identified using PCR analyses of 16S rRNA (Perez *et al.*, 2005; Garcia-Vasquez *et al.*, 2006 ; Akimoto *et al.*, 2002). In the same way, Aranishi (2006) has identified single eggs of walleye Pollack (*Theragra chalcogramma*) , and Hyde *et al.* (2005) have distinguished blue marlin (*Makaira nigricans*), dolphinfish (*Coryphaena equiselis* and *Coryphaena hippurus)*, shortbill spearfish (*Tetrapturus belone*), swordfish (*Xiphias gladius*) and wahoo (*Acanthocybium solandri*) eggs in one step-PCR (multiplex PCR) both based the cytochrome *b* gene. TaqMan DNA technology has also been successfully applied on cod eggs (Fox, 2005a) or to distinguish cod, haddock and whiting eggs (Goodsir, 2007). PCR-RFLP is a simple, easy, robust and inexpensive method which can be applied to routine surveys. This technique has already been applied using the cytochrome *b* gene in order to identify eggs of eighteen Gadidae species (Calo-Mata *et al.*, 2003) or to distinguish Alaska pollack, Pacific cod and Atlantic cod (Aranishi *et al.*, 2005a) or to differentiate walleye pollack from cod fish (Aranishi *et al.*, 2005b).

Here we describe the implementation of a cytochrome *b* PCR-RFLP method to identify gadoid formalin-fixed fish eggs and to use the DNA-based data to correct spawning distribution maps in the Eastern English Channel and the Southern part of the North Sea. Geostatistical interpolation techniques were applied in order to produce the spatial pattern of each species identified with visual methods (Carpentier *et al.*, 2005 and Martin *et al.*, 2007) and that of data obtained by DNA analyses.

METHOD

Data collection and conservation

All samples were collected during an IBTS (International Bottom Trawl Survey) survey organized under the aegis of the International Council for the Exploration of the Sea (ICES) to estimate the abundance and recruitment level of the main commercial fish species exploited in the North Sea. The present study is based on the data collected during the first quarter of the year 2008 (Fig. 1).

The French research vessel "Thalassa" from IFREMER samples the Southern part of the North Sea each year during January and February. Fish eggs were collected using the CUFES (Continuous Underway Fish Eggs Sampler) for which a detailed description has been provided by Checkley *et al.* (1997). Briefly, the system consists of a pump situated under the boat drawing water from five meters below the sea surface, a stirrer with a sieve to assist in screening and concentrating eggs and a collector. Both the concentrator sieve and the collector filter have a mesh size of 500 µm. Samples were filtered over 30 min periods (approximately 20 m^3), 24 hours per day during the entire survey. The exact volume filtered for each sample depended both on sample duration and pump flow.

Geneticists traditionally preserve samples in alcohol. However, alcohol deteriorates the morphological criteria of eggs. As in this study, fish eggs were previously identified microscopically according to morphological characteristics before being analysed by DNA techniques, egg samples were fixed and conserved in formalin solution (Mastail and Battaglia, 1978; modified by Bigot, 1979). Briefly, a BHA solution was made up by adding 8 g BHA (buthylhydroxyanisol) to 500 mL monopropylen glycol and an EDTA solution was made by adding 20 g EDTA to 500 mL distilled water. 2 L of commercial formalin (36%) were buffered to pH 7 with sodium glycerophosphate and EDTA solution was added, while stirring. BHA solution and then 2 g ascorbic acid were added to distilled water up to 5 L to obtain a stock solution at 14.4% of formalin buffered at pH 7. The stock solution was agitated for about 20 minutes. Sea water was added to 2.4 mL of this solution to have a 40 mL of solution at 0.864 % formalin in pill jars. This fixation solution allowed a better pigment conservation. After two to three weeks fixation, samples were transferred in storage solution without formalin (Steedman, 1976), containing 0.5 mL of propylene phenoxetol, 4.5 mL of propylene glycol and 95 mL of distilled water to limit DNA damaged due to formalin.

Fish egg identification

Fish eggs were identified under a binocular microscope according to morphological criteria, mainly the shape of the eggs (spherical for the majority of fish eggs or ovoid), egg diameter (measured by micrometer), the presence of an oil globule such as in rockling species and pigmentation as a function of the stage of development (Russell, 1976; Munk and Nielsen, 2005; Lelièvre, 2007). Three stages were identified: (1) stage 1, without embryo, (2) stage 2 with the beginning of the development of an embryo and (3) stage 3 with a well developed embryo. Species identification proved difficult. Many species which spawn in the Eastern Channel and the southern North Sea, during the study period, have the same range of size (Table 1). So two groups of fish eggs, which

can be observed in the study area, were identified: group 1 including eggs of three species of rocklings and group 2 including eggs of other Gadidae species: cod, haddock and whiting. Another group including dab (*Limanda Limanda*), flounder (*Platichthys flesus*) and *Trisopterus sp*. was identified but not analysed in this study. The three rockling species cannot be distinguished by visual methods so rockling eggs were recorded as rockling sp. No confusion was possible between these two groups.

Molecular biology

DNA extraction

Three DNA extraction methods were tested: the first was a method based on phenol/chloroform/isoamyl alcohol (PCI) extraction (Quintero *et al*., 1998). DNA from adult fish muscles stored in alcohol was extracted as described by Jérôme *et al*. (2003a). The second method based on Chelex® 100 Resin (Biorad) was tested for individual egg DNA extraction with the protocol described by Jérôme et al. (2003b). However the yield of DNA extraction was too low to amplify egg DNA, therefore this method was not used in our study. A third method based on magnetic bead technology (ChargesSwitch® Forensic DNA Purification Kit, Invitrogen, Carlsbad, United States) was finally used for DNA extraction from individual eggs, previously categorized by visual identification. The standard protocol of this method has been slightly modified for fish egg DNA extraction. The magnetic bead-based technology provides a switchable surface charge dependent on the pH from the surrounding buffer to facilitate nucleic acid purification. First , eggs were crushed in 500 µL of lysis buffer added with 10 µL of proteinase K (20 mg/mL) in 1.5 mL microcentifuge tubes and incubated for one hour at 55°C. Then, 200 µL of purification buffer and 20 µL of magnetic beads were added to the microcentifuge tubes and left at room temperature for 1 minute. The tubes were then placed on a magnetic rack and after immobilization of the beads, the supernatant was removed and discarded. Beads were washed two times using 500 µL washing buffer each time. DNA was eluted by adding 100 µL of elution buffer and recovered after 1 minute of incubation at 55°C, and stored at 4°C for subsequent analyses.

PCR amplification and DNA sequencing of reference specimens

To select a fragment of the mitochondrial gene coding for the cytochrome *b* for egg identification, complete cytochrome *b* (1141 base pair, bp) sequences were obtained from muscle tissue of adult specimens of target species sampled during the IBTS research campaigns. The entire gene was amplified by PCR using the external primers Fishcytb-F and TruccytB-R (giving fragment of 1158 bp) (Sevilla *et al*., 2007).The PCR reactions were carried out in a total volume of 50 µL: 0.5 to 2.5 µL DNA template were added to a PCR mix consisting of 0.2 mM dNTP each (Interchim), 2 mM

$MgCl_2$, 10 µL 5X buffer, 1.25 units of GoTaq® polymerase (Promega), 0.2 µM of each primer and molecular biology grade water (Interchim) to adjust to the final volume. Polymerase chain reactions were carried out in a MyCycler™ thermocycler (BioRad). Conditions of cycling were as follows: a preheating step at 94 °C for 7 minutes, 35 cycles of amplification (94°C for 45 s, 50°C for 45 s, 72°C for 60 s) and a final extension step at 72°C for 7 min.

The DNA amplification was controlled on 1.5% agarose gels (Interchim), using TAE buffer (2 mM EDTA, 40 mM Tris acetate, pH 8.5). GelRed™ (Interchim) was used for band visualization via ultraviolet transillumination (Image Master VDSCL, Amersham Pharmacia Biotech). The size of the expected PCR products was estimated using the GeneRuler™ 100bp DNA ladder plus (MBI Fermentas). For sequencing reactions, the DNA template was quantified using the MassRuler™ DNA ladder low range (MBI Fermentas) on agarose gels.

Before sequencing, double-stranded PCR products were purified by filtration through a Qiagen QIA quick column according to the manufacturer's protocol. PCR fragments were used for direct cycle sequencing with the dye terminator cycle sequencing kit (Beckman) according to the manufacturer's protocol. Sequencing analysis was performed with a Beckman Coulter CEQ 8000 DNA sequencer in both directions with the primers used for PCR amplification. The DNA sequences were edited with BioEdit software (Hall, 1999).

PCR amplification of fish eggs

Based on the sequences obtained from the DNA of adult fish and from those downloaded from GenBank and FishTrace (http://fishnet.jrc.it/fishtrace_int/) databases, primers were designed to amplify a 5' cytochrome *b* fragment for rockling labelled eggs (group 1: 460 base pair (bp)) and for cod, haddock or whiting labelled eggs (group 2: 424 bp). Fishcytb-F (Sevilla *et al.*, 2007) for group 1, F-cb-SPF-GAD for group 2 and R2-cb-plat-428 for the two groups as reverse primer (Table 2) were used. Conditions of PCR reaction mixtures were as described above. Conditions of cycling for DNA amplification for both groups were as follows: a preheating step at 94 °C for 5 minutes, 35 cycles of amplification (94°C for 30 s, 48°C for 30 s, 72°C for 40 s) and a final extension step at 72°C for 7 min.

RFLP analysis

Restriction maps of a total of 45 DNA sequences (10sequences from this study – 35 sequences retrieved from Fish Trace and GenBank databases) were generated using the tools on the website of Rebase, restriction enzyme database (Roberts *et al.*, 2010). Restriction enzymes were selected, based on their ability to generate characteristic restriction profiles for each species with band sizes easily distinguishable on agarose gels. A total of 10 µL of PCR products was digested with two

units of enzyme in the buffer recommended by the manufacturer (Ozyme) in a final total volume of 20 µL at 37°C for 3 h. The reaction was stopped by heating at 65°C for 20 min. DNA restriction fragments were separated on 1.5% agarose gels as described above. The DNA fragments with sizes below 50 bp generated in the restriction digestion were not used for the identification because the the agarose gel conditions used did not allow their visualization.

Geostatistical analyses and interpolated map

The geo-referenced abundance data enable the distribution of fish eggs to be visualizes qualitatively using simple maps and quantitatively using geostatistical analyses (Petitgas, 1993 and Petitgas, 2001). Geostatistics (Webster and Oliver, 2001) may be used to study the spatial distribution of eggs using the experimental variogram calculated on log-transformed abundances ($\log_{10}(x+1)$) of eggs or on the relative occurrence of each species in the analysed samples (Vaz *et al*., 2005). The variogram is a model of the spatial auto-correlation pattern of the variable of interest that summarises how the variance of a variable changes as the distance and direction separating any two points varies.

The separation distance was calculated using latitude and corrected longitude (longitude x cos ((latitude x π)/180) of the sampling location. In the presence of local trends or drift, the spatial trend was modelled by fitting a low-order polynomial (linear or quadratic regression) of the spatial coordinates to the abundance or relative occurrence data using the least-square regression method. The experimental variogram was then calculated on the residuals if the fitted polynomial accounted for over 20 % of the variance.

A theoretical model, chosen among exponential, circular, spherical and pentaspherical forms was adjusted to the experimental variogram to determine the nugget, sill and range parameters that characterise the shape of the variogram. These four models were tested using least-square regression and the one which explained the most variation of the experimental variogram was retained as the theoretical variogram model. The theoretical variogram was used to estimate egg abundances on the knots of a regular grid by using the ordinary kriging interpolation method or the universal kriging in the case of a spatial drift (Cressie, 1993 and Carpentier *et al*., 2005). The geostatistical analyses were made using Genstat (GenStat Release 7.1., 2004) on abundance data (obtained from microscope identification) for each species cod, haddock and rockling sp. and for relative occurrence data (obtained from molecular identification). Interpolated maps of relative occurrence of each species obtained from molecular identification were applied to the interpolated map of total abundance of each group by simple multiplication as a correction factor. The resulting maps obtained reflected the distribution of each fish species.

RESULTS

Three methods were tested for DNA extraction from formalin eggs. Phenol/chloroform/isoamyl alcohol (PCI) extraction (Quintero *et al*., 1998; Jérôme *et al*., 2003a), a Chelex (BioRad) based method (Jérôme et *al*., 2003b) and magnetic bead-based technology (ChargesSwitch® Forensic DNA Purification Kit, Invitrogen). Among the methods tested, the last method was the easiest and quickest method for DNA extraction from single fish egg, and showed extraction-purification yield good enough to enable PCR-amplification of DNA. Therefore, this method was chosen for the egg DNA extraction with slight improvements as described in the materiel and methods section.

Complete cytochrome *b* sequences (10 sequences) obtained from reference samples (adult specimens) (data not shown) will be further uploaded to the GenBank database. Alignment of those sequences and of 35 sequences retrieved from FishTrace and GenBank databases indicated that the 5' fragment of the cytochrome *b* gene was polymorphic enough between the three rocklings on the one hand, and between the three other Gadidae species on the other to discriminate between the different species through specific cut sites of restriction enzyme.

These enzymes have a simple recognition sequence. The DNA fragments generated after the digestion were compatible with the expected size on the basis of the restriction maps (Fig. 2).

Molecular analysis validation

Among the 404 eggs tested, 80% of positive amplification was obtained for rocklings eggs and 86.36 % for Gadidae eggs. Some DNA egg extracts gave weak or no amplification by PCR. 105 eggs identified as cod following morphological criteria were confirmed on 107 eggs analysed by molecular analyses, the two eggs misidentified were in fact whiting eggs. This corresponds to 98.13 % of accurate identification. Among the 76 whiting pre-sorting eggs analysed by molecular analyses, 54 were correctly identified by the visual method and 22 were in fact cod eggs. This corresponds to 71.05 % of correct identification. Eggs analysed represent 36 location stations. Errors of identification concerned 7 sites so 19.5 % of the analysed stations. These locations were some of the areas where cod and whiting were present; these locations seem to be spread randomly so there is not, in this study, a specific area of error.

Interpolated map

Group 1: rockling species

Overall, 113 stations (1 573 Rockling sp. eggs, all stages combined) were identified during the 2008 IBTS sea survey, by binocular microscope. A total of 221 eggs belonging to 81 stations covering all the study area were analysed among which,75 eggs were identified as *Ciliata mustela*, 36 eggs as *Ciliata septentrionalis* and 110 eggs as *Enchelyopus cimbrius* (Figure 3)

Geostatistical analyses were performed on log-transformed abundance data for the rockling group (all species combined as eggs cannot be identified to species level by visual method). Similar analyses and interpolations were carried out on the relative occurrence of each species eggs obtained by molecular analyses (Fig. 4).

The relative occurrence maps of the three rockling species were used as correction factors and applied to the rockling group abundance map obtained by visual identification in order to obtain the distribution of eggs of the three rockling species in the entire study area and to analyse the spawning ground of each species (Fig. 5).

The three species covered a large proportion of the study area. *Ciliata mustela* eggs were present mainly in the eastern inshore region, near France, Belgian, Netherlands and German coasts. *Ciliata septentrionalis* eggs were present in the south of the study area, mainly in the Channel and the south of the North Sea. *Enchelyopus cimbrius* eggs were mainly offshore, in the centre of the study area and near the Dutch coasts, and totally absent from the Channel. The Dutch coasts seemed to be a large spawning ground for the three rockling species.

Group 2 : Other gadidae

Overall 262 stations (5 708 cod eggs and 25 597 whiting eggs, all stages combined) were identified by binocular microscope during the 2008 IBTS sea survey. 36 stations were analysed by molecular analyses. Figure 6 illustrates the station locations of each sample identified by visual method and by molecular analyses. A total of 183 eggs were analysed among which 107 eggs were identified as *Gadus morhua* and 76 eggs as *Merlangius merlangus.* No haddock eggs were found in the study area.

Geostatistical analyses were performed on log-transformed abundance data for cod and whiting eggs and on the relative number of cod and whiting eggs identified by molecular analyses. Similar analyses and interpolations were carried out on the relative occurrence of each species eggs obtained by molecular analyses (Fig. 7).

The relative occurrence maps of each species were used as correction factors and applied to the Gadidae group abundance map obtained by visual identification in order to obtain the distribution of eggs of the cod and whiting species in the entire study area and to depict the spawning ground of each species (Fig. 8).

Together the two species covered a large proportion of the study area but cod and whiting seemed to have separate spawning grounds. Cod spawned offshore of the German and Danish coasts, in the centre of the study area, while whiting was found more inshore, near the French, Belgian and Dutch coasts.

DISCUSSION

Early stock investigations, based on morphological characteristics of eggs, could lead to inaccurate spawning maps. Applications of DNA-based methods to large-scale distribution mapping of marine plankton have been hampered by the need to analyse very large numbers of specimens, by the relatively high cost per sample and by the time and specialist skills needed (Lindeque *et al.*, 2006). Therefore, the application of PCR-RFLP methods as described here combined with pre-sorting by morphological identification can be complementary. Many fish have a winter spawning period and some fish eggs have the same range of size such as cod, haddock and whiting, which spawn at the same time in the North Sea. Molecular analyses would be performed only on uncertain eggs and would allow the occurrence of eggs to be mapped across the study area. The molecular techniques used in this study on formalin-fixed eggs have proven their applicability to improve the accuracy of spawning maps.

Many studies have shown the problem of DNA damaged with formalin-fixed tissues (Chang *et al.*, 1994, Skage *et al.*, 2007 and Sawada *et al.*, 2008,). This fixation method reduces, particularly, the efficiency of extraction (Caldarelli-Stefano *et al.*, 1999). This problem has been encountered for fish eggs identification by molecular analyses (unpublished results). Perez *et al.* (2005) demonstrate the possibility of DNA extraction with formalin fixed hake (*Merluccius merluccius*) and megrim (*Lepidorhombus whiffiagonis* and *Lepidorhombus boscii*) eggs. In this study, the fixation solution has been improved to be efficient with less than 1% of formalin and the storage solution for the formalin-fixed eggs did not contain formalin in order to avoid any DNA damage.

The most widely used methods for extracting DNA from formalin-fixed eggs are based on phenol/chloroform/isoamyl alcohol (PCI) extraction (Quintero *et al.*, 1998) or Chelex (Aranishi, 2006). However, extraction of DNA by means of the PCI method is time-consuming and requires the use of potentially dangerous chemical reagents (Caldarelli-Stefano *et al.*, 1999). An alternative method, based upon magnetic bead technology was recently implemented because it allows rapid and efficient purification of DNA (in less than 15 minutes following egg preparation and lysis) and can be used in routine analyses. Although less DNA was obtained by the magnetic bead extraction method than by PCI extraction, DNA purified by magnetic beads gave after PCR slightly stronger

and cleaner bands on agarose gel than DNA extracted with the phenol/chloroform method (Caldarelli-Stefano *et al*., 1999).

Perez *et al*. (2005) have genetically identified hake and megrim eggs using fragment size of the 16S rRNA gene with a good PCR amplification success from 85 % formaldehyde-fixed eggs. Karaiskou *et al*., 2007 obtained 100% of PCR amplification of ethanol-preserved eggs while PCR amplification from formalin-preserved eggs was 20%. Fox *et al*. (2005a) found that only 6% of formalin-fixed cod egg DNA failed to be amplify. In the present study, amplification rate was quite good, 80 % for rockling eggs and 86% for other Gadidae eggs.

The sampled eggs analysed by the PCR-RFLP method did not show variation in the restriction profile, although the possible existence of variations in the recognition sites cannot be dismissed. However, the high number of reference samples (adult fish muscle) that were taken into account in the development of the methodology assured high degree of reliability for the method developed.

Rockling species eggs cannot be distinguished morphologically, but PCR-RFLP applied to rockling eggs allowed identifaction of the three rockling species present in the Channel and Southern North Sea: *Ciliata mustela, Ciliata septentrionalis* and *Enchelyopus cimbrius*. Eggs of rockling species, showed increased abundance towards the surface (Conway *et al*., 1997) which ensured that they were sampled adequately by CUFES. Rocklings are known to spawn mostly from January to September in the English Channel and North Sea with a peak in April and May so only the early stages of their spawning distribution are shown in the present study. Nevertheless, the rockling species spawning grounds found in this study are similar to those described in Martin *et al*. (2007) and in Carpentier *et al.* (2009). Eggs of *Gadus morhua, Merlangius merlangus* and *Melanogrammus aeglefinus* are hard to distinguish by visual identification (Taylor *et al*., 2002). During January and February, cod, haddock and whiting can spawn simultaneously in the study area. Eggs of *Gadus morhua* and *Merlangius merlangus* were identified by morphological identification but no *Melanogrammus aeglefinus* eggs were found; this was confirmed by molecular analyses. Cod and whiting seem to have two separate spawning grounds. Whiting spawn from January in the Southern North Sea to July in the Northern part (Russell, 1978; Svetnovidov, 1986) while Munk and Nielsen (2005) reported that whiting spawning took place from March to June. Whiting eggs were found south of the Dogger Bank and to the east of the Shetland Isles but were absent from the central North Sea (Fox *et al*., 2005b). Daan (1978) identified the main spawning areas of cod around the Southern flank of the Dogger Bank and a smaller patch in the German Bight (Fox *et al*., 2008). Moreover, Harding and Nichols (1987) identified cod eggs off the Western edge of the Dogger Bank. Spawning of cod begins in the south-east of the Dogger Bank (Martin *et al*., 2007; Fox *et al*., 2008). A survey in spring 2004 applying DNA-techniques for species identification (Fox *et al*., 2008) showed hot spots of cod egg production around the southern

and eastern edges of the Dogger Bank, and egg abundance at some locations in the southern North Sea was much lower than in earlier years (Daan, 1978).

Atlantic cod is classified as 'vulnerable' species by the IUCN[1] (Fox *et al.*, 2008) so their management has become necessary. Population recovery is related to stock-recruitment relationship but spawning biomass and egg production are expected to build up rapidly when fishes are allowed to grow as fecundity is a cubic function of fish length (Docker *et al.*, 1990; Clay, 2008). As a consequence, fishery management may use size limits to protect adults and to ensure that they are given the opportunity to spawn at least once (Fuiman and Werner, 2002). The precise knowledge of spawning areas obtained by this study may be used to determine the potential location and size of marine protected areas (MPAs). Managers may then decide to close off a given area or time of year to fishing, in order to protect spawning and settlement and with the aim of maintaining productive fisheries. Ichthyoplankton surveys may be a powerful tool to monitor spawning areas (Fox *et al.*, 2005a). Application of molecular analyses for fish eggs increases the accuracy of spawning maps and thus both stock assessment and sustainable management of species producing pelagic eggs which cannot be distinguished by visual method. However this PCR-RFLP method is quite expensive and lengthy because it necessitates egg per egg analysis, so it cannot replace visual identification. But both methods are complementary, molecular analyses could be performed only on uncertain eggs. In the same way, we are studying the feasibility of an image analysis based method using an integrated system which allows automatic counting, measurement and thus identification of fish eggs. All these methods will be complementary to improve knowledge of spawning grounds, to have a better understanding of fish population spatial dynamics and to implement spatially explicit protection schemes.

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[1] www.iucn.redlist.org

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Tables and figures legends

Table 1. Target species, their egg size diameter and their spawning period

Table 2. Primer sequences for PCR and enzyme for RFLP analysis for each group

Fig. 1. Location of CUFES (Continuous Underway fish Egg Sampler) samples during IBTS (International Bottom Trawl Survey) 2008

Fig. 2. Expected size of DNA fragments after digestion by restriction enzymes StuI or HpyCH4IV.
Group 1 (rockling restriction enzyme: StuI): *Enchelyopus cimbrius* (Ec) 460 bp, *Ciliata mustela* (Cm) 335-125 bp, *Ciliata septentrionalis* (Cs) 189-146-125 bp.
Group 2 (Gadidae restriction enzyme: HpyCH4IV): *Merlangius merlangus* (Mm) 213-211 bp, *Gadus morhua* (Gm) 424 bp, *Melanogrammus aeglefinus* (Ma) 330-58-36 bp

Fig. 3. Location of rockling species eggs analysed by PCR-RFLP: *Ciliata mustela* (CILIMUS), *Enchelyopus cimbrius* (ENCHCIM) and *Ciliata septentrionalis* (CILISEP)

Fig. 4. Relative occurrence distribution of each rockling species eggs identified by molecular analyses a) *Ciliata mustela*, b) *Ciliata septentrionalis*, c) *Enchelyopus cimbrius*

Fig. 5. Rockling species egg distribution a) *Ciliata mustela*, b) *Ciliata septentrionalis*, c) *Enchelyopus cimbrius*

Fig. 6. Locations of Gadidae eggs identified by molecular analyses: *Gadus morhua* (GADUMOR) and *Merlangius merlangus* (MERNMER)

Fig. 7. Relative occurrence distribution of cod and whiting eggs identified by molecular analyses a) *Gadus morhua*, b) *Merlangius merlangus*

Fig. 8. Cod and whiting egg distribution a) *Gadus morhua*, b) *Merlangius merlangus*

Table 1. Target species, their egg size diameter and their spawning period

	Scientific name	Common name	Egg size diameter	Spawning period
Group 1	*Enchelyopus cimbrius*	Fourbeard rockling	0.66-0.98 mm	January-July
	Ciliata mustela	Fivebeard rockling	0.66-0.98 mm	January-July
	Ciliata septentrionalis	Northern rockling	0.70-0.88 mm	January-July
Group 2	*Merlangius merlangus*	Whiting	0.97-1.32 mm	March-June
	Gadus morhua	Cod	1.16-1.89 mm	January-May
	Melanogrammus aeglefinus	Haddock	1.20-1.70 mm	February-July

Table 2. Primers sequences for PCR and enzyme for RFLP analysis for each group

	PCR		**RFLP**
	5' fragment cytochrome *b* gene		
	forward primer	**reverse primer**	**Restriction enzyme**
Group 1 Rockling	Fishcyt b-F: ACCACCGTTGTTATTCAACTACAAGAAC	R2-cb-plat428: GAGGACAAATGTCGTTCTGAGGTGC	StuI
Group 2 Gadidae	F-cb-SPF-GAD: CCAGCCTTCGGAAAACCCATCC	R2-cb-plat428: GAGGACAAATGTCGTTCTGAGGTGC	HpyCH4IV

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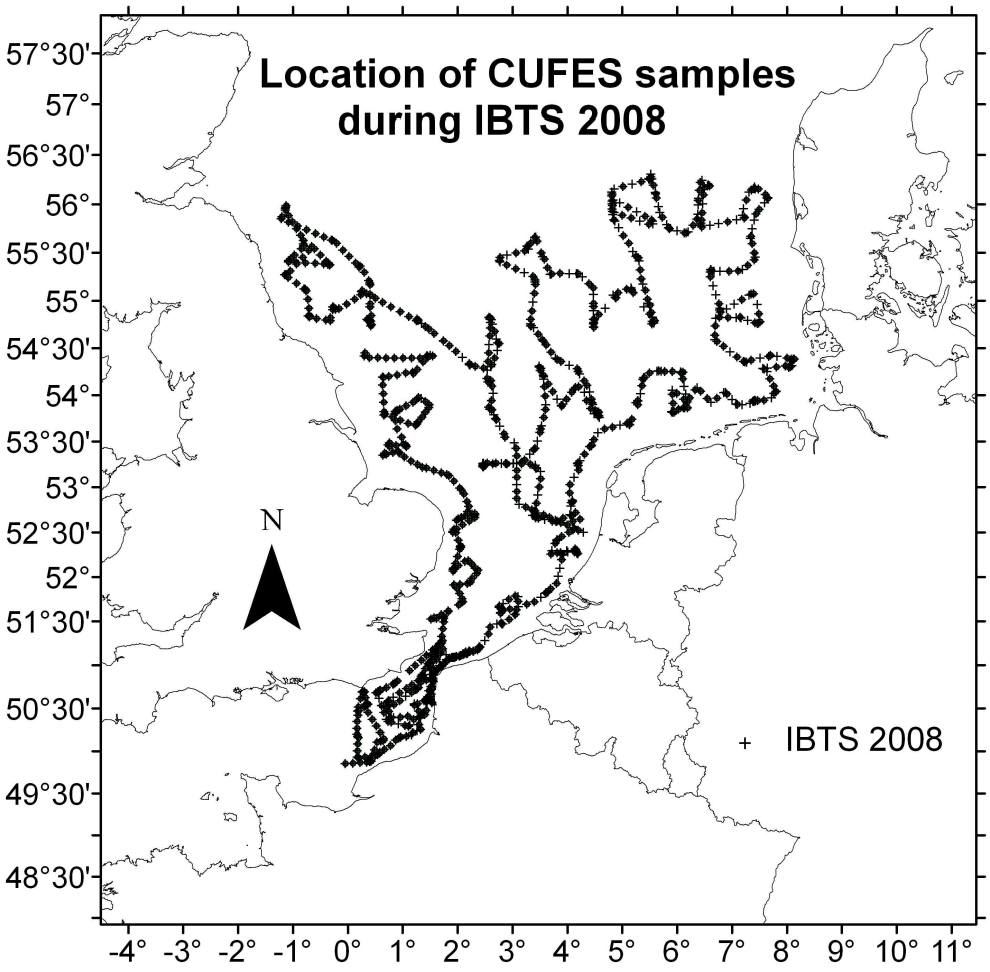

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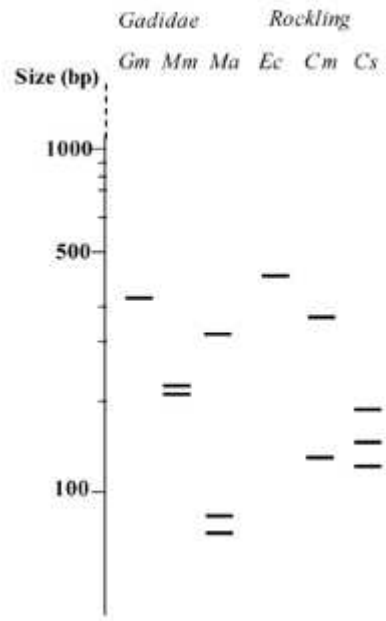

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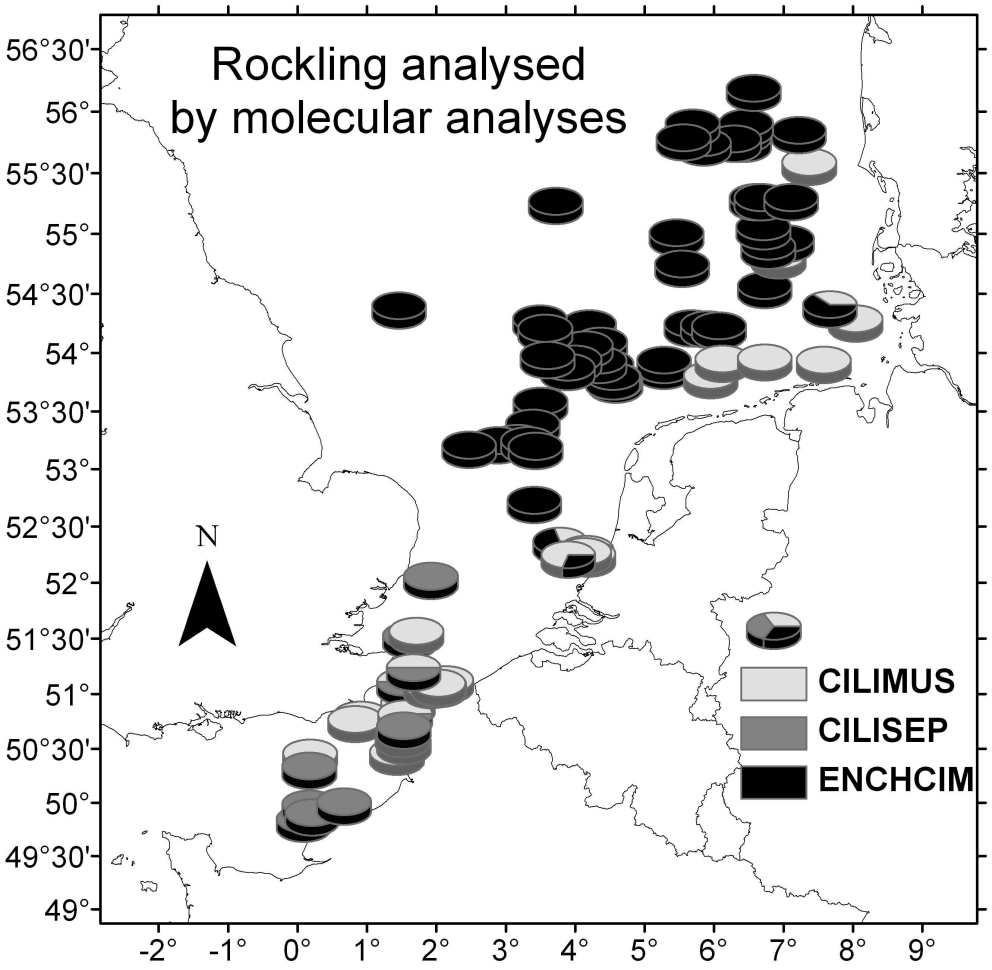

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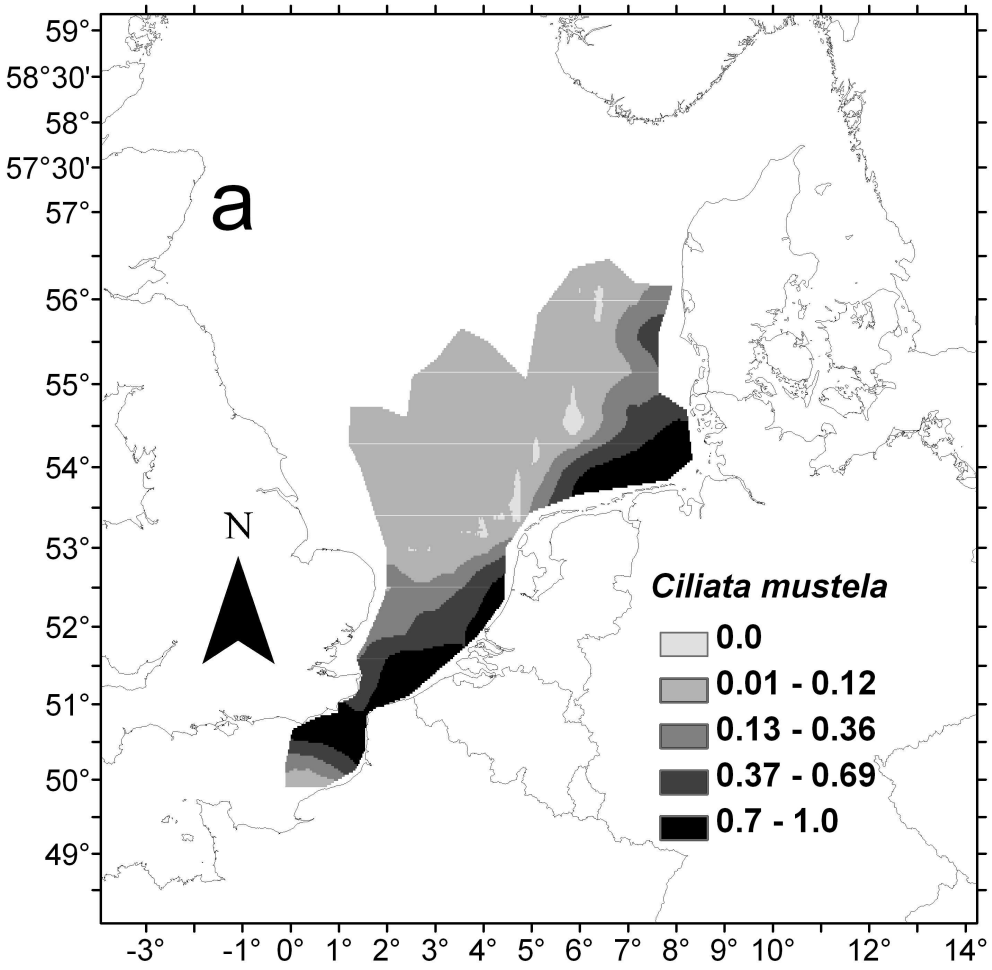

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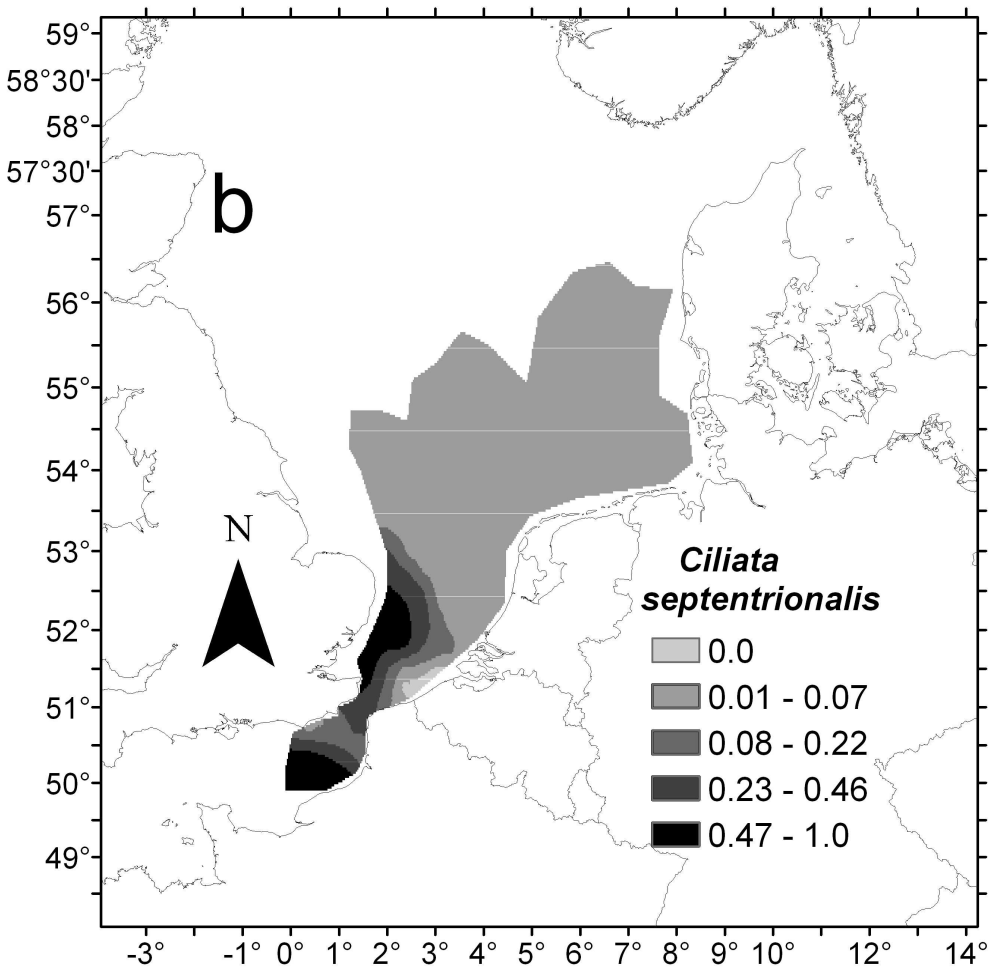

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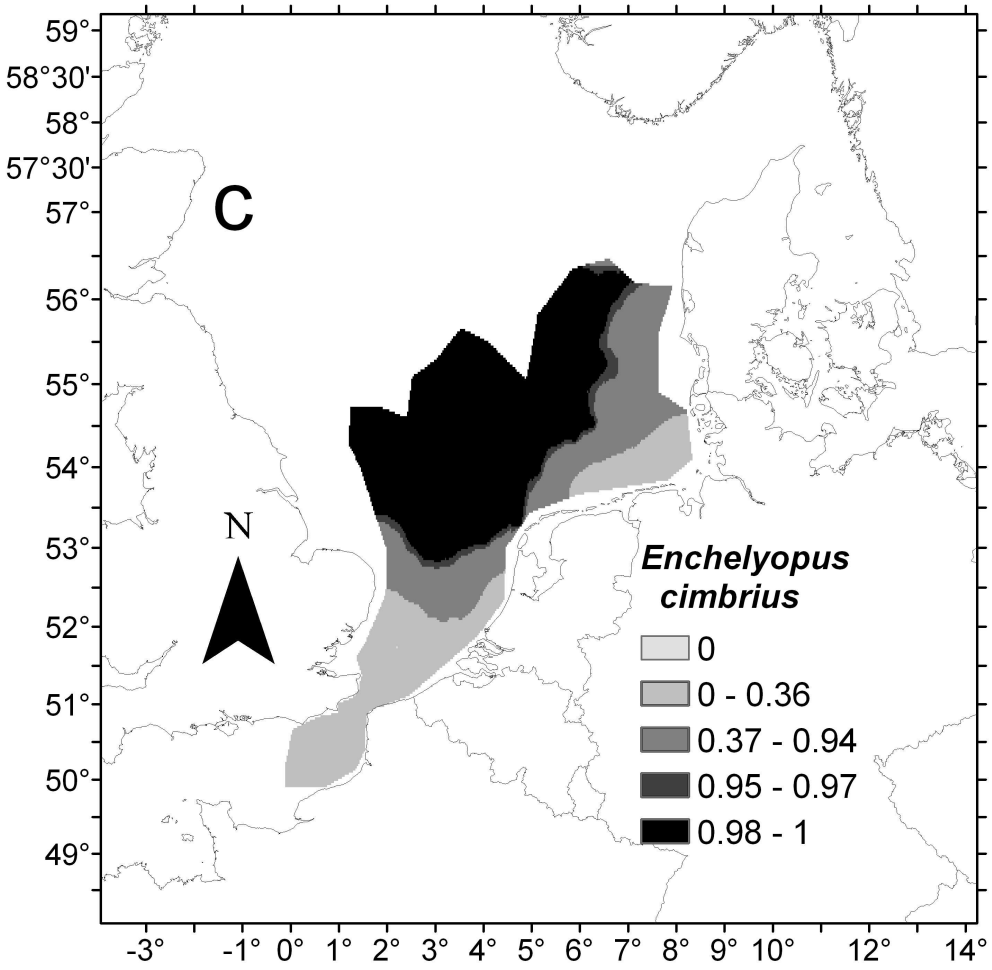

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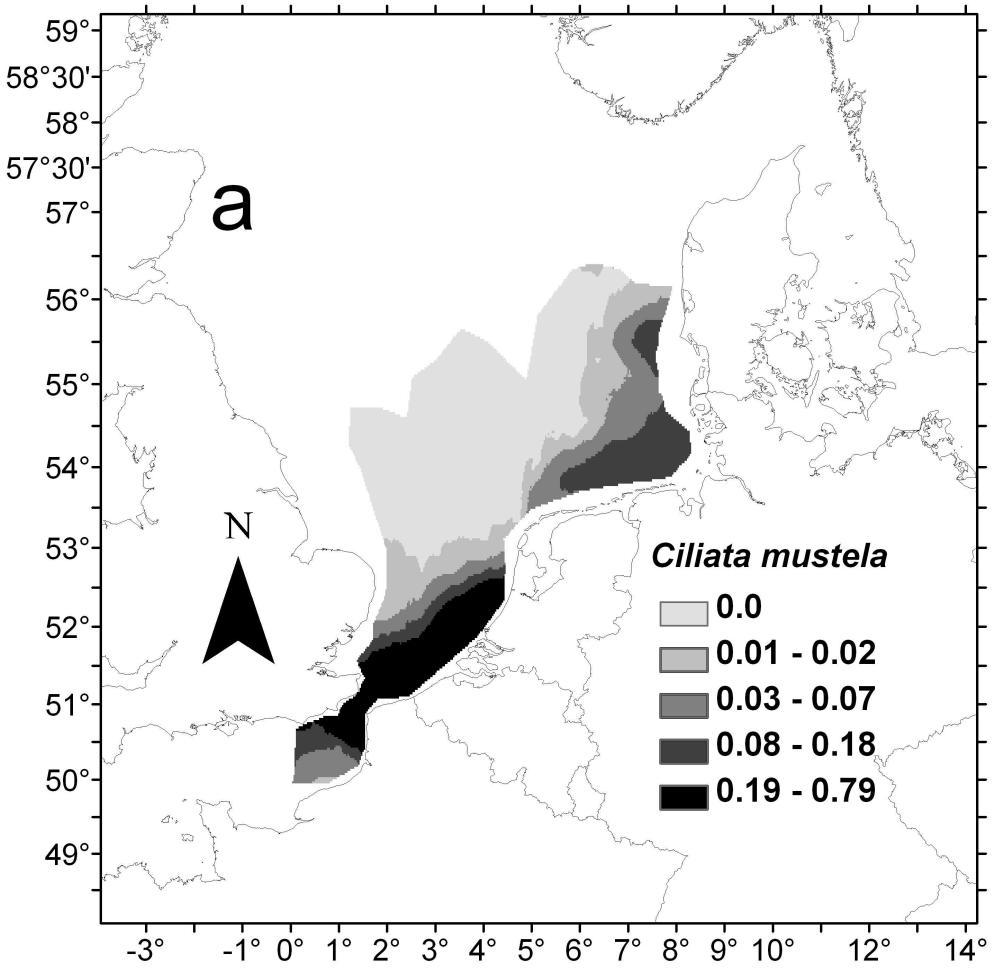

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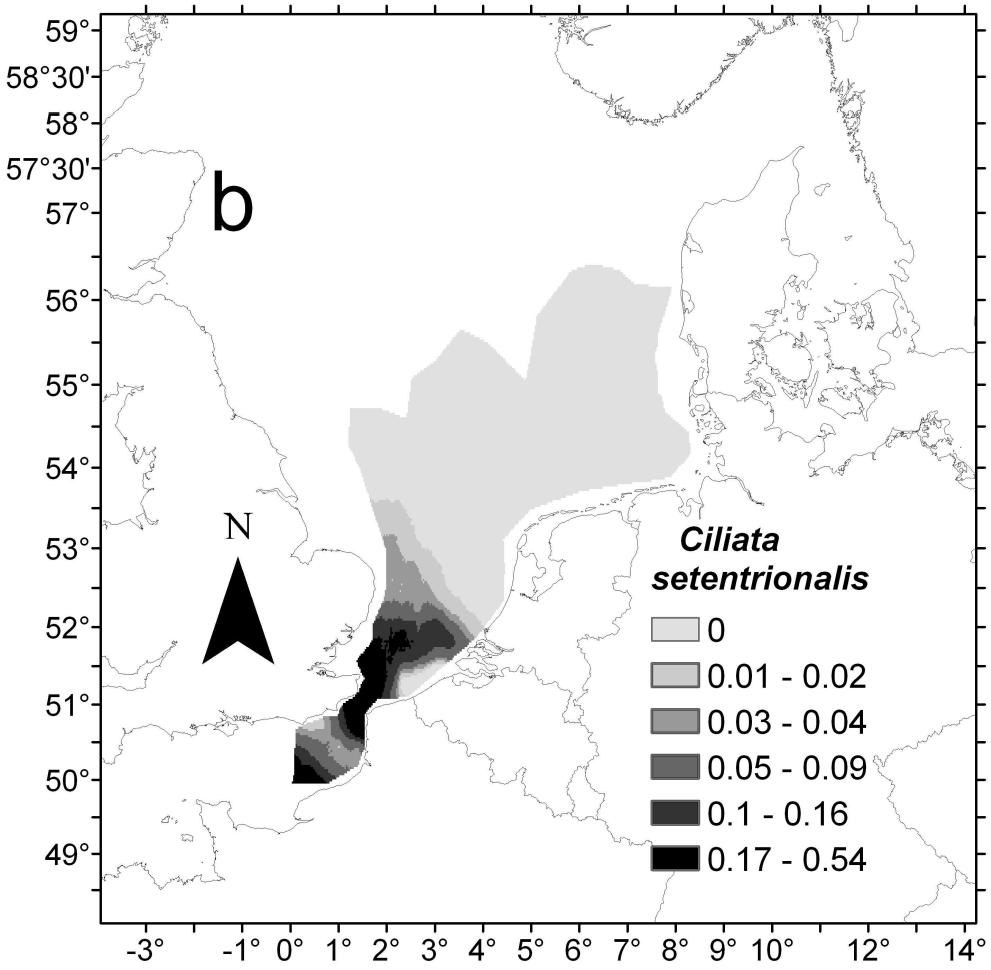

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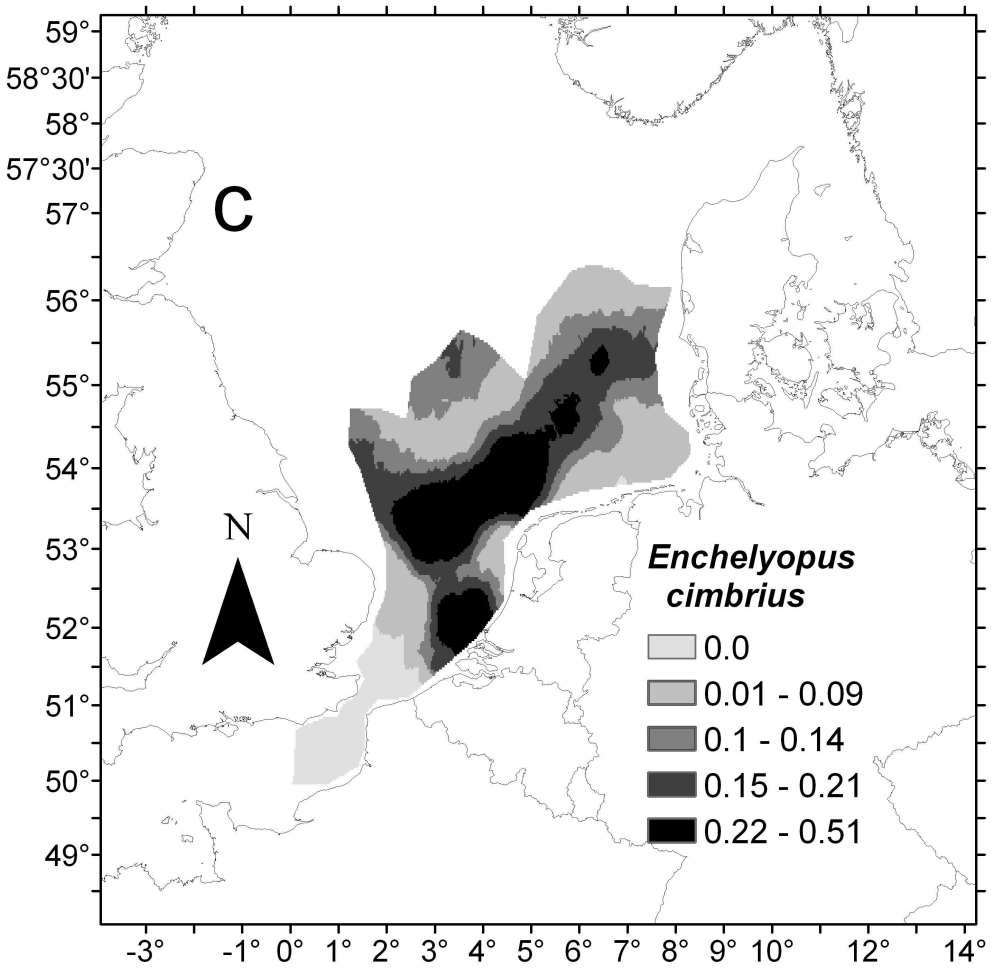

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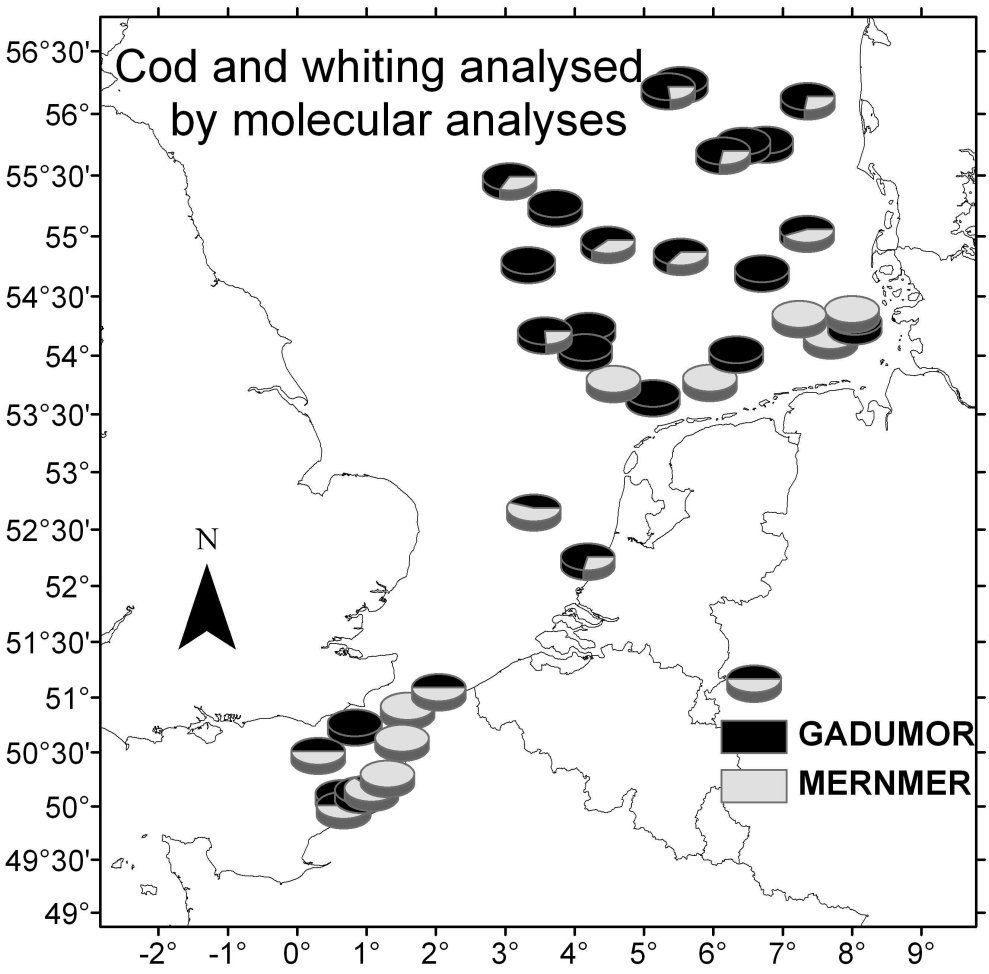

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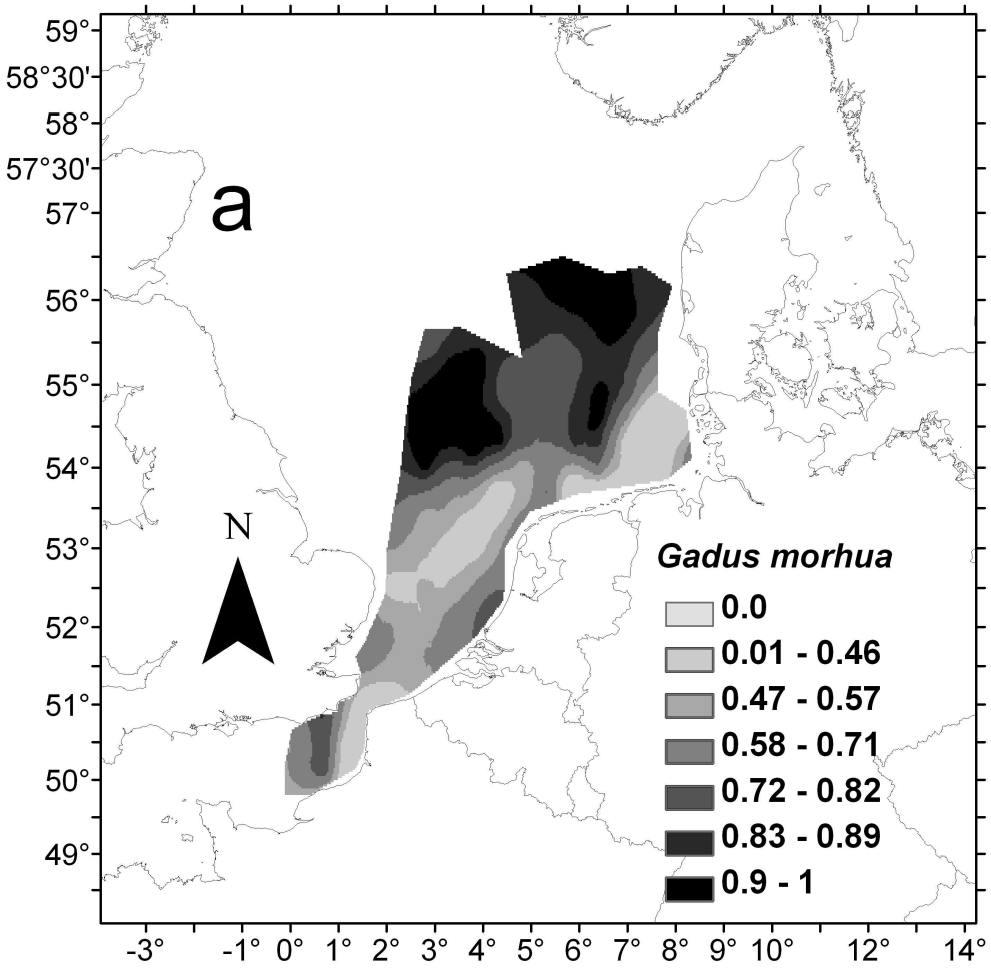

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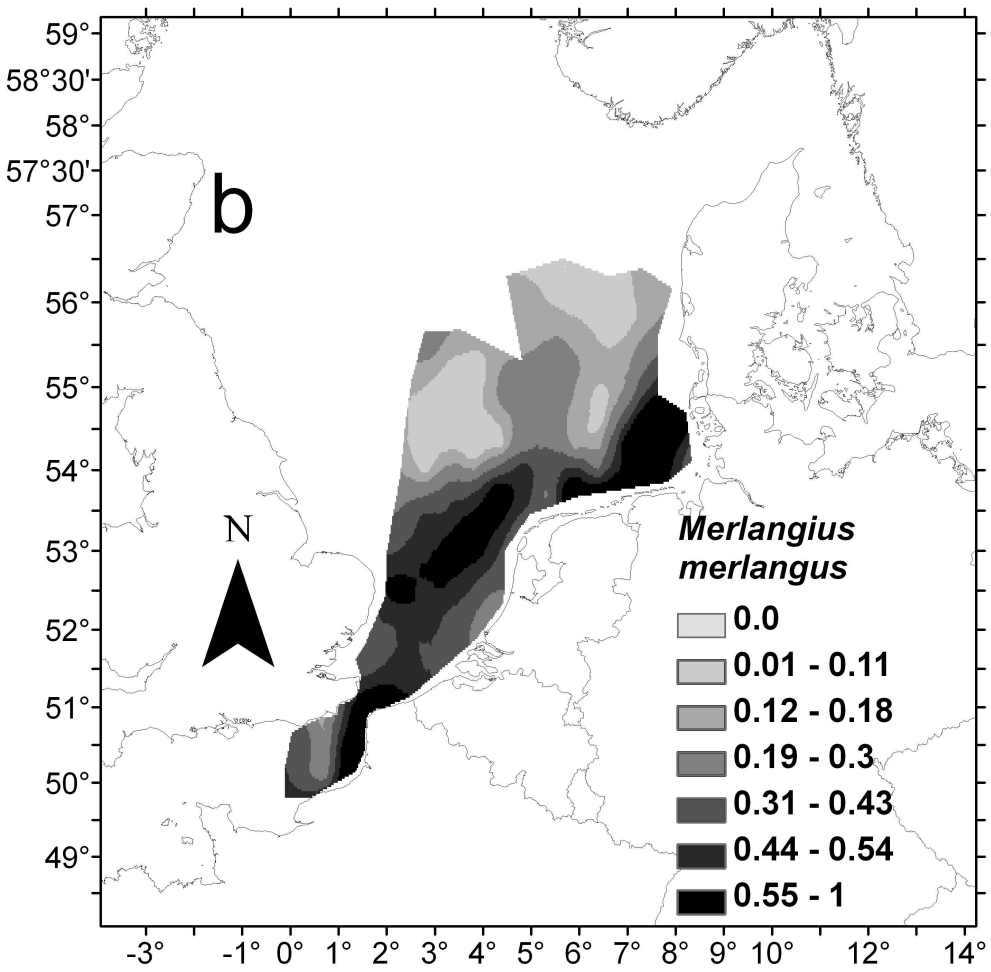

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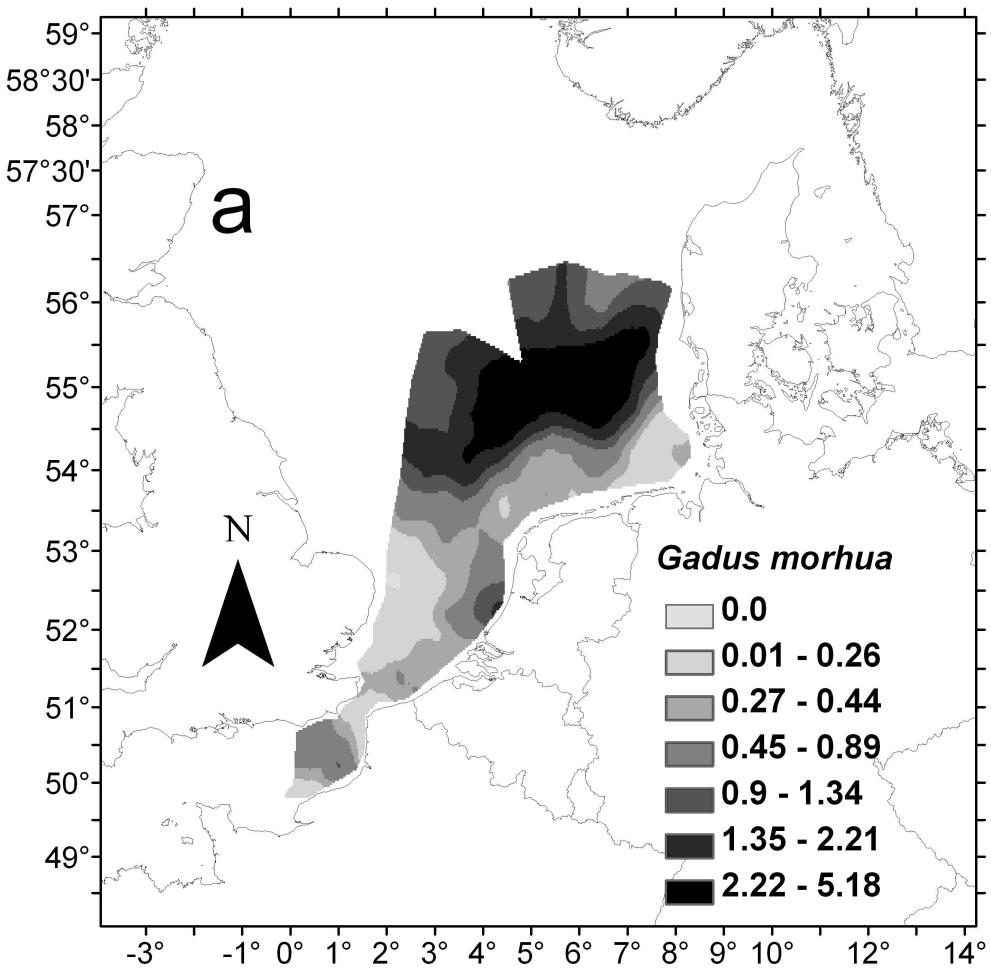

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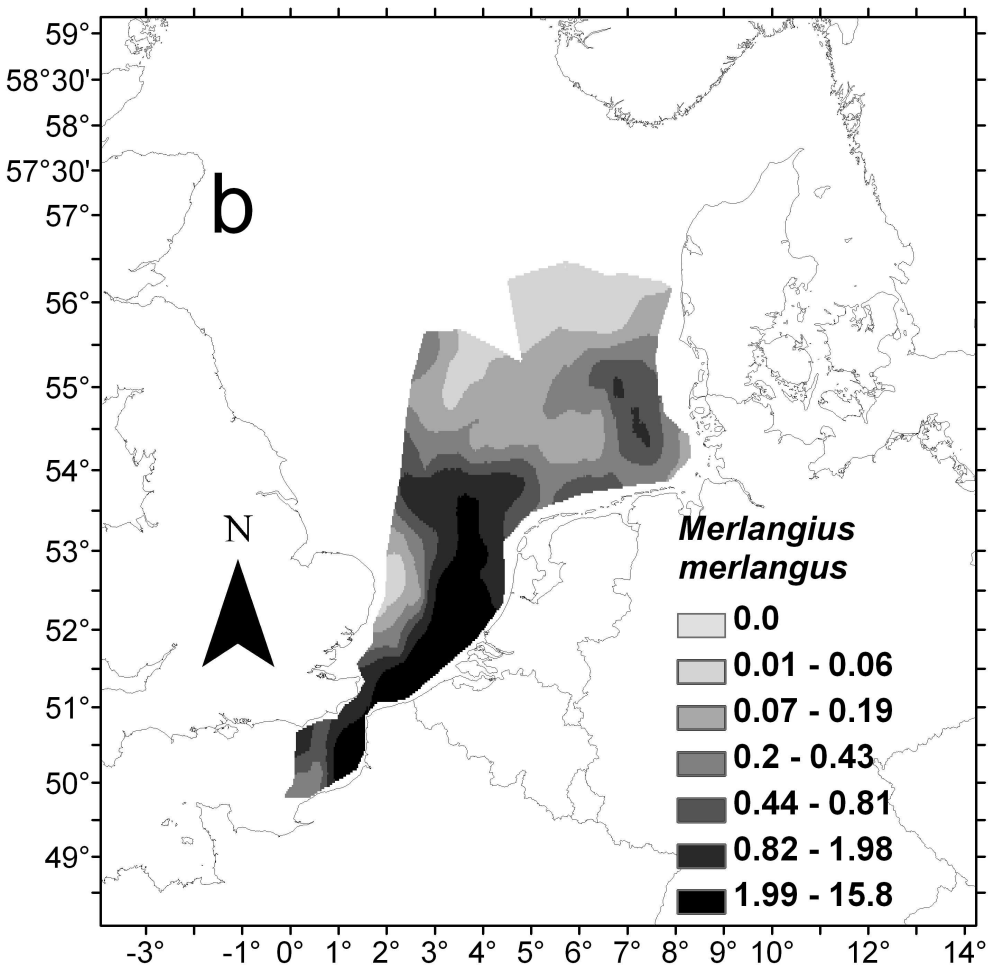

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