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VIABILITY OF THE NORTHEAST ATLANTIC HARBOUR PORPOISE AND SEAL POPULATION (GENETIC AND ECOLOGICAL STUDY)

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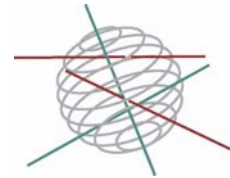
Part 2:
Global change, Ecosystems and Biodiversity

FINAL REPORT

VIABILITY OF THE NORTHEAST ATLANTIC
HARBOUR PORPOISE AND SEAL POPULATION
(GENETIC AND ECOLOGICAL STUDY)

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Abstract

Harbour porpoises (*Phocoena phocoena*) and harbour seals (*Phoca vitulina*) are far more abundant along our coast compared to the beginning of the nineties. Human impact on these species is however hard to establish, mainly due to lack of information on marine mammal population ecology, density, distribution and diversity.

This project aims to gain further knowledge on the viability of the harbour porpoise and harbour seal populations in the North Sea (focusing mainly on its southern Bight) through
- The characterisation of their genetic structure and diversity (through mtDNA and microsatellites in harbour porpoises)
- A better understanding of their feeding ecology (through $\delta^{13}C$ and $\delta^{15}N$ measurements in muscles)
- The assessment of their susceptibility of being trapped accidentally in fishing nets (post-mortem investigations)

Harbour porpoise and harbour seal occupied the top trophic levels but displayed different feeding habits as inferred from their $\delta^{13}C$ and $\delta^{15}N$ mean values. Harbour porpoises displayed lower mean $\delta^{15}N$ values suggesting a lower trophic position likely oriented towards small planktivorous fish such as herring and lesser sandeel. However, both their recent high abundance and their dietary preferences might lead to a higher susceptibility to by-catch as revealed by the significant emergence of net entrapment and net marks revealed by post-mortem investigations. The question rises about the sustainability of these incidental captures. Furthermore, genetic investigations revealed a higher fragmentation of the porpoises collected along the coast of France, Belgium and Netherlands. This apparent fragmentation is of particular importance from a conservation point of view and enhances the fact to protect in priority these last populations.

Our study showed importance of multidisciplinary approaches (post-mortem investigations, stable isotope measurements ($\delta^{13}C$ and $\delta^{15}N$ measurements) and genetic investigations using mtDNA and microsatellites) to apprehend the question of marine mammal survival in our waters.

Key words: Harbour porpoise - harbour seal – North Sea – stable isotopes – Genetic- post mortem investigation – by-catch - fishery

1. Introduction and objectives

Marine mammals of the North Eastern Atlantic are all listed among vulnerable, declined, or threatened species by international directives. Among these species, the harbour porpoise (*Phocoena phocoena*) and the harbour seal (*Phoca vitulina*) are especially concerned due to their coastal distribution, in direct contact with shipping, fishing, and noise pollution and to their overall presence in reasonable number along the European coasts.

The harbour porpoise (figure 1.1.) is a coastal cetacean species, limited to the cold temperate and subarctic waters of the Northern Hemisphere. In the Eastern North Atlantic it ranges from the Kara Sea south to Senegal, Africa, including the North Sea, the Baltic Sea and the Western Mediterranean. There is an isolated population in the Black Sea. In the Western North Atlantic, this species ranges from southern Greenland to North Carolina, USA. There is also a population around Iceland. In the Eastern Pacific, it ranges from Alaska (up to Point Barrow) south to California. In the Western Pacific, the species ranges from the Bering Sea to northern Japan (Klinowksa, 1991; Donovan and Bjorge, 1995; Bjorge and Tolley, 2002). There is no clear migration. Most of the travelling seems to be related to movement of food resources.

Figure 1.1. The harbour porpoise Phocoena phocoena
(picture from: http://ourworld.compuserve.com/homepages/jaap/phocoena.htm)

The harbour seal (figure 1.2.) also known as the common seal occurs over a great latitudinal range and in many different coastal and insular habitats around the rims of both the north Atlantic and the North Pacific regions (Burns, 2002). The North Sea contains around 10 % of the world population (North Sea Task Force, 1993). Counts of common seal numbers (1994-1996) estimate the current North Sea population at 36 000 seals. The harbour seal breeds along the coast of the North Sea. In 1988 and 2002, two outbreaks of Phocine distemper virus ravaged populations of harbour

seals in Europe (Harding et al. 2002). The death rate varied from 15% to 58% among regions (Harding et al. 2002). It seemed that populations have recovered between these two events: since 1989 (and before 2002), numbers have increased to more than 14 000 (Reijnders and Reineking, 1999).

Influences of human activities on harbour seal populations are abundant. There is a competition for resources with commercial fisheries. Consequently also net entanglement of seal occurs. Several areas, especially the Wadden Sea and the Baltic, are severely polluted and negative effects on the seal have been previously demonstrated Tidal flat areas, where harbour seals haul out at low tide, are often visited by tourists, which create disturbances (Reijnders 1986; Reijnders and Reineking 1999; Reinders and Aguilar 2002).

**Figure 1.2. The harbour seal, *Phoca vitulina*
(picture kindly provided by A. Dupont)**

Whereas some aspects such as the xenobiotic pollution are already largely studied, human impact is however hard to establish, mainly due to a blatant lack of information on marine mammal population ecology, density, distribution and diversity.

This project aims to assess the viability of the harbour porpoise and harbour seal populations in the North Sea (focusing mainly on its southern Bay) through

1- The characterisation of their genetic structure and diversity
2- A better understanding of their feeding ecology
3- The assessment of their susceptibility of being trapped accidentally in fishing nets

1.1. The characterisation of their genetic structure and diversity

The aim of this section was to determine the population genetic structure of two marine mammal species: the harbour seal (*Phoca vitulina*) and the harbour porpoise (*Phocoena phocoena*). Indeed, the understanding of the population structure of these species is a crucial point within the context of their long-term conservation. This is particularly true for the porpoise as it presently experiences high rates of incidental

mortality in commercial fisheries and urgent conservation efforts are needed to protect this species. As the population structure of the harbour seal is presently well documented (e.g. Goodman et al. 1998), we mainly focused our researches on the harbour porpoise populations.

1.2. The use of stable isotope in feeding ecology

Assessing the diet and trophic position of a top-level marine predator is important to the understanding of the ecology of marine food webs (Herman et al. 2005). Despite regular and new observations of harbour porpoises and harbour seals (and other marine mammal species) in the Southern North Sea, few data dealing with their diet within this area are available (Prime & Hammond 1995, Santos 1998, Santos et al. 1999; Santos & Pierce 2003).

The diet of the harbour porpoise is varied and differs geographically and seasonally (reviewed recently by Santos and Pierce, 2004). Common prey species include herring, sandeels, hake, lantern fish, capelin as well as cephalopod, anchovy (Sekiguchi, 1995; Palka et al, 1996). Total food intake is between 4 and 9.5% of the total body weight, representing between 8000 and 25000 kJ/day (Kastelein et al. 1997).

Feeding forays of harbour seals can be close to haul-out sites or many miles distant, either along the coast or seaward (Burns 2002). They are capable of feeding at considerable depths (to 500+ m). Prey items are small to medium size fish, such as various members of Gadidae family, hake, mackerel, herring, sardines, smelts, shad, capelin, sandlance, sculpins, a variety of flatfishes, salmonids and many others. The diet of the harbour seals from the south-western North Sea included whiting and sole and to a lesser extent other flatfish and gadoid species as well as sandeels (Hall et al. 1998).

Strandings offer a good opportunity for scientists to collect biological data but in most cases, either stranded animal stomachs are empty or digested material is not suitable for diet research (Santos et al. 1994, Jauniaux et al. 2002). Moreover, strandings might represent potentially biased samples of animals as sick or injured animals may not be feeding normally prior to death (Sekiguchi et al. 1992, Santos et al. 1994, Santos & Pierce 2003).

The use of naturally occurring stable isotopes of carbon and nitrogen has provided complementary data to marine mammal feeding ecology (Hobson & Welch 1992, Abend & Smith 1995, Smith et al. 1996, Hobson et al. 1997, Burns et al. 1998, Hobson & Schell 1998, Das et al. 2000, 2003a,b; 2004a,b). Indeed, the carbon and nitrogen isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in a consumer reflect those of its diet with a slight selective retention of the heavier isotope and excretion of the lighter one (figure 1.3).
The isotopic ratio of nitrogen shows enrichment in the tissue of a predator as compared to its prey. This element thus represents a good indicator of the trophic level of food chain constituents. On the other hand, carbon is weakly enriched from one trophic level to the other and rather gives an indication of the source of primary production of the assimilated organic matter (Gannes et al. 1998; Kelly 2000).The application of stable isotopes at the natural abundance level as indicators of the origin of organic matter and

of trophic interactions is based upon three important hypotheses (reviewed by Bouillon et al., 2002):

1. Differences (may) exist in the $\delta^{13}C$ and/or $\delta^{15}N$ signatures of different primary producers,
2. These differences are maintained or altered in a sufficiently predictable way during degradation processes,
3. Consistent and predictable changes in the isotopic signature occur during transfer to higher trophic levels.
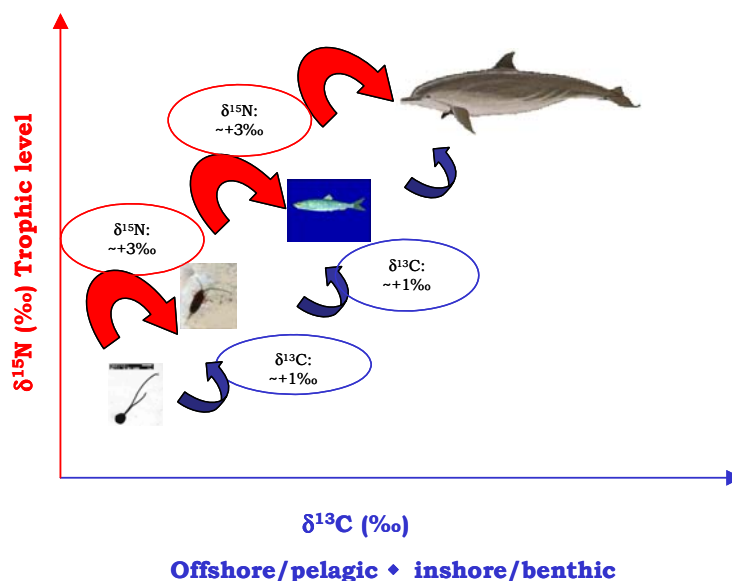
Figure 1.3. Enrichment of $\delta^{13}C$ and $\delta^{15}N$ from one trophic level to the other one (after Das et al. 2002)

Systematic enrichment in ^{13}C values in marine food chains has been reported (Rau et al., 1983; Boutton, 1991). Most studies however, indicated that in marine environment, ^{13}C enrichment occurred at low trophic levels but not among vertebrate consumers (Rau et al., 1983; Wada et al., 1987; Fry, 1988; Hobson and Welch, 1992; Hobson, 1993; Hobson et al., 1993). This minor stepwise trophic enrichment of the carbon isotope ratio that has been documented among vertebrate consumers limits its use in assessing trophic level. However, this characteristic enhances the utility of carbon-isotope ratios for tracking carbon sources through a food chain (Peterson and Fry, 1987; Michener and Schell, 1994). Specifically, because there is little enrichment with increase in trophic level the carbon isotope signature of secondary and tertiary consumers should reflect the source of carbon (C3, C4 or marine plants) at the base of the food chain.

The variations in nitrogen isotopic composition are largely determined by biologic reactions. Catabolic pathways favour the excretion (through urine, for example) of the lighter isotope, resulting in an enrichment in animal tissues in ^{15}N relative to plants. This progressive enrichment increases along advancing trophic levels. (Ehleringer and Rundel, 1989; Minagawa and Wada, 1984 quoted by Lesage et al., 2001; Peterson and Fry, 1987).

An important advantage of this approach is that the isotope ratios from the tissues are derived from assimilated food (and not just ingested food) and therefore reflects dietary input over time. This approach can also be a drawback, however, as it does not allow us to identify exactly which species has been consumed. Previous knowledge of possible prey species is therefore necessary to complement the results obtained through these analyses for trophic level studies.

The use of stable isotopes to study marine mammals is recent, but not altogether new. In the past decade we have witnessed the increase in the use of this technique for studies concerning migrations, trophic ecology, pollution and even paleontology.

Hobson and Welch (1992) characterized the trophic relationships of the food web in the Canadian arctic region of Lancaster Sound using stepwise increase in ^{15}N (and to a lesser extent ^{13}C) to determine the trophic level of the animal concerned (zooplankton to marine mammals). Lesage et al. (2001) published a study on marine mammals and the community structure of the Estuary and Gulf of St Lawrence in Canada, also based on stable isotope analysis. They showed that marine mammals occupy the highest trophic levels in the food web (using ^{15}N), although overlapping one another, and that an enrichment in ^{13}C occurs in animals from the Estuary relative to those from the Gulf.

Atwell et al. (1998) used δ^{15}N to characterise the trophic relationships in an arctic food web (ranging through particulate organic matter to seals and polar bears) in order to determine that food web's relationship with total Hg concentrations in the same tissues, with the objective of studying Hg bioaccumulation along the food chain and with age.

Schell and Haubenstock (1989) measured ^{13}C values in keratin in bowhead whale baleen from the western arctic environment (obtained from hunting records and museums, records extending to 1947), which can be found in growth layers. These data are representative of secondary production in the Bering Sea ecosystem and show that the ecosystem has undergone a significant decrease in average primary productivity.

In recent decades, the use of stable isotope analysis for ecological studies has been steadily increasing:

- tracer studies to determine migration patterns (reviewed by Hobson and Wasseneaer 1999) as 'animals that move between isotopically different food webs can retain information of previous feeding locations for periods that depend on the elemental turnover rate for the tissue of interest';

- trophic ecology (Hobson and Welch, 1992; Hobson, 1999; Das et al. 2000; Kelly, 2000; Lesage et al., 2001; Polischuk et al., 2001) to understand the relationships between organisms;

- pollution studies (e.g. Atwell et al., 1997; Thompson et al., 1998a,b; Bearhop et al., 2000; Das et al. 2004a,b) especially pollutant transfer through the ecosystem.

Although this dramatic increase in the application of stable isotope analyses to ecological research, this field is still in its infancy and continuous data are needed before getting a better understanding of the fundamental principles governing the

behaviour of stable isotopes in ecosystems and their potential use in solving ecological questions (Hobson and Wassenaar, 1999).

1.3. The assessment of their susceptibility of being trapped accidentally in fishing nets

The incidental capture of marine animals in commercial fishing gear occurs worldwide (Harwood 1983). The extent of this problem is widely recognised in cetaceans (e.g. Perrin 1988) and culminated in the International Whaling Commission Symposium and Workshop on the Mortality of Cetaceans its Passive Fishing Nets and Traps, La Jolla, California, October 1990 (IWC 1990). Pinnipeds are also taken in commercial fishing operations, yet little information on such incidental catches is available (Harwood 1983, Lien et al. 1988; Woodley and Lavigne 1991).

In the past, the harbour porpoise has been hunted throughout its range for food and oil. There has been a major fishery for this species in the Lille Bælt in Denmark from the 1830s until the Second World War, in which several hundred to more than thousand animals were taken annually. There has also been a drive fishery in the Black Sea. Before the Second World War, this fishery took 100,000-300,000 animals per year, which declined to 5,000-7,000 per year in the mid-1960s. This fishery targeted harbour porpoises, common dolphins and bottlenose dolphins. The composition of the catch is unknown. The Turkish fishery, which was suspended in 1983, took 34,000-44,000 animals per year between 1976 and 1981. Harbour porpoises made up about 80% of the total catch (Klinowksa, 1991)

Currently, the main threat for the harbour porpoise is incidental capture in fishing nets (commonly named by-catch). The International Whaling Commission (1996) reports the following by-catch estimates for the North Atlantic:

Table 1.1. : Harbour porpoise annual by-catches by Region (IWC, 1996)

North Sea: 4,000-5,000

Other:

Gulf of Maine - Bay of Fundy: 1,000-4,000
Greenland: > 700
Kattegat, Skagerrak and Bælt areas: > 250
Baltic Sea: ~ 10
Ireland and Western UK: 2,000

The IWC concluded that the current by-catch in the North Sea may not be sustainable. In the area where most of the by-catch occurs, 3.1% of the population is killed this way annually. Recently, the Agreement on the Conservation of Small Cetaceans of the Baltic and North Seas (ASCOBANS) was set up to provide an international platform for the conservation of among others the harbour porpoise. A number of countries already signed and ratified this agreement.

2. Material and Methods

2.1. Genetic structure

As Cetaceans could have wide territories and can move a lot during their life, it was quite difficult to develop a genetic study on the harbour porpoise only in the southern Bay of North Sea without taking into account their possible relationships with populations living in more northern and southern regions. Therefore, we preferred to enlarge our study to populations of the North-East Atlantic, starting from Spain in the South to Norway, in the North. This would help to determine more precisely the origin of populations living in the southern Bay of North Sea as well as their contacts with other populations. This is particularly important to estimate the level of genetic variability within and among these populations. Moreover, to estimate the level of "genetic health" of North-East Atlantic porpoises, we compared the obtained results with an "outgroup" corresponding to animals living in another European region, the Black Sea.

- Mitochondrial DNA

Samples of by-caught or stranded harbour porpoises from the countries bordering the southern North Sea (France (n=49), Belgium (n=19), Netherlands (n=28)) and adjacent waters not yet investigated (North Sea (n=47), Portugal (n=14) have been analyzed in collaboration of Dr Kristal Tolley (University of Stellenbosh, South Africa). Total genomic DNA was extracted using DNAeasy extraction kit (Quiagen®) according to manufacturer's protocols.
The phylogeographic history has been investigated by analyzing sequences (365 base pairs) of the mitochondrial DNA (mtDNA) control-region using different statistical methods. Haplotype diversity (δ) and nucleotide diversity (π) were estimated in Arlequin 2.0 (Schneider et al., 2000). An analysis of molecular variance (AMOVA) was conducted to estimate the degree of differentiation among the six sampling areas using two measures of genetic differentiation (F_{ST} and Φ_{ST}) in Arlequin 2.0. Φ_{ST} was estimated using a Tamura-Nei model of evolution with a gamma correction (α= 0.17) as estimated by maximum likelihood in PAUP*4.0b10 (Swofford, 2002). A spatial analysis of molecular variance (SAMOVA) was used to identify the possible geographic groupings of sampling areas that form genetically homogeneous populations that are maximally different from each other (Duplanoup et al., 2002).

- Nuclear DNA (microsatellites)

To precise the fine-scale structure and dynamics of the harbour porpoise populations or sub-groups, a total of 772 harbour porpoise coming from different regions of the North-East Atlantic (comprising 191 animals of the southern Bay of the North Sea) as well as from the Black Sea (See table 2.1 and figure 2.1) were analysed using 12 nuclear microsatellite loci previously defined (Rosel et al. 1999, Andersen et al. 2001). Amplified products were analyzed using an automatic sequencer MegaBACE 1000 (Amersham Biosciences®).

Table 2.1. Global sampling analysed for the microsatellite study

countries	n	Sources
Turkey	19	Turkish Marine Research Fundation
Bulgaria	10	BREMA
Ukraine	42	BREMA
Géorgia	9	BREMA
Portugal	13	Instituto da Conservação da Natureza
Spain	23	CEMMA
France	78	CRMM & MARIN
Belgium	**58**	**MARIN**
Netherlands	34	Drs. Addink & Smeenk
Germany	47	FTZ
Denmark	5	FTZ & Dr. Andersen
Ireland	46	Dr. Rogan
Scotland	19	
Feroes islands	9	Museum of Natural History
Norway	166	Institute of Marine Research
Iceland	194	Marine Research Institute
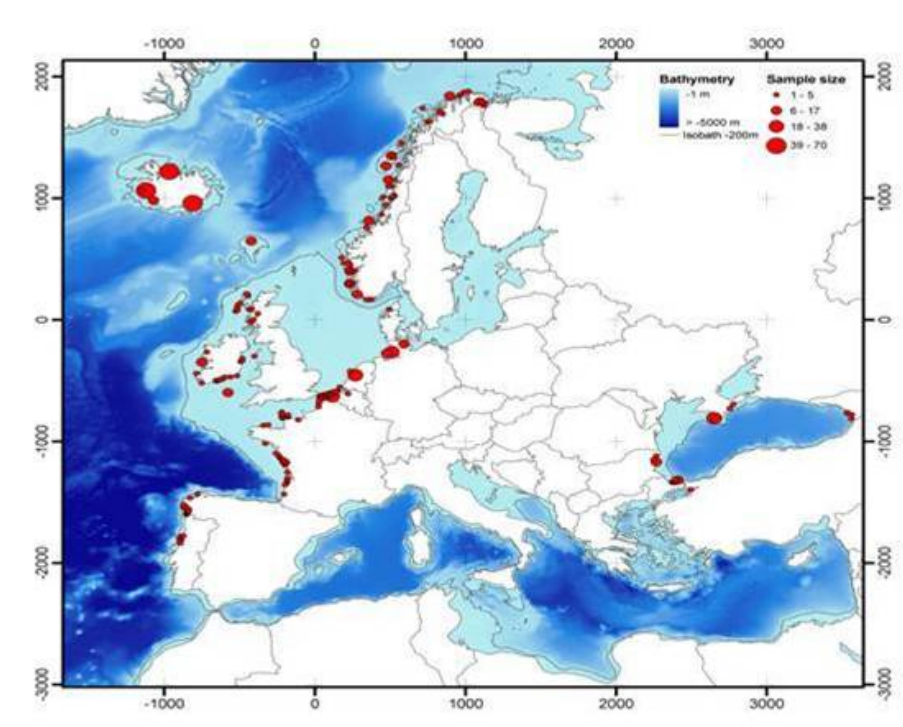
Figure 2.1. Approximate geographic locations of the samples, sample sizes per location, and bathymetric features. Geographic locations are based on GPS coordinates or reported discovery location. The map is projected using a gnomonic projection centred on the sampling centroïd (scale units in kilometres).

Species such as small cetaceans, difficult to observe and distributed over a continuous habitat, presents special analytical challenges because there is generally little *a priori* information on what could be a "population"(Waples and Gaggiotti, 2006). Until recently, most genetic studies conducted on cetaceans and especially on

harbour porpoises (Andersen, 2003) have applied classic genetic approaches which require *a priori* definition of population boundaries generally unknown for cetaceans. To overcome these difficulties, we used recently developed individual-based genetic approaches that do not require such information on population units and limits (Manel et al., 2003; Manel et al., 2005; Rousset, 2000). We first applied a Bayesian model-based clustering algorithms to infer population structure and to assign individuals (probabilistically) to populations based on their multilocus genotypes: *Geneland 1.05 (Guillot et al., 2005a; Guillot et al., 2005b).* This clustering approach therefore assumes that populations are discrete genetic units with distinct allele frequencies. However, Isolation By Distance (IBD) may be superimposed on this structure. We tested this hypothesis using the individual-based approach developed by Rousset (Rousset, 1997; Rousset, 2000), which involves regression of an index of genetic differentiation (a_r) between pairs of individuals, analogous to $F_{st}/(1-F_{st})$ between pairs of populations, on marine geographic distance

2.2. Stable isotope analysis

After drying at 50°C (48 h), muscle samples were ground into an homogeneous powder and treated with a 2:1 chloroform:methanol solution to remove lipids. Carbon dioxide and nitrogen gas were analysed on a V.G. Optima (Micromass) isotope ratio-mass spectrometer (IR-MS) coupled to an N-C-S elemental analyser (Carlo Erba). Routine measurements are precise to 0.3‰ for both 13-carbon and 15-nitrogen. Stable isotope ratios were expressed in delta notation according to:

$$\delta X = [(R_{sample}/R_{standard})- 1] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio ($^{13}C/^{12}C$ or $^{15}N/^{14}N$).

Carbon and nitrogen ratios are expressed relative to the V-PDB (Vienna Peedee Belemnite) standard and to atmospheric nitrogen respectively. Reference materials were IAEA CH-6 (sucrose) ($\delta^{13}C = -10.4 \pm 0.2$ ‰) and IAEA-N1 ($\delta^{15}N = +0.4 \pm 0.2$ ‰) respectively.

2.3. The susceptibility to incidental capture

In 2004 and 2005, 182 marine mammals were collected along the Belgian and northern France coast, from the Belgian border to Dunkerque. All the carcasses were forwarded to the Department of Veterinary Pathology of the University of Liege. For porpoises, three age categories based on body length were recognized: neonatal (≤ 90 cm), immature (91-130 cm) and mature (>130 cm) (Lockyer, 1995).

In total, for the two years, 155 cetaceans (including 147 porpoises) and 27 seals (including 20 harbor seals) were subjected to necropsy and sampled by a standard procedure (Jauniaux et al., 2001; 2002a,b). Briefly, they were measured and weighted, and the blubber thickness was measured dorsally. After external examination, the abdominal and thoracic cavities were opened and the skull was sown longitudinally to expose the brain.

Table 2.2. Marine mammals stranded in 2004 and 2005

Species	2004	2005	Total
Porpoise *Phocoena phocoena*	54	93	**147**
White-beaked dolphin *Lagenorhynchus albirostris*	3	1	**4**
Striped dolphin *Stenella coeruleoalba*	1	/	**1**
Fin whale *Balaenoptera physalus*	1	/	**1**
Minke whale *B. acutorostrata*	1	/	**1**
Sperm whale *Physeter macrocephalus*	1	/	**1**
Harbor seal *Phoca vitulina*	12	8	**20**
Grey seal *Halichoerus grypus*	3	3	**6**
Hooded *seal Cystophora cristata*	1	/	**1**

For histopathology, samples of eye, skin, liver, lymph nodes, gonad, reproductive tract, oesophagus, stomach, intestine, kidney, urinary bladder, pancreas, lung, heart, thyroid, thymus and brain, and all tissues with lesions were fixed in 10% buffered formalin and embedded in paraffin wax by routine procedures. Sections (5 µm) were cut and stained with haematoxylin and eosin (HE). Selected sections were also stained with Masson trichrome, periodic acid Shiff (PAS) for fungi and Ziehl-Neelsen stain for acid-fast organisms. In addition, immunohistochemical examination with a monoclonal antibody against the glycosylated haemagglutinin protein of phocine distemper virus (clone 1.3) (Trudgett et al, 1991; Kennedy et al, 1998) was performed on sections of lungs, pulmonary lymph nodes and all lesions suspected to have a viral origin. The other immunohistochemical reagents used were those included in the Enhanced Polymer One-Step Staining Procedure (Dako Envision™, Dako, Glostrup, Denmark) (Jauniaux et al., 2000 and 2001).

For bacteriology, tissue samples were collected aseptically and incubated under aerobic and anaerobic conditions on Columbia blood agar containing sheep blood 5 % (Becton Dickinson Europe, Meylan Cedex, France) and on a selective medium for Enterobacteriaceae (Gassner agar, Oxoid, Gent, Belgium).

Parasites were collected and preserved in 70 % ethanol containing glycerin 5 %.

3. Results

3.1. Genetic investigations

<u>- Mitochondrial DNA</u>

The results obtained using the mitochondrial control region evidenced that this marker more reflects the ancient demographic history (Quaternary glaciations period) of the porpoise than its contemporaneous one. This is mainly the result of the slower genetic evolutionary rate characterising the mtDNA control region for the Cetaceans. Indeed, the studied populations are weakly differentiated and characterised by a single majority haplotype present in all the regions as well as by several rare closely related haplotypes (star like topology). However, southern populations (Iberian Peninsula) appear more differentiated as compared to the northern ones. This phylogeographic pattern seems to be the results of population's contractions during the last glacial maximum in waters south of the English Channel which were followed by a northward post-glacial population expansion after the ice retreat, 10 000 years ago. This scenario also explains in part the lack of population structure within the southern North Sea. This study was developed in close collaboration with Dr K. Tolley who kindly accepted to share her mtDNA porpoise sequences with ours. This led to an oral presentation in the international congress of La Rochelle (Fontaine *et al.* 2005)

<u>- Nuclear DNA (microsatellites)</u>

Geneland identified three spatially coherent clusters: the first gathers all porpoises from the Black Sea and Marmara Sea genetically isolated from those in the Atlantic by the Mediterranean (Figure 3.1a); the second gathers the porpoises from the Iberian peninsula genetically isolated from samples further north by a barrier to gene flow located in the southern Bay of Biscay (Figure 3.1b); and the third is unequivocally composed of the samples further north in Atlantic widely distributed from the French coast of the Bay of Biscay to the Arctic waters of Iceland and Norway (Figure 3.1c). The genetic barriers identified coincide with deep/strong oceanographic changes both characterised by steep variation in the bathymetry and in temperature and thus of food quantity and availability.

We found a significant isolation by distance among porpoises north of the Bay of Biscay barrier to arctic waters. Samples collected in the south bay of the North Sea are thus incorporated in a genetic continuum that widely extends from the French coast of the Bay of Biscay to the Arctic waters of Iceland and Norway. While this suggest there are few, if any, potential barriers to porpoise's dispersal from the northern Bay of Biscay up to Arctic waters, the heterozygosity deficit related to the detected isolation by distance shows nevertheless that porpoises do not mate randomly over that extended area and that gene flow is spatially restricted.
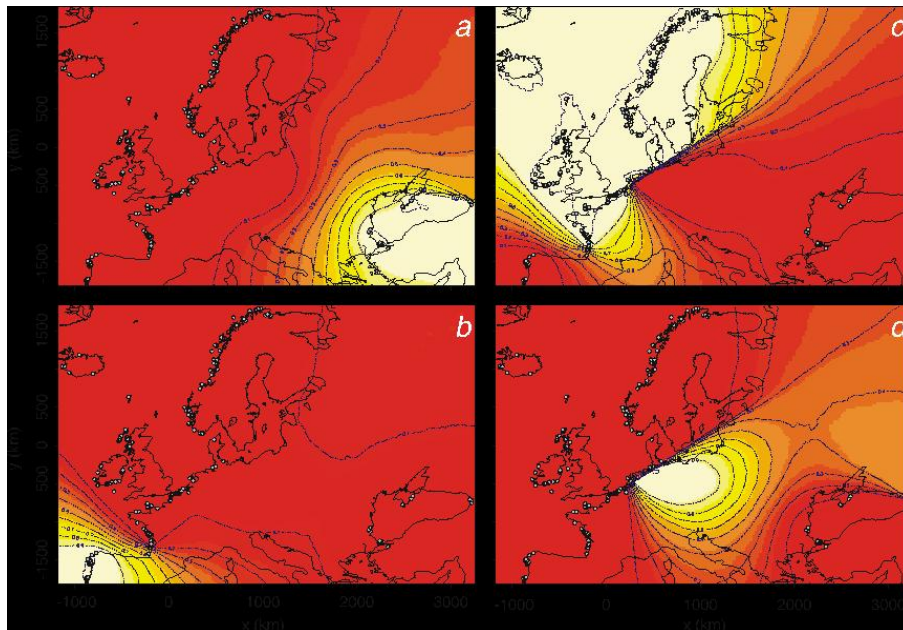
Figure 3.1. Geneland individual assignments to population

Map of the posterior probability of belonging to each cluster inferred by *Geneland* analysis for *K*=4. The plot is based on the highest-probability run (of ten) at that value of *K.*

3.2. Stable isotope investigations

$\delta^{13}C$ and $\delta^{15}N$ analyses were performed in muscles of 38 harbour porpoises (*Phocoena phocoena*), 10 harbour seals (*Phoca vitulina*), 1 grey seal (*Halichoerus grypus*) and 3 hooded seals (*Cystophora cristata*) and results are presented in table 3.1 and table table 3.2).

Stable isotope ratios acquired on marine mammals collected between 1994 and 2004 were integrated in this study (Das et al. 2003a; Das et al. 2004b) to allow a time comparison over these last decade.

Table 3.1. δ^{13}C and δ^{15}N (‰) muscle values in seal species stranded along the Belgian coasts (2003-2005)

A03/1321	*Phoca vitulina*	-15.5	16.0
A03/504	*Phoca vitulina*	-15.6	17
A03/521	*Phoca vitulina*	-14.7	18.7
A03/0084	*Phoca vitulina*	-14.2	18.7
A03/0089	*Phoca vitulina*	-15.8	18.3
A03/0069	*Phoca vitulina*	-16.6	18.5
A03/488	*Phoca vitulina*	-16.1	18.8
A03/0051	*Phoca vitulina*	-14.5	18.8
A04/484	*Phoca vitulina*	-15.4	20.5
A04/335	*Phoca vitulina*	-14.8	18.7
Mean	***N = 11***	**-15.7 ± 1**	**18.1 ± 1.3**
A03/1064	*Halichoerus grypus*	-15.6	17.5
A04/088	*Cystophora cristata*	-18.1	12.6
A04/242	*Cystophora cristata*	-19.5	14.2
A04/144	*Cystophora cristata*	-18.3	12.8

Harbour seals and the grey seals collected these last two years displayed high δ^{15}N values compared to harbour porpoises and the hooded seals. The three hooded seals are also ^{13}C-depleted compared to the harbour porpoise, the harbour seal and the grey seal.

Table 3.2. $\delta^{13}C$ and $\delta^{15}N$ (‰) muscle values in harbour porpoise stranded along the Belgian coasts (2003-2005)

ID	Species	$\delta^{13}C$	$\delta^{15}N$
A03/1180	*Phocoena phocoena*	-17.7	16.1
A03/1238	*Phocoena phocoena*	-17.0	12.3
A03/1353	*Phocoena phocoena*	-16.8	14.0
A03/1380	*Phocoena phocoena*	-17.5	15.4
A03/1381	*Phocoena phocoena*	-17.8	14.9
A03/1517	*Phocoena phocoena*	-16.5	14.7
A03/1521	*Phocoena phocoena*	-17.5	14.6
A03/1562	*Phocoena phocoena*	-16.4	14.7
A03/1571	*Phocoena phocoena*	-17.3	14.5
A04/1384	*Phocoena phocoena*	-16.9	18.5
A04/1383	*Phocoena phocoena*	-16.9	16.7
A04/1335	*Phocoena phocoena*	-17.4	16.8
A04/1317	*Phocoena phocoena*	-17.0	17.0
A04/1336	*Phocoena phocoena*	-16.8	16.9
A04/1020	*Phocoena phocoena*	-16.1	16.1
A04/1001	*Phocoena phocoena*	-16.2	15.4
A04/987	*Phocoena phocoena*	-17.1	15.6
A04/1318	*Phocoena phocoena*	-17.3	16.9
A04/1187	*Phocoena phocoena*	-16.8	14.9
A04/1313	*Phocoena phocoena*	-17.2	17.0
A04/914	*Phocoena phocoena*	-16.2	17.2
A04/XX	*Phocoena phocoena*	-16.4	16.6
A04/1649	*Phocoena phocoena*	-17.0	15.8
A04/1709	*Phocoena phocoena*	-16.7	16.0
A04/1644	*Phocoena phocoena*	-16.8	17.2
A04/1432	*Phocoena phocoena*	-17.3	17.8
A04/017	*Phocoena phocoena*	-16.4	14.8
A04/018	*Phocoena phocoena*	-16.2	11.9
A04/083	*Phocoena phocoena*	-16.7	14.6
A04/1021	*Phocoena phocoena*	-17.1	14.3
A04/1312	*Phocoena phocoena*	-17.4	14.6
A04/1648	*Phocoena phocoena*	-16.4	15.7
A05/519	*Phocoena phocoena*	-16.7	15.6
A05/886	*Phocoena phocoena*	-16.2	15.7
A05/885	*Phocoena phocoena*	-17.2	14.7
A05/520	*Phocoena phocoena*	-15.9	14.8
A05/955	*Phocoena phocoena*	-17.2	15.0
A05/884	*Phocoena phocoena*	-16.5	15.4
Mean :	N= 38	**-16.8 ± 0.5**	**15.5 ± 1.4**

3.3. Susceptibility to fishing nets

3.3.1. Harbour porpoise strandings

Most of porpoises stranded on Belgian coast (n= 157) comparatively with animals stranded on northern France coastline (n= 25) and most of stranding occurred during winter period (figure 3.2).
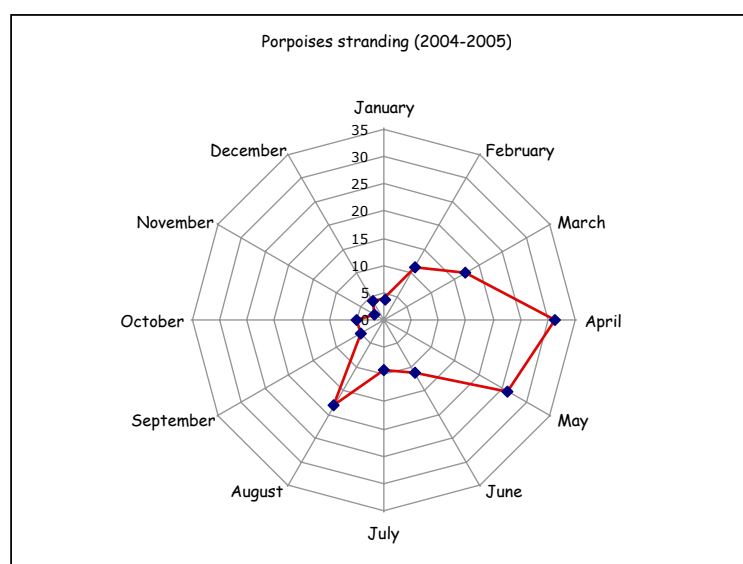

Figure 3.2. Repartition of the stranding of porpoises in 2004 and 2005, month by month

108 porpoises were enough fresh (conservation code 1 to 3) to be necropsied and a cause of death determined.

Net entrapment

Amongst observed lesions, those related to net entrapment were significantly emerging, reaching 40% comparatively with the mean of 20% for previous years (figure 3.3.). In addition, that cause of death was mainly diagnosed from February to May with 12/17 and 16/24 by-caught porpoises in March and April respectively (Figure 2).

There was 21 females and 22 males, most juveniles (n= 41) with a mean body length of 109,6 cm (min. : 92 cm ; max : 130 cm) ; mean weight of 21,4 kg (min. : 11 kg ; max : 36 kg), with a mean blubber thickness of 16,6 mm (min. : 5 mm ; max : 40 mm). Only 2 adults were by-caught: a lactating female of 75 kg, 170 cm and a blubber thickness of 13 mm and male of 30 kg, 137 cm and a blubber thickness of 8 mm
Most of those animals had lesions related to net entrapment: superficial skin cuts on mouth or fins; incision into body cavity; amputated fin, fluke or tail; hypohema (presence of blood in the eye); subcutaneous hematomas; lung edema and congestion without evidence of inflammatory or infectious process. Nevertheless some animals had lesions before net entrapment: emaciation; parasitosis and mild pneumonia. In 17 cases, stomachs were empty.
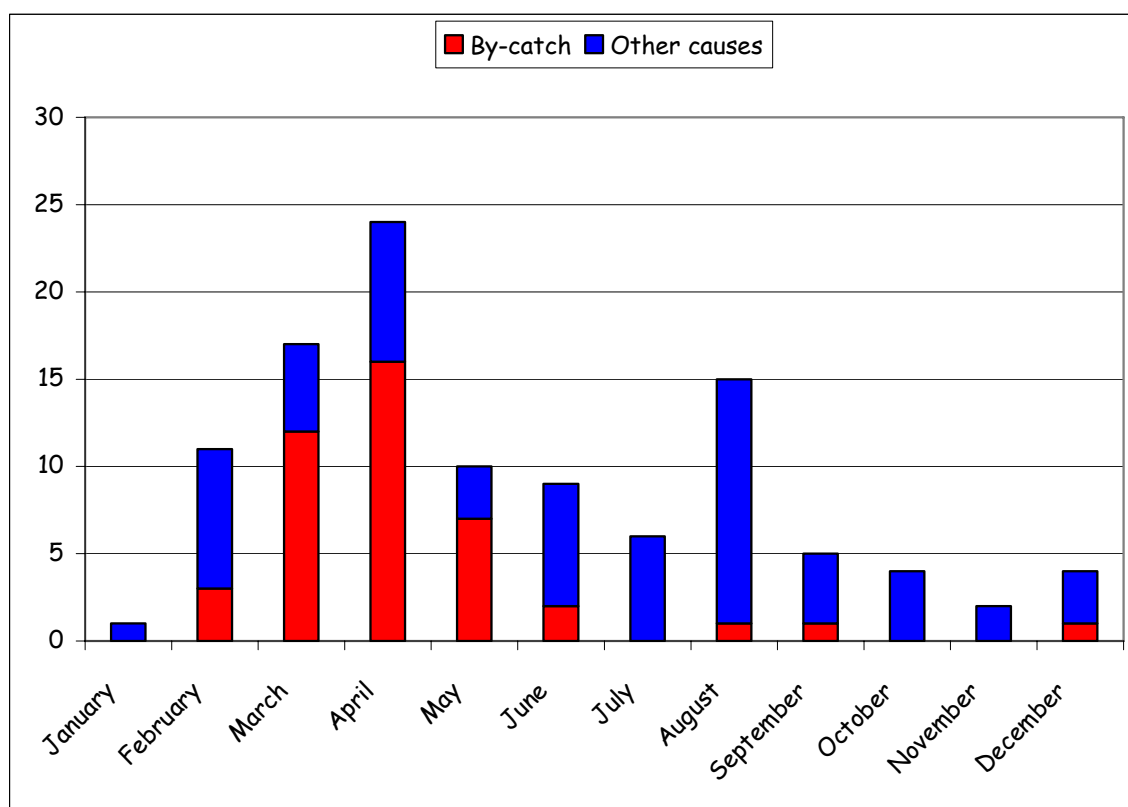
Figure 3.3. Comparison between net entrapment and other cause of death of harbour porpoise, month by month in 2004 and 2005

Other causes of death

In 65 cases, most frequent post-mortem lesions were mild to severe emaciation (n= 56), severe and multisystemic parasitosis (n= 32) and acute exsudative pneumonia (n= 20). Frequently the 3 lesions were present simultaneously.
They were 12 adults with a mean body length of 148,5 cm (min. : 134 cm ; max : 160 cm) ; with a mean body weight of 39,74 kg (min. : 31 kg ; max : 56 kg) and a mean blubber thickness of 14,9 mm (min. : 5 mm ; max : 26 mm). In addition, 51 juveniles stranded with a mean body length of 111,5 cm (min. : 86 cm ; max : 130 cm) ; a mean body weight of 18,05 kg (min. : 11 kg ; max : 39,8 kg) and a mean blubber thickness of 10,5 mm (min. : 1 mm ; max : 20 mm). Finally, 5 new-born or very young porpoises stranded in 2004 and 2005 with a maximum size of 88 cm and maximum body weight of 7,5 kg. The blubber thickness was less or equal to 8 mm. The stomachs were empty in 41 cases

Severe emaciation was characterized by weight loss, reduced blubber layer thickness and dorsal muscle atrophy. Severe parasitosis consisted of heavy infestations of multiple organs, with associated lesions of bronchopneumonia, chronic ulcerative gastritis and chronic hepatitis. Bronchopneumonia was associated with nematode infestations of airways and pulmonary blood vessels (*P. inflexus*), including pulmonary arteries and the right ventricle of the heart. Chronic ulcerative

gastritis was frequently associated with nematode (*Anisakis simplex*) infestation. Usually several hundred or more nematodes were present. Chronic nodules (1 to 2 cm in diameter) in the wall of the second stomach had a thick, white, fibrous capsule and a core containing trematodes (*Pholeter gastrophilus*). Extensive chronic portal hepatitis associated with fibrosis of bile ducts was observed in some cases of severe liver trematode (*Campula oblonga*) infestation. Flukes were observed in these damaged bile ducts and, in some cases, in the ductus choledochus. Frequently, spherical nodules (up to 3 cm in diameter) with a thick, fibrous, partly calcified wall, and dark green to black caseous contents were distributed throughout the liver. Adults and larvae of *Stenurus minor* were frequently observed in the middle ear. Bronchopneumonia was frequently associated with parasitosis or emaciation, or both. These are chronic, debilitating processes which might predispose to fatal bronchopneumonia, lung parasitism, leading to secondary bacterial infection and bronchopneumonia.

3.3.2 Seal strandings

Net entrapment

On the 27 pinnipeds necropsied in 2004, 7 harbor seals and 4 gray seals were considered as being dead following trauma (net entrapment or collision). Three harbor seals were ringed (BE073 and BE085). The first one was released in the Western Scheld on January 30, 2004 and found dead in Oostend on May 21; the second one was released on March 17, 2004 on the beach of Heist and was found dead on March 23. A gray seal was also ringed (BE078).

   All by-caught seals stranded between February and May, no case being reported, for harbor seals, after May.

   All the by-caught animals had similar lesion: hypohema, lung congestion and edema with in some cases evidence of emphysema, froth in airways. No other lesions were observed and most of them were in good nutritional status with a mean blubber thickness of 29.,1 mm (min.: 20; max.: 35). In 3 cases (including BE073), abundant fresh preys were found in the stomach, in 3 other cases, gastric cavity was empty. Finally, one seal (BE085), had few otolithes and fishbones within stomach.

Other causes of death

In 6 cases, animals were severely emaciated with among those, 3 seals with evidence of acute exsudative pneumonia but, most of harbor seals were in bad conservation status, hampering a clear identification of lesions and cause of death.

4. Discussion

4.1. Genetic investigations

In the marine realm, community structure is shaped heavily by physical processes (Li, 2002; Longhurst, 1998). In this study we report for the first time, with the example of the harbour porpoises, that physical forcing and especially the temperature has major impacts on the demographic and genetic structure of a cetacean. The small body size of harbour porpoises undoubtedly has profound consequences at all levels of their biology and we can reasonably expect that this will be also applicable to other cetaceans of similar body size, habitat and thermoregulation constraints. The ancient isolation of the Black Sea population and the recent isolation of the Iberian population indicate that fragmentation of harbour porpoise range is under way and that it is likely to continue with the predicted changes in climate. Our findings have important conservation implications as harbour porpoises are furthermore strongly impacted by commercial fisheries (Stenson, 2003). The population structure we have highlighted here should help in assessing the impact of this human induced mortality on local densities and in designing appropriate management strategies. This study will lead to a publication presently in the final stage of preparation:
- Rise of Oceanographic Barriers in Continuous Populations of a Cetacean: The Harbour Porpoise in Old World Waters. (Fontaine et al. to be submitted in PloS Biology).

Concerning more precisely the populations of the South Bay of the North Sea, our results evidenced that they does not appear to be differentiated to the other populations living in southern or northern regions and that they belong to a huge population that widely extends from the French coast of the Bay of Biscay to the Arctic waters of Iceland and Norway. Therefore, they should not be considered as distinct populations. However, isolation by distance analyses showed that porpoises do not mate randomly over that extended area. This would be explained by the fact that animals from a same region will have more chances to reproduce together than with others living in more distant areas. Moreover, more precise analyses evidenced that this isolation by distance is more important in the French and Belgian coasts until North Netherlands, whereas more northern populations appears less marked by such phenomenon. As isolation by distance is directly associated to population's densities, this observation would be a signal of higher fragmentation of populations from France, Belgium and Netherlands. This information is particularly important on the conservation point of view and enhances the fact to protect in priority these last populations.

As the study of "neutral" markers (mtDNA and microsatellites) appeared much more complex and highly interesting as previously expected, these last two years were mainly focused to develop such way of research. Therefore, we had less time to perform the study based on "selected" markers associated to the immune system, such as the Major Histocompatibility Complex (MHC) genes. However, the first tests on the three MHC exons (the sub-unit alpha, the DRB1and the DQB) selected for this study are presently finalised and this study will start next month on an extensive sampling.

4.2. Stable isotope ratios of harbour porpoises and seals

Previous studies on marine mammals from the southern North Sea documented large variations in their feeding ecology inferred from stable isotope ratios (Das et al. 2003a,b; Das et al. 2004b). The results acquired on marine mammals collected during this research program confirmed these first data.

Within the southern North Sea, harbour seal and grey seal seem to occupy a similar trophic position at the top of the food web as suggested by their high δ^{15}N values in muscles (figure 3.2).

A third seal species was analysed for stable isotopes : the hooded seal *Cystophora cristata*. Its presence in the southern part of the North Sea is quite unusual. Indeed, the hooded seal is a large northern phocid with a range that encompasses a large sector of the North Atlantic (Greenland, Iceland, New Foundland…). The hooded seal follow an annual movement cycle that keeps them in close association with drifting pack ice (Kovacs, 2002). However record of hooded seals being found outside their normal range is not so uncommon; young animals in particular are great wanderers. Juveniles have been found as far as Portugal and Florida in the Atlantic Ocean and in California on the Pacific side (Kovacs, 2002). The low δ^{13}C values measured in the muscles of these three hooded seals can be related to a more oceanic feeding regime (figure 3.2). Stable carbon isotope ratios have proven most useful in identifying where particular organisms feed as δ^{13}C values are typically higher in species from coastal or benthic food webs than in offshore food webs (Lesage et al. 2001; Das et al. 2003b).

In Norwegian waters, the squid *Gonatus fabricii* constituted 79% of the prey biomass found in hooded seal stomach (Potelov et al. 1997). 1000 000 t of *G. fabricii* is believed to be consumed by the hooded seal in the area off east Greenland (Bjorke et al. 2001). The hooded seal may also feed on a variety of deep-waters fishes, including Greenland halibut (*Reinhardtius hippoglossoides*) and a variety of redfishes fishes (*Sebastes sp*) and several squid species (Kovacs, 2002). Herring (*Clupea harengus*), capelin (*Mallotus villosus*), and various gadoid fishes, including Atlantic cod (*Gadus morhua*), have also been found in hooded seal stomach (Kovacs, 2002). From our isotopic data, it appears that despite recent strandings of hooded seals along the Southern North Sea coasts, they do not feed mainly within this area.
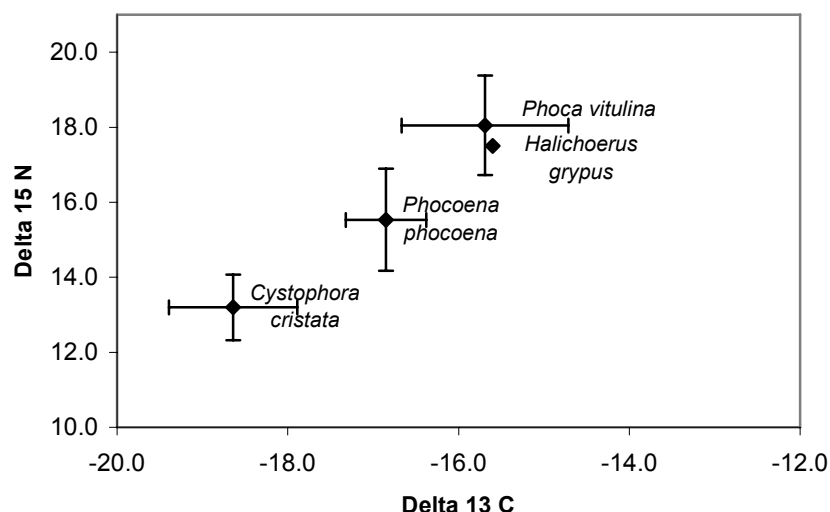
Figure 4.1. Muscle $\delta^{15}N$ and $\delta^{13}C$ (in ‰) in harbour porpoise and seals collected along Belgian North Sea coasts between 2003 and 2005.

Estimated trophic levels calculated by Pauly et al. (1998), based on stomach content data, indicate that trophic levels of harbour porpoise, harbour seal and grey seal are similar ranging between 4.0 and 4.2. From our isotopic data, it appears that harbour porpoise occupy a lower trophic position than harbour seal and grey seal. This discrepancy reflects a high proportion of prey displaying a low trophic level such as zooplanktivorous fish in the diet of harbour porpoise from the Southern North Sea (Das et al. 2003b). Furthermore, porpoises are opportunist feeders taking advantage of local abundance of prey (Santos et al. 2004; Santos and Pierce; 2003). This opportunistic strategy might also explain the large interannual variations observed between 1994 and 2004 (figure 4.2 and figure 4.3). Harbour porpoises collected in 1998 displayed high $\delta^{15}N$ values compared to porpoises collected these last two or three years. Harbour porpoise from Scottish waters displayed also interannual, seasonal and regional variation in its diet (Santos et al. 2004). The most important prey types, in terms of contribution by number and mass, were whiting (*Merlangius merlangus*) and sandeels (Ammodytidae). Sandeel was most often taken during the summer months (Santos et al. 2004). The authors quoted the fact that importance of clupeids such as the herring *Clupea harengus* may have decreased since the 1960's, mirroring the decline in North Sea herring abundance.

The North Sea herring population was seriously affected by over-fishing in the 1970s (Santos et al. 2004). The closure of the North Sea fishery between 1978 and 1982 allowed stocks to recover. From the late 1980s there was another decline in stocks of North Sea herring. This recovered again from the mid-1990s and in 2004 the stock was at the highest level recorded for 40 years, but declined slightly in 2005. Both Norwegian spring-spawning herring and North Sea herring recovered from complete collapses in the 1960s and 1970s and now with improved management they continue to support productive fisheries. This success was partly put down to several years of good recruitment of young fish to the stocks, possibly aided by favourable environmental conditions. Another reason was that the by-catch of herring in other

fisheries was restricted so that when the fisheries targeting herring were closed, the pressure on the stock stopped immediately (Fletcher 2005). Herring is, to a large extent, fished in mixed fisheries, i.e., with small-meshed trawls targeting sprat with by-catches of herring and with herring trawls yielding by-catches of sprat. A fraction of the landed herring is taken with trapnets / pound nets and gillnets during spawning time (Quéro and Vayne 1997).

However, the recovery of the North Sea herring stock in recent years is no yet reflected in Scottish porpoise diet (Santos et al. 2004). Concerning Belgian harbour porpoises, their $\delta^{15}N$ value is consistent with a diet oriented towards zooplanktivorous fish such as sandeel and herring (Das et al. 2003b). Both these two fish species displayed low $\delta^{15}N$ (mean values: 15.6 $^0/_{00}$ for lesser sandeels and around 13 $^0/_{00}$ for herrings; Das et al. 2003b). Fatty acid analyses in harbour porpoise and harbour seal blubber (and potential prey) should give further information on herring contribution to their diet, especially for individuals collected these last few years.
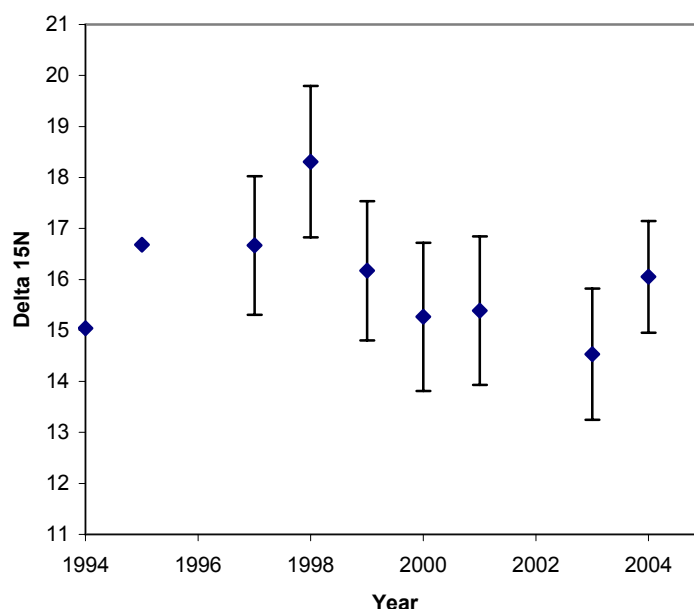

Figure 4.2. $\delta^{15}N$ annual value (mean ± standard deviation) in the muscles of harbour porpoises collected between 1994 and 2005
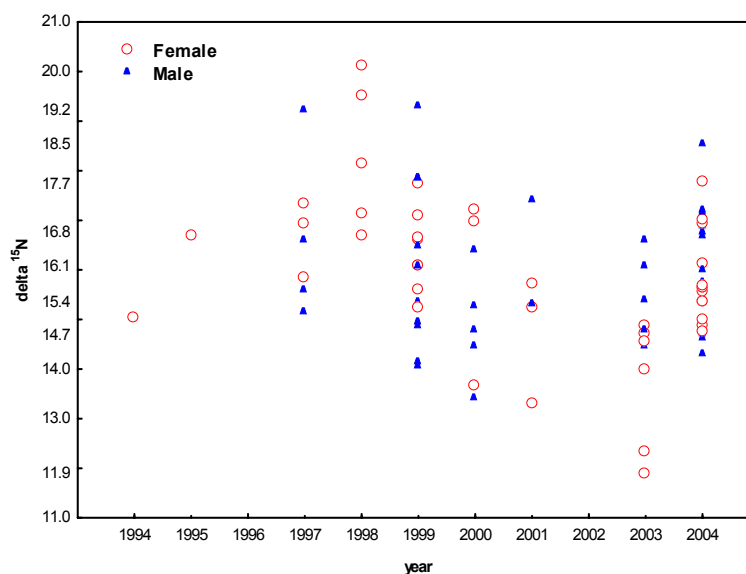
Figure 4.3. $\delta^{15}N$ annual values (individual values) in the muscles of harbour porpoises collected between 1994 and 2005

4.3. By-catch susceptibility of harbour porpoise and harbour seal

There are many threats to small cetaceans in European waters including pollution, noise disturbance and capture in fishing nets. Incidental captures, or by-catch, in fishing nets is of particular concern and thousands of small cetaceans are taken annually in European waters. Agreement on the Conservation of Small Cetaceans in the Baltic and North Seas (ASCOBANS) advised that the maximum annual by-catch for harbour porpoises should not exceed 1.7% of the population size. The International Whaling Commission (IWC) has stated that if the number of small cetaceans captured should not be greater than 1%. Therefore, estimates of abundance are essential to assessment and management of by-catch. Estimates of animal abundance of the harbour porpoise encountered during SCANS in 1994 gave very low densities in the southern part of the North Sea (around zero in the Channel, Belgian coasts, Hammond et al. 2002).

However, preliminary data extracted from the new project SCAN II revealed increase of abundance within this area (Macleod et al. 2006) in good agreement with the recent number of stranding observed these last years along the Belgian coast (figure 4.4.). Between 1990 and 1996, three to six stranded porpoises were reported every year. From 1997 to 2004, this figure rose to more than 60 a year (Jauniaux et al. 2002; Haelters and Kerckhof 2004). No abundance data are yet available for the harbour seal, but evidences from stranding suggest also some increase along our coastline.
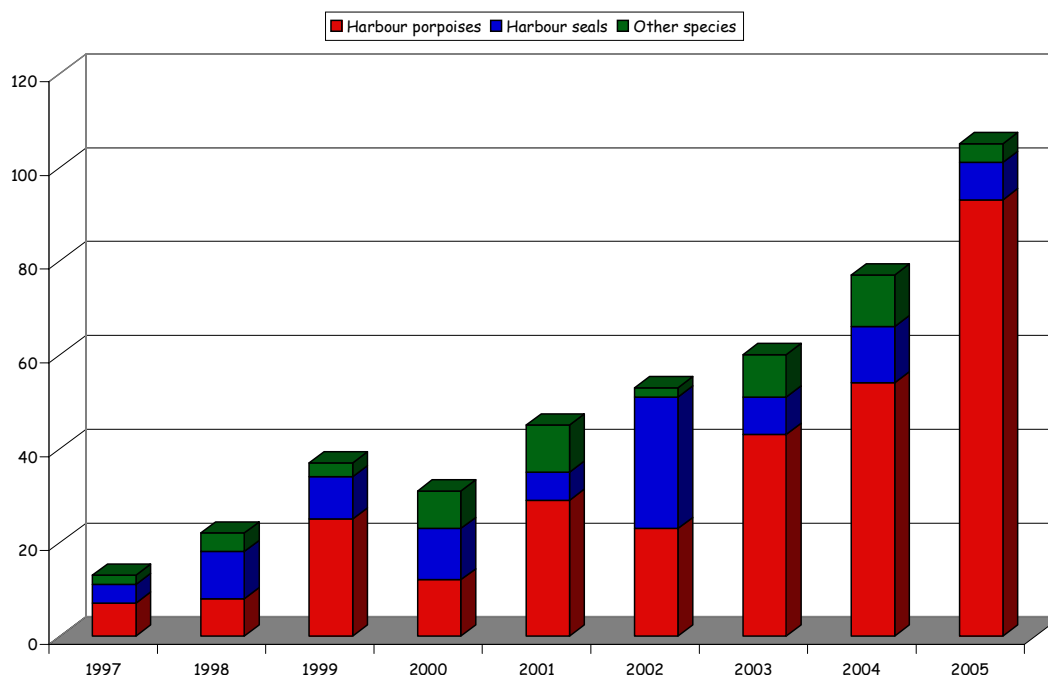
Figure 4.4. Annual repartition of marine mammals stranded on the Belgian and northern France coastline between 1997 and 2005

Unfortunately, along with this recent increase of abundance, fishing marks are also more frequently observed during necropsies suggesting some impact of local fishery. Net entrapment appeared as emerging threat, responsible for the death of 40% of the harbour porpoises and 35% of harbour seals collected on our coastline.

Previously net entrapment was considered as being the cause of death of 20% on stranded porpoises (Jauniaux et al. 2000) and 29% for stranded seals (Jauniaux et al. unpublished data). The average percentage of stranded porpoises showing signs of net entanglement was 34% on the British coasts from 1990 to 1996 (Jepson *et al.,* 2000), 50% on the German coasts from 1991 to 1993 (Benke *et al.,* 1998), 10 - 20 % for the Dutch coast from 1990 to 1994 (Addink and Smeenk, 1999), and 24% for the French part of the Channel from 1970 to 1994 (A. Collet, unpublished data).

We have considered that the low level of net entrapment for Dutch, Belgian and northern France coastlines comparatively with other countries bordering the North Sea, where by-catch is considered to be the main threat for cetaceans, may be due to different fishery practices (Jauniaux *et al.,* 2002). Indeed, the use of gill nets (figure 4.5.) was relatively infrequent in Belgium and the Netherlands (Reijnders *et al.,* 1996).
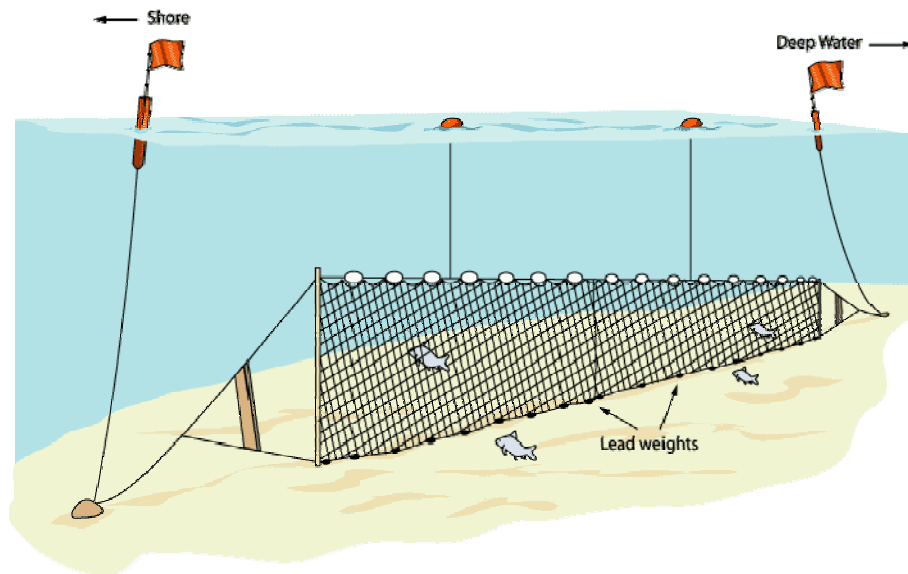
Figure 4.5. Gill net (picture from http://www.miseagrant.umich.edu/nets/largegill.html)

Beyond the territorial limits, however, Dutch and Belgian waters are intensively used by gill net fishermen from other European nations, especially France, Denmark and the United Kingdom. The emergence of by-caught could be related to modification of fishing practice in conjunction with relative abundance of marine mammals along the continental coastline of the southern North Sea. Indeed, for porpoises and seals, there is a clear temporal preference, all being stranded from February to May and most of the observations of living cetaceans were done during the first months of the year (for further information:http://www.mumm.ac.be/FR/Management/Nature/strandings.php)

By the necropsy, it is not possible to determine what type of net or fishery is responsible. But, most of the animals were very fresh and may have died shortly before their stranding. Beach net or fishery activity at proximity of the shore could be considered as responsible. To confirm such affirmation, it was reported that a porpoise was directly caught in beach net (Jan Haelters, pers. comm.).
Stranding can not give information of the total number of by-caught porpoises. Indeed, bodies are drifting following wind direction and sea current and frequently, fishermen are opening largely body cavities to promote sinking of the animal.

5. Conclusions and recommandations

Harbour porpoises and harbour seals are indubitably part of the southern North Sea ecosystem. Harbour porpoises and harbour seals are far more abundant along our coast compared to the beginning of the nineties. They occupy the top trophic levels but displayed different feeding habits as inferred from their δ^{13}C and δ^{15}N mean values. Harbour porpoises displayed lower mean δ^{15}N values suggesting a lower trophic position likely oriented towards small planktivorous fish such as herrings and lesser sandeels. Increase harbour porpoise abundance might be linked also to the recent come back of the herring in the southern part of the North Sea. However, both their high abundance and their dietary preferences might lead to a higher susceptibility to by-catch as revealed by the significant emergence of net entrapment. The question rises about the sustainability of these incidental captures. Furthermore, genetic investigations revealed a higher fragmentation of the porpoises collected along the coasts of France, Belgium and Netherlands. This apparent fragmentation is of particular importance from a conservation point of view and enhances the fact to protect in priority these last populations. These findings have important conservation implications as harbour porpoises are strongly impacted by commercial fishery. The population structure we have highlighted here should help in assessing the impact of this human induced mortality on local densities and in designing appropriate management strategies.

Our study showed importance of multidisciplinary approaches (post-mortem investigations, stable isotope measurements and genetic investigations using mtDNA and microsatellites) to apprehend the question of marine mammal survival in our waters.

In this framework, following recommendations and perspectives have been drawn:

1. Both genetic investigations and increase evidences of by-catch suggest that arrangements are necessary to enhance protection and conservation of cetaceans and pinnipeds within Belgian waters.
2. Genetic investigations should be continued using "selected" markers associated to the immune system, such as the Major Histocompatibility Complex (MHC) genes. Preliminary results dealing with three MHC exons (the sub-unit alpha, the DRB1and the DQB) are presently finalized.
3. Harbour porpoise by-catch suggests also new interactions with fishery in this southern part of the North Sea. Further knowledge of their diet is necessary to better apprehend the risks linked to their feeding habits. Fatty acid analyses in the blubber (and prey) would be an additional tool often used for diet reconstruction. Combining the two methods (stable isotope ratios and fatty acid analyses) will increases the information gained.
4. Finally, the causes of death of stranded marine mammals should be investigated continuously using a multidisciplinary approach to highlight main mortality processes.

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