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INTERNATIONAL WHALING COMMISSION

UNUSUAL MASS MORTALITY EVENT OF RORQUAL WHALES IN THE GULF OF PENAS (PATAGONIA, CHILE) DURING 2015

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ABSTRACT

In May 2015, a group of scientists from “Huinay Scientific Field Station” studying marine invertebrates, made a report regarding an unusual mortality event of unidentified whales (in excess of 20 individuals) in Slight Bay, located south of Gulf of Penas, in southern Chile. In response to the above, the Chilean Navy made a ship available, in order to make a first official expedition to the area, along with a multidisciplinary group of professionals and scientists from Universidad Austral de Chile (UACH), Universidad de Chile (UCHILE), Instituto Antártico Chileno (INACH), Centro de Estudios del Cuaternario (CEQUA), WWF Chile, Centro Ballena Azul (CBA), Universidad Santo Tomas (UST), Brigada de Delitos Ambientales (BIDEMA), Huinay Scientific Field Station (HSFS) and TV media, coordinated and led by the Fisheries and Aquaculture Service of Chile (SERNAPESCA). The aim of the expedition, which took place in April 2015, was to determine the proximate causes of death and stranding of this group of whales in the Gulf of Penas. Later on, during mid June 2015, HSFS made a private overflight of the area and verified through aerial photographs the existence of more than 300 specimens of dead whales stranded along an unsurveyed adjacent bay in the Gulf of Penas, fact that was reported to Sernapesca in November of 2015. Further on, in January 2016, the Chilean Navy made available an airplane to make an official overflight to the area to check what HSFS had reported. It was only in February of 2016 when the Chilean Navy made available another ship to revisit the area as part of a second official expedition together with a small group of professionals and scientists from Universidad de Chile (UCHILE), Huinay Scientific Field Station (HSFS), Consejo de Monumentos Nacionales (CMN), Brigada de Delitos Ambientales (BIDEMA), again organized and led by SERNAPESCA. Within the framework of a formal investigation associated with this unusual mass mortality event of whales in the Gulf of Penas, this document provides the technical and scientific background, together with the basis of possible causes of death of these animals.

KEY WORDS: MYSTICETES; SEI WHALES, SOUTH AMERICA, BALAENOPTERA BOREALIS

INTRODUCTION

Nearly 50% of all existing species of marine mammals in the world live in or visit the coasts of Chile. One of the emblematic species that visit the waters of the Chilean coast includes the sei whale (*Balaenoptera borealis*). This species is distributed throughout the world's oceans (Rice 1998), however there is little knowledge regarding their populations and their migratory patterns. As for other large baleen whales, data obtained during the whaling era suggest a seasonal latitudinal migration (summer feeding in sub-Antarctic waters and reproduction during winter in tropical waters). This migratory pattern in conjunction with other ecological, biological and geographical characteristics results in a temporary separation of the different groups or sub-populations of sei whales in both hemispheres. However genetic studies have not clearly defined separation of the populations from both hemispheres (Kanda et al. 2006). For management purposes, populations have been arbitrarily separated over the basis of different biological characteristics and migration for other baleen whales. In the southern hemisphere six populations (stocks) are assumed (Donovan 1991), two or three populations in the North Pacific, while in the North Atlantic, *ca*. eight populations have been suggested (but only three are considered for management purposes).

Sei whale population size in the Southern Hemisphere was estimated at *ca.* 191,000 individuals for the 1940s, before commercial catches began. Subsequently, after the ending commercial catches in 1984, the Scientific Committee of the International Whaling Commission calculated a remaining population of 37,000 individuals (Gambel, 1985). Between 1929 and 1983, catches of this species within the Chilean EEZ accounted for 17.3 % of total Mysticeti captured after the fin (*Balaenoptera physalus*) and blue (*Balaenoptera musculus*) (Aguayo-Lobo 1974).

Large sei whales aggregations in southern Chile have been known for a long time, particularly in waters adjacent to the Gulf of Penas (44°- 48° S). Aguayo-Lobo (op. cit.), recorded around 244 individuals in March 1966, later in October that same year an additional 399 individuals and in December, a further 142 animals. Two years later, Japanese whalers working in Chile, explored marine waters between 40° and 48°S, recording many sightings of this species and reporting that the highest concentration of them were found in waters adjacent to the Taitao Peninsula in the northern limit of the Gulf of Penas, where hundreds of individuals and a great abundance of plankton had been recorded (Pastene and Shimada 1999). These authors concluded that sei whales tend to aggregate during summer months in the Gulf of Penas to feed. Subsequently, in May 2001, Aguayo et. al. (2006) suggests that this species penetrates the bays and archipelago’s channels for protection from storms or as potential areas of permanent or occasional feeding during migration. Recent records of researchers from the Universidad Austral de Chile and Centro Ballena Azul, report a growing number of sightings of sei whales in the waters corresponding to Los Lagos and Aysen regions of Chile (43°- 49°S), and a similar situation occurs in the southernmost region of Chile (Magallanes), where sightings have also been increasing (CEQUA Foundation). After 32 years of ceasing sei whale commercial catches off Chile, the species could slowly be showing signs of recovery in the eastern South Pacific. However, recent findings regarding mass mortalities of the species in fjords and channels off Patagonia, Chile, are concerning on these regards. Here we report on those unusual mortality events witnessed in southern Chile during 2015 and provide detailed information regarding expeditions and analyses performed and underway.

Generally speaking, strandings are recognized as important events where important scientific information can be collected. These events have permitted the description of new species, provided information on growth rates, reproduction, feeding habits, population structure and mortality factors, such as toxins, pollutants and infections. Recently, information gained from dead and stranded animals has increased our understanding of the impacts of sonar and underwater detonations.

Likewise, over long periods of time, the scientific study of strandings has allowed increasing our knowledge on species diversity and its dynamics.

Marine mammals, as predatory species of mid to high trophic levels, are sentinels and indicator species of ecosystem health. Thus, to determine the causes of death and stranding of marine mammals is of essential importance because it assists to better understand the biology of the animals concerned. Also, if deaths have been caused directly or indirectly by actions and / or human activities, the information gathered can be useful to generate and develop action and contingency plans to prevent and mitigate the impacts of such activities.

MATERIALS AND METHODS

As part of the organization of both official expeditions, prior to shipment Sernapesca requested the participating scientists to submit a summary of their skills and capabilities for research analysis so that a multidisciplinary group was formed to approach the event.

FIRST EXPEDITION:

During the cruise performed for the first expedition (April 2015) two formal meetings were carried out to establish the objectives and goals of the expedition, to analyze the available information, allocate roles and responsibilities and assimilate the protocol data collection (photos, GPS location , biological sampling), storage and subsequent distribution of samples.

Once in the targeted area of investigation, the biological sampling followed a hierarchical pattern so as to evaluate the event as organized as possible, appointing the most suitable people for each function.

Thus, the expedition took place in six stages:

1. Preliminary exploration of the area;
2. Development of a first-level sampling to individuals in better condition;
3. Development of a coastal circuit (by zodiac) for sampling the remaining individuals;
4. Water and potential vectors for biotoxins sampling;
5. Search for other debris or corpses during the return journey;
6. Analysis of samples from vectors and stomach contents for detection of Domoic Acid (amnesic poison) and Saxitoxin (paralytic poison).

1. Preliminary exploration of the area

We defined performing a preliminary study of the area in a first official approach to the place of stranding, previously defined as Slight Bay and Caleta Buena, where we expected to find most of the stranded and dead specimens based on available information. Photographic, planimetric and georeferencing records were made as well as estimation of the number of individuals eligible for sampling and analysis.

2. First level sampling

It was possible to detect 39 cetacean corpses, 29 of which corresponded to a recent mortality event while the remaining 10 specimens corresponded only to bone structures, which probably were from a past event.

3. **Coastal Circuit**

Previously conformed working teams performed assigned tasks before different scenarios expected:

- Necropsy and sampling,
- Only sampling if some kind of tissue is left
- Study of samples from phytoplankton and filtering vectors

4. **Sampling water (phytoplankton), vectors (bivalves) and fluids (gastrointestinal)**

Analysis of water samples and vectors:

- Water samples, vectors (46 specimens of clams), sediment (from inside and out of the bay), stomach and fecal contents from some specimens were obtained to determine the presence of amnesic and/or paralyzing toxins, after discarding human causes, predators and geographical reasons among others (Figure 1).

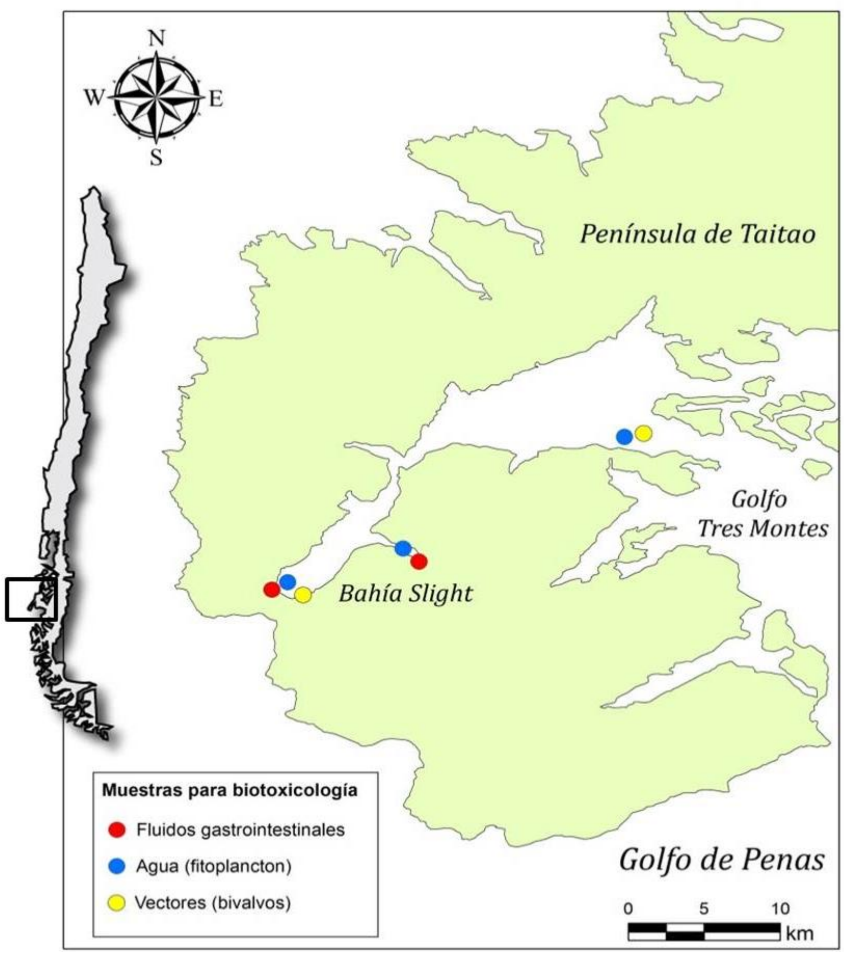

Figure 1. Study area located in southern Chile indicating sampling locations where gastrointestinal fluids, water and vectors were obtained.

5. **Search of other debris or corpses during the return journey**

- On the return journey there were seven specimens of a recent event of death in the Gulf of Tres Montes area of which only photographic records were obtained due to meteorological constraints.

Post cruise

Back at the port of departure in Puerto Montt, the samples were transferred to the Universidad Austral de Chile, in the city of Valdivia for storage in a suitable freezer. The samples were issued by Sernapesca under formal receipt by standard protocol.

6. ***Ex situ* analysis of water, vector samples and stomach contents for detection of Domoic Acid and Saxitoxin** using rapid tests based on antibodies produced by Scotia Rapid Testing Ltd.

- Water samples and gastro-intestinal contents were transferred to Santo Tomas University in Santiago and analyzed by microscopy
- Water samples for determining the presence of toxic phytoplankton by microscopy were preserved in a saturated solution of iodine.

Other *ex situ* analysis

- One periotic bone was transferred to the University of Chile in Santiago, for performing CT volumetric tomography to analyze the internal anatomy of the ear for possible hearing damage (bone microfracture in the inner ear).
- For genetics it was used Ross et al 2003 methods, DNA Surveillance: Web-based molecular identification of whales, dolphins and porpoises and Basic Local ALIGMENT Search tool (BLAST**.**
- Determination of orientations of the carcasses in the field using Rose Diagram

RESULTS

The death of 369 individuals in the span of several months in the Gulf of Penas is a phenomenon of great concern. Fortunately, in four of the studied specimens through necropsy during the first expeditions to the Gulf of Penas and in one specimen from the second expedition ((Hucke-Gaete *et al.* 2015; Ulloa *et al*. 2016), no signs of human interaction were determined in the death of these animals.

In May 2015, one of us (Häussermann), from the Huinay Scientific Center in Chile made a report about a mass mortality of baleen whales in the area of Slight Bay and Gulf of Penas during the development of research on marine invertebrates in the area. After this report, Sernapesca (National Fisheries Service of Chile) joined a lawsuit before the Regional Prosecutor of Aysen (D.A. office) to whoever was responsible for this mass mortality of whales. Then the regional prosecutor instructed the Chilean Navy to make a ship available in order to undertake an official expedition to the area, along with a multidisciplinary group of professionals and scientists from Universidad Austral de Chile, Universidad de Chile, Chilean Antarctic Institute, Huinay Scientific Center, WWF Chile, Blue Whale Center, Center for Quaternary Studies and the Environmental Crimes Brigade, under the coordination and leadership of Sernapesca. The aim of the expedition was to investigate the causes of death of these whales.

In the framework of the formal investigation associated with the mortality of whales in the Gulf of Penas in the southern region of Aysen, Chile, the following report provides the technical and scientific background together with the basis of possible causes of death of these animals in the area interest.

Number of dead animals found and sampled

Counting the number of skulls was used (considering that the carcasses kept skulls articulated) for determining the minimum number of individuals (NMI). 39 rests of whales belonging to at least two different mortality events (carcasses and skeletal rests) were observed (fig 2). The recently dead whales, with an approximate date of 2-3 months had a state of decay that exposed internal tissues such as fat, muscle, and even parts of organs and stomach contents. However, in some cases even skin debris was observed in some individuals. In other cases, only skeletal rests were found, without soft tissue present, denoting a greater antiquity of these rests (Table 2). It was determined that the presence of a skull (or much of it) represented a particular individual. A total of 22 different whales were sampled (Table 1). The remaining bodies were not sampled for being out of reach or because they were only skulls (watched from the ship).

The position of orientation of the carcasses on the beaches, which were plotted in a **Rose Diagram** (Figure 3) using the program PAST (3.07) (Hammer et al.) was recorded. A preferential direction of the carcasses in the quadrants NE-SW (14 of 18) can be observed, although this trend was not significant in post hoc test of Von Mises ($p = 0.08$), probably due to the small number of samples and also to the presence of complex marine currents, wind and tide patterns within the channel.

Table 1. Summary of number of rests of whales and whales found and sampled in Slight bay and surrounding areas.

WHALE MORTALITY (CURRENT AND PREVIOUS)	n
Number of recent whales (mortality event: March-April?)	29
Number of previous whales only with the presence of skeletal remains (skulls)	10
Number of whales from which a sample was obtained (from all events)	22
Total number of whales found	**39**

Table 2. Preservation condition of carcasses found in Slight bay and adjacent areas.

Carcass condition	*n*
Complete and intact	3
Complete, bones exposed	8
Disjointed, not intact	2
Completely disjointed, associated	5
Isolated bones (skulls, vertebrae, etc.)	13

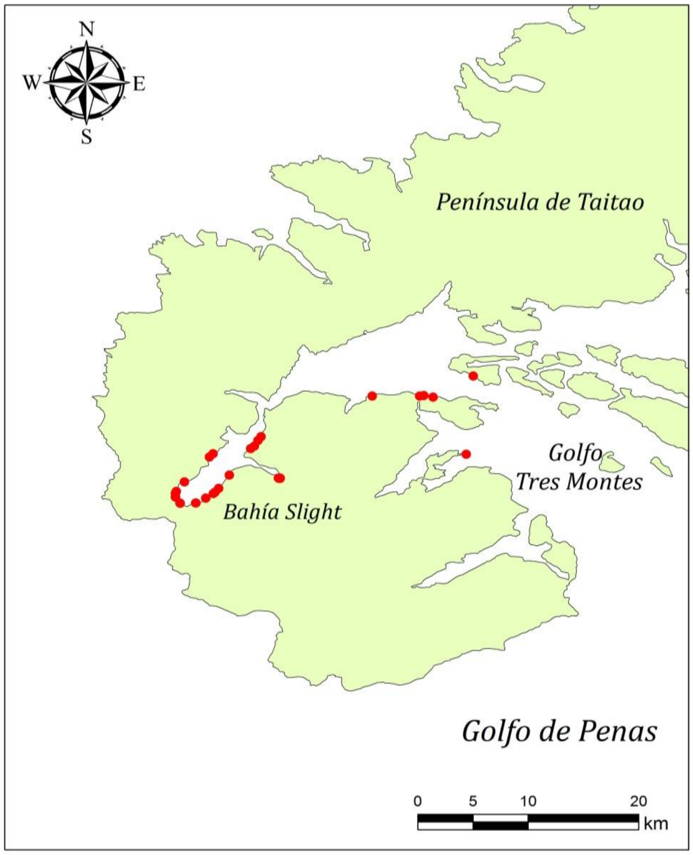

Figure 2. Geographical location of dead whales in Slight Bay and adjacent areas, both recent and former events.

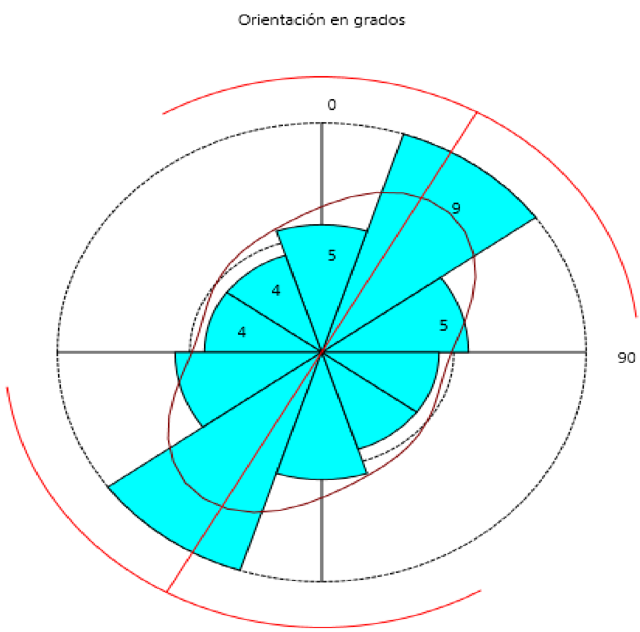

Figure 3. Rose diagram showing orientations of the carcasses in the channel, expressed in degrees, where 0 is the geographical North. The numbers indicate the number of cases in each quadrant oriented and red oval indicates the interval of confidence (95 %) of the distribution of the data.

Species determination

Anatomical description:

Four whales were fully examined in order to determine the species involved. The total length was measured with standardized metrics as described in Clark and Aguayo-Lobo (1965); pictures were taken as well as several baleen plates from each individual for further descriptive analysis (See annex 1). Considering that the ventral grooves do not reach the umbilicus, the triangular shape and almost complete black color (with white scripts on the inner edge) of the baleen, and the characteristics of the fringes, all diagnostics features of *Balaenoptera borealis* allow us to determine that Sei Whale was the species involved. This was afterwards confirmed by genetics studies.

Genetic analyses:

Taxonomic corroboration was performed in 15 out of 39 sampled individuals using genetic analyses in the Laboratory of Molecular Ecology at the University of Chile. For the above DNA tissue (skin, muscle and /or gum) was extracted, part of the control region of mitochondrial DNA (mtDNA) was amplified, region normally used for the identification of cetacean species due to its accelerated mutation rate which results in a high variation, thus allowing discrimination at the species level (Ross *et al*. 2003) .The amplification of the control region (or D-loop) was performed using preferentially the primers M13 Dlp1.5 5´ TGTAAAACGACAGCCAGTTCACCCAAAGCTGRARTTCTA-3' and 8G 5´GGAGTACTATGTCCTGTAACCA (Dalebout et al. 2005), since it amplifies a larger and more informative fragment size. Secondarily, primers DLP-10 5'-CCACAGTACTATGTCCGTATT-3' and Dlp-5 5'-CCATCGWGATGTCTTATTTAAGRGGAA-3' (Baker *et al*. 1993).
PCR products were purified and sequenced in both directions in Macrogen, Korea using an automatic sequencer 3730xl. The resulting sequences were aligned and assembled manually by 2.91 ProSeq program (Filatov, 2002).

Identification to species was carried out using two programs of comparative sequence analysis (1) "Basic Local ALIGMENT Search tool (BLAST. www.blast.ncbi.nlm.nih.gov) and (2) DNA Survillance (Ross *et al*. 2003).

BLAST identifies regions of local similarity between sequences by comparing nucleotide or protein sequences with sequence databases and estimates the degree of similarity between them. This program can be used to infer functional and evolutionary relationships between sequences as well as helping identify members of gene families. Meanwhile, DNA Surveillance is a service for determining the identity of cetacean species using phylogenetic methods. This program aligns a DNA sequence "problem" against a set of reference DNA sequences of cetacean species and then builds a phylogenetic tree (based on genetic distances). The user can then make inferences about the identity of the unknown species by their position in the phylogenetic tree (Ross et al, 2003). In the latter program, the entire database of mitochondrial Control region has at least one sequence validated, *i.e.*, from individuals with confirmed identification, which provides a comparative advantage over BLAST

In most of tissue samples analyzed (14 of 15 individuals) sufficient quantity and quality of DNA was obtained that allowed the amplification of a larger fragment of mtDNA Control region (primers M13 Dlp1.5 5 'and 8G, Dalebout *et al*. 2005) . For the remaining individual it was feasible to amplify a fragment with fewer pairs of bases as previously described (primers Dlp -10 and Dlp -5 , Baker *et al.* 1993) .

BLAST software obtained 99 % reliability that all sequences analyzed and compared with that database corresponded to sei whale, *Balaenoptera borealis* (Figure 4).

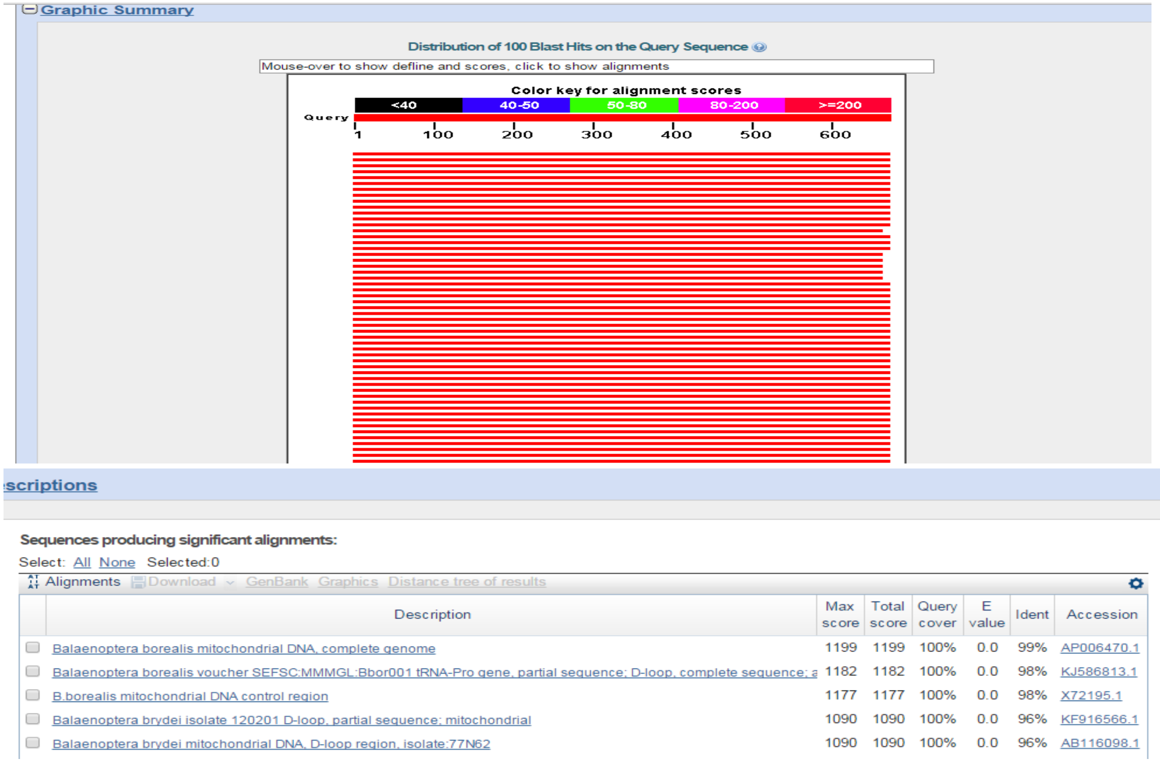

Figure 4. Example of a Profile obtained for one of the sequences analyzed and compared with BLAST data base. The "Ident" column gives the probability value (99 %) the species corresponding to the species *Balaenoptera borealis.*

In the same way, using the DNA - Surveillance program it was obtained that the lowest genetic distance (5%, Table 3), and therefore greater similarity between the sequences analyzed and the sequences of the corresponding database to *Balaenoptera borealis*. The result was similar when comparing the sequences at both Order (Cetacea) (Figure 5) and Suborder level (Mysticeti) (Figure 6).

Table 3. Genetic distances obtained in the comparison of the analyzed sequence (randomly selected as example) and the DNA Surveillance database. It is evident that the Compared Sequence has the lowest genetic distance (0.05) (*i.e*. the greatest similarity) with the species *Balaenoptera borealis.*

ID	Common Name	Distance
BborNA5.UA	Sei Whale	0.0501
BedeSA6.UA	Bryde's Whale (Common form)	0.0929
BedeJP1.HY	Bryde's Whale (Kochi form)	0.1066
BmusIC4.UA	Blue Whale	0.1631
BphyIC5.UA	Fin Whale	0.1696
ErobNEP397	null	0.1727
ErobWS03	Gray Whale	0.1729
MnovSEA9	Humpback Whale	0.1756
BacuNPSWFC	North Pacific Minke Whale	0.1771
BacuNA6.UA	North Atlantic Minke Whale	0.1794
BbonSO8.RH	Antartic Minke Whale	0.1855
CmarNZ1.SB	Pygmy Right Whale	0.1887
EglaNA9.SM	North Atlantic Right Whale	0.2126
CheBP03D	Hector's Dolphin	0.2333
EausSP16NP	Southern Right Whale	0.2384
BmysNEPSB1	Bowhead	0.2417
CcomL2.FP	Commerson's Dolphin	0.2443
LobsPE2.FC	Dusky Dolphin	0.2552
LacuNF6.FC	Atlantic White-Sided Dolphin	0.2554
KbreNZ01SP	Pygmy Sperm Whale	0.2573
TtruT2.JW	Bottlenose Dolphin (truncatus form)	0.2711
DcapCA1.PR	Common Dolphin (Long-Beaked)	0.272
DdelCA6.PR	Common Dolphin (Short-Beaked)	0.2788
GgriNZ1.MD	Risso's Dolphin	0.2788
LobINP8.FC	Pacific White-Sided Dolphin	0.283
BbaSW4965	Baird's Beaked Whale	0.2836
GmelNA8.LS	Long-Finned Pilot Whale	0.2949
PspiPE4.PR	Burmeister's Porpoise	0.295

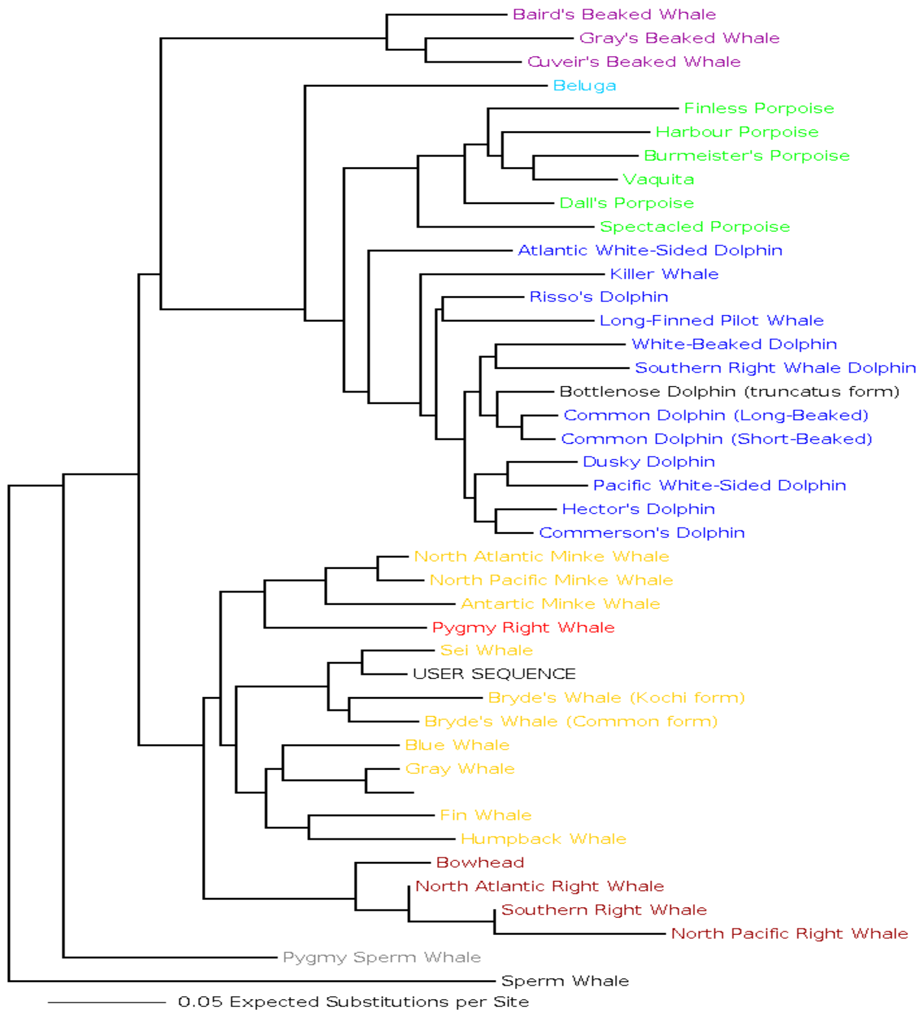

Figure 5. Location of one of the sequences analyzed (see arrow) in a genetic distance tree constructed with information from the order Cetacea in DNA Surveillance.

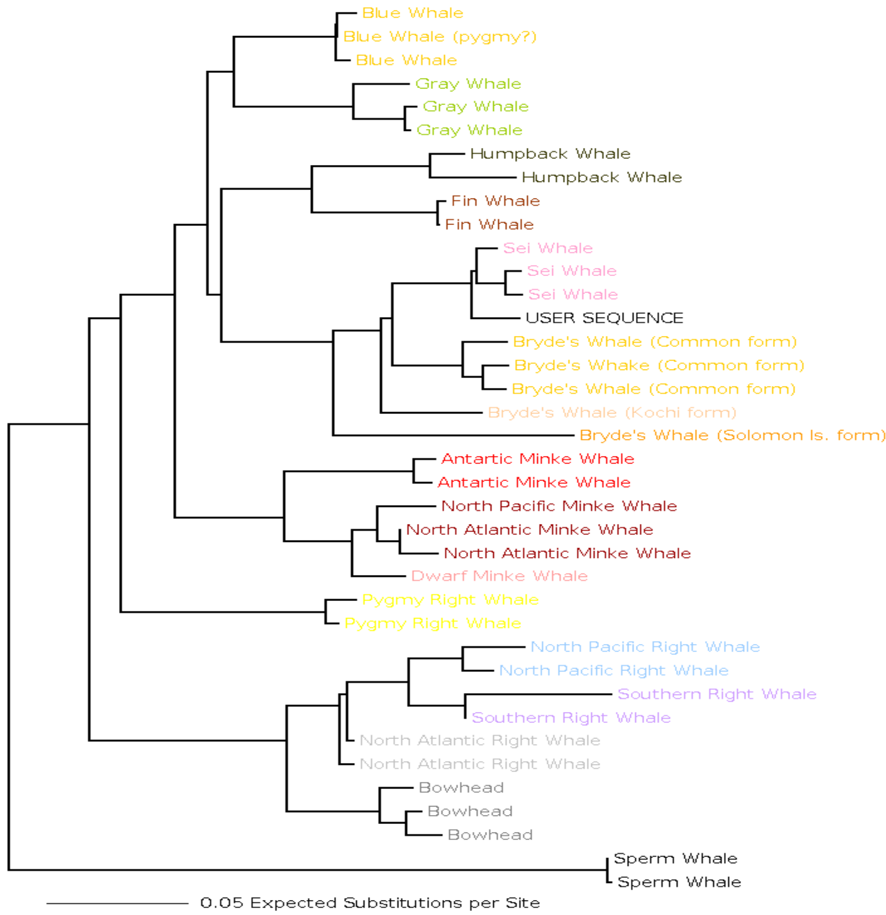

Figure 6. Location of one of the sequences analyzed (see arrow) in a genetic distance tree constructed with information from the order suborder Mysticeti in DNA Surveillance.

Table 4. List of codes of stranded and sampled whales in the Gulf of Penas and their specific identification. In the column species is showed that all individuals genetically analyzed (15) corresponded to the species *Balaenoptera borealis.*

ID	Fecha	Familia	Especie
B1	27-05-2015	Balaenopteridae	
B2	27-05-2015	Balaenopteridae	
B3	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B4	27-05-2015	Balaenopteridae	
B5	27-05-2015	Balaenopteridae	
B6	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B7	27-05-2015	Balaenopteridae	
B8	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B9	27-05-2015	Balaenopteridae	
B10	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B11	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B12	27-05-2015	Balaenopteridae	
B13	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B14	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B15	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B16	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B17	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B18	27-05-2015	Balaenopteridae	
B19	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B20	27-05-2015	Balaenopteridae	
B21	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B22	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B23	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B24	27-05-2015	Balaenopteridae	*Balaenoptera borealis*
B25	27-05-2015	Balaenopteridae	
B26	28-05-2015	Balaenopteridae	
B27	28-05-2015	Balaenopteridae	
B28	29-05-2015	Balaenopteridae	
B29	29-05-2015	Balaenopteridae	
B30	29-05-2015	Balaenopteridae	
B31	29-05-2015	Balaenopteridae	
B32	29-05-2015	Balaenopteridae	
B33	29-05-2015	Balaenopteridae	
B34	29-05-2015	Balaenopteridae	
B35	29-05-2015	Balaenopteridae	
B36	29-05-2015	Balaenopteridae	
B37	29-05-2015	Balaenopteridae	
B38	29-05-2015	Balaenopteridae	
B39	29-05-2015	Balaenopteridae	

Microfracture analysis in the middle and inner ear

The analysis of the bones of the middle and inner ear (only a periotic bone in this case) extracted from the specimen B8 did not show any microfractures in the spiral lamina (inner ear) and demonstrated that the small bone (stirrup, middle ear) remained jointed to the oval window of the periotic bone, showing no signs of alteration that may have been caused by mechanical or acoustic impacts. Similarly, the region of the semicircular channels also showed anatomically intact, showing no damage (Figure 7).

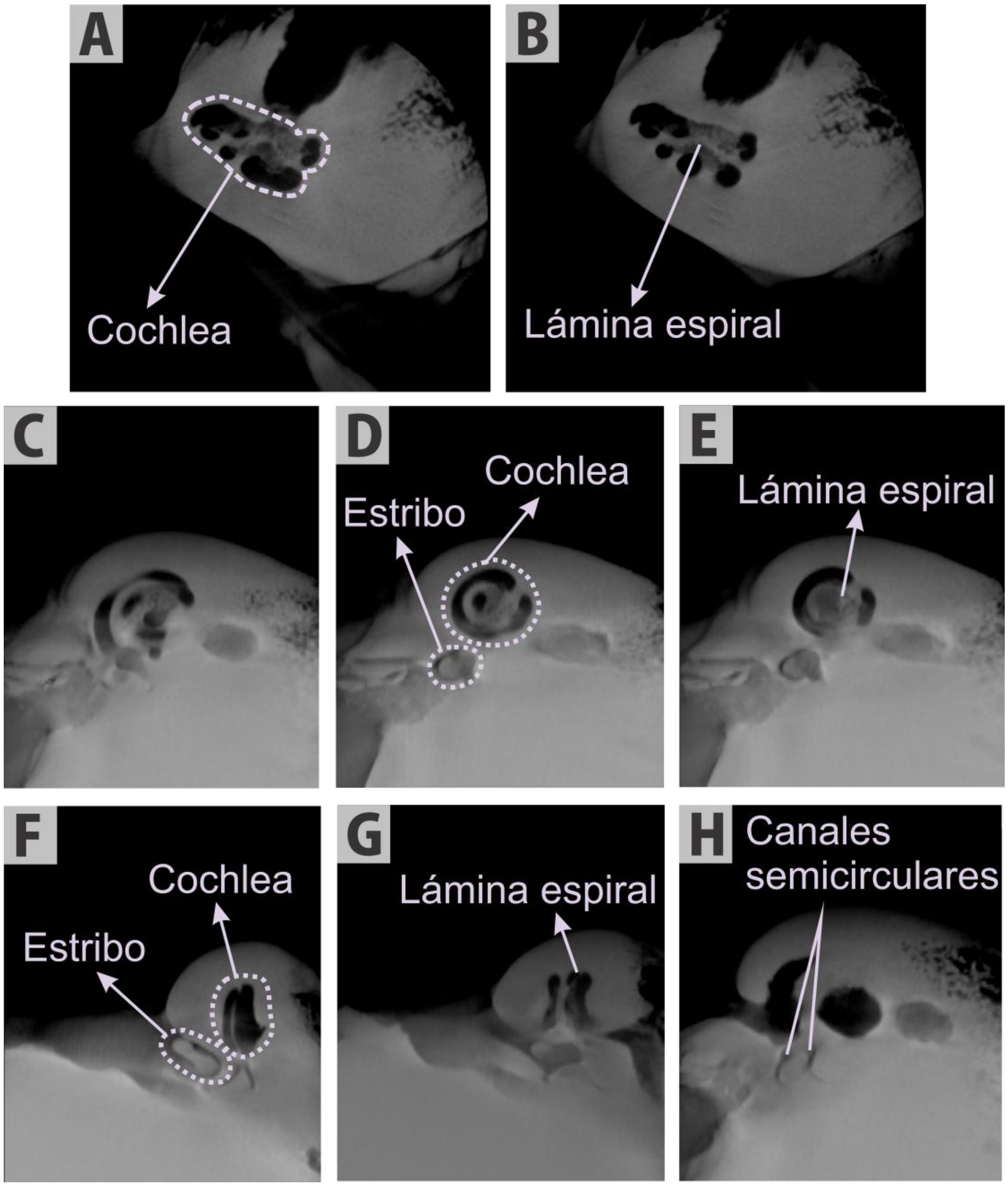

Figure 7. Selected cuts of images obtained by volumetric tomography scans performed in Morita (box of 60 mm, 500 slices). All structurally fragile acoustic anatomical structures of the middle ear (stirrup) and inner ear (cochlea: Spiral lamina), beside the semicircular canals are observed in perfect conditions.

3. Analysis of biotoxins and phytoplankton in water, vectors and whales (Table 5).

1. A quantitative analysis of water samples by microscopy for determination of phytoplankton species
2. Qualitative tests, Scotia Rapid Testing for detection of Saxitoxin and Domoic Acid in stomach contents.
3. Qualitative tests, Scotia Rapid Testing for detection of Saxitoxin and Domoic Acid in vectors extracted during the expedition (clams and mussels)
4. A qualitative analysis by microscopy (presence /absence of cells) in intestinal contents.

Table 5. Toxicological test results.

Test	Results
Presence of harmful phytoplankton (water samples)	**Negative** All water samples (6) were negative for the presence of harmful species of the genus Pseudonitzschia (Diatom) and the genus Alexandrium (Dinoflagellate)
Toxin in vectors (samples of clams and mussels)	**2 positives for Saxitoxin** **2 negatives for Domoic Acid** Homogenized samples of mussels (4 *Perumytilus purpuratus*) from Slight Bay and clam samples (43 of several species) from Peninsula Tres Montes, both positive for Saxitoxin and negative for DA
Toxin in stomach content	**Positive (whale 8) for DA** Positive result, more than 20 ug of Domoic Acid per 100 g of sample **Positive (whale 21) for Saxitoxin** Positive result, but in low level, semiquantitative estimate of 60 ug equivalent of Saxitoxin per 100 g of sample --- 1 whale tested per case, one stomach content sample per whale, one biotoxin test per sample Whale 21 from Caleta Buena, whale 8 from Slight Bay
Presence of harmful phytoplancton in intestinal content	**Positive for *Pseudonitzschia*** Positive for both whales (#8 y #21), medium abundance (relative scale) stool and stomach contents samples analized for both whales.

SECOND EXPEDITION:

During the cruise of the second expedition (February 2016) several formal meetings were carried out to contextualize the new participants according to the information gathered from the first expedition as well as establishing the objectives and goals of the expedition, to analyze the available data, allocate roles and responsibilities and assimilate the protocols for data collection (photos, GPS location , biological sampling, phytoplankton sampling), storage and subsequent distribution of samples.

Once in the targeted area of investigation, as this time we only expected to find skeletons from the mortality event in 2015, the expedition took place in four stages.

1. Development of a first-level sampling to freshly dead individuals in case found.
2. Development of a second level sampling to skeletons with some skin tissue left
3. Water and potential vectors for biotoxins sampling
4. Analysis of samples from water, vectors and stomach contents for detection of Domoic Acid (amnesic poison) and Saxitoxin (paralytic poison) in case fresh animals are found.

Biotoxin analysis for paralytic and amnesic poison was performed using rapid tests based on antibodies produced by Scotia Rapid Testing Ltd.
Water samples for determining the presence of toxic phytoplankton by microscopy were preserved in a saturated solution of iodine for subsequent ex situ analysis at the Universidad Santo Tomas.
For genetics it was used Ross et al 2003 methods, DNA Surveillance: Web-based molecular identification of whales, dolphins and porpoises and Basic Local ALIGMENT Search tool (BLAST).

Development of a first-level sampling to freshly dead individuals in case found.

On February 12 the first inspection in the field was done, identifying a specimen of cetacean in very good state of conservation in the area of Caleta Buena. This specimen was identified as B1, corresponding to a female, total length of 12.40 m, which was necropsied and sampled (Figures 8, 9, 10 and 11).

Figure 8

Figure 9

Figure 10

Figure 11

Development of a second level sampling to skeletons with some skin tissue left

On February 13th and 14th an inspection of Seno Newman was developed, identifying a total of 38 specimens (mainly skeletons) in different states of decomposition (Figures 12 and 13).

Figure 12

Figure 13

As part of the second level sampling to skeletons with some skin tissue left, biological samples were obtained from the specimens (Table 6).

Table 6. Biological sampling records obtained during the 2nd expedition.

ID	Total length	Skin	Muscle	Blubber	Bullae	Periotic	Stomach content	Rectal content	Duodenum content	Baleens	Internal organs	Bone marrow
B1	12.40	X	X	X	-	-	X	X	X	X	X	X
B2	10.60	X	-	-		-	-	-	-	-	-	X
B3	12.60	X	-	-		-	-	-	-	-	-	X
B4	-	X	-	-	2	2	-	-	-	X	-	X
B5	-	X	-	-	-	-	-	-	-	-	-	
B6	-	X			1	-	-	-	-	-	-	X
B7	12.90	X	-	-	2	-	-	-	-	-	-	
B8	12.80	X	-	-	-	1	-	-	-	-	-	X
B9	-	X	-	-	-	-	-	-	-	-	-	-
B10	10.10	X	-	-	2	-	-	-	-	-	-	-
B11	10.10	X	-	-	2	-	-	-	-	-	-	-
B12	-	X	-	-	1	-	-	-	-	-	-	-
B13	15.90	X	-	-	2	1	-	-		-	-	-
B14	-	X	-	-	-	-	-	-	-	-	-	-
B15	-	X	-	-	2	-	-	-	-	-	-	-
B16	-	X	-	-	-	-	-	-	-	-	-	-
B17	-	X	-	-	-	-	-	-	-	-	-	-
B18	-	X	-	-	-	-	-	-	-	-	-	-
B19	-	X	-	-	-	-	-	-	-	-	-	-
B20	-	X	-	-	-	-	-	-	-	-	-	-
B21	-	X	-	-	-	-	-	-	-	-	-	-
B22	-	X	-	-	-	-	-	-	-	-	-	-
B23	-	X	-	-	-	-	-	-	-	-	-	-
B24	-	X	-	-	-	-	-	-	-	-	-	-
B25	-	X	-	-	X	-	-	-	-	-	-	-
B26	-	X	-	-	X	-	-	-	-	-	-	-

B27	-	X	-	-	-	-	-	-	-	-	-	-
B28	-	X	-	-	-	-	-	-	-	-	-	-
B29	-	X	-	-	-	-	-	-	-	-	-	-
B30	-	X	-	-	-	-	-	-	-	-	-	-
B31	-	X	-	-	-	-	-	-	-	-	-	-
B32	-	X	-	-	-	-	-	-	-	-	-	-
B33	-	X	-	-	-	-	-	-	-	-	-	-
B34	-	X	-	-	-	-	-	-	-	-	-	-
B35	-	X	-	-	-	-	-	-	-	-	-	-
B36	-	X	-	-	-	-	-	-	-	-	-	-
B37	-	X	-	-	-	-	-	-	-	-	-	-

Complementary to biological sampling, water samples were taken for detection of zooplankton and phytoplankton in five locations (Figure 14) according to the established protocol (Table 7).

Table 7. Water sampling records.

Area	Name	East	North	Date	Time	Cuantitative sample	Cualitative sample
Puerto Sligth	Agua Slight	456560	4816010	02/13/2016	12:58:35	x	x
Seno	N1	493070	4838395	02/14/2016	15:38:43	x	x
Seno	N2	497768	4842364	02/14/2016	15:58:31	x	x
Seno	N3	496888	4838665	02/15/2016	18:27:48	x	x
Canal Chaycayec	Agua Chaycayec	485553 485553	4832890 4832890	02/15/2016 02/15/2016	19:33:51 19:33:51	x x	x x

Figure 14 Water sampling stations.

Phytoplankton and marine biotoxins analysis (Table 8)

Qualitative samples: Five phytoplankton samples obtained with a net of 20 µm mesh size were analyzed using light microscopy.

In water sampling stations N1 , N2, N3 and Chaycayec Channel large abundances of detritus, zooplankton and their feces were observed, along with a very low abundance of phytoplankton. Phytoplankton identified in these samples was composed exclusively of dinoflagellates, mainly by members of the genus *Ceratium* typical of marine waters. Among these dinoflagellates, low abundances of toxic species *Alexandrium catenella* (saxitoxin) in N1 and N2 stations were also found as well as *Dinophysis hastata* (diarrheic poison) at the station Canal Chaycayec.

Zooplankton observed at stations N1, N2, N3 and Chaycayec Channel was mainly composed of calanoid copepods in high abundance and good physical condition. No individuals were observed in reproductive stages. Zooplankton from Chaycayec Channel station was the most diverse and included zoeas of decapod crustaceans, chaetognat worms, gelatinous organisms (salps or micromedusas), plus numerous calanoid copepods.

This indicates that there was a high predation pressure on the existing phytoplankton, which was composed of marine species of relatively warm waters, with little freshwater or estuarine influence at these stations. The sample from Puerto Slight station was significantly different from the others not showing zooplankton, low amounts of organic detritus, and the dominance of scarce phytoplankton diatoms (*Chaetoceros*), with the presence of ciliated organisms. This indicates a more estuarine influence, with input and mixing of fresh and marine water than in the other stations.

Quantitative samples: quantitative analysis of surface samples indicated that phytoplankton was extremely low in all samples collected. The possible explanation is that the high abundance of zooplankton observed in net samples was able to remove most of existing populations in the area.

Biotoxin analysis: The stomach contents obtained from the whale B1 was analyzed to determine the presence of Saxitoxin and Domoic Acid by rapid tests based on specific antibodies. The test for DA yielded negative results indicating no presence of domoic acid in the stomach contents analyzed. The test for Saxitoxin was positive indicating the presence of more than 80µg/100g of saxitoxin and its derivatives.

Table 8. Biotoxin and phytoplankton analysis from the 2nd expedition**.**

Test	Results
Presence of harmful phytoplankton (water samples)	**Negative** Stations Puerto Slight and N3 were negative for the presence of harmful species of the genus *Pseudonitzschia* (Diatom) and the genus *Alexandrium* (Dinoflagellate). **Positive** Stations N1 and N2 for the presence of *Alexandrium catenella* (saxitoxin), low abundance. Station Canal Chaycayec *for the presence of Dinophysis hastata* (diarrheic poison).
Toxin in vectors (samples of mussels)	**3 negatives for Domoic Acid and Saxitoxin** Homogenized samples of mussels (*Perumytilus purpuratus*) from Seno Newmann from Stations B38, B10 and B6.
Toxin in stomach content of whale B1	**Negative for DA** **Positive for Saxitoxin** Positive result: more than 80ug / 100g of saxitoxin and its derivatives.

The results obtained from the analysis of water samples taken in February 2016 indicate that there was a very low abundance of phytoplankton in the area. The low level of phytoplankton was dominated by dinoflagellates of marine habitat, including *Alexandrium catenella* , the causative agent of Saxitoxin. The presence of Saxitoxin in the stomach contents of whale B1 suggests that the cause of death could be the ingestion of these toxins.

Genetic analysis: Regarding the results of determination of species for the latest samples obtained (33) it was feasible to positively identify eight skeletons and one fresh individual B1 as sei whales. The remaining samples are still under laboratory scrutiny since amplification has not worked so far (Table 9).

Table 9. Identification of nine individuals as sei whales.

ID	Date	Species
1	Feb-16	***Balaenoptera borealis***
2	Feb-16	Not determined yet
3	Feb-16	Not determined yet
4	Feb-16	Not determined yet
5	Feb-16	Not determined yet
6	Feb-16	Not determined yet
7	Feb-16	Not determined yet
8	Feb-16	Not determined yet
9	Feb-16	Not determined yet
10	Feb-16	***Balaenoptera borealis***
11	Feb-16	***Balaenoptera borealis***
12	Feb-16	Not determined yet
13	Feb-16	Not determined yet
14	Feb-16	Not determined yet
15	Feb-16	Not determined yet
16	Feb-16	***Balaenoptera borealis***
17	Feb-16	Not determined yet
18	Feb-16	Not determined yet
19	Feb-16	Not determined yet

ID	Date	Species
20	Feb-16	Not determined yet
21	Feb-16	Not determined yet
22	Feb-16	Not determined yet
23	Feb-16	Not determined yet
24	Feb-16	Not determined yet
25	Feb-16	Not determined yet
26	Feb-16	Not determined yet
27	Feb-16	Not determined yet
28	Feb-16	Not determined yet
29	Feb-16	Not determined yet
30	Feb-16	***Balaenoptera borealis***
31	Feb-16	Not determined yet
32	Feb-16	***Balaenoptera borealis***
33	Feb-16	***Balaenoptera borealis***
34	Feb-16	Not determined yet
35	Feb-16	Not determined yet
36	Feb-16	Not determined yet
37	Feb-16	***Balaenoptera borealis***
38	Feb-16	***Balaenoptera borealis***

POTENTIAL CAUSES OF MORTALITY OF WHALES

According to field diagnoses and photographic analysis, no direct action from human activity was observed (wounds, obvious mechanical trauma or entanglement evidence) nor obvious contamination by oil or other chemicals was observed.

From the position of the whales on the shore (skull perched on its dorsal part), it is assumed that animals apparently died while still in the water and sea currents and winds dragged them ashore. This is partly because rorquals tend to think after death due to low fat levels in comparison to other species. After the onset of decomposition gases generated mostly in the abdominal section, the carcasses return to the surface and remain floating with the ventral portion upwards until these gases are released or until stranding on the coast. Therefore, in this paper we refer to the event as mass mortality rather than multiple stranding.

There are some features that make this mortality event particular, and allows excluding some causes of death and support others. Given the absence of marks or cuts and the negative results of acoustic and mechanical impact analysis scan of one of the periotic bones, mortality by anthropogenic action has been potentially ruled out (*e.g.* noise impact or collision with vessels).

In addition, the multiple nature of the finding makes unlikely any of the specific causes, such as boat collisions and-natural death or senility. The presence of a dead pinniped in the proximal study area (very fresh skeletal rests) and the optimal physical condition of the freshly dead whales make it unlikely to attribute the cause of death to disease. The positioning of the carcasses (ventral side up) strongly suggests that the death agent was out or inside the channel where they were found, *i.e*. allochthonous. The determination of an allochthonous agent of death, the number of individuals and the presence of at least one recent skeleton of another taxonomic category (South American sea lion, *Otaria byronia*) in combination with the results of the biotoxin analyses, determined that the strongest hypothesis being evaluated is that the death of whales occurred by exposure and subsequent poisoning of animals due to toxins produced by harmful phytoplankton blooms (microalgae). The more likely vectors of the toxins (consumers and filter feeders) include sardines (*Sprattus fueguensis*) and/or lobster krill in its pelagic larval phase (*Munida spp.).* Both vectors are key part of the food chain of these fjords and thus a potentially important link for whales in this region.

Although the results of water samples yielded negative for the presence of the expected phytoplankton (normal after 2-3 months of blooms occurrence), traces of toxin were found in the vectors and in the stomach contents and rests of *Pseudonitzschia* cells in intestinal contents (Tables 5 and 8).

The results obtained during the expedition, plus the high presence of *Pseudonitzschia* during February and March 2015 measured in the monitoring stations of the Chilean Red Tide Monitoring Program enhance the possibility of supporting the hypothesis that harmful algal blooms (HABs) might be responsible for the mass whale mortality. However, mainly due to the poor state of conservation of the animals that hindered a good sampling for further analysis and the limited time spent in the area of study (1.5 days) to perform a more comprehensive work, the results presented here are necessarily inconclusive. The presence of toxins in vectors in the area where the dead whales were or the presence of toxins in three of the 30 whales sampled with soft tissues cannot be considered conclusive evidence. The evidence presented here are rather a gathering of factors that allow building several hypotheses to be studied more rigorously in the near future.

There is no certainty that the assembly of whales represents an alochthonous death and latter stranding due to the non-significant results of the test for the orientation of carcasses, but most importantly, because sei whales in this area tend to come close to shore to feed and could have died when feeding on dense aggregations of prey within the enclosed bay where they were found. Also, the number of sampled and registered individuals in this first expedition represented only a fraction of the total number of dead animals (>360 whales from one single event, Haussermann et al.). The complex geomorphology of the coast, isolation and weather conditions generated unfavorable conditions for searching and finding more animals. This added to the possible existence of other individuals that can be found under the water surface line and therefore “invisible" to a marine survey like this.

The mass mortality event described in this document demonstrates an unprecedented event in Chile and the world, in terms of the number of affected individuals. A total of 23 individuals genetically analyzed from both expeditions corresponded to the sei whale species *Balaenoptera borealis* (Tables 4 and 9).

However, the mortality of whales in the region is not new. There are at least four previously recorded events that have not been documented in detail (Hucke-Gaete *in litt.*). The most recent one occurred between January and March 2014 and was partially documented through photographs and stranding records. It consisted of ca. five whale specimens, possibly from the species Minke (*Balaenoptera acutorostrata*) or juvenile of another species of baleen whale, two in an inner channel Kent Island, located hundreds of meters apart, and other three further south, near Bernardo Glacier, these distributed only a few meters apart (C. Christie, UACH, pers. commn.).

On the other hand, mass mortality from poisoning caused by harmful algal blooms has been described for two other species of cetaceans around the world, but mainly for the Pacific Northeast (*e.g.* Geraci *et al*. 1989 ; Durbin *et al.* 2002; Doucette *et al*. 2006). This is of great importance for the effect that it could have at the populations level, particularly for those species listed in some threatened category (vulnerable or endangered, as is the case of many species of whales).

That is why the creation of a standard protocol for response to marine mammal stranding is not only important for the standardization of methodologies and collecting key information, but also is of great value to understand the causes of death, whether natural or human actions. Likewise, good planning and action is of great importance to the coordinating agencies so that these samples are made in a responsible, efficient and organic way and that those responsible for obtaining this information have the experience and scientific support that deserves such work.

Everything suggests that the cause of death of these cetaceans is not directly related to human actions. So far the strongest hypothesis to explain the death of these 360 or more specimens of baleen whales is poisoning by marine biotoxins.

Annex I

REPORT OF PARTIAL NECROPSIES MADE IN FOUR SEI WHALES (*Balaenoptera borealis)* FOUND IN SLIGHT BAY, NORTHWESTERN GULF OF PENAS, XI REGION OF CHILE

Introduction

The objective of this report (Annex), is to show whether there was evidence of human interactions in the death of four specimens of sei whale in the Gulf of Penas, Aysen Region that were examined during the May 27, 28 and 29 of 2015; after about 30 days of being observed by staff of the Huinay Research Station. Moreover, we consider it necessary to note, that the goal of a full necropsy is to know the cause of death of an organism. Consequently, it seems necessary to distinguish two Protocols: The first, seeking the cause of death (necropsy) and the second, which seeks signs of human interactions in the death of the whales (Illicit Environmental Protocol). This difference is essential to differentiate the causes of death or causes of strandings of cetaceans; since it is accepted as stranding of cetaceans, when one or more copies arrive alive to the coast and then die on land (Perrin and Geraci, 2002; Aguayo-Lobo, 2013; 2015 Haro et al.).

Development of necropsies

Out of the 18 complete specimens of whales of the genus Balaenoptera , 16 they were able to be measured and only 12 were sexed. Of these, 6 were male (specimens 2, 10, 17, 21, 23 and 34) and 5 females (specimens 3 , 11, 12, 15 and 22) and 1 of unknown sex (specimen 8). Of these 11 sexed specimens it was possible to perform an external examination to know whether there was an environmental unlawful, and partial or incomplete necropsies , in order to try to determine the cause of death of four specimens; specimens 8, 21 , 22 and 23. Beside, 12 skulls, belonging to the genus *Balaenoptera* were reported, of which 9 were measured, with the exception of 3 units (13, 18 and 27). Due to topographical difficulties, there was no access to 9 specimens (28, 29, 30 , 31, 32, 36 , 37, 38 and 39).

Specimens examined and sampled

<u>Specimen N° 8 : Genus Balaenoptera</u>

The animal examined corresponds to a specimen of *Balaenoptera borealis*, considering that some of the body measurements such as length of the thoraco - ventral grooves, relative to the total length , do not reach the umbilicus, which is one of the diagnostic features of this species. Furthermore, the shape, color and characteristics of the baleen fringes confirm the identification of this species (Figure 1) .

External examination

The external examination of the specimen No. 8, of 15.27 meters of total length (approximate measure due to torsion of the caudal peduncle); unknown sex, due to the advanced decomposition of the genital area (Figure 2). The skin was of an orange and white tone, indicating the partial or total loss of epidermis by areas with no evidence of fresh wounds or old scars or trauma, tumors, inflammations or external parasites, and no evidence of fishing nets that could have triggered the stranding and subsequent death of the specimen, or that the specimen died at sea and was taken, in that condition, drifting by the prevailing currents in the Gulf of Penas.

Considering that the animal was on right lateral decubitus on a sandy beach of fine-grained, it was not possible to see the right side of it. Accordingly, the left side, back and belly of the animal; ie all parts of the body examined, showed no signs of anthropogenic lesions (Figure 3). It is noteworthy that no traces of oil, oily substances or other harmful chemicals was found near the specimen

Necropsy

Given the state of decomposition of the specimen and the technical difficulties in handling an animal of this size, only a partial necropsy was done. An incision was made from the back of the chest to the front of the abdomen (Figure 4), then samples of skin, fat, muscle, ribs, stomach wall, stomach contents, rectal content and sloughed whiskers from the mouth were obtained for subsequent laboratory analysis. Stomach content samples and rectal content obtained were used for analysis of possible red tide poisoning. When removing the muscle layer of the left chest area, a section of muscle of darker coloration was observed, with accumulation of blood clots by gravity (Figure 5 a) and b)), showing that there was a trauma, which may have be present pre or post mortem. No esophagus, trachea or heart was observed in the thoracic cavity; or liver and intestine in the abdomen, without being able to examine the abdominal and pelvic cavity thoroughly.

Instead, an incision near the jaw joint was successfully done in order to extract the bull eardrum-periotic. Unfortunately, it was not possible to obtain the full auditory complex.

Figure 1. Baleen of 56 cm length and 25 cm width corresponding to whale #8 (15.27 m).

Figure 2. Photography of genital area from individual #8 where the advanced state of decomposition is evident.

Figure 3. Specimen #8, in right lateral decubitus where the length of the thoraco-ventral grooves are observed.

Figura 4. Thoraco-abdominal incision performed in specimen N° 8.

Figure 5. View of clots accumulated in ventral area by the action of gravity (left); View of haematoma observed in the thoracic area (right).

<u>Specimen N° 21: Genus *Balaenoptera*</u>

Like the previous specimen (#8), based on the same diagnostic characteristics it has been determined that the specimen corresponds to the species *Balaenoptera borealis* (Figure 6 and 7).

External examination

The external examination of the specimen #21 (male of 8.3 meters long). This specimen exhibited similar coloration to previous one orange white (Figure 8), indicating a partial or complete loss of the epidermis. There is no evidence of recent or old scars wounds or trauma, tumors, inflammations or external parasites, or fishing nets or nets that can be associated with the death of the animal. Considering that the animal was left lateral decubitus on a rocky and partially covered by vegetation substrate, it was not possible to see signs or symptoms of any anthropogenic participation whether in the belly, head of the animal. It is noteworthy that no traces of oil, oily substances or other harmful chemicals were found near the specimen.

Necropsy

For sampling and access to internal viscera an incision covering the back of the chest to the front of the abdomen (Figure 9) was performed. Samples of skin, blubber, muscle, stomach contents and sloughed whiskers from the mouth were obtained. Esophagus, trachea, heart, spleen, kidneys, liver and intestines were not observed. Samples from stomach contents were taken for further analysis of the presence of phytoplankton cells or poisoning toxins indicating red tide.

Figure 6. Photograph of whale #21 showing the end of the thoraco-ventral grooves anterior to the umbilicus.

Figure 7. Baleen (20 cm lenght and 7 cm width) of Individual # 21 (8,3 m).

Figure 8. Specimen # 21 on left lateral decubitus.

Figura 9. Incision covering the back of the chest to the front of the abdomen for sampling andaccess to internal viscera.

Specimen N° 22: Genus *Balaenoptera*

As the above specimens (# 8 and 21) this one also corresponds to the species *Balaenoptera borealis*, based on the same diagnostic features already mentioned (Figure 10).

External examination

The external examination of the specimen #21 (female, 10 meters long) showed that the skin had an orange white coloring, indicating the partial or complete loss of the epidermis. In the abdominal region an advanced state of decomposition and a loss of muscle tissue due to the action of carrion birds in place (Figure 11 and 12) were observed. There was no evidence of recent or old scars wounds or trauma, tumors, inflammations, external parasites, fishing nets or nets that can be associated with the death of the animal. Considering that the animal was left lateral decubitus on a rocky presence of vegetation and substrate, it was not possible to see signs or injuries caused by the actions of third parties. It is noteworthy that no traces of oil, oily substances or other harmful chemicals were found near the specimen.

Necropsy

An incision was made from the front of the abdomen (Figure 13) to the front of a large wound caused by scavengers. Then an incision was made in the rectal area. Subsequently samples of skin, muscle, stomach contents, intestine, intestinal contents, intestinal parasites and kidneys were obtained. No samples of esophagus, liver, heart, spleen, uterus and gonads were obtained.

Figure 10. Baleen (43 cm lenght and 18 cm width) corresponding to specimen #22 (10 m long).

Figure 11. Left lateral decubitus position and evidence of carrion birds in the area.

Figure 12. Photography showing the high degree of decomposition in the abdominal area of the specimen.

Figura 13. Abdominal incision of the specimen #22.

Specimen N° 23: Genus *Balaenoptera*

As the above specimens (# 8, 21 and 22), this one corresponds to the species *Balaenoptera borealis*, based on the same evidence shown above (Figure 14).

External examination

The external examination of the specimen N° 23 (male, 11.5 meters long) shows the skin exhibiting similar coloration to the preceding, possibly indicating at least one or two months of death. There is no evidence of trauma, tumors, inflammations or external parasites, fishing nets or nets that can be

associated with the death of the animal. Considering that the specimen was on right lateral decubitus (Figure 15), on a rocky beach with partial vegetation and exposed to tide position, it was not possible to see the right side of it. Consequently, it is not possible to state categorically that no external injuries attributable to anthropogenic actions are absent on the left side of it.
Not hydrocarbon residues, oily substances or other harmful chemicals were found near the specimen.

Necropsy

An incision was made from the back of the chest to the front of the pelvis (Figure 16). Then samples of skin, bubble, muscle, stomach and stomach contents were obtained. In addition adjacent baleens were collected; however, it is not possible to determine with certainty if they belong to specimen N° 23. Heart, liver, spleen, pancreas, kidneys and gonads were not inspected.

Figure 14. Baleen (41 cm length and 18,6 cm width) from specimen # 23.

Figure 15. Specimen # 23 on right lateral decubitus position.

Figure 16. Thoraco-abdominal incision of specimen # 23.

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