



First Case of Brevetoxicosis Linked to Rough-Toothed Dolphin (*Steno bredanensis*) Mass-Mortality Event in Eastern Central Atlantic Ocean: A Climate Change Effect?

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Harmful algal blooms (HABs) have been increasingly recorded over the last decades and much work has linked these events to multiple oceanographic and climate disturbances. HABs can affect ecosystems either as events that affect dissolved oxygen, clog fish gills, or smother corals or through the production of biotoxins which affect living marine resources through food web transfers or aerosols. HAB represent a natural driver of decline and potential extinction of aquatic organisms, from invertebrates to mammals, which may offer little evolutionary adaptation particularly in very high and long-lasting exposures. Despite numerous multispecies mass-mortality events linked to HAB-associated biotoxins globally, there are no records in cetaceans off the central eastern Atlantic Ocean. Herein, we report the epidemiology, pathologic, microbiologic and toxicologic investigation results attesting to the first documentation of cetacean mass-mortality in European waters associated with brevetoxins. Twelve rough-toothed dolphins (*Steno bredanensis*) were found dead adrift or beached along the southwestern coast of Gran Canaria from April 28th to May 7th, 2008. Although pathologic examinations were limited by moderate to advanced autolysis and decomposition of the carcasses, consistent findings included multisystemic hemorrhage and undigested ingesta within the gastric compartments, mainly salema porgy (*Sarpa salpa*). Toxicologic analysis of gastric contents identified PbTx2 and PbTx3 brevetoxins. Our results provide compelling toxicopathologic evidence of fatal brevetoxicosis in a cohort of rough-toothed dolphins. These data add to the limited knowledge on pathology of HAB in cetaceans and provide the first account of brevetoxicosis in European waters. No other mass-mortality or individual fatality of any cetacean species has been linked to brevetoxicosis in the Canary Islands since this event.

Keywords: biotoxin, *Karenia brevis*, Harmful algal bloom, climate change, PbTx

INTRODUCTION

Harmful algal blooms (HABs) are increasingly frequent worldwide, occurring in lake, riverine, coastal and pelagic waters (Lowenstine, 2007). Different species of microalgae in these blooms, including diatoms and dinoflagellates, produce a variety of biotoxins potentially detrimental for aquatic organisms and human beings. HABs can affect ecosystems either as events that affect dissolved oxygen, clog fish gills, or smother corals or through the production of biotoxins which affect living marine resources through food web transfers or aerosols. HAB represent a natural driver of decline and potential extinction of aquatic organisms, from invertebrates to mammals, which may offer little evolutionary adaptation particularly in very high and long-lasting exposures (Lowenstine, 2007). While morbidity and mortality are among the grave outcomes linked to ingested biotoxin exposures (Geraci et al., 1989; Scholin et al., 2000; Flewelling et al., 2005), more subtle sequela may occur as result of prolonged, non-lethal exposure to different biotoxins (Brodie et al., 2006; Goldstein et al., 2008; Zabka et al., 2009). Massive die-offs involving many different fish species, manatees, sea lions, odontocetes, mysticetes and giant squids have been reported (Lowenstine, 2007). Furthermore, many of these biotoxins pose concerns for public health through ingestion of biotoxins in food or inhalation of biotoxins downwind of the bloom (Gerssen and Gago-Martinez, 2019).

Naturally occurring brevetoxicosis fatalities have involved aquatic invertebrates, fish, sea turtle, seabirds, marine mammals (pinnipeds, cetaceans, and sirenians), terrestrial mammals (coyotes, domestic dogs), green tree frogs, and ground squirrels (Castle et al., 2013; Buttke et al., 2018). Brevetoxins have had the greatest impact in aquatic organisms as they may vector into the food chain, killing fish, invertebrates, birds, sea turtles, and marine mammals (Lowenstine, 2007). Several mortality events in odontocete species, primarily involving common bottlenose dolphins (*Tursiops truncatus*) have been recorded in the Gulf of Mexico, mostly along the west coast of Florida (Fire et al., 2021). Brevetoxins are produced primarily by dinoflagellates during “red tides,” predominantly by members of genus *Karenia*. The latter encompasses eight species: *K. brevis* (formerly known as *Gymnodinium breve* and *Ptychodiscus brevis*), *K. mikimotoi*, *K. brevisulcata*, *K. longicanalis*, *K. digitata*, *K. papilionacea*, *K. selliformis*, and *K. bidigitata*. Reliable identification of these species may require a combination of morphologic features and molecular analysis (Haywood et al., 2004). *K. bicuneiformis* (syn. *K. bidigitata*), *K. papilionacea*, and *K. brevis* have been recorded in the Canarian archipelago (Ojeda, 2005, 2013). Specifically, *K. papilionacea* is able to produce neurotoxins and *K. brevis* produces neurotoxins (designated PbTx_s) and hemolytic toxins (Fire et al., 2008a, 2015). Other potentially toxic metabolites, such as hemi-brevetoxins, brevenals, brevisamides, brevisins, tamulamides, gymnocins, gymnodimines, brevisucenals, and brevisulcatic acids, can be produced by *Karenia* species (Caruana and Amzil, 2018; Hort et al., 2021). Intoxication occurs primarily by ingestion of contaminated prey or marine vegetation or by inhalation of toxins in aerosol during algal blooms. Limited clinical signs have been recorded in cetaceans with brevetoxicosis,

mainly due to the peracute nature of the neurotoxicity. There are records of low levels of PbTx_s in stranded dolphins without specific pathologic findings, and acute neurologic signs have been observed in some dolphins during *Karenia* blooms (Rowles, T., personal communication). Herein, we report the epidemiology, pathologic, microbiologic and toxicologic investigations results attesting to the first episode of brevetoxicosis-related cetacean mass-mortality event in European waters involving 12 rough-toothed dolphins (*Steno bredanensis*).

MATERIALS AND METHODS

Stranding Epidemiology

Twelve rough-toothed dolphins were found adrift or beached along the southeastern coastline of Gran Canaria over a 10 day-period, from April 28th to May 7th, 2008. Carcasses were retrieved and submitted for necropsy to the Institute for Animal Health and Food Safety, University of Las Palmas of Gran Canaria. Biologic data [age class, sex, body condition (BD), conservation status (CS), morphometrics] and stranding conditions for each individual were recorded systematically (Table 1). Age class was based on total body length and gonad development, including fetus/neonate/calf, juvenile/subadult, and adult (Geraci and Lounsbury, 2005; Arbelo et al., 2013; Diaz-Delgado et al., 2018). BD was subjectively classified into good, moderate, poor, and emaciated according to anatomic parameters such as the osseous prominence of the spinous and transverse vertebral processes and ribs, the mass of the epaxial musculature, and the amount of fat deposits, taking into account the species and the age of the animal (Geraci and Lounsbury, 2005; Arbelo et al., 2013; Diaz-Delgado et al., 2018). Carcasses were classified as very fresh, fresh, moderate autolysis, advanced autolysis or very advanced autolysis (Arbelo et al., 2013; Diaz-Delgado et al., 2018). The permission required for the handling of stranded cetaceans within the Canarian archipelago was issued by the environmental department of the Canary Islands' Government. No experiments

TABLE 1 | Life history data of 11 rough-toothed dolphins (*Steno bredanensis*) found adrift off or beached along the Southeastern coast of Gran Canaria.

| Case | Sex | Age | SD | TS | ND | CS |
|------|-----|-----|---------|----|----------|----|
| 1 | M | Ad | 4/24/08 | D | 4/25/08 | 4 |
| 2 | F | Ad | 4/28/08 | D | 4/30/08 | 4 |
| 3 | F | Sub | 4/29/08 | D | 4/30/08 | 5 |
| 4 | F | Sub | 4/29/08 | D | 4/30/08 | 5 |
| 5 | F | Sub | 4/30/08 | D | 4/30/08 | 4 |
| 6 | M | Sub | 4/30/08 | D | 4/30/08 | 4 |
| 7 | M | Sub | 5/2/08 | D | 5/2/08 | 5 |
| 8 | M | Ad | 5/5/08 | D | 5/5/08 | 5 |
| 9 | M | Ad | 5/5/08 | D | 5/5/08 | 5 |
| 10 | M | Sub | 5/5/08 | D | 5/6/08 | 5 |
| 11 | F | Ad | 5/7/08 | D | 5/7/08 | 5 |
| 12 | ND | Neo | 5/7/08 | D | 16/05/18 | 4 |

Sex: F, female; M, male. Age: Sub, subadult; Ad, adult; SD, Stranding date; TS, Type of stranding; ND, Necropsy date; CS, Conservation status.

were performed on live animals because our work was based on dead stranded cetaceans.

Pathologic Examinations

Necropsies followed standardized protocols (Geraci and Lounsbury, 2005; Arbelo et al., 2013; Diaz-Delgado et al., 2018). Representative tissue samples were collected and fixed in 10% neutral buffered formalin. All these tissues were processed routinely, embedded in paraffin-wax and 5 μm -thick sections were stained with hematoxylin and eosin for microscopic analysis. For histopathologic analysis, formalin-fixed tissues were embedded in paraffin-wax, sectioned at 5 μm , and stained with hematoxylin-eosin. To rule out fat embolism, osmium tetroxide (post-fixation) was used on selected lung tissue sections (Arregui et al., 2020).

Chemical Analysis

Fresh samples (stomach content, skeletal muscle, liver, kidney) from seven animals were collected during necropsy. No water samples were collected. The samples were stored frozen (-80°C) and were submitted to liquid chromatography-mass spectrometry (LC-MS) analysis for the identification of brevetoxins (PbTx). Brevetoxins extraction from homogenized tissue was carried out according to McNabb et al. (2012). Briefly, homogenized sample tissue (2.0 ± 0.1 gr) was weighted into 50 mL centrifuge tube, a 9 mL volume of 80% (v/v) aqueous methanol was added and the content was mixed using a vortex during 1 min. The sample was then heated in a water bath at $60 \pm 2^{\circ}\text{C}$ for 20 min. The sample was cooled in an ice bath and centrifuged at 3,000 g for 10 min. The supernatant was pipetted and transferred into another 50 mL centrifuge tube, and the tissue pellet re-extraction was repeated. The final extract volume was adjusted into 20 mL with Milli Q water and the final extract solution was mixed very well; 8 mL of the liver extract was transferred to a 50 mL pyrex tube and partitioned with 10 mL of n-hexane. The hexane layer containing lipid compounds was removed and MilliQ water was added to 5 mL of methanolic extract until achieve 20 mL of final volume extract for purification [solid-phase extraction (SPE) procedure].

PbTx sample cleanup was carried out according to McNabb et al. (2012) with slight modifications. Strata-X SPE column (60 mg, 3 mL) was conditioned with 3 mL of methanol and 3 mL of 25% (v/v) aqueous methanol. A total of 20 mL of extract was loaded onto conditioned SPE cartridges at flow rate approximately one to two drops per second followed by 2 mL/min. The stationary phase was subsequently washed with 4.5 mL of 25% (v/v) aqueous methanol at the same flow rate. Graduated tubes (15 mL) were placed inside the manifold for elution volume collection; the retained PbTx in SPE stationary phase was eluted with 4.5 mL of methanol. The final volume was made up to 5.0 mL with MilliQ and mixed; 1 mL of purified extract was filtered with 0.22 μm Millipore membrane and injected into LC-MS system.

PbTx analysis was performed by LC-MS using a single Quadrupole, Mass Spectrometer under the conditions initially described in McNabb et al. (2012) with some slight modifications. A LC/MS Hewlett Packard (HP) System equipped with LC

Binary Pump HP Series 100 coupled to Single Quadrupole Mass Spectrometer 1,100 Series MSD using Electrospray (ESI) interface was used. PbTx chromatographic separation was carried out using Luna C18 Reverse Phase LC column (50×2 mm, 3 μm , Phenomenex) as stationary phase. PbTx elution was conducted by using mobile phase A (33 mM of ammonium hydroxide and 500 mM ammonium formate in 50% v/v of MeOH) and mobile phase B (33 mM of ammonium hydroxide and 500 mM ammonium formate in 90% v/v of MeOH). The gradient is recorded in **Supplementary Table 1**. The flow rate was 0.20 mL/min, the injection volume was 20 μL , and the column was set at room temperature. Positive electrospray ionization was used with the following parameters: nitrogen (N_2) gas flow 9 L/h, drying gas temperature 350°C , capillary voltage 3,500 V, and fragmentor 120 V. Single Quadrupole mass analyzer was operated in SIM (Single Ion Monitoring) mode. PbTx2 was monitored select m/z (mass/charge) 896 and PbTx3 898, both corresponding to $[\text{M} + \text{H}]^+$ protonated molecule fragment ion. PbTx2 and PbTx3 standard solutions were kindly provided by Dr. Robert W Dickey (Marine Sciences, University of Texas, United States); 1 $\mu\text{g}/\text{mL}$ of individual toxin standard solution was prepared in methanol (grade LC-MS) and used for the identification and quantitation of brevetoxins in dolphin samples.

Diet (Prey Items) Analysis

Gastric contents including intact or fragments of teleost fishes as well as squid beaks and otoliths were collected and submitted for taxonomic identification using national and regional catalogs and identification keys.

Microbiologic Analysis

Fresh tissue samples (liver, kidney, and lung) from six animals were collected routinely during necropsy, were frozen (-80°C) and selectively submitted for bacteriologic analysis. These included routine culture and surface plating on routine media, e.g., Columbia blood agar and preliminary identification of isolates via API[®] system (API[®] 20E, API[®] Rapid 20E, API[®] Staph, API[®] 20 Strep, API[®] Coryne, API[®] 20A). PCR targeting the 16S rRNA gene coupled with pulsed-field gel electrophoresis were performed on selected isolates.

Virologic Analysis

At necropsy, selected samples (liver, kidney, lymph nodes, brain) were collected for virologic analyses and stored frozen at -80°C until processing for molecular virology testing. Approximately 0.5 gr of fresh-frozen tissue sample from each animal was mechanically macerated in lysis buffer and subsequently centrifuged. DNA/RNA extraction was carried out from each 300 μL macerated sample by pressure filtration method, using a QuickGene R Mini 80 nucleic acid isolation instrument, using the DNA Tissue Kit S (QuickGene, Kurabo, Japan) according to the manufacturer's instructions with modifications: RNA carrier (Applied Biosystems[™], Thermo Fisher Scientific Waltham, Massachusetts, United States.) was added during the lysis step (Sacristan et al., 2015). Herpesvirus DNA was detected by conventional nested PCR using degenerate primers designed to amplify a region of the DNA polymerase

gene (VanDevanter et al., 1996). Molecular detection of cetacean morbillivirus (CeMV) was performed by one or more of four different PCR methods: one-step RT-PCR of a 426-bp conserved region of the phosphoprotein (P) gene (Reidarson et al., 1998), one-step real-time RT-PCR that detect the most common CeMV strains known to circulate in the Atlantic Ocean targeting the P gene (Groch et al., 2020), RT-PCR using nested primers targeting the P gene (Sierra et al., 2014), and one-step real-time RT-PCR to detect sequences in a conserved region (192 bp) of the fusion protein (F) gene (Sacristan et al., 2015).

RESULTS

Epidemiology, Pathologic Investigations, and Diet Analysis

On April 15th, a group of approximately 50 rough-toothed dolphins was sighted in the area where carcasses were later found (27°48'15"N; 15°46'46"W). This group is well known in the area by whale-watching ships as they could be found close to shore (< 2–3 nautical miles). That day the animals were observed as usually, in a very cohesive group including adults and calves. On April 16th, biologists working on board (whale-watching ships) informed about seagrass floating in that area; no alterations on behavior or social structure were noticeable in the group of dolphins. However, on April 17th, the group had dispersed in small groups or individually. One of the dolphins was very quiet for a long time and behaved oddly (as per on board biologist). On April 23rd evening, one dolphin was found dead floating with moderate autolysis around the same location as previous sightings. On April 24th, a group of dolphins ($n = 30$) was observed (within < 1 nautical mile) displaying normal cohesive behavior including adults and juveniles. On April 27th, a second dolphin was found dead floating. Additional carcasses were recovered on the following days, two on April 29th, two on April 30th, one on May 1st, two on May 4th, and one on May 5th, 7th, and 10th, each, mostly with advanced/very advanced autolysis.

These 12 dolphins were investigated. All the dolphins were in apparent good body condition. Age classes were: neonate ($n = 1$), adult ($n = 5$), subadult ($n = 6$); sex was: male ($n = 6$), female ($n = 5$). Nine were found adrift and three beached dead along the coast. Four presented advanced autolysis and eight had very advanced autolysis (Table 1) at necropsy. Pathologic examinations were limited by carcass preservation status; however, varying degrees of multisystemic hemorrhage within the subcutis and axial muscles in occipital, intermandibular, thoracic, and abdominal regions, as well as hemorrhage in the rete mirabile, stomach, esophagus, larynx, and lung, were seen (Figure 1). Most of the animals presented undigested or partially digested prey items within the gastric compartments, primarily *Sarpa salpa* (Figure 2). Pathologic findings are recorded in Table 2.

A total of 28 samples from 7 dolphins was examined (Table 3). The retention times observed for each toxin were 13.42 min for PbTx3 and 13.81 min for PbTx2 (Figure 3). Positive samples for PbTx were those in which PbTx2 and PbTx3 were detected; chromatographic data and m/z fragment ion values for these two

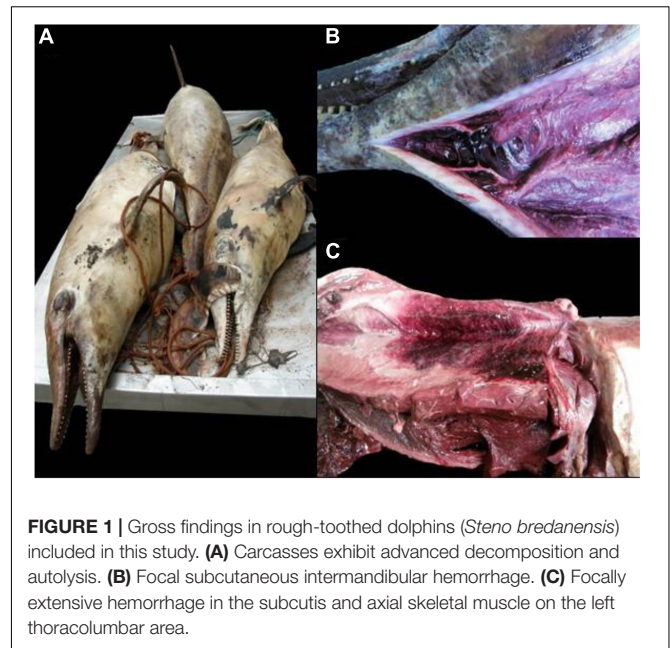


FIGURE 1 | Gross findings in rough-toothed dolphins (*Steno bredanensis*) included in this study. **(A)** Carcasses exhibit advanced decomposition and autolysis. **(B)** Focal subcutaneous intermandibular hemorrhage. **(C)** Focally extensive hemorrhage in the subcutis and axial skeletal muscle on the left thoracolumbar area.

toxins, for which reference standards were available, were taken into account. Qualitative LC-MS analysis results obtained for seven dolphins tested (cases 190 through 196) are summarized in Table 3. PbTx2 and PbTx3 were detected in gastric contents of 3/4 and 4/4 animals, respectively. An example of LC-MS chromatogram corresponding to the analysis of liver extract from case #196 where PbTx2 and PbTx3 were detected at trace level is recorded in Figure 4.

Microbiologic Analysis

Bacterial culture of liver, kidney, and lung from six animals yielded *Clostridium novyi*. Animals tested were negative for CeMV and HV.

DISCUSSION

Brevetoxicosis was first suspected and later confirmed to result in cetacean fatalities since 1946, primarily in manatees and to a lesser extent in common bottlenose dolphins in the Gulf of Mexico (Gunter et al., 1948; Van Dolah, 2005). Several UME involving common bottlenose dolphins have been described ever since, primarily in the west coast of Florida and Florida Panhandle regions yet fewer fatalities have been seen in the Atlantic coast of Florida (Mase et al., 2000; Flewelling et al., 2005; Gaydos, 2006; Twiner et al., 2012; Litz et al., 2014; Fire et al., 2015). Mortalities of dolphins with multiple biotoxins including brevetoxins found in the carcasses have been documented (Fire et al., 2011). Little is known about the effects of exposures to multiple biotoxins and whether the toxins are synergistic, additive or neutral. Limited clinical signs have been documented in cetaceans with brevetoxicosis, mainly due to the peracute and fatal nature of the toxicity. Clinical signs may include stranding, seizures, and incoordination, leading to

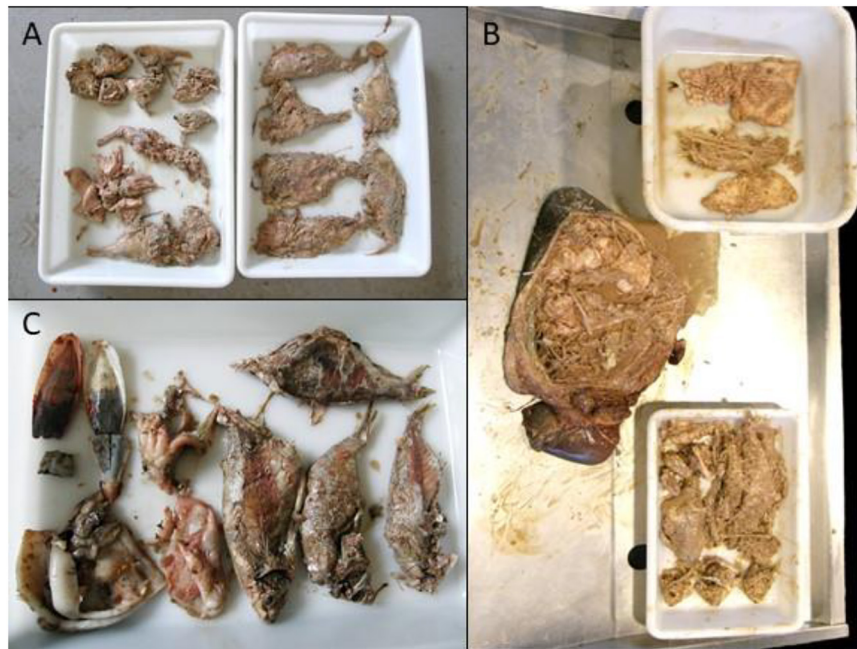


FIGURE 2 | Gastric contents in case 9 (A), case 8 (B) and case 5 (C).

death (Fauquier and Landsberg, 2018). Furthermore, brevetoxins have been shown to interfere with lymphocyte function *in vitro* (Gebhard et al., 2015), which could be a potential sublethal effect. This remains to be proven *in vivo*. Pathologic findings in cetaceans known to have succumbed to brevetoxins are largely lacking, often due to the poor preservation status of the carcasses at necropsy or to the peracute neurotoxic death, although even fresher carcasses may lack pathologic findings attributable to PbTx (Mase et al., 2000; Fauquier and Landsberg, 2018).

The pathogenic mechanisms of brevetoxins are not fully elucidated (Twiner et al., 2012). Brevetoxins act on voltage-gated sodium channels (Catterall and Gainer, 1985) by binding to neurotoxin receptor site 5, resulting in persistent channel activation (Jeglitsch et al., 1998). This action is particularly prominent in parasympathetic nerves and adrenergic nerve fibers. Bronchospasm ensues when the toxin is inhaled due to release of acetylcholine in postganglionic parasympathetic nerve endings (Smart, 1995). Studies suggest these toxins inhibit neurotransmission to skeletal muscles, yet paralysis has not been documented (Smart, 1995). Also, brevetoxins are potent cardiac toxins (Kirkpatrick et al., 2004). Furthermore, cutaneous exposure to brevetoxins in aerosol may induce irritation, cough, shortness of breath and tearing; ingestion of brevetoxins may result in gastrointestinal and peripheral and central nervous system injury. In our study, it is possible that a presumptive *K. brevis*-hemolytic toxin (Tatters et al., 2010) could have played a role; we cannot further evaluate this hypothesis because we did not investigate this specific hemolytic toxin. Interestingly, *C. novyi* was identified in a subset of animals. While *C. novyi* subtypes may produce several toxins and may be associated with necrotizing and hemorrhagic lesions in muscle and other

organs in terrestrial mammals (Uzal et al., 2016), its pathogenic role remains unknown in marine mammals (Li et al., 2019). Typing and toxinotyping analyses were not conducted on these isolates, thus, further conclusions cannot be drawn. It is possible that autolysis and decomposition phenomena (carcasses were probably sunken for several days and then resurfaced) have favored this probable commensal or environmental bacterium to appear in various tissues. Noteworthy, we detected a still birth that could have potentially resulted from abortion secondary to brevetoxicosis. Brevetoxins have been detected in fetal and neonate dolphins, providing evidence of maternal transfer of the toxin in wild populations (Fire et al., 2015).

Diagnosis of brevetoxins may rely on enzyme linked immunosorbent assay, radioimmunoassay, electrochemiluminescence essays, electrochemical immunoanalysis, electrochemical impedance spectroscopy, spectroscopic ellipsometry, liquid chromatography-mass spectrometry, and immunohistochemistry (Bossart et al., 1998). In this study, PbTx2 and PbTx3 were identified and confirmed in gastric contents of 3/4 and 4/4 animals. Studies have reported a wide range of PbTx in bottlenose dolphin gastric contents (174–6,235 ng/g) and livers (37–278 ng/g) (Flewelling et al., 2005; Fire et al., 2008b, 2015; Flewelling, 2008; Twiner et al., 2012), however, the lethal dose remains elusive (Hort et al., 2021). Comparatively, experimental studies in mice have shown a median lethal dose value (LD₅₀) of 520 mg/kg bodyweight for PbTx-3 and 66,000 mg/kg bodyweight for PbTx-2 (Baden and Mende, 1982). Furthermore, in humans, it is estimated that an exposure of 2–3 µg/kg bodyweight for PbTx-3 may produce non-lethal toxic effects (Cunningham et al., 2021). While PbTx have been detected and quantified in various organic matrices,

TABLE 2 | Pathologic findings in rough-toothed dolphins (*Steno bredanensis*) included in this study.

| Case | Gross | Microscopic | Gastric content |
|------|--|--|--|
| 1 | Moderate number of <i>Xenobalanus</i> sp. in pectoral fins; Mild cutaneous infection by <i>Phyllobotrium delphini</i> at anogenital and peduncle region; Mild infection by <i>Monorygma grimaldi</i> within peritoneal cavity; Multifocal acute hemorrhage in right lateral thorax, peritoneum, intercostal muscles, diaphragm; Serosanguineous fluid within thoracic cavity; Multiple accessory spleens. | Mild, multifocal renal cortical and medullary tubular mineralization | Abundant undigested ingesta in pharynx, esophagus and gastric compartments (fish) |
| 2 | Multifocal acute subcutaneous and muscular hemorrhage in suprascapular, intercostal dorsal, cervical, base of tongue, larynx, lung, kidney, diaphragm, and rete mirabile; Mild cutaneous infection by <i>P. delphini</i> at anogenital and peduncle region; Mild infection by <i>M. grimaldi</i> within peritoneal cavity; Serosanguineous fluid within thoracic and peritoneal cavities; Multifocal granulomatous gastritis with <i>Pholleter gastrophilus</i> in glandular compartment; Moderate hepatobiliary infection by <i>Brachycladiidae</i> ; Multiple accessory spleens; Multifocal suppurative mastitis. | <i>Nasitrema</i> sp. eggs within alveoli; Mild, multifocal renal cortical and medullary tubular mineralization | Moderate undigested ingesta in keratinize gastric compartment (fish, spines, lenses, otoliths) |
| 3 | Multifocal acute subcutaneous and muscular hemorrhage in ventro-abdominal muscles, scapular, right lateral thorax, intercostal, pharynx, peritoneum, lung, rete mirabile, and kidney; Serosanguineous fluid within thoracic cavity; Laryngeal edema; Abdominal viscera evisceration through the umbilicus; Multifocal granulomatous gastritis with <i>P. gastrophilus</i> in glandular compartment; Multiple accessory spleens; | NSFO | Mild undigested ingesta within esophagus and gastric compartments (fish head, otoliths). |
| 4 | Abdominal viscera evisceration through the umbilicus; Mild cutaneous infection by <i>P. delphini</i> at anogenital and peduncle region; Multifocal acute subcutaneous and muscular hemorrhage in thoracic, intercostal muscles, peritoneum, rete mirabile, diaphragm and mediastinum, kidney; Pulmonary edema; Serosanguineous fluid within thoracic cavity; Marked dental wear, fracture and tooth loss. | Granulomatous dermatitis and panniculitis with extracellular and phagocytized yellow globules; Intrasinus trematode egg within prescapular lymph node. | Mild undigested ingesta within gastric compartments (scales, spines, squid). |
| 5 | Diffuse skin loss (postmortem sloughing); loss of fluke and cephalic soft tissue; Multifocal acute subcutaneous and muscular thoracolumbar hemorrhage, bilateral | NSFO | Moderate undigested ingesta within gastric compartments. |
| 6 | Multifocal acute subcutaneous and muscular hemorrhage in thorax and abdomen; Skin loss (postmortem sloughing); loss of fluke. | Multifocal intrahepatic vascular trematode eggs. | NR |
| 7 | Multifocal acute subcutaneous and muscular hemorrhage in left thorax, ventro-abdominal, keratinized gastric compartment, larynx, lung; Mild cutaneous infection by <i>P. delphini</i> at anogenital and peduncle region; Mild degenerative joint disease of the scapula-humeral joint; Serosanguineous fluid within thoracic cavity; Focal accessory spleen; Multifocal granulomatous gastritis with <i>P. gastrophilus</i> in glandular and pyloric compartments. | Intrasinus trematode egg within gastric lymph node. | Abundant undigested ingesta in gastric compartments (fish, spines, otoliths) |
| 8 | Diffuse skin loss (postmortem sloughing); Abdominal viscera evisceration through the inguinal region; Mild cutaneous infection by <i>P. delphini</i> at anogenital and peduncle region; Multifocal acute subcutaneous and muscular hemorrhage in cervical region, esophageal, and tracheal serosae, lung; Moderate serosanguineous fluid within peritoneal cavity; Moderate <i>Nasitrema</i> sp. infection in pterygoid sinuses. | Mild, multifocal, chronic mononuclear interstitial nephritis | Abundant undigested ingesta in gastric compartments |
| 9 | Multifocal acute subcutaneous and muscular hemorrhage in thoracolumbar, bilateral, left scapula-humeral joint, intermandibular, esophagus, larynx, trachea, lungs, mediastinal and prescapular lymph node, pterygoid sinuses; Laryngeal edema and congestion; Serosanguineous fluid within peritoneal cavity; Severe infection by <i>M. grimaldi</i> within peritoneal cavity; Severe <i>Nasitrema</i> sp. infection in pterygoid sinuses. | NSFO | Oral seagrass; Small number of plastic particles among teeth; Abundant undigested ingesta in gastric compartments (fish, squid). |
| 10 | Seagrass in caudal fin; Intestine evisceration through ventral region; Multifocal acute subcutaneous and muscular hemorrhage in thoracolumbar bilateral, retromandibular, intercostal, dorsal and ventral lumbar, lung; laryngeal edema; Abundant serosanguineous fluid within peritoneal and thoracic cavities; Mild cutaneous infection by <i>P. delphini</i> at anogenital and peduncle region; Moderate <i>Crassicauda</i> sp. and <i>Nasitrema</i> sp. coinfection in pterygoid sinuses. | NSFO | Abundant undigested ingesta in gastric compartments (fish, squid, scales, otoliths; intact fish in esophagus). |
| 11 | Abdominal viscera evisceration through the ventro-abdominal region; Multifocal acute subcutaneous and muscular hemorrhage in thoracolumbar bilateral, periscapular, pharynx, epaxial and hypaxial muscles, lungs, mediastinal; Mild cutaneous infection by <i>P. delphini</i> at anogenital and peduncle region; Moderate infection by <i>M. grimaldi</i> within peritoneal cavity; Mild intestinal infection by unidentified nematodes; Abundant serosanguineous fluid within peritoneal and thoracic cavities; Mild mammary infection by <i>Crassicauda</i> sp.; Diffuse mammary gland hyperplasia (compatible with postpartum hyperplasia); Mild <i>Nasitrema</i> sp. infection in pterygoid sinuses. | NSFO | Lack of ingesta in gastric chambers |
| 12 | Multifocal acute subcutaneous and muscular hemorrhage in thoracolumbar, left pectoral fin, and peritoneal wall; Fetal bodily folds; Flaccid caudal fin; Abdominal viscera evisceration through the umbilicus (aborted fetus); diffuse bilateral pulmonary atelectasis; persistent ductus arteriosus; Incompletely fused fontanel. | NSFO | Small amount of sand within the proximal esophagus. |

NSFO, no significant findings observed.

TABLE 3 | Results obtained for brevetoxins screening analysis by LC-MS of dolphin samples collected in Canary Island (Spain).

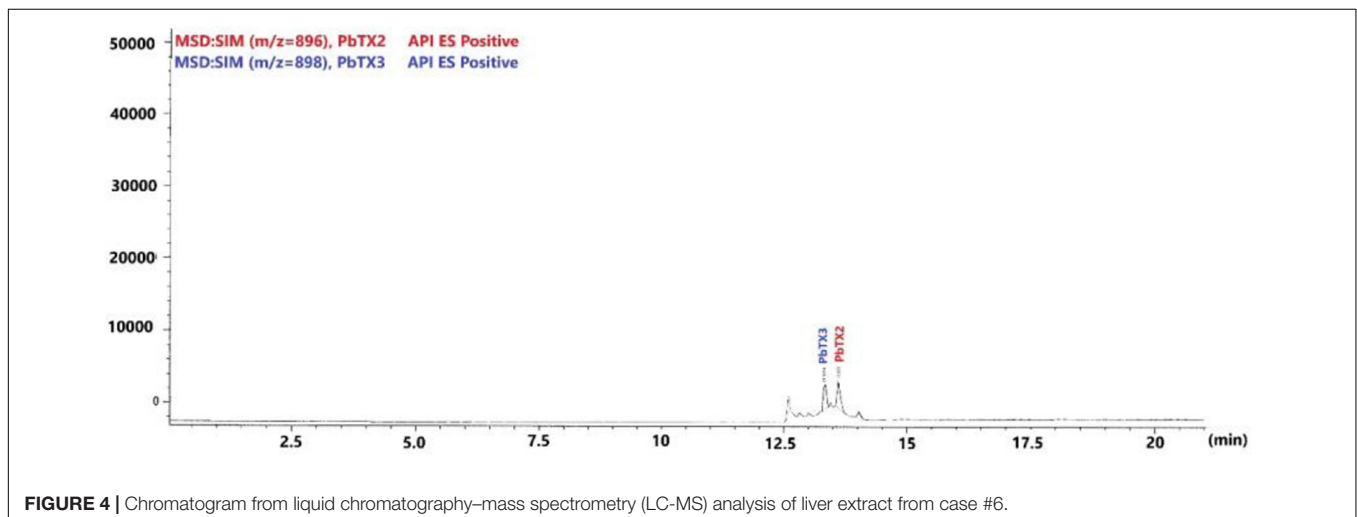
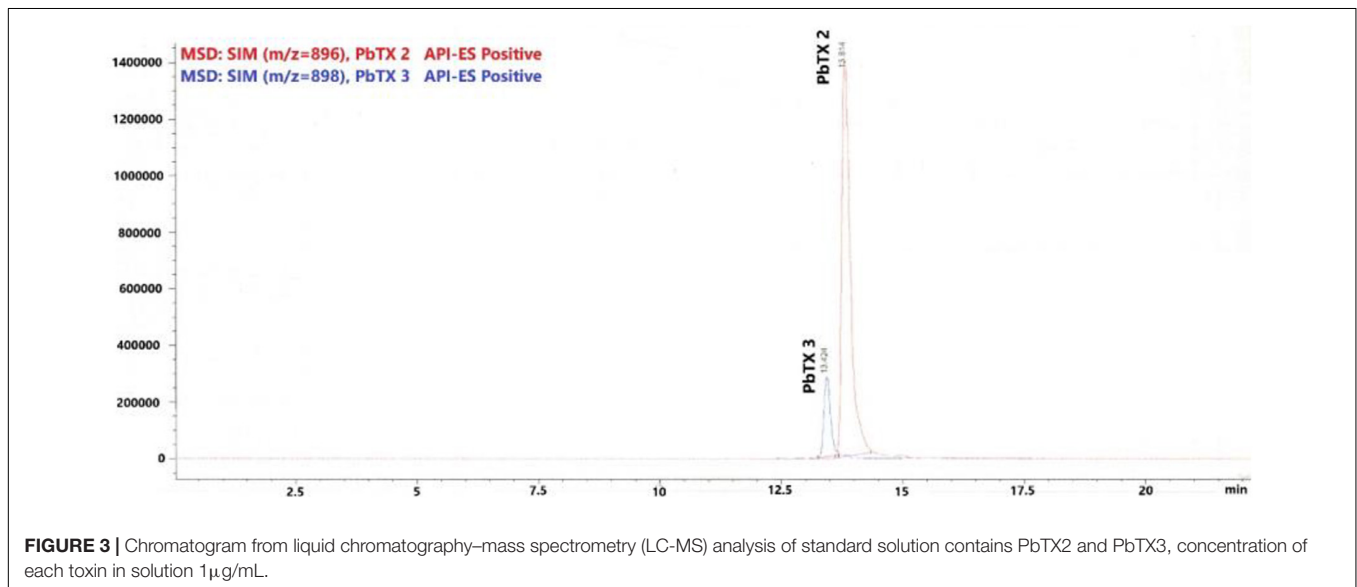
| Case no. | Sample (n = 28) | | | |
|----------|-----------------|--------|-------|--------|
| | Stomach content | Muscle | Liver | Kidney |
| 190 | + | + | + | + |
| 192 | ND | + | + | + |
| 195 | ND | ND | + | + |
| 196 | + | ND | + | + |
| 197 | ND | + | + | + |
| 198 | + | + | + | + |
| 199 | + | + | + | + |

ND, Not detected; +, PbTx 2 and 3 were detected in sample extract.

gastric content is regarded as the most reliable matrix for acute brevetoxin exposure determination, yet other matrices allow for diagnosis at lower sensitivities, e.g., feces, urine, liver

(Fire et al., 2015). Unfortunately, no quantification analysis was conducted on these samples, therefore, conclusions on this regard cannot be drawn.

Transmission is via inhalation (Bossart et al., 1998) or ingestion (Flewelling et al., 2005; Capper et al., 2013). Toxins remain stable in the environment and within seagrass, and mass mortalities can be seen long after and/or remotely located from a known algal bloom (Flewelling et al., 2005; Capper et al., 2013). PbTx are released upon algal cell lysis and can be aerosolized in the surf zone, by waves, or by wind, and present a risk of exposure by inhalation (Pierce, 1986). Finfish species (Fire et al., 2008a; Twiner et al., 2011) and certain types of seagrass can accumulate or vector brevetoxins and play a primary role in brevetoxin-induced marine mammal mortality events or strandings (Flewelling et al., 2005). In this study, most of the animals presented undigested prey items (in agreement with previous observations) within the gastric compartments wherein salema porgy was overrepresented. The latter is a



herbivorous teleost fish that preferentially feeds on the seagrass *Posidonia oceanica* through the year (Peirano et al., 2001; Prado et al., 2008) and is used for human consumption. It is found over rocky substrates and sandy area with algal growth. Young mainly carnivorous on crustaceans, adults almost exclusively herbivorous, feed on seaweeds. Interestingly, consumption of this fish is inadvisable in certain periods of the year (spring and summer) because of ciguatoxins (Raikhlín-Eisenkraft et al., 1988; Chevaldonne, 1990). As in previous reports (Fire et al., 2021), our data suggest dietary exposure rather than inhalation of brevetoxin was the primary route of intoxication for these cetaceans.

Oceanographic investigations (data not shown) including biogeochemical parameters, surface water temperature and primary production analyses did not identify HAB or specific singularities, except for mildly increased chlorophyll levels. No phytoplankton data from the water body were available for the study period. It is believed that satellite (two)-resolution limitations (1 km²) hampered detection of HAB in this event (González-Ramos, personal communication). There are records of *Karenia* and other dinoflagellates in neritic and oceanic waters off central eastern Atlantic Ocean, including Canarian waters since 2004 (Ojeda, 2005, 2013), likely conditioned by global climate change, with a slow and progressive warming of the waters that has led to the settlement of species from warmer areas.

In summary, we documented the first cetacean mass-mortality event linked to brevetoxicosis in the eastern Atlantic Ocean. Epidemiologic data, including strandings or retrieval dates as well as carcass decomposition clearly indicated the cluster of deaths occurred at around the same time and area. Pathologic examinations, although limited by carcass preservation status, demonstrated consistent multisystemic hemorrhage and undigested or partially digested prey items, predominantly salema porgy. Toxicologic analyses of gastric contents detected PbTx2 and PbTx3. No other biotoxin related mortality has been recorded in the Canary Islands since this event. Brevetoxins should be considered in the differential diagnosis of cetacean mortality in the eastern Atlantic Ocean. These results raise concern for public health. Therefore, future monitoring for this type of events throughout the stranding programs or observation networks in Europe and/or partnership with the Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB) would be of great value.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

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ETHICS STATEMENT

Ethical review and approval was not required for the animal study because ethical review and approval were waived for this study, as neither animals were sacrificed nor experiments were performed with live animals. The permission for the management of stranded cetaceans was issued by the Spanish Ministry of Environment.

AUTHOR CONTRIBUTIONS

AF, ES, MAO, YB, and JDD contributed to the conception and design of the study. AF, ES, MAO, and YB performed the necropsy examination. AF, ES, MAO, and JDD conducted histopathologic examinations. AG-M, JL, NG-Á, and MAI conducted chemical analyses. ES conducted virologic analysis. AV conducted bacteriological investigations. AF and JDD wrote the first draft of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.834051/full#supplementary-material>

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