



Clinical signs and histopathology associated with domoic acid poisoning in northern fur seals (*Callorhinus ursinus*) and comparison of toxin detection methods

Kathi A. Lefebvre^{a,*}, Alison Robertson^a, Elizabeth R. Frame^a, Kathleen M. Colegrove^b, Shelly Nance^a, Keri A. Baugh^a, Heather Wiedenhoft^a, Frances M.D. Gulland^c

^a NOAA Fisheries, Northwest Fisheries Science Center, Marine Biotoxins Program, 2725 Montlake Blvd. East, Seattle, WA 98112, USA

^b Zoological Pathology Program, University of Illinois College of Veterinary Medicine, LUMC, Bld. 101, Rm 0745, 2160 S. First Ave., Maywood, IL 60153, USA

^c The Marine Mammal Center, Marin Headlands, 2000 Bunker Road, Fort Cronkhite, Sausalito, CA 94965, USA

ARTICLE INFO

Article history:

Received 11 September 2009

Received in revised form 21 January 2010

Accepted 21 January 2010

Keywords:

Algal toxin
Biotoxin detection methods
Biotoxin trophic transfer
Callorhinus
Domoic acid
Northern fur seal

ABSTRACT

Between July 2005 and March 2009, 33 northern fur seals (*Callorhinus ursinus*) were collected after stranding along the central California coast between Sonoma and San Luis Obispo counties. Of these, 26 were collected live and could be observed for signs of neuroexcitotoxicity. Approximately half exhibited the classic clinical signs of domoic acid (DA) toxicosis including muscle twitches and ataxia, to seizures and coma, and had lesions in the central nervous system and heart. Several biological fluids were collected for DA analysis including aqueous humor, serum, stomach contents, feces, urine, abdominal fluid, amniotic fluid and milk. Four analytical methods were employed including receptor binding assay (RBA), enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC-UV) and ultra performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS). The DA concentrations determined by each method were positively correlated. Domoic acid was detected in 83% of fecal samples collected from northern fur seals in the present study and in one animal was calculated to contain up to 18.6 $\mu\text{g DA/g}$. Interestingly, DA was detected and confirmed in the aqueous humor of the only animal this sample-type was collected from, suggesting that this may prove to be a useful diagnostic body fluid for algal toxin detection in marine mammal mortality events. These data document for the first time that northern fur seals are impacted by DA-producing harmful algal blooms along the California coast.

Published by Elsevier B.V.

1. Introduction

Domoic acid (DA) is a potent marine neurotoxin produced by some diatom species of the genus *Pseudo-nitzschia* and impacts of these toxic species appear to be becoming more frequent along the West Coast of the United States (Fryxell et al., 1997; Van Dolah, 2000). This is a concern for mammal health because DA is an excitatory amino acid that has a high affinity for the α -amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) and kainate subclasses of glutamate receptors that are present in the mammalian central nervous system. The interaction of DA and these glutamate receptors causes cell depolarization, dysfunction and death (Olney et al., 1979; Jeffery et al., 2004). In humans, DA is the cause of a well-described neurotoxic illness termed amnesic shellfish poisoning (ASP), first recognized in Canada in 1987

following consumption of contaminated shellfish (Perl et al., 1990). Clinical signs in human poisoning events included gastrointestinal distress, seizures, agitation, coma and death (Teitelbaum et al., 1990).

Domoic acid toxicosis was first reported in wildlife in 1991 when pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) died in Monterey Bay (Work et al., 1993). It was then documented in California sea lions (*Zalophus californianus*) in 1998 (Scholin et al., 2000), and every year since then sea lions have stranded displaying severe neurological signs associated with acute and long term effects of DA exposure (Gulland et al., 2002; Goldstein et al., 2008). Lesions in sea lions that die following acute intoxication of DA are characterized by ischemic neuronal necrosis in the hippocampal formation, especially granular cells in the dentate gyrus and pyramidal cells in sectors CA4, CA3 and CA1, and progress to neuronal loss with parenchymal atrophy and marked gliosis in sea lions dying after 15 days (Silvagni et al., 2005; Goldstein et al., 2008). A degenerative cardiomyopathy has also been reported in California sea lions, and

* Corresponding author. Tel.: +1 206 302 2454; fax: +1 206 860 3335.
E-mail address: Kathi.Lefebvre@noaa.gov (K.A. Lefebvre).

likely results from direct activation of cardiac glutamate receptors by DA (Zabka et al., 2009).

Despite the well-documented effects of DA on California sea lions and its increased detection in the marine food web (Lefebvre et al., 1999), effects on other marine wildlife are poorly understood. The toxin has been detected in feces of live swimming blue (*Balaenoptera musculus*) and humpback (*Megaptera novaeangliae*) whales (Lefebvre et al., 2002) as well as stranded pygmy and dwarf sperm whales (*Kogia* spp.) (Fire et al., 2009). Cetacean mortality, especially of common dolphins (*Delphinus* spp.) in southern California in 2002, has been temporally associated with DA producing *Pseudo-nitzschia* blooms (Torres de la Riva et al., 2009). Domoic acid exposure has also been associated with mortality and risk of development of cardiomyopathy in southern sea otters (*Enhydra lutris*) (Kreuder et al., 2003). However, in marine mammals other than California sea lions, the association between DA exposure and abnormal clinical signs and lesion development has been limited to epidemiological associations rather than direct measurement of DA in body fluids of affected animals. In the present study, we examined stranded northern fur seals (*C. ursinus*) along the California coast to identify potential exposure and health impacts of DA in this species.

Northern fur seals range widely across the North Pacific Ocean from California to Japan, but breed at a limited number of sites (rookeries) in July where they give birth to a single pup, lactate for 3–4 months, then disperse at sea until the following breeding season (Gentry and Kooyman, 1986). The main rookeries in the eastern North Pacific are on San Miguel Island off California (Fig. 1), and the Pribilof Islands in the Bering Sea. The populations on the Pribilof Islands are declining annually at approximately 5% per year, whereas the population on San Miguel Island has increased since the 1980s; reasons for these different population changes are unclear (Towell et al., 2006; Sinclair et al., 2008; Lea et al., 2009).

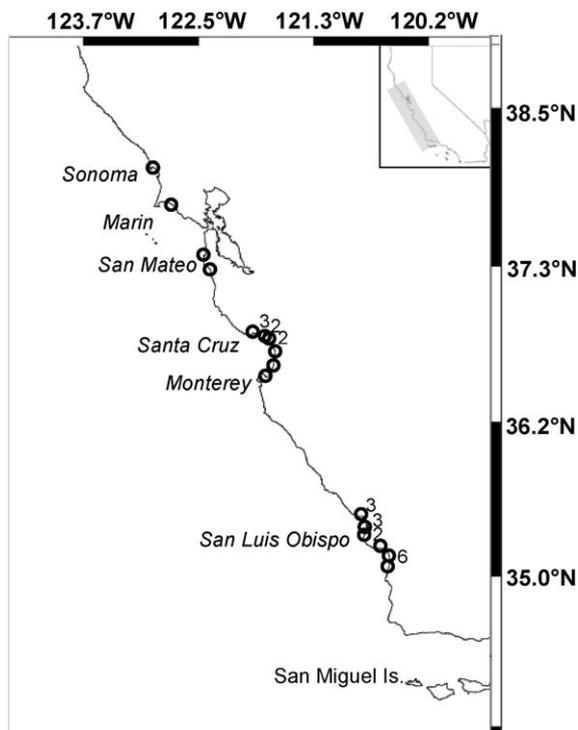


Fig. 1. Locations of the 33 northern fur seals (*Callorhinus ursinus*) used in this study. Open circles indicate the general stranding area of each animal. If more than one animal stranded in an area, that number is indicated to the right of the circle. County names are indicated in italics. Samples were collected from July 2005 to March 2009. The southernmost rookery for northern fur seals in the eastern North Pacific is located on San Miguel Island.

Fur seals migrate long distances between breeding seasons, with females traveling over 9000 km to forage along continental margins and frontal regions (Ream et al., 2005). They currently feed predominantly on pollock, gonatid squid, mackerel, capelin and salmon, although prey choice varies amongst individuals and across years (Sinclair et al., 2008). Domoic acid has been reported in prey and water samples from within the foraging range of this species, and has the potential to cause mortality and reduce reproduction in otariids (Scholin et al., 2000; Brodie et al., 2006). Here we present the first available data on the presence of DA in northern fur seals and document the associated clinical signs and histopathological lesions in tandem with validated toxin detection methods for multiple sample matrices.

2. Methods

2.1. Sample collection and assessment of northern fur seals

All northern fur seals collected for this study were found stranded along the California coast (between Sonoma and San Luis Obispo Counties) from July 2005 to March 2009, and were transported to The Marine Mammal Center, Sausalito, CA, for rehabilitation (Fig. 1). Animals were evaluated clinically and treatment was administered as dictated by clinical signs (Gulland et al., 2002). Animals that died or were euthanized due to deteriorating clinical signs had a complete necropsy and tissues were fixed in 10% formalin for histological examination. Where possible, select fluids including urine, feces, stomach contents, serum, abdominal fluid, amniotic fluid, aqueous humor, and milk were collected in plastic vials or bags and stored at -20°C until extraction and DA analysis could be performed.

2.2. Sample preparation and extraction

All samples analyzed for DA were extracted in 50% aqueous MeOH at a ratio of 4 ml/g. Fecal and stomach content extracts were homogenized for approximately 60 s using a Brinkman Polytron[®] PT3000 homogenizer. Homogenized samples were centrifuged at $10,000 \times g$ in a Sorvall[®] RC 5C Plus centrifuge for 20 min at 4°C . Aliquots of the supernatant were subsequently filtered (Millipore Ultrafree[®]-MC centrifugation device, Durapore[™] membrane, $0.22 \mu\text{m}$) in a desk-top microcentrifuge (Eppendorf model 5415C) at room temperature for 10 min at 14,000 rpm. Urine, serum, milk, amniotic fluid, abdominal fluid, and aqueous humor extracts were sonicated for 60 s with a Branson probe Sonifier 450 at 50% duty cycle (power setting 5). Sonicated extracts were centrifuged at $10,000 \times g$ in a Sorvall[®] RC 5C Plus for 20 min at 4°C . The supernatant was then filtered through a 25 mm diameter, $0.45 \mu\text{m}$ pore size syringe filter (Pall Gelman Acrodisc[®] PSF GxP with GHP membrane). Samples were stored at 4°C until analysis.

Select samples were cleaned up by solid phase extraction (SPE) for analysis by receptor binding assay (RBA) and high performance liquid chromatography (HPLC). These samples were extracted and prepared as described above with the addition of an SPE clean-up step using a quaternary amine (N+) disposable extraction column (JT Baker[®]). The SPE cartridge was conditioned with 6 ml of 100% MeOH, followed by 6 ml of water, and 6 ml of 50% MeOH. Sample aliquots (2 ml) were loaded onto the column and allowed to filter slowly, and were then followed by a 5 ml wash with 0.1 M NaCl. Domoic acid was eluted from the column in 5 ml of 0.5 M NaCl in 10% acetonitrile.

2.3. Enzyme-linked immunosorbent assay (ELISA) for domoic acid

Biosense[®] ELISA measurements of DA were performed according to the manufacturer's instructions (Biosense[®] Laboratories, Bergen,

Norway). Samples were diluted with sample buffer (10% MeOH in PBS-T) as determined by the matrix assessment (described below). In addition, a 10-point standard curve, an A_{\max} (maximum binding of anti-DA-HRP conjugate, yielding maximum absorbance) and a blank (background absorbance of TMB peroxidase substrate, no anti-DA-HRP conjugate, yielding minimum absorbance) were run with each assay. All samples, standards, A_{\max} , and blanks were analyzed in duplicate. As detailed in the kit instructions, pre-coated plate wells were soaked in washing buffer for 10 min, buffer was removed and sample added to duplicate wells. Diluted anti-DA-HRP conjugate was added to each well (except blank) and the plate was sealed and incubated for 1 h in the dark at room temperature. Following incubation, plates were washed in a Bio Tek[®] ELX50 plate washer and TMB peroxidase substrate was then added for an incubation period of 15 min. The reaction was stopped following the addition of sulfuric acid (0.3 M) for 2–5 min and absorbance measured at 450 nm on a VERSAmax microplate reader (MDS Analytical Technologies).

Matrix effects were evaluated using a sample of northern fur seal fecal material (prepared in 50% aqueous MeOH) that had been verified to be negative for DA by all methods. A titration curve was generated using the sample extract at the following dilutions: 1, 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, and 5120 (Fig. 2). From this curve, the minimum dilution required to avoid matrix effects was determined. Assay interference by MeOH was ruled out as all sample dilutions, with the exception of the undiluted extract, contained approximately 10% MeOH.

For each assay performed, a standard calibration curve was constructed and data were fit to a four-parameter logistic curve. Quantification of DA was then calculated using the template provided by the manufacturer (Biosense). Based on a working range determined by the I_{20} and I_{80} values from the calibration curve, DA values in samples were flagged as “within range”, “too dilute” or “too concentrated”. Any values flagged “within range” were accepted. Values flagged as “too dilute” were assigned a value of “below detection limit” (bdl) whilst samples which were flagged as “too concentrated” were diluted and re-analyzed until a “within range” value was obtained. The average CV for duplicate samples was $7.6 \pm 7.9\%$ (mean \pm sd) for all positive samples ($n = 34$).

2.4. Receptor binding assay (RBA) for domoic acid

RBA was used as a rapid screening tool to quantify DA activity in a selection of northern fur seal samples that were cleaned by SPE. This filtration assay determines the competitive displacement of a given sample to tritiated kainic acid (³H-KA) from a cloned glutamate receptor as previously described (Baugh et al., 2004; Van Dolah et al., 1997). Nine standard concentrations of DA in water (0.1–1200 ng/ml) plus a blank (distilled water) were used to generate a standard curve for each assay. Competition binding curves were fit with GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA). All samples and standards were treated with glutamate dehydrogenase to remove glutamate (Baugh et al., 2004). All samples were analyzed in triplicate and the mean values were used for quantification of unknowns. All extracts deemed positive were subsequently diluted to obtain a fraction bound value within the linear portion of the standard curve which was defined as that part of the curve between 20% and 80% of the total binding. The minimum detection limit in the extracts was 5 ng DA/ml, which is 50 ng DA/ml in the original sample material. Final concentration values were then adjusted to account for dilution factors from extraction and assay procedures.

2.5. High performance liquid chromatography (HPLC)

Samples that were cleaned by SPE were analyzed by (HPLC) using the isocratic method detailed by Quilliam (2003). A Hewlett-

Packard (HP) Model 1090 liquid chromatography system equipped with degasser, three-solvent system, autosampler, column oven and diode-array detector (DAD) was used for these analyses. Chromatographic separation was performed on a reverse phase C18 column (Vydac 5 μ m, 4.6 mm \times 150 mm) with guard, maintained at 40 °C with a flow rate of 0.4 ml/min. Isocratic analysis was performed with a mobile phase containing 10% aqueous acetonitrile containing the ion pairing agent, trifluoroacetic acid (TFA; 0.1%). An ultraviolet (UV) detection wavelength characteristic for DA was set at 242 nm (10 nm bandwidth), with a confirmatory wavelength set at 262 nm, and a reference wavelength at 450 nm (100 nm bandwidth).

Despite the use of SPE and sample dilution, these primary analyses were confounded by the presence of substantial matrix effects so a gradient method was formulated to improve the separation of DA from the matrix peaks. For these analyses, the stationary phase and conditions remained the same but analytes were separated using a mobile phase consisting of water (A) and 25% aqueous acetonitrile containing 0.1% trifluoroacetic acid (B). Samples and standards were then eluted with a linear gradient of 10–100% B over 12 min. A subsequent hold step at 100% B (3 min) and re-equilibration to 10% B (5 min) was added to flush all remaining matrix compounds from the column and ensure that starting conditions were established for each successive run. The level of DA detection in matrix by this method was determined to be 8 ng/ml (signal/noise = 3).

All solvents and water used for our analyses were of HPLC grade and purchased from JT Baker Laboratory Chemicals (Phillipsburg, NJ). A certified DA reference standard (DACS-E) was purchased from the National Research Council Certified Reference Materials Program (NRC-CRMP, Halifax, NS) for calibration, matrix assessment and spiking. Domoic acid was confirmed by retention time, spiking and assessment of the characteristic UV absorption spectrum ($\lambda_{\max} = 242$). Data analysis including calibration curve construction, peak integration and spectral interrogation was performed using Chemstation software (Agilent).

2.6. Ultra performance LC–tandem mass spectrometry (UPLC–MS/MS)

Selected northern fur seal samples were analyzed by UPLC–MS/MS in positive ion mode using multiple reaction monitoring (MRM) for the confirmation of DA. All MS experiments were performed using a Waters UPLC[®] system coupled to a Quattro micro triple quadrupole tandem mass spectrometer (MicroMass, Waters, US) fitted with an electrospray ionization interface (capillary: +2.6 kV, cone: +30 V). UPLC separation was achieved on an Acquity BEH C18 micro-particulate column (1.7 μ m, 50 mm \times 2.1 mm, Waters, MA) with a VanGuard[™] pre-column, both maintained at 35 °C. A flow rate of 0.8 ml/min resulted in a stable backpressure of approximately 10,000 psi and was used for all analyses. Samples (10 μ l injection volume) were separated using a shallow linear gradient from 5% to 15% B for 3 min, followed by a 30 s hold at 15% B, then a 90 s re-equilibration at 5%. The mobile phase consisted of water (A) and acetonitrile (B) both containing 50 mM formic acid.

Method optimization, calibration, retention time verification, matrix effects and characteristic MS/MS fragmentation of DA were determined using a certified DA reference standard (DACS-E) from NRC-CRMP (Halifax, NS). Identification of suspect toxin peaks was undertaken using multiple reaction monitoring (MRM) for three characteristic transition ions of the protonated DA including; 312 > 266 (collision: 15 eV), 312 > 248 (collision: 20 eV), and 312 > 161 (collision: 25 eV). The relative ion ratios for these three transitions was also recorded and used for additional verification of DA. All MS/MS parameters were optimized prior to analysis and

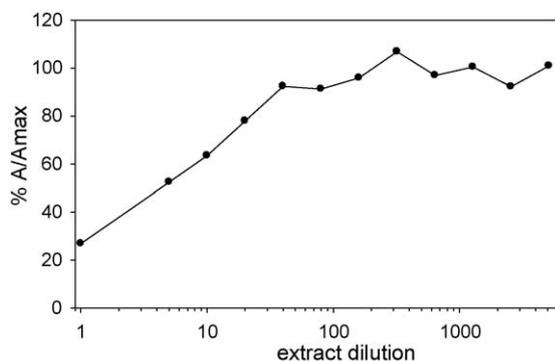


Fig. 2. Matrix effects in extracts of northern fur seal fecal samples at various dilutions using Biosense[®] ASP direct cELISA kits. Based on this curve a minimum dilution of 1:50 of the sample extract in Biosense sample buffer was used.



Fig. 3. Photo of one of the sixteen malnourished northern fur seal (*Callorhinus ursinus*) pups sampled in this study.

quantification was determined using an 8-point calibration curve (1 ng/ml–1 µg/ml DA). Matrix effects were assessed by spiking and standard addition experiments and samples were subsequently diluted as required to eliminate any observed effects such as ionization suppression and DA retention shifts. Data analysis including peak integration and quantitation was performed using MassLynx software (version 4.1; Waters Laboratory Informatics).

Mass spectrometer source and analyzer parameters were as follows: Extractor 11 V; RF Lens 0.5 V; source temperature 125 °C; desolvation temperature 390 °C; cone gas flow 200 L/h; desolvation gas flow 550 L/h; collision cell entrance –5 V and exit 1 V; and multiplier at 650 V.

3. Results

3.1. Clinical signs of ASP in northern fur seals

Thirty-three northern fur seals which stranded along the central California coast between July 2005 and March 2009 from San Luis Obispo County in the south to Sonoma County in the north were tested for the presence of DA in body fluids (Fig. 1). These animals were mostly adult females or newly weaned pups (Table 1), with the exceptions of one juvenile male stranded in Sonoma with a gunshot wound, and two *in utero* fetuses. One adult

and one pup had pink flipper tags identifying them as born and tagged on San Miguel Island (Fig. 1). From July 20–August 21, 2005, a cluster of nine adult females stranded showing neurological signs varying from muscle twitches and ataxia, to seizures and coma. Seizures became more frequent until death in four cases, whereas in five other cases seizures were controlled by medication with lorazepam (0.1 mg/kg intramuscularly) and phenobarbital (2 mg/kg twice daily intramuscularly), and the animals recovered within a week. One of the animals had a fish hook embedded in her skin and fishing line around the neck that was removed. All nine animals had detectable levels of DA in serum, urine or feces (Table 1). Two animals had DA in milk and one animal had detectable levels of DA in aqueous humor as well as in serum, stomach contents, feces, and milk (Table 1). A second cluster of three adult female fur seals stranded in April 2007. All three were pregnant; one aborted a dead fetus and lived, the other two died with dead fetuses *in utero*. Domoic acid was detected in fetal feces and stomach contents, and amniotic fluid from these cases (Table 1). A fourth pregnant female carcass was recovered from a fishing vessel off California in April 2008 and DA was detected in feces from this animal; however it was too decomposed for useful histological examination.

In the Fall months of October–December of each year, 16 emaciated recently weaned pups that stranded were tested for the presence of DA: one in 2005, nine in 2006, two in 2007, and four in 2008 (Table 1 and Fig. 3). No lesions or clinical signs were observed in the seven of these pups that died, other than severe atrophy of fat and muscle consistent with malnutrition. However, DA was detected at low levels in five of these pups and at moderate levels in three others (Table 1). One pup (NFS 189) stranded twice and was sampled both times for DA in urine (Table 1). Interestingly, this pup did not have quantifiable levels of DA in urine when sampled the first time it stranded in October 2006, but was found to have a DA concentration of 43 ng/ml in the urine (as determined by ELISA) when it re-stranded five months later in April. This pup died 10 days after its stranding the second time and histological examination revealed myocardial fibrosis, but no lesions were detected in the brain.

3.2. Histopathology of ASP in northern fur seals

Histopathologic findings in the female fur seals that died or were euthanized between 2005 and 2007 included hippocampal atrophy, extensive loss of granular cells in the dentate gyrus, loss of hippocampal pyramidal neurons in cornu ammonis (CA) sectors CA4, CA3, and CA1, intense gliosis throughout the hippocampus and adjacent temporal lobe, edema of the neuropil, and occasional acute neuronal necrosis (Fig. 4 and Table 2). Lesions were unilateral or bilateral. Perivascular cuffing with lymphocytes was less commonly noted, compared to lesions associated with DA in California sea lions (Silvagni et al., 2005). In one female that died in April 2007, the interventricular septum of the heart had a focal area of myocardial vacuolar degeneration and necrosis with replacement of lost myocytes by adipocytes (Fig. 5). Pregnant females or females that had aborted prior to death had varying degrees of acute endometritis.

3.3. Comparison of domoic acid detection methods

In general, DA values quantified via ELISA, UPLC–MS/MS, RBA and HPLC were in qualitative agreement when moderate to high levels of toxin were present in northern fur seal samples (Fig. 6). When DA values were ≥ 46 ng/g as measured by ELISA, all other methods also yielded positive DA measurements. At values below 46 ng/g, several samples were not quantifiable by RBA or UPLC–MS/MS. In comparisons with samples that were quantifiable by

Table 1
Domoic acid (DA) levels quantified via enzyme-linked immunosorbent assay (ELISA) ultra performance liquid chromatography–tandem mass spectrometry (LC–MS/MS), Receptor binding assay (RBA), and HPLC in thirty-three stranded northern fur seals (*Callorhinus ursinus*). Age class categories as follows: A: Adult, J: Juvenile, P: Pup, Fe: Fetus. Domoic acid levels are reported as ng DA/gram sample matrix (F: feces, U: urine, S: serum, SC: stomach contents, M: milk, AF: amniotic fluid, AbF: abdominal fluid, AH: aqueous humor).

ID#	Admit date	Age class	Sample type	Days after admit	[DA] (ng/g)			
					ELISA	LC–MS/MS	RBA	HPLC
NFS 151	7/20/05	A	U	One	46	371	512	398
NFS 152	7/27/05	A	U	One	192	352	563	458
NFS 153	7/28/05	A	AH	Two	249	167	na	na
NFS 153	7/28/05	A	F	Two	6730	54,730	na	na
NFS 153	7/28/05	A	M	Two	155	229	na	na
NFS 153	7/28/05	A	S	Two	134	828	na	na
NFS 153	7/28/05	A	SC	Two	65	466	na	na
NFS 155	8/5/05	A	F	Nine	33	na	na	na
NFS 155	8/5/05	A	U	Nine	bdl	bdl	bdl	bdl
NFS 157	8/7/05	A	U	One	2784	5630	12,693	13,661
NFS 158	8/8/05	A	S	One	286	811	na	na
NFS 158	8/8/05	A	SC	One	154	650	na	na
NFS 159	8/9/05	A	U	Same day	10	bdl	bdl	bdl
NFS 163	8/20/05	A	F	Eight	5	na	na	na
NFS 163	8/20/05	A	M	Eight	1	na	na	na
NFS 164	8/21/05	A	U	Same day	956	Trace	1169	3843
NFS 167	10/9/05	P	F	Four	bdl	bdl	bdl	bdl
NFS 171	10/16/06	P	F	Seven	244	1020	na	na
NFS 172	10/17/06	P	F	Six	27	na	na	na
NFS 175	10/30/06	P	F	One	bdl	na	bdl	bdl
NFS 177	11/1/06	P	U	Two	bdl	bdl	bdl	bdl
NFS 180	11/7/06	P	U	Two	bdl	bdl	bdl	190
NFS 189 ^a	11/17/06	P	U	Same day	bdl	bdl	bdl	bdl
NFS 190	11/18/06	P	U	Same day	bdl	bdl	bdl	300
NFS 191	11/18/06	P	F	Two	bdl	bdl	bdl	bdl
NFS 198	11/30/06	P	U	One	0	na	na	na
NFS 201	1/5/07	P	F	Three	2	bdl	bdl	0
NFS 203	4/6/07	A	F	Two	18,600	13,948	46,472	2796
NFS 203	4/6/07	A	S	Same day	2	bdl	na	na
NFS 204	4/8/07	A	F	Two	3	bdl	na	bdl
NFS 204F	4/8/07	Fe	AbF	Three	5	bdl	bdl	bdl
NFS 204F	4/8/07	Fe	AF	Three	20	bdl	bdl	bdl
NFS 204F	4/8/07	Fe	F	Three	3	bdl	bdl	bdl
NFS 205	4/14/07	A	F	Five	458	436	bdl	529
NFS 205F	4/14/07	Fe	F	Three	7	bdl	bdl	bdl
NFS 205F	4/14/07	Fe	SC	Three	bdl	bdl	bdl	bdl
NFS 205F	4/14/07	Fe	S	Three	bdl	bdl	na	na
NFS 189 ^a	4/18/07	P	U	One	43	na	na	na
NFS 207	8/9/07	J	S	Three	bdl	na	na	na
NFS 207	8/9/07	J	U	Three	bdl	na	na	na
NFS 209	10/28/07	P	U	Two	bdl	na	na	na
NFS 218	4/26/08	A	F	Same day	88	na	na	na
NFS 218	4/26/08	A	SC	Same day	bdl	na	na	na
NFS 218F	4/26/08	Fe	AF	Same day	bdl	na	na	na
NFS 219	10/4/08	P	U	Same day	1	na	na	na
NFS 224	10/16/08	P	U	Same day	3	na	na	na
NFS 231	11/3/08	P	U	Eighteen	1	na	na	na
NFS 232	11/22/08	P	U	Two	1	na	na	na
NFS 235	3/10/09	A	F	Two	6	na	na	na
NFS 235	3/10/09	A	S	Same day	bdl	na	na	na

bdl: Below detection limit and na: not analyzed.

^a A pup that stranded twice.

both ELISA and the other methods, DA values were positively correlated but only significantly in the ELISA vs RBA comparison ($n = 5$, $p = 0.001$, $r^2 = 0.98$, Fig. 6b).

A gradient UPLC–MS/MS method was developed and implemented during this study as the confirmatory method for analysis of DA. Domoic acid was detected in six matrices from the northern fur seal samples including feces, urine, serum, stomach contents, milk, and aqueous humor (see Fig. 7).

4. Discussion

The presence of DA in 83% of fecal samples (range 2–18,600 ng/ml) collected from northern fur seals in this study confirms that this species is at risk for DA exposure via the diet. In addition, the

detection of DA in 10 of 17 urine samples (range 1–2784 ng/ml) and three of six serum samples (range 2–286 ng/ml) further substantiates that the toxin is absorbed from the digestive tract, enters the blood stream and therefore could be transported to target receptors in the central nervous system. Finally, of the 33 animals examined, 26 were collected live and could be observed for behavioral signs of neuroexcitotoxicity. Of those animals, approximately half exhibited the classic clinical signs of DA toxicosis and lesions in the central nervous system at post mortem necropsy as described previously in California sea lions (Scholin et al., 2000; Gulland et al., 2002). Collectively, these data document for the first time that northern fur seal populations are impacted by DA-producing *Pseudo-nitzschia* blooms along the California coast.

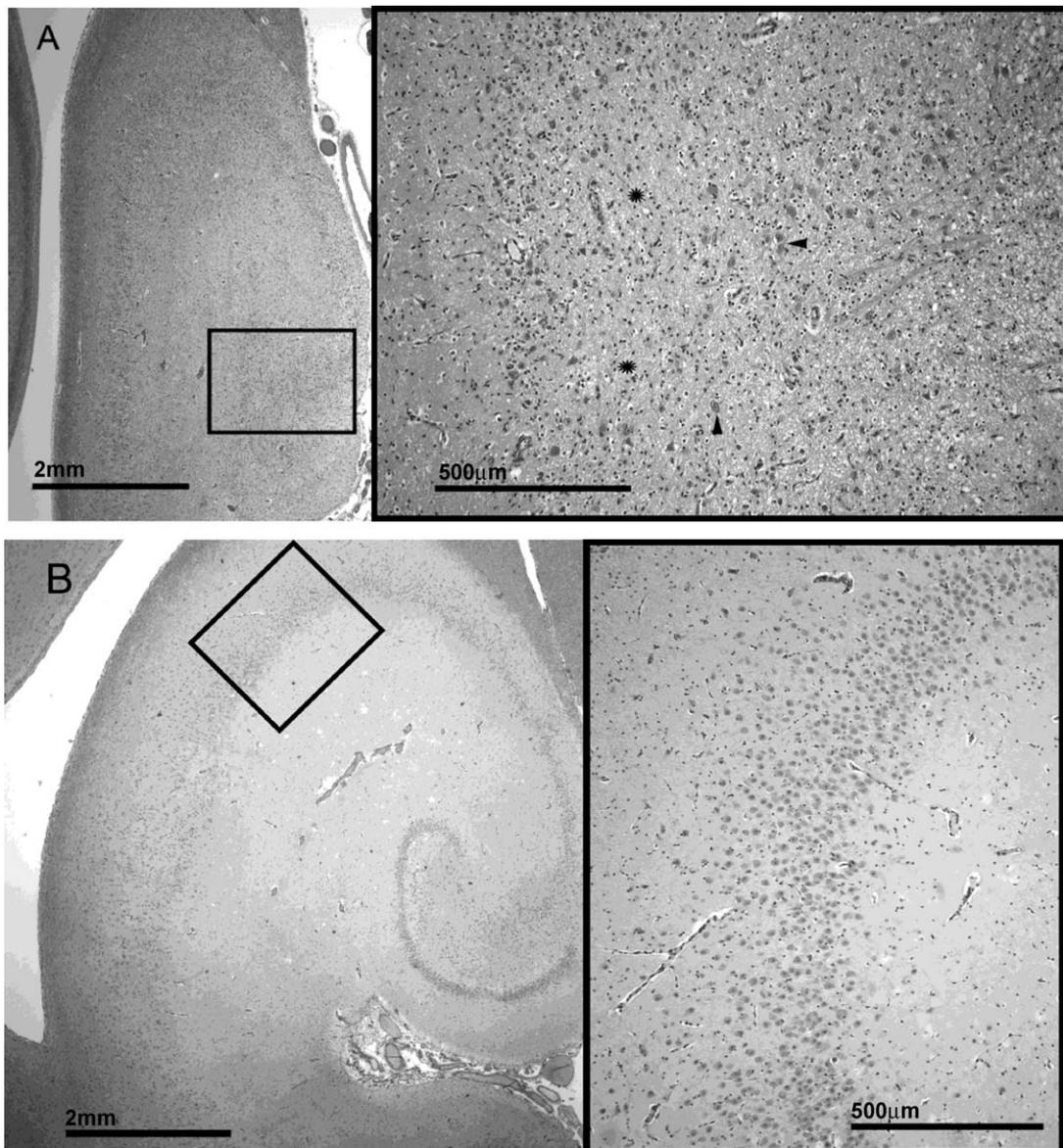


Fig. 4. (A) Left panel: haematoxylin and eosin stained section of an atrophied hippocampus from a northern fur seal with domoic acid poisoning. Right panel: higher magnification of region indicated by box in left panel. There is marked loss of neurons (arrowheads) and increased numbers of glial cells (asterisks) throughout the hippocampus. (B) Left panel: section of a normal hippocampus from a California sea lion for comparison. Right panel: higher magnification of normal region in box indicated in left panel.

4.1. Clinical signs of domoic acid toxicosis in northern fur seals

Clinical signs of ataxia and seizures, and lesions in the hippocampus and heart observed in these northern fur seals were similar to those characterized in California sea lions intoxicated with DA (Gulland et al., 2002; Silvagni et al., 2005; Zabka et al., 2009), indicating that manifestations of DA toxicosis are consistent between these two otariid species. Feeding ranges of California sea lions and northern fur seals overlap along the West Coast of the U.S., with some prey items shared by these two marine mammal species. Domoic acid toxicosis is a recurring problem for California sea lions and this is likely not the first DA poisoning event in northern fur seals. In spring of 1980, 32 northern fur seals stranded along the outer coast of Washington and Oregon with neurological signs consistent with domoic acid exposure (Brent Norberg, pers. comm.). However, no brain tissue or fluids are available for retrospective examination to determine the role DA exposure may have played in this event. The location and age of stranded animals

likely reflects their foraging behavior, with adult females and pups stranding relatively close to the breeding rookery on San Miguel Island around the breeding or weaning seasons.

4.2. Trophic pathways for domoic acid exposure in northern fur seals

The trophic pathway of DA is well documented in California sea lions with anchovies being the direct link between toxin-producing *Pseudo-nitzschia* and California sea lions (Lefebvre et al., 1999). Northern fur seal diets overlap with those of California sea lions, with both species being generalists (Sinclair et al., 2008). Fur seals tend to eat highly concentrated prey such as surface-schooling fish over the shelf or those that migrate with the deep scattering layer when off the shelf break (Gentry, 1998). This patchiness in prey coupled with flexibility in diet might help account for the variability in DA levels measured in fur seals that strand during the same time period and at the same location.

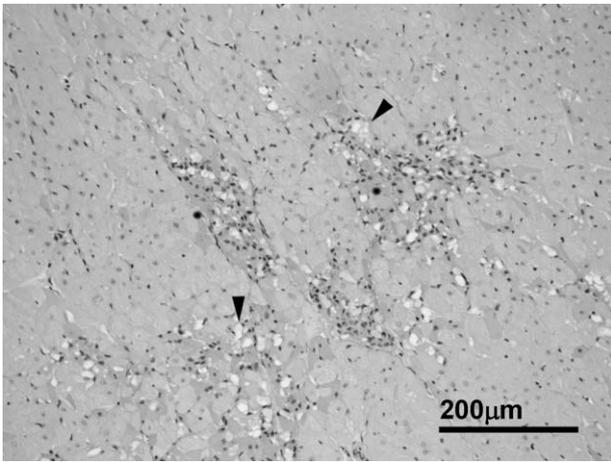


Fig. 5. Haematoxylin and eosin stained section of interventricular septum from a northern fur seal with domoic acid-associated degenerative cardiomyopathy. There are multiple necrotic myocytes (asterisks), mild leukocyte infiltrate, loss of myocytes, and replacement by adipocytes (arrowheads).

A direct link between *Pseudo-nitzschia* and measurable DA in northern fur seals was most evident in the four animals that stranded during the first two weeks of April 2007. *Pseudo-nitzschia* spp. were abundant along most of the California coast in March 2007 and increased throughout April with one of the highest population-percentages of *Pseudo-nitzschia* along the California coast recorded in San Luis Obispo on April 5th (Langlois, MBMP Annual Report 2007). Measurable DA in mussels also increased during April, indicating that these *Pseudo-nitzschia* cells were producing DA. This toxin increase was sudden in some locations. For example, mussel samples from Santa Barbara were negative for DA when tested on April 4th, but had DA levels of 62 ppm just one week later (Langlois, MBMP 2007). Three adult female northern fur seals stranded in San Luis Obispo during this time period and the female with the highest DA level measured in our study stranded on April 6th, while a stranded animal from April 8th had low detectable DA, and a stranding from April 14th had moderate levels of DA (all from fecal samples). It is also notable that on April 18th a female which stranded as a pup five months earlier in San Luis Obispo re-stranded further north in San Mateo county with measurable DA in its urine (a sample type which typically has lower values than feces). The tight temporal coupling between *Pseudo-nitzschia* abundance and measurable DA in this group of stranded fur seals indicates a short trophic pathway, likely via ingestion of fish feeding directly on *Pseudo-nitzschia*.

The link between *Pseudo-nitzschia* and DA was not as direct for the nine animals that stranded in July/August 2005. The relative abundance of *Pseudo-nitzschia* in coastal waters of San Luis Obispo and Santa Barbara counties increased in June 2005, with higher levels offshore around the Channel Islands, and with shellfish in the area having low levels of DA in June (Langlois, MBMP Annual Report 2005). However, when the animals stranded in late July and August, *Pseudo-nitzschia* levels were not very high in the water column, and shellfish tested negative for DA. It is possible the fur seals exposed during this time period were acquiring DA from food items further trophically and temporally removed from *Pseudo-nitzschia*. Gonatid squid could be the vector, since squid feed on euphausiids, a documented DA vector in this area (Bargu et al., 2002) as well as copepods, chaetognaths, and small fish. It is also possible that there are cryptic or patchy *Pseudo-nitzschia* blooms, or that phytoplankton sampling sites are too limited to accurately monitor all toxin sources for marine mammal prey.

Trophic links for pups are more complicated since they can acquire DA from their mothers *in utero* or via milk while nursing;

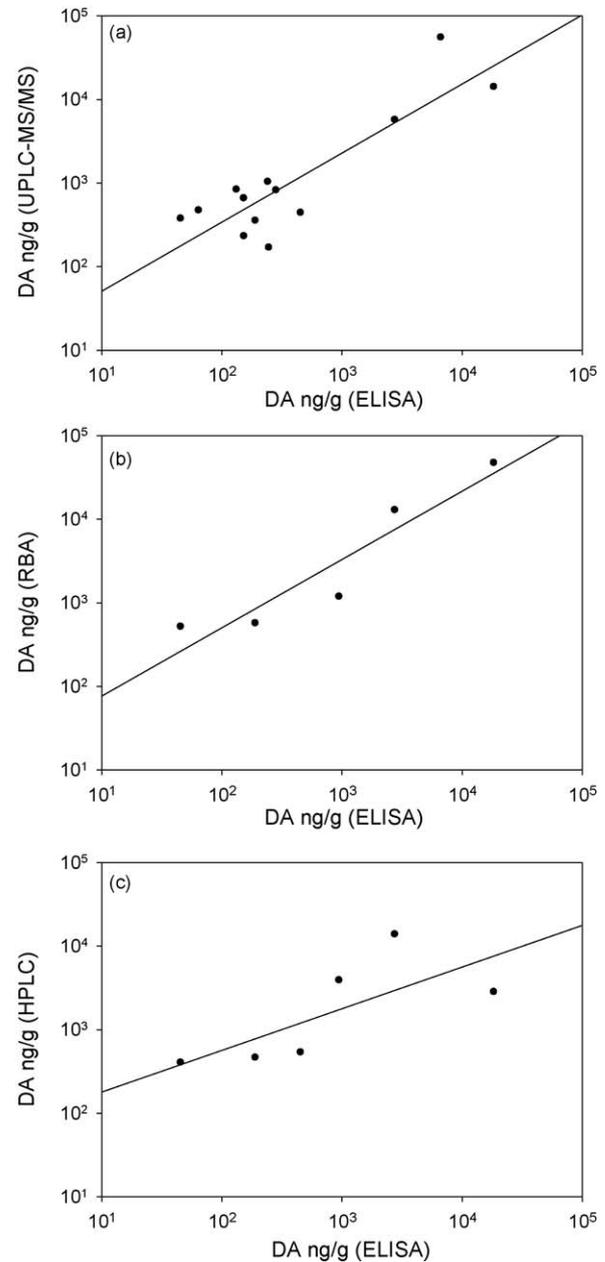


Fig. 6. Comparison of domoic acid (DA) concentration as measured by Biosense[®] ASP direct cELISA kits (ELISA) vs (a) ultra performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS), (b) receptor binding assay (RBA), and (c) high performance liquid chromatography (HPLC). Only samples containing levels of DA quantifiable by both methods were used for the comparisons ($n = 13$ for ELISA vs UPLC–MS/MS, $n = 5$ for ELISA vs RBA, and $n = 6$ for ELISA vs HPLC).

or directly from prey once they are weaned. All pups in the present study stranded during October and November after weaning and appeared extremely malnourished. Over half of these pups (9/16) stranded in late 2006, with an additional pup stranding in early January 2007. The pups with the highest DA levels were from this late 2006–early 2007 time period. While *Pseudo-nitzschia* cells were present along the coast they were not abundant in the area and shellfish sampled did not yield significant DA values. However, spiny lobster viscera from the Channel Islands (near the rookery on San Miguel) in late December did have very high levels of DA (Langlois, MBMP Annual Report 2006), indicating that DA was present in the food web, but not necessarily in the water column. The benthic

Table 2

Clinical signs of illness, outcome, and pathology results (if applicable) for each animal in the study. Domoic acid levels were determined by the ELISA method. If more than one sample type was available for an animal, the highest DA value is reported here (F: feces, U: urine, S: serum, and AF: amniotic fluid). Stranding location is reported by county (SLO: San Luis Obispo, SB: Santa Barbara, Mo: Monterey, SC: Santa Cruz, SM: San Mateo, Ma: Marin, So: Sonoma, and OS: Off Shore), outcome (R: released, RS: restrand, DOA: died in treatment, DIT: died in treatment, E: euthanized, MD: maternal death, and C: carcass).

ID #	County	Sample type	[DA] (ng/g)	Clinical signs	Outcome	Pathology results
NFS 151	SLO	U	46	Muscle twitches	R	na
NFS 152	SLO	U	192	Seizures, recovered	R	na
NFS 153	SLO	F	6730	Dead on beach	C	Microvesicular degeneration in the hippocampus
NFS 155	SLO	F	33	Seizures	DIT	Died in treatment
NFS 157	SLO	U	2784	Head weaving, seizures	R	na
NFS 158	SLO	S	286	Seizures, died in transport	DOA	Neuronal necrosis in hippocampus C4, necrotic myocardial fibers
NFS 159	SLO	U	10	Fish hook in skin	R	na
NFS 163	SLO	F	5	Seizures	DIT	Hippocampal necrosis, abscess in skeletal muscle
NFS 164	SB	U	956	Seizures	R	na
NFS 167	SLO	F	bdl	Malnutrition	DIT	Emaciation, hepatic lipidosis
NFS 171	SLO	F	244	Malnutrition	DIT	Emaciation, brain normal on histology
NFS 172	Mo	F	27	Malnutrition	DIT	Emaciation, brain normal on histology
NFS 175	SC	F	bdl	Malnutrition, abscess	R	na
NFS 177	SC	U	bdl	Malnutrition, pneumonia	R	na
NFS 180	SLO	U	bdl	Malnutrition	R	na
NFS 189 ^a	SC	U	bdl	Malnutrition	R	na
NFS 189 ^a	SM	U	43	Malnutrition	RS	Myocardial fibrosis
NFS 190	SC	U	bdl	Malnutrition	E	Fat atrophy, pulmonary edema
NFS 191	SC	F	bdl	Malnutrition,	R	na
NFS 198	SM	U	0	Malnutrition	C	na
NFS 201	Mo	F	2	Malnutrition, trauma to flipper	R	na
NFS 203	SLO	F	18600	Thin, coma, tremors, disoriented	R	na
NFS 204	SLO	F	3	Coma	DIT	Neuronal loss and necrosis, hippocampal degeneration
NFS 204F	Ma	AF	20	Aborted	MD	Autolyzed
NFS 205	SLO	F	458	Coma, emaciation	DIT	Unilateral hippocampal atrophy, gliosis, myocardial degeneration
NFS 205F	Ma	F	7	Prematurity	MD	na
NFS 207	So	U	bdl	Gunshot, bullet in head	E	Cerebral hemorrhage, fractured skull
NFS 209	SC	U	bdl	Malnutrition	DIT	Emaciation, no lesions on histology
NFS-218	OS	F	88	Fisheries interaction	C	na
NFS 218F	OS	AF	bdl	Maternal death	MD	na
NFS 219	SC	U	1	Malnutrition	R	na
NFS 224	Mo	U	3	Malnutrition	R	na
NFS 231	Ma	U	1	Malnutrition, cerebral hemorrhage	DIT	na
NFS 232	SC	U	1	Malnutrition	DIT	na
NFS 235	SLO	F	6	Coma, died in transport	DOA	na

bdl: Below detection limit and na: not analyzed.

^a A pup that stranded twice.

environment may provide a reservoir of DA (Kvitek et al., 2008), allowing animals such as spiny lobster which forage on benthic organisms to be exposed to DA even if *Pseudo-nitzschia* do not appear abundant in the water column, possibly showing a lag between toxic cells in the water column and DA exposure of marine animals.

4.3. Fetal and pup exposure risks to DA

In addition to the traditional route of food web transfer of DA through prey items such as planktivorous fish and shellfish (Perl et al., 1990; Lefebvre et al., 1999), DA may also be transferred via milk and across the placenta, thereby presenting exposure risks to the developing fetus and nursing pups. In this study, DA was detected in milk sampled from two adult field-collected females that were exhibiting signs of DA toxicosis (Table 1). This finding is consistent with a previous laboratory study in which DA was quantified in milk after intraperitoneal injection of DA in lactating female adult rats (Maucher and Ramsdell, 2005) and verifies this route of neonatal exposure under ecologically relevant conditions. The presence of DA in the amniotic fluid of a pregnant female (Table 1) represents a fetal exposure risk and the potential for exposure of the developing brain. Further studies are needed to determine how important these risks are to northern fur seal populations, but the evidence is clear that fetal and neonatal exposure occur.

4.4. Sub-clinical signs of domoic acid exposure in pups

The presence of DA in eight of 16 malnourished northern fur seal pups suggests that subclinical DA exposure occurs and could impact the health status of young animals. Of 14 adult northern fur seals observed in this study, 11 exhibited classic clinical signs of DA toxicosis, yet of the 16 pups examined, none exhibited neurobehavioral signs of toxicity, although 50% of them contained low but detectable levels of the toxin in urine and feces. The significance of this low level exposure on health in young fur seals is unclear. Lefebvre et al. (2009) have reported that subclinical exposure to DA (doses below those that cause overt neurobehavioral signs of toxicity) induces significant changes in gene expression in brain tissue of exposed vs control zebrafish and that changes induced via subclinical exposure were different from those induced by acute DA exposure (doses that cause clinical signs of DA toxicosis). Further research is needed to determine how these gene expression changes relate to overall health status. For example, genes involved in important subcellular processes including immune function, RNA processing, ion transport, metabolism, and signal transduction were altered with both subclinical and acute DA exposures. However, the direction of regulation was different between treatments, with low exposure having a higher percentage of downregulation and the acute dose having a higher percentage of upregulation suggesting that there are dose dependent modes of toxicity (Lefebvre et al., 2009).

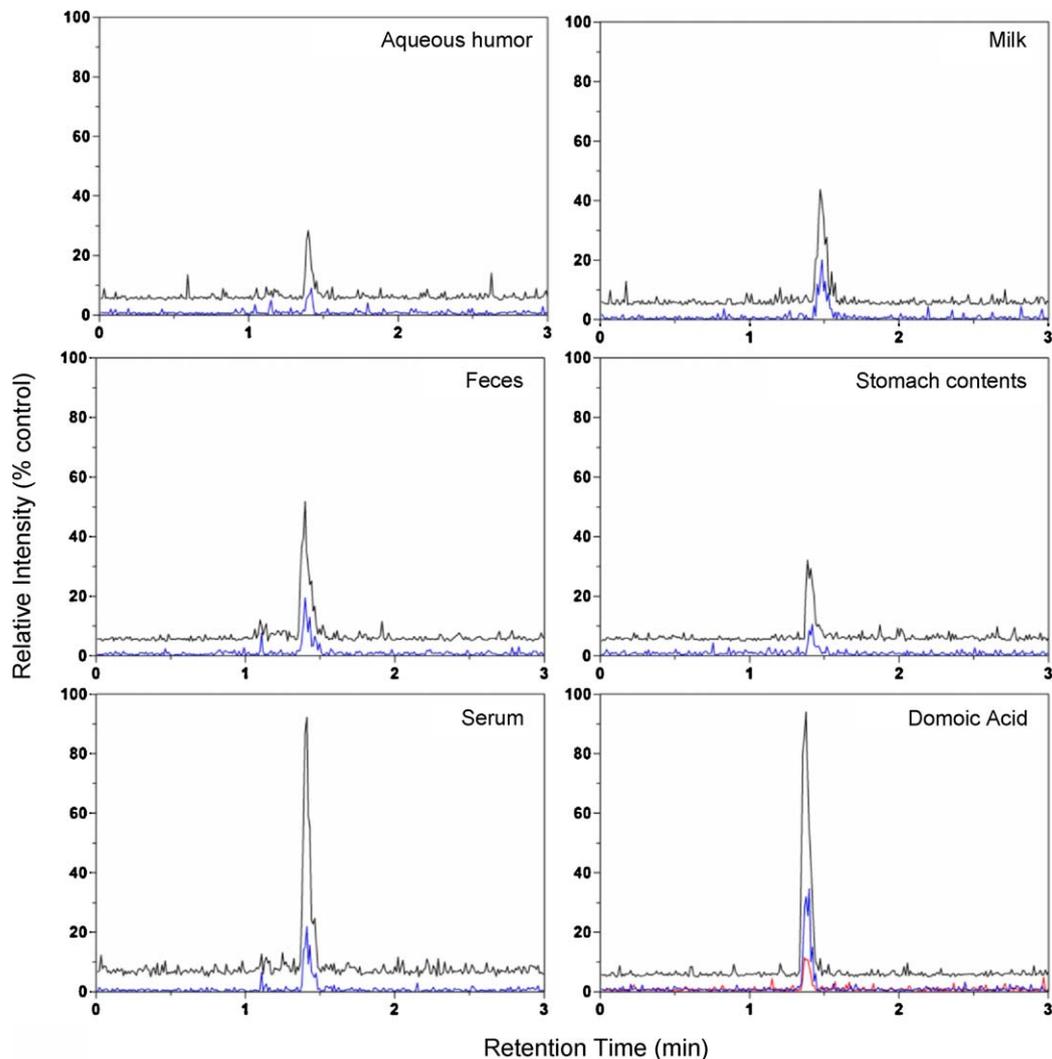


Fig. 7. Ultra performance LCMS/MS chromatograms obtained from multiple reaction monitoring experiments showing confirmation of domoic acid in a selection of matrices from one northern fur seal (NFS 153) compared to an authentic DA standard (25 ng/ml). The presence of a peak at the same retention time as DA in multiple ion transitions and ratio provided sufficient information for structural confirmation. Two ion transitions of the protonated DA are displayed in all cases including 312 > 161 (black) and 312 > 266 (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

In addition to differences in dose associated with size, there may be age specific differences in susceptibility to DA neurotoxicity in the fur seal central nervous system, as has been documented in laboratory rodents (Kerr et al., 2002). Brain lesions were not observed in any of the exposed pups, however one pup did present myocardial fibrosis which is consistent with DA toxicity (Zabka et al., 2009).

4.5. Diagnostic tools for the detection of domoic acid in stranded marine mammals

Accurate and reliable detection of DA in marine mammal fluid and tissue samples is an integral part of diagnosing an algal toxin-related mortality event. Previous investigations have typically employed HPLC-UV and/or LCMS/MS techniques for DA quantification in marine mammal urine, serum and fecal samples (Lefebvre et al., 1999, 2002; Sierra-Beltran et al., 2004; Fire et al., 2009). The RBA method is not as commonly employed due to its utilization of radioisotopes but it is a valuable technique because, unlike the other methods, it measures functional activity. In this study we utilized four analytical techniques; HPLC-UV, UPLC-MS/MS, RBA, and ELISA and examined eight fur seal sample matrices; feces, urine, blood serum, stomach contents, milk, amniotic fluid,

abdominal fluid, and aqueous humor. With the use of a recently developed highly sensitive ELISA kit that is commercially available via Biosense[®] Laboratories (Bergen, Norway) DA was detected in all eight sample matrices (Table 1). Many of these samples had DA levels that were below the detection limits of the other more traditional techniques and therefore would not have been identified previously.

The Biosense[®] ELISA kit has been validated by the manufacturer for shellfish homogenates, but has not been validated for marine mammal samples. The use of any analytical technique for novel sample matrices requires careful assessment of the potential for matrix effects to cause false positives. In this study, a matrix test was included for the use of the ELISA method with northern fur seal fecal samples to determine the appropriate minimum dilutions required for sample preparation. A dilution of 1:50 of the 1:4 50% MeOH extracts proved to be effective for eliminating matrix effects (Fig. 2). Additionally, DA was structurally confirmed via UPLC-MS/MS in six of the sample matrices including feces, urine, blood serum, stomach contents, milk, and aqueous humor (Table 1 and Fig. 7). Amniotic fluid and abdominal fluid samples had ELISA DA levels below the detection limit of the UPLC-MS/MS and therefore could not be confirmed in this instance (Table 1).

The novel detection and confirmation of DA in aqueous humor (Fig. 7) revealed a potentially valuable diagnostic tool for examining *post mortem* marine mammals. Aqueous humor is a thin, watery fluid filling the anterior chamber of the eye that is well-protected anatomically and therefore undergoes minimal *post mortem* change (Sarran et al., 2008). This fluid intermixes with the bloodstream (Johnson and Kamm, 1983) via ultrafiltration making it a very “clean” matrix that is easy to analyze and readily available in dead stranded animals. Aqueous humor has been used as a surrogate for blood serum in humans, domestic animals and California sea lions for the detection of toxins and antibody titers (Sarran et al., 2008). The identification of aqueous humor as a potential diagnostic bodily fluid for algal toxin detection in marine mammals will be useful to wildlife veterinarians and animal health researchers for investigating the role of DA in mortality events.

4.6. Summary

Collectively, the data presented here characterize the clinical signs and histopathological lesions associated with DA toxicosis in northern fur seals, provide species-specific validated protocols for DA detection, and document for the first time that northern fur seal populations are impacted by DA. It is still unclear how DA levels that are detected in animal fluids relate to DA toxicosis. Further research is needed to characterize the “background” levels of DA that are present in species of concern before we can effectively use toxin levels as biomarkers of DA-related disease in the absence of clinical signs and lesions. Additionally, further development of species and matrix-specific DA detection protocols for marine mammal tissues and fluids, which is currently underway in the Marine Biotoxins Program at the Northwest Fisheries Science Center, will provide a useful standardized toxin detection approach for the national and international HAB community.

Acknowledgements

The authors would like to thank Mark Myers, Vera Trainer, and Tracy Collier (all of the Northwest Fisheries Science Center) for thoughtful review of the manuscript.[SS]

References

- Bargu, S., Powell, C.L., Coale, S.L., Busman, M., Doucette, G.J., Silver, M.W., 2002. Krill: a potential vector for domoic acid in marine food webs. *Mar. Ecol. Prog. Ser.* 237, 209–216.
- Baugh, K.A., Spencer, S., Wekell, J.C., Trainer, V.L., 2004. An alternative method for domoic acid determination in seawater particulates: a receptor binding assay using glutamate dehydrogenase. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), *Harmful Algae 2002*. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, pp. 228–230.
- Brodie, E.C., Gulland, F.M.D., Greig, D.J., Hunter, M., Jaakola, J., St. Leger, J., Leighfield, T., Van Dolah, F.M., 2006. Domoic acid causes reproductive failure in California sea lions (*Zalophus californianus*). *Mar. Mamm. Sci.* 22, 700–707.
- Fire, S.E., Wang, Z., Leighfield, T., Morton, S., McFee, W., McLellan, R., Litaker, W., Tester, P., Hohn, A., Lovewell, G., Harms, C., Rotstein, D., Barco, S., Costidis, A., Sheppard, B., Bossart, G., Stolen, M., Durden, W., Van Dolah, F.M., 2009. Domoic acid exposure in pygmy and dwarf sperm whales (*Kogia* spp.) from southeastern and mid-Atlantic U.S. waters. *Harmful Algae* 8, 658–664.
- Fryxell, G.A., Greta, A., Villac, M.C., Shapiro, L.P., 1997. The occurrence of the toxic diatom genus *Pseudo-nitzschia* (Bacillariophyceae) on the West Coast of the USA, 1920–1996: a review. *Phycologia* 36, 419–437.
- Gentry, R.L., Kooyman, G.L., 1986. *Fur Seals: Maternal Strategies on Land and Sea*. Princeton University Press, New Jersey.
- Gentry, R.L., 1998. *Behavior and Ecology of the Northern Fur Seal*. Princeton University Press, New Jersey.
- Goldstein, T., Mazet, J.A., Zabka, T.S., Langlois, G., Colegrove, K.M., Silver, M., Bargu, S., Van Dolah, F., Leighfield, T., Conrad, P.A., Barakos, J., Williams, D.C., Dennison, S., Haulena, M., Gulland, F.M., 2008. Novel symptomatology and changing epidemiology of domoic acid toxicosis in California sea lions (*Zalophus californianus*): an increasing risk to marine mammal health. *Proc. R. Soc. B* 275, 267–276.
- Gulland, F.M., Haulena, M., Fauquier, D., Langlois, G., Lander, M.E., Zabka, T., Duerr, R., 2002. Domoic acid toxicity in Californian sea lions (*Zalophus californianus*): clinical signs, treatment and survival. *Vet. Rec.* 150, 475–480.
- Jeffery, B., Barlow, T., Moizer, K., Paul, S., Boyle, C., 2004. Amnesic shellfish poison. *Food Chem. Toxicol.* 42, 545–557.
- Johnson, M.C., Kamm, R.D., 1983. The role of Schlemm’s canal in aqueous outflow from the human eye. *Investig. Ophthalmol. Vis. Sci.* 24, 320–325.
- Kerr, D.S., Razak, A., Crawford, N., 2002. Age-related changes in tolerance to the marine algal excitotoxin domoic acid. *Neuropharmacology* 43, 357–366.
- Kreuder, C., Miller, M., Jessup, D.A., 2003. Patterns of mortality in southern sea otters (*Enhydra lutris nereis*) from 1998 to 2001. *J. Wildl. Dis.* 39, 495–509.
- Kvitek, R.G., Goldberg, J.D., Smith, G.J., Doucette, G.J., Silver, M.W., 2008. Domoic acid contamination within eight representative species from the benthic food web of Monterey Bay, California. *Mar. Ecol. Prog. Ser.* 367, 35–47.
- Lea, M.A., Johnson, D., Ream, R., Sterling, J., Melin, S., Gelatt, T., 2009. Extreme weather events influence dispersal of naive northern fur seals. *Biol. Lett.* 5, 252–257.
- Lefebvre, K.A., Bargu, S., Kieckhefer, T., Silver, M.W., 2002. From sanddabs to blue whales: the pervasiveness of domoic acid. *Toxicol.* 40, 971–977.
- Lefebvre, K.A., Powell, C.L., Busman, M., Doucette, C.J., Moeller, P.D.R., Sliver, J.B., Miller, P.E., Hughes, M.P., Singaram, S., Silver, M.W., Tjeerdema, R.S., 1999. Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event. *Nat. Toxins* 7, 85–92.
- Lefebvre, K.A., Tilton, S.C., Bammler, T.K., Beyer, R.P., Srinouanprachan, S., Stapleton, P.L., Farin, F.M., Gallagher, E.P., 2009. Gene expression profiles in zebrafish brain after acute exposure to domoic acid at symptomatic and asymptomatic doses. *Toxicol. Sci.* 107, 65–77.
- Maucher, J.M., Ramsdell, J.S., 2005. Domoic acid transfer to milk: evaluation of a potential route of neonatal exposure. *Environ. Health Perspect.* 113, 461–464.
- Olney, J.W., Fuller, T., de Gubareff, T., 1979. Acute dendrotoxic changes in the hippocampus of kainate treated rats. *Brain Res.* 176, 91–100.
- Perl, T.M., Bedard, L., Kosatsky, T., Hockin, J.C., Todd, E.C., Remis, R.S., 1990. An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *N. Engl. J. Med.* 322, 1775–1780.
- Quilliam, M.A., 2003. The role of chromatography in the hunt for red tide toxins. *J. Chromatogr. A* 1000, 527–548.
- Ream, R., Sterling, J., Loughlin, T.R., 2005. Oceanographic features related to northern fur seal migratory movements. *Deep-Sea Res. II* 52, 823–843.
- Sarran, D., Greig, D.J., Rios, C.A., Zabka, T.S., Gulland, F.M.D., 2008. Evaluation of Aqueous Humor as a Surrogate for Serum Biochemistry in California Sea Lions (*Zalophus californianus*). *Aquat. Mamm.* 34, 157–165.
- Scholm, C.A., Gulland, F., Doucette, G.J., Benson, S., Busman, M., Chavez, F.P., Cordaro, J., DeLong, R., De Vogelaere, A., Harvey, J., Haulena, M., Lefebvre, K., Lipscomb, T., Loscutoff, S., Lowenstine, L.J., Marin, R., Miller, P.E., McLellan, W.A., Moeller, P.D., Powell, C.L., Rowles, T., Silvagni, P., Silver, M., Spraker, T., Trainer, V., Van Dolah, F.M., 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 403, 80–84.
- Sierra-Beltran, A.P., Gallo-Reynoso, J.P., Egado-Villarreal, J., Blanco-Parra, M.P., Nino-Torres, C.A., Lluch-Cota, S.E., 2004. Marine mammals stranding at Bahia San Jorge, Caborca, Sonora during 2004 are toxic diatom blooms recurrent? In: *Proceedings of the 11th International Conference on Harmful Algal Blooms*, p. pp. 337.
- Silvagni, P.A., Lowenstine, L.J., Spraker, T., Lipscomb, T.P., Gulland, F.M.D., 2005. Pathology of domoic acid toxicity in California sea lions (*Zalophus californianus*). *Vet. Pathol.* 42, 184–191.
- Sinclair, E.H., Vlietstra, L.S., Johnson, D., Zeppelin, T.K., Bryd, G.V., Springer, A.M., Ream, R., Hunt, G.L., 2008. Patterns in prey use among fur seals and seabirds in the Pribilof Islands. *Deep-Sea Res.* II 55, 1897–1918.
- Teitelbaum, J.S., Zatorre, R.J., Carpenter, S., Gendron, D., Evans, A.C., Gjedde, A., Cashman, N.R., 1990. Neurologic sequelae of domoic acid intoxication due to the ingestion of contaminated mussels. *N. Engl. J. Med.* 322, 1781–1787.
- Torres de la Riva, G., Kreuder Johnson, C., Gulland, F.M.D., Langlois, G.W., Heyning, J.E., Rowles, T.K., Mazet, J.A.K., 2009. Association of an unusual marine mammal mortality event with *Pseudo-nitzschia* spp. blooms along the southern California coastline. *J. Wildl. Dis.* 45, 109–121.
- Towell, R.G., Ream, R.R., York, A.E., 2006. Decline in northern fur seal (*Callorhinus ursinus*) pup production on the Pribilof Islands. *Mar. Mamm. Sci.* 22, 486–491.
- Van Dolah, F.M., 2000. Marine algal toxins: origins, health effects, and their increased occurrence. *Environ. Health Perspect.* 108, 133–141.
- Van Dolah, F.M., Leighfield, T.A., Haynes, B.L., Hampson, D.R., Ramsdell, J.S., 1997. A microplate receptor assay for the amnesic shellfish poisoning toxin, domoic acid, utilizing a cloned glutamate receptor. *Anal. Biochem.* 245, 102–105.
- Work, T.M., Barr, B., Beale, A.M., Fritz, L., Quilliam, M.A., Wright, J.L.C., 1993. Epidemiology of domoic acid poisoning in brown pelicans (*Pelicanus occidentalis*) and Brandt’s cormorants (*Phalacrocorax penicillatus*) in California. *J. Zoo Wildl. Med.* 24, 54–62.
- Zabka, T.S., Goldstein, T., Cross, R.W., Mueller, C., Kreuder Johnson, C., Gill, S., Gulland, F.M.D., 2009. Characterization of a degenerative cardiomyopathy associated with domoic acid toxicity in California sea lions (*Zalophus californianus*). *Vet. Pathol.* 46, 105–119.