Novel symptomatology and changing epidemiology of domoic acid toxicosis in California sea lions (*Zalophus californianus*): an increasing risk to marine mammal health

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Harmful algal blooms are increasing worldwide, including those of *Pseudo-nitzschia* spp. producing domoic acid off the California coast. This neurotoxin was first shown to cause mortality of marine mammals in 1998. A decade of monitoring California sea lion (*Zalophus californianus*) health since then has indicated that changes in the symptomatology and epidemiology of domoic acid toxicosis in this species are associated with the increase in toxigenic blooms. Two separate clinical syndromes now exist: acute domoic acid toxicosis as has been previously documented, and a second novel neurological syndrome characterized by epilepsy described here associated with chronic consequences of previous sub-lethal exposure to the toxin. This study indicates that domoic acid causes chronic damage to California sea lions and that these health effects are increasing.

Keywords: amnesic shellfish poisoning; California sea lions; domoic acid; hippocampal atrophy; seizures; harmful algal blooms

1. INTRODUCTION

Domoic acid is a potent marine neurotoxin produced by some diatom species of the genus *Pseudo-nitzschia* that appear to be becoming more frequent along the California coast (Allen 1922; Fryxell *et al.* 1997; Jeffery *et al.* 2004). Blooms of other toxigenic algae worldwide also appear to be increasing in extent, frequency and type in recent years, although the reasons for this are unclear (Van Dolah 2000; HARNESS 2005). Possible explanations include oceanographic regime shifts, overfishing, movement and discharge of ballast waste, eutrophication of marine waters and global climate change (Anderson 1997; Landsberg 2002; Chavez *et al.* 2003). As harmful algal blooms increase, it is likely that their impacts on marine fauna and human health will also increase, yet data on these impacts are rare due to the logistical difficulties associated with studying these effects.

Domoic acid is water soluble and has a high affinity for the α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) and kainate subclasses of neuronal glutamate ionotropic receptors (Jeffery *et al.* 2004). Following binding of glutamate receptors, domoic acid acts as an excitotoxin by causing massive cell depolarization with subsequent increase in intracellular calcium, resulting in cell dysfunction and death (Olney *et al.* 1979). Domoic acid is the cause of amnesic shellfish poisoning in humans, first recognized in Canada in 1987 following consumption of contaminated shellfish (Perl *et al.* 1990). Clinical signs included gastrointestinal distress and neurological symptoms including seizures, agitation, coma and death (Teitelbaum *et al.* 1990). Histological changes in four people who died 7–90 days after the event were consistent with exposure to an excitotoxin and included neuronal necrosis or loss in all sectors of the hippocampal formation except in *cornu ammonis* sector CA2, and astrocytosis in one patient (Teitelbaum *et al.* 1990). One year after the acute episode had resolved, one patient developed complex partial seizures (Cendes *et al.* 1995). Electroencephalograms (EEG) showed periodic epileptiform discharges over both temporal lobes and magnetic resonance imaging (MRI) revealed bilateral hippocampal atrophy. Autopsy over 3 years following intoxication revealed bilateral hippocampal sclerosis (i.e. astrocytosis), demonstrating that long-term neurological damage persisted years after initial exposure to domoic acid.

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Domoic acid has more recently been associated with neurological signs in seabirds and California sea lions off the California coast (Work *et al.* 1993; Scholin *et al.* 2000). In 1998, it was estimated that over 400 sea lions were exposed to the toxin through contaminated prey. During the event, domoic acid was detected in anchovies, sardines, phytoplankton, and blood and urine samples from affected sea lions (Scholin *et al.* 2000). Lesions in sea lions that died acutely were consistent with limbic system injury characterized by ischaemic neuronal necrosis in the hippocampal formation, especially granule cells in the dentate gyrus and pyramidal cells in sectors CA4, CA3 and CA1, and progressed to neuronal loss with parenchymal atrophy and marked gliosis (glial cell type not specified) in sea lions dying after 15 days (Silvagni *et al.* 2005).

Experimental exposure of laboratory animals to domoic acid has produced clinical signs and brain lesions mostly consistent with those in naturally exposed humans and sea lions, and the effects of acutely lethal toxic doses of domoic acid have been well described in multiple species (Tryphonas *et al.* 1990; Scallet *et al.* 1993; Schmued *et al.* 1995). However, the effects of low doses and repeated exposures are not as well defined, with single and repeated low-dose exposure experiments conducted in rats, mice and primates obtaining varied results. Low doses administered by systemic injection produced clinical signs as well as mild localized lesions and gliosis in the hippocampus (Scallet *et al.* 1993; Sobotka *et al.* 1996). However, oral dosing produced mild to no clinical signs and histological examination revealed minimal to no morphologic changes in the brain (Truelove *et al.* 1996, 1997). Peng *et al.* (1997) did observe clinical signs and associated changes at low doses in mice, but repeated exposures failed to enhance symptomatic toxicity.

This paucity of information on the effects of single and repeated low doses of domoic acid on mammals is of concern, because as the frequency of domoic acid-producing algal blooms increases, the likelihood also increases that humans and marine mammals may ingest prey contaminated with low levels of the toxin below the currently accepted dose for seafood closures to prevent acute poisoning. California sea lions range from Baja California to British Columbia and feed on species that often enter the human seafood market, such as anchovies, sardines, hake, rockfish, salmon and market squid (King 1983). This prey base that is shared with humans, and their accessibility, mammalian physiology and longevity make California sea lions excellent sentinels of the potential effects of toxins in seafood on human health. The United States' sea lion population breeds primarily on the California Channel Islands between May and July, which numbers approximately 200 000 animals (Forney *et al.* 2000). Adult females from San Miguel Island, the most northwestern of the rookery islands, primarily forage north of the island from Point Conception along the coast of central California (Melin *et al.* 2000), but the foraging range of juveniles is unknown. Little information is available on bloom dynamics in the areas where sea lions feed, however, conditions currently appear to favour the dominance of *Pseudo-nitzschia australis* and production of domoic acid off the central and southern California coast (Anderson *et al.* 2006). This may be related to algal nutrient physiology and availability associated with wind-driven upwelling, as well as eddy circulation and entrainment promoting conditions for bloom formation and triggers for toxin production. Therefore, sea lions are likely to be naturally exposed to varying levels of domoic acid in their prey.

Here we present data from a decade of monitoring California sea lion health which suggest changes in the symptomatology and epidemiology of domoic acid toxicosis associated with increasing harmful algal blooms.

2. MATERIAL AND METHODS

(a) *Animals*

Sea lions found ill along the California coast between Ventura and Humboldt Counties were taken to The Marine Mammal Center in Sausalito, CA (TMMC) for clinical examination, treatment and rehabilitation for release back to the wild when possible. Three additional cases that originally stranded in Los Angeles county were transferred from another facility for treatment. Sex determination was based on genital morphology and age class was estimated using a combination of body length, tooth size and stage of sagittal crest development (Greig *et al.* 2005). Animals were housed in individual pens with fresh water pools, observed hourly 07.00–23.00, and fed thawed, previously frozen, herring offered three times daily. Blood samples were collected for haematology, serum biochemistry and serology (Dierauf & Gulland 2001).

Sea lions that exhibited chronic neurological signs were evaluated clinically and by EEG and MRI. Animals were sedated for EEG with medetomidine at a dose of $0.07 \, \text{mg kg}^{-1}$ intramuscularly. Needle electrode (Grass/Astro-Med, Inc.) placement was based on that used in canine patients (Holliday & Williams 1998). Fifteen EEG, four electro-ocular (EOG), two electrocardiac (EKG) and one ground electrode were placed for each recording. A Nihon Kohden model 2110 digital EEG system was used for recording all EEGs which were displayed via a modified double banana montage. Display sensitivities varied between 7 and $20 \, \mu\text{V mm}^{-1}$, with the time constant at 0.1 s and a high-frequency cut-off of 70 Hz. Duration of EEG recordings ranged from 10 to 61 min.

MRI was performed following anaesthesia using medetomidine ($0.07 \, \text{mg kg}^{-1}$) and tiletamine–zolazepam ($1 \, \text{mg kg}^{-1}$) as described by Haulena & Gulland (2001). All MR studies were performed on a 1.5-T General Electric MR system by using a quadrature knee coil. Following the localizer scan, five sequences were acquired, including coronal fast spin echo (FSE), fat-suppressed (FS) T2, FS FSE proton density (PD), fluid-attenuated inversion recovery (FLAIR), sagittal FSE T2 and FS three-dimensional spoiled gradient-recalled echo T1 (3D volume SPGR T1). The first three sequences (FSE T2, FSE PD and FLAIR) were obtained along a plane perpendicular to the long axis of the temporal lobe and ventricular horn. This oblique dorsal (coronal) orientation was selected to optimize the characterization of the hippocampal formation, as well as to align MR images with pathological sectioning. Hippocampal changes were classified as normal, mild, moderate or severe atrophy based on subjective analysis using the following imaging characteristics: generalized or focal volume loss, corresponding *ex-vacuo* temporal ventricular horn enlargement and loss of grey white differentiation of hippocampal structures. The MR images were interpreted by a neuroradiologist (J.B.) without knowledge of the clinical or pathological data. To determine the effect of intraobserver variation on the imaging interpretations, all examinations were re-reviewed by the same neuroradiologist three months later and interpreted in a blinded fashion.

Urine and serum samples submitted for domoic acid testing were screened by direct competitive domoic acid ELISA (Biosense Laboratories, Bergen, Norway), and positive samples were confirmed by liquid chromatography–tandem mass spectrometry (LC-MS/MS; Van Dolah *et al.* 1997; Maucher & Ramsdell 2005). Faeces were examined for toxic *P. australis* diatoms by scanning electron microscopy and positive samples were tested for domoic acid using high-performance liquid chromatography (HPLC; Lefebvre *et al.* 1999).

After the animals died, a necropsy was performed and representative tissue samples of brain, eyes and all viscera were collected, immersed in 10% neutral buffered formalin, paraffin embedded, sectioned at 4–5 μm, stained with haematoxylin and eosin and examined by light microscopy. Brains were serially sectioned and gross abnormalities were documented when examined by a pathologist. Brain sections sampled consistently and examined histologically were regions incorporating (i) anterior frontal lobes, (ii) anterior cingulated gyrus, (iii) both anterior to mid hippocampal formations--parahippocampal gyri–thalamus–insular gyrus, (iv) midbrain, (v) medulla oblongata at the level of the anterior velum, (vi) cerebellum, and (vii) cervical spinal cord. Additional sections sampled in some cases included regions incorporating (i) amygdaloid nuclei, (ii) olfactory structures at the level of the olfactory tubercle and gyrus, (iii) optic structures at the level of the optic tract and chiasm, (iv) posterior hippocampal formation, and (v) occipital lobes.

(b) *Domoic acid-producing algal blooms*

The California Department of Public Health (CDPH) manages a volunteer-based programme that monitors for the occurrence of toxic algal blooms along the California coast as a part of the Marine Biotoxin Monitoring Program. There were 230 sampling sites within the study range and these were sampled at varying frequencies, from 1 to 490 times over the 8 years of this study. An estimate of the relative abundance of *Pseudo-nitzschia* spp. was reported based on sampling effort, per cent composition and cell mass of the diatom in each sample. Two index sites, Santa Cruz pier (Santa Cruz county, centre of sea lion stranding range) and Goleta pier (Santa Barbara county, southern edge of sea lion stranding range), were chosen for comparison of monthly *Pseudo-nitzschia* spp. abundance estimates in water samples with sea lion stranding data as both were sampled consistently during the study period. Domoic acid analyses were performed on mussel samples (collected at 11 sites between Marin and Los Angeles counties) according to the methods of Quilliam *et al.* (1995) by CDPH when blooms of the diatoms were detected.

(c) *Statistical analysis*

The distribution by year, sex and age class for animals that stranded with acute toxicosis and with chronic neurological signs were evaluated by the two-sided Chi-square test for independence and with the Fisher's exact test when an expected cell frequency was less than 5 using EPI INFO 2000 software (v. 1.1.2, June 2000, Centers for Disease Control and Prevention, Atlanta, GA, USA). Strength of associations was estimated by the odds ratio. Temporal associations between stranding of acute and chronic neurological cases were evaluated using time-series cross-correlation analysis (Box & Jenkins 1976; SPSS for Windows, v. 11.0, SPSS, Inc., Chicago, IL). The number of strandings for both groups was summed for each week and month, and cross-correlations were evaluated

for 12 forward and 12 backward week and month time periods. Temporal associations between the monthly relative abundance index of *Pseudo-nitzschia* spp. and sea lions stranding with acute toxicity were also evaluated using time-series cross-correlation analysis. Cross-correlation coefficients were considered to be significant at $p < 0.05$. Santa Cruz & Goleta pier counts were used for comparison when acute events occurred north or south of Point Sur (Monterey County), respectively. Spatial and temporal clustering was evaluated for stranding events associated with acute toxicosis. The space–time statistic with the Space-Time Permutation model (Kulldorff *et al.* 2005) was used to test whether these cases were randomly distributed along TMMC's response area and in time (SATSCAN Software: Kulldorff M. and Information Management Services, Inc. SATSCAN v. 7.0: software for the spatial and space-time scan statistics).

3. RESULTS

(a) *Clinical case characterization*

A total of 2963 sea lions were admitted to The Marine Mammal Center during the study period. Examination of 715 sea lions stranding along the California coast with neurological signs between 1998 and 2006 revealed two separate clinical syndromes: acute domoic acid toxicosis as documented by Scholin *et al.* (2000) and Gulland *et al.* (2002; $n = 551$); and a novel chronic epileptic syndrome characterized by behavioural changes, seizures and atrophy of the hippocampal formation ($n = 164$). Acute cases were characterized by clinical signs that included ataxia, head weaving, seizures or coma which varied in severity but were continuous during the period of toxicosis, lasting about one week followed by recovery, if treated, or death (Gulland *et al.* 2002); they stranded in clusters and had histopathological findings that included hippocampal neuronal necrosis. Chronic neurological cases were characterized by animals that developed intermittent seizures (suffering from seizures at least two weeks apart and/or seizures after at least two weeks following admission to The Marine Mammal Center) but were asymptomatic between seizures, exhibited unusual behaviours, stranded individually and/or had chronic pathological changes. The latter were consistent and more extensive than those previously described for acute cases that survived longer in rehabilitation, which were described as neuronal loss with hippocampal atrophy and marked gliosis (glial cell types not specified; Silvagni *et al.* 2005).

Clinical signs in the 164 animals with chronic neurological disease included seizures, periods of marked lethargy and inappetence, vomiting, muscular twitching, central blindness blepharospasm (often unilateral) and abnormal behaviour. Duration of clinical signs from initial presentation to death varied from 25 to 1525 days. Seizures were observed in 140 of these cases at intervals varying from hours to weeks, often progressing from simple (not impairing the level of consciousness), partial (focal) to secondary (following a simple seizure) generalized (involving loss of consciousness) seizures. Partial seizures were characterized by muscle twitching of the head (sometimes just the eyelids and vibrissae) or flippers and were occasionally unilateral. Generalized seizures were most commonly clonic–tonic, characterized by lateral recumbency and extended flippers and neck. Seizures were often preceded by vomiting, pacing or swimming in tight circles.

Figure 1. Electroencephalogram (EEG) tracing from two sea lions recorded during slow-wave sleep. (a) Shows a short segment of an EEG recorded from a sea lion without signs of neurological disease and (b) is an EEG from a sea lion with chronic neurological disease showing epileptiform activity, consisting primarily of numerous high-amplitude spike-and-wave discharges (note the sensitivity has been decreased by half of that used in a). These discharges appear both generalized (oval) and somewhat lateralized over the midline and left hemisphere (boxes). A single right caudal discharge is circled. This paroxysmal activity is consistent with a diagnosis of epilepsy.

Despite treatment with diazepam, lorazepam and phenobarbital as described in Gulland et al. (2002), seizures became progressively more frequent and severe, resulting in *status epilepticus* and spontaneous death in some animals, or euthanasia due to poor prognosis for release in others. Abnormal behaviours included stranding in atypical locations (sleeping in a public restroom, climbing onto police cars, found up to 100 miles inland in an artichoke field, car dealership or walking down the road; $n=36$); repetitive behaviours (chewing on a flipper, tail or inanimate objects, swimming in tight circles, circling backwards on the pen floor, frantic pacing, rocking along the pen floor; $n=70$); and abnormal aggression towards conspecifics or humans ($n=25$). Clinical examination, haematology, serum biochemistry, radiography and serology did not reveal evidence of traumatic (i.e. fractures, foreign bodies) or infectious aetiology (septicaemia, leptospirosis, or bacterial, viral or protozoal encephalitis), or any electrolyte imbalance or abnormality of organ function (Dierauf & Gulland 2001). All but 1 out of 29 EEGs were abnormal, showing numerous epileptiform discharges (figure 1). These events were primarily multifocal, though some appeared generalized. Discharges consisted of short

duration, high-voltage spikes or sharp waves. Intermittent rhythmic delta activity was also a common feature and was often followed by brief background attenuation. These features were localized to the posterior aspects of both hemispheres with voltage maxima in one hemisphere or the other. Forty-one out of 42 animals examined by MRI had abnormal findings (14 of which also were examined by an EEG). An abnormal hippocampal formation to amygdala or parenchymal signal on T2-weighted images was defined as increased signal (T2 hyper-intensity) of the affected tissues relative to other grey matter structures (figure 2). There were varying degrees of hippocampal atrophy (mild 24%, $n=10$; moderate 34%, $n=14$; severe 41%, $n=17$), often accompanied by thinning of the parahippocampal gyrus ($n=28$), increases in ventricle (temporal horn) size ($n=34$) and T2 hyperintensity in the parahippocampus ($n=19$). Lesions were unilateral in 24 cases (left 11, right 13) and bilateral in 17 cases, but asymmetrically severe in 6 of the latter cases. The one sea lion with no detectable changes by MRI had histological evidence of bilateral hippocampal atrophy due to neuronal necrosis and astrocytosis extending to both parahippocampal gyri when it died three weeks following the MRI, during which time it had continued to have seizures.

Eighty-nine of the chronic neurological cases died. Histological examination of 87 of these cases (tissues were not collected from two animals) revealed chronic lesions affecting first the hippocampal formation and then the parahippocampal gyrus (figure 3). Sixty-eight cases had atrophy of the parenchyma due to neuronal loss and astrocytosis, which in 12 cases was confounded by active neuronal necrosis. Neuronal loss with attending oligodendrogliosis often was segmental and affected to varying degrees the granule cells of the dentate gyrus, pyramidal cells of sectors CA1-4 and subiculum, and the parahippocampal gyrus, most often of the mid-deep cortical layers. Of all regions, the dentate gyrus and sector CA3 were affected most consistently and severely and could be affected independently of each other. Oligodendrogliosis and astrocytosis followed the pattern of neuronal loss. In the hippocampal formation, astrocytosis was equal to or less severe than the most severe segment of neuronal loss; however, in the majority of affected parahippocampal gyri, astrocytosis was more pronounced than neuronal loss, which may be due to the difficulty of detecting neuronal loss by routine histopathology or to the pathogenesis of seizure propagation. Lesion symmetry was infrequent, however asymmetrical bilateral atrophy of the hippocampal formation occurred in 23 cases and unilateral atrophy occurred in 48 cases, 18 on the left and 30 on the right. Gross lesions of hippocampal to parahippocampal atrophy and sclerosis were apparent on the serial section of formalin-fixed brains when examined by a pathologist. Cardiac lesions were documented in 102 cases (67 acute, 35 chronic neurological). The cardiomyopathy varied from mild to severe and acute to chronic active and was characterized by cardiomyocyte vacuolar to hyaline degeneration and necrosis to apoptosis with a minimal reactive leucocyte infiltrate, adipocyte replacement and less often fibroplasias to fibrosis (T. Zabka 2007, personal observation). Lesions were not found in eyes from any of the animals to explain the clinically apparent intermittent central blindness.

Minimal or no typical domoic acid-related lesions were found upon examination of the brains of 19 out of the 89
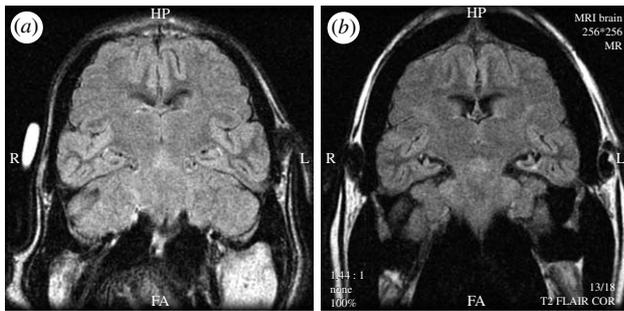
Figure 2. The magnitude of hippocampal atrophic changes was classified from normal to severe. T2-weighted images showing a sea lion with (*a*) bilaterally normal hippocampi and (*b*) a chronic neurological case with severe bilateral hippocampal atrophy, moderate bilateral thinning of the parahippocampus accompanied by bilateral temporal horn enlargement.

animals that died with chronic neurological signs, all of which were immature animals (1 pup, 3 yearlings and 15 juveniles or subadults). Lesions in these 19 sea lions were non-specific, including focal meningeal haemorrhage, mild to severe diffuse brain oedema and mild scattered non-suppurative meningoencephalitis limited to extra-(para) hippocampal regions such as the medulla oblongata, cingulate gyrus, thalamus and occipital lobes. These findings were milder than expected in the light of the abnormal neurological presentation of these cases including seizures, and severe behavioural abnormalities. Also, these lesions were present variably in chronic cases with typical brain lesions.

Of the 164 chronic neurological cases, 112 developed epilepsy (intermittent seizures) while under care, 52 had severe behavioural abnormalities and 68 out of the 87 examined by histology had chronic histopathological changes. Additionally, 32 animals initially admitted and treated for acute domoic acid exposure developed epilepsy between 16 and 85 days after apparent recovery from acute toxicosis. An additional six animals that had detectable domoic acid in clinical samples upon their first admission tested negative when readmitted following their second stranding and were exhibiting epilepsy and abnormal behaviour.

When possible, samples were collected within 24 hours of admission from animals upon admission for domoic acid testing. Domoic acid was detected in 54% (60/112) of samples collected from 80 out of the 551 acute cases (urine 43, serum 10, faeces 7) and from only 5 out of 31 samples submitted from cases with chronic neurological sequellae (urine 2, faeces 2, serum 1; table 1). Twenty out of these 80 acute animals that tested positive went on to develop neurological sequellae typical of chronic cases.

(b) *Epidemiology*
For epidemiological analyses, an acute domoic acid stranding event was identified when at least five cases were admitted into rehabilitation over 48 hours which exhibited signs as described previously by Gulland *et al.* (2002), the strandings were clustered spatially within 80 km of coastline of each other and histopathological findings included hippocampal necrosis in at least one case. The above criteria were chosen based on estimates in humans and primates for time from ingestion of the toxin to manifestation of neurological signs (within
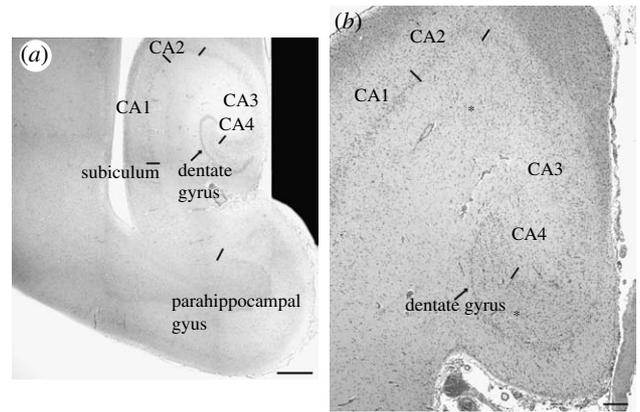

Figure 3. Sea lion brain, HE stain. (*a*) Normal hippocampal formation and parahippocampal gyrus to demonstrate the hippocampal substructures, as demarcated by lines and arrow, and the relative numbers of neurons and glial cells for comparison with chronic histopathology from domoic acid exposure (scale bar = 500 μm). (*b*) Chronic domoic acid-associated histopathology in the hippocampal formation with substructures demarcated by lines and arrow. Characteristic features that cause parenchymal atrophy, which is especially notable as contraction at the medial aspect, are the loss of granule cells in the dentate gyrus and pyramidal cells in all CA sectors, with the most notable loss in the dentate gyrus and CA3, and the astrocytosis (asterisks) that contributes to the overall increased cellular appearance, most prominent in regions with greatest neuronal loss. Increased cellularity also is due to oligodendrogliosis that attends the neuronal loss (scale bar = 300 μm).

48 hours; Teitelbaum *et al.* 1990; Tryphonas *et al.* 1990), the distance off the coast where sea lions may feed (30–60 km) and the distance they can travel in 24 hours (60–80 km; Feldcamp *et al.* 1989; Melin *et al.* 2000; Weise *et al.* 2006). Animals that stranded with neurological signs within two weeks before and after the cluster of cases were included in the event as Torres de la Riva *et al.* (in review) have found this time lag to correlate significantly with associated sea lion strandings during a bloom. Often clusters were adjacent to each other resulting in events that spanned multiple two-week periods.

The 551 sea lions that stranded with acute toxicity during a bloom event and 164 sea lions that stranded as chronic neurological cases between 1998 and 2006 were found along the California coast between 38.78° N, 123.54° W and 34.21° N, 199.26° W. Overall, the prevalence of domoic acid-related strandings increased significantly from 1998 to 2006 ($\chi^2 = 27.66$, $p < 0.001$). The number of acute cases fluctuated each year and did not show an overall increasing trend, whereas the number of chronic cases increased significantly each year ($\chi^2 = 71.82$, $p < 0.001$). There was a four-month time lag ($p = 0.003$) between acute events and strandings of subsequent chronic neurological cases (figure 4). Five significant clusters ($p < 0.01$) of acute domoic acid-related stranding events were identified, one in the spring of 1998, while the other four occurred in the mid to late summer of 2000, 2001, 2002 and 2005 (figure 5). The 1998 event was centred in Monterey Bay (figure 5*a*), while the other four were all centred off the coast of San Luis Obispo and Santa Barbara counties (figure 5*b*,*c*; data not shown for 2000 and 2002). The 1998 stranding event was tightly correlated temporally with the phytoplankton bloom off Monterey Bay detected
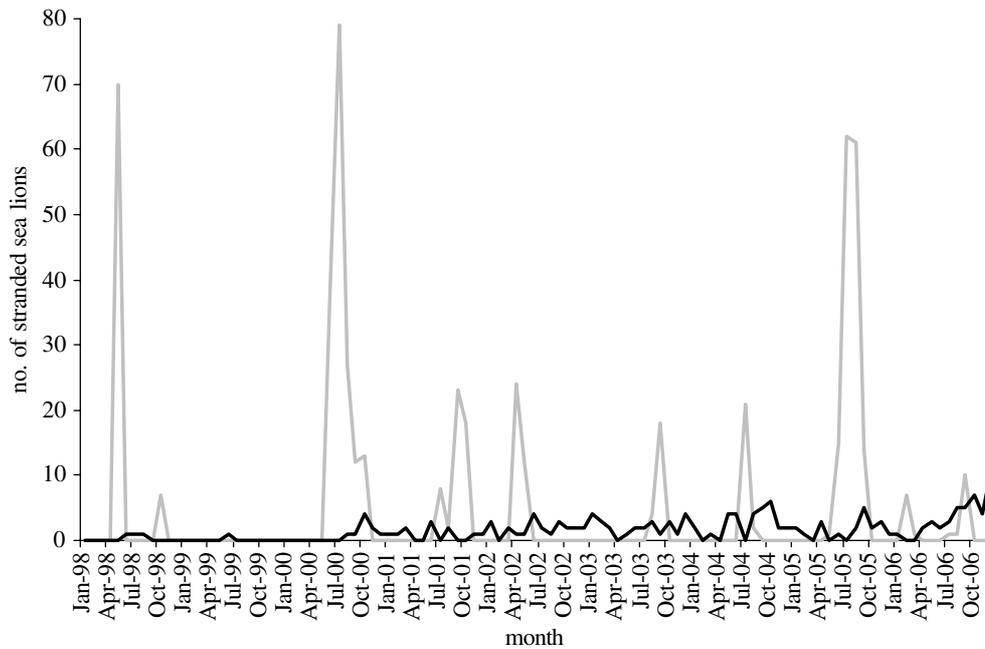
Figure 4. Comparison of monthly acute (grey line) and chronic (black line) neurological cases admitted to The Marine Mammal Center from 1998 to 2006.

Table 1. Domoic acid concentrations measured in samples collected from stranded California sea lions suffering from acute or chronic effects of domoic acid toxicity. (Note. Urine and serum were tested by direct competitive domoic acid ELISA followed by LC-MS/MS for confirmation and faeces were examined for toxic *P. australis* diatoms by scanning electron microscopy and were tested for domoic acid using HPLC.)

sample type	acute toxicity		chronic effects	
	no. positive/no. tested	domoic acid concentration ($\mu g\, ml^{-1}$)	no. positive/no. tested	domoic acid concentration ($\mu g\, ml^{-1}$)
faeces	7/16	1–82.02	2/4	1.6–4.15
urine	43/67	0.01–3.72	2/14	0.002–0.11
serum	10/29	0.004–0.2	1/13	0.003

by monthly cell counts off Santa Cruz pier (figure 5*a*). After 1998, the relationship between bloom activity off the coast and acute stranding events became less clear, as in some years the stranding clusters occurred not only at the same time as the blooms, as measured by samples collected from Goleta pier, but also up two months prior to and/or after the peak (figure 5*b*), or with no apparent temporal association with the bloom (figure 5*c*). In contrast to the acute stranding events, chronic neurological cases stranded individually in varied locations, both inland and along the coast, throughout the year following an acute event (figure 5*a–c*) and the number of these cases increased each year from 4 in the year following the 1998 event to 45 in the year following the 2005 event.

Sea lions with acute domoic acid toxicity were five times more likely to be adult females (OR =5.20, 95% CI=3.50 to 7.73; table 2*a*). In contrast, chronic animals were significantly more likely to be subadult females, juvenile males and yearlings of both sexes ($p<0.001$, table 2*b*). Almost one-third (47/167) of the immature animals did not have seizures upon admission, and instead exhibited milder clinical signs such as tremors, ataxia or severe depression.

Of the 551 acute cases, 209 (38%) died or were euthanized due to poor prognosis and 342 (62%) were released. Eleven (3%) of the released animals restranded and

were readmitted for care. In contrast, more of the chronic cases died or were euthanized (89, 54%) than were released (75, 46%). Additionally, many of the surviving chronic cases were not successful following release as over two-thirds of them restranded at least once (53/75, 71%) and were readmitted for care. Twenty-four per cent (106/434) of all adult females admitted with neurological signs had evidence of reproductive failure. Evidence included 14 live aborted premature pups, 25 stillborn pups, 29 dead *in utero* pups, 34 lactating adult females and an additional 4 adult females with dilatation of one uterine horn with associated mucosal haemorrhage noted at necropsy.

4. DISCUSSION

The frequency of domoic acid-associated California sea lion strandings has increased since 1998, and a novel neurological syndrome associated with chronic changes from sub-lethal exposure to the toxin is now recognized. Since the number of toxigenic blooms has also become more frequent along the California coast (Allen 1922; Fryxell *et al.* 1997; Jeffery *et al.* 2004), these events are likely causally related. The subset of 20 sea lions that tested positive for domoic acid upon admission to rehabilitation and later developed epilepsy indicate that a progression from an 'acute' case to a 'chronic' case is possible. The
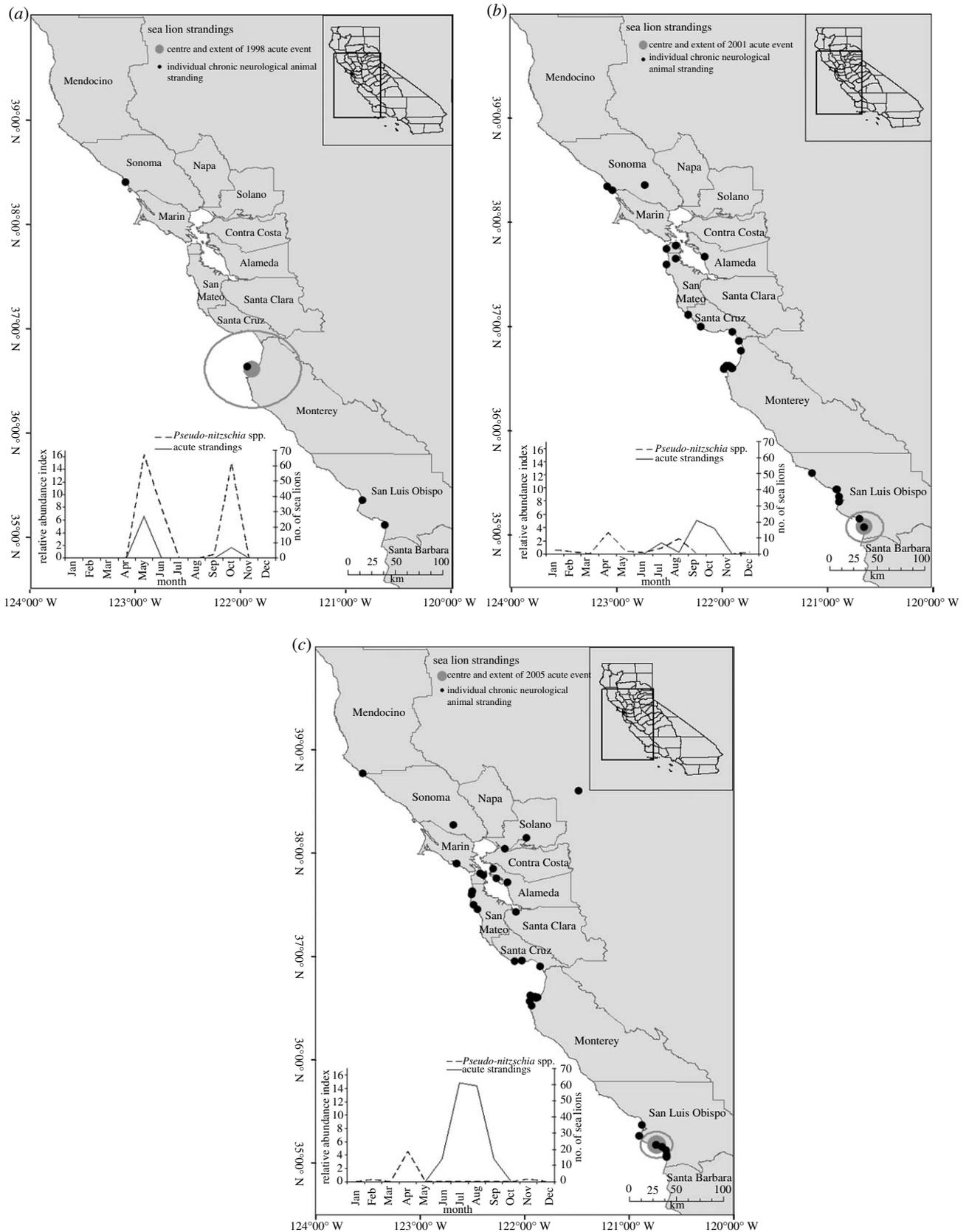
Figure 5. Central California showing the centre and extent of significant clusters of acute domoic acid stranding events and individual locations of chronic neurological animal stranding within 1 year after the acute event. Inset graphs show monthly comparisons of estimates of *Pseudo-nitzschia* spp. abundance and number of sea lions stranding with acute toxicity: (*a*) 1998, monthly relative abundance index for *Pseudo-nitzschia* spp. sampled from Santa Cruz Pier; (*b*) 2001, monthly relative abundance index for *Pseudo-nitzschia* spp. sampled from Goleta Pier and (*c*) 2005, monthly relative abundance index for *Pseudo-nitzschia* spp. sampled from Goleta Pier. Note: inland stranding locations represent sites where sea lions swam up rivers or inlets and strayed further onto land.

Table 2. (*a*) Summary of age class and sex distribution of stranded California sea lions with acute domoic acid toxicity from 2000 to 2006. (*b*) Summary of age class and sex distribution of stranded California sea lions suffering from chronic effects of domoic acid toxicity from 2000 to 2006. (Note: age classes were defined as: pup (0–1 year), yearling (1–2 years), juvenile male (2–4 years), subadult male (4–8 years), subadult female (2–5 years), adult male (8 + years) and adult female (5 + years).)

year	adult M	adult F	subadult M	subadult F	juvenile M	yearling M	yearling F	pup M	pup F	total
(*a*)										
1998		59	6	4	5	1	2			77
1999										0
2000	7	136	10	7	7		2			169
2001		39		4	3		5			51
2002	1	30		2		1	2			36
2003		17	2	1	2					22
2004	1	15	1		4	1	1			23
2005	2	80	20	4	25	7	14		1	153
2006	1	8	4	1	3		2		1	20
total	12	384	43	23	49	10	28	0	2	551
% total	2	70	8	4	8	2	5	0	1	
(*b*)										
1998		1	1				2			4
1999		1								1
2000	1	4	1	1	1		1			9
2001	2	4	1	3			1			11
2002		5	2	3	6	2	5			23
2003		5	2	6	4	3	4		2	26
2004		10	4	2	8	2	4			30
2005	2	10	1		3		4			20
2006	3	10	7	7	6	2	3	1	1	40
total	8	50	19	22	28	9	24	1	3	164
% total	5	30	12	13	17	5	15	1	2	

atrophy with sclerosis of the hippocampal formation, involvement (predominantly astrocytosis) of the extra-hippocampal regions and recurrence of complex partial seizures after initial exposure is reminiscent of the development of temporal lobe epilepsy observed in a human following natural exposure to the toxin (Cendes *et al.* 1995). Notable differences in sea lions include more consistent involvement of the dentate gyrus, lesser involvement of sector CA1 and involvement of the subiculum. Also, different was the frequent unilateral or asymmetrical occurrence. The abnormal burst activity seen by EEG demonstrated that these animals continued to experience subclinical seizures which probably resulted in the structural changes that occurred in the hippocampus and other regions of the brain shown by MRI and histological examination. These lesions are likely a result of a combination of the initial exposure to the toxin causing excitatory damage to the limbic system of these animals and the progressive effects of ongoing seizure activity.

Disease progression requires time to occur and explains the lack of association between sea lion strandings with chronic signs and bloom location. In contrast, sea lions stranding with acute toxicity tended to be clustered spatially and temporally. The lack of a tight relationship between these acute strandings and bloom activity observed in some years probably resulted from a combination of bloom patchiness, bloom distribution (with offshore blooms more likely to go undetected due to much lower sampling effort offshore), variable oceanographic conditions that may or may not transport blooms onshore, as well as the spatial variability of prey distribution and sea lion feeding. For example, the coastal sampling sites in 2005 did not identify bloom activity when large numbers of sea lions were stranding with neurological signs. In 2006, phytoplankton sampling increased offshore and as a result there were periods when bloom activity was identified at these sites, closer to where sea lions feed, but not at the coastal sites where monitoring had occurred in previous years. This example illustrates the difficulties associated with monitoring bloom activity using manual water sampling and suggests that sea lions may, at times, be a more sensitive and reliable indicator of the presence of domoic acid-producing *Pseudonitzschia* blooms off the California coast, as has also been suggested by Torres de la Riva *et al.* (in review).

Not only has there been an increase in domoic acid-associated sea lion strandings in recent years, but in particular there has been an increase in the number of stranded animals suffering from chronic effects. In recent years, the increased number of identified cases may be partly attributed to the awareness of the new neurological syndrome in sea lions and therefore an increased effort to classify and diagnose these cases. However, the higher number of chronic neurological cases observed in 2001 compared with those in 1998 is striking, suggesting that this increase is not due to a change in observer effort alone. Stranded sea lions have been collected and examined systematically since 1990, and signs suggestive of neurological disease were rarely observed prior to 1998 (Greig *et al.* 2005). The demographics of the affected population have also changed. The largest proportion of sea lions admitted with acute domoic acid toxicity remain adult females; however, the immature age classes of both sexes are increasingly represented with neurological disease. The significant increase in the number of affected immature sea

lions in recent years is of interest as some of these animals differed from adults both in clinical presentation and structural changes seen in the brains. The reasons for these differences may be related to dose; age; seizure frequency, intensity and duration; or differential distribution of brain receptors for the toxin. Age and dose effects associated with domoic acid exposure have been demonstrated in humans, rats and monkeys, with adults being shown to be more susceptible to the toxin than juveniles (Scallet *et al.* 1993; Truelove *et al.* 1996). Additionally, both intrauterine and neonatal exposure in rats resulted in permanent behavioural and morphologic changes associated with reduced seizure threshold and decreased ability to perform memory tasks (Xi *et al.* 1997; Doucette *et al.* 2004). Morphologic changes included hippocampal neuronal loss and mossy fibre sprouting (indicative of new synapse formation) in the rat neonates and the authors hypothesize that this reorganization provided a substrate for hippocampal excitability, resulting in the observed behavioural changes. Previous work has shown that domoic acid crosses the placenta of California sea lions as the toxin has been detected in amniotic fluid, foetal urine and foetal gastric fluid (Brodie *et al.* 2006). Thus it is possible that in exposed cases where the sea lion foetus survives to birth, exposure to the toxin *in utero* could result in developmental abnormalities leading to neurological and behavioural deficits as the animals age. Additionally, neonatal sea lions may be exposed to the toxin after birth by exposure in milk, which has been demonstrated to be possible in rats (Maucher & Ramsdell 2005). Further work is warranted to examine the effects of domoic acid on brain development as well as association between behavioural abnormalities and hippocampal and extra-hippocampal lesions in sea lions.

The reasons for the occurrence of unilateral brain lesions in the chronic animals are unclear but may explain, in part, the varied behavioural presentations noted in affected animals. More sea lions exhibited unilateral damage than bilateral damage, and right-sided hippocampal atrophy was most common. Changes in hippocampal volume have been associated with navigational ability in humans (Burwell *et al.* 2004; Maguire *et al.* 2006*a,b*). Thus, unilateral atrophy of the hippocampal formation may have important implications on their ability to navigate and could explain the unusual stranding locations and abnormal behaviour following release in some of these animals.

In conclusion, although the history of exposure to domoic acid in individual wild sea lions is mostly unknown, this study demonstrates that impacts of domoic acid on California sea lions are increasing and that effects can be more subtle than death, such as epilepsy and severe behavioural changes. Atrophy of the hippocampal formation and parahippocampal gyrus results from a combination of neuronal loss and astrocytosis and may be a result of the initial toxic insult and the progressive and cumulative effects from seizure propogation. Neurological and behavioural abnormalities are now observed in free-ranging juvenile animals rather than primarily in adult females, suggesting that toxin exposure *in utero* and through milk may be important. These cases of epilepsy in sea lions suggest that this free-living marine mammal naturally exposed to domoic acid in its environment may serve as a useful model for the pathogenesis of medial temporal lobe epilepsy in humans, especially the sclerotic form, as well as serve as an important

food safety sentinel for the presence of harmful algal blooms off the California coast.

REFERENCES

Allen, W. E. 1922 Observations on surface distributions of marine diatoms between San Diego and Seattle. *Ecology* **3**, 140–145. (doi:10.2307/1929148)

Anderson, D. M. 1997 Turning back the harmful red tide. *Nature* **6642**, 513–514. (doi:10.1038/41415)

Anderson, C. R., Brzezinski, M. A., Wasburn, L. & Kudela, R. 2006 Circulation and environmental conditions during a toxigenic *Pseudo-nitzschia australis* bloom in the Santa Barbara Channel, California. *Mar. Ecol. Prog. Ser.* **327**, 119–133. (doi:10.3354/meps327119)

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. (doi:10.1111/j.1748-7692.2006.00045.x)

Burwell, R. D., Saddoris, M. P., Bucci, D. J. & Wigg, K. A. 2004 Corticohippocampal contributions to spatial and contextual learning. *J. Neurosci.* **24**, 3826–3838. (doi:10.1523/JNEUROSCI.0410-04.2004)

Cendes, F., Andermann, F., Carpenter, S., Zatorre, R. J. & Cashman, N. R. 1995 Temporal lobe epilepsy caused by domoic acid intoxication: evidence for glutamate receptor mediated excitotoxicity in humans. *Ann. Neurol.* **37**, 123–126. (doi:10.1002/ana.410370125)

Chavez, F. P., Ryan, J., Lluch-Cota, S. E. & Niquen, C. M. 2003 From anchovies to sardines and back: multidecadal changes in the Pacific Ocean. *Science* **299**, 217–221. (doi:10.1126/science.1075880)

Dierauf, L. & Gulland, F. M. D. (eds) 2001 *Handbook of marine mammal medicine*, p. 10633. Boca Raton, FL: CRC Press.

Doucette, T. A., Bernard, P. B., Husum, H., Perry, M. A., Ryan, C. L. & Taker, R. A. 2004 Low doses of domoic acid during postnatal development produce permanent changes in rat behaviour and hippocampal morphology. *Neurotox. Res.* **6**, 555–563.

Feldcamp, S. D., Delong, R. L. & Antonelis, G. A. 1989 Diving patterns of the California sea lion, *Zalophus californianus*. *Can. J. Zool.* **67**, 872–883.

Forney, K. A., Barlow, J., Muto, M., Lowry, M., Baker, J., Cameron, G., Mobley, J., Stinchcomb, C. & Carretta, J. V. 2000 *U.S. Pacific marine mammal stock assessments: 2000*, pp. 1–7. NOAA-TM-NMFS-SWFSC-300, Washington, DC: NOAA.

Fryxell, G. 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.

Greig, D. J., Gulland, F. M. D. & Kreuder, C. 2005 A decade of live California sea lion (*Zalophus californianus*) strandings along the central California coast: causes and trends, 1991–2000. *Aquat. Mamm.* **31**, 40–51.

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.

HARNESS 2005 Harmful Algal Research and Response National Environmental Science Strategy 2005–2015. National Plan for Algal Toxins and Harmful Algal Blooms, (eds D. M. Anderson, J. S. Ramsdell & P. M. Glibert). *Oceanography* **18**, 238–245.

Haulena, M. & Gulland, F. M. D. 2001 Use of medetomidine–zolazepam–tiletamine with and without atipamezole reversal to immobilize captive California sea lions. *J. Wildl. Dis.* **37**, 566–573.

Holliday, T. A. & Williams, D. C. 1998 Interictal paroxysmal discharges in the electroencephalograms of epileptic dogs. *Clin. Tech. Small Anim. Pract.* **13**, 132–143. (doi:10.1016/S1096-2867(98)80034-0)

Jeffery, B., Barlow, T., Moizer, K., Paul, S. & Boyle, C. 2004 Amnesic shellfish poison. *Food Chem. Toxicol.* **42**, 545–557. (doi:10.1016/j.fct.2003.11.010)

King, J. E. 1983 In *Seals of the World*, pp. 240. St. Lucia, Australia: University of Queensland Press.

Kulldorff, M., Heffernan, R., Hartman, J., Assunção, R. M. & Mostashari, F. 2005 A space-time permutation scan statistic for the early detection of disease outbreaks. *PLoS Med.* **2**, 216–224. (doi:10.1371/journal.pmed.0020059)

Landsberg, J. H. 2002 The effects of harmful algal blooms on aquatic organisms. *Rev. Fish. Sci.* **10**, 113–390. (doi:10.1080/20026491051695)

Lefebvre, K. A. *et al.* 1999 Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event. *Nat. Toxins* **7**, 85–92. (doi:10.1002/(SICI)1522-7189(199905/06)7:3<85::AID-NT39>3.0.CO;2-Q)

Maguire, E. A., Nannery, R. & Spiers, H. J. 2006*a* Navigation around London by a taxi driver with bilateral hippocampal lesions. *Brain* **129**, 2894–2907. (doi:10.1093/brain/awl286)

Maguire, E. A., Woollett, K. & Spiers, H. S. 2006*b* London taxi drivers and bus driver: a structural MRI and neuropsychological analysis. *Hippocampus* **16**, 1091–1101. (doi:10.1002/hipo.20233)

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.

Melin, S. R., Delong, R. L., Thomason, J. R. & Vanblaricom, G. R. 2000 Attendance patterns of California sea lion (*Zalophus californianus*) females and pups during the non-breeding season at San Miguel Island. *Mar. Mamm. Sci.* **16**, 169–185. (doi:10.1111/j.1748-7692.2000.tb00911.x)

Olney, J. W., Fuller, T. & de Gubareff, T. 1979 Acute dendrotoxic changes in the hippocampus of kainate treated rats. *Brain Res.* **176**, 91–100. (doi:10.1016/0006-8993(79)90872-2)

Peng, Y. G., Clayton, E. C., Means, L. W. & Ramsdell, J. S. 1997 Repeated independent exposures to domoic acid do not enhance symptomatic toxicity in outbred or seizure-sensitive inbred mice. *Fundam. Appl. Toxicol.* **40**, 63–67. (doi:10.1006/faat.1997.2360)

Perl, T. M., Bedard, L., Kosatsky, T., Hockin, J. C., Todd, E. C., McNutt, L. A. & Remis, R. S. 1990 An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *New Engl. J. Med.* **322**, 1775–1780.

Quilliam, M. A., Xhie, M. & Hardstaff, W. R. 1995 Rapid extraction and cleanup for liquid chromatographic determination of domoic acid in unsalted seafood. *J. AOAC Int.* **78**, 543–555.

Scallet, A. C., Binienda, Z., Caputo, F. A., Hall, S., Paule, M. G., Rountree, R. L., Schmued, L., Sobotka, S. & Slikker Jr, W. 1993 Domoic acid-treated cynomolgus monkeys (*M. fascicularis*): effects of dose on hippocampal neuronal and terminal degeneration. *Brain Res.* **627**, 307–313. (doi:10.1016/0006-8993(93)90335-K)

Schmued, L. C., Scallet, A. C. & Slikker, W. 1995 Domoic acid-induced neuronal degeneration in the primate forebrain revealed by degeneration specific histochemistry. *Brain Res.* **695**, 64–70. (doi:10.1016/0006-8993(95)00799-V)

Scholin, C. A. *et al.* 2000 Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* **403**, 80–84. (doi:10.1038/47481)

Silvagni, P., Lowenstine, L. J., Spraker, T., Lipscomb, T. P. & Gulland, F. M. 2005 Pathology of domoic acid toxicity in California sea lions (*Zalophus californianus*). *Vet. Pathol.* **42**, 184–191. (doi:10.1354/vp.42-2-184)

Sobotka, T. J. *et al.* 1996 Domoic acid: neurobehavioural and neurohistological effects of low-dose exposure in adult rats. *Neurotoxicol. Teratol.* **18**, 659–670. (doi:10.1016/S0892-0362(96)00120-1)

Teitelbaum, J. S., Zatorre, R. J., Carpenter, S., Gendron, D., Evans, A. C., Gjedde, A. & Cashman, N. R. 1990 Neurologic sequellae 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., Heyning, J., Rowles, T. & Mazet, J. K. In review. *Pseudo-nitzschia* blooms associated with marine mammal strandings along the Southern California. *J. Wildl. Dis.*.

Truelove, J., Mueller, R., Pulido, O. & Iverson, F. 1996 Subchronic toxicity study of domoic acid in the rat. *Food Chem. Toxicol.* **34**, 525–529. (doi:10.1016/0278-6915(96)81814-X)

Truelove, J., Meuller, R., Pulido, O., Martin, L., Fernie, S. & Iverson, F. 1997 30-Day oral toxicity study of domoic acid in cynomolgus monkeys: lack of overt toxicity of doses approaching the acute toxic dose. *Nat. Toxins* **5**, 111–114. (doi:10.1002/nt.5)

Tryphonas, L., Truelove, J., Todd, E., Nera, E. & Iverson, F. 1990 Experimental oral toxicity of domoic acid in cynomolgus monkeys (*Macaca fascicularis*) and rats. Preliminary investigations. *Food Chem. Toxicol.* **28**, 707–715. (doi:10.1016/0278-6915(90)90147-F)

Van Dolah, F. M. 2000 Marine algal toxins: origins, health effects, and their increased occurrence. *Environ. Health Perspect.* **108S1**, 133–141. (doi:10.2307/3454638)

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. (doi:10.1006/abio.1996.9889)

Weise, M. J., Costa, D. P. & Kudela, R. M. 2006 Movement and diving behaviour of male California sea lion (*Zalophus californianus*) during anomalous oceanographic conditions of 2005 compared to those of 2004. *Geophys. Res. Lett.* **33**, L22S10. (doi:10.1029/2006GL027113)

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 (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) in California. *J. Zool. Wildl. Med.* **24**, 54–62.

Xi, D., Peng, Y.-G. & Ramsdell, J. S. 1997 Domoic acid is a potent neurotoxin in neonatal rats. *Nat. Toxins* **5**, 74–79.