Development, comparison and validation using ELISAs for the analysis of domoic acid in California sea lion body fluids

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Abstract

California sea lion (*Zalophus californianus*) mortality attributed to the neurotoxin domoic acid (DA) produced by the diatom *Pseudo-nitzschia* has occurred repeatedly along the United States' (US) west coast since the late 1990s. The purpose of this study was to provide a comparison of DA concentrations for several method platforms in the analysis of sea lion body fluids and to validate a new preparation protocol. The amount, quality, and type of body fluid available for DA analysis from an individual animal are variable and highly dependent on the health of the animal upon arrival at rehabilitation facilities. Additionally, differences in analytical materials, equipment, technical capability, budgets, and objectives of the various groups and/or agencies involved in this work have influenced current DA quantification platforms. The goal of the present study was to compare the performance of two commercially available enzyme-linked immunosorbent assays (ELISA) for the analysis of DA in a spectrum of California sea lion body fluids, then compare those results with results obtained using liquid chromatography-mass spectrometry (LC-MS) on the same samples. These methods were capable of detecting DA in California sea lion fluids without introducing a significant risk of false positives. The platforms demonstrated relatively good agreement (high R^2 values) with known DA concentrations added to sea lion body fluid samples. Also, the linearity observed when platform results were directly compared verified that the magnitude of DA concentrations measured by each platform were comparable. Urine was the exception; all platforms performed poorly in this matrix, likely due to matrix effects, suggesting that sea lion urine should not be used to quantify DA and care should be taken when comparing data from existing datasets.

Introduction

The diatom *Pseudo-nitzschia* has been known as a common member of the phytoplankton community in California since the early 1900s (Allen 1934, 1936; Fryxell *et al.* 1997), however the capability of *Pseudo-nitzschia* to produce the neurotoxin domoic acid (DA) and the threat that toxin can pose for human and wildlife health was not identified until the end of the century. Domoic acid can bio-

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accumulate in organisms feeding directly on toxic *Pseudo-nitzschia* cells such as zooplankton (Bargu *et al.* 2002, Leandro *et al.* 2010), shellfish (Bates *et al.* 1989, Wright *et al.* 1989, Blanco *et al.* 2006) and planktivorous fish (Work *et al.* 1993, Lefebvre *et al.* 1999, Lefebvre *et al.* 2001, Busse *et al.* 2006, Del Rio *et al.* 2010), which in turn serve as vectors transporting the toxin to higher trophic levels of the food web. The first outbreak of human illness occurred in 1987 on Prince Edward Island, Canada, when over 100 people became ill and three died after consuming DA contaminated blue mussels (Bates *et al.* 1989, Wright *et al.* 1989, Perl *et al.* 1990). Since the initial Canadian outbreak, DA has not caused any widespread human illnesses, most likely due to extensive coastal monitoring programs for DA implemented by health departments worldwide since 1987. Conversely, DA continues to be the cause of marine bird (Fritz *et al.* 1992, Work *et al.* 1993, Sierra Beltran *et al.* 1997) and marine mammal (Scholin *et al.* 2000, Torres de la Riva *et al.* 2009, Fire *et al.* 2010, Leandro *et al.* 2010, Wang *et al.* 2012) mortality events in areas where *Pseudo-nitzschia* occurs. These mortalities are often the first sign of an emerging DA event in a given area.

The marine mammal predominately associated with DA mortality events on the US west coast is the California sea lion (*Zalophus californianus*), undoubtedly due to large population sizes and overlapping distribution with *Pseudo-nitzschia* in coastal waters (Scholin *et al.* 2000, Bejarano *et al.* 2008, Torres de la Riva *et al.* 2009, Bargu *et al.* 2010). Currently, there are two types of DA exposure identified in sea lions: acute DA toxicosis that occurs when a sea lion is exposed to a single high dose of DA and chronic DA toxicosis that occurs when a sea lion is repeatedly exposed to sub-lethal concentrations of DA (Gulland *et al.* 2002, Goldstein *et al.* 2008). Presumably these different types of DA exposure influence the magnitude of DA concentrations present in the body fluids of stranded animals at the time of rescue. The majority of sea lion strandings involve females, and DA exposure not only threatens their health, but the health of fetuses they may be carrying (Brodie *et al.* 2006, Ramsdell and Zabka 2008, Goldstein *et al.* 2009). The impact of DA on sea lions following rehabilitation can be seen in alterations of sea lion behavior, movement, dive pattern, and survival (Gulland *et al.* 2002, Thomas *et al.* 2010).

Identifying strong positive correlations between the presence of DA producing *Pseudo-nitzschia* and sea lion strandings is impeded by several factors: the type of exposure (acute or chronic), the amount of time between exposure and the time of stranding and rescue, the health of the animal upon arrival at the rehabilitation center, the vector responsible for the exposure, the amount of DA produced by *Pseudo-nitzschia,* and the abundance of the *Pseudo-nitzschia* cells producing DA. Unlike dinoflagellates, that often form conspicuous blooms capable of altering the color of the water, *Pseudo-nitzschia* does not typically reach cell abundances large enough to be visibly noticed. Moreover, coastal monitoring programs for *Pseudo-nitzschia* and DA are routinely established at surface water stations that are accessible from shore where DA in phytoplankton and/or shellfish is most likely to overlap with human activities. Yet, marine animals can come in contact with *Pseudo-nitzschia* blooms present offshore and/or in thin layers (McManus *et al.* 2008, Rines *et al.* 2010), and strandings of these animals often are the first sign of an emerging DA event prior to the *Pseudo-nitzschia c*ells being physically transported to shore and subsequently detected by a coastal monitoring program.

The range of DA concentrations reported for fluids and solids collected from stranded pinnipeds vary in range, fluid type, collection, and DA quantification protocols (Table 1). Multiple platforms exist for the measurement of DA including mouse bioassay, high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), receptor binding assay (RBA) and enzyme linked immunosorbent assay (ELISA). Each method has its own array of advantages and potential shortcomings, and laboratories generally choose the methodology that will best suit their needs in terms of cost, technical sophistication and research goals. For example, commercially available ELISAs generally offer lower cost alternatives to analytical chemical approaches (i.e., HPLC, LC-MS) that require a significant investment of equipment and technical expertise. Several studies have implemented the rapid, lower-cost methodologies (i.e., RBA and ELISA) as pre-screening tools prior to analysis by chemical methods (Brodie *et al.* 2006, Goldstein *et al.* 2008, Goldstein *et al.* 2009).

The primary objective of the present study was to compare the performance of several of the methodologies available for quantification of DA

Table 1. Summary of findings from previous studies of domoic acid (DA) concentrations in pinniped species using several different analytical methods. HPLC = high performance liquid chromatography; RBA = receptor binding assay; LC-MS = liquid chromatography-mass spectrometry; and ELISA = the enzyme linked immunosorbent assay.

Species	AF (ng ml^{-1})	Feces (µg g^{-1})	Gastric Fluid (ng ml^{-1})	Serum (ng ml^{-1})	Urine (ng ml^{-1})	Method	Literature Cited
California sea lions (*Zalophus californianus*)		2.5 - 152.0				HPLC	Lefebvre et al. 1999
		1.3 - 182.0				RBA	Lefebvre et al. 1999
		1.31 - 182.01 µg ml^{-1}		170.0 - 200.0	30.0 - 3720.0	RBA	Scholin et al. 2000
	4.0 - 34.0		0.5 - 15.0		7.0 - 261.0	LC-MS	Brodie et al. 2006
		1.0 - 82.02				HPLC	Goldstein et al. 2008
				3.0 - 200.0	2.0 - 3720.0	LC-MS	Goldstein et al. 2008
	3.0 - 9.3		0.3 - 44.0		2.0 - 17.6	LC-MS	Goldstein et al. 2009
		1.4 - 96.8				HPLC	Bargu et al. 2010
		0.2 - 96.8				HPLC	Bargu et al. 2012
Harbor seals (*Phoca vitulina*)	10	0.002 - 0.063		8.0 - 10.0	2.0 - 16.0	ELISA	Hall and Frame 2010
Northern fur seals (*Callorhinus ursinus*)	20.0 ng g^{-1}	0.002 - 18.6		2 - 286 ng g^{-1}	1.0 - 2784.0 ng g^{-1}	ELISA	Lefebvre et al. 2010
		0.53 - 2.80			190.0 - 13661.0 ng g^{-1}	HPLC	Lefebvre et al. 2010
		0.44 - 54.73		811.0 - 828.0	371.0 - 5630.0 ng g^{-1}	LC-MS	Lefebvre et al. 2010
		46.47			512 - 12693 ng g^{-1}	RBA	Lefebvre et al. 2010

in sea lion body fluids, thereby enabling some degree of extrapolation across existing datasets, and to provide context for comparisons to previously published studies. This objective was met through: 1) validating a protocol adapted for the measurement of DA by ELISA in sea lion body fluids that minimized the sample volume required and reduced sample handling procedures; 2) comparing the performance of two commercially available ELISAs: the monoclonal antibody ELISA manufactured by Mercury Science, Inc. (MS) and the polyclonal antibody ELISA manufactured by Biosense (BS) using the modified protocol; and 3) comparing the results from ELISA platforms with a well-established analytical method utilizing LC-MS. The sea lion body fluids used in the validation study include amniotic fluid (AF), cerebral spinal fluid (CSF), serum, and urine.

Methods

DA Quantification Methods

Three method platforms for the analysis of DA (two commercially available ELISAs and LC-MS) were compared for their ability to accurately measure DA in sea lion body fluids and to provide information on how to compare data collected using these different platforms. Each fluid type selected (amniotic fluid, cerebral spinal fluid, serum, and urine) has unique properties that may cause interferences for ELISA or LC-MS methodologies, warranting examination of each fluid individually. The Mercury Science ELISA is a monoclonal antibody assay developed by the National Oceanographic and Atmospheric Association Centers for Coastal Ocean Science, National Ocean Service, the Northwest Fisheries Science Center with Mercury Science, Inc., (Durham, NC). It has been validated for the analysis of DA in shellfish tissues and in dissolved and particulate phytoplankton samples (Litaker *et al.* 2008, Seubert *et al.* 2012). The Biosense ELISA is a polyclonal antibody based assay developed by Biosense Laboratories (Bergen, Norway) that has been validated by both single and inter-laboratory studies for the analysis of DA in shellfish tissues (Kleivdal *et al.* 2007) and for the analysis of DA concentrations present in rat serum and brain samples (Hesp *et al.* 2005). An Agilent 6130 LC-MS system operated in positive electrospray ionization mode with an Agilent Zorbax Rapid Resolution column and Selected Ion Monitoring of DA (312 amu) was used for LC-MS analysis generally following the method of Wang *et al.* (Wang *et al.* 2007). Quantification was based on peak area and an external standard curve using National Research Council Canada Certified Reference Materials-DA-f standards. Peaks were confirmed based on the presence of daughter fragments at 266 and 248 amu. Since the objective

of the study was to compare methods and matrices, the unknown samples were run blind and not corrected for matrix effects using standard addition or an internal standard.

Sample Collection and Selection for Validation Study

Samples of amniotic fluid, cerebral spinal fluid, serum and urine were obtained from stranded sea lions treated by the Pacific Marine Mammal Center (PMMC; Laguna Beach, CA) during 2007 and 2009. Following collection, samples were stored at -20°C at PMMC, transported frozen to the University of Southern California (USC; Los Angeles, CA) and once again stored at -20°C until analysis via ELISA. Samples collected in 2007 were initially analyzed using the Biosense ELISA, and samples collected in 2009 were initially analyzed using the Mercury Science ELISA; all samples were initially analyzed typically within one month following receipt at USC. Fluid samples that were determined to be below the detection limit of the respective ELISA platforms and contained sufficient volume after the initial analysis were employed in a 'spike and recovery' study that utilized simultaneous analysis by all three platforms in the fall of 2009. Samples collected in 2009 that yielded measurable DA concentrations using the Mercury Science ELISA and contained sufficient remaining volume to be analyzed simultaneously on all three platforms were stored and included in the fall 2009 study to allow for comparison of naturally DA positive fluid samples across all three platforms. These samples also allowed determination of any DA degradation that may have occurred in the samples during storage.

Modified ELISA Protocol

A modified ELISA protocol was developed in 2009 with the primary goal of minimizing the required sample volume and reducing sample preparation. The methanol extraction step, typically used in the extraction of DA from solid matrices (i.e., phytoplankton cells, shellfish tissues), was omitted for fluid samples in this study as the DA was assumed to be in the dissolved form. Fluid samples were vortexed for 1 minute, diluted 1:25 with the sample buffer provided by the respective ELISA manufacturer and the diluted sample briefly vortexed immediately prior to pipetting onto the ELISA plate. The expected limit of detection for each ELISA platform was calculated from the plate sensitivity reported by the manufacturer and adjusted for the 1:25 minimum dilution. The Biosense ELISA is reported to have a 0.01 ng ml^{-1} plate sensitivity, and the limit of detection for a sample diluted 1:25 is expected to be 0.25 ng ml^{-1}. The Mercury Science ELISA is reported to have a 0.1 ng ml^{-1} plate sensitivity, and the limit of detection expected is 2.5 ng ml^{-1}.

Method Comparison Study

In October 2009, at the University of California in Santa Cruz, analyses of sea lion body fluids using the modified protocol for both ELISAs and LC-MS were performed over the course of one week in order to minimize degradation of DA that may occur in samples over long storage periods. Samples selected for the spike and recovery portion of the comparison study were previously determined to be below detection of the respective ELISA platform during their initial receipt and analysis in 2007 or 2009. A portion of the samples collected in the spring of 2009 that had been initially identified as having quantifiable concentrations of DA were re-analyzed simultaneously on all three platforms. This study was designed to allow the following comparisons: 1) performance of the modified ELISA protocol on the analysis of DA concentrations in amniotic fluid, cerebral spinal fluid, serum, and urine samples spiked with known concentrations of DA; 2) comparison of the results obtained with the modified protocol by the Mercury Science and Biosense ELISAs; 3) comparison of results obtained from solid-phase extraction (SPE) cleaned spiked samples analyzed by LC-MS and the Mercury Science ELISA; and 4) comparison of LC-MS results on SPE cleaned samples to Mercury Science and Biosense ELISA results without SPE cleaning (Figure 1).

Preparation of Standards

The DA standard used to spike sea lion fluid samples and to prepare standard curves of LC-MS analysis was obtained from the National Research Council, Canada (Certified Reference Materials-DA-*f*; Ottawa, Ontario). Two standard curves were prepared for the LC-MS analysis: one in Milli-Q water and the other in LC-MS grade 50% methanol. A subset of the Milli-Q standards were SPE-cleaned (see section below) prior to analysis by LC-MS in order to quantify the amount of DA lost during the cleanup procedure. The LC-MS standard curves were made through serial dilution with final concentrations of 1, 2, 5, 20, 50, 100, 250, and 500

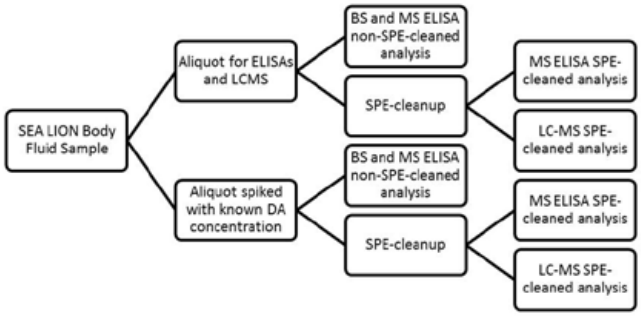

Figure 1. Flow diagram outlining the validation design for California sea lion body fluid samples.

ng ml^{-1}. A standard of 1000 ng ml^{-1} was prepared in Milli-Q to use in the spiking of AF, CSF, serum, and urine samples to the following concentrations: 12.5, 15, 18, 21.5, 26, 31, 37, 44.5, 53.5, 64, 77, 92.5, and 110 ng ml^{-1}. A matrix free 1 ng ml^{-1} Milli-Q standard was used for assessing ELISA platform performance during the study.

LC-MS Sample Handling Procedure

The procedure used for LC-MS analysis was modified from the previously used procedure for seawater and phytoplankton samples described in Wang *et al.* (2007). The same samples analyzed using ELISA methods were also analyzed using LC-MS after they were cleaned using Bond Elut SPE columns with large reservoir capacity C18 resin (Varian, Inc., now Aglient Technologies, Santa Clara, CA). The columns were conditioned by vacuum filtering 10 ml of 100% methanol followed by 10 ml of LC-MS grade water (Fisher Scientific, Pittsburgh, PA) prior to the addition of samples. Samples were acidified with 0.5 ml 2:5:93 formic acid:methanol:water, and 4 ml 5% formic acid was added prior to introduction to the SPE column. Samples were pipetted into the column, and 4 ml of 1.5% formic acid was added to the sample followed by vacuum filtration and extract disposal. The final extraction step used vacuum filtration of 3 ml of 50% methanol onto the column, with the resulting extract collected for analysis. The method detection limit of 0.48 ng ml^{-1} was determined using seven spiked Milli-Q samples with SPE cleanup.

Analysis of Spike and Recovery of DA Results

The quality of the fluid samples collected by rehabilitation centers can be impacted by multiple factors, as discussed in the introduction, and fluid samples from a number of individual animals were employed in the spike and recovery portion of the study to account for this variability. The samples were divided into two portions; one portion of each sample remained unspiked, and the other portion was spiked with DA standard at known concentrations (Figure 1). The unspiked portions were run on each platform; non-SPE cleaned samples analyzed using the Mercury Science and Biosense ELISAs, and SPE-cleaned samples analyzed by Mercury Science ELISA and LC-MS. The remaining spiked portion of each sample type was vortexed for 1 minute, then analyzed as noted in the previous sections detailing the modified ELISA protocol and LC-MS sample handling procedure.

ELISA samples were run in triplicate and spread across two plates to account for any inter- or intra-plate variability that may have arisen during commercial fabrication. DA concentrations for each ELISA sample were determined from the average results of duplicate wells and calculated using Excel spreadsheets provided by the respective manufacturers. Biosense ELISA results were quantified using an Excel Macro that employs a 4-parameter logistics curve-fitting model to produce a standard curve for each plate and sample concentrations were determined by extrapolating from the standard curve. Mercury Science ELISA results were calculated using an Excel spreadsheet that used a ratio between the maximal absorbance signal (a control containing no DA) and the absorbance signal of the sample in conjunction with constants for the midpoint and slope of a standard curve determined by the manufacturer when the method was developed. Both quantification spreadsheets automatically identified samples outside of the working range of the ELISA (i.e., too dilute or too concentrated) and calculated coefficients of variation (CV) for the duplicate sample wells. Samples with CVs greater than 15% were eliminated as recommended by the manufacturers because the high amount of variation confounds identification of duplicate wells that can be used to calculate an accurate DA concentration.

The measured DA concentrations determined by the respective platforms were plotted against the known DA concentration spiked into each sample. The ELISA and LC-MS samples were treated blindly, with no correction of ELISA or LC-MS results for the known DA concentrations spiked into the samples. This was done in order to more appropriately mimic the manner in which

sea lion body fluid samples of an unknown DA concentration might be handled in different laboratories. The slopes and R^2 values of the linear regressions plotted for each platform were recorded and the slopes statistically compared. The linear regressions were carried out with SigmaPlot (v 11.0.0; Systat Software, Inc., Chicago, IL). A Student's *t* test was computed as the difference between the two slopes being compared and divided by the standard error of the difference between the slopes. The comparison of slopes using the Student's *t* test has been identified as reliable for determining agreement between two methods and agreement to the ideal equality line with a slope of 1 (Westgard and Hunt 1973, Bland and Altman 1986). Deming regressions were performed to test for linear relationships between platform results using the XLSTAT Macro for Microsoft Excel (v 2012; Addinsoft SARL, New York, NY).

Results and Discussion

Spike and Recovery of DA in Sea Lion Amniotic Fluid Samples

The DA concentrations measured by each method for the spiked sea lion amniotic fluid samples were plotted versus the known DA concentrations added to each sample and linear regressions performed to determine the R^2 and slope (Figure 2a; Table 2). The highest R^2 value of 0.94 was obtained for the Mercury Science ELISA results using non-SPE-cleaned samples, indicating good linearity across the range of DA concentrations examined. The slope obtained for the Mercury Science non-SPE-cleaned regression line was 0.67, lower than and statistically different from the ideal slope of 1 ($t_{0.05,2,8}$ >2.31). The Biosense ELISA results using non-SPE-cleaned samples also showed good linearity across the range of DA concentrations as indicated by an

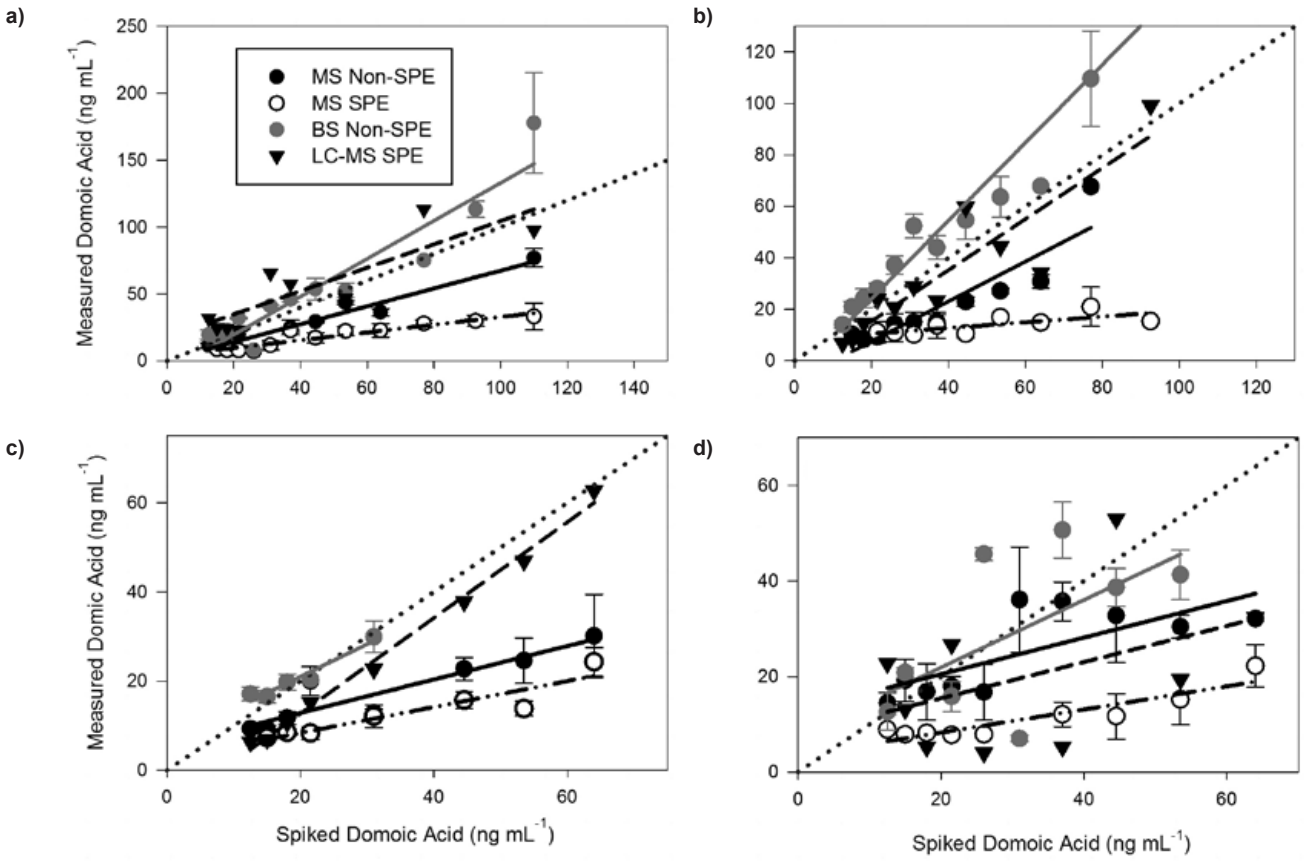

Figure 2. Results of DA measurements using different analytical platforms for samples spiked with known concentrations of DA standard in amniotic fluid (a), cerebral spinal fluid (b), serum (c), and urine (d). The dotted lines in all graphs show the expected DA concentration based on the concentration of DA added to each sample. Error bars represent standard deviations of triplicate replicates. The results for Mercury Science ELISA non-SPE-cleaned are represented by black circles and the solid black line, with the Mercury Science (SPE-cleaned results represented by open circles and the long-dashed and dotted line; Biosense ELISA non-SPE-cleaned results are represented by gray circles and the gray line; and LC-MS results are represented by black triangles and the long-dashed line.

Table 2. The R^2 and slope values for each method platform and fluid obtained from linear regression with the known (spiked) concentration of DA.

Fluid Type	Mercury Science ELISA				Biosense ELISA		LC-MS	
	Non-SPE Cleaned		SPE Cleaned		Non-SPE Cleaned		SPE Cleaned	
	R^2	Slope	R^2	Slope	R^2	Slope	R^2	Slope
Amniotic	0.94	0.67	0.88	0.29	0.89	1.42	0.75	0.87
Cerebral Spinal	0.82	0.78	0.55	0.11	0.92	1.52	0.48	0.7
Serum	0.85	0.38	0.86	0.29	0.94	0.73	0.99	1.07
Urine	0.55	0.38	0.84	0.24	0.36	0.71	0.19	0.38

R^2 of 0.90. The slope of the regression line was 1.4, higher than and statistically different from the ideal slope of 1 ($t_{0.05,2,10}$ >2.23). The results from the SPE-cleaned samples analyzed on the Mercury Science ELISA had a high R^2 (0.88), but the slope of the linear regression line was 0.29, much lower than and statistically different from the ideal slope of 1 ($t_{0.05,2,10}$ >2.23). The slope of 0.87 obtained for the LC-MS measured SPE-cleaned samples was not statistically distinguishable from the ideal slope of 1 ($t_{0.05,2,7}$ <2.37), and the R^2 value of 0.75 indicated a relatively good linearity across the range of DA concentrations tested.

DA concentrations quantified by the Biosense and Mercury Science ELISAs were the result of averaged duplicate wells with CVs less than the manufacturer recommended 15%, with each sample run in triplicate. The CVs calculated for amniotic fluid samples using the Biosense ELISA were commonly higher than the Mercury Science ELISA, and as a consequence more Biosense measured results were rejected than Mercury Science. The Biosense ELISA results on the non-SPE-cleaned spiked amniotic fluid samples had an average CV of 12% and 10 sample results were discarded because of CVs >15%. The Mercury Science ELISA results had an average CVs of 5% for the non-SPE-cleaned spiked amniotic fluid samples and 4% for SPE-cleaned samples, with only one sample replicate yielding a CV >15%.

Comparison of the slopes calculated using each analytical approach with the amniotic fluid spiked samples revealed that the slope obtained using the Mercury Science ELISA on non-SPE-cleaned samples (0.67) was not statistically different from the slope of the LC-MS regression line (0.87; $t_{0.05,2,15}$ <2.13), while the slopes obtained for the remaining platforms were found to be statistically different from one another (Figure 2a; Table 2). The latter result was not unexpected given the basic dissimilarities in sample preparation and analytical methods.

The results for the SPE-cleaned amniotic fluid samples measured by LC-MS and the Mercury Science ELISA were compared and had an R^2 of 0.73 (Table 3). These results demonstrated relatively good linearity between the two methods, further confirmed through a Deming regression with computed p-value of 0.34, greater than $\alpha = 0.05$, supporting the null

Table 3. Comparison of the ELISA method results with LC-MS results for each sea lion fluid matrix. The R^2 and slope values are reported from linear regression analysis.

Method	Fluid Type LC-MS							
	Amniotic		Cerebral Spinal		Serum		Urine	
	R^2	Slope	R^2	Slope	R^2	Slope	R^2	Slope
Mercury Science ELISA (SPE cleaned)	0.73	0.25	0.15	0.03	0.89	0.27	0.14	0.12
Mercury Science ELISA (non-SPE cleaned)	0.60	0.68	0.54	0.38	0.29	0.35	0.05	0.12
Biosense ELISA	0.56	1.13	0.59	0.84	0.92	0.77	0.06	-0.22

hypothesis that the relationship was linear. The low slope of 0.27 for the regression line indicated significant underestimation of DA concentrations on SPE-cleaned amniotic fluid samples by the Mercury Science ELISA. Underestimation of DA concentrations in the SPE-cleaned amniotic fluid samples was also evidenced by the slope of 0.28 obtained when these values were plotted versus the known spike concentrations (Table 2). The SPE-cleaning produced an extract comprised of 50% methanol, which may have an inhibitory effect on ELISA antibodies and therefore underestimate DA concentrations. The low slope values obtained when plotting the Mercury Science ELISA results for SPE-cleaned samples of amniotic fluid (Tables 2 and 3) indicate that SPE-cleaning may be contraindicated for analysis of amniotic fluid samples using an ELISA platform.

The results obtained by both ELISAs with non-SPE-cleaned amniotic fluid samples were compared with the LC-MS results on SPE-cleaned amniotic fluid samples (Table 3). The R^2 values for the regression lines of the Mercury Science and Biosense ELISA results were 0.60 and 0.56, respectively, indicating a moderate linearity between the ELISA and LC-MS measured values. Deming regressions of the ELISA results versus the LC-MS results produced p-values of 0.34 in each regression and supported the null hypothesis that the ELISA results on non-SPE-cleaned amniotic fluid samples have a linear relationship with the LC-MS SPE-cleaned results.

The comparison of the results for the Mercury Science ELISA (non-SPE-cleaned samples) and the Biosense ELISA results are shown in Table 4. The R^2 of 0.89 indicated good linearity between the results of the two ELISA methods, and a Deming regression analysis was performed to confirm a linear relationship. The computed p-value of 0.95 for the Deming regression is greater than $\alpha = 0.05$, and supported the null hypothesis that the relationship between the Mercury Science and Biosense ELISAs (non-SPE-cleaned) amniotic fluid results was linear. The slope of the Deming regression was 2.16, most likely influenced by the Mercury Science ELISA slightly underestimating DA concentrations and the Biosense ELISA overestimating DA concentrations for the higher concentrations investigated.

Table 4. Direct comparison of the Biosense and Mercury Science (non-SPE cleaned) ELISA platform results.

Fluid Type	R^2	Slope
Amniotic	0.89	2.16
Cerebral Spinal	0.92	1.43
Serum	0.08	0.35
Urine	0.08	0.52

Spike and Recovery of DA in Sea Lion Cerebral Spinal Fluid Samples

DA concentrations measured by each method for the spiked sea lion cerebral spinal fluid samples were plotted versus the known DA concentrations and linear regression performed to determine the R^2 and slope values (Figure 2b; Table 2). The highest R^2 value of 0.92 was tabulated for the Biosense ELISA results on non-SPE-cleaned cerebral spinal fluid samples, indicating good linearity across the range of DA concentrations measured. The slope of the Biosense ELISA regression line was 1.5, greater and statistically different than the ideal slope of 1 ($t_{0.05,2,10} > 2.23$). The Biosense ELISA overestimated DA concentrations in cerebral spinal fluid samples, similar to the result obtained for the amniotic fluid samples analyzed by the Biosense ELISA discussed above. The slope of the regression line of Mercury Science ELISA results on non-SPE-cleaned cerebral spinal fluid samples was 0.78 and not statistically different than the ideal slope of 1 or the slope of the LC-MS regression line (0.70; $t_{0.05,2,18} < 2.10$, $t_{0.05,2,8} < 2.31$, respectively). The R^2 of 0.82 for the non-SPE-cleaned cerebral spinal fluid samples analyzed using the Mercury Science ELISA indicated a relatively good linearity across the range of DA concentrations tested. The LC-MS regression line had a relatively poor R^2 value (0.48), but the slope of the regression line was not statistically different than the ideal slope of 1 (0.70; $t_{0.05,2,6} < 2.45$). The low R^2 value may have been a consequence of matrix influence on the ability of the LC-MS to quantify DA concentrations, or DA lost in the SPE-cleanup procedure. This could be addressed in the future by including standard additions for the unknown samples, or by using the same matrix for the standard curve. The SPE-cleaned cerebral spinal fluid samples that were analyzed on the Mercury Science ELISA platform exhibited relatively good linearity with a R^2 value of 0.55, but the slope of the regression line was relatively flat (0.11) and statistically different from

the ideal slope of 1 ($t_{0.05,2,5}$ >2.57). Whether these results were a consequence of the impact of 50% methanol extract on the ELISA or the SPE- cleanup procedure itself is unknown. However, these results verified previous findings that the SPE-cleanup step was contraindicated for ELISA.

The CVs calculated for non-SPE-cleaned cerebral spinal fluid samples were higher using the Biosense ELISA than the Mercury Science ELISA, similar to the results obtained with the analysis of the amniotic fluid samples discussed above. Non-SPE-cleaned and SPE-cleaned samples measured by the Mercury Science ELISA both had average CVs of 4%, and no results were discarded for CVs >15%. In contrast, the non-SPE-cleaned samples analyzed by the Biosense ELISA had an average CV of 14%, with 20 results having CVs >15% (and were therefore discarded).

The Mercury Science ELISA (SPE cleaned) and LC-MS results were compared and an R^2 and slope value calculated (Table 3). Although samples were handled in an identical manner prior to analysis, the SPE-cleaning only appeared to impact the results of the Mercury Science ELISA analysis, presumably due to the effect of the 50% methanol extract produced by SPE-cleaning. The impact on toxin detection capabilities of SPE-cleaned cerebral spinal fluid samples analyzed by the Mercury Science ELISA was reflected in the slope value of 0.03 and confirmed the finding that SPE was contraindicated for the analysis of DA samples by ELISA. A Deming regression computed a p-value of 0.51 ($>\alpha = 0.05$) and supported the null hypothesis that the relationship between the LC-MS and Mercury Science ELISA results was linear, in spite of the flat slope and a low R^2 value of 0.15.

The results of non-SPE-cleaned cerebral spinal fluid samples using both ELISAs were compared with the LC-MS results and Deming regressions performed to test for linear relationships. R^2 values of 0.59 and 0.54 were obtained for the Biosense and Mercury Science ELISA results of non-SPE-cleaned cerebral spinal fluid samples, respectively (Table 3). The moderate linearity indicated by the R^2 value for the Biosense ELISA results was confirmed with a Deming regression with a computed p-value of 0.70 ($>\alpha = 0.05$) supporting the null hypothesis of a linear relationship. The Deming regression computed a p-value of 0.99 ($>\alpha = 0.05$) to support the null hypothesis that the non-SPE-cleaned Mercury Science ELISA results had a linear relationship with the SPE-cleaned LC-MS results.

The slopes of the regression lines obtained for the non-SPE-cleaned samples analyzed by the Mercury Science and Biosense ELISAs plotted against known DA concentrations were found to be statistically different ($t_{0.05,2,18}$ >2.13). The absolute values obtained for the two ELISA methods slightly overestimated (Biosense) or underestimated (Mercury Science) the DA concentrations relative to the known amounts of DA spiked into these samples. When the results for each ELISA were plotted against each other (Table 4), the R^2 of 0.92 indicated good linearity across the range of DA concentrations measured by each platform; however, the Biosense ELISA yielded values that were consistently higher than values obtained using the Mercury Science ELISA. A Deming regression confirmed the null hypothesis of a linear relationship between the Mercury Science and Biosense ELISA results with a computed p-value of 0.082 ($>\alpha = 0.05$).

Spike and Recovery of DA in Sea Lion Serum Samples

The DA concentrations measured by each method for spiked sea lion serum samples were plotted against known DA concentrations, and linear regression was performed to determine the R^2 and slope (Figure 2C; Table 2). The regression line slope of DA concentrations in SPE-cleaned serum samples analyzed using LC-MS was 1.07 and not statistically different from the ideal slope of 1 ($t_{0.05,2,6}$ <2.45; R^2 value was 0.99). The absolute values obtained by LC-MS were all slightly less than the expected (spiked) values, indicating a slight loss of DA, presumably during the SPE cleaning, or possibly suppression of ionization due to matrix effects; again this could be corrected in the future with the use of matrix-specific standard curve or with internal spikes of known concentrations. The Biosense ELISA results on non-SPE cleaned serum samples returned the second highest R^2 values with a 0.94 and a slope of 0.73 that was not statistically different than the ideal slope of 1 ($t_{0.05,2,3}$ <3.18). The absolute toxin concentrations for the Biosense ELISA method were all very similar to the expected (spiked) concentrations of DA, but the results may be misleading because the number of samples plotted was reduced considerably by the elimination of sample results yielding poor CV values. The CVs calculated for the Biosense ELISA averaged 18%, with 23 results discarded (CVs

>15%). The frequency of high CV values obtained on the Biosense ELISA may have been influenced by the color of the serum samples analyzed. Serum is blood plasma with the fibrinogens removed and should not contain any red or white blood cells. The removal of red and white blood cells is affected by the efficiency of the centrifugation and the quality of the serum sample collected from the animal. From this study, it is speculated that the presence of red blood cells (or material from lysed cells) in a serum sample may interfere with colorimetric determination of DA concentration determination by ELISA methods. However, this effect was not observed for the Mercury Science ELISA analysis on either the non-SPE or SPE-cleaned serum samples. The non-SPE and SPE-cleaned sample CVs for the Mercury Science ELISA averaged 4% and there were no results with high CVs, therefore no results were discarded.

The R^2 value determined for the Mercury Science ELISA for non-SPE-cleaned serum samples was 0.85 and the SPE-cleaned samples returned a R^2 value of 0.86 (Table 2). The R^2 values indicate good linearity across the range of DA concentrations measured, however all the DA concentrations analyzed by using the Mercury Science ELISA platform were less than the expected values, and the slopes of 0.38 and 0.29, respectively, were statistically different than the ideal slope of 1 ($t_{0.05,2,6}$ >2.45, $t_{0.05,2,4}$ >2.78; Figure 2C and Table 2).

The regression for the SPE-cleaned serum samples measured by LC-MS and the Mercury Science ELISA yielded an R^2 of 0.89, indicating a good linearity between the measured DA concentrations (Table 3). However, most of the Mercury Science ELISA values were less than the values obtained using LC-MS, particularly for samples with high concentrations of DA added, as indicated by the slope of the regression line (0.27). Deming regression analysis computed a p-value of 0.98 ($>\alpha = 0.05$) supporting the null hypothesis of a linear relationship between SPE-cleaned serum samples analyzed by the Mercury Science ELISA and LC-MS.

The results obtained from non-SPE-cleaned serum samples using the two ELISA were compared to the LC-MS results (Table 3). The R^2 for the Biosense ELISA analysis was 0.92, indicating good linearity between the measured LC-MS and Biosense ELISA measured DA concentrations. However, relatively few samples were compared because many of the Biosense ELISA results were discarded due to high CVs, and those samples that were compared had higher values than the LC-MS results. The R^2 value for the Mercury Science ELISA (non-SPE cleaned) results was 0.29, indicating poor linearity between DA concentrations in spiked serum samples measured by LC-MS and the Mercury Science ELISA. Deming regressions computed p-values of 0.76 ($>\alpha = 0.05$) for the Mercury Science and Biosense ELISA non-SPE-cleaned serum results in comparison to the SPE-cleaned LC-MS results. The null hypothesis was supported in both instances: non-SPE-cleaned Mercury Science ELISA results had a linear relationship with the LC-MS SPE-cleaned results, and the non-SPE-cleaned Biosense ELISA results had a linear relationship with the LC-MS SPE-cleaned results.

The R^2 of 0.08 and slope of 0.35 obtained when plotting the results of the non-SPE-cleaned serum samples measured using both ELISAs indicated a lack of agreement between the two platforms (Table 4). However, the number of samples included in the comparison plot was small (4) because many of the samples measured using the Biosense ELISA were discarded due to high CVs >15%. Deming regression analysis computed a p-value of 0.76 ($>\alpha = 0.05$) supporting the null hypothesis that there is a linear relationship between the non-SPE-cleaned serum samples analyzed by the Mercury Science and Biosense ELISAs.

Spike and Recovery of DA in sea lion Urine Samples

The DA concentrations measured by each method for the spiked sea lion urine samples were plotted against known DA concentrations, and linear regressions were performed to determine the R^2 and slope values (Figure 2d; Table 2). The urine sample results had the lowest R^2 values for all platforms tested when compared to results for the other sea lion fluids. The highest R^2 (0.84) was obtained for values determined using the Mercury Science ELISA on SPE-cleaned urine samples. However, the regression line slope of 0.24 was lower than and statistically different from the ideal slope of 1, and all absolute values were considerably less than values anticipated from the DA concentrations added to the samples. The non-SPE-cleaned samples analyzed by the Mercury Science and Biosense ELISAs were 0.55 and 0.36, respectively, but the absolute values obtained were generally more similar to the expected

(spiked) concentrations of DA. Therefore, the SPE-cleaning improved the precision of replicate samples analyzed by the Mercury Science ELISA, but the results underestimated the known DA concentrations, especially at the higher concentrations of DA used in the spiked samples. The R^2 value of 0.19 obtained for the LC-MS measured results was the lowest R^2 value.

The poor performance of the urine analysis by the three platforms may be attributable to the high salt content of sea lion urine, which is 2.5 times more concentrated than seawater and 7 to 8 times more concentrated than sea lion blood. The concentrated urine produced by these animals is a mechanism to rid their bodies of excess salt and reduce freshwater loss. It is possible that the salt inhibits antibody performance in the ELISA platforms, affects the loss of DA from samples during the SPE cleaning procedure and/or impacts the efficiency of ion formation during LC-MS analysis. SPE cleaning is recommended for the analysis of DA concentrations in seawater by LC-MS as the salt presence impacts Mercury Science signal stability (Wang *et al.* 2007).

The results of the Mercury Science and Biosense ELISAs compared with the LC-MS results are shown in Table 3. The highest R^2 was 0.14 for the Mercury Science ELISA SPE-cleaned. The other platforms, Biosense and Mercury Science (non-SPE-cleaned) ELISAs yielded very low (0.06 and 0.05) R^2 results, highlighting the poor relationships identified between each of the methods. Further, a large number of samples were discarded from the ELISA results due to high CVs. The non-SPE-cleaned samples analyzed using the Mercury Science ELISA had an average CV of 6%, but five sample results were discarded for CVs exceeding 15%. The SPE-cleaned Mercury Science samples had better CVs, 3% on average, and no results were discarded. The Biosense ELISA results (non-SPE-cleaned) averaged CVs were 14% and 24 sample results were discarded due to high CVs (>15%).

The direct comparison of the non-SPE-cleaned urine samples analyzed by Mercury Science and Biosense ELISAs are shown in Table 4. The R^2 was 0.08, and the regression line slope was 0.52.

Analysis of DA in Sea Lion Body Fluids from Natural Samples

The modified DA protocol described in this study for the analysis of DA in sea lion body fluids was adopted at USC in 2009. Samples collected from stranded sea lions in Orange County, California, by Pacific Marine Mammal Center were analyzed within one month of sample receipt using the modified protocol on the Mercury Science ELISA. Samples with DA concentrations thought to be significant enough to survive storage at -20°C until the planned validation study were set aside to be reanalyzed by the three platforms simultaneously in the fall of 2009. Domoic acid degradation in particulate phytoplankton samples has been shown to be highly variable (Quay *et al.* 2011) and capable of potentially influencing successful quantification of DA concentrations of sea lion body fluids after long-term storage. Amniotic fluid samples collected in the spring of 2009 were below the detection limit of the Mercury Science ELISA, and therefore no naturally positive amniotic fluid samples were available for analysis.

Available fluid samples from 11 different animals were analyzed simultaneously using the 4 methods (Table 5). The original DA concentrations measured using the Mercury Science ELISA (non-SPE cleaned) on samples within one month of receipt at USC are summarized as well as the values reported during this validation study in the fall 2009. The majority of the samples (eight animals) were stored at -20°C for six months, samples from two animals were stored for eight months and samples from one animal were stored for nine months. The amount of DA degradation observed in individual samples analyzed by the Mercury Science ELISA using the modified protocol was highly variable. There was a 49% decrease in the DA concentration measured in the urine sample stored for nine months, an average decrease 40% in DA concentrations for urine samples stored for eight months and an average 43% decrease in DA concentrations for urine samples stored for six months, for an overall average loss of 44%. The individual percent decreases in DA concentrations measured by the Mercury Science ELISA in urine samples ranged from 4 to 72%, and in two instances the DA concentrations measured were higher than the original DA concentrations measured from fresh samples. Four serum samples were stored for six months and losses of DA ranged from 3 to 64% when measured by Mercury Science ELISA. Two of the four samples decreased in DA concentration during storage to levels below the detection of the Mercury Science ELISA. One of the cerebral

Table 5. Measurements of naturally present DA concentrations in California sea lion fluids compared among the three platforms. The original DA concentration determined by Mercury Science ELISA with the modified DA analysis protocol performed within one month of receipt of samples at USC (MS NonSPE (Orig)) is shown. Domoic acid concentrations detected during this validation study are reported by fluid type for the Mercury Science ELISA without SPE cleaning (MS NonSPE), Biosense ELISA without SPE cleaning (BS NonSPE), and Mercury Science SPE cleaned (MS SPE) results. Reported values are averages of triplicate replicates.

Animal	Cerebral Spinal Fluid (ng ml^{-1})					Serum (ng ml^{-1})					Urine (ng ml^{-1})				
	MS NonSPE (Orig)	MS NonSPE	BS NonSPE	MS SPE	LC-MS	MS NonSPE (Orig)	MS NonSPE	BS NonSPE	MS SPE	LC-MS	MS NonSPE (Orig)	MS NonSPE	BS NonSPE	MS SPE	LC-MS
14											547.7	281.9 ±57.6	335.3 ±56.2	117.6 ±25.4	410.87
32											603.5	293.6 ±97.9	362.5 ±40.3	158.4 ±32.9	500.46
33											366.7	260.8 ±0.0	265.5 ±0.0	191.5 ±4.3	358.43
58											202.4	194.3 ±20.0		108.2 ±32.6	239.67
65						13.9	bd	8.6 ±1.1	bd	8.79	4904.9	1366.7 ±71.7	1838.5 ±371.7	906.1 ±0.0	1828.9
66	74.6	34.1 ±5.0	55.6 ±1.3	bd	4.92						12519.5	4483.0 ±429.6		2800.0 ±325.5	6396.4
67						15.6	16.1 ±4.5	18.1 ±3.4	12.6 ±1.9	16.56	1119.8			323.9 ±17.0	1059.8
68											49.8	54.5 ±9.5		23.8 ±2.0	106.84
72											894.5	2884.0 ±806.1		228.4 ±109.2	2273.8
76	10.3	bd	3.4 ±0.09	bd	bd	20	7.3 ±0.9	12.8 ±1.2	10.0 ±0.0	7.75	7456.5	4345.0 ±527.5	4903.4 ±83.0	1817.3 ±227.0	4401.8
78						9.4	bd	bd	bd	29.10	692.9	462.4 ±45.9		108.8 ±7.7	2275.5

spinal fluid samples stored for six months decreased 54% in DA concentration by Mercury Science ELISA analysis, and the other decreased to below the detection limit of the Mercury Science ELISA. It is recommended that body fluid samples be analyzed for DA content promptly following receipt as DA degradation in samples during storage was found to be highly variable.

The impact of SPE-cleaning of naturally positive samples intended for ELISA analysis was compared and summarized in Table 5. The SPE-cleaned urine samples averaged 51% lower (range = 9 to 92% lower) in DA concentrations than the non-SPE-cleaned samples analyzed by the Mercury Science ELISA. The SPE-cleaned urine samples analyzed by the Mercury Science ELISA were consistently lower than the LC-MS measured DA concentrations for the same sample. The Mercury Science ELISA SPE-cleaned samples averaged 67% (range = 47 to 95% lower) of the LC-MS measured concentrations. These results were consistent with the results obtained from the spike and recovery portion of this validation study in that SPE-cleaning did not improve DA analysis of sea lion body fluids for ELISA methods. The 50% methanol extract obtained from the SPE clean-up appeared to inhibit antibody performance leading to underestimation of the DA concentrations.

Summary of Platform Performance

The modified protocol described in the present study for the analysis of sea lion body fluids by an ELISA platform was validated as a protocol for analysis of DA concentrations. SPE-cleaning was shown to be contraindicated for the removal of matrix effects stemming from the different body fluid matrices, because the 50% methanol extract was more inhibitive to antibody performance than the individual fluid types. False positives were not observed using either ELISA platform, regardless of sample type, using a 1:25 minimal dilution without a methanol extraction step. Overall, the Biosense ELISA yielded accurate DA concentrations or slightly overestimated DA concentrations, possibly due to the multiple

epitopes targeted by the polyclonal antibodies. Whether a slightly higher dilution of body fluid samples would have overcome this inhibition was not tested. Overestimation of DA concentrations using the Biosense ELISA to analyze body fluids has also been noted in previous studies comparing Biosense ELISA measured results of DA in rat serum and brain samples, relative to LC-MS measurements (Hesp *et al.* 2005). In general, the Mercury Science ELISA slightly underestimated DA concentrations relative to the polyclonal antibodies of the Biosense ELISA, possibly due to greater sensitivity of the monoclonal antibody to the body fluid matrices,. However, the Biosense ELISA results were less reproducible than Mercury Science ELISA results, with the Biosense ELISA values yielding higher standard deviations and CVs. A total of 77 samples analyzed using the Biosense ELISA were discarded due to high CVs, while only 6 samples analyzed using the Mercury Science ELISA were discarded. High CVs can be a consequence of operator error during analysis (i.e., pipette error, use of an un-homogenized sample, procedural error), matrix impacts on antibody performance, and/or a manufacturing error. It is unknown if matrix effects were solely responsible for the higher CVs observed in the Biosense ELISA analysis.

In summary, the purpose of this study was to provide a comparison of DA concentrations reported in sea lion body fluids across methods, laboratories, and datasets for a spectrum of matrices. The amount, quality, and type of body fluid available for DA analysis from an individual animal are variable and highly dependent on the health of the animal upon arrival at rehabilitation facilities. In addition, animals suffering from chronic DA toxicosis could become sick at lower concentrations of DA in their body than animals afflicted with acute DA toxicosis. Accurate determination of toxin concentration is useful in order to determine the treatment for California sea lions suffering from DA toxicosis.

The ELISA and LC-MS methods described in the present study have been shown to be capable of detecting DA in California sea lion fluids without introducing a significant risk of false positives. The platforms demonstrated relatively good agreement (high R^2 values) with known DA concentrations added to sea lion body fluid samples, and the linearity observed when platform results that were directly compared verified that the magnitude of DA concentrations measured by each platform was comparable. As the sole exception, urine was identified as a complicated matrix, most likely due to the high salt content, and further investigation is needed to determine if additional sample preparation and/or clean-up procedures might reduce the interference observed in the urine results of the present study. It is therefore recommended that urine samples be analyzed for presence or absence of DA (as opposed to DA concentration), regardless of the platform being used for the analysis.

Literature Cited

Allen, W. 1934. Marine plankton diatoms of lower California in 1931. *Botanical Gazette* 95:485-492.

Allen, W. 1936. Occurrence of marine plankton diatoms in a ten-year series of caily catches in southern California. *American Journal of Botany* 23:60-63.

Bargu, S., C. Powell, S. Coale, M. Busman, G. Doucette and M. Silver. 2002. Krill: A potential vector for domoic acid in marine food webs. *Marine Ecology Progress Series* 237:209-216.

Bargu, S., M. Silver, T. Goldstein, K. Roberts and F. Gulland. 2010. Complexity of domoic acid-related sea lion strandings in Monterey Bay, California: Foraging patterns, climate events and toxic blooms. *Marine Ecology Progress Series* 418:213-222.

Bates, S., C. Bird, A. de Freitas, R. Foxall, M. Gilgan, L. Hanic, G. Johnson, A. McCulloch, P. Odense, R. Pocklington, M. Quilliam, P. Sim, J. Smith, D. Subba Rao, E. Todd, J. Walter and J. Wright. 1989. Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. *Canadian. Journal of Fisheries and Aquatic Sciences* 46:1203-1215.

Bejarano, A., F. Gulland, T. Goldstein, J. St. Leger, M. Hunter, L. Schwacke, F. Van Dolah and T. Rowles. 2008. Demographics and spatio-temporal signature of the biotoxin domoic acid in California sea lions (*Zalophus californianus*) stranding records. *Marine Mammal Science* 24:899-912.

Blanco, J., C. Acosta, C. Marino, S. Muniz, H. Martin, A. Morono, J. Correa, F. Arevalo and C. Salgado. 2006. Depuration of domoic acid from different body compartments of the king scallop

Pecten maximus grown in raft culture and natural bed. *Aquatic Living Resources* 19:257-265.

Bland, J. and D. Altman. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1:307-310.

Brodie, E., F. Gulland, D. Greig, M. Hunter, J. Jaakola, J. St Leger, T. Leighfield and F. Van Dolah. 2006. Domoic acid reproductive failure of California sea lions (*Zalophus californianus*). *Marine Mammal Science* 22:700-707.

Busse, L., E. Venrick, R. Antrobus, P. Miller, V. Vigilant, M. Silver, C. Mengelt, L. Mydlarz and B. Prezelin. 2006. Domoic acid in phytoplankton and fish in San Diego, CA, USA. *Harmful Algae* 5:91-101.

Del Rio, R., S. Bargu, D. Baltz, S. Fire, G. Peterson and Z. Wang. 2010. Gulf menhaden (*Brevoortia patronus*): A potential vector of domoic acid in coastal Louisiana food webs. *Harmful Algae* 10:19-29.

Fire, S., Z. Wang, M. Berman, G. Langlois, S. Morton, E. Sekula-Wood and C. Benitez-Nelson. 2010. Trophic transfer of the harmful algal toxin domoic acid as a cause of death in a Minke whale (*Balaenoptera acutorostrata*) stranding in southern California. *Aquatic Mammals* 36:342-350.

Fritz, L., M. Quilliam, J. Wright, A. Beale and T. Work. 1992. An outbreak of domoic acid poisoning attributed to the pennate diatom *Pseudo-nitzschia australis*. *Journal of Phycology* 28:439-442.

Fryxell, G., M. Villace and L. Shapiro. 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.

Goldstein, T., J. Mazet, T. Zabka, G. Langlois, K. Colegrove, M. Silver, S. Bargu, F. Van Dolah, T. Leighfield, P. Conrad, J. Barakos, D. Williams, S. Dennison, M. Haulena and F. Gulland. 2008. Novel symptomatology and changing epidemiology of domoic acid toxicosis in California sea lions (*Zalophus californianus*): An increasing risk to marine mammal health. *Proceedings of the Royal Society B* 275:267-276.

Goldstein, T., T. Zabka, R. DeLong, E. Wheeler, G. Ylitalo, S. Bargu, M. Silver, T. Leighfield, F. Van Dolah, G. Langlois, I. Sidor, J. Dunn and F. Gulland. 2009. The role of domoic acid in abortion and premature parturition of California sea lions (*Zalophus californianus*) on San Miguel Island, California. *Journal of Wildlife Disease* 45:91-108.

Gulland, F., M. Haulena, D. Fauquier, G. Langlois, M. Lander, T. Zabka and R. Duerr. 2002. Domoic acid toxicity in California sea lions (*Zalophus caliornianus*): Clinical signs, treatment and survival. *Veterinary Record* 150:475-480.

Hesp, B., J. Harrison, A. Selwood, P. Holland and D. Kerr. 2005. Detecton of domoic acid in rat serum and brain by direct competitive enzyme-linked immunosorbent assay (cELISA). *Analytical and Bioanalytical Chemistry* 383:783-786.

Kleivdal, H., S. Kristiansen and M. Nilsen. 2007. Single-Laboratory validation of Biosense direct competitive enzyme-linked immunosorbent assay (ELISA) for determination of domoic acid toxins in shellfish. *Journal of Association of Official Analytical Chemists International* 90:1000-1010.

Leandro, L., R. Rolland, P. Roth, N. Lundholm, Z. Wang and G. Doucette. 2010. Exposure of the North Atlantic right whale *Eubalaena glacialis* to the marine algal biotoxin, domoic acid. *Marine Ecology Progress Series* 398:287-303.

Lefebvre, K., S. Dovel and M. Silver. 2001. Tissue distribution and neurotoxic effects of domoic acid in a prominent vector species, the northen anchovy *Engraulis mordax*. *Marine Biology* 138:693-700.

Lefebvre, K., C. Powell, M. Busman, G. Doucette, P. Moeller, J. Silver, P. Miller, M. Hughes, S. Singaram, M. Silver and R. Tjeerdema. 1999. Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event. *Natural Toxins* 7:85-92.

Litaker, R., T. Stewart, B. Eberhart, J. Wekell, V. Trainer, R. Kudela, P. Miller, A. Roberts, C. Hertz, T. Johnson, G. Frankfurter, G. Smith, A. Schnetzer, J. Schumacker, J. Bastian, A. Odell, P. Gentien, D. Le Gal, D. Hardison and P. Tester. 2008. Rapid enzyme-linked immunosorbent assay for detection of the algal toxin domoic acid. *Journal of Shellfish Research* 27:1301-1310.

McManus, M., R. Kudela, M. SIlver, G. Steward, P. Donaghay and J. Sullivan. 2008. Cryptic blooms:

Are thin layers the missing connection? *Estuaries and Coasts* 31:396-401.

Perl, T., L. Bedard, T. Kosatsky, J. Hockin, E. Todd and R. Remis. 1990. An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *New England Journal of Medicine* 322:1775-1780.

Quay, J., K. Hayashi and R. Kudela. 2011. Watch what you watch for: Degradation of particulate domoic acid (pDA) field samples. *in*: Sixth Symposium of Harmful Algae in the US. University of Texas. Austin, TX.

Ramsdell, J. and T. Zabka. 2008. *In utero* domoic acid toxicity: a fetal basis to adult disease in the California sea lion (*Zalophus californianus*). *Marine Drugs* 6:262-290.

Rines, J., P. Donaghay, M. Dekshenieks, J. Sullivan, and M. Twardowski. Thin layers and camouflage: Hidden *Pseudo-nitzschia* spp. (Bacillariophyceae) populations in a fjord in the San Juan Islands, Washington, USA. *Marine Ecology Progress Series* 225:123-137.

Rines, J., M. McFarland, P. Donaghay and J. Sullivan. 2010. Thin layers and species-specific characterization of the phytoplankton community in Monterey Bay, California, USA. *Continental Shelf Research* 30:66-80.

Scholin, C., F. Gulland, G. Doucette, S. Benson, M. Busman, F. Chavez, J. Cordaro, R. DeLong, A. De Vogelaere, J. Harvey, M. Haulena, K. Lefebvre, T. Lipscomb, S. Loscutoff, L. Lowenstine, R. Marin III, P. Miller, W. McLellan, P. Moeller, C. Powell, T. Rowles, P. Silvagni, M. Silver, T. Spraker, V. Trainer and F. Van Dolah. 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 403:80-84.

Seubert, E., S. Trussell, J. Eagleton, A. Schnetzer, I. Cetinic, P. Lauri, B. Jones and D. Caron. 2012. Algal toxins and reverse osmosis desalination operations: Laboratory bench testing and field monitoring of domoic acid, saxitoxin, brevetoxin and okadaic acid. *Water Research* 46:6563-6573.

Sierra Beltran, A., M. Palafox-Uribe, J. Grajales-Montiel, A. Cruz-Villacorta and J. Ochoa. 1997. Sea bird mortality at Cabo San Lucas, Mexico: Evidence that toxic diatom blooms are spreading. *Toxicon* 35:447-453.

Thomas, K., J. Harvey, T. Goldstein, J. Barakos and F. Gulland. 2010. Movement, dive behavior and survival of California lions (*Zalophus californianus*) posttreatment for domoic acid toxicosis. *Marine Mammal Science* 26:36-52.

Torres de la Riva, G., C. Johnson, F. Gulland, G. Langlois, J. Heyning, T. Rowles and J. Mazet. 2009. Association of an unusual marine mammal mortality event with *Pseudo-nitzschia* spp. blooms along the southern California coastline. *Journal of Wildlife Disease* 45:109-121.

Wang, Z., K. King, J. Ramsdell and G. Doucette. 2007. Determination of domoic acid in seawater and phytoplankton by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* 1163:169-176.

Wang, Z., J. Maucher-Fuquay, S. Fire, C. Mikulski, B. Haynes, G. Doucette and J. Ramsdell. 2012. Optimization of solid-phase extraction and liquid chromatography-tandem mass spectrometry for the determination of domoic acid in seawater, phytoplankton and mammalian fluids and tissues. *Analytica Chimica Acta* 715:71-79.

Westgard, J. and M. Hunt. 1973. Use and interpretation of common statistical tests in method-comparison studies. *Clinical Chemistry* 19:49-57.

Work, T., B. Barr, A. Beale, L. Fritz, M. Quilliam and J. Wright. 1993. Epidemiology of domoic acid poisoning in brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax pencillatus*) in California. *Journal of Zoo Wildlife Medicine* 24:54-62.

Wright, J., R. Boyd, A. de Freitas, M. Falk, R. Foxall, W. Jamieson, M. Laycock, A. McCulloch, A. McInnes, P. Odense, V. Pathak, M. Quilliam, M. Ragan, P. Sim, P. Thibault and J. Walter. 1989. Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. *Canadian Journal of Chemistry* 67:481-490.

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