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Development of a protocol for determination of domoic acid in the sand crab (*Emerita analoga*): a possible new indicator species☆

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Abstract

The aim of this study was to begin evaluating the utility of sand crabs (*Emerita analoga*) as an indicator species for the algal neurotoxin, domoic acid (DA), in Monterey Bay, California, USA, a site of recurrent blooms of the DA-producing diatom, *Pseudo-nitzschia*. One of the current sentinel organisms, the sea mussel (*Mytilus californianus*), has shown minimal or undetectable toxicity during some local bloom events. As a critical step in assuring the accuracy of DA determinations in *E. analoga*, we have developed and validated a highly efficient extraction protocol that yields toxin recoveries of 97 ± 2.9%. We also determined by HPLC-UV and receptor binding assay, with confirmation by LC-MS/MS, that sand crabs accumulated measurable amounts of DA during toxic *Pseudo-nitzschia* blooms, while the sea mussel showed no detectable toxin. In addition, a comparison of inter-animal variability in DA content revealed values ranging from ca. 0.5 to 5 μg DA g^{-1} tissue and no consistent trend with size class, based on either animal weight or length. These data on the toxicity of individual animals will be useful in designing an appropriate sampling strategy for monitoring DA and, importantly, indicate that sand crabs do not appear to progressively bioaccumulate DA with age. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Domoic acid; *Pseudo-nitzschia*; Indicator species; Sentinel species; *Emerita*; Harmful algal blooms; Toxin extraction; Toxin trophic transfer

1. Introduction

The transfer of algal toxins through marine food webs has important implications not only for public health, but also the health of ecosystems and their trophic structure (Boesch et al., 1997). Impacts of algal toxin trophic transfer are manifested as contaminated commercial and recreational fishery resources (Shumway, 1988; Castonguay et al., 1997; Horner et al., 1997; Lefebvre et al., 1999), as well as mortality events involving a variety of birds and mammals (Geraci et al., 1989; Work et al., 1993; Bossart et al., 1998; Scholin et al., 2000). Protection of public health requires identification of an indicator or sentinel species that rapidly accumulates the algal toxin upon its appearance in local waters, while monitoring the movement of toxin through food webs requires identification of potential points of entry and thus vectors for toxin transfer. In the case of domoic acid (DA), sand crabs (*Emerita analoga*) have the potential to serve both as a sentinel species and as a vector for trophic transfer of this potent neurotoxin produced by members of the diatom genus, *Pseudo-nitzschia*. Sand crabs are suspension feeders, found in dense aggregations along the surf zone from California to Alaska, that can directly consume *Pseudo-nitzschia* cells and are common prey for many larger crabs and sea birds. Interestingly, *E. analoga*

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was previously suggested as an indicator species for another class of algal toxins, the saxitoxins, which are responsible for paralytic shellfish poisoning in humans. Sand crabs were shown to accumulate high concentrations of these toxins and exhibited toxicity levels similar to the mussel, *Mytilus californianus*, as determined by mouse bioassay (Sommer 1932).

Monterey Bay, California is a site of recurrent, toxic and non-toxic *Pseudo-nitzschia* blooms (e.g. Buck et al., 1992; Scholin et al., 2000), as well as extensive sand crab populations (unpubl. observ.). This location was therefore selected to begin evaluating the utility of *E. analoga* as an indicator for DA presence in local waters. Moreover, one of the current sentinel organisms, the sea mussel (*M. californianus*), has shown minimal or undetectable toxicity during some bloom events in Monterey Bay (e.g. Scholin et al., 2000). It was anticipated that data collected during this project would provide information on the efficacy of sand crabs as a vector for DA transfer to higher trophic levels.

The efficiency of extracting algal toxins from diverse sample types is highly variable and depends on a variety of factors, including the individual sample matrix and the method employed. As a critical step in assuring the accuracy of DA determination in sand crabs, we initiated an effort to optimize the toxin extraction using a conventional aqueous methanol-based method (Quilliam et al., 1995) as a starting point. We report here on the development and validation of a highly efficient protocol for extracting DA from whole sand crabs and its measurement by HPLC-UV, the method currently used to monitor DA levels for regulatory purposes. We also confirmed by tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS) that sand crabs did, in fact, accumulate DA during toxic *Pseudo-nitzschia* bloom events. In addition, the protocol developed herein was used for assessing inter-animal variability in DA content, which will aid in designing an appropriate sampling strategy and, importantly, in determining whether sand crabs progressively bioaccumulate DA with age.

2. Materials and methods

2.1. Source material

All sand crabs employed in this study were collected from populations in the Monterey Bay region of central California, USA during the time period from October 1998 to May 1999. Following collection, specimens were stored at −80 °C at California State University (Monterey Bay, CA, USA), shipped on dry ice by overnight courier to the NOAA/National Ocean Service laboratory (Charleston, SC, USA), and once again stored at −80 °C until extracted and analyzed.

2.2. Homogenization

The initial step in this extraction procedure was to thoroughly homogenize the animals prior to treatment with an extraction solvent. Two approaches were tested during this study, both involving the homogenization of five whole animals (i.e. including carapace) per extract. Each of the two tests involved four replicate determinations. The first employed a hand-operated garlic press, in which each of five sand crabs was individually passed through the press and the resulting extruded material, collected, weighed, and combined with an equal weight of Milli-Q water (Millipore Inc., Bedford, MA, USA) to yield the homogenate. The second approach involved placing five animals in a tared blender cup (37 ml capacity), recording the weight, adding an equal weight of Milli-Q water, and blending for 2 min to yield the homogenate.

2.3. Extraction

All chemicals used in the extraction, sample clean-up, and DA analysis were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise noted. Samples were kept on an ice/water slurry during all extraction manipulations to minimize any heat build-up. A total of 8 g of sample homogenate prepared using either of the two methods described earlier were placed in a 50 ml plastic conical tube and combined with 8 ml of methanol (HPLC grade) and 4 ml of Milli-Q water. The homogenate suspension was further disrupted with a Polytron (PowerGen 700; Fisher Scientific, Pittsburgh, PA, USA) using a 10×195 mm sawtooth generator probe for 1 min at 10,000 rpm to facilitate extraction of DA by the methanolic solvent. The mixture was then either centrifuged for 10 min at $3200 \times g$ (10 °C) or sonicated (Branson 450; Branson Ultrasonics Corp., Danbury, CT, USA) for 1 min using a microprobe at maximal setting and then centrifuged as described previously. The supernatant, representing the sample extract, was removed, filtered through a 0.45 μm pore diameter nylon syringe filter (Whatman GD/X; Whatman Inc., Clifton, NJ, USA), and stored in a glass vial (teflon septum) at either −20 °C (short-term) or −80 °C (long-term). Sonication of the homogenate prior to addition of the aqueous methanol extraction solvent was also tested during method development.

In an attempt to assess the quantitative recovery of DA from sand crabs, exhaustive extractions were conducted. Following centrifugation and removal of the supernatant from the initial extract, the pellet remaining in the 50 ml conical tube was re-extracted in 8 ml of methanol and 8 ml of Milli-Q water using the Polytron and sonicator as outlined earlier. The 50 ml conical tube was then centrifuged and the supernatant removed, filtered, and stored frozen as before.

2.4. Sample clean-up and domoic acid analysis

Sand crab samples were tested for the presence of DA either as crude extracts or following the application of a

solid phase extraction (SPE) clean-up method. Extracts subjected to SPE clean-up were processed according to the method of Hatfield et al. (1994), employing a strong anion exchange (SAX) cartridge (BakerBond; J.T. Baker, Phillipsburg, NJ, USA). Briefly, SAX cartridges were preconditioned with 6 ml of methanol, then 3 ml of Milli-Q water, followed by 3 ml of 50% aqueous methanol. Once cartridges were conditioned, 2 ml of extract were applied at a flow rate of approximately one drop per second. Cartridges were then washed with 5 ml of 0.1 M NaCl in 10% aqueous acetonitrile (MeCN) and the DA eluted by application of 5 ml of 0.5 M NaCl in 10% aqueous MeCN. Spike-recovery tests were conducted to confirm the performance of the SAX cartridges and yielded DA recoveries of 99% ($n = 1$) for the 50% aqueous methanol extraction solvent and $94 \pm 2\%$ ($n = 2$) for sand crab extract.

Both crude and cleaned extracts were analyzed for DA by the HPLC-UV method described by Quilliam et al. (1995). All analyses were conducted on an LC Module 1 (Waters Inc., Milford, MA, USA), with reverse phase chromatographic separations performed on a Vydac 201TP54 C_{18} column (4.6×250 mm; Separations Group, Hesperia, CA, USA) preceded by a Vydac C_{18} guard column (5 μm particle diameter). Isocratic separations were conducted using a mobile phase of water/MeCN/TFA (90/10/0.1) applied at a flow rate of 1 ml min^{-1}. Quantification of DA was based on the DACS-1C certified DA reference standard available through the Institute for Marine Biosciences, National Research Council of Canada (Halifax, NS, Canada). This reference standard was used to generate a calibration curve comprising 0.5, 1, 2, 5, and 10 μg DA ml^{-1}. All sample values derived from this calibration curve were expressed as either percent recoveries in cases of spike-recovery experiments or as μg DA g^{-1} tissue if the toxin was naturally incurred.

For comparison with the HPLC-UV method, three SAX-cleaned sand crab extracts containing naturally incurred DA were tested using a receptor binding assay based on the method of Van Dolah et al. (1997) with several modifications. In order to remove ambient glutamate, which can yield false positive responses, 50 μl aliquots of each extract were incubated with 10 μl glutamate decarboxylase (100 units ml^{-1} in 10 mM citrate, pH 5.0) and 40 μl citrate buffer (10 mM citrate, pH 5.0; 2 mM pyridoxal 5-phosphate; 200 mM NaCl) for 30 min on ice. Receptor assays were performed in 96-well microtiter filtration plates (Millipore Inc., Bedford, MA, USA) and employed a cloned GluR6 glutamate receptor expressed in SF9 insect cells using a bacculovirus expression system. The radioactive endpoint of this competitive binding assay was determined by microplate scintillation counting (Microbeta 1450; Perkin Elmer Wallac Inc., Gaithersburg, MD, USA). Additional details of the DA receptor binding assay are provided in Lefebvre et al. (1999).

These same three samples with naturally incurred toxicity as tested by HPLC-UV and receptor binding assay were also examined using liquid chromatography coupled with tandem mass spectrometric detection (LC-MS/MS) to obtain absolute confirmation of DA presence. Liquid chromatographic separation was performed on a Vydac 201TP52 C_{18} column (2.1×250 mm; Separations Group, Hesperia, CA, USA) with an elution gradient of 1–95% methanol in 0.1% TFA run over 35 min at a flow rate of 0.2 ml min^{-1}. Following separation, the eluent was introduced into a PE SCIEX API-III triple quadrupole mass spectrometer (SCIEX Instruments, Thornhill, Ontario, Canada) operating in positive ion spray mode and using compressed air as the nebulization gas. Confirmation of DA was performed by selective ion monitoring and based on the MS/MS fragmentation pattern for this toxin given by Quilliam (1996), including the pseudo-molecular ion (312 m/z) and two diagnostic fragment ions produced by collisionally induced dissociation (161 and 266 m/z).

2.5. Percent recovery and inter-animal variability

Sand crabs used in development of the extraction protocol were control animals verified by receptor binding assay to contain less than 70 ng DA equiv./g tissue extracted, the limit of detection for this method following SAX clean-up. All control animals were injected individually with 25 μl of DA (Sigma; St Louis, MO) made up to a working concentration of 1.4 ± 0.23 mg ml^{-1} in 1:9 MeCN:H_2O as determined by LC-MS/MS. Several additional trials were performed in which DA reference standard was added following the homogenization step, rather than injecting individual animals prior to homogenizing. Percent recovery values determined by HPLC-UV reflect the quantity of toxin recovered following homogenization and extraction expressed as a percentage of the total amount of DA injected. Each permutation of the homogenization/extraction protocol, including the SAX clean-up, was performed in quadruplicate with each replicate extract comprising five whole animals.

In order to assess the inter-animal variability in naturally incurred DA content for sand crabs in the Monterey Bay region, individual animals were homogenized and extracted employing the method determined to yield the highest toxin recoveries. As described in Section 3, the highest recovery rates were obtained following homogenization by blender and extraction using the Polytron/sonication technique outlined earlier. This approach was, therefore, used to extract individual animals, and the extracts were tested for DA by HPLC-UV (described earlier). A total of 12 sand crabs representing each of the following size classes were analyzed: 1.0–1.49, 1.5–1.99, 2.0–2.49, 2.5–2.99, and 3.0–3.49 cm body length (60 animals total).

3. Results and discussion

3.1. Protocol development

The sand crab, *E. analoga*, presents a unique challenge for the extraction and measurement of DA. Most other

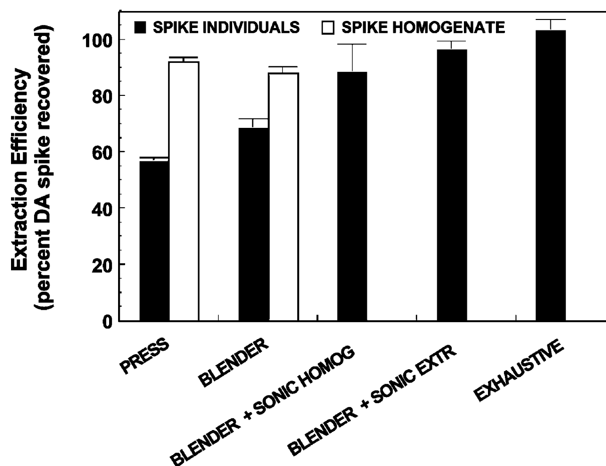

Fig. 1. Bar graph showing DA extraction efficiencies of various homogenization and extraction techniques. Solid bars indicate tests for which individual sand crabs were injected with DA before homogenization, while unfilled bars represent spiking with DA following homogenization but prior to extraction. 'Sonic homog' = sonication of homogenate; 'Sonic extr' = sonication of extract. Values are mean ± S.D. ($n = 4$). See text for additional details.

animals that are routinely tested for this toxin, primarily bivalve molluscs and larger crabs (e.g. dungeoness crab), are cleaned free of shell and carapace before initiating the extraction protocol on the remaining soft components. In contrast, because of the sand crab's small size (i.e. 1–3.5 cm in this study) and overall morphology, removal of the carapace prior to extraction is not practical and thus whole animals must be processed. It is therefore most critical that the animals, including their carapace, be homogenized thoroughly in order to maximize exposure to the aqueous methanol extraction solvent. Although efficient disruption of the carapace proved to be difficult, resulting toxicity values reflective of the entire animal are probably more informative in the context of DA trophic transfer, since sand crabs are generally consumed whole by their predators.

In this study, two homogenization techniques were tested in conjunction with the Polytron-based extraction protocol (i.e. no sonication step), one employing a hand-operated garlic press and the other a blender fitted with a mini-sample cup (37 ml capacity). When individually spiked animals were used, homogenization in the blender yielded a recovery of 69 ± 3.1%, while the garlic press appeared somewhat less efficient with a recovery of 57 ± 0.9% (Fig. 1). The 20% greater recovery realized by using the blender lead us to select this method for homogenizing the sand crabs. Both methods showed considerably higher recovery percentages if the DA reference standard was added following homogenization instead of directly injecting animals before homogenizing (blender: 88 ± 2.2, press: 92 ± 1.6; Fig. 1). We would thus argue that by injecting individual sand crabs with DA a more realistic situation is achieved in which the toxin must be recovered from an intact animal, as would be the case with naturally incurred contamination.

In our previous experience, once a thorough homogenization of the sample has been achieved, extraction of DA in 50% aqueous methanol using a Polytron generally yields recoveries of 85–95% (e.g. Lefebvre et al., 1999; unpubl. observ.). However, as noted earlier, even when sand crabs were homogenized using a blender, DA recoveries following Polytron-based extraction in 50% aqueous methanol did not exceed 70%. Suspecting that at least some of the unrecoverable portion of the toxin spike may have been adsorbed to the carapace and other "non-tissue" components, we tested the efficacy of probe sonication in releasing this DA into the extraction solvent. A sonication step was first added immediately following homogenization but before the methanol extraction (animals spiked individually), resulting in a recovery of 88 ± 9.8%—an increase of almost 30% over the non-sonicated homogenate (Fig. 1). We anticipated that sonication in the presence of the methanol extraction solvent would enhance the effectiveness of this step, so we instead applied the probe sonicator to the sample extract immediately following solvent extraction using the Polytron. This modification to the protocol increased the recovery of DA to a very acceptable 97 ± 2.9%, a further 10% increase compared to sonication of the homogenate prior to addition of the methanol (Fig. 1). Exhaustive extraction of the homogenate (i.e. a re-extraction of the tissue/carapace mass), including sonication of the methanol extract, slightly increased the recovery to 103 ± 3.7% (Fig. 1), demonstrating quantitative recovery of the DA reference standard but not justifying the additional effort for routine extractions.

Given that the HPLC-UV method for DA analysis can be susceptible to interference from many naturally occurring substances, the SAX-SPE clean-up cartridges are frequently employed to remove such material from

Table 1
Comparison of percent recovery values (mean ± SD; $n = 4$) determined by HPLC-UV for 50% aqueous methanol extracts in either crude form (= No clean-up) or following clean-up using a SAX-SPE cartridge (= SAX clean-up). Sample extracts were produced by the following three methods: blender homogenization/Polytron extraction (column 1); blender-sonication homogenization/Polytron extraction (column 2); blender homogenization/Polytron-sonication extraction (column 3) (see text for protocol details)

	Blender/polytron	Blender-sonicate/polytron	Blender/polytron-sonicate
No clean-up	69 ± 3.1	88 ± 9.8	97 ± 2.9
SAX clean-up	66 ± 1.9	85 ± 5.4	92 ± 4.3

extracts. In the case of sand crab extracts, chromatograms were surprisingly clean and relatively free of interfering peaks, including tryptophan, which is known to elute close to DA. Nonetheless, in order to confirm that interpretation of our chromatograms was not influenced by contaminants, extracts were compared before and after SAX-SPE clean-up for samples homogenized by blender and extracted in methanol using a Polytron, both with and without sonication. The results, shown in Table 1, demonstrate clearly that extracts not subjected to the SAX-SPE clean-up protocol are not different than those same extracts following clean-up. We are therefore confident that crude, 50% aqueous methanolic extracts provide reasonably accurate estimates of DA levels contained in sand crabs, thereby eliminating a costly and time-consuming clean-up step from routine analyses.

3.2. LC-MS/MS confirmation and method comparison

Upon completing optimization of the extraction protocol using negative control animals spiked with a DA reference standard, we focused on toxin measurements in sand crabs with naturally incurred toxicity. We first used LC-MS/MS to confirm the presence of DA in these animals collected from the Monterey Bay region. As can be seen from the spectral traces (Fig. 2), the intact DA pseudo-molecular ion (312 *m*/*z*), as well as the two diagnostic fragment ions (161 and 266 *m*/*z*) are clearly present, thereby providing absolute confirmation of this toxin's presence in sand crabs.

Although the HPLC-UV is currently the method of choice for monitoring DA in indicator or sentinel species, the receptor binding assay is attracting increased attention from regulatory agencies. The rapid, high-throughput receptor assay provides an economical and efficient approach to

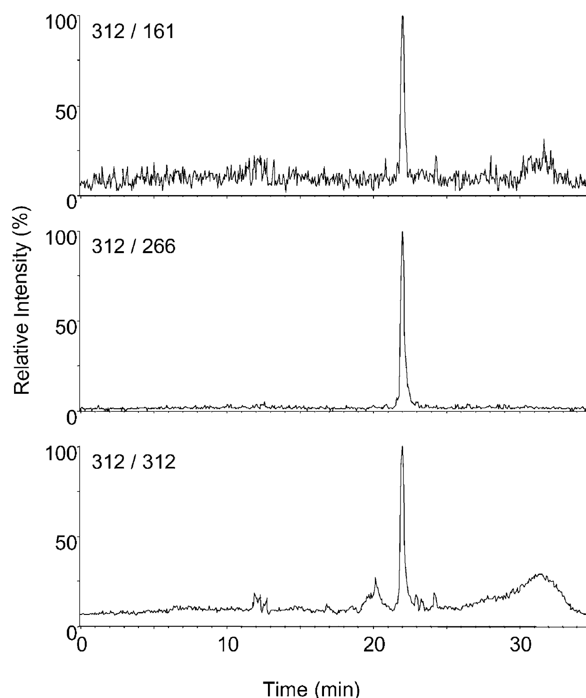

Fig. 2. Selected ion monitoring LC-MS/MS chromatograms of DA contained in sand crabs of naturally incurred toxicity. The top two panels represent the two primary diagnostic fragment ions (nominally of 161 and 266 *m*/*z*) resulting from the collisionally induced dissociation of the DA $(M + H)^+$ ion. The bottom panel shows the residual unfragmented DA $(M + H)^+$ pseudo-molecular ion nominally of 312 *m*/*z*.

Table 2
Comparison of DA measurements for three sand crab samples containing naturally incurred toxin and collected from Monterey Bay, CA. Each sample extract was tested using the following three methods: HPLC-UV, receptor binding assay, and liquid chromatography coupled with tandem mass spectrometric detection (LC-MS/MS). Values are mean ± SE ($n = 2$)

	HPLC-UV (μg DA g^{-1})	Receptor assay (μg DA equiv. g^{-1})	LC-MS/MS (μg DA g^{-1})
Sample 1	4.1 ± 0.09	4.4 ± 0.61	3.8 ± 0.33
Sample 2	1.7 ± 0.02	1.8 ± 0.05	1.8 ± 0.17
Sample 3	4.0 ± 0.06	3.2 ± 0.44	3.2 ± 0.58

toxin testing, and has compared favorably with HPLC-based methods for the quantification of DA in toxic *Pseudo-nitzschia* (Van Dolah et al., 1995; Powell and Doucette, unpubl. observ.) and highly complex matrices such as sea lion feces (Lefebvre et al., 1999). In the present study we compared the receptor assay with the HPLC-UV method as well as quantification using the LC-MS/MS technique. The results of this comparison (Table 2) demonstrate exceedingly good agreement among these very different approaches for determining DA concentration or DA-like activity, in the case of the receptor assay, for sand crab extracts. Values for an individual sample varied by no more than 20% between the three methods and in most cases less than 10%. The LC-MS/MS approach detects specifically the DA molecule and is capable of discriminating between this compound and its less toxic congeners reported previously from contaminated shellfish (Wright et al., 1990). The close quantitative relationship between the tandem mass spectrometric and receptor assay results, and thus between DA quantity and toxic activity, respectively, would suggest that DA itself accounts for the majority of toxin contained in the sand crabs examined. Moreover, excellent agreement with the HPLC-UV technique provides strong evidence that receptor assay data accurately reflect DA levels as they are now measured for regulatory purposes by state monitoring programs.

3.3. Inter-animal variability

An especially important consideration in establishing a sampling protocol for a new sentinel species is the inter-animal variability in toxin content. Specific issues that must be considered include not only to what extent toxin levels vary from animal to animal, but also whether a relationship exists between animal size and toxin content. For example, a positive correlation between sand crab size and toxicity may be indicative of a progressive bioaccumulation of toxin over an animal's life span, thus making it difficult to discern 'new' toxin associated with an ongoing outbreak from 'old' remaining from a previous event. The latter is a critical distinction if an organism is to be employed as an indicator of impending toxicity from a developing algal bloom.

Our analysis of inter-animal variability in DA content spanned five size classes of sand crab, ranging from 1 to 3.5 cm in body length (i.e. 1.0–1.49, 1.5–1.99, 2.0–2.49, 2.5–2.99, and 3.0–3.49 cm). Results shown in Table 3 indicate that toxin levels were highly variable within a size class (coefficient of variation, ca 30–50%), yet on average did not differ appreciably among the various size classes. A plot of all 60 data points shows DA content varied by over an order of magnitude (ca 0.5–5 μg DA g^{-1} tissue) and demonstrates clearly the lack of a relationship between the size of a sand crab and its toxin content ($r^2 = 0.002$; Fig. 3). Similar results were obtained when DA content was plotted as a function of animal weight (0.2–4.9 g; $r^2 = 0.003$; data not shown). This finding suggests that sand crabs in the Monterey Bay region do not continue to accumulate DA as they grow, but rather appear to depurate the toxin once the source of toxin is removed (e.g. termination of a bloom event). Further support for this argument was provided recently by the year-long study of Ferdin et al. (in prep), during which DA was detected in sand crabs (HPLC-UV analyses) only when toxic *Pseudo-nitzschia* cells approached or exceeded 5×10^4 cells l^{-1}. Moreover, when the DA source organism was no longer present, toxin levels quickly dissipated to below the detection limit. These same authors also determined that *M. californianus*, the current sentinel species for DA in this region, showed no measurable toxin at times when DA was easily detected in sand crabs.

4. Conclusions

We have developed and validated a highly efficient

Table 3
Comparison of DA concentrations in various size classes of sand crab containing naturally incurred toxin and collected from Monterey Bay, CA. Values are mean ± SD ($n = 12$) and expressed as μg DA g^{-1} tissue

	1.0–1.49 cm	1.5–1.99 cm	2.0–2.49 cm	2.5–2.99 cm	3.0–3.49 cm
DA concentration	2.2 ± 0.82	3.1 ± 0.89	2.4 ± 0.80	2.8 ± 0.80	2.3 ± 1.26

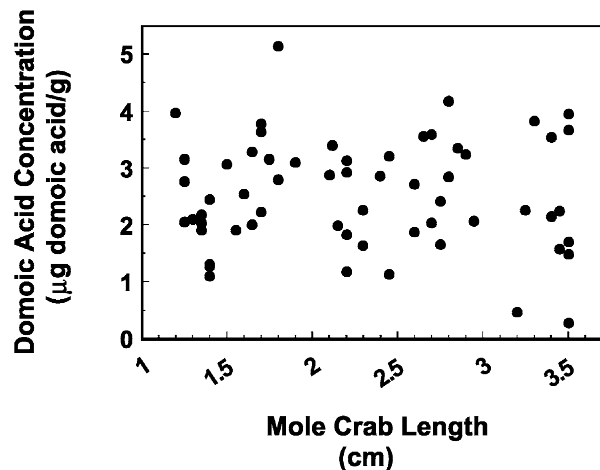

Fig. 3. Scatter plot showing DA concentration (µg DA per gram of tissue) for individual sand crabs with naturally incurred toxicity. Data are given according to sand crab length (cm). A similar pattern was observed when DA concentration was expressed according to sand crab weight (data not shown).

DA extraction protocol for the sand crab (*E. analoga*) that yields toxin recoveries of 97 ± 2.9%. It was also determined by HPLC-UV and receptor assay, with confirmation by LC-MS/MS analyses, that sand crabs accumulate measurable amounts of DA during toxic *Pseudo-nitzschia* blooms occurring in Monterey Bay, CA. The DA content of these animals ranged from ca 0.5 to 5 µg DA/g tissue, with no consistent trend as a function of size class, based on either animal weight or length. Our results indicate that sand crabs do not accumulate DA with age, but rather depurate the toxin in the absence of a toxin source such as a toxic *Pseudo-nitzschia* bloom. Moreover, failure to detect DA during a bloom event in the current sentinel species, *M. californianus*, when toxin was easily measured in sand crabs, suggests that these filter-feeding crustaceans be considered as sensitive indicators of impending DA outbreaks. Finally, our data characterizing the inter-animal variability in toxin content will be useful in designing an appropriate sampling strategy for monitoring DA in this region.

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