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**TROPHIC TRANSFER, TISSUE DISTRIBUTION, AND NEUROTOXIC
CONSEQUENCES OF THE PHYCOTOXIN, DOMOIC ACID, IN
NORTHERN ANCHOVIES (*Engraulis mordax*)**

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by


Katherine Ann Lefebvre

September 2001

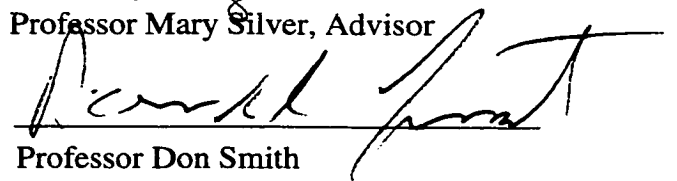
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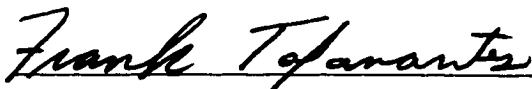
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TABLE OF CONTENTS

LIST OF FIGURES	v
LIST OF TABLES	vi
OVERALL ABSTRACT	vii
ACKNOWLEDGEMENTS	ix
OVERALL INTRODUCTION	1
CHAPTER ONE: Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event	
ABSTRACT	2
INTRODUCTION	3
MATERIALS AND METHODS	4
RESULTS AND DISCUSSION	10
CONCLUSIONS	19
CHAPTER TWO: Tissue distribution and neurotoxic effects of domoic acid in a prominent vector species, the northern anchovy (<i>Engraulis mordax</i>)	
ABSTRACT	20
INTRODUCTION	21
MATERIALS AND METHODS	24
RESULTS	30
DISCUSSION	34
CONCLUSIONS	39

CHAPTER THREE: Domoic acid in planktivorous fish in relation to toxic *Pseudo-nitzschia* cell densities

ABSTRACT	41
INTRODUCTION	42
MATERIALS AND METHODS	44
RESULTS	49
DISCUSSION	54
CONCLUSIONS	59
OVERALL CONCLUSIONS	61
APPENDIX A: Examination of brain slices from control and domoic acid-intoxicated northern anchovies	62
REFERENCES	69

LIST OF FIGURES

CHAPTER ONE:

Figure 1.	HPLC chromatogram of anchovy body tissue	12
Figure 2.	SEM micrograph of <i>Pseudo-nitzschia</i> frustules	13
Figure 3.	LC-MS/MS trace of domoic acid in sea lion feces	15
Figure 4.	HPLC chromatograms of tryptophan and domoic acid	18

CHAPTER TWO:

Figure 1.	Dose-response curve for domoic acid	35
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CHAPTER THREE:

Figure 1.	Map of phytoplankton and fish collection sites	46
Figure 2.	Phytoplankton cell densities and domoic acid levels in fish	50

LIST OF TABLES

CHAPTER ONE:

Table 1.	Domoic acid levels in anchovies	11
Table 2.	Inter-laboratory comparison of domoic acid levels in feces	16

CHAPTER TWO:

Table 1.	Tissue distribution of domoic acid	31
Table 2.	Gavage experiment	33

CHAPTER THREE:

Table 1.	Comparison of domoic acid levels in anchovies and sardines ...	52
Table 2.	<i>Pseudo-nitzschia</i> in anchovy and sardine stomach contents	53
Table 3.	Domoic acid levels in viscera and body tissue of anchovies	55
Table 4.	Domoic acid levels in viscera and body tissue of sardines	56

Abstract

**TROPHIC TRANSFER, TISSUE DISTRIBUTION, AND NEUROTOXIC
CONSEQUENCES OF THE PHYCOTOXIN, DOMOIC ACID, IN
NORTHERN ANCHOVIES (*Engraulis mordax*)**

Katherine Ann Lefebvre

Over the past decade the consumption of planktivorous northern anchovies containing the diatom-produced neurotoxin, domoic acid (DA), has been responsible for severe neurologic illness and mass mortality events involving hundreds of sea birds and marine mammals in Monterey Bay, California. This research characterizes the dynamics of DA transfer, accumulation, and neurotoxicity in this pivotal and dangerous DA vector fish species. Chapter one documents a DA-poisoning event in which dozens of California sea lions died as a result of consuming DA-contaminated anchovies in Monterey Bay. DA levels in sea lion feces and anchovies were attained using HPLC-UV and microplate receptor binding assays, with absolute confirmation by tandem mass spectrometry. The presence of toxic *Pseudo-nitzschia* frustules in sea lion feces and anchovy gut contents was determined using scanning electron microscopy. Chapter two presents data from an intracoelomic injection study in which anchovies were shown to be neurologically susceptible to DA excitotoxicity and to have a similar sensitivity to the toxin as mammals. Excitotoxic symptoms are described and confirm that DA crosses the blood brain barrier in fish. Chapter three characterizes the dynamics of DA accumulation in small planktivorous fish in relation

to ecologically relevant toxic diatom densities. Toxic *Pseudo-nitzschia* densities and DA levels in anchovies and sardines were measured from samples collected weekly throughout Monterey Bay from 10/8/99 to 10/8/00. DA levels in fish were estimated via HPLC-UV and toxic cell densities were determined via whole cell hybridization with species-specific large subunit rRNA-targeted fluorescent probes. DA levels accumulated in fish viscera tracked toxic cell densities in surface waters confirming that anchovies and sardines regularly consume toxic diatoms when present in Monterey Bay. Fish viscera DA levels harmful to piscivorous predators occurred when toxic cell densities exceeded 10^4 cells liter⁻¹. Anchovies accumulated more DA than sardines in viscera, however neither species appears to accumulate significant levels of toxin in body tissue. Finally, Appendix A includes results from histologic examination of brain sections taken from control and DA-intoxicated anchovies and stained with haemotoxylin and eosin dyes. Although intoxicated fish exhibited seizures, brain lesions characteristic of DA excitotoxicity were not found.

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INTRODUCTION

The neurotoxin, domoic acid (DA), is naturally produced by some diatoms of the genus *Pseudo-nitzschia* and is responsible for a severe neurologic and gastrointestinal illness called Amnesic Shellfish Poisoning (ASP). ASP was first observed in 1987 in humans after the consumption of DA-contaminated blue mussels. Subsequent ASP events have revealed that small planktivorous fish, such as anchovies, are potent vectors of DA to higher level consumers. DA-contaminated anchovies were responsible for mass mortality events involving hundreds of sea birds and marine mammals on various occasions in Monterey Bay, California. This research documents the first ASP event observed in marine mammals and addresses various aspects of relationships between the movement and toxicity of DA in anchovies. In addition, this work describes the relationship between toxic *Pseudo-nitzschia* cell densities and DA presence in planktivorous fish in real field conditions. Each chapter contributes to the understanding of the relationship between DA and anchovies, beginning with an ecological level study of food web transfer of DA in chapter one, moving to an organismal level study defining neurotoxic sensitivity and effects of DA in fish in chapter two, and ending with a comprehensive field study documenting the presence of DA in planktivorous fish in relation to toxic *Pseudo-nitzschia* cell densities in surface waters described in chapter three.

Chapter One

DETECTION OF DOMOIC ACID IN NORTHERN ANCHOVIES AND CALIFORNIA SEA LIONS ASSOCIATED WITH AN UNUSUAL MORTALITY EVENT

Abstract

The occurrence of an unusual mortality event involving California sea lions (*Zalophus californianus*) along the central California coast in May 1998 was recently reported. The potent neurotoxin domoic acid (DA), produced naturally by the diatom *Pseudo-nitzschia australis* and transmitted to the sea lions via planktivorous northern anchovies (*Engraulis mordax*), was identified as the probable causative agent. Details of DA analyses for anchovy tissues and sea lion feces are described. Domoic acid levels were estimated in anchovy samples by HPLC-UV, and in sea lion feces using the same method as well as a microplate receptor binding assay, with absolute confirmation by tandem mass spectrometry. The highest DA concentrations in anchovies occurred in the viscera (223 ± 5 ug DA/g), exceeding values in the body tissues by seven-fold and suggesting minimal bioaccumulation of DA in anchovy tissue. HPLC values for DA in sea lion fecal material (ranging from 2.5 to 152 ug DA/g) required correction for interference from an unidentified compound. Inter-laboratory comparisons of HPLC data showed close quantitative agreement. Fecal DA activity determined using the receptor binding assay corresponded with HPLC values to within a factor of two. Finally, our detection of *P. australis* frustules, via scanning electron microscopy, in both anchovy viscera and fecal material from sea lions exhibiting seizures provided corroborating evidence that this toxic algal species was involved in this unusual sea lion mortality event.

Introduction

Domoic acid (DA) is a potent neuroexcitatory amino acid naturally produced by diatoms of the genus *Pseudo-nitzschia*. During toxic diatom blooms DA has been found to accumulate in the digestive tracts of filter-feeding marine species such as, northern anchovies (*Engraulis mordax*), blue mussels (*Mytilus edulis*), and razor clams (*Siliqua patula*) (Quilliam and Wright, 1989; Wekell et al., 1994; Altwein et al., 1995). These species may subsequently act as vectors of the toxin to organisms at higher trophic levels. The first documented case of DA poisoning occurred in 1987 when three people died and over ninety others suffered neurological problems after consuming DA-containing cultured blue mussels harvested from eastern Prince Edward Island, Canada (Quilliam and Wright, 1989; Perl et al., 1990). The condition was termed amnesic shellfish poisoning (ASP) and has not been reported in humans since. However, subsequent DA poisoning events attributed to the consumption of fish containing toxic *Pseudo-nitzschia* have occurred, resulting in the deaths of hundreds of brown pelicans (*Pelecanus occidentalis*) in Monterey Bay, California in 1991 (Work et al., 1993) and in Cabo San Lucas, Mexico in 1996 (Beltran, 1997).

In addition to humans and sea birds, acute toxic effects of DA have been demonstrated in mice, rats and monkeys in the laboratory (Grimmell et al., 1990; Tasker et al., 1991; Truelove and Iverson, 1994; Tryphonas et al., 1990a and 1990b). The mechanism of DA excitotoxicity is initiated by kainate subtype receptor activation and requires subsequent activation of NMDA subtype receptors (Debonnel et al., 1989; Berman and Murray, 1997). DA is a rigid structural analog of glutamate and is 25 times more potent than kainic acid in inducing excitotoxic effects (Dakshinamurti et al., 1993; Dakshinamurti et al., 1991). Outward symptoms of DA toxicity in mammals

include vomiting, diarrhea, confusion, disorientation, memory loss, scratching, seizures and coma (Truelove and Iverson, 1994).

During May 1998 approximately 70 California sea lions stranded along central California beaches from Santa Cruz to San Luis Obispo exhibiting signs of neurological disorder in the form of seizures (Gulland, 1999). Stomachs of all animals were empty indicating recent vomiting. Forty-seven sea lions died, while 23 recovered and were released after treatment at the Marine Mammal Center in Sausalito, CA (Gulland, 1999). This unusual mortality event occurred concurrently with the presence of a toxic *P. australis* bloom detected off the central California coast (Scholin et al., 2000). Our detection of DA and *P. australis* frustules in anchovy and sea lion fecal samples provided corroborative evidence that DA produced by *P. australis* was most likely the toxic agent responsible for the observed neurological symptoms exhibited by the sea lions and that the probable route of toxin transfer between trophic levels was the northern anchovy, a common prey item of California sea lions (Lowry et al., 1991). Here we describe details of DA analysis for anchovy tissues and sea lion feces, including an inter-laboratory comparison of independent analysis, the use of two detection methods, and a description of problems encountered while testing these sample matrices.

Materials and Methods

Sample Collection

Anchovies were collected by local fishermen from Monterey Bay on May 22, 1998 during the toxic *P. australis* bloom. Control anchovies were collected from the anchovy exhibit in the Monterey Bay Aquarium which receives only filtered sea water. A second set of control anchovies was collected from Santa Cruz harbor live bait tanks

in late July 1998. Fecal samples from eleven sea lions exhibiting neurotoxic symptoms were collected between May 22 and 29, 1998. Nine control California sea lion fecal samples were obtained; two from captive animals at Long Marine Lab (LML) in Santa Cruz, CA, three from captive animals at Marine World in Vallejo, CA, three from captive animals at the Marine Mammal Center in Sausalito, CA, and one from a wild animal collected two months after the bloom (had died from a gun shot wound). All captive animals had been fed frozen atlantic herring. Animals from Marine World are maintained in artificial sea water, while LML animals are maintained in sand filtered sea water taken from Monterey Bay.

Chemical Reagents

DACS-1C certified domoic acid standard (Canadian National Research Council, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, N.S., Canada B3H 3Z1) and 90% pure domoic acid reagent (Sigma Chemical Company, St. Louis, MO 63178) were obtained for calibration standard preparation and spike/recovery calculations. Trifluoroacetic acid (TFA), analytical grade sodium chloride (NaCl), and Optima grade methanol (MeOH) and acetonitrile (MeCN) were obtained from Fisher Scientific (Pittsburg, PA). Nanopure water was used for solution preparation. Standards were kept refrigerated and in the dark when not in use.

Domoic Acid Detection Via HPLC-UV

Anchovy and sea lion fecal samples were analyzed for the presence of DA using an isocratic elution profile on a Hewlett-Packard 1090 manual injection HPLC equipped with a diode array detector (DAD) set at 242 nm and 280 nm with a bandwidth of 10nm (reference signal was set at 450 nm with a bandwidth of 10 nm). Monitoring at 280 nm

in addition to the standard 242 nm was essential for California sea lion fecal analysis due to the presence of an unidentified compound in some fecal samples that eluted at the same retention time as DA. Details on correction for this interference are explained in the Results and Discussion section on sea lion fecal material. A reverse phase Vydac C₁₈ column (catalog #201TP52, 2.1 mm X 25 mm, Separations Group, Hesperia, CA 92345) equipped with a Vydac guard column (particle size 5 µm) was used. The mobile phase (90/10/0.1, water/ MeCN/ TFA) was degassed with helium for 10 minutes prior to analysis. A calibration curve generated using DACS-1C DA standards of 0.2, 0.5, 1.0, 2.0, 4.0 and 8 µg/ml ($r= 0.99$). The lowest detectable standard was 0.1 µg DA/ml. The instrument detection limit, which was equivalent to the concentration that corresponded to three times the standard deviation of the signal from the smallest detectable standard, was 0.2 µg DA/ml. All injections were 20 µl with a flow rate of 0.3 ml/min. Retention time for DA ranged from 9-11 minutes originally, then changed to 11-13 minutes for analyses performed after the guard column was changed.

Sample Extraction

Three types of anchovy samples were prepared for analysis; whole anchovies, anchovy viscera only, and anchovy body tissue only. Pooled anchovy and individual sea lion fecal samples were first ground in a blender and aliquots (usually 4 grams) were taken for DA extraction using procedures described by Quilliam et al. (1995) and Hatfield et al. (1994). A 1:4 ratio of sample to extraction solvent (50% MeOH) was homogenized for 3 minutes on the highest setting with a homogenizer probe. The resulting slurry was centrifuged for 20 minutes at 4,000 x g. The supernatant was passed through a 0.22 µm filter (Millipore Corp., Bedford, MA) and collected for solid phase extraction (SPE) clean-up.

Solid Phase Extraction (SPE)

Supelco (Supelco Park, Bellefonte, PA) strong anion exchange (SAX) SPE cartridges from lot # SP1691E were used for all samples analyzed. Because SAX columns from different lot numbers and manufacturers can have variable recoveries (Hatfield et al., 1994), two spike and recovery experiments using Sigma DA standards were performed and resulted in 95 and 91% recoveries. Cartridges were preconditioned with 6 ml MeOH, then 3 ml water, followed by 3 ml 50% aqueous MeOH. All solutions were added to the cartridge when the meniscus of the previous solvent first touched the top of the column, thus never allowing the the column to run dry. After conditioning, 2.0 ml of filtered sample were eluted at a rate of one drop per second, followed by 5 ml of wash (10% MeCN). Finally, 5 ml of 0.5 M NaCl in 10% MeCN were used to elute off the DA into a 5 ml graduated centrifuge tube. Some fecal samples were prepared with an additional wash (0.1 M NaCl) step before the final elution step, which is proposed to further eliminate interfering peaks (Hatfield et al., 1994). The collected eluate was vortexed and 20 µl was injected for HPLC analysis. To determine DA concentrations per gram sample the following equations were used;

- 1) $X \mu\text{g DA/ml} = (\text{Area} + 1.14) \div 157.83$
- 2) $(X \mu\text{g DA/ml}) (5 \text{ ml}/2 \text{ ml}) (20 \text{ ml}) = \text{Total } \mu\text{g DA}$
- 3) $\text{Total } \mu\text{g DA} \div 4 \text{ grams feces or tissue} = \mu\text{g DA/ gram feces or tissue}$

Equation 1 was used to calculate DA concentration in the 20 µl injection in terms of µg DA/ml (X). It is the linear regression determined from the standards and calculated using Chemstation Software (Hewlett-Packard Inc., Palo Alto, CA). Equation 2 multiplies DA concentration (X µg DA/ml) by the extraction dilution factor to give total

DA in anchovy and sea lion fecal samples. The third equation divides total DA by the number of grams of sample prepared to give $\mu\text{g DA/g sample}$. Spike and recovery experiments using the DACS-1C certified standard yielded an estimated 88 ± 3.5 percent ($n = 3$) efficiency of extraction.

Inter-laboratory Comparison

Fecal samples found positive for DA, along with two control samples, were sent to the NOAA/NOS laboratory (Charleston, SC) and re-analyzed by the same HPLC-UV method (Hatfield et al., 1994; Quilliam et al., 1995). In addition, the fecal extracts were tested for DA activity using a receptor binding assay described by Van Dolah et al. (1997) with several modifications. Ambient glutamate was removed from SAX-cleaned fecal extracts by incubating 50 μl extract with 40 μl buffer (10 mM citrate, pH 5.0; 2 mM pyridoxal 5-phosphate; 200 mM NaCl) and 10 μl glutamate decarboxylase (100 units/ml in 10 mM citrate, pH 5.0) for 30 min on ice. Assays were performed in a 96-well microtiter filtration plate (cat. no. MAFC NOB50, Millipore Inc., Bedford, MA) by adding sequentially 35 μl of 5 nM [^3H] kainic acid (New England Nuclear, Boston, MA), 35 μl DA standard (10^{-6} to 10^{-11} M "in assay" concentrations; DACS-1C reference standard, NRC/IMB, Halifax, Canada) or sample, and 140 μl SF9 insect cell membranes containing GluR6 glutamate receptors (0.1 mg protein/ml), followed by incubation for 1 hr at 4 °C. Membranes were washed of unbound [^3H] kainic acid and DA by rinsing once with 200 μl ice-cold 50 mM Tris, pH 7.4 over a MultiScreen TM vacuum manifold (Millipore Inc.). To each well were added 25 μl of scintillant (OptiPhase, Wallac Inc., Gaithersburg, MD). The plate incubated at room temperature for 30 min. and then counted on a microplate scintillation counter (Microbeta 1450, Wallac Inc.). The standard curve was derived by a four parameter logistic fit of DA

standard values, and unknown sample concentrations were calculated from the linear portion of this curve using MultiCalc software (Wallac Inc.). The limit of detection for fecal extracts was 10 ng DA equivalents/mL and μg DA equiv./g tissue were determined for each sample.

Tandem Mass Spectrometry

An aqueous methanolic extract of sea lion fecal material (CSL #3758) prepared as described above (see Sample Extraction) was analyzed by tandem mass spectrometry (LC-MS/MS) to obtain absolute confirmation of DA presence in this sample. The extract was first subjected to chromatographic separation on a C18 column using a gradient of 1-95% methanol in 0.1% TFA. The eluent was then introduced into a PE SCIEX API-III triple quadrupole mass spectrometer, with the ionspray source operated in positive ion mode and compressed air serving as the nebulization gas. The first quadrupole was used to selectively pass ions of nominal 312 m/z , representing the DA parent ion. These ions were then fragmented by collisionally induced dissociation in the second quadrupole. By operating the third quadrupole in multiple ion monitoring mode, fragment ions of 161 and 266 m/z , as well as residual DA parent ions (312 m/z), were allowed to pass to the ion detector.

Diatom Frustule Analysis

Anchovy gut and sea lion fecal samples were prepared for scanning electron microscopy (SEM), using a method described by Miller and Scholin (1998). Samples were diluted with DI water, placed in filter tubes on a vacuum manifold and four drops of potassium permanganate saturated DI water were added. Once the potassium permanganate solution was filtered, concentrated HCl was added continuously until

samples became colorless. Once colorless, samples were rinsed three times with DI water and the entire method was repeated. Filters were then glued, with colloidal graphite in isopropanol, to SEM plugs and analyzed. Diatom frustules were identified to species by observing frustule characteristics unique to *P. australis*.

Results and Discussion

Anchovy Samples

Monterey Bay anchovies collected during May 1998 contained 223 ± 5 , 55 ± 1 , and 39 ± 0.7 $\mu\text{g DA/g}$ in viscera, whole anchovy and body tissue samples, respectively, as determined by HPLC-UV (Table 1). Anchovy matrices presented no detectable interferences for DA analysis with the HPLC-UV method (Figure 1). In addition to high DA concentrations, diatom frustules found in anchovy gut contents were identified to species and revealed almost complete dominance of *P. australis* (Figure 2A). DA was not detected in control anchovy viscera, whole body or body tissue samples (Figure 1A).

Although there are abundant laboratory data reporting DA-induced neuroexcitotoxicity in mammals (Iverson et al., 1989; Iverson et al., 1990; Peng and Ramsdell, 1996; Perl et al., 1990; Preston and Hynie, 1991; Tasker et al., 1991; Tryphonas et al., 1990a and 1990b) and field data in birds (Beltran, 1997; Work et al., 1993; Fritz et al., 1992), to date there has been no evidence of neurotoxic effects in anchovies or other fish exposed to DA. This has raised a question as to whether fish are capable of transporting DA from the digestive tract into the blood stream and accumulating it in tissues, or if they act only as a vector, passing the toxin through without absorbing it. A study by Hardy et al. (1995) addressed this question by feeding rainbow trout (*Oncorhynchus mykiss*) domoic acid contaminated fish meal. No

Table 1. Domoic acid concentrations in anchovy samples determined via HPLC-UV.

Source	Viscera ($\mu\text{g DA /g} \pm \text{sd}$)	Whole Fish ($\mu\text{g DA /g} \pm \text{sd}$)	Body Tissue ($\mu\text{g DA /g} \pm \text{sd}$)
Bloom	223 \pm 5	55 \pm 1	39 \pm 0.7
Control 1	0	0	0
Control 2	0	0	0

Bloom: Anchovies collected from Monterey Bay (May 1998) during diatom bloom.

Control 1: Monterey Bay Aquarium captive anchovies.

Control 2: Anchovies collected from Monterey Bay (July 1998) after diatom bloom.

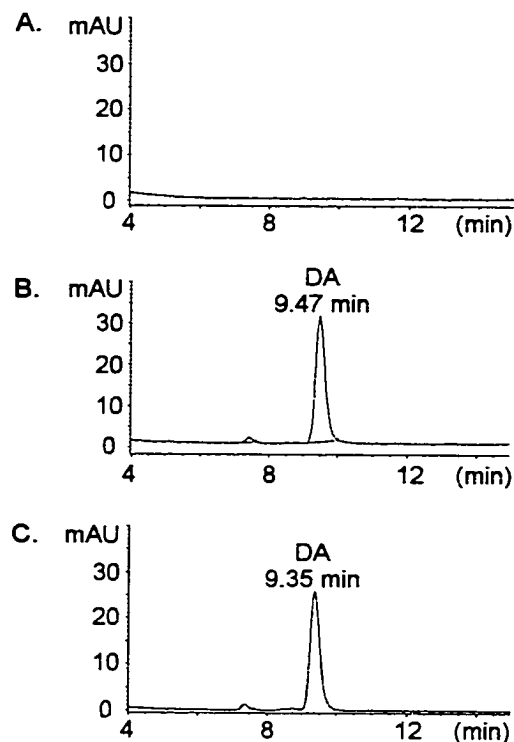


Figure 1. **A)** HPLC chromatogram of body tissue from control anchovies collected from Monterey Bay in July 1998 (after the diatom bloom). **B)** HPLC chromatogram of the same control sample as in (A), spiked with Sigma DA ($3.97 \mu\text{g DA/ml}$). Retention time (RT) = 9.47 min. **C)** HPLC chromatogram of body tissue from anchovies collected from Monterey Bay in May 1998 (during the diatom bloom). Sample contained $3.18 \mu\text{g DA/ml}$ or $35.8 \mu\text{g DA/g tissue}$. RT = 9.35 min.

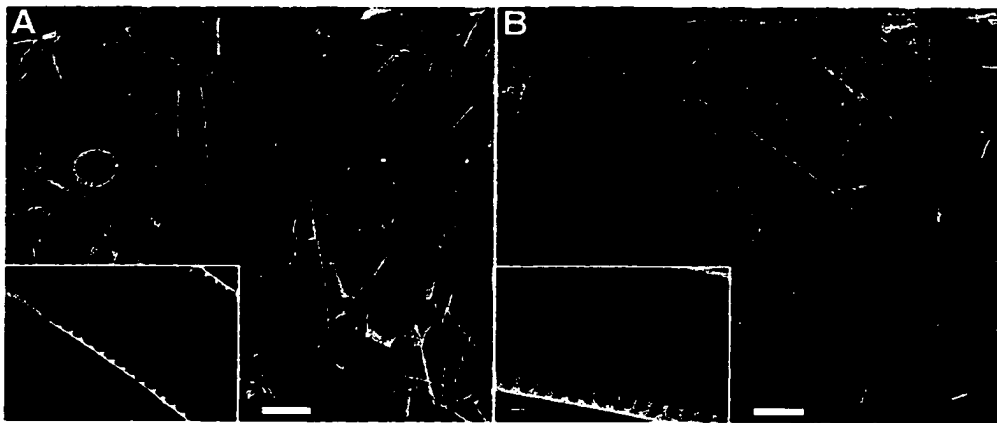


Figure 2. A) SEM micrograph of gut contents taken from an anchovy collected during the diatom bloom (May 1998) from Monterey Bay. Diatom frustules were identified to species revealing almost complete dominance of *P. australis*. B) SEM micrograph showing *P. australis* frustules isolated from fecal material collected May 26, 1998 from a California sea lion (CSL) exhibiting seizures (CSL#3783). Scale bars = 10 μm on main SEM micrographs and 1 μm on insets.

mortality, signs of toxicity, nor neurological symptoms were observed. DA was present in feces and viscera, but not in blood or body tissue, suggesting that DA in fish feed is not readily absorbed by rainbow trout. In contrast, our detection of DA in anchovy body tissue (Table 1, Figure 1C) suggests that DA may have been absorbed from the digestive tract in these fish. We plan to pursue this issue further by exposing anchovies to DA-producing diatoms in controlled laboratory experiments, followed by immediate analysis of isolated body tissue.

Sea Lion Samples

Three of eleven sea lion fecal samples collected from seizing animals contained detectable levels of DA by the HPLC-UV method. Tandem mass spectrometry performed on one fecal sample from this event (CSL #3758) unambiguously confirmed that DA was present (Figure 3). Inter-laboratory comparisons of two control and the three DA-positive fecal samples by the HPLC-UV method gave similar quantitative results for samples prepared and tested at each laboratory (Table 2). Microplate receptor binding assays yielded DA activities that followed the same trend in order of potency and agreed quantitatively with HPLC-UV values to within about a factor of two (Table 2). In addition to high concentrations of DA, California sea lion (CSL) # 3783 and # 3758 were found to contain *P. australis* frustules (Figure 2B). Unfortunately, there was not enough sample left of CSL # 3734 after HPLC methods to analyze for frustules. The lack of detectable DA and diatom frustules in the other eight fecal samples taken from seizing sea lions may be explained by rapid rates of digesta passage in California sea lions. Digesta passage rates for captive sea lions fed Pacific herring (*Clupea harengus*) average less than 5 hours (Helm, 1983). These rates may be even faster in the DA-exposed sea lions due to

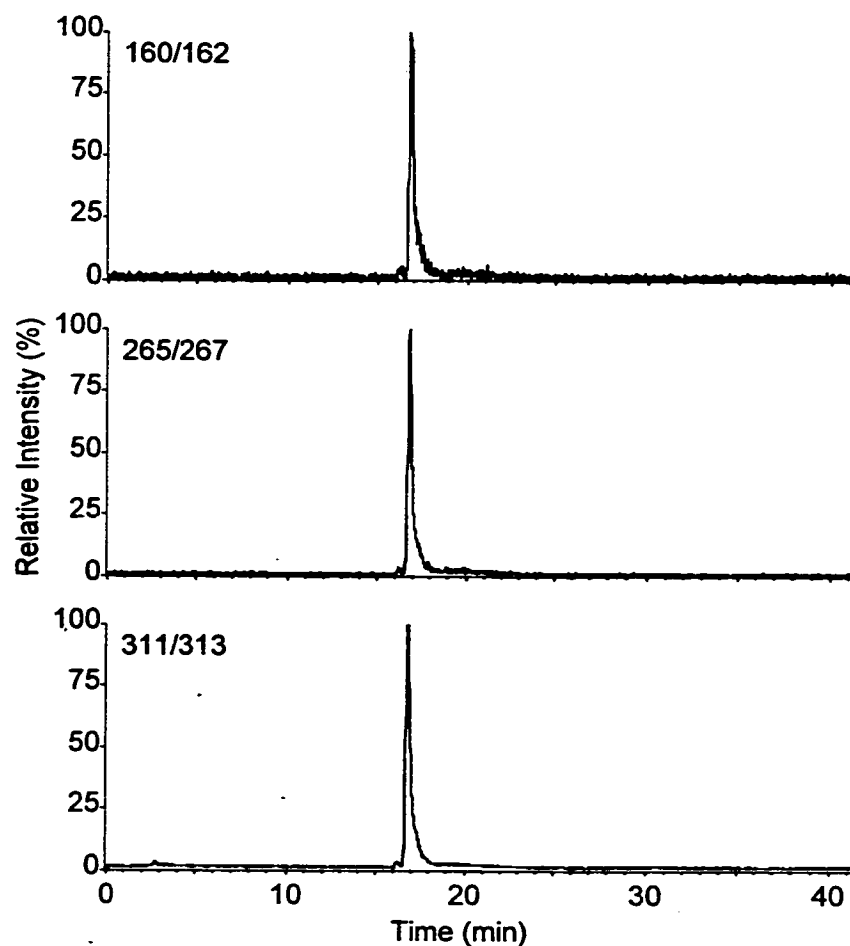


Figure 3. LC-MS/MS chromatographic traces for sea lion fecal sample (CSL#3758). Each trace represents the detection of a selected ion from the tandem MS experiment. Traces "160/162" and "265/267" represent characteristic product ions from the collisionally induced dissociation of the domoic acid $(M+H)^+$ ion nominally of 161 and 266 m/z , respectively. The trace "311/313" represents detection of residual unfragmented domoic acid $(M+H)^+$ ion nominally of 312 m/z .

Table 2. Inter-laboratory comparison of domoic acid concentrations in CSL feces.

*CSL#	<u>Laboratory # 1</u>	<u>Laboratory # 2</u>	
	HPLC-UV ($\mu\text{g DA /g} \pm \text{sd}$)	HPLC-UV ($\mu\text{g DA /g} \pm \text{sd}$)	Microplate Receptor Assay ($\mu\text{g DA equiv./g} \pm \text{sd}$)
3783	127 \pm 2	152 \pm 9	182 \pm 36
3758	50 \pm 2	36 \pm 3	96 \pm 4
3734	3.1 \pm 0.3	2.5 \pm 0.5	1.3 \pm 0.1
Control 1	0	0	0
Control 2	0	0	0

Laboratory 1: University of California at Santa Cruz (UCSC)

Laboratory 2: NOAA/NOS Charleston, SC

*CSL: California Sea Lion

toxin induced diarrhea. DA and diatom frustules were not detected in any of the nine control sea lion fecal samples.

Sea lion fecal material proved to be a more difficult matrix to work with than anchovy tissues. There was an unidentified compound present in trace amounts after extraction in some CSL fecal samples, including controls, that had the same retention time as DA (Figure 4). When present, this compound was barely detectable (near the lowest instrument detection limit) and may not be detected on other instruments with lower sensitivity. Fortunately, this compound could be distinguished from DA by monitoring at 280 nm as well as the traditional 242 nm wavelength during HPLC-UV analysis. Maximum absorption for DA occurred at wavelength 242 nm (Falk et al., 1989) with no detectable absorbance at 280 nm (Figure 4A, A'). The unknown compound had a higher absorbance at 280 nm than at 242 nm (Figure 4B, B'). Enlargement of the peak at 242 nm occurred when spiked with DA (Figure 4C, C'). Tryptophan, a potential interfering compound, also has a higher absorbance at 280 nm than at 242 nm (Quilliam et al., 1989; Figure 4A, A'). Results of HPLC analysis of combined standards of tryptophan (RT = 10.4 min) and DA (RT = 12.19 min) showed that the unknown compound (RT = 12.0) was not tryptophan (Figure 4A, A'). A 0.1M NaCl wash step employed before the final 0.5M NaCl elution step during DA extraction procedures has been shown by Hatfield et al., (1994) to remove interfering peaks. We prepared fecal samples both with the 0.1M NaCl wash step and without it. The wash was not able to remove or decrease the peak representing the unidentified compound.

Current HPLC-UV detection methods for DA dictate monitoring at 242 nm. Six fecal samples from seizing animals analyzed for this study had higher peak areas at 280 nm than 242 nm at the DA retention time (peak enlargement occurred when spiked with DA standard in all of these samples). This would have lead to false

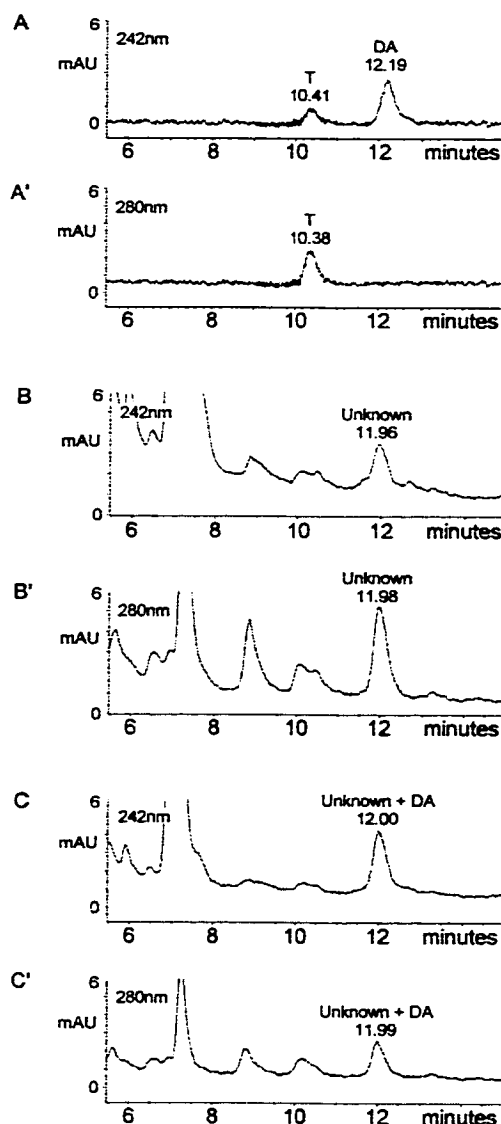


Figure 4. (A, A') HPLC trace of a combined standard of tryptophan (T) (RT = 10.41, 10.38 min) and DACS-1C domoic acid (DA) (RT = 12.19 min) monitored at (A) 242 nm and (A') 280 nm. Traces show that tryptophan does not interfere with DA. (B, B') HPLC trace of a control fecal sample containing the unidentified interfering compound monitored at (B) 242 nm (area = 54) and (B') 280 nm (area = 108). Traces show that the unknown compound has a higher absorbance at 280 nm. (C, C') HPLC trace of the same control sample as in (B) spiked with DACS-1C. Traces show peak enlargement at (C) 242 nm but not at (C') 280 nm. To correct for the interfering compound, the area at 280 nm is subtracted from the area at 242 nm before calculating DA concentration.

positives for DA if only the 242 nm wavelength was monitored. To prevent false detection and over-estimation of DA in fecal samples, the area at 280 nm was subtracted from the area at 242 nm before DA concentration was calculated. Using three control samples containing the unknown compound, a ratio of the areas (242 nm to 280 nm) for the unknown was calculated as 0.56 ± 0.03 . Because the unknown compound has a higher absorbance at 280 nm, subtracting this area results in a conservative estimate of DA concentration, and may partially explain the generally higher DA values obtained by the receptor binding assay, which was not influenced by this factor. Although presence of the interfering compound does not permit exact measurement of DA, the HPLC-UV technique is useful for determining presence or absence of this toxin and its relative concentrations in fecal samples.

Conclusions

Three-tiered trophic transfer of the neurotoxin DA, from diatoms to anchovies to sea lions, was the probable cause of mortality and neuroexcitotoxicity witnessed in 70 sea lions in central California during May 1998 (Scholin et al., 2000). In the current study, HPLC-UV and microplate receptor binding assays were used successfully by independent laboratories to detect and estimate levels of DA in fecal samples collected from sea lions exhibiting seizures. Moreover, the presence of DA in sea lion fecal material was confirmed unambiguously by tandem mass spectrometry. Our detection of both DA and *P. australis* diatom frustules in sea lion fecal material provided corroborating evidence of DA intoxication in this marine mammal species.

Chapter Two

TISSUE DISTRIBUTION AND NEUROTOXIC EFFECTS OF DOMOIC ACID IN A PROMINENT VECTOR SPECIES, THE NORTHERN ANCHOVY (*Engraulis mordax*)

Abstract

The planktivorous northern anchovy is a prominent vector of the phycotoxin domoic acid (DA) to organisms at higher trophic levels, including fish-eating seabirds and mammals. Although there are abundant data reporting DA-induced excitotoxic symptoms in higher vertebrates, to date there has been no reported evidence of neurotoxic effects in lower vertebrate vectors such as fish. To explain this apparent lack of toxicity, it has been suggested that DA may not reach the brain in anchovies and/or that fish are not as sensitive neurologically to DA. In the present study, intracoelomic (IC) injection of DA, at doses ranging from 1- 14 $\mu\text{g DA g}^{-1}$ total fish weight, resulted in severe neurotoxic symptoms such as spinning, disorientation, inability to school, and mortality, indicating that anchovies are neurologically susceptible and that DA crosses the blood-brain barrier in fish. An ED₅₀ of 3.2 $\mu\text{g DA g}^{-1}$ total body weight was determined via IC injection of DA in 83 anchovies. Comparable intraperitoneal (IP) injection studies with mice, rats, and monkeys report similar DA-induced neurotoxic symptoms at doses near 3.2 $\mu\text{g DA g}^{-1}$, suggesting a similar neurologic sensitivity and mechanism of toxicity between anchovies and mammals. DA tissue distribution measurements from freshly collected field-exposed anchovies and orally gavaged anchovies indicate that DA uptake from the gastrointestinal tract does occur. Levels as high as 1175 $\mu\text{g DA g}^{-1}$ were measured in anchovy viscera, while muscle and brain tissue DA levels were three orders of magnitude lower, indicating low but measurable

DA uptake. Further evidence is needed to confirm that uptake is sufficient during field events to induce symptoms in anchovies. Our work provides the first reported evidence of neurotoxic symptoms in fish and suggests that anchovies may be affected by DA during toxic diatom blooms. If sufficient uptake occurs, DA-induced neurotoxic symptoms and mortality may make fish easier prey targets, thereby selecting for the highest toxin levels transferred, as well as providing a possible pathway for the transfer of DA to benthic communities.

Introduction

Amnesic Shellfish Poisoning (ASP) is a neurotoxic illness resulting from consumption of the naturally-produced phycotoxin, domoic acid (DA). Several diatoms of the genus *Pseudo-nitzschia* have been shown to produce this neuroexcitatory amino acid (Subba Roa et al. 1988; Bates et al. 1989; Douglas and Bates et al. 1992; Garrison et al. 1992). Filter-feeding marine species, such as northern anchovies (*Engraulis mordax*), blue mussels (*Mytilus edulis*), and razor clams (*Siliqua patula*), have been found to accumulate the toxin in their digestive tracts (Quilliam and Wright 1989; Wekell et al. 1994; Altwein et al. 1995; Lefebvre et al. 1999). When DA levels are high enough, these species can act as vectors of DA to organisms at higher trophic levels, resulting in severe neurological disorder and mass mortality. DA was first recognized as a harmful food web-transferred phycotoxin in 1987, when at least four people died and over 100 others suffered neurological problems after consuming DA-containing cultured blue mussels harvested from eastern Prince Edward Island, Canada (Perl et al. 1990; Quilliam and Wright 1989).

Subsequent ASP events involving seabirds and marine mammals have elucidated the importance of fish as vectors of DA. In 1991, the northern anchovy was identified

as a DA vector resulting in mortality and illness in over 100 brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) in Monterey Bay, California (Work et al. 1993). In 1996, mackerel (*Scomber japonicus*) was reported as the vector of DA to pelicans in Cabo San Lucas, Mexico, also resulting in mass mortality (Sierra-Beltran et al. 1997). In 1998, over 400 California sea lions died, and several others displayed seizures, along beaches on the central California coast, again attributed to the consumption of anchovies containing DA (Lefebvre et al. 1999; Scholin et al. 2000).

Observable symptoms of ASP reported from field events involving seabirds and mammals included vomiting, diarrhea, confusion, disorientation, scratching, seizures, coma, and death (Perl et al. 1990; Todd 1993; Gulland 2000; Scholin et al. 2000). DA-induced neurotoxic symptoms occur because DA is a rigid structural analog of glutamate, the major excitatory neurotransmitter in the brain (Levitan and Kaczmarek 1991; Dakshinamurti et al. 1991; 1993). Excitotoxicity is initiated by activation of AMPA/kainate receptors, a type of glutamate receptor found in high concentrations in the brain (Debonnel et al. 1989; Berman and Murray 1997). Unique lesions in the hippocampus of mammals are characteristic of DA toxicity (Sutherland et al. 1990; Strain and Tasker 1991; Dakshinamurti et al. 1993; Scholin et al. 2000), suggesting that DA is not only absorbed from the digestive tract into the blood stream, but that it crosses the blood-brain barrier in affected organisms.

Neurotoxic effects resulting from DA consumption in seabirds and mammals are obvious and severe. However, over the 13 years that scientists have been studying toxic diatom blooms involving DA there has been no documented evidence of neurotoxic symptoms in fish, even though DA has been detected in both viscera and body tissue samples of vector fish species (Work et al. 1993; Wekell et al. 1994;

Lefebvre et al. 1999). The most prominent vector fish species in central California is the northern anchovy, which is included in the diets of nearly every predatory fish, bird, and mammal species in the California Current (Kucas 1986; Fritz et al. 1992; Work et al. 1993). The fact that no negative effects have been observed in anchovies after DA ingestion has led to the prevailing belief that fish may be acting only as a vector of the toxin without being affected themselves. In the only reported laboratory study with fish, rainbow trout (*Oncorhynchus mykiss*) were fed DA-contaminated fish meal for several weeks (Hardy et al. 1995). After a 24 hour fast, fish were sampled and DA was detected in feces and viscera, but not in blood or body tissue, suggesting that DA in fish feed is not readily absorbed by rainbow trout. In addition, no mortality, signs of toxicity, nor neurological symptoms were observed throughout the 15 week period (Hardy et al. 1995). This work has supported the growing paradigm that fish are not affected by DA.

The objective of this research was to determine whether an ecologically important vector species, the northern anchovy, is affected by DA. In order to accomplish this objective, we addressed the following questions:

- 1) Do anchovies absorb DA from the digestive tract?
- 2) Does DA reach the brain in anchovies?
- 3) Does DA exposure cause observable neurotoxic symptoms in anchovies?
- 4) If such symptoms occur, what DA concentration is necessary to induce them and how does that compare with DA concentrations required to produce neurotoxic symptoms in mammals?

The answers to these questions were obtained using a combination of field collections of naturally DA-exposed *Engraulis mordax* and laboratory studies of tank-reared specimens exposed directly to DA in known dosages. To determine ecologically

relevant DA levels in various anchovy tissues, we collected and examined fish from Monterey Bay, California during two toxic *P. australis* blooms. We obtained additional information on the uptake of DA into the liver from an oral gavage experiment with anchovies reared in the laboratory. Intracoelomic (IC) injection experiments were used to determine if neurotoxic symptoms due to DA exposure occur in anchovies and if so, at what concentrations. Results of this study provide the first evidence of DA-induced neuroexcitotoxicity in fish.

Materials and Methods

Field Sample Collection

The first set of field-exposed anchovies was obtained from Monterey Bay, California during the May 1998 toxic *P. australis* bloom. Maximum recorded density of *P. australis* was 1.3×10^5 cells l^{-1} and estimates of cellular DA levels ranged from 7 - 32 pg DA equivalents per diatom cell (Scholin et al. 2000). Fish were caught by hook and line, kept alive in bait wells, and immediately placed in ice upon landing. They were then stored in a frost-free freezer for three months, followed by storage at $-20\text{ }^{\circ}\text{C}$ for eight months before analysis. A second set of field-exposed anchovies was obtained on September 8, 2000 from Monterey Bay during a research cruise coinciding with a toxic *P. australis* bloom. At the time of collection, *P. australis* density was over 1×10^5 cells l^{-1} . Fish were caught in a net, frozen alive at $-20\text{ }^{\circ}\text{C}$ onboard the vessel, and analyzed within five days.

DA analysis was performed on whole viscera (which included all internal organs in the intracoelomic cavity), brain, and body tissue samples. Anchovies collected in May 1998 were analyzed individually ($N = 10$). Fish were thawed, followed by the removal of the entire visceral mass and brain. Brains were weighed and rinsed three times with

saline solution. To avoid analyzing body tissue contaminated with DA leaked directly from the digestive tract, body tissue samples were taken by removing fillets from the posterior dorsal side of the fish near the tail and rinsed with saline. Six anchovies collected in September 2000 were pooled in pairs resulting in three replicates of each tissue type. These fish were not allowed to thaw before the whole viscera was removed. Body tissue and brain samples were taken in the same way as the first set of anchovies except that the saline rinse was not applied. Hemoglobin concentrations were not measured in brain tissues, therefore blood contamination could be a confounding factor for measured brain DA concentrations.

Live Anchovy Maintenance

Approximately 500 live anchovies were obtained from bait fishermen in Morro Bay, California and maintained at Long Marine Laboratory in Santa Cruz, California. Fish were fed Mazuri Koi food[®] (PMI Feeds Inc. St. Louis, MO) and kept in 700 liter circular tanks (1.5 m diameter) with running, sand-filtered seawater. Captive anchovies were used for DA oral gavage and intracoelomic (IC) injection experiments.

Feeding Experiment

In an attempt to determine DA uptake and depuration rates in fish, we originally performed a laboratory feeding experiment in which anchovies were fed DA-containing *P. multiseriis*. The *P. multiseriis* clone (mu-1) was isolated in 1995 from Monterey Bay by Dr. Peter Miller and identified by scanning electron microscopy and muD1 probe following procedures described in Miller and Scholin (1996 and 1998). Over 400 l of culture, grown in Fritz f/2 Algae media[®] (Fritz Industries Inc. Dallas, TX) was used in the feeding experiment at approximately mid-stationary phase (15 days).

Diatom densities were 10^6 - 10^7 cells l^{-1} and cellular DA levels ranged from 0.2-0.4 pg DA cell $^{-1}$ throughout the three day feeding period. DA levels in phytoplankton were determined using HPLC-FMOC methods described by Pocklington et al. 1990. Fish were sampled every four hours throughout the experiment and dissected into brain, viscera, and body tissue. None of the fish samples from this feeding experiment accumulated detectable levels of toxin using HPLC-UV methods. We believe the main reasons DA was not accumulated in detectable levels were that the fish did not feed actively under these laboratory conditions and that the cell levels of DA in the cultured *Pseudo-nitzschia* were exceptionally low, as compared with typical values measured in the field (i.e. 7 - 32 pg DA cell $^{-1}$ Bates 1998; Scholin et al. 2000). We observed only short sporadic feeding throughout the experiment, while obvious feeding behavior was observed before and after the experiment when fish food was used. Because of the difficulty of getting diatoms to produce ecologically relevant DA levels, and the unreliability of fish feeding in the original experiment, we changed to a gavage experiment in which DA was placed directly into the gut.

Gavage Experiment

To prepare for gavage, the water level in the anchovy holding tank was siphoned to 20 cm. Anchovies were scooped out with a large round bottom plastic bag. The entire bag containing the fish was then placed in a small holding cooler. Each fish was then scooped individually into a small Ziplock[®] (SC Johnson Inc. Racine, WI) bag, the water was drained, and the fish was held tightly in the bag. The corner of the bag near the mouth was cut open and a smooth plastic gavage tube connected to a syringe was inserted into the mouth, down the esophagus, and into the pyloric stomach of the anchovy. The contents of the syringe were slowly injected. Six anchovies were orally

gavaged with 200 μl nanopure water containing a total of 800 μg DA (Sigma Chemical Company, St. Louis, MO). The gavage dose of 800 μg was chosen because it is within the range of DA concentrations previously detected in the guts of field-exposed fish. Since anchovy viscera weights average 1 to 2 g, the resulting gavage dose was 400 to 800 μg DA g^{-1} viscera. Levels as high as 2300 μg DA g^{-1} viscera have been reported in field-exposed anchovies (Trainer et al. 2000). DA concentrations were confirmed by HPLC-UV (Hatfield et al. 1994; Quilliam et al. 1995). After ten minutes, three fish were sampled by removing the liver, separating out the digestive tract (esophagus to anus), and taking body tissue fillets. After six hours, the final three fish were sampled in the same way. Liver, digestive tract, and body tissue samples were analyzed for the presence of DA.

Domoic Acid Detection via HPLC-UV

Anchovy whole viscera, digestive tract, brain, liver, and body tissue samples were analyzed for the presence of DA using an isocratic elution profile on a Hewlett-Packard 1090 HPLC equipped with a diode array detector (DAD) set at 242 nm with a bandwidth of 10 nm (reference signal was set at 450 nm with a bandwidth of 10 nm). A reverse phase Vydac C₁₈ column (catalog #201TP52, 2.1 mm X 25 mm, Separations Group, Hesperia, CA) equipped with a Vydac guard column (particle size 5 μm) was used. The mobile phase (90/10/0.1, water/ MeCN/ TFA) was degassed with helium for 10 minutes prior to analysis. A calibration curve was generated using DACS-1C DA standards of 0.3, 0.5, 1.0, 2.0, 4.0, 8.0, and 16 μg ml^{-1} ($r= 0.99$). The lowest detectable standard was 0.1 μg DA/ml. The instrument detection limit, which was equivalent to the concentration that corresponded to three times the standard deviation of the signal from

the lowest detectable standard, was $0.2 \mu\text{g DA ml}^{-1}$. Injections were $20 \mu\text{l}$ or $50 \mu\text{l}$, with a flow rate of 0.3 ml min^{-1} .

Sample Extraction

DA extraction in whole viscera, digestive tract, and body tissue followed procedures described by Quilliam et al. (1995) and Hatfield et al. (1994). Individual brain and liver samples, mean weight $0.060 \pm 0.02 \text{ g}$ ($N = 20$) and $0.149 \pm 0.01 \text{ g}$ ($N = 3$) respectively, required a variation in extraction procedure. For these samples, the entire organ was homogenized in 1 ml extraction solvent for 3 min then pipetted into a microcentrifuge tube. The homogenizer probe and tube were rinsed with 0.5 ml extraction solvent which was also pipetted into the microcentrifuge tube and centrifuged for 20 min. The supernatant was passed through a $0.2 \mu\text{m}$ Millex-GS filter (Millipore S.A. Molsheim France) along with another 0.5 ml rinse with extraction solvent, bringing the total extraction volume to 2 ml.

Solid Phase Extraction (SPE)

JT Baker™ strong anion exchange (SAX) SPE cartridges, reported to have the highest recovery by Hatfield et al. (1994), were used for all samples analyzed. Solid phase extraction procedures described in Lefebvre et al. (1999) were followed with a few exceptions. In fish from May 1998, 1.5 ml of filtrate was passed through the SAX column followed by elution with a 0.5 M NaCl in 10% MeCN solution in a volume of 3 ml for viscera and 2 ml for body and brain samples. Spike and recovery experiments using DA standards yielded an estimated 80 ± 16.9 , 70 ± 17.5 , 66 ± 5.5 , and 64 ± 4.7 % ($N = 3$ for each matrix) efficiency of extraction in viscera, body tissue, brain, and liver matrices, respectively. Method detection sensitivities were 1.0, 0.4, and $2.7 \mu\text{g DA g}^{-1}$

for viscera, body tissue, and brain and liver samples, respectively. In fish from September 2000, viscera and body tissue samples were eluted with 5 ml, and brain tissue was eluted with 3 ml. For body tissue, 6 ml of extraction filtrate was passed through the SAX column instead of the standard 2 ml. Efficiency of extraction ($N = 3$) was 92.7 ± 4.6 , 89 ± 5 , and 97 ± 2.8 %, and method detection sensitivities were 0.5, 0.2, and $0.9 \mu\text{g DA g}^{-1}$ for viscera, body tissue, and brain tissue, respectively. HPLC-UV analysis of the raw extract (no SAX clean-up) from three body tissue samples yielded 98 ± 1.2 % recovery, suggesting that most of the DA loss occurred at the SAX clean-up step.

Chemical Reagents

DACS-1C certified DA standard (National Research Council of Canada, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, NS, Canada) and 90% pure DA reagent (Sigma Chemical Company, St. Louis, MO) were obtained for calibration standard preparation and spike/recovery calculations. Trifluoroacetic acid (TFA), analytical grade sodium chloride (NaCl), and Optima grade methanol (MeOH) and acetonitrile (MeCN) were obtained from Fisher Scientific (Pittsburgh, PA). Nanopure water was used for solution preparation. Standards were kept refrigerated and in the dark when not in use.

Intracoelomic (IC) Injection Experiments

IC injection experiments were performed with 178 anchovies. Fifteen to 20 control and DA-dosed fish were used in each experiment. Control and DA-dosed fish were placed into separate tanks (1.5 m diameter) for observation. Control anchovies ($N = 95$, mean wt = 19.6 ± 4.3 g) were injected with 300 μl nanopure water. DA-dosed anchovies ($N = 83$, mean wt = 20.6 ± 4.4 g) were injected with 300 μl nanopure water

containing DA (Sigma). Absolute DA injection doses, confirmed by HPLC-UV were 38.6 ± 0.9 (N = 3), 81.7 ± 2.4 (N = 3), 148 (N = 1), and 194 (N = 1) μg DA. The exposure dose for each fish was determined by dividing the absolute DA injection dose by the total body weight of each fish, resulting in exposure groups of 1.8 ± 0.2 , 2.7 ± 0.3 , 3.7 ± 0.2 , 4.3 ± 0.1 , 5.7 ± 0.4 , 6.5 ± 0.3 , 7.7 ± 0.2 , 8.5 ± 0.3 , 9.5 ± 0.3 , 10.6 ± 0.1 , 11.4 ± 0.1 , 12.5 ± 0.5 , and $\geq 14.7 \mu\text{g DA g}^{-1}$ body wt (Figure 1). For injection, fish were scooped individually into Ziplock[®] bags and injected through the bag at a point between the pectoral fins, with the needle angled toward the posterior end. Contents were slowly injected into the intracoelomic cavity. After injection, fish were released into tanks and observed for six hours. Behavioral toxicity was quantified by the presence or absence of "spinning" behavior (see results section). The brains of eight anchovies injected with DA were removed one hour after injection and analyzed for DA by HPLC-UV.

Results

DA Tissue Distribution During Toxic *P. australis* Blooms

All ten whole viscera and body tissue samples from fish sampled in 1998 contained detectable levels of DA by HPLC-UV. Seven of ten brain samples contained detectable levels of DA ranging from 2.1 to $9.7 \mu\text{g DA g}^{-1}$. Interestingly, the fish collected in 2000 had viscera DA levels over 70 times higher than the 1998 fish, yet body and brain levels much lower (Table 1). Because of sample storage differences, we question the validity of the DA measurements in tissues from the 1998 fish (see discussion). Whole viscera, body tissue, and brain sample matrices contained no interfering factors for DA detection using HPLC-UV methods.

Table 1: Tissue distribution of domoic acid in field-exposed anchovies (*Engraulis mordax*) collected for this study and reported in previous literature. (NA = not analyzed; nd = not-detectable)

Date Collected	Viscera ($\mu\text{g DA g}^{-1}$)	Body Tissue ($\mu\text{g DA g}^{-1}$)	Brain Tissue ($\mu\text{g DA g}^{-1}$)	Reference
April 1991	169-215	22-33*	NA	Wekell et al.1994
Sept 1991	275	77*	NA	Quilliam et al.1991
Sept 1991	190	40*	NA	Work et al. 1993
May 1998	223	39*	NA	Lefebvre et al.1999
May 1998 (N = 7)	15.7 \pm 5	6.7 \pm 2*	6.2 \pm 3*	this study
Sept 2000	1050	1.2	nd	this study
Sept 2000	1050	0.6	1.4	
Sept 2000	1175	1.2	nd	
Detection Limit	0.5	0.2	0.9	

*Questionably high DA levels possibly due to post-mortem leakage from the digestive tract (see discussion).

DA Uptake During Gavage Experiment

Of the 800 µg of DA orally gavaged in each of six anchovies, only 3-25 % was retained in the digestive tract of the three fish sampled at the end of ten minutes (Table 2). In one case, DA was not detected in the digestive tract after ten minutes post-gavage. During pilot studies, in which dye was added to the gavage solution, we could see that most fish vomited out a portion of the gavage dose. By chance, it appears that two of the fish sampled at 10 min had vomited out a larger portion of the gavage dose than fish sampled at 6 hours (Table 2). For this reason, the gavage method was not useful for calculating quantitative uptake rates of DA, but did provide corroborating laboratory evidence that DA uptake from the digestive tract does occur in anchovies. DA was not detected in body tissue or liver samples in anchovies sampled ten minutes post-gavage, but was detected in all three liver samples from anchovies sampled six hours post-gavage (Table 2). DA concentrations in liver samples increased, along with those in the digestive tract (Table 2). One body tissue sample (out of three) from a fish taken six hours post-gavage contained 1.6 µg DA g⁻¹ (Table 2). Care was taken to ensure that puncturing of the digestive tract did not occur by using a smooth plastic gavage tube. Also, during method development the gavage tube was inserted in ten anchovies, followed by dissection to see if perforation occurred. No break in the gut lining was observed. The length of the tube was marked at the point where it entered the pyloric stomach. This measurement was used for the test anchovies. These preliminary results suggest that DA is absorbed from the digestive tract within six hours and that the liver is contaminated first, before the muscle tissues of the body. Throughout the six hour experiment, no neurotoxic symptoms nor mortality were observed.

Table 2: Tissue distribution of domoic acid in six anchovies (*Engraulis mordax*) after oral gavage with 200 µl nanopure water containing 800 µg of domoic acid. Anchovies 1-3 were sampled ten minutes post-gavage and anchovies 4-6 were sampled six hours post-gavage. The digestive tract was separated from the other internal organs before analysis. (NA = not analyzed; nd = not-detectable)

Fish	Time Post-Gavage	Digestive Tract (µg DA total)	Body Tissue (µg DA g⁻¹)	Liver (µg DA g⁻¹)
1	10 min	23	nd	nd
2	10 min	nd	nd	NA
3	10 min	199	nd	nd
4	6 hours	100	1.6	2.7
5	6 hours	138	nd	3.9
6	6 hours	144	nd	6.1
Matrix Detection Limit		1.0	0.4	2.7

Neurotoxic Symptoms Resulting From Intracoelomic (IC) Injection

Approximately 40 min after IC injection of DA, fish exhibited severe neurological disturbance in the form of altered swimming behavior and death. First, fish appeared disoriented and unable to school. As symptoms progressed, individual fish begin to spin rapidly in small circles at the surface or bottom of the tank. This spinning behavior was the most obvious and consistent neurotoxic symptom. Other symptoms included spiral swimming, upside-down swimming, listing to the side, gaping of the mouth, and head shaking. Many fish exhibiting spinning symptoms began to sink to the bottom immediately and eventually died. However, some fish exhibiting spinning recovered and were swimming normally when observed again after 24 hours. Control fish maintained schooling ability and had 99% survival after IC injection. None of the control anchovies exhibited spinning, spiral swimming, or upside-down swimming. All fish with exposure doses $\geq 4.3 \pm 0.1 \mu\text{g DA g}^{-1}$ total body weight exhibited neurotoxic symptoms (Figure 1). After combining all exposure doses, an ED₅₀ of $3.2 \mu\text{g DA g}^{-1}$ was calculated using the best-fit sigmoidal equation (Figure 1). An ED₅₀ refers to the exposure dose at which 50% of the fish show symptoms. For our purposes, the most severe symptom (spinning) was used to determine whether a fish was affected by DA or not. The brains of eight anchovies exhibiting severe spinning behavior were sampled one hour after IC injection and analyzed for the presence of DA by HPLC-UV. In all eight brains, DA concentration was below detectable levels.

Discussion

Excitotoxic symptoms observed after intracoelomic (IC) injection of DA confirm that anchovies are neurologically sensitive to DA. The fact that all fish injected with exposure doses $\geq 4 \mu\text{g DA g}^{-1}$ exhibited neurotoxic effects, suggests that the

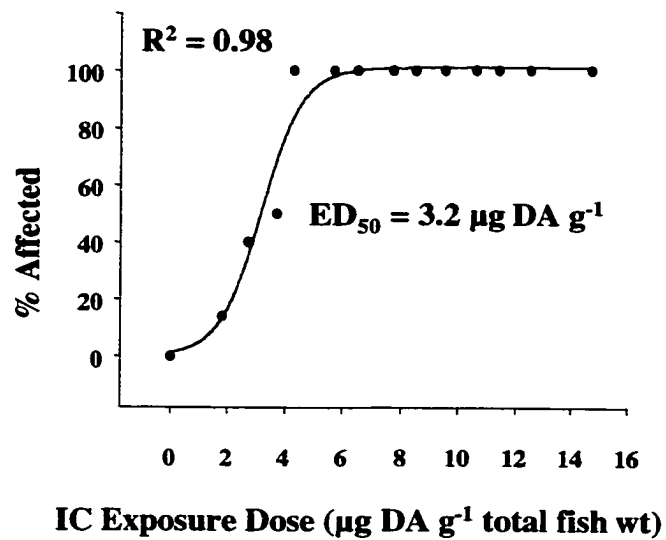


Figure 1. Dose-response curve showing the relationship between Intracoelomic (IC) dose of domoic acid and the percent of anchovies (*Engraulis mordax*) affected at each dose. Spinning behavior was Used to determine whether fish were affected by domoic acid.

sensitivity of anchovies to DA is similar to that of mammals. Comparable intraperitoneal (IP) injection studies report tremors in *Cynomolgus* monkeys after IP doses of $4.0 \mu\text{g DA g}^{-1}$ (Tryphonas et al. 1990; Todd 1993), and scratching and seizures in rats with IP doses of $2.0\text{-}2.7 \mu\text{g DA g}^{-1}$ (Todd 1993). In addition, the ED₅₀ (exposure dose affecting 50 % of the population) of $3.2 \mu\text{g DA g}^{-1}$ calculated in this study (Figure 1) is similar to the ED₅₀ of $4.0 \mu\text{g DA g}^{-1}$ reported for mice in a previous IP injection study (Strain and Tasker 1991). Excitotoxic symptoms, such as seizures in mammals (Grimmelt et al. 1990; Tryphonas et al. 1990; Tasker et al. 1991; Truelove and Iverson 1994; Scholin et al. 2000), head shaking in birds (Work et al. 1993), and spinning, head shaking, and disorientation in fish (this study), suggest that the mechanism of DA-induced excitotoxicity involves the central nervous system (CNS) in each of these groups and that DA crosses the blood brain barrier in affected organisms.

Although we have shown that anchovies are affected by DA and have a similar sensitivity as mammals as measured by IP doses, excitotoxic symptoms have yet to be observed in fish after oral doses. DA tissue distribution measurements from field-exposed anchovies collected in May 1998 for this study, along with previously reported DA levels in fish muscle tissues would suggest that anchovies do absorb enough DA to induce symptoms during toxic diatom blooms (Table 1). However, the fact that anchovies collected in May 1998 had brain DA levels ranging from 2.1 to $9.7 \mu\text{g DA g}^{-1}$ and did not exhibit neurotoxic symptoms, while non-detectable brain DA levels (N = 8) caused spinning behavior in anchovies after IC injection, causes us to question the validity of measurements from field-collected fish. Because the May 1998 anchovies were stored for three months in a frost-free freezer, followed by eight months at $-20 \text{ }^\circ\text{C}$ before analysis, we suggest that direct leakage of DA from the digestive tract may have

occurred, resulting in erroneously high concentrations of DA in brain and body tissue samples. Authors of the studies listed in Table one have been contacted and in all cases they were concerned that factors such as duration between death and freezing, storage duration, thawing, and sample preparation, may have contributed to DA contamination of the tissues via post-mortem leakage from the digestive tract.

Although previously reported DA tissue levels in fish may be erroneously high, tissue distribution measurements from freshly collected field-exposed anchovies, as well as from our gavage experiment, suggest that some DA uptake does occur. Anchovies collected in September 2000 were caught live in a net, frozen alive at -20°C , never allowed to thaw, and analyzed within five days. Muscle fillets were taken from the dorsal side of the fish near the tail (as far away from the digestive tract as possible). DA values detected in viscera were among the highest previously reported ($1092 \pm 72 \mu\text{g DA g}^{-1}$), while muscle and brain tissue values were three orders of magnitude lower than viscera levels (Table 1), suggesting that DA uptake is low but measurable. Results from the gavage experiment provide corroborating evidence for low DA uptake and suggest that DA accumulates in the liver first before muscle tissues (Table 2). The low uptake of DA in anchovies observed in this study is consistent with uptake levels reported in other orally dosed animals such as mice, rats, and monkeys. In laboratory studies, oral doses much higher than intravenous (IV) or IP doses were required to induce the same excitotoxic effects, indicating that DA is poorly absorbed from the gastrointestinal tract and mostly excreted in the feces (Todd 1993).

Additional evidence is required to determine if anchovies absorb sufficient levels of DA to induce neurotoxic symptoms during toxic diatom blooms. In the only reported oral-dose DA study with fish, rainbow trout were fed DA-contaminated fish meal ($58 \mu\text{g DA g}^{-1}$) for 15 weeks and showed no signs of toxicity (Hardy et al. 1995).

However, we calculated that at a feeding rate of 10% of the trout body weight per day, the daily DA dose obtained from fish meal used in that study would have been below the doses found to induce neurotoxic symptoms in this study. In fact, viscera levels were significantly lower than those reported here in field-collected anchovies.

Anchovies collected in September 2000 had a mean viscera level of $1092 \pm 72 \mu\text{g DA g}^{-1}$, which is equivalent to 2,184 $\mu\text{g DA}$ per fish since individual viscera weights were 2 g. Gut levels of DA in field-exposed anchovies can also be predicted based on measurements of anchovy filtering rates, their gut evacuation rates, cell densities of *P. australis* in seawater, and per cell concentrations of DA in *P. australis*. For example, to estimate ecologically relevant maximum gut levels of DA, we assume maximum *P. australis* densities of $10^6 \text{ cells l}^{-1}$ (Scholin unpublished observation) and DA values of 75 pg cell^{-1} (Scholin et al. 2000) found in Monterey Bay and we assume steady state (DA entering the gut equals DA leaving the gut). These data, along with the observed anchovy gut evacuation rate of 0.42 hr^{-1} (Tudela and Palmera 1995), and the maximum clearance rate of filter feeding anchovies of 2.3 l min^{-1} (Leong and O'Connell 1969), imply an anchovy stomach reservoir of 319 l worth of *P. australis* cells (23,925 $\mu\text{g DA}$ per fish). This calculation assumes DA is only found in diatoms in the gut and not transferred to body tissues: if tissue retention occurs, overall DA levels in the fish could be higher. At more common bloom levels of *P. australis*, i.e. $10^5 \text{ cells l}^{-1}$ and toxin levels of 32 pg cell^{-1} (Scholin et al. 2000), together with mid-level feeding rates of 1 l min^{-1} (Leong and O'Connell 1969), the stomach reservoir of toxin would be 447 $\mu\text{g DA}$ per fish, using the same value for gut evacuation rate. These calculations identify ecologically relevant anchovy gut exposure levels ranging from 447 - 23,925 $\mu\text{g DA}$ during various bloom conditions. These massive oral doses may be sufficient to induce neurotoxicity even with low DA uptake. Alternatively, filter-feeding marine herbivores

may have evolved mechanisms to avoid DA toxicity via their limited uptake or metabolic detoxification if selective pressure is sufficiently high.

If sufficient doses of DA are absorbed from the gut in anchovies after oral ingestion, then excitotoxic symptoms induced by DA, such as disorientation, spinning, and inability to school, would make anchovies easier prey targets in the field. The spinning and state of confusion brought on by DA in anchovies appears to leave the fish oblivious to predators as well as vulnerable due to the loss of schooling ability. As a result, the most toxic fish would be the easiest prey items and could select for the transferal of the highest possible toxin levels to predators of anchovies, including pelicans, cormorants, and sea lions, thereby maximizing the detrimental effects of toxic diatom blooms. In addition, the lack of a swim bladder in anchovies results in rapid sinking upon death. This may provide a pathway for the rapid transfer of DA to deep pelagic or benthic communities, thus exposing not only surface communities, but also those throughout the water column and benthos.

Conclusions

Anchovies are neurologically susceptible to DA excitotoxicity and have a similar sensitivity as mammals as modeled by mice, rats, and monkeys in IP injection experiments. Excitotoxic symptoms, such as spinning, head shaking, and disorientation, are similar to previously reported symptoms witnessed in birds and mammals, indicating that DA crosses the blood brain barrier and affects the CNS in each group. DA uptake is low in anchovies, as it is in mammals, but additional evidence is needed to determine if symptom-inducing DA doses are absorbed in anchovies during field events. If so, neurotoxic symptoms, such as spinning at the surface and inability to school, would make intoxicated anchovies easier prey items and could select for the most toxic

specimens being consumed. Additionally, if sufficiently dosed, anchovies die and sink, where they may rapidly transfer DA to deep water and benthic communities. Because of the similarity between anchovies and mammals in terms of neurologic sensitivity, symptomology, and low uptake, we suggest that DA may play a role in shaping anchovy populations, as it does in bird and mammal populations.

Chapter Three

DOMOIC ACID IN PLANKTIVOROUS FISH IN RELATION TO TOXIC *PSEUDO-NITZSCHIA* CELL DENSITIES

Abstract

In at least two mass mortality events in Monterey Bay, California planktivorous fish were implicated as vectors of the neurotoxin domoic acid (DA) from diatoms to sea birds and marine mammals. While the transfer of DA from planktivorous fish to piscivorous predators has been well established, the relationship between toxin levels in plankton-feeding fish and the regional abundance of DA-producing diatoms has not been documented. Here we present data from an extensive field study in which cell densities of toxic *Pseudo-nitzschia* species and DA levels in anchovies and sardines were measured from samples collected weekly throughout Monterey Bay from 10/8/99 to 10/8/00. Four distinct blooms were documented with cell densities ranging from 3.2×10^3 to 5.0×10^5 cells liter⁻¹. DA was detected in fish viscera samples whenever toxic diatom densities reached $\geq 10^3$ cells liter⁻¹ in surface waters, suggesting that anchovies and sardines regularly consume toxic diatoms when present. Fish contained DA levels above the regulatory limit ($20 \mu\text{g DA g}^{-1}$ whole fish or $222 \mu\text{g DA g}^{-1}$ viscera) only when toxic cell densities exceeded 10^4 cells liter⁻¹. DA was only detected in fish when toxic diatom species were also present in the water, suggesting that the toxin is quickly depurated and that fish are only dangerous vectors during the bloom period. Anchovies appear to be more potent vectors than sardines as they consistently contained more DA than sardines collected simultaneously. Maximum DA levels detected in fish were $1815 \mu\text{g DA g}^{-1}$ in anchovy and $728 \mu\text{g DA g}^{-1}$ in sardine viscera samples. In fish with high

viscera levels of DA, corresponding body tissues contained 0.2 to 2.2 $\mu\text{g DA g}^{-1}$, suggesting that DA is not accumulated in edible body tissues to levels that threaten human consumers. Results from this study suggest that anchovies may be a valuable indicator species for assessing the risk of DA intoxication to higher level consumers during toxic *Pseudo-nitzschia* blooms in Monterey Bay.

Introduction

Toxins associated with harmful algal blooms (HABs) have long been recognized as a serious threat to public health, wildlife, and fisheries (Taylor and Seliger 1979; Shumway 1990; Anderson et al. 1993; Boesch et al. 1997). Toxin accumulation in filter feeding organisms can result in devastating effects on higher level predators such as humans, sea birds, and marine mammals. One such toxin, domoic acid (DA), is of particular concern in Monterey Bay, California where DA-producing diatoms commonly occur (Horner et al. 1997; Scholin et al. 2000). DA is naturally produced by some species of the diatom genus *Pseudo-nitzschia* and is responsible for a neurotoxic illness called amnesic shellfish poisoning (ASP) (Subba Rao et al. 1988; Bates et al. 1989; Wright et al. 1989; Garrison et al. 1992). ASP was first recognized in Canada in 1987 when several people became ill and at least 3 died after consuming DA contaminated blue mussels (*Mytilus edulis*) (Perl et al. 1990; Quilliam and Wright 1989). Since that event there has been extensive research focusing on DA accumulation in bivalve vector species (Wohlgeschaffen et al. 1992; Wekell et al. 1994; Jones et al. 1995; Douglas et al. 1997) and neurotoxic effects in mammals (Grimmelt et al. 1990; Tasker et al. 1991; Truelove and Iverson 1994; Scholin et al. 2000), as well as the establishment of safety standards for harvested seafood (20 $\mu\text{g DA g}^{-1}$ mussel tissue is

the upper limit for shellfish consumption established by Health and Welfare Canada) (Todd 1990).

Although bivalves were the vector in the first ASP event, as is the case with many algal toxins, subsequent DA poisoning events have revealed that fish can also be prominent vectors of DA. In fact, it appears that filter feeding fish are more potent vectors of DA than bivalves in Monterey Bay. These fish link primary algal producers directly to higher level consumers. Two species of planktivorous fish, northern anchovy (*Engraulis mordax*) and Pacific sardine (*Sardinops sagax*) dominate surface waters of the California Current and Monterey Bay posing a serious threat to secondary consumers during toxic *Pseudo-nitzschia* blooms. In two documented cases of ASP in Monterey Bay, transfer of the toxin occurred when *Pseudo-nitzschia* cells accumulated in the gastrointestinal (GI) tracts of planktivorous fish and were then passed to piscivorous predators. In 1991, the deaths of several hundred brown pelicans (*Pelecanus occidentalis*) and Brant's cormorants (*Phalacrocorax penicillatus*) were attributed to the consumption of anchovies containing toxic *Pseudo-nitzschia* (Fritz et al. 1992; Work et al. 1993). The second event occurred in 1998, when DA intoxication killed hundreds of California sea lions (*Zalophus californianus*) and left many seizing on the beaches. Anchovies were, again, identified as the vector species for the transfer of DA from primary diatom producers to higher trophic levels (Lefebvre et al. 1999; Scholin et al. 2000).

The most well studied vectors of algal toxins are bivalve molluscs (Kvitek 1991; Wohlgeschaffen et al. 1992; Cembella et al. 1994; Douglas et al. 1997), while little is known about the movement of algal toxins through other members of the marine food web such as filter feeding herbivorous fish. For the Monterey Bay region, understanding the dynamics of DA movement and accumulation in planktivorous fish is

paramount to understanding the risks associated with *Pseudo-nitzschia* blooms to various members of the local marine food web. Here we present a comprehensive study detailing the presence of DA in one level of the food web under “real” bloom conditions.

The goal of this study was to characterize the dynamics of DA accumulation in small planktivorous fish in relation to ecologically relevant toxic *Pseudo-nitzschia* densities experienced in Monterey Bay. In order to achieve this goal, we addressed the following questions:

- 1) Is there a relationship between toxic *Pseudo-nitzschia* cell densities in surface waters and DA levels detected in the GI tracts of planktivorous fish collected from Monterey Bay?
- 2) At what toxic cell densities do fish obtain DA levels potentially harmful to piscivorous predators ($\geq 20 \mu\text{g DA g}^{-1}$ whole fish, based on seafood safety limits)?
- 3) In the absence of toxic *Pseudo-nitzschia* in surface waters, do fish retain DA in viscera and/or body tissues?
- 4) Do planktivorous fish of different species contain equivalent levels of toxin?

To answer these questions we performed a field study in which toxic *Pseudo-nitzschia* densities and DA levels in anchovies and sardines were measured over a one year period from samples collected weekly throughout Monterey Bay.

Materials and Methods

Phytoplankton Density Samples

Water samples were collected in Monterey Bay, California from October 1999 through October 2000. Weekly samples were collected from the Santa Cruz Municipal Wharf. Other sites were opportunistically sampled in response to reports of phytoplankton blooms. All sites are shown in Figure one. In the laboratory, water

column abundance's of toxic *Pseudo-nitzschia* species, *P. australis* and *P. multiseriis* were determined using whole cell hybridization with species-specific large subunit (LSU) rRNA-targeted fluorescent probes. Based on the unique LSU ribosomal RNA nucleotide signatures of different species of *Pseudo-nitzschia*, fluorescently labeled oligonucleotide probes were developed that recognize these unique sequences (Miller and Scholin, 1998). The whole cell hybridization method used here is described in detail in Miller and Scholin (1998) and a brief description follows. Five aliquots (10 to 30 ml) from surface sea water samples were filtered onto 1.2 μm Isopore polycarbonate filters (Millipore Corporation, Bedford, MA) and preserved with a saline ethanol solution for at least one hour. After rinsing with hybridization buffer, the samples were incubated with species specific probes for *P. australis*, *P. multiseriis*, a positive control probe which hybridizes with RNA sequences common to all known small subunit rRNA molecules, and a negative control probe designed for a LSU rRNA sequence specific for a strain of *Alexandrium tamarense*. The later control is directed against a sequence of LSU rRNA that is in a position of the molecule similar to that targeted by the *P. australis* probe. A third control consisted of sample with no probe added. After one hour, filters were rinsed and placed on microscope slides. Intact cells that retained the fluorescein labeled probe were then counted on a Zeiss Standard 18 compound microscope, equipped with epifluorescence. The entire area of each filter was counted. *P. australis* and *P. multiseriis* counts were combined to give the total number of toxic *Pseudo-nitzschia* cells present per liter of surface seawater at each collection date.

Anchovy and Sardine Samples

Anchovies and/or sardines were obtained weekly from landings of commercial boats at Moss Landing harbor (Figure 1). The catches were made in various sectors in

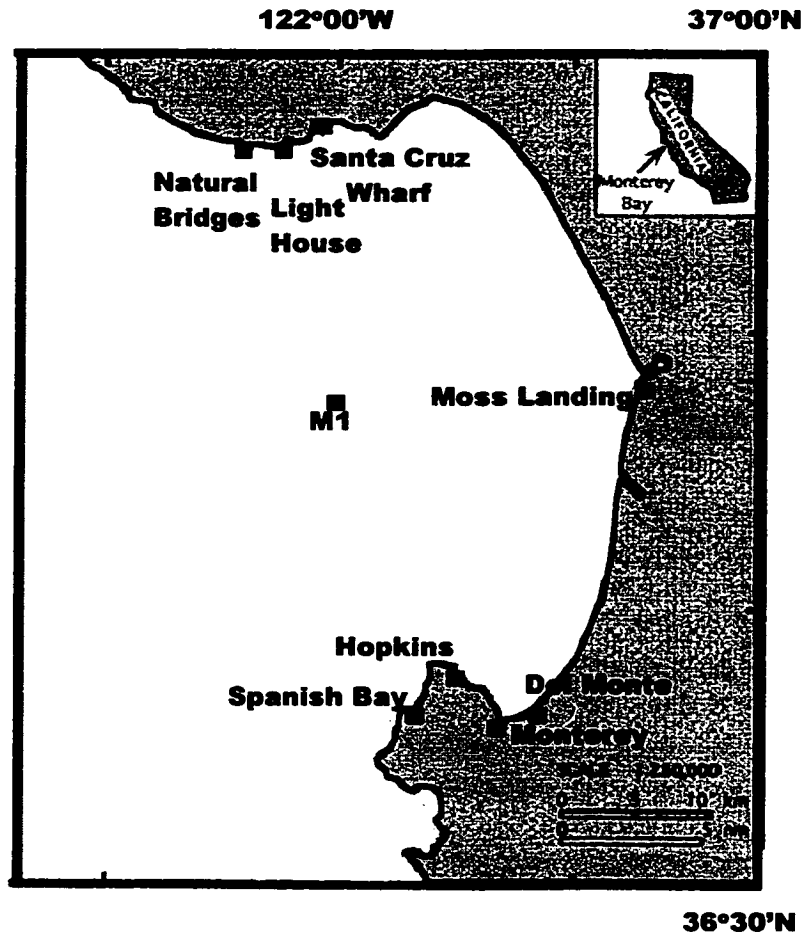


Figure 1. Figure shows the nine sample sites in Monterey Bay, California where surface water samples used for toxic *Pseudo-nitzschia* cell counts were collected. Anchovies (*Engraulis mordax*) and sardines (*Sardinops sagax*) were caught in various sectors of Monterey Bay by commercial boats and landed at Moss Landing harbor.

Monterey Bay, California from October 1999 through October 2000. At the dock, five to ten live fish of each type were removed from bait wells, packaged in ziplock® bags, and placed directly into a -20°C freezer at the site. Fish viscera samples (N = 66) were analyzed for the presence of DA via HPLC-UV (Hatfield et al. 1994; Lefebvre et al. 1999). In fish with high viscera DA levels, body tissues were also analyzed for the presence of DA. Anchovy and sardine samples collected simultaneously at seven distinct time points were examined for the presence of *Pseudo-nitzschia* in gut contents using a compound microscope and diatoms were identified to genus level only.

The established seafood safety level of 20 µg DA g⁻¹ refers to whole organisms. In fish, most of the toxin is found in the gut (Lefebvre et al. 1999; Vale and Sampayo 2001). In order to determine which viscera DA levels detected in this study resulted in whole fish DA levels above the regulatory limit, mean percent viscera (viscera weight ÷ whole fish weight X 100%) was determined for anchovies (N = 39) and sardines (N = 12).

Chemical Reagents

DACS-1C certified DA standard (National Research Council of Canada, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, NS, Canada) and 90% pure DA reagent (Sigma Chemical Company, St. Louis, MO) were obtained for calibration standard preparation and spike/recovery calculations. Trifluoroacetic acid (TFA), analytical grade sodium chloride (NaCl), and Optima grade methanol (MeOH) and acetonitrile (MeCN) were obtained from Fisher Scientific (Pittsburgh, PA). Nanopure water was used for solution preparation. Standards were kept refrigerated and in the dark when not in use.

DA Detection in Fish Viscera and Body Tissue Samples via HPLC-UV

Fish viscera and body tissue samples were analyzed for the presence of DA using an isocratic elution profile on a Hewlett-Packard 1090 HPLC equipped with a diode array detector (DAD) set at 242 nm with a bandwidth of 10 nm. The reference signal was set at 450 nm with a bandwidth of 10 nm. A reverse phase Vydac C₁₈ column (catalog #201TP52, 2.1 mm X 25 mm, Separations Group, Hesperia, CA) equipped with a Vydac guard column (particle size 5 µm) was used. The mobile phase (90/10/0.1, water/ MeCN/ TFA) was degassed with helium for 10 min prior to analysis. A calibration curve was generated using DACS-1C DA standards of 0.15, 0.3, 0.5, 1.0, 2.0, 4.0, 8.0, and 16 µg ml⁻¹ (r= 0.99). The lowest detectable standard was 0.15 µg DA ml⁻¹. The instrument detection limit, which was equivalent to the concentration that corresponded to three times the standard deviation of the signal from the lowest detectable standard (N=3), was 0.2 µg DA ml⁻¹. Injections were 10 µl or 25 µl with a flow rate of 0.3 ml min⁻¹.

Sample Extraction and Solid Phase Extraction

The entire visceral mass (digestive tract and all internal organs except gonads) was dissected from 2 to 10 fish from each date, then pooled and homogenized with a mortar and pestle. Sixteen ml of 50% MeOH was added to a 4 g aliquot of homogenized viscera, vortexed for 30 sec, and sonicated for 4 min in a water bath. Following sonication, the sample was vortexed for 30 sec, homogenized for 1 min with a homogenizer probe on ice, and vortexed again for 30 sec. The resulting slurry was centrifuged for 20 min at 4000 rpm and the supernatant passed through a 1.0 µm filter (Millipore Corp., Bedford, MA, USA). Two ml of filtrate was then passed through a

strong anion exchange (SAX) solid phase extraction (SPE) column (JT Baker™) which was preconditioned with 6 ml nanopure water, followed by 3 ml 100% methanol, and finally 3 ml 50% MeOH (Lefebvre et al. 1999). After washing the column with 5 ml of 10% MeCN, DA was eluted with 5 ml of 0.5 M NaCl in 10% MeCN at a rate of one drop per sec. The column was not allowed to run dry at any time during solid phase extraction. Analysis of body tissue samples followed the same procedure as viscera samples except that 2 grams of tissue were homogenized in 8 ml of 50% MeOH, and 6 ml of filtrate was eluted with 5 ml 0.5 M NaCl. Efficiency of extraction was $95 \pm 9.1 \%$ ($N = 5$) and $90 \pm 4.5 \%$ ($N = 4$) for viscera and body tissue samples, respectively. Method detection sensitivities were $0.5 \mu\text{g DA g}^{-1}$ for viscera and $0.2 \mu\text{g DA g}^{-1}$ for body tissues.

Results

Toxic Pseudo-nitzschia Densities and Domoic Acid Levels in Fish Viscera

Toxic *Pseudo-nitzschia* species reached bloom conditions ($> 10^3$ cells liter⁻¹) at four distinct times in Monterey Bay over the one year sampling period (Figure 2A). Domoic acid was detected in fish viscera samples only at times when *Pseudo-nitzschia* blooms were also observed, suggesting that the two are tightly temporally coupled (Figure 2A,B). The highest toxic cell densities counted during each bloom period were 3.4×10^4 cells liter⁻¹ on December 17, 1999, 6.8×10^4 cells liter⁻¹ on March 24, 2000, 3.2×10^3 cell liter⁻¹ on June 16, 2000, and 5.0×10^5 cells liter⁻¹ on September 12, 2000 (Figure 2A). The highest DA levels detected in viscera samples during each bloom period were $23 \mu\text{g DA g}^{-1}$ on December 17, 1999, $200 \mu\text{g DA g}^{-1}$ on April 13, 2000, $4.3 \mu\text{g DA g}^{-1}$ on June 22, 2000, and $1815 \mu\text{g DA g}^{-1}$ on September 10, 2000 (Figure 2B). Maximum fish viscera DA levels detected during each bloom period were positively

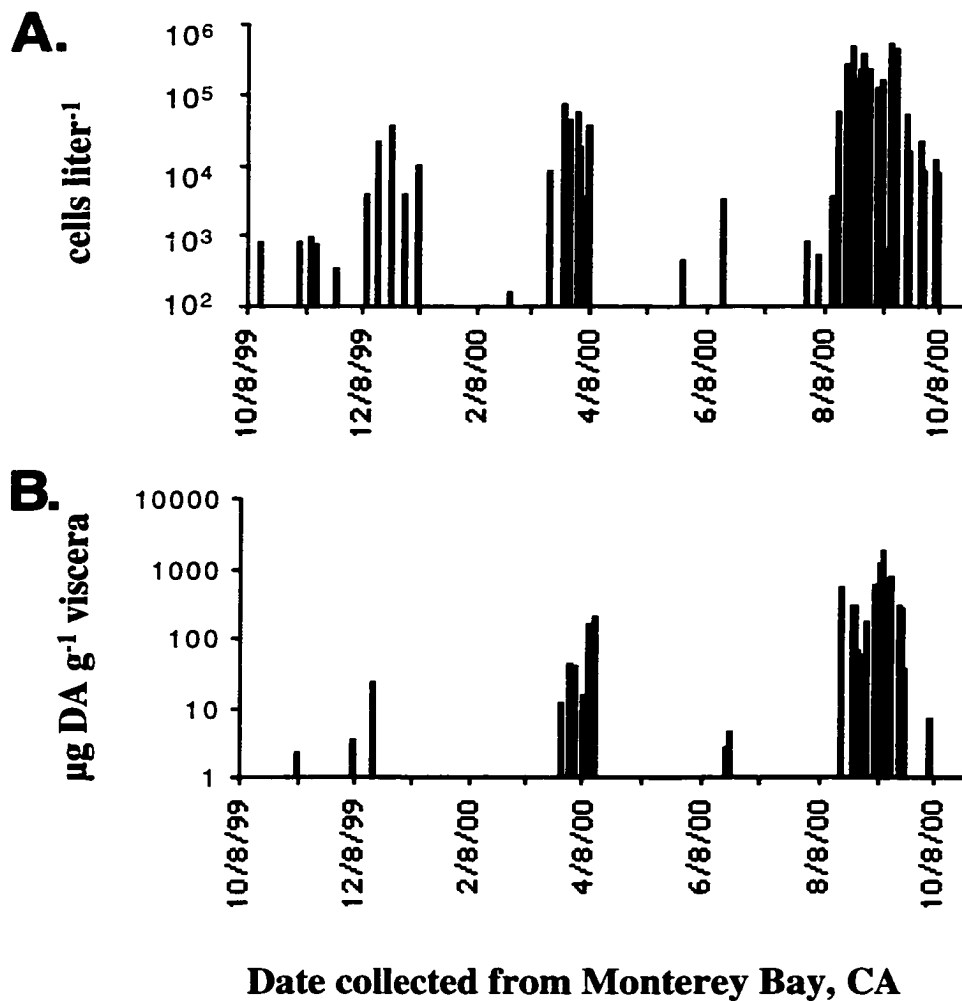


Figure 2. A) Number of toxic *Pseudo-nitzschia* cells liter⁻¹ in surface water collected from Monterey Bay, California from 10/8/99 to 10/8/00. B) Domoic acid concentrations in viscera from anchovies (*Engraulis mordax*) and sardines (*Sardinops sagax*) collected in Monterey Bay from 10/8/99 to 10/8/00.

correlated with maximum total toxic cell densities counted at corresponding bloom periods.

Mean (\pm SD) visceral masses were 9.4 ± 1.2 % and 8.9 ± 1.3 % of total fish weight for anchovies (N = 39) and sardines (N = 12), respectively. Using 9 % to estimate percent viscera for both species, the regulatory limit of $20 \mu\text{g DA g}^{-1}$ whole fish was converted to $222 \mu\text{g DA g}^{-1}$ for viscera samples. Although moderate DA levels were detected in fish viscera samples several times throughout the year, DA levels exceeding seafood safety standards ($20 \mu\text{g DA g}^{-1}$ whole fish or $222 \mu\text{g DA g}^{-1}$ viscera) were detected only during the largest bloom which occurred in August and September of 2000.

Domoic Acid Levels Detected in Anchovy and Sardine Viscera

On twelve occasions throughout this study, anchovies and sardines were collected simultaneously from the same location. A comparison of DA levels detected in anchovy and sardine viscera is shown in Table 1. When DA was detectable, anchovy viscera contained more DA than sardine viscera in eight out of nine cases (Table 1). A two-tailed binomial test confirmed that anchovies had significantly higher DA viscera values than sardines ($p < 0.05$). Viscera DA levels detected in anchovies were up to 39 times higher than those detected in sardines for the same date. *Pseudo-nitzschia* frustules were observed in five of seven anchovy and sardine stomach content samples analyzed (Table 2).

Table 1: A comparison of domoic acid levels in anchovies (*Engraulis mordax*) and sardines (*Sardinops sagax*) collected simultaneously from Monterey Bay, California. Domoic acid values are in $\mu\text{g DA g}^{-1}$ viscera wet weight. nd = not-detected. Anchovies contained significantly higher DA levels than sardines ($p < 0.05$) using a two-tailed binomial test (Zar, 1984) with paired samples in which at least one contained detectable levels of DA (N=9).

Date Collected	Anchovy Viscera ($\mu\text{g DA g}^{-1}$)	Sardine Viscera ($\mu\text{g DA g}^{-1}$)
12-7-99	3.3	nd
5-23-00	nd	nd
6-5-00	nd	1.0
6-16-00	nd	nd
6-20-00	2.5	nd
6-22-00	4.3	nd
7-9-00	nd	nd
7-20-00	23	nd
8-24-00	288	28
9-8-00	1175	279
9-10-00	1815	46
11-3-00	30	1.2

Table 2: Presence of *Pseudo-nitzschia* in stomach contents of seven anchovy (*Engraulis mordax*) and sardine (*Sardinops sagax*) samples collected simultaneously. (+) = yes present (-) = not present

Date Collected	<i>Pseudo-nitzschia</i> present in anchovy stomach contents	<i>Pseudo-nitzschia</i> present in sardine stomach contents
12-7-99	-	-
5-2-01	+	+
6-5-01	+	+
6-16-01	+	+
6-20-01	+	+
6-22-01	+	+
7-9-01	-	-

Domoic Acid Levels Detected in Fish Body Tissue

In anchovies containing viscera levels $\geq 288 \mu\text{g DA g}^{-1}$, five out of eight contained detectable levels of DA in body tissue (Table 3). In seven sets of sardine samples containing viscera levels $\geq 169 \mu\text{g DA g}^{-1}$, all contained detectable levels of DA in body tissue (Table 4). Viscera DA levels in anchovy and sardine samples were 250 to 1800 times higher than corresponding body tissue DA levels, suggesting that DA accumulation in body tissue is minimal (Tables 3, 4). All body tissue DA levels detected in fish ($N = 12$) were less than $3 \mu\text{g DA g}^{-1}$, far below the established seafood safety limit of $20 \mu\text{g DA g}^{-1}$, suggesting that DA is not accumulated to dangerous concentrations in edible fish tissue.

Discussion

Results from this year long survey reveal that planktivorous fish such as anchovies and sardines are regular and consistent carriers of DA when toxic diatom blooms are present in Monterey Bay, California. Fish viscera DA levels not only tracked cell densities of *P. australis* and *P. multiseriis*, but DA was detected in viscera every time toxic diatom populations reached densities of $\geq 10^3$ cells liter⁻¹ in surface waters. The fact that DA was only present in viscera when toxic diatom species were present and not at other times, suggests that this water soluble toxin is quickly depurated and that fish vectors present a threat to higher level predators only as long as a bloom persists. In addition to being temporally coupled, the amount of toxin detected in viscera samples was correlated with the magnitude of cell density of toxic species. Although DA was present in fish several times throughout the year, it only reached levels at or above seafood safety limits when cell densities exceeded 10^4 cells liter⁻¹,

Table 3: Domoic acid levels in viscera and body tissues from anchovies (*Engraulis mordax*) collected during toxic *Pseudo-nitzschia* blooms in Monterey Bay, California. Domoic acid values are in $\mu\text{g DA g}^{-1}$ viscera or body tissue from 2 to 8 pooled anchovies. nd = not-detected

Date Collected	Viscera ($\mu\text{g DA g}^{-1}$)	Body Tissue ($\mu\text{g DA g}^{-1}$)
4-13-00	131	nd
7-5-00	128	nd
8-19-00	507	nd
8-24-00	288	1.1
8-26-00	288	nd
8-26-00	230	nd
9-8-00	1050	1.2
9-8-00	1050	0.6
9-8-00	1175	1.1
9-10-00	1815	nd
9-10-00	1600	1.0

Table 4: Domoic acid levels in viscera and body tissues from sardines (*Sardinops sagax*) collected during a toxic *Pseudo-nitzschia* bloom in Monterey Bay, California. Domoic acid values are in $\mu\text{g DA g}^{-1}$ viscera or body tissue wet weight from 2 pooled sardines. nd = not-detected

Date Collected	Viscera ($\mu\text{g DA g}^{-1}$)	Body Tissue ($\mu\text{g DA g}^{-1}$)
9-1-00	169	0.2
9-1-00	216	0.8
9-6-00	558	0.5
9-7-00	588	0.5
9-7-00	551	2.2
9-8-00	279	0.2
9-8-00	386	0.5

suggesting that bloom densities at or below 10^4 cells liter⁻¹ may not present a threat to piscivorous sea birds and mammals.

Stomach content analyses reveal that both anchovies and sardines consume *Pseudo-nitzschia* and can therefore act as DA vectors (Table 2; McGinness et al. 1995; Lefebvre et al. 1999; Scholin et al. 2000). However, our data on viscera DA levels suggest that anchovies may be more potent vectors than sardines. When both species were collected simultaneously, anchovy viscera consistently contained more DA than sardine viscera, indicating that anchovies generally consume more toxic cells than sardines (Table 1). Maximum DA levels detected in fish over the entire study were $1815 \mu\text{g DA g}^{-1}$ in anchovy and $728 \mu\text{g DA g}^{-1}$ in sardine viscera samples. Previous work, based on stomach content analysis, reveals that both anchovies and sardines are omnivorous with the ability to feed on phytoplankton and zooplankton via filter feeding or particulate/selective feeding modes (Radovich 1952; Loukashkin 1970). The consistently higher DA levels detected in anchovies suggests that during *Pseudo-nitzschia* blooms in Monterey Bay anchovies may filter feed more exclusively on diatoms, while sardines may target zooplankton thereby accumulating *Pseudo-nitzschia* secondarily or in lower quantities as zooplankton fulfill their dietary needs.

Dangerous levels of DA ($\geq 222 \mu\text{g DA g}^{-1}$ viscera) are commonly detected in fish viscera samples, yet DA accumulation in body tissue of anchovies and sardines appears to be negligible (Tables 3, 4). Although unsafe DA levels ($\geq 20 \mu\text{g DA g}^{-1}$ body tissue) have been previously reported for anchovy body tissues (Quilliam et al. 1991; Work et al. 1993; Wekell et al. 1994; Lefebvre et al. 1999), those measurements are questionable due to possible leakage of DA from the GI tract during sample collection, storage, and processing procedures (Lefebvre et al. 2001). In the present

study, all DA levels detected in body tissue samples (N = 12) were $\leq 2.2 \mu\text{g DA g}^{-1}$, while viscera levels ranged from 169 to 1,600 $\mu\text{g DA g}^{-1}$ (Tables 3,4). This indicates that DA is not accumulated to levels that threaten human consumers of fish body tissue. However, even though DA uptake appears to be low, it may be sufficient to induce neurotoxic symptoms in the fish themselves (Lefebvre et al. 2001). If DA is not accumulated to toxic levels in edible body tissues, then fish that are gutted and cleaned may not be a threat to human consumers even when caught during high density toxic diatom blooms. In all documented cases of ASP in which fish were identified as the DA vector, entire fish were consumed by affected sea birds and marine mammals (Fritz et al. 1992; Work et al. 1993; Sierra-Beltran et al. 1997; Lefebvre et al. 1999; Scholin et al. 2000). Since intoxication occurs from consumption of whole fish, care should also be taken when utilizing small pelagic filter-feeding fish for non-human consumption. Fish such as anchovies, sardines, and herring are often used to feed captive animals including sea lions, seals, dolphins, and penguins in zoos, aquariums, and marine laboratories, and therefore could pose threats to these animals if obtained from areas with dense blooms of toxic diatoms. Furthermore, the processing of whole fish for other purposes, such as for fishmeal, could result in a product with DA levels exceeding the recommended toxin limits for safe consumption.

Since anchovies have a central position in the marine food web, contain DA levels that track *Pseudo-nitzschia* blooms, and appear to be more potent vectors than sardines, they may be the most valuable indicators of potential DA intoxication of higher level predators, including sea birds and marine mammals. However, not all toxic diatom blooms in Monterey Bay result in obvious symptoms at higher trophic levels. Our data suggest that many blooms occur throughout the year with no observed adverse affects

on wildlife. Many factors, such as bloom density, duration, per cell toxicity of *Pseudo-nitzschia*, and the presence or absence of filter-feeding fish, are likely to influence the impact a particular bloom will have on various predators. Our data suggest that the density of toxic cells is more influential than the bloom duration, since fish contained unsafe levels of DA within a few days of the onset of high toxic cell densities in August 2000. Even though fish and surface water samples were taken from different places in Monterey Bay (due to logistic restraints), a tight temporal relationship between viscera DA levels and cell densities was observed (Figure 2). Monitoring DA levels in anchovies when toxic diatoms are present in Monterey Bay will allow regulators to predict potential intoxication events involving sea birds or marine mammals, as well as provide information for warning fisheries of potential contamination in whole processed fish.

Conclusions

DA levels accumulated in fish viscera track toxic cell densities in surface waters confirming that anchovies and sardines regularly consume toxic diatoms when present in Monterey Bay. Although DA was detected in fish viscera samples several times throughout the year, fish appear to accumulate levels of DA harmful to piscivorous predators only when toxic cell densities exceed 10^4 cells liter⁻¹. It is likely that DA is quickly depurated and that fish are only dangerous vectors during a bloom period, since DA was only present in fish when toxic cells were also present in the water. In addition, anchovies appear to be more potent vectors than sardines. Both species accumulate toxic levels of DA in the GI tract, but neither appear to accumulate DA in edible body tissues to levels that are unsafe for human consumption. Although DA uptake into body tissue is low, it may be sufficient to induce neurotoxic symptoms in fish. Because

whole fish can be potent vectors of DA, attention should be paid to monitoring fish collected during toxic diatom blooms to be used for feeding of captive animals or for converting to fish meal. Finally, anchovies may be a valuable indicator species for assessing the risk of DA intoxication to higher level consumers during toxic *Pseudo-nitzschia* blooms in Monterey Bay.

CONCLUSIONS

Collectively, this research characterizes the role of anchovies in the transfer of DA through the food web, defines the sensitivity of anchovies to DA in comparison to mammals, describes the neurotoxic consequences of DA exposure via intraperitoneal injection in anchovies, and reveals the temporal relationship between DA levels in planktivorous fish and toxic *Pseudo-nitzschia* densities in surface waters. This work shows that anchovies are potent vectors of DA in marine food webs and regularly consume DA-producing diatoms when present. Chapter one describes an event in which three-tiered trophic transfer of the neurotoxin DA, from diatoms to anchovies to sea lions, was identified as the probable cause of mortality and neuroexcitotoxicity witnessed in 70 sea lions in central California during May 1998. Chapter two confirms through intracoelomic injection experiments that fish are neurologically susceptible to DA excitotoxicity and have a similar sensitivity to DA as mammals. Excitotoxic symptoms indicated CNS disturbance and included spinning, upside down swimming, spiral swimming, and inability to school. Finally, chapter three reveals that DA levels in planktivorous fish are tightly temporally coupled to toxic *Pseudo-nitzschia* densities in surface waters, anchovies contain more DA than sardines, cell densities exceeding 10^4 cells liter⁻¹ are required before fish accumulate viscera levels of DA harmful to piscivorous predators, and DA does not appear to accumulate in fish body tissues to levels that are unsafe for human consumption.

APPENDIX A:
EXAMINATION OF BRAIN SECTIONS FROM CONTROL AND DOMOIC
ACID-INTOXICATED ANCHOVIES

Introduction

Excitotoxicity as a result of DA exposure results in distinct morphological damage in the brain of affected mammals (Sutherland et al. 1990; Tryphonas et al. 1990; Strain and Tasker 1991; Dakshinamurti et al. 1993; Scholin et al. 2000). Simple staining techniques are used to identify this damage and the lesions have been well characterized in the mammalian system. However, to date there has been no histologic examination of fish brains after DA exposure. Here a preliminary examination of brain sections from control and DA-exposed anchovies is described. Although neuropathological features were not observed, this Appendix provides valuable information for future studies in which DA-induced neuropathology in fish is addressed.

Methods

Brain Preservation

Brains from control (N = 31) and DA-dosed (N = 38) anchovies used in the intracoelomic (IC) injection experiments described in chapter two were examined to determine whether DA excitotoxicity caused brain lesions in fish. DA doses in exposed fish ranged from 5 to 12 $\mu\text{g DA g}^{-1}$ total body weight and all fish exhibited “spinning” behavior. Control and exposed fish were sampled at 4, 24, and 48 hours after IP injection. For control fish N = 18, 8, and 5, and for DA-exposed fish N = 29, 5, and 3, for the 4, 24, and 48 hour time points, respectively. Brains were fixed in a phosphate buffered formalin solution within 1 minute of death. The formalin solution was prepared by mixing 100 ml of 40% formaldehyde, 4 g Sodium dihydrogen phosphate monohydrate, 6.5 g di-sodium hydrogen phosphate anhydrous, and 900 ml nanopure water. Immediately after a fish was sacrificed, the skull cap was removed to

expose the brain, formalin was added directly to the skull cavity, and the entire head was stored in a centrifuge tube containing 30 ml of formalin solution.

Brain Sectioning

Each brain was excised from the skull and trimmed into sections. Four transverse incisions were made from the spinal cord to the frontal lobe resulting in five brain sections. Incisions were made between the medulla oblongata and the cerebellum, between the cerebellum and the optic lobes, through the center of the optic lobes, and between the optic lobes and the olfactory lobes. Trimmed sections were placed in foam padded holders, clamped down, and placed in a Citadel 1000 autoprocessor (Shandon Inc., Pittsburg, PA 15275). The following sequence was run to prepare tissues for paraffin embedding; 4 hours in 70 % IMS (industrial methylated spirits (Chemix LTD. Standish, NR Wigan, UK)), 3 hours in 90 % IMS, 4 hours in 99 % IMS, 4 hours in technical grade Xylene (Chemix LTD. Standish, NR Wigan, UK), and 5 hours in wax (56 C Formula 'R'TM purified paraffin wax and select polymers (Surgipath® USA)). Sections were then placed in order (from spinal cord to frontal lobe) and proper orientation in a metal holder with hot paraffin and allowed to cool. Embedded blocks were trimmed and brain sections of 5 µm were cut using an Anglia Scientific microtome (Cambridge, England). Cut sections were placed in a hot water bath and floated onto APS treated slides. APS treatment included coating Surgipath® precleaned microscope slides with a freshly prepared 2 % solution of 3-aminopropyltriethoxysilane (Sigma) in dry acetone, rinsing twice in acetone, then twice in nanopure water, and finally allowing slides to dry overnight. Two full sets of slides were prepared, each containing all 31

control and 38 DA exposed brains. One full set was stained with Haematoxylin & Eosin (H & E) and the second set was stained with Luxol fast blue & Cresyl fast violet.

Staining Procedures and Analysis of Brain Sections

Slides were placed in a slide holder and dipped in ordered containers containing various solvents and dyes. For H & E staining the dipping sequence was 3 min Xylene, 2 min Xylene, 2 min 99 % IMS, 2 min 99 % IMS, 1 min water, 1 min Haematoxylin, 2 min running water, 20 sec differentiator (2 % Acetic acid), 1 min water, 20 sec bluing agent (2 % NaHCO₃, sodium hydrogen carbonate), 1 min water, 1 min 99 % IMS, 30 sec Eosin, 30 sec 99 % IMS, 30 sec 99 % IMS, 2 min Xylene, and finally 2 min Xylene. Luxol fast blue & Cresyl fast violet staining procedures followed those outlined in Kluver and Barrera (1953). Immediately after staining, slides were cover-slipped with Depex mounting medium (Gurr BDH Chemicals, Poole, England). To determine whether DA exposure caused morphological damage to CNS neurons in affected fish, all stained slides were examined with a compound light microscope. Each brain section was examined carefully for any type of morphologic damage unique to DA exposed brains in comparison to control brains.

Results and Discussion

Of the stained transverse sections examined, no neuropathological findings were observed. There was no evidence of edema, hemorrhage, necrosis/apoptosis, macrophage infiltration, or neuronal cell thinning. Commonly when CNS neurons are damaged, glial cells proliferate to fill in damaged areas. There was no evidence of a glial response in the anchovy brains examined here, suggesting that no significant damage had occurred. The absence of an obvious neuropathological response in anchovies may

be due to several factors such as DA dose, duration of excitotoxicity, and/or brain structure.

Exposure Dose

This study was modeled after a previous experiment in which Strain and Tasker (1991) exposed mice to DA via intraperitoneal (IP) injection at a dose of $4 \mu\text{g DA g}^{-1}$ total body weight. Obvious damage was observed in the form of vacuolation of the hippocampal neuropil (Strain and Tasker 1991). These lesions are typical of the DA-induced damage reported in other mammals such as macaques, sea lions, and humans (Sutherland et al. 1990; Scholin et al. 2000). In the present study, DA doses (5 to $12 \mu\text{g DA g}^{-1}$ total body weight) were greater than those administered in the mouse study, confirming that the doses given to fish were sufficient to induce lesions in mammalian systems.

Duration of Excitotoxicity

Although the DA exposure dose given to anchovies was sufficient to induce lesions in mice, the duration of DA exposure was significantly less in the fish study. Exposure duration ranged from 4 to 48 hours in this study and 4 to 168 hours in the mammalian study. The most significant damage, measured as the percentage of damaged cells in an area, was observed after 48 hours or longer after IP injection in mice (Strain and Tasker 1991). Mice showed approximately 4, 27, 21, 50, 62, and 82 percent damaged cells in the hippocampal region at 4, 24, 48, 72, and 168 hours, respectively (Strain and Tasker 1991). This suggests that the length of time following injection, during which excitotoxic symptoms occur, plays a significant role in lesion formation. In fact, it is the seizures themselves that contribute to cell damage via release

of additional neurotransmitters as a result of excessive excitation. In the study with mice, it took over 48 hours of excitotoxic symptoms for 50 % damage to be observed. A longer exposure time of 168 hours resulted in 82 % damage. It is likely that the shorter exposure period utilized in this study is the primary reason lesions were not present in fish. Unfortunately, longer time points were not available because fish died after 48 hours, most likely due to the higher doses used (5 to 12 $\mu\text{g DA g}^{-1}$ total body weight). In future studies, exposure doses close to 4 $\mu\text{g DA g}^{-1}$ total body weight should be used, as well as exposure durations of 48 to 168 hours.

Brain Structure

DA-induced brain lesions are well characterized in the mammalian system. In laboratory animals, sea lions, and humans, neuronal vacuolation occurs in the hippocampus, primarily in the CA 3 region and to some extent in the dentate gyrus (Sutherland et al. 1990; Tryphonas et al. 1990; Strain and Tasker 1991; Dakshinamurti et al. 1993; Scholin et al. 2000). Anchovies, like other teleosts, lack a hippocampus which makes finding potential lesion sites more difficult. In a current study with sea birds, DA-induced lesions have been identified in brain regions other than those described for mammals (Salvagni and Lowenstine, unpublished data). Since fish, like sea birds, exhibit neurotoxic symptoms indicative of CNS disturbance, it is likely that DA-induced damage does occur at some region of the fish brain. In contrast to humans and laboratory animals, there are currently no stereotaxic atlases to help facilitate a detailed examination of the neuroanatomy and neurohistology of the anchovy brain. Characterization of the neuroanatomy and neurohistology of *Engraulis* will require much future effort.

Conclusions

IP injection of DA resulted in neurotoxic symptoms characteristic of CNS excitotoxicity, yet no neuropathological signs were observed in stained brain sections from affected anchovies. Future fish studies are needed in which longer exposure durations and doses of $4 \mu\text{g DA g}^{-1}$ total body weight are utilized. However, before damaged neuronal cell bodies can be localized to a particular group of brain cell nuclei or laminae of neurons, the neuroanatomy of the anchovy brain must be characterized.

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