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SANTA CRUZ

**NOVEL INSIGHTS ON THE DYNAMICS AND CONSEQUENCES OF
HARMFUL ALGAL BLOOMS IN THE CALIFORNIA CURRENT SYSTEM:
FROM PARASITES AS BLOOM CONTROL AGENTS TO HUMAN TOXIN
EXPOSURE**

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

DOCTOR OF PHILOSOPHY

in

OCEAN SCIENCES

by

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June 2011

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ABSTRACT

NOVEL INSIGHTS ON THE DYNAMICS AND CONSEQUENCES OF HARMFUL ALGAL BLOOMS IN THE CALIFORNIA CURRENT SYSTEM: FROM PARASITES AS BLOOM CONTROL AGENTS TO HUMAN TOXIN EXPOSURE

by
Fernanda da Frota Mattos Mazzillo

This dissertation provided novel insights on the dynamics and consequences of harmful algal blooms (HABs) in the California Current System (CCS). Parasitism is described as a biological control agent of harmful dinoflagellate blooms and referred to as a novel factor influencing HAB dynamics in coastal upwelling environments. Chapter 1 documented, for the first time, the presence of *Amoebophrya*, an endoparasitic dinoflagellate that infects and kills 7 bloom-forming dinoflagellate host species that occur throughout the CCS. Chapter 1 also discussed parasitism effects on (1) host population dynamics, (2) dinoflagellate species diversity, (3) net phytoplankton community composition and (4) trophic web structure within the planktonic community of an upwelling environment. Chapter 2 specifically verified the role of such a parasite in controlling harmful dinoflagellate blooms caused by one of its hosts in Monterey Bay. Findings of Chapter 2 suggested that blooms might develop when the host ‘escapes’ parasitism by *Amoebophrya*. In contrast, epidemic parasitic outbreaks may contribute to stopping or preventing the occurrence of dinoflagellate harmful blooms. Chapter 3 and 4 focused on the consequences of HABs caused by diatoms of the genus *Pseudo-nitzschia*, which cannot be parasitized by *Amoebophrya* and produces the neurotoxin domoic acid (DA). Chapter 3 verified that human consumers of recreationally-caught fishes can be

exposed to asymptomatic doses of domoic acid. Lastly, Chapter 4 dealt with the hypothesis that the pelagic predator Humboldt squid (*Dosidicus gigas*), which recently invaded the domains of CCS, is exposed to domoic acid. However, beach strandings of this species in Southern California could not be linked to domoic acid exposure and was likely related to other unknown causes.

For my parents Tania and Sergio Mazzillo,
who inspired me and supported me to pursue my dreams.

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DISSERTATION INTRODUCTION

A glance at a net plankton sample collected in the coastal domains of California Current System may reveal the presence of a fairly diverse diatom and dinoflagellate assemblage. The fact that multiple plankton species can coexist in a relatively homogenous and unstructured environment, all competing for the same resources (light and nutrients), is referred to as the paradox of the plankton (Hutchinson 1961). Later, Slobodkin (1989) presented the idea that a phytoplankton bloom, where one species occurs in high densities and thus dominates the net phytoplankton community, might be regarded as the null case of Hutchinson's paradox of the plankton and suggested that blooms should be frequent and what was needed was to explain their absence. Indeed, a net plankton sample collected from the CCS coastal domain will often be dominated by a single (or a few) diatom or dinoflagellate species.

Some of these bloom-forming species may negatively impact the health of humans and marine fauna via toxin production. Additionally, other bloom-forming species can impact marine fauna by depleting oxygen levels in the water column, causing mechanical or physical damage (e.g. fish gill particle irritation or clogging) or through excess production of nontoxic compounds. Toxin producing species present in the California Current System include diatom species of the genus *Pseudo-nitzschia*, dinoflagellate species from the genera *Alexandrium* and *Dinophysis*, and *Lingulodinium polyedrum*. Note that *Alexandrium* or *Dinophysis* species typically do

not need to achieve high cell densities to pose a threat to the marine fauna or humans, given the high potency of their toxin. Several other species of dinoflagellate and diatoms that occur throughout the CCS may harm marine fauna by means other than toxin production (Horner et al. 1997). Additionally, some nontoxic dinoflagellate blooms may discolor surface seawater and are commonly referred to as “red tides”.

Most of the research done on the ecology and oceanography of harmful algal bloom dynamics is restricted to the study of “bottom up” processes (i.e., physico-chemical processes) that stimulate the growth and accumulation of cells whereas the “top down” control of blooms by bacterial, parasitic or viral infections has likely been underestimated (Elbrachter and Schnepf 1998). The endoparasitic dinoflagellate, *Amoebophrya* can invade and kill free-living dinoflagellate species (Cachon 1964). Thus, a more careful look at a net plankton sample from the CCS may also reveal the presence of phytoplankton parasites. Further, phytoplankton parasitism may perhaps be an explanation for the absence of blooms, as parasites are known to regulate host abundance in other environments (Chambouvet et al. 2008). Therefore, the primary goal of this thesis is to investigate the occurrence of *Amoebophrya* parasitism in bloom-forming dinoflagellate species that occur in Monterey Bay, an open embayment on the California coast influenced by upwelling dynamics of the CCS and to understand the ecological role of parasitism within the planktonic community of this system (see Chapter 1). Specifically, Chapter 2’s goal is to verify whether such a parasite is able to control dinoflagellate blooms that occur in an environment influenced by upwelling dynamics and whether parasitism can influence net

phytoplankton composition.

However, as mentioned above, such a parasite only attacks dinoflagellate species, and toxic blooms caused by diatoms are also frequent in the CCS coastal domains. The only toxin producing diatom species that occur in the CCS belongs to the genus *Pseudo-nitzschia*. Toxic blooms caused by *Pseudo-nitzschia* spp. cannot be prevented by *Amoebophrya* infections. Moreover, exposure to the *Pseudo-nitzschia* produced toxin, domoic acid, causes severe neurological symptoms and 3 human deaths have been reported for humans that consumed domoic acid-contaminated shellfish (Perl et al. 1990). Marine animals affected by this toxin may become disoriented and found stranded in large numbers after consuming DA-contaminated fish (Scholin et al. 2000). Therefore, Chapter 3 investigated whether humans can be exposed to DA by consuming recreationally caught fish (in addition to being exposed when consuming shellfish). Lastly, Chapter 4's goal was to determine if the pelagic predator, Humboldt squid (*Dosidicus gigas*), which recently invaded the domains of the CCS, might be exposed to DA. Investigation of this possibility may help us understand whether domoic acid poisoning is a contributing factor to the increasingly reported strandings of Humboldt squid along coastal domains of the CCS.

CHAPTER ONE

Parasitic infections in bloom-forming dinoflagellates from the California Current System

Abstract

Amoebophrya is an endoparasitic dinoflagellate that infects and kills free-living dinoflagellates. This study reports for the first time the presence of *Amoebophrya* infections in 6 bloom-forming dinoflagellate species, including some that are toxic and harmful in Monterey Bay, an open coastal embayment on the U.S.A. west coast that is influenced by the upwelling dynamics of the California Current System (CCS). Infections were detected with 3 different methodologies (i.e., parasite green autofluorescence, DAPI staining, and rRNA-based fluorescent in situ hybridization assays) in weekly samples collected from 3 Aug 2005 to 28 July 2010 at the Santa Cruz Wharf, and from 21 and 28 October 2008 on an inshore transect in the northeastern shelf of Monterey Bay. Confirmation of the parasite presence was obtained using transmission electron microscopy. Unique parasite morphological structures and the effects of infection in host cell morphology are hypothesized to be adaptations that allow the parasite to produce hundreds of infective dinospores. The presence of *Amoebophrya* infections in bloom-forming dinoflagellate species that occur in Monterey Bay, and likely the broader CCS, and its ability to kill the host while producing hundreds of new infective dinospores suggest that this parasite may

prevent the occurrence and/or facilitate the end of toxic dinoflagellate blooms and “red tides” that occur in pelagic ecosystems influenced by the CCS. Additionally, *Amoebophrya* may also influence dinoflagellate species diversity, contribute in the ascendancy of diatoms versus dinoflagellates and promote temporary changes in trophic web structure of pelagic ecosystems during epidemic parasitic outbreaks.

Introduction

Amoebophrya is an intracellular parasitic dinoflagellate. Species within *Amoebophrya* can parasitize a variety of marine planktonic organisms including acantharians, radiolarians, ciliates, tintinnids, and other dinoflagellates (Cachon and Cachon 1987). *A. ceratii* has been documented in at least 40 dinoflagellate species, including toxic and harmful dinoflagellate species (Park et al. 2004). However, studies using 18S rRNA (ribosomal ribonucleic acid) gene sequences of *Amoebophrya* infecting some of these dinoflagellate species support the hypothesis that *Amoebophrya ceratii* is actually a complex of several species (Coats et al. 1996, Gunderson et al. 2002).

Epidemic outbreaks of *Amoebophrya* can influence dynamics of dinoflagellate populations and help prevent or end dinoflagellate blooms in estuarine systems (Nishitani et al. 1985, Chambouvet et al. 2008). *Amoebophrya*'s ability to influence dynamics of dinoflagellate population is probably a result of its life cycle, which is divided into a free-living (sporont) phase and an intracellular parasitic (trophont) phase (Cachon and Cachon 1987). The free-living dinospore, a bi-flagellate, tear-

shaped cell, is the starting point of the *Amoebophrya* life cycle. Once a suitable dinoflagellate host is found, the dinospore uses its pointed end to perforate the host membrane and to squeeze itself into the host cytoplasm (Cachon 1964). Once inside the host, the dinospore is referred to as the trophont. The trophont becomes multinucleated and each parasite nucleus corresponds to a future infective dinospore. Once the dinospores are mature, the parasite leaves the host as a multicellular worm-like structure, named the vermiform. The vermiform consists of hundreds of dinospores, which rapidly separate themselves into new infective, free-living, single-cell dinospores (Cachon and Cachon 1987). Therefore, one infected host produces 60 - 400 new infective dinospores in ~1-2 days whereas free-living dinoflagellates typically have a mean growth rate of 0.6 doublings per day (Tang 1996, Coats and Bockstahler 1994, Chambouvet et al. 2008).

Although it has been suggested that *Amoebophrya* is likely to have a worldwide distribution (Park et al. 2004) and has an important role in regulation of dinoflagellate blooms, this genus has not been observed in coastal ecosystems associated with the California Current System (CCS). The CCS extends approximately from Vancouver Island, Canada to Baja California, Mexico and it is formed by the California Current, a major eastern boundary current, the California Undercurrent, the Davidson Current and the Southern California Counter Current (Hickey 1978, Strud et al. 2002). The coastal domains influenced by the CCS are subject to coastal upwelling dynamics and dinoflagellate blooms are frequent and intense in these locations (Curtis et al. 2008; Jester et al. 2009a). However, the

present state of knowledge about dinoflagellate bloom dynamics in coastal locations influenced by the CCS has focused mostly on to physical and chemical drivers of dinoflagellates blooms (Bolin and Abbott 1963; Ryan et al. 2008; Kudela et al. 2010).

The goal of this study was to determine whether *Amoebophrya* can infect dinoflagellates in the CCS within the U.S.A. coastal zone, namely in Monterey Bay and thereby potentially control their populations. Monterey Bay is an open embayment located on the central coast of California and its oceanography is highly influenced by the upwelling dynamics of the CCS. Another objective was to understand and discuss the ecological role of parasitism in shaping population dynamics of these bloom-forming dinoflagellates, as well as its possible role in driving dinoflagellate species diversity and the implications for the net phytoplankton community structure of this productive, seasonal pelagic environment.

Material and Methods

Water samples

Surface seawater samples were collected at the Santa Cruz Wharf (SCW) and on a 4 station transect along the 12m isobath at the northeastern shelf of Monterey Bay (36.95 N, 122.02 W) (Fig. 1). Samples from the SCW were collected weekly from 3 Aug 2005 to 28 Jul 2010 by net tow (20 μ m) and surface bucket. Aliquots of 100 mL from bucket samples and 20 mL of net material were preserved in 4% formaldehyde final concentration and stored in the dark at 4°C. Surface bucket samples from the northeastern Bay shelf were collected on 21 and 29 Oct 2008 and

aliquots of 100 mL were preserved in 1% glutaraldehyde stored in the dark at 4°C. Aliquots of 200 ml from bucket samples collected on 21 and 29 Oct 2008 and from 19 Oct 2009 to 28 July 2010 at all stations were also preserved in saline EtOH. Saline EtOH fixative was prepared by adding 165 mL of dH₂O, 100 mL 25X SET buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris HCl, pH 7.8) and 735 mL of 95% ethanol (Gold Shield Chemical Co., Hayward, California, U.S.A.) to a 1L polypropylene bottle and stored at room temperature.

Phytoplankton species composition

Phytoplankton species composition was determined for all samples from 3 Aug 2005 to 28 Jul 2010. Live net tow material was examined in the laboratory using a dissecting scope (Olympus SZH Stereozoom) and magnified 64X. The net phytoplankton (>20µm) community was characterized by identifying taxa to genus or species level and estimating contribution of each taxon to the total relative abundance of the sample. Relative abundance categories used were: “dominant” (taxa represented >45% of the total net phytoplankton community), “common” (taxa contributed <45% but >10%) and “present” (taxa contributed <10% of the total net phytoplankton community).

Amoebophrya detection methods.

Amoebophrya infections were detected using the following: (1) parasite green autofluorescence, (2) rRNA-based, fluorescent in situ hybridization (FISH) probes (OligosEtc Inc., Wilsonville, OR, USA) or (3) DAPI (Pierce Biotechnology Inc., Rockford, IL, USA). Parasite green autofluorescence was used to monitor for the

presence of infected host cells or the vermiform stage of *Amoebophrya* in live net tow samples from 19 Oct 2009 to 28 July 2010. Observations of parasite autofluorescence were made on an Olympus IX70 inverted epifluorescent microscope fitted with a chlorophyll (wavelength excitation (λ_{ex}) 440-470nm, wavelength emission (λ_{em}) >515nm) bandpass filter set and a 50W light source. Photographs were taken using a Nikon E995 digital camera. FISH probes were used for samples from this same period (19 Oct 2009 to 28 June 2010) from the SCW and also for samples collected in Oct 2008 from the 4-station transect. A probe specific to *Amoebophrya* strains (ALV01) (Chambouvet et. al 2008) was tested in *Amoebophrya* strains from Monterey Bay. Such a probe was also used because parasite green autofluorescence may only be detected during late stages of the parasite development within the host (Coats and Bockstaheler 1994) thus the probe allowed for detection of early and late infection stages of the parasite (see probe hybridization method below). DAPI was the most appropriate method to quickly screen for infections in species that occurred in archived weekly samples from 3 Aug 2005 to 12 Dec 2007 in addition to those present in Monterey Bay from 19 Oct 2009 to 28 June 2010. DAPI was also used to provide an approximate number of parasite nuclei during the late trophont phase. Subsamples of 25-75mL from 45 formaldehyde preserved samples were filtered on 5 μ m polycarbonate black filters and DAPI (final concentration of 500mg mL⁻¹) was added at 40 μ l.

Probe hybridization

Probe hybridizations were done with fluorescein-tagged rRNA-based oligonucleotide UniC (5'- GWA TTA CCG CGG CKG CTG – 3') and *Amoebophrya* general (ALV01) (5' – GCC TGC CGT GAA CAC TCT – 3') probes. UniC probes were used for nuclear infections. UniC probes target a universally conserved sequence of the SSU rRNA (519r, Field et al. 1988, Embley et al. 1992 *in* Miller & Scholin, 1996). This rRNA sequence is found in the cytoplasm and in the nucleolus (where rRNA is transcribed) of eukaryotic cells. Thus, the nucleus of a cell without *Amoebophrya* nuclear infection appears dark when probed with UniC, with the exception of a few spots in the nucleolus (Fig. 2A,C) whereas cells with *Amoebophrya* nuclear infection have source of rRNA in the nucleus (i.e., the parasite rRNA) thus the UniC probes accumulate in the nucleus of the host. As a result, a strong UniC signal is observed as a “glowing” nucleus in infected cells (Fig. 2B, D). The ALV01 general probe can detect nuclear and cytoplasmatic infections. Aliquots of 50mL of bucket samples were used for both FISH probes assays following Miller and Scholin (1996, 1998), except hybridization was carried out for 7-16hrs at 45°C when using ALV01 probe. After hybridization, filters were mounted on microscope slides. Observations were carried out on a Zeiss Axio Imager fitted with a fluorescein (λ_{ex} 460-500nm, λ_{em} 510- 560nm) and 2 DAPI (λ_{ex} 350nm, λ_{em} > 420nm and (λ_{ex} 350nm, λ_{em} > 460nm) bandpass filter sets and a 50W light source. Photographs were taken using an Axio Cam HRC camera system.

TEM analysis

Transmission electron micrographs were obtained on material in samples collected at the 4-station transect located on the northeastern shelf of Monterey Bay (Fig. 1) and preserved in 1% glutaraldehyde. Samples from all 4 stations were combined and centrifuged for 10min at 4000rpm and sent to the electron microscopy facility at Beckmam Research Institute, Duarte, California for subsequent preparation. At the facility, post-fixation was carried out with 1% OsO₄ in 0.1M Cacodylate buffer for 30 min and specimens then were washed 3x with the buffer. Cells were dehydrated through 60%, 70%, 80%, 95% EtOH, 100% EtOH and propylene oxide (PO) twice, and left in PO/Eponate (1:1) overnight, sealed and at room temperature. Eppendorf tubes opened for 2-3 hrs for the PO to evaporate. At the end of the day the material was transferred to capsules with 100% fresh Eponate and the capsules were polymerized at ~64°C for 2 day. Ultrathin ~65 nm sections were cut using a Leica Ultra cut UCT ultra microtome with a diamond knife and placed on 300 mesh Copper grids. Grids were stained with 5% uranyl acetate for 15min, followed by 2min staining with Reinhold's lead citrate. The grids were examined using a JEOL 1200EX transmission electron microscope (TEM) equipped with CCD camera running at 80 KeV.

Results

Species parasitized by Amoebophrya

Amoebophrya was detected in 6 dinoflagellate species from Monterey Bay: *Akashiwo sanguinea*, *Alexandrium* sp., *Dinophysis* sp., *Neoceratium furca*, *Neoceratium fusus* and *Prorocentrum micans* (*Neoceratium* is the former marine *Ceratium* Gómez et al . 2010). Infections were either exclusively detected in one host or simultaneously detected in 2 or 3 different host species in a given sample (Fig. 3). The infected species represented 23% of the Monterey Bay typical dinoflagellate assemblage and included thecate and athecate dinoflagellate species (Table 1).

Parasitized species *Dinophysis* sp., *Neoceratium furca* and *Prorocentrum micans* were among those that were most frequently observed during the period of 19 Oct 2009 to 28 July 2010 when infections were weekly monitored. However, other species were also frequently present (*Dinophysis fortii*, *Preperidinium* sp., *Protoperidinium* spp. and *P. stenii*) or dominated (*Neoceratium* cf. *divaricatum*) the net phytoplankton community during this period, but were not parasitized (Table 1).

Further, from 19 Oct 2009 to 28 July 2010, parasitism occurred when the host species was present, common or a dominant taxon of the net phytoplankton, but infections were more frequently detected when the hosts were common or dominated the net phytoplankton assemblage (Table 2). Additionally, observations of archived samples from 3 Aug 2005 to 12 Dec 2007 revealed that *Alexandrium* sp. and *Neoceratium fusus* were observed with infections when they contributed to <10% of the total net phytoplankton community (25 Oct 2006 and 16 Nov 2005, respectively).

Light microscopy observations

Figure 4 shows infections detected with parasite autofluorescence (A, B, C), DAPI (D, E, F, G, H) and the *Amoebophrya* general probe (ALV01) (I, J, K). All *Amoebophrya* infections were within the host cell membrane and possibly within the host nucleus. Enlargement of the host due to infection was most evident on the chain of *Alexandrium* where uninfected cells were also present (Fig. 4B and E), and enlargement of the host is typical in all infected species. The numerous parasite nuclei, estimated to range between 50 -100 among infected species, are shown in Figs. 4F and G and appear as small round structures along the outer and inner surfaces of the mastigocoel. The mastigocoel is a cavity that has its inner and outer surfaces raised in coiled ridge-like structures and resembles a beehive shape when observed with phase contrast (Taylor, 1968). The adapted sketch from Cachon (1964) shows parasite nuclei arranged between the ridges and along the inner and outer surfaces of the mastigocoel (Fig. 5). The beehive shape of the mastigocoel is visible in *Amoebophrya ex P. micans* (the term “ex” is a standard parasitological notation for “from” or “out of”) (Fig. 6). The spiral or coil shape of the ridges is observed in Fig. 4J

A few dinospores were observed in live net samples, where trophonts of *Amoebophrya ex P. micans* were exclusively detected. These dinospores were oval-shaped and autofluoresced green when observed using UV light and chlorophyll bandpass filter set. They swam in a spiral pattern and the swimming speed appeared faster relative to other dinoflagellate species in the samples. Only one free-living

vermiform was briefly observed in a field sample, and it also autofluoresced green. The vermiform life-stage rapidly separated into swarmers and subsequently, individual dinospores. Fig 7 shows the last stages of vermiform fragmentation, the swarmer splitting into individual free-living dinospores.

Transmission electron microscopy observations

Figures 8A, B and C show uninfected and infected specimens of *A. sanguinea* from Monterey Bay samples. On Fig. 8B, the host nucleus is enlarged and mostly occupied by the trophont (t), as opposed to the smaller nucleus of the uninfected cell (Fig. 8A), where the typical dinoflagellate condensed chromosomes (ch) occupied the entire nucleus and are evident as circular, elongated or oval structures. The trophont observed in Fig 8B was likely in mid development stage and was multinucleate. The denser areas indicated by arrowheads may correspond to the trophont's heterochromatin (Fig. 8B). The less dense fibrous appearing matrix (f) that surrounds the heterochromatin region could represent the trophont's nucleoplasm (Fig. 8B). (W. Coats pers. comm.). A spiral structure (s) was observed among the remains of the host chromosomes (ch) (Fig. 8B). The structure could represent the degradation of the host nucleus as the parasite grows (W. Coats pers. comm.). Fig. 8C shows a more advanced stage of infection and this particular TEM section could correspond to the section indicated by the dashed line in Fig. 5 adapted from Cachon (1964). The trophont in Fig. 8C occupied the entire host nucleus and the large structure in the center of the trophont could represent a developing mastigocoel cavity (m) where each parasite nucleus is attached between the mastigocoel ridges (arrows).

Discussion

The goal of the present study was to determine whether the marine parasite *Amoebophrya* was present in dinoflagellates that occur in Monterey Bay, a well studied region of the California Current System (CCS) and a major upwelling environment where dinoflagellate blooms are frequent. In particular, the study goal was to gain perspective on whether the parasites, if present, might act as agents to control or limit dinoflagellate blooms, as some of the blooms can have significant ecological consequences. Infections were detected in 6 dinoflagellate species, representing 23% of the typical dinoflagellate assemblage of Monterey Bay. In addition, *Amoebophrya* also has been detected in *Lingulodinium polyedrum* from samples collected on 15 Mar 2010 at the Scripps Pier in La Jolla Bay, California (F. Mazzillo pers. obs.). Horner et al. (1997) listed 10 dinoflagellate genera that can be constituents of “red tide” populations or toxin producers in coastal regions influenced by the CCS. According to observations during this study, *Amoebophrya* can infect 60% of these genera (including *Cochlodinium*, which was not listed in Horner et al. 1997). Among the recorded infected species, *L. polyedrum* and species within the genera *Alexandrium* and *Dinophysis* are able to produce toxins that can be transferred in the food web and thereby negatively impact marine ecosystems and human health when sufficiently abundant (Howard et al. 2008; Sutherland 2008; Jester et al. 2009b,c). *A. sanguinea*, *N. furca*, *N. fusus*, *L. polyedrum* and *P. micans* are “red tide” formers and harm marine fauna by oxygen depletion or production of harmful compounds (Horner et al. 1997, Jessup et al. 2009).

Except for previous observations of *Amoebophrya* ex *A. sanguinea* (the term “ex” is a standard parasitological notation for “from” or “out of”) in Monterey Bay (C. Scholin pers. comm.), this is the first report of *Amoebophrya* in the U.S.A. coastal domains of the CCS. Other published studies of *Amoebophrya* in the North Pacific Ocean include the work of Taylor (1968), Nishitani et al. (1985), who observed infections in *A. catenella*, *A. sanguinea*, *Scrippsiella trochoidea* at Sequim Bay and Puget Sound, Washington, and the more recent work of Lizarraga and Beltrones (2003) who reported *Amoebophrya* ex *Neoceratium furca* in Baja California, Mexico.

Amoebophrya has largely gone unnoticed in dinoflagellates from the CCS probably because early stages of infection of *Amoebophrya* are difficult to detect with brightfield microscopy and because the features of the mature trophont (i.e., beehive shape) can only be recognized at high magnification (Coats and Bockstahler 1994). However, infections were easily detected in live net tow material by examining the parasite’s green autofluorescence on an epifluorescence microscope using a chlorophyll bandpass filter set (Fig. 4A and C). Parasite green autofluorescence was detectable in 4% formaldehyde samples stored at 4°C, but the signal was not as strong as from live samples (Fig. 4B). Alternatively, if immediate analysis of live samples is not feasible, parasites can be detected by DAPI staining or FISH probes, which target specific RNA sequences of the parasite or can “highlight” excessive RNA in the host species’ nucleus. DAPI staining allows visualization of the mature trophont only whereas FISH probes are more effective as they allow detection of the early infective stages as well as the mature trophont.

Parasitism influence in host population dynamics

Amoebophrya parasitism may represent a previously undescribed mechanism of top down control of dinoflagellate populations in the CCS. Observations of DAPI stained cells indicated the presence of at most 100 parasite nuclei in an infected host. Such a high number of offspring suggests that *Amoebophrya* likely impact the host's population, given that the host grows at approximately the same rate as the parasite, but generates only 2 daughter cells with each cellular division (Coats and Bockstahler 1994, Tang 1996). Accordingly, mathematical models have suggested that *Amoebophrya* has the potential to eliminate dinoflagellate blooms and that such a parasite may have a greater impact on dinoflagellate populations than grazing microzooplankton (Montagnes et al. 2008). Moreover, *Amoebophrya*'s control of dinoflagellate populations has been documented in estuarine environments (Nishitani et al. 1985, Chambouvet et al. 2008) and is presently being investigated in upwelling systems (Chapter 2).

Indeed *Amoebophrya* adaptations to produce large numbers of offspring when invading each host cell were observed with light and electron microscopy in the present study. These include (1) the enlargement of the host cell (Fig. 4B and E), which suggests the parasite ability to take full advantage of the host's intracellular space in order to produce the largest number of dinospores possible and (2) the development of a ridged mastigocoel cavity by the parasite. The ridges of the mastigocoel cavity may increase the parasite's internal space for dinospores within the host, thus providing enough physical space for a large number of parasite

dinospores. Fig. 8C shows parasite nuclei, which will develop into infective dinospores, inserted between the ridges of the mastigocoel. These are unique features of *Amoebophrya*, which makes them morphologically dissimilar from a free-living dinoflagellate during the trophont phase, but may enable *Amoebophrya* to thrive as a marine parasite.

Amoebophrya genetic diversity and host specificity may contribute to dinoflagellate species diversity

Whether or not the 6 dinoflagellate host species observed here were infected by different strains of *Amoebophrya* is unknown. The occurrence of different *Amoebophrya* strains infecting specific dinoflagellates species within the same environment has been documented in other environments (Chambouvet et al. 2008). However, strains of *Amoebophrya* may have varying degrees of host specificity, ranging from extremely species-specific, moderately specific, to rather nonspecific (Kim 2006). The presence of a host specific strain in the CCS may be indicated in cases when infections were observed exclusively in one host species (e.g. *A. sanguinea* or *P. micans*) but other potential dinoflagellate host species were present in the same sample in high relative abundances. Indeed, a high degree of host specificity has been observed for *Amoebophrya* ex *A. sanguinea* from Chesapeake Bay (Coats et al. 1996, Coats and Park 2002), and *Amoebophrya* strain isolates from athecate hosts, in general, are considered to have higher specificity levels than do thecate hosts (Kim 2006). However, high host specificity of *Amoebophrya* strains isolated from athecate host does not seem to be the case in Monterey Bay because *P. micans*, which

is a thecate dinoflagellate, seemed to be infected by a specific strain of *Amoebophrya*. Additionally, *Amoebophrya* ex *P. micans* preliminary cross-infection experiments with cultures of *A. catenella* isolated from Monterey Bay were unsuccessful, further indicating some degree of host specificity for *Amoebophrya* ex *P. micans* from Monterey Bay (F. Mazzillo pers. obs.).

The presence of *Amoebophrya* strains with low host specificity was indicated by the fact that infections were sometimes detected in the same sample in 2 or more hosts (Fig. 3). Indeed, low host specificity, i.e., the ability to infect 3 to 5 dinoflagellate genera, has been reported for isolates of *Amoebophrya* ex *Alexandrium tamarense* and *Amoebophrya* ex *Gonyaulax polygramma* (thecate hosts) during laboratory experiments (Sengco et al. 2003, Kim 2006). Additionally, isolates from *Amoebophrya* ex *Alexandrium affine* successfully infected other species within the genus *Alexandrium* (Kim 2006).

Different *Amoebophrya* strains, ones with low or high host specificity, therefore may be infecting a variety of dinoflagellate species in the CCS. The impact of parasites with low or high host specificity has been discussed for terrestrial plant species and it is believed that host specific parasites may increase species diversity in a community (Mills and Bever 1998; Packer and Clay 2000; Hudson et al. 2005). Perhaps such a concept may be extended to dinoflagellate assemblages from coastal pelagic systems where parasitism is present. Evidences from previous studies indicated that while host specific strains of *Amoebophrya* keep a specific host population in low density levels, other dinoflagellate species that are immune to the

dinospores being produced via the infected hosts can grow (Chambouvet et al. 2008; Chapter 2) and therefore increasing dinoflagellate species diversity within the planktonic community. In contrast, *Amoebophrya* strains with low host specificity may have the opposite effect, as they would be able to infect several dinoflagellate species keeping their population at low levels, and thus decreasing dinoflagellate species diversity.

Verifying *Amoebophrya* genetic diversity and host specificity for dinoflagellate species from the CCS is also important to determine the viability of using *Amoebophrya* as a natural control agent of “red tides” or toxic dinoflagellate blooms (Taylor 1968). Accordingly, Slobodkin (1989) argued that a highly specific infectious disease could be used as a “magic bullet” to help control algal blooms. *Amoebophrya* could potentially be such a “magic bullet” to end dinoflagellate blooms in the CCS, if host specific strains are present. Studies on *Amoebophrya* species diversity and host specificity, however, are still missing for dinoflagellate strains from the CCS.

Parasitism role in shaping net phytoplankton community structure

Parasitism, as a top down control mechanism, may occur more often in dinoflagellates than in diatoms, the other frequently dominant net phytoplankton bloom taxon in the CCS, and thus may help shape the overall composition of the biomass dominant members of phytoplankton from coastal upwelling systems. More than 50 dinoflagellate species are known to be parasitized by *Amoebophrya* (Park et al. 2004; Siano et al. 2011). Additionally, the parasitic dinoflagellates *Duboscquella*

and *Coccoldinium* and the recently described species of the parasitic perkinsozoan flagellate *Parvilucifera* can infect ~19 free-living dinoflagellates (Park et al. 2004; Figueroa et al. 2008). Conversely, fewer diatoms species (~17 marine species) have been documented with parasitic infections (Jonhson 1966; Schweikert and Schnepf 1997; Hanic et al. 2009). Unlike dinoflagellates, diatoms may be protected against parasitic infections by their hydrated glass ($\text{SiO}_2 \cdot \text{H}_2\text{O}$) frustule (Drum and Gordon 2003). The surface of the frustule possibly provides protection against parasitic invasions, as diatom endoparasites penetrate specific sites within the frustule (e.g. rimoportulae, strutted processes, or in gaps between the girdle bands) using specialized adaptations such as the pseudopodium, a tube feeding structure developed specifically by these parasites to penetrate the frustule and feed on diatom cellular material (Schnepf et al. 1990; Debres et al. 1996; Schnepf and Kuhn 2000;). Thus, since dinoflagellates seem to be more vulnerable to parasitism than diatoms in the marine environment, it is possible that in addition to environmental factors such as water column stability and nutrient levels (Margalef 1978), *Amoebophrya* parasitism in dinoflagellate species may also contribute to the ascendancy of diatoms in coastal upwelling regions.

Temporal changes in trophic web structure due to parasitism

Amoebophrya parasitism may induce a temporary change in trophic web structure of coastal upwelling systems. For example, during a parasitic epidemic outbreak, the host biomass is converted to dinospore biomass, a shift from large food particles (host cells measuring $>20\mu\text{m}$) to smaller food particles (dinospores

measuring $\sim 8\mu\text{m}$) (Tillman et al. 1999). Accordingly, Chambouvet et al. (2008) detected high dinospore abundances after epidemic outbreaks of *Amoebophrya*. This switch to higher nanoplankton (i.e., cells $< 20\mu\text{m}$) biomass may impact higher trophic levels that selectively prey on either large (netplankton, cells between $20\mu\text{m}$ and $200\mu\text{m}$) or smaller cells (nanoplankton). Consequently, a change from a system where energy flows from netplankton to multicellular grazers (i.e., copepods, fish larvae, anchovies) to a system where energy flows from nanoplankton to unicellular grazers (i.e., ciliates) (Johanson and Coats 2002) is likely to occur as a result of dinoflagellate parasitism by *Amoebophrya*. However, the duration of that shift would likely be short since dinospores only survive from hours to days without the host (Park et al. 2004).

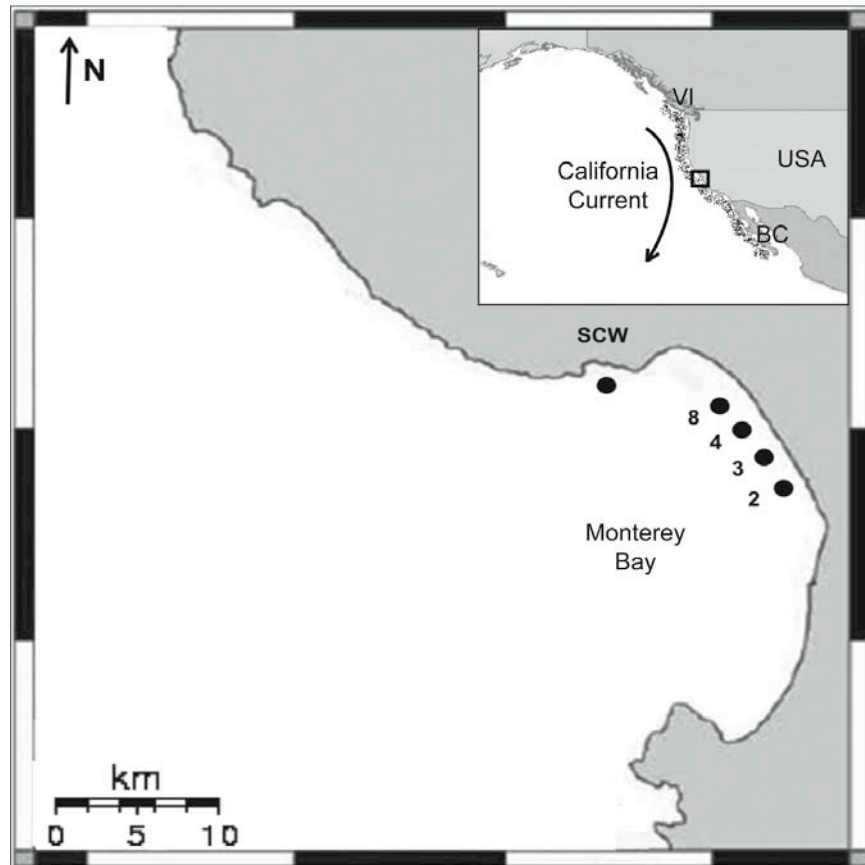


Fig. 1. Map of Monterey Bay showing location of sampling stations: the Santa Cruz Wharf (SCW) and the 4-station transect over the 12m isobath. Inset show location of Monterey Bay (box) within the coastal upwelling domain (dotted area from Vancouver Island (VI), Canada to Baja California (BC), Mexico) of the California Current System.

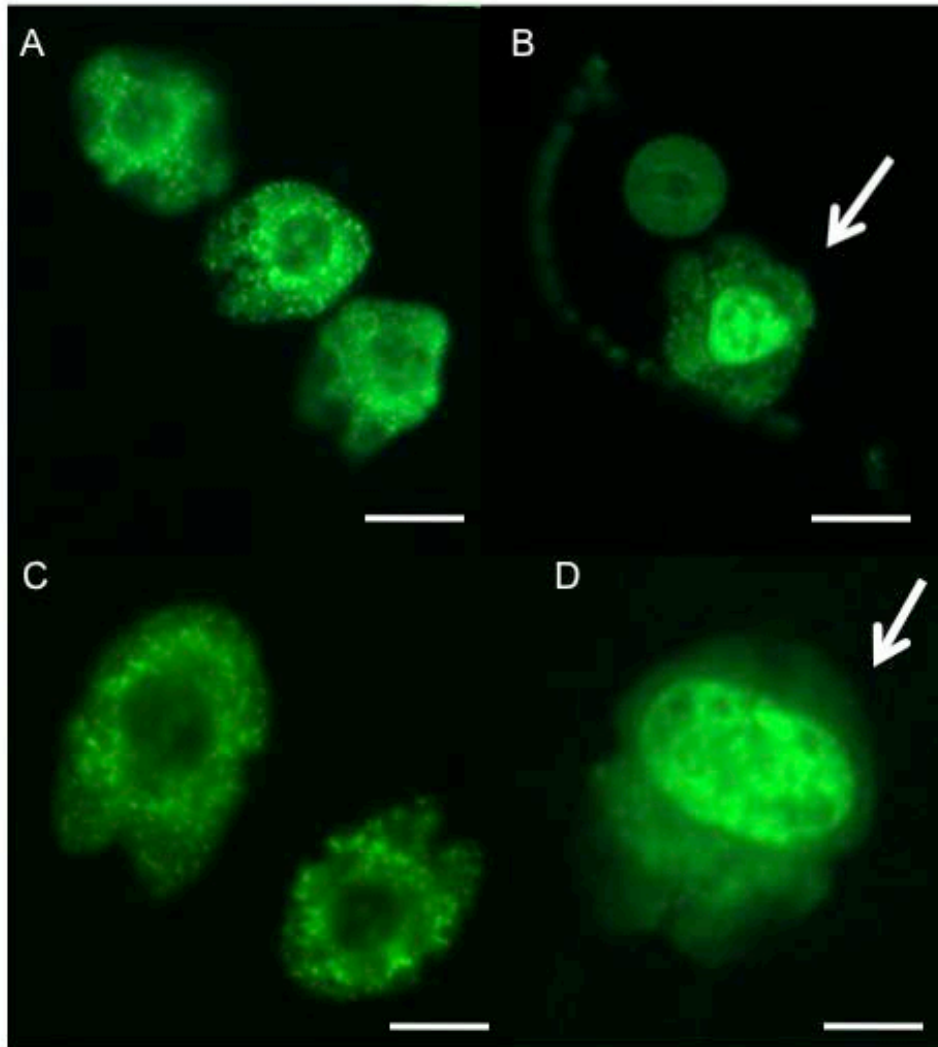


Fig. 2. *A. sanguinea* cells probed with UniC. (A) Uninfected *A. sanguinea* cells and (B) infected cells (white arrows) from Monterey Bay field samples. (C) Cultured uninfected *A. sanguinea* isolated from Monterey Bay, (D) Cultured *A. sanguinea* isolated from Chesapeake Bay infected with *Amoebophrya*. Scale bars 20 μ m.

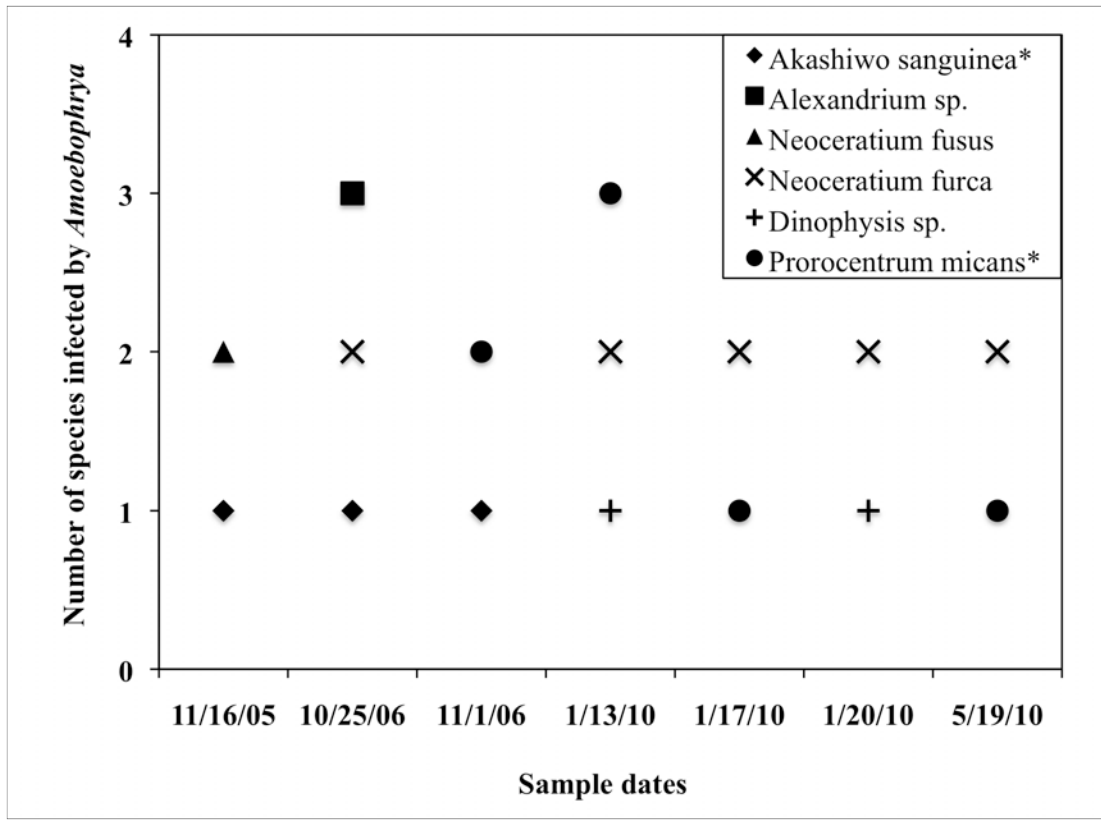


Fig. 3. Dates when *Amoebophrya* was simultaneously detected in more than one host species. Species marked with a star (*) were sometimes detected exclusively with *Amoebophrya* when other potential hosts were rare or dominated the net phytoplankton community. Samples collected in 2010 were analyzed with *Amoebophrya* specific probe, ALV01, which allows recognition of otherwise undetectable, early-stage infection of *Amoebophrya*.

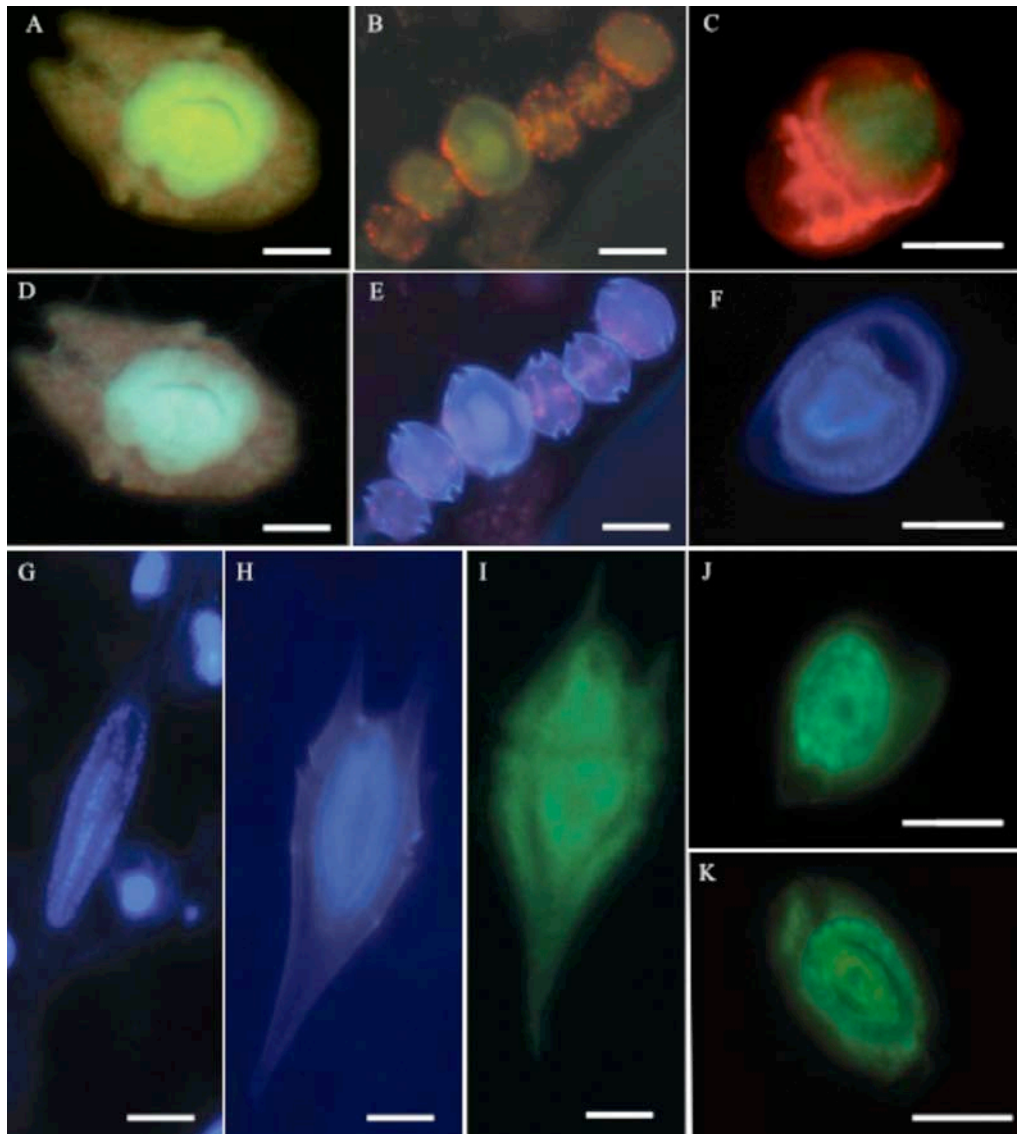


Fig. 4. *Amoebophrya* infections in 6 dinoflagellate species from Monterey Bay detected with different methods. (A), (B), (C) Parasite green autofluorescence and host chlorophyll in red for (A) *Amoebophrya* ex. *A. sanguinea*, (B) ex. *Alexandrium* sp., (C) ex. *P. micans*. (D), (E), (F), (G), (H) DAPI staining of (D) *Amoebophrya* ex. *A. sanguinea*, (E) ex. *A. catenella*, (F) ex. *P. micans*, (G) ex *N. fusus*, (H) ex *N. furca*. (I), (J), (K): ALV01 probe, which detects multiple *Amoebophrya* strains in (I) *Amoebophrya* ex *N. furca*, (J) *Amoebophrya* ex *P. micans*, (K) *Amoebophrya* ex *Dinophysis* sp.. Scale bars 20μm

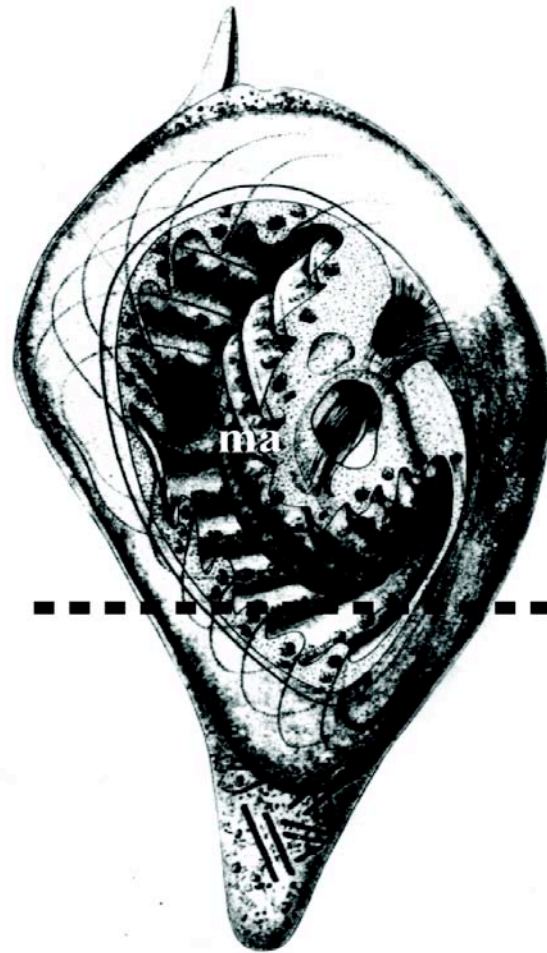


Fig. 5. Sketch adapted from Cachon (1964) showing developing trophont in *Amoebophrya* ex. *Prorocentrum micans*. Parasite nuclei are arranged between the several ridges of the mastigocoel. Dashed line indicates possible TEM section seen in Fig. 8C cutting through the mastigocoel cavity (ma).

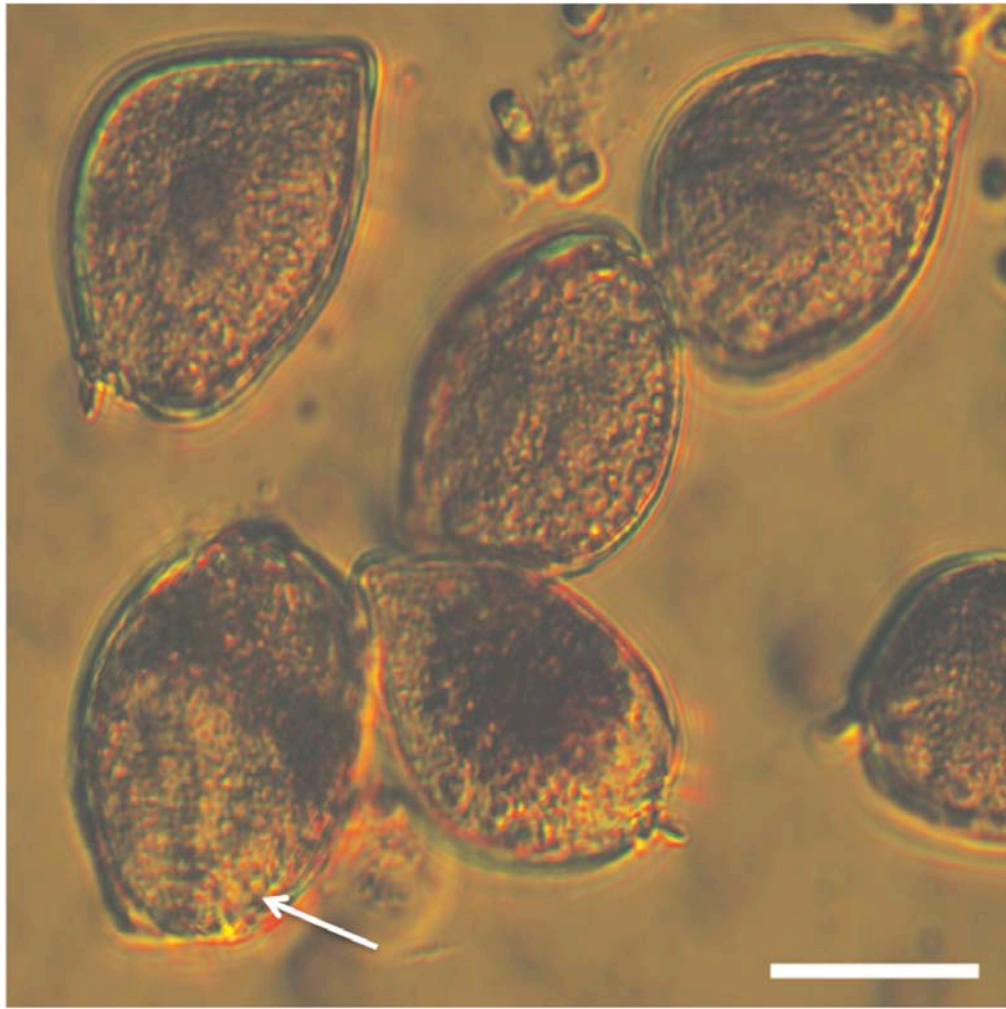


Fig. 6. Beehive shape of the mastigocoel of *Amoebophrya* trophont ex *Prorocentrum micans* (arrow). Scale bar 20 μ m.

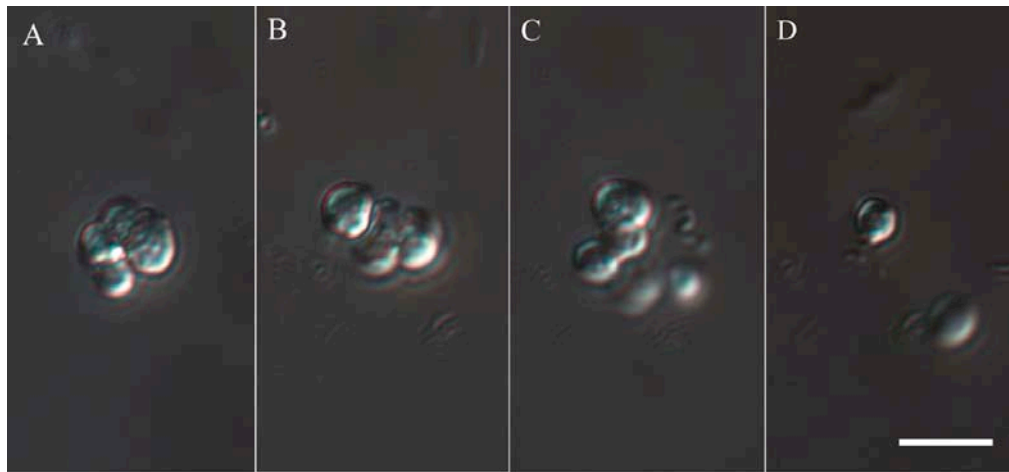


Fig. 7. (A): Swarmer, one of the very last stages of vermiform fragmentation. (B) and (C): individual free-living dinospore separation from swarmer. (D) individual dinospore. Scale bar 10 μ m.

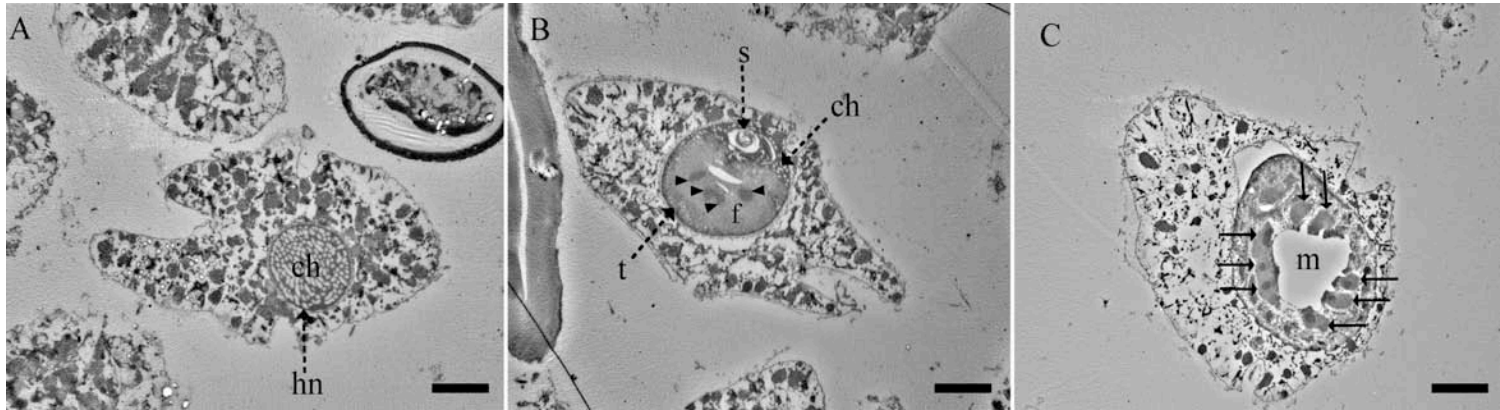


Fig. 8. (A). Uninfected specimen of *A. sanguinea* with typical dinoflagellate chromosomes (ch) occupying the entire host nucleus (hn). (B) Nuclear infection of *Amoebophrya* ex. *A. sanguinea*. Multinucleated trophont (t) in mid development stage occupied most of the host nucleus. Dense areas indicated by arrowheads correspond to heterochromatin and less dense areas fibrous area (f) correspond to the nucleoplasm. Spiral (s) may represent digestion of the remaining of host chromosomes (ch) by the parasite. (C) A more advanced *Amoebophrya* ex. *A. sanguinea* trophont stage. Arrows indicate several parasite nuclei inserted between ridges of the developing mastigocoel cavity (m). Scale bars 5 μ m.

Table 1: Percent frequency of occurrence of parasitized and non parasitized dinoflagellate species in net phytoplankton weekly surface samples from the Santa Cruz Wharf (SCW) from 19 Oct 2009 to 28 July 2010 (N = 35). *species able to produce toxins. **species able to form “red tides” within the CCS. ♦ species detected with infections once in archived samples. Species listed as 100% “not present” during this study period but observed in the Bay in other times.

Dinoflagellate species observed in Monterey Bay	Dinoflagellates % frequency of occurrence				Thecate (t) or athecate (a) species	
	not present	present	common	dominant		
Parasitized species	<i>Akashiwo sanguinea</i> **	83	17	-	-	a
	<i>Neoceratium furca</i> **	39	49	9	3	t
	<i>Dinophysis</i> sp.	48	46	6	-	t
	<i>Prorocentrum micans</i> **	16	49	26	9	t
	<i>Alexandrium</i> sp.* ♦	100	-	-	-	t
	<i>Neoceratium fusus</i> **♦	86	11	3	-	t

	Non parasitized species	<i>Neoceratium</i> cf. <i>divaricatum</i> **	62	26	3	9
<i>Neoceratium lineatum</i>		86	14	-	-	t
<i>Neoceratium</i> spp.		83	17	-	-	t
<i>Cochlodinium</i> **		100	-	-	-	a
<i>Dinophysis caudata</i>		83	14	3	-	t
<i>Dinophysis fortii</i> *		51	49	-	-	t
<i>Dinophysis rotundatum</i>		77	23	-	-	t
<i>Dinophysis tripos</i>		83	17	-	-	t
<i>Gyrodinium</i> spp.		89	11	-	-	a
<i>Gonyaulax</i> sp.		89	11	-	-	t

Table 1: Continued

	Dinoflagellate species observed in Monterey Bay	Dinoflagellates % frequency of occurrence			Thecate (t) or athecate (a) species	
		not present	present	common		dominant
Non parasitized species	<i>Lingulodinium polyedrum</i> **	88	9	3	-	t
	<i>Noctiluca scintilans</i> **	100	-	-	-	a
	<i>Oxytoxum</i> spp.	97	3	-	-	t
	<i>Polykrikos</i> sp.	91	9	-	-	a
	<i>Preperidinium</i> sp.	54	46	-	-	t
	<i>Prorocentrum gracile</i>	97	3	-	-	t
	<i>Protoperidinium</i> spp.**	20	60	17	3	t
	<i>Protoperidinium stenii</i>	49	46	5	-	t
	<i>Pyrocystis lunula</i>	74	26	-	-	t or a
	<i>Scrippsiella trochoidea</i>	91	9	-	-	t

Table 2: Percent frequency of occurrence of *Amoebophrya* infections in host's relative abundance categories. Observations were done in net phytoplankton weekly surface samples from the Santa Cruz Wharf (SCW) from 19 Oct 2009 to 28 July 2010. N indicates the number of samples from 19 Oct 2009 to 28 July 2010 where host was detected in each relative abundance category.

Hosts	Frequency of <i>Amoebophrya</i> infections in host's relative abundance categories			
	Not present	Present	Common	Dominant
<i>Akashiwo sanguinea</i>	-	17% (N =6)	-	-
<i>Neoceratium furca</i>	-	12% (N = 17)	33% (N =3)	100% (N =1)
<i>Dinophysis</i> sp.	-	19% (N =16)	100% (N =2)	-
<i>Prorocentrum micans</i>	-	24% (N =17)	78% (N =9)	67% (N =3)

¹*Alexandrium* sp. and *Neoceratium fusus* were observed to be infected once, when present in preserved samples from 16 Nov 2005 and 25 Oct 2006, respectively.

CHAPTER TWO

Parasitism as a biological control agent of dinoflagellate blooms in the California Current System

Abstract

Amoebophrya is a marine parasite recently found to infect and kill bloom-forming dinoflagellates in the California Current System (CCS). However, it is unknown whether parasitism by *Amoebophrya* can control dinoflagellate blooms in major eastern boundary upwelling systems, such as the CCS. We quantified the abundance of a common bloom-forming species *Akashiwo sanguinea* and prevalence of its parasite (i.e., % infected cells) in surface water samples collected weekly from Aug 2005 to Dec 2008 at the Santa Cruz Wharf (SCW), Monterey Bay, California. Additionally, we measured physical and chemical properties at the SCW and examined regional patterns of wind forcing and sea surface temperature. Relative abundance of the net phytoplankton species was also analyzed to discern whether parasitism influences net phytoplankton community composition. Epidemic infection outbreaks (>20% parasite prevalence in the host species) ended or prevented the occurrence of *A. sanguinea* blooms, whereas low parasite prevalence was associated with short-term (≤ 2 weeks) *A. sanguinea* blooms. The complete absence of parasitism in 2007 was associated with an extreme *A. sanguinea* bloom. Anomalously strong

upwelling conditions were detected in 2007, suggesting that *A. sanguinea* was able to outgrow *Amoebophrya* and ‘escape’ parasitism. We conclude that parasitism can strongly influence dinoflagellate bloom dynamics in upwelling systems. Moreover, *Amoebophrya* may indirectly influence net phytoplankton species composition, as species that dominated the net phytoplankton and developed algal blooms never appeared to be infected.

Introduction

Biological control processes, more specifically parasitism, may strongly influence the dynamics of dinoflagellate blooms. Slobodkin (1989) argued that, except for a highly specific infectious disease, there is no “magic bullet” to end algal blooms. Recently, it has been demonstrated that the marine parasitic dinoflagellate, *Amoebophrya*, which infects free-living dinoflagellates, can have high or moderate host specificity (Kim et al. 2008). Moreover, such a parasite is able to retard or prevent dinoflagellate blooms through epidemic infection outbreaks in estuarine systems (Nishitani et al. 1985; Coats et al. 1996; Chambouvet et al. 2008). Accordingly, Montagnes et al. (2008) showed through mathematical models that marine parasitism by *Amoebophrya* might have a greater impact on the demise of toxic dinoflagellate blooms than do microzooplankton grazers.

The ability of *Amoebophrya* to efficiently control dinoflagellate populations is likely the result of a faster growth rate and higher offspring production of the parasite than of the host. For example, an average intracellular development time of 2.16 days

has been estimated for *Amoebophrya* infecting *Akashiwo sanguinea* populations from Chesapeake Bay (Coats and Park, 2002). As it kills and leaves the host in ~2 days, *Amoebophrya* can release up to hundreds of infective dinospores (Chambouvet et al. 2008), while healthy free-living dinoflagellates have a mean growth rate of 0.6 doublings per day (Tang, 1996).

Amoebophrya infections in bloom-forming dinoflagellates species from the California Current System (CCS) north of Baja California have only been recently observed (Chapter 1). Our goal in the present study was to investigate whether or not *Amoebophrya* can regulate dinoflagellate blooms that occur in locations influenced by the CCS. Our observations were made in Monterey Bay, an open embayment in central California that is highly influenced by the upwelling dynamics of the CCS. We focused on a common dinoflagellate *A. sanguinea* (previously known as *Gymnodinium sanguineum* and *G. splendens*), which often forms red tides in coastal locations influenced by major eastern boundary current systems (Trainer et al. 2010). Red tides are defined here as dinoflagellates blooms ($> 10^4$ cells L⁻¹) that may discolor surface seawater. Although this particular species is not known to produce toxins, mass mortality of marine birds and increase in acute upper respiratory symptoms (i.e., sinus congestion) in humans have been documented during an *A. sanguinea* red tide in Monterey Bay (Jessup et al. 2009; C. O'Halloran, pers. comm.). Additionally, abalone mariculture farms are likely threatened by *A. sanguinea*, as this dinoflagellate can prey on abalone larvae (Botes et al. 2003). Thus *Amoebophrya* may

help reduce negative effects of *A. sanguinea* blooms, when it controls the abundance of this common red tide former.

Physico-chemical processes in major coastal upwelling systems that directly influence red tide dynamics have been extensively studied (Bolin and Abbot, 1963; Kudela et al. 2005; Kudela et al. 2010; Pitcher et al. 2010). For example, in Monterey Bay, physical processes that may influence bloom initiation are associated with the upwelling and downwelling circulation of the California Current (CC) and include development of vertical density stratification (which may be followed by the intrusion of CC warm offshore waters) and convergent frontal zones that may aggregate dinoflagellates (Ryan et al. 2005; 2010a). In addition, upwelling and downwelling circulation can spread and disperse red tides that are initiated in the Bay (Ryan et al. 2009). Thus, an additional goal was to evaluate the role of parasitism within the physico-chemical scenario in which *A. sanguinea* red tides occur. Upwelling circulation patterns that influence Monterey Bay were inferred from wind speed and direction and sea surface temperatures measured at 2 moorings located in the outer Bay region. Additionally, we measured sea surface temperature, salinity and inorganic nutrients (nitrate-nitrite and phosphate) at our long-term, nearshore study site, the Santa Cruz Wharf, where we monitored *A. sanguinea* abundance and infection levels.

We also investigated the influence that parasitism might have on net phytoplankton community composition. In a recent study in a marine coastal estuary, Chambouvet et al. (2008) found that when parasites exhibit high host specificity, the

release of dinospores from one infected species may not suppress the bloom of other local dinoflagellates. Therefore, parasitism may shape phytoplankton community composition by selectively infecting species that might otherwise dominate the community. The specific questions being addressed in the present study are: 1) Does density variation of *A. sanguinea* populations from Monterey Bay, California correlate with *Amoebophrya* infections and/or with physical and chemical variables? 2) Can *Amoebophrya* parasitism influence the net phytoplankton community composition?

Material and Methods

Water sample collection

Surface seawater samples were collected weekly from 3 August 2005 through 10 December 2008 at the Santa Cruz Wharf (SCW) (36.95 N, 122.02 W) (Fig.1). Samples were collected by net tow (35 μ m mesh) and surface bucket. Bucket samples provided sub-samples for host and parasite enumeration, temperature, salinity and nutrient analyses.

Enumeration of A. sanguinea and Amoebophrya

Aliquots of 100ml from bucket samples were preserved in 4% formalin final concentration for host and parasite enumeration. Enumeration of *A. sanguinea* (the host) and parasite prevalence (i.e., % of *A. sanguinea* infected by *Amoebophrya*) was done in samples where *A. sanguinea* was recorded as the dominant net phytoplankton species (see phytoplankton composition, below). To observe the progress of

infections at the start and end of *A. sanguinea* blooms, samples collected one week after and before *A. sanguinea* was recorded as the dominant species of the net phytoplankton were also selected for host and parasite enumeration. As a result, 5 study periods (Aug-Dec 2005, Jan-Apr 2006, Jun-Dec 2006, Sep-Dec 2007, Sep-Dec 2008) were weekly monitored for parasite prevalence and host abundance.

To detect *Amoebophrya* infections within hosts, subsamples of 25-75mL were filtered on 5µm polycarbonate black filters and DAPI (final concentration of 500mg mL⁻¹) was added at 40µl. DAPI stains allowed us to identify only the mature trophont stage of *Amoebophrya*, also know as the “beehive” stage and hereafter referred to as such. To estimate parasite prevalence, a minimum of 100 - 200 total *A. sanguinea* cells were counted. Thus, our limit of detection for parasite prevalence was 1 - 0.5%. Enumeration of healthy and infected *A. sanguinea* cells was done on an epifluorescent compound microscope (Zeiss Axio Imager) using a 10X objective (40X was used when needed).

Parasite daily induced mortality

The percentage of a given host population killed per day by *Amoebophrya* was adapted from Coast and Bockstahler (1994) and estimated as:

$$\text{Daily parasite induced mortality} = \frac{(\% \text{ of infected host cells estimated with DAPI} * 1.97)}{\text{infection time in days}}$$

We calculated a correction factor of 1.97 to account for host cells parasitized with early life history stages of *Amoebophrya* since DAPI stains allowed us to detect only the mature beehive life history stage. In contrast, quantitative protargol staining

(QPS) allows the observation of initial stages of infection as well as the beehive stage. Thus one sample from each year where infections were previously detected with DAPI was also analyzed with quantitative protargol staining (QPS) as described in Montagnes and Lynn (1993) and Coats and Bockstahler (1994). The correction factor of 1.97 was then calculated as the averaged ratio between parasite prevalence detected with QPS and DAPI in 3 samples (17 Aug 2005, 29 Mar 2006 and 16 Sep 2008).

Infection time of 1.42 days for *Amoebophrya* in Monterey Bay was calculated using average intracellular phase time of 2.16 days (Coats and Park, 2002), corrected for Monterey Bay average temperature from days where infection was detected (13.9°C) and using a Q_{10} of 2.

Physicochemical analysis

Water temperature was measured with a digital thermometer upon sample collection. Samples for salinity and nutrient analysis were prepared by filtering 200mL through a 25mm Whatman GF/F filter. Salinity samples were tested using a portable salinometer (Guildline Portasal mod. 8410) shortly after collection. Nutrient samples were frozen at -20°C and tested later for nitrate-nitrite and phosphate concentrations on a LaChat Instrument automated ion analyzer (8000 series) using standard methods (Diamond, 2003a, b).

Moorings observations

Two moorings, one at the mouth of Monterey Bay (M1) and another 23 km further offshore (M2) provided hourly data on sea surface temperature and surface

wind, respectively, from 1995 to 2009 (Fig. 1). Surface wind direction and speed were measured hourly on M2 (36.7 N, 122 W) by a RM young model 05103 wind monitor. Sea surface temperatures (SST) from the same time period were obtained from M1 (36.7 N, 122 W) by temperature sensor at 3.5m. SST anomalies were calculated by the difference between the average SST from 1995 to 2009 from the same time period that *A. sanguinea* abundance and parasite prevalence were monitored in 2005 (Aug-Dec), 2006 (Jan-Apr and Jun-Dec), 2007 (Sep-Dec) and 2008 (Aug-Dec) and the mean SST from the actual study periods of 2005, 2006, 2007 and 2008.

Satellite remote sensing

To place our Monterey Bay observations in a greater regional context during the biologically and physically anomalous 2007 study period, we examined satellite sea surface temperature (SST) data from the Advanced Very High Resolution Radiometer (AVHRR) satellite sensor. SST anomaly patterns were computed for the 2007 study period as the average SST at each pixel during the study minus the average for the same annual period averaged between 2004 and 2007. AVHRR processing methods are published (Ryan et al. 2010b).

Phytoplankton species composition

Live net tow material was examined in the laboratory using a dissecting scope (Olympus SZH Stereozoom) and magnified 64X. The net phytoplankton community (>20 μ m) was characterized by identifying taxa to genus or species level and estimating the relative abundance contribution of each individual taxon to the total net

phytoplankton taxa in the sample. Relative abundance categories used were: dominant (taxa contributing $\geq 45\%$ of the total cell number within the net phytoplankton community), common (taxa contributing $\leq 44\%$ but $\geq 11\%$) and present (taxa contributing $\leq 10\%$ of the total net phytoplankton community). Taxa were identified to genus level to avoid misidentification of species, except for the case of *A. sanguinea*, which was an easily recognized species. Diatom presence in the net phytoplankton community was noted, but is not described here, as it was not directly relevant to the focus of this paper.

Statistical analysis

Systat was used for linear and multiple regressions and ANOVA analyses. Linear regression was used to evaluate whether *A. sanguinea* (i.e., host) density was correlated with *Amoebophrya* infections. A multiple linear regression model was used to determine whether *A. sanguinea* density was correlated with physicochemical variables (i.e., temperature, salinity, nitrate-nitrite and phosphate). Finally, ANOVA was used to evaluate whether sea surface temperatures differed among years when *Amoebophrya* was detected. A value below the detection limit was added to host densities and nitrate samples to avoid the use of zeros in models. For each of these analysis, nitrate-nitrite and phosphate were $\log(x+1)$ transformed, host densities were log transformed, and the % of infected *A. sanguinea* was fourth root transformed to comply with linearity and normality assumptions of the respective tests.

Results

Akashiwo sanguinea red tides and *Amoebophrya* infections

Red tides of *A. sanguinea* ($> 10^4$ cells L⁻¹) were frequently detected in surface water samples collected in the summer at the Santa Cruz Wharf during 2005, 2006, 2007 (Fig. 2A). Additionally, red tides were detected in the fall of 2005 and 2007, and throughout all seasons during 2006. The highest density of *A. sanguinea* was observed in the fall of 2007 (i.e., $>10^6$ cells L⁻¹). During that time, *A. sanguinea* cell concentration remained above the red tide threshold for 8 consecutive weeks (17 Oct to 5 Dec). In 2008, *A. sanguinea* populations remained just below red tide threshold.

Parasite prevalence ranged between 0.5 and 10% with a maximum of 40%. Fig.2B shows parasite prevalence from Aug 2005 to Dec 2008 after fourth root transformation. Average parasite prevalence was lowest during red tides and vice-versa (Fig. 3). The highest parasite induced daily mortality rate of 56% was calculated for 17 August 2005 and minimum rate of 0% throughout the fall of 2007.

Oceanographic conditions during A. sanguinea red tides

At the Santa Cruz Wharf, sea surface temperature ranged from 10.9 to 18.1 °C and salinity from 30.63 - 33.56‰ during *A. sanguinea* red tides ($>10^4$ cells L⁻¹) in 2005, 2006 and 2007 (Fig. 4). Nitrate and phosphate concentrations ranged from 11.07 μM to undetectable and from 3.97 μM to 0.31 μM, respectively (Fig. 2 C, D).

During the study periods of 2005, 2006, 2007 and 2008, mean surface winds were upwelling favorable (NW). Consistent with the exceptionally high abundances of *A. sanguinea* and the absence of parasites during the fall of 2007 (Fig. 2A, 2B),

regional wind and SST patterns were unique during the 2007 study period. While average winds during study periods in 2005, 2006 and 2008 were very similar to the long-term (1995 to 2009) average for the same annual period as the studies, or weaker than average during the early 2006 study period, average winds during the 2007 study period were stronger than average by ~ 1 m/s (Fig. 5). The alongshore, equatorward wind direction (from the northwest) indicates anomalously strong upwelling during the 2007 study period. Accordingly, sea surface temperature (SST) anomalies calculated at M1 for the study periods indicated the coldest anomalies, $\sim 1^\circ\text{C}$, during the 2007 study period (Fig. 6). An anomaly map computed from satellite SST data revealed that during the 2007 study period cold anomaly extended throughout Monterey Bay and was part of a regional pattern detected along much of the central California coast (Fig. 7).

***A. sanguinea* abundance correlation with parasitism and/or environmental variables**

Linear regression results indicated significant and negative correlation between log of host density and parasite prevalence (Fig. 8). Likewise, multiple linear regression results indicated that environmental variables (i.e., temperature, salinity, log nitrate and log phosphate) were significantly correlated with the log of *A. sanguinea* density (Table 1). Furthermore, higher R^2 and lower p values were obtained when *A. sanguinea* densities were significantly related to *Amoebophrya* parasitism but not to other variables (Table 1). An additional model that combined both biological and environmental variables, however, explained even more of the

variability of *A. sanguinea* densities than the models that considered these variables separately (Table 1).

Parasitism influence in net phytoplankton species composition

In 83% of the days when *A. sanguinea* densities were above the red tide threshold ($> 10^4$ cells L⁻¹) and low averaged infection rates were recorded, *A. sanguinea* remained the dominant species of net phytoplankton community and sometimes co-dominated with *Cochlodinium* sp. or *Ceratium* spp. (Figs. 9, 11 and 12). In the remaining 17% of these samples, *A. sanguinea* was a common species and no dominant species was observed (Figs. 9 and 12).

Conversely, on 75% of the days when *A. sanguinea* densities were below the red tide threshold and infections were detectable, the net phytoplankton community was dominated by species other than *A. sanguinea* (i.e., *Cochlodinium* sp., *Ceratium* spp. or diatoms) (Figs. 11 and 13) or no dominant species was detected (Figs. 9, 10 and 13). In the remaining 25%, *A. sanguinea* was dominant (Figs. 9 and 10) or co-dominant (Fig. 10).

Discussion

The dinoflagellate *A. sanguinea* has been known to cause red tides along the coast of California since the early 1970s (Kiefer and Larsen, 1975; Fiedler, 1982). Although this species does not appear to produce toxins, it can negatively affect marine fauna, human health and mariculture (Botes et al. 2003; Jessup et al. 2009; C. O'Halloran, pers. comm.). The goal of the present study was to determine whether or not the marine parasite *Amoebophrya* can regulate the abundance of *A. sanguinea* in Monterey Bay, California as well as to understand the role of parasitism compared to that of environmental variables in controlling red tides in a coastal region strongly influenced by upwelling dynamics. An additional objective was to discern the consequences to the net phytoplankton community composition of parasitism of one of its dominant constituents, *A. sanguinea*.

Parasitism as a biological control of A. sanguinea population

Interestingly, highest *A. sanguinea* cell densities at the Santa Cruz Wharf (SCW) were observed in the absence of *Amoebophrya* infections, in the fall of 2007 (Fig. 1D). Early and late stages of infection were not observed in net samples from 2007 that were examined using quantitative protargol staining (QPS). Moreover, infections were not detected in water samples collected at the offshore Monterey Bay station M1 or in water samples collected along an inshore transect located on the northeastern shelf of the Bay during the 2007 study period (data not shown), suggesting that the 2007 red tide occurred as *A. sanguinea* was released from parasitism. Such a red tide caused harm in a previously undocumented manner. Specifically, organic matter from

the senescing *A. sanguinea* bloom coated feathers of birds, fouling the insulating function of their feathers and causing morbidity and mortality via hypothermia (Jessup et al. 2009).

The factors responsible for the absence of *Amoebophrya* infections in the 2007 fall bloom are unknown. Mechanisms that may lead the host to escape parasitism and, consequently, develop red tides may include (I) a low encounter rate of dinospore and host due to low host density or spatial segregation (Coats and Bockstahler, 1994); (II) ciliate and other microzooplankton grazing pressure on dinospores (Johansson and Coats, 2002); or (III) an environmental change that negatively affects parasite fitness and at the same time leads to a faster growth rate of the host. Alternative I may be rejected in this case, as high host densities were recorded in 2007, the Santa Cruz Wharf site is shallow (~9m) and relatively well mixed making spatial segregation of host and dinospores unlikely. Alternative II could not be evaluated, as we did not recorded zooplankton biomass. On the other hand, anomalous environmental conditions were observed during the 2007 red tide and may have influenced host growth rate and/or parasite fitness.

During the 2007 red tide, the lowest sea surface temperatures (SST) along with the highest salinity values were recorded relative to temperature and salinity levels during other *A. sanguinea* bloom events in this study (Fig. 4). A comparison with mean climatological data for 15 years (1995 to 2009) indicated that during the 2007 red tide period, upwelling-favorable winds were stronger than average by 1m/s and anomalously cold SSTs were detected throughout Monterey Bay (Fig. 5, 6, 7).

Accordingly, climatic conditions of the California Current System (CCS) reflected a strong and persistent La Niña marked by low SST and above-normal upwelling volumes in 2007 (McClatchie et al. 2007). Such upwelling conditions could have increased the growth rate of *A. sanguinea* by increasing the supply of nutrients. Likewise, Cloern et al. (2007) found that the shift to La Niña (cold) conditions, which implied increased upwelling intensity along with strengthened southerly flows transporting subarctic waters, was linked to an increase in the occurrence of red tides (including those caused by *A. sanguinea*) in San Francisco Bay.

While the cold, nutrient-rich upwelled waters may have “fueled” the bloom, it is also possible that an unknown mechanism may have decreased the growth rate of the parasite relative to the host growth rate. For example, the low SST could have altered *Amoebophrya* dinospore physiology. Accordingly, ANOVA results showed that mean SST measured at the SCW significantly differed among the years when *Amoebophrya* was detected (i.e., 2005, 2006, 2007 and 2008) ($p < 0.001$, $F_{3,30} = 7.65$). Pairwise comparison analysis further indicated that SST in 2007 was significantly lower than SST in 2006 ($p < 0.05$) and 2008 ($p < 0.01$), and lower than in 2005 ($p = 0.05$). Therefore, low SST could have signaled the presence of a habitat that reduced *Amoebophrya* infection ability.

An alternative hypothesis for the absence of infections in 2007 is that the observed bloom could have been caused by a strain of *A. sanguinea* resistant to *Amoebophrya* infections. Although different strains of *A. sanguinea* have not been identified in Monterey Bay, 6 different strains of this species have been identified in

US waters (data from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton) and several strains of the same dinoflagellate species are common in coastal embayments (Martinez et al. 2006, Touzet et al. 2007).

Moreover, *Amoebophrya*, which was originally described as one species (i.e. *A. ceratii*), is actually a complex of several species as shown by studies using 18S rRNA gene sequences of *Amoebophrya* that infect different dinoflagellate species (Cachon, 1964; Janson et al. 2000; Gunderson et al. 2002; Salomon et al. 2003; Kim et al. 2008), and some of these strains can be host-specific while others are not (Coats and Park, 2002; Kim, 2006, Chambouvet et al. 2008; Kim et al. 2008). *Amoebophrya* infections have been detected in 6 dinoflagellate species from Monterey Bay and simultaneously in at least 2 dinoflagellate species (Chapter 1). The latter observation suggests lack of host specificity (assuming that the same species of *Amoebophrya* were detected). However, preliminary cross-infection laboratory experiments using dinospores of *Amoebophrya* ex. *Prorocentrum micans* into an *Alexandrium catenella* culture isolated from Monterey Bay were unsuccessful, and indicated some degree of host specificity - at least for the host-parasite system tested (FM, pers. obs.). Thus, whether different strains of *A. sanguinea* are present in Monterey Bay and whether the 2007 bloom was caused by a strain resistant to infections of *Amoebophrya* strains present in Monterey Bay are questions that have not yet been answered.

Our data suggest that *Amoebophrya* epidemic infection outbreaks could have been responsible for stopping or preventing *A. sanguinea* red tides. Epidemic infection outbreaks are considered when >20% of the host is parasitized and thus most of the

host population is killed due to infections (Coats et al. 1996). An epidemic outbreak in the summer of 2005 was estimated to remove 56% of the host population per day and thus could have been responsible for the short-lived red tide (Fig. 9). Another possible epidemic infection outbreak was observed in September 2008. Although 9.0% and 9.3% parasite prevalence was recorded in September 2008, our methodology allowed us to detect only the *Amoebophrya* beehive stage (i.e., the mature trophont). This life-history stage corresponds to less than half of *Amoebophrya* total generation time (see calculation of correction factor of 1.97 on methods) and thus the actual parasite prevalence could have been ~20% in September 2008. In this case, the calculated parasite-induced mortality rate indicated that 13% of the host was being killed daily. Thus, high levels of parasitism may have been responsible for the demise and prevention of *A. sanguinea* red tides in Monterey Bay.

Parasitism as another piece of the red tide dynamics puzzle

Red tides dynamics are likely to be influenced by the synergism among environmental and biological processes. Although a significant negative correlation was observed between infections and host abundance, parasitism may have been one of several factors preventing the occurrence of red tides in Monterey Bay.

Additionally, results of our multiple linear regression model that included both parasitism and environmental variables as predictors of *A. sanguinea* density variability were the most predictive, as opposed to models that used parasitism and environmental variables separately to predict changes in *A. sanguinea* density (Table 1). Similarly, an inverse correlation between parasite prevalence and host abundance

was reported in an *Alexandrium* (= *Gonyaulax*) *catenella* population from Puget Sound, Washington, where the decline of an *A. catenella* bloom was associated with high infection rates as well as decreased host growth rate and depleted nutrients (Nishitani et al. 1985).

Other biological interactions that potentially influence red tide dynamics, such as grazing, also should be considered. Regarding grazers of *A. sanguinea*, Fiedler (1982) observed that zooplankton avoided grazing *A. sanguinea* during a red tide. Likewise, experimental studies showed that some heterotrophic dinoflagellates have difficulty capturing/ingesting *A. sanguinea* (Jeong and Latz, 1994; Jeong et al. 1999). Additionally, the production of anti-grazing substances may diminish grazer control (Smayda, 2008). To date *A. sanguinea* anti-grazing substances have not been reported in the literature, but surfactant-like proteins were detected in the surface foam generated at the end of the 2007 bloom period (Jessup et al. 2009). Whether or not surfactants produced during the 2007 bloom acted as an anti-grazing substance and aided in the bloom development is unknown.

Parasitism influence in net phytoplankton species composition

Amoebophrya parasitism may indirectly affect net phytoplankton community species composition by keeping species that are susceptible to infection at low densities and allowing species that are resistant to infections to reach bloom levels ($>10^4$ cells L⁻¹). In most of the samples where *A. sanguinea* was parasitized, another species dominated the net phytoplankton community. These included 2 dinoflagellates species not known to be parasitized by *Amoebophrya* (i.e., *Ceratium*

divaricatum, *Cochlodinium* sp.) in Monterey Bay or elsewhere (Park et al. 2004, Chapter 1), and species of the diatom genus *Pseudo-nitzschia*, which cannot be infected by *Amoebophrya*. Accordingly, the other dinoflagellate species detected throughout the study never dominated the community and are susceptible to *Amoebophrya* infections (Chapter 1).

It has been suggested that if parasitism specificity is high, the efficiency of natural biological control is reduced for exotic (enemy release hypothesis) (Kean and Crawley, 2002) or for rare species that become abundant due to environmental change (such as coastal eutrophication or climate change) (Chambouvet et al. 2008). The latter may be the case for the observed *Cochlodinium* bloom in 2006. *Cochlodinium* was regarded as a rare species in Monterey Bay prior to 2004 (Curtis et al. 2008). This dinoflagellate is adapted to low-nutrient environments, but also is capable of responding to eutrophication (Kudela et al. 2008). Moreover, *Cochlodinium* has similar ecophysiological characteristics to those of *A. sanguinea* (Smayda, 2002). Thus, it is possible that during the fall of 2006, while *Amoebophrya* controlled *A. sanguinea* populations, *Cochlodinium* was able to exploit an “open niche” and dominated the phytoplankton assemblage.

Conclusions

Fatal parasitic infections by *Amoebophrya* can contribute to the dynamics of red tides that occur in coastal locations influenced by upwelling, as exemplified by red tides of *A. sanguinea* in Monterey Bay, California. While *Amoebophrya* epidemic infection outbreaks may end or prevent the occurrence of red tides, strong and persistent upwelling events may cause the host to grow at a faster rate than the parasite, which then escapes parasitism and, consequently develops extreme red tides. These findings suggest that red tide dynamics can be controlled both by environmental (bottom up) and biological (top down) processes, and that the importance of each of these processes may vary temporally. Additionally, throughout this study, only dinoflagellate species that were free of *Amoebophrya* infections (i.e. *Cochlodinium* sp., *Ceratium divaricatum*) developed red tides in the Monterey Bay, further suggesting *Amoebophrya*'s significant role in indirectly contributing to the net phytoplankton species composition.

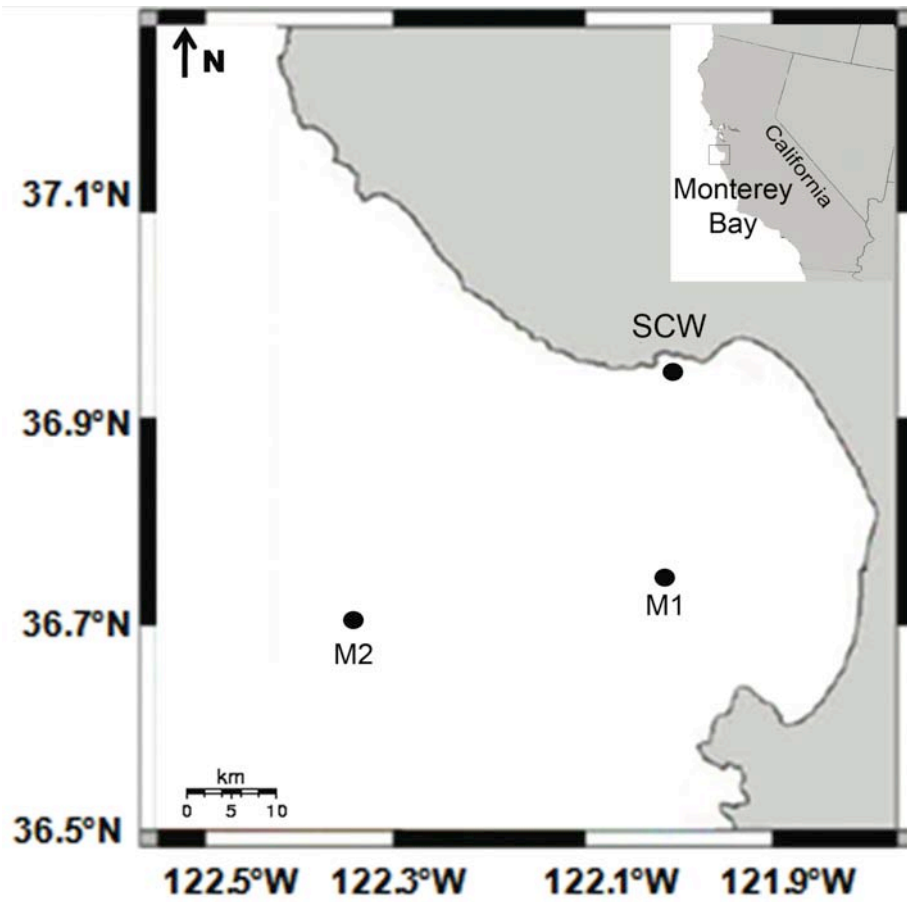


Fig. 1. Monterey Bay, California, showing sampling sites, the Santa Cruz Wharf (SCW), and the moorings M1 and M2.

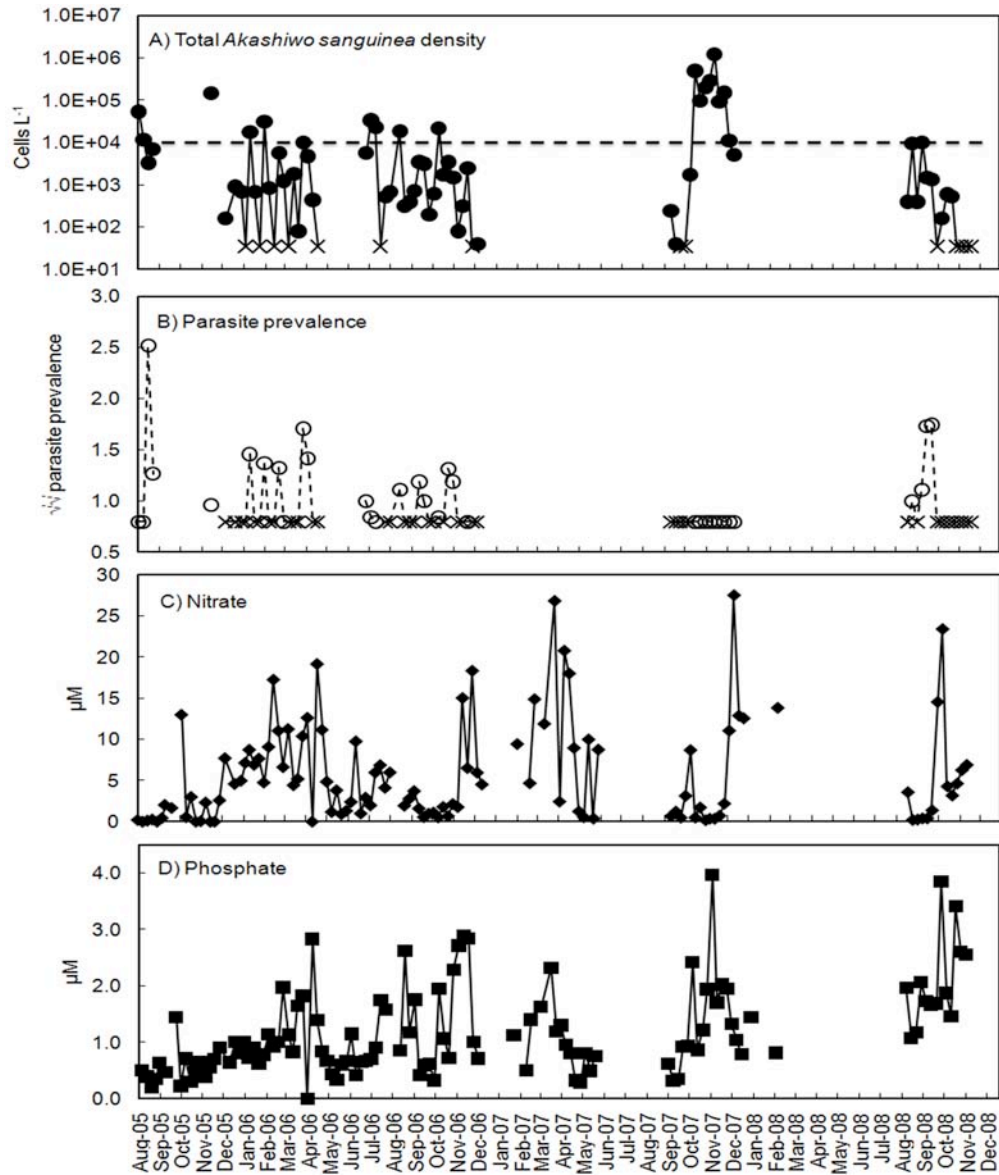


Fig. 2. *A. sanguinea* density (closed circles) (A), parasite prevalence (open circles) (B), nitrate (C) and phosphate (D) at the Santa Cruz Wharf from 08/2005 to 12/2008. Crosses indicate samples below limit of detection (LOD) of 40 cells L⁻¹ on (A) and 0.5% parasite prevalence on (B). Dashed line on (A) indicates red tide threshold of >10⁴ cells L⁻¹.

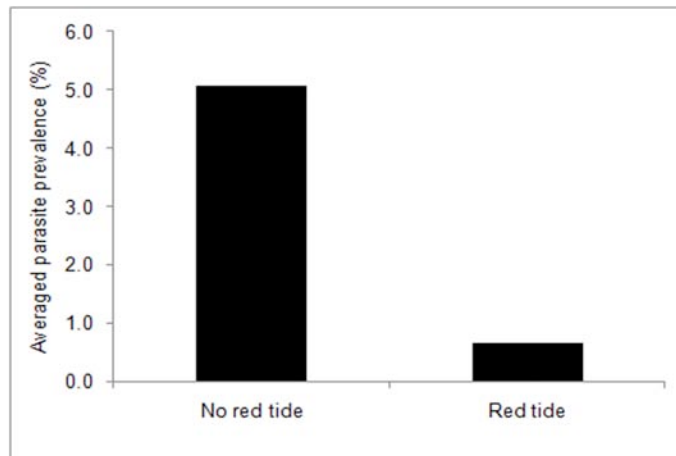


Fig. 3. Averaged parasite prevalence during days without a red tide ($<10^4$ cells L^{-1}) and days with red tide ($>10^4$ cells L^{-1}) at the SCW from study periods in 2005, 2006, 2007 and 2008.

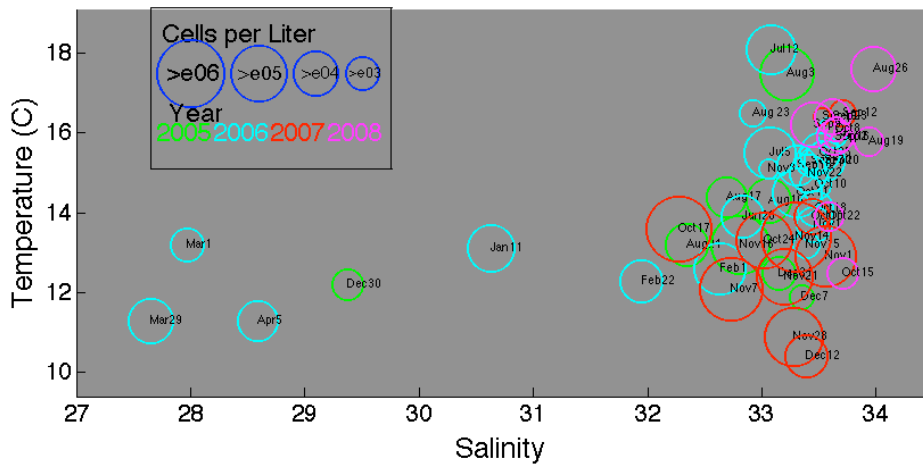


Fig 4. Sea surface temperature and salinity relative to *A. sanguinea* abundance at the Santa Cruz Wharf surface waters on 2005, 2006, 2007 and 2008. Note that highest cell densities were observed in the coldest and saltiest waters during 2007.

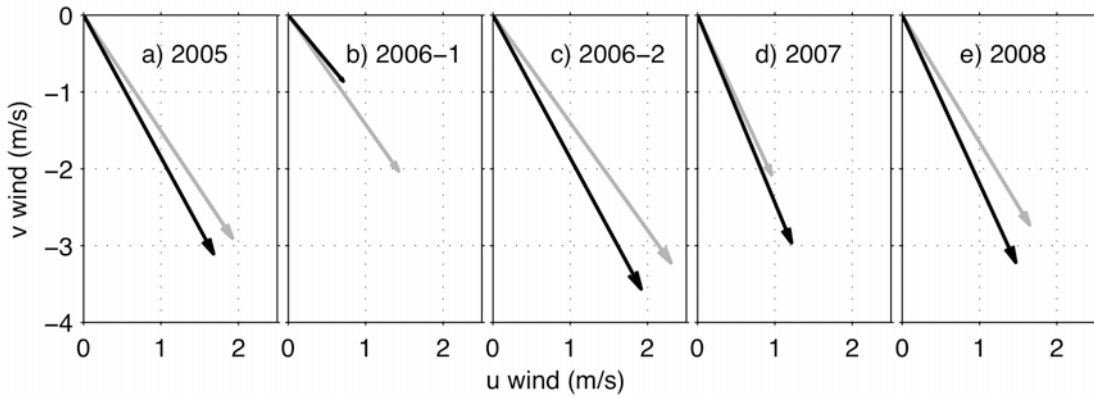


Fig. 5. Surface wind vector plots showing a comparison between mean wind direction and speed at M2 (Fig. 1) from 1995 to 2009 (gray arrow) during study periods in 2005, 2006-1 (Jan-Apr), 2006-2 (Jun-Dec), 2007 and 2008, and average winds (black arrow) during the actual study periods.

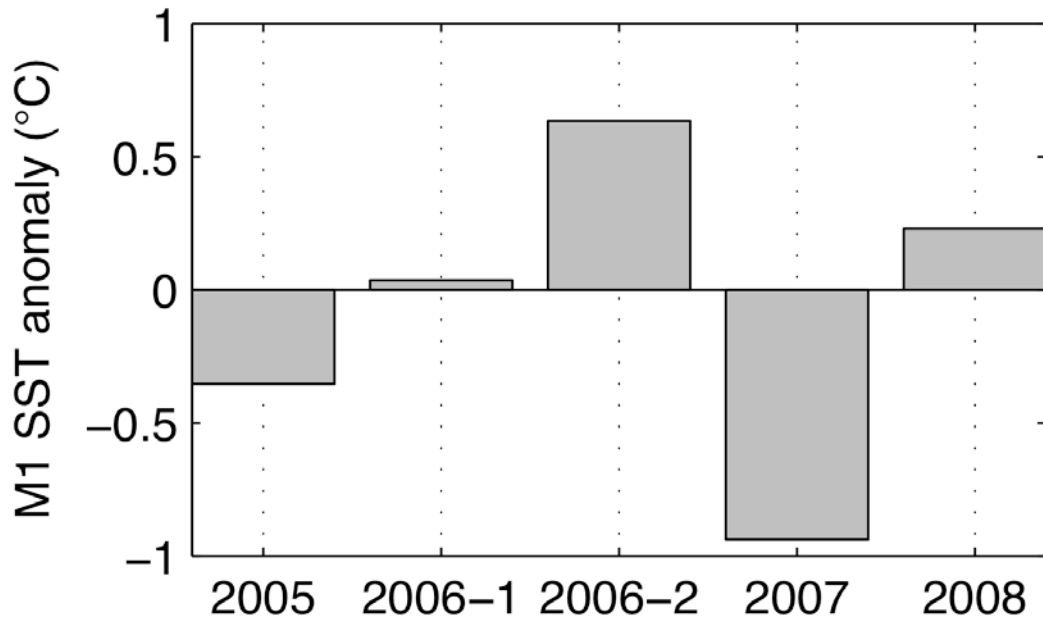


Fig. 6. Sea surface temperature (SST) anomaly at M1 (Fig. 1) during study periods in 2005, 2006-1(Jan-April), 2006-2 (Jun-Dec), 2007 and late summer of 2008.

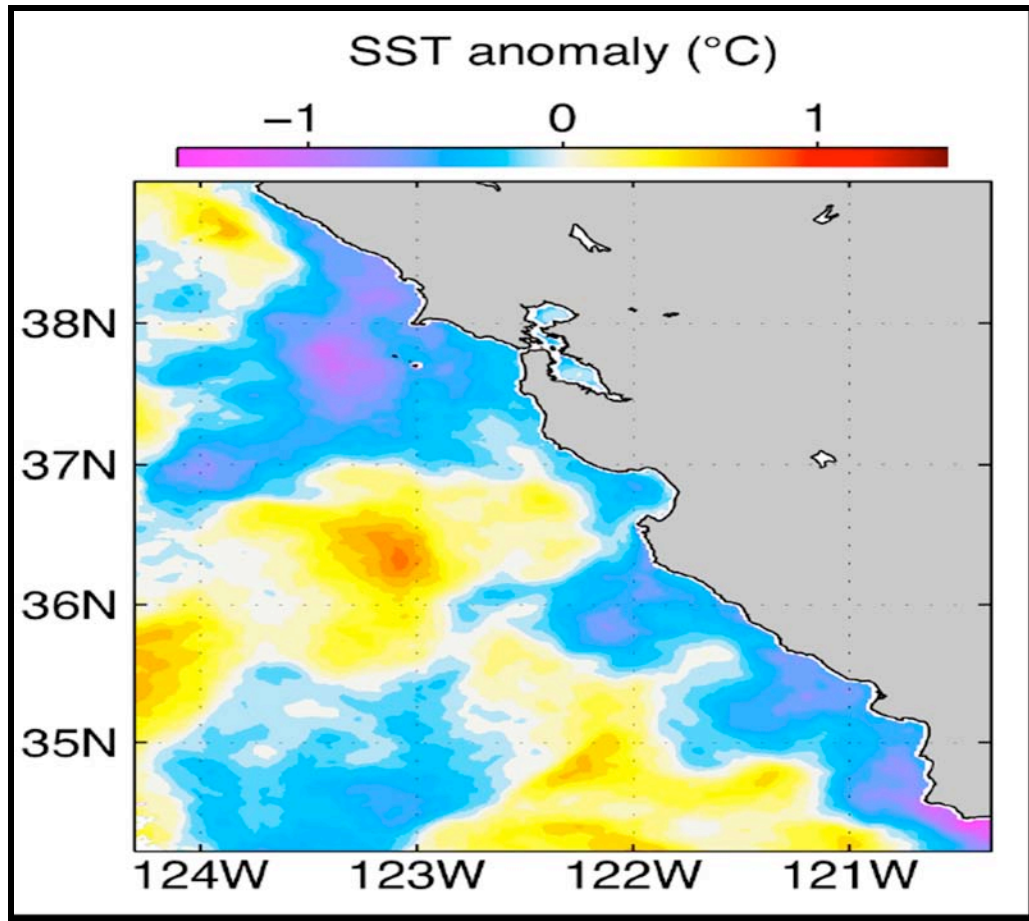


Fig. 7. Regional sea surface temperature (SST) anomaly during the 2007 study period.

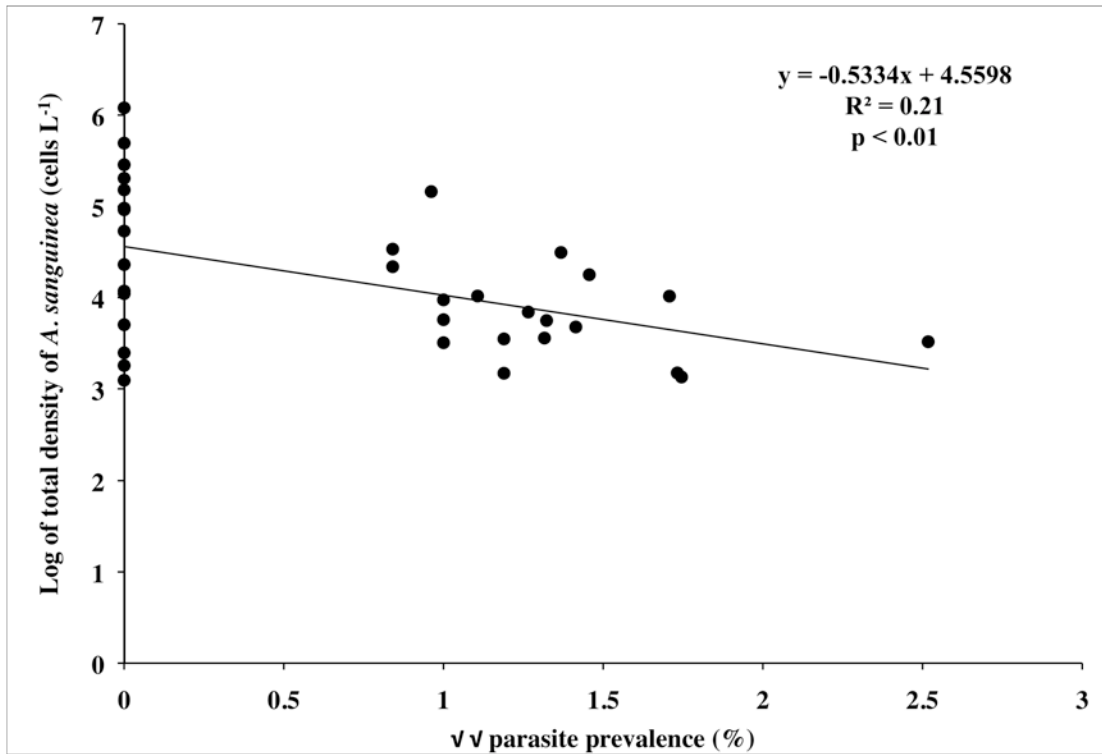


Fig. 8. Linear regression results (adjusted $R^2 = 0.21$, $df = 32$, $p < 0.01$, $N = 34$, $F = 9.79$) from log of host density (cells L⁻¹) versus parasite prevalence (4th root transformed) in surface water samples collected from study periods in 2005, 2006, 2007 and 2008 at the Santa Cruz Wharf. (Note: removal of highest parasite prevalence of 40% (i.e. 2.5% after 4th root transformation) changes results to adjusted $R^2 = 0.20$, $df = 31$, $p < 0.01$, $N = 33$, $F = 8.92$)

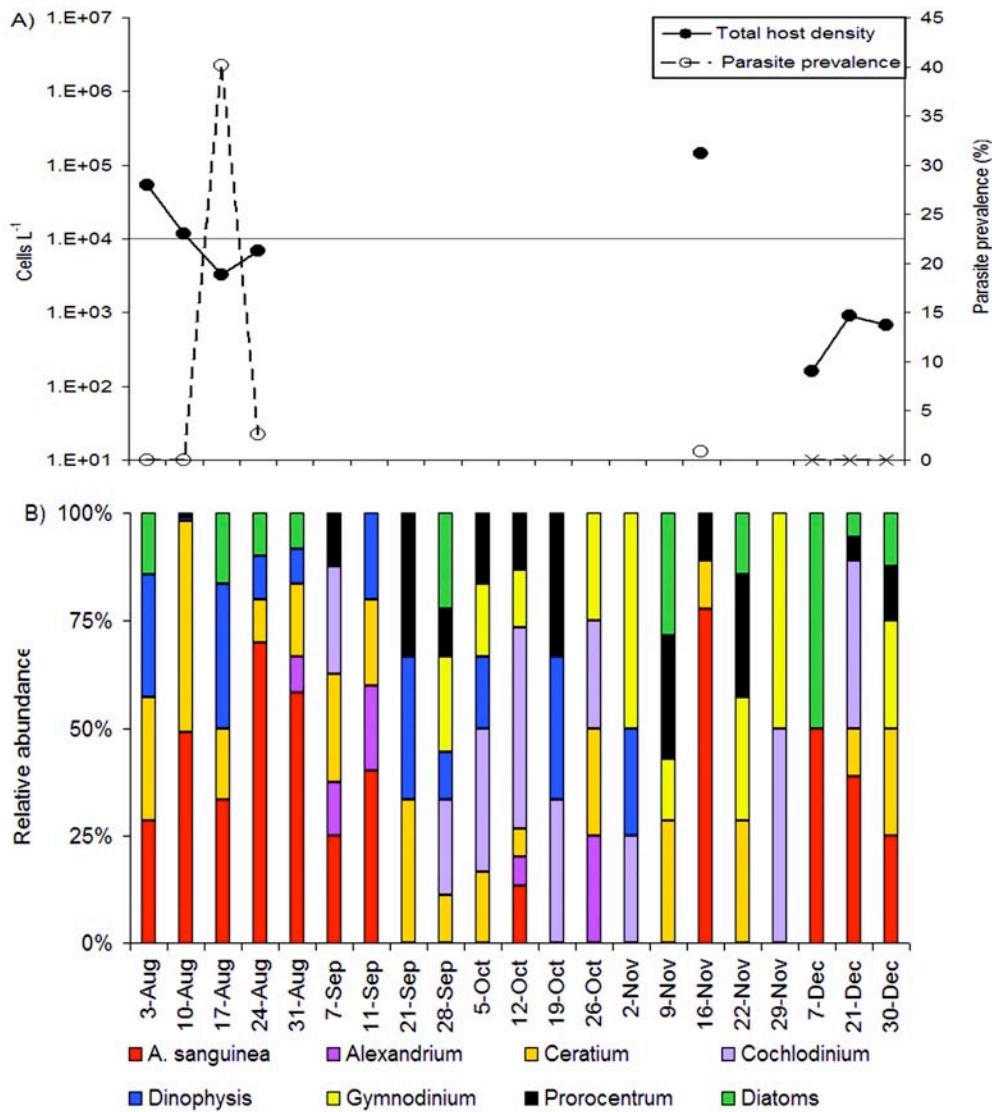


Fig. 9. A) *A. sanguinea* total density (cells L⁻¹) (closed circles) and parasite prevalence (open circles) for 2005 study period. Crosses indicate samples below the detection limit. Line indicates red tide threshold. B) Dinoflagellates (by genus) and diatoms (all genera combined) relative abundance patterns from Aug-Dec 2005 at the Santa Cruz Wharf

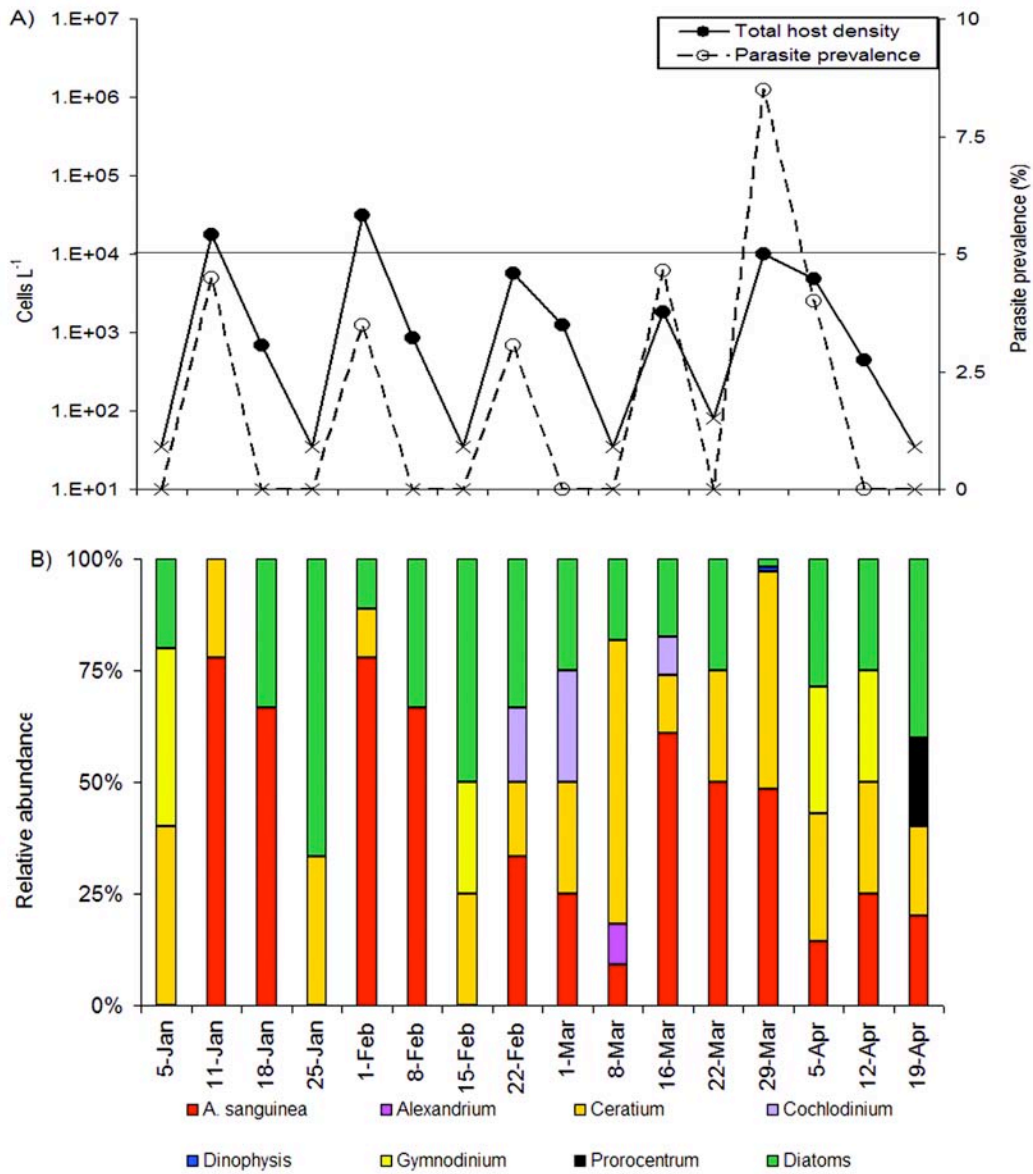


Fig. 10. A) *A. sanguinea* total density (cells L⁻¹) (closed circles) and parasite prevalence (open circles) for 2006 study period (winter and spring). Crosses indicate samples below the detection limit. Line indicates red tide threshold. B) Dinoflagellates (by genus) and diatoms (all genera combined) relative abundance patterns from Jan-Apr 2006 at the Santa Cruz Wharf

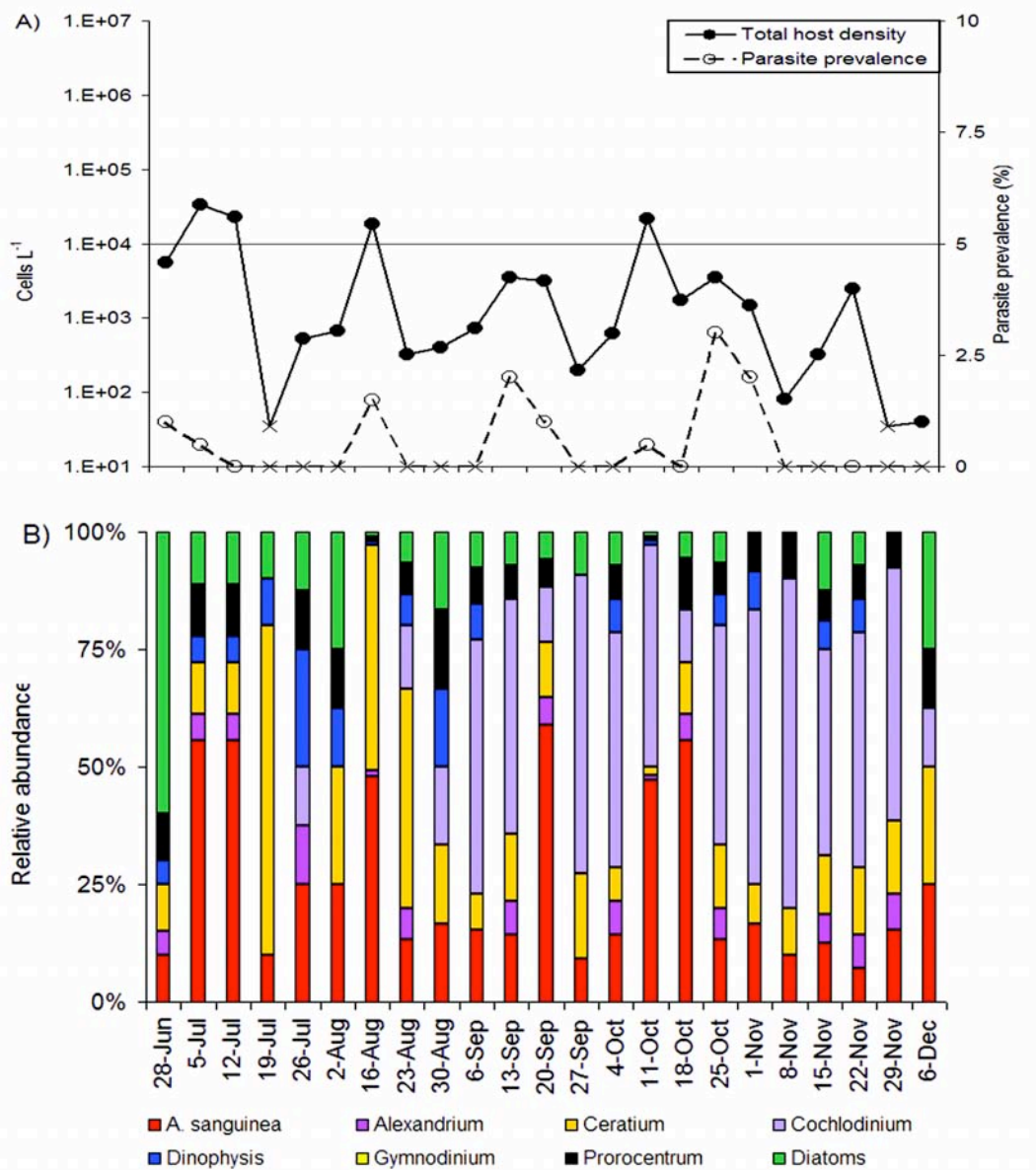


Fig. 11.A) *A. sanguinea* total density (cells L⁻¹) (closed circles) and parasite prevalence (open circles) for 2006 study period (summer and fall). Crosses indicate samples below the detection limit. Line indicates red tide threshold. B) Dinoflagellates (by genus) and diatoms (all genera combined) relative abundance patterns from Jun-Dec 2006 at the Santa Cruz Wharf.

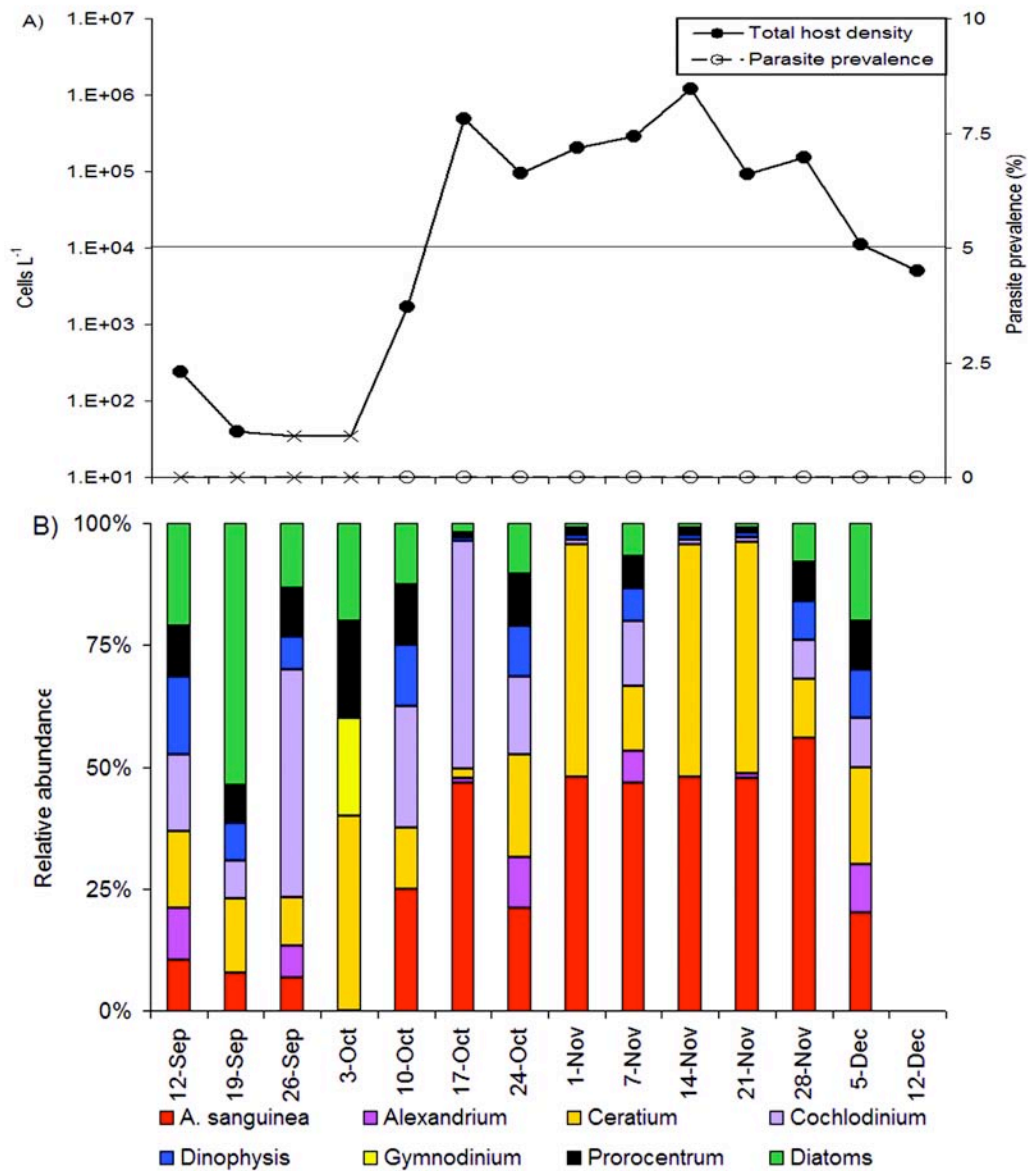


Fig. 12. A) *A. sanguinea* total density (cells L⁻¹) (closed circles) and parasite prevalence (open circles) for 2007 study period. Crosses indicate samples below the detection limit. Line indicates red tide threshold. B) Dinoflagellates (by genus) and diatoms (all genera combined) relative abundance patterns from Sep-Dec 2007 at the Santa Cruz Wharf

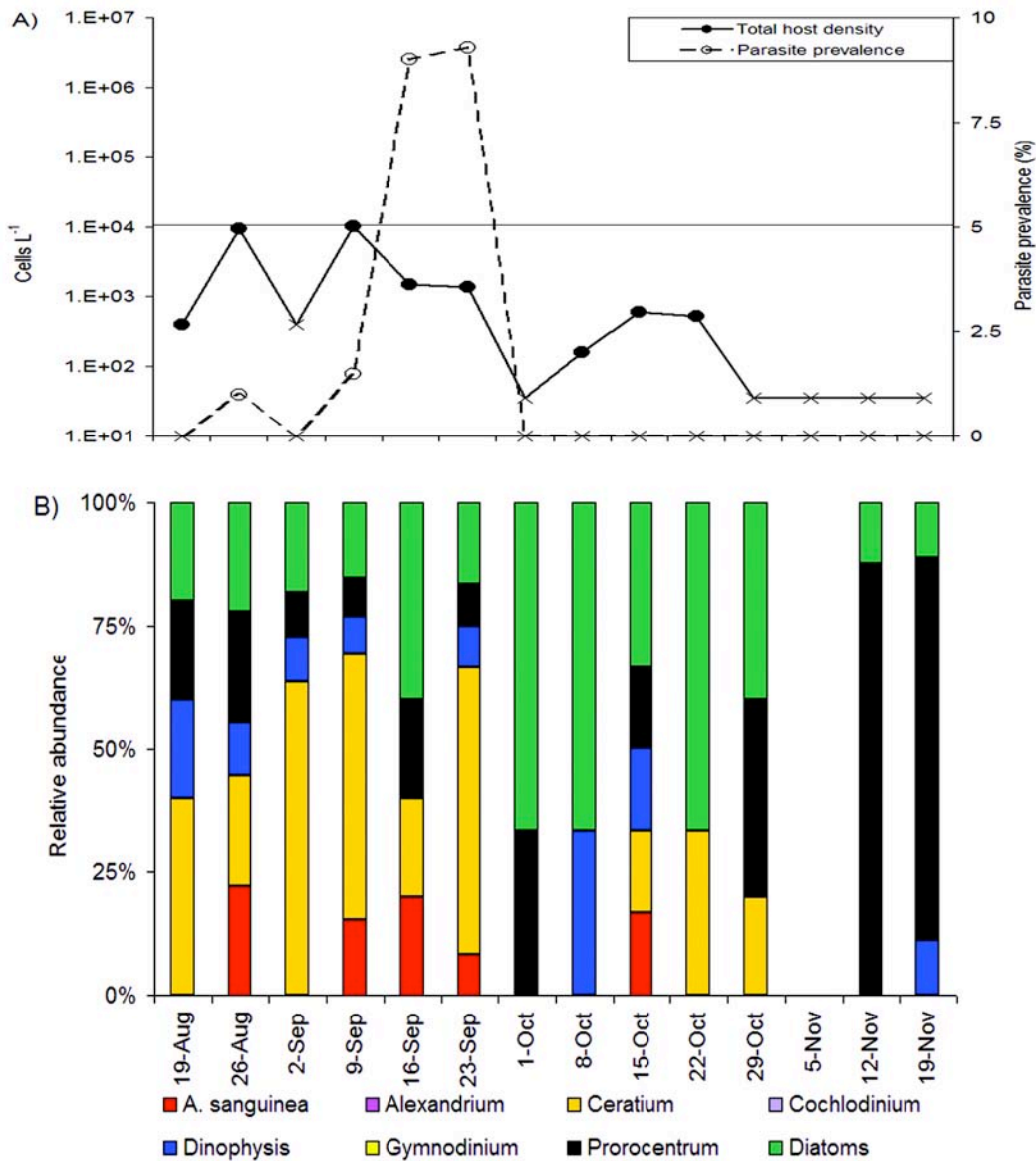


Fig. 13. A) *A. sanguinea* total density (cells L⁻¹) (closed circles) and parasite prevalence (open circles) for 2008 study period. Crosses indicate samples below the detection limit. Line indicates red tide threshold. B) Dinoflagellates (by genus) and diatoms (all genera combined) relative abundance patterns from Aug-Nov 2008 at the Santa Cruz Wharf.

Table 1: Results from linear and multiple regression models that used $\sqrt{\sqrt{\text{parasite prevalence}}}$ and/or environmental variables as predictors of log of *A. sanguinea* density variation.

Predicting variables of log of <i>A. sanguinea</i> density used in regression models	R²	df	P	N	F
$\sqrt{\sqrt{\text{Parasite prevalence}}}$	0.21	32	0.00	34	9.79
Environmental variables (temperature, salinity, log nitrate, log phosphate)	0.2	29	0.04	34	2.99
$\sqrt{\sqrt{\text{Parasite prevalence}}}$ and environmental variables	0.36	28	0.00	34	4.76

CHAPTER THREE

Angler exposure to domoic acid via consumption of contaminated fishes

Abstract

Domoic acid (DA) is a neurotoxin that causes Amnesic Shellfish Poisoning, and fish are recognized vectors of DA to marine fauna. However, the exposure of anglers through consumption of DA-contaminated fish is unknown. We measured DA in 11 fish species targeted by Santa Cruz Wharf (SCW) anglers in Monterey Bay, California, USA and surveyed anglers regarding their fish consumption patterns. In addition, we used California mussel (*Mytilus californianus*) DA data provided by the state of California and our measurements of DA in seawater to examine the associations between DA in fish viscera versus DA in mussels and seawater. DA was detected in the viscera of 7 fish species commonly consumed by anglers, and fish species' toxin intake levels varied according to their diet. Toxin was almost entirely in the viscera, with low DA detected in the muscle tissue. The majority of anglers (58% of 565) reported consuming their catch, with a small fraction ingesting the viscera. Total DA concentrations in fish decreased significantly after 11 months' storage in a -20°C freezer. DA concentration in seawater and California mussels was correlated with DA levels in the viscera of some but not all fish groups. We conclude that SCW anglers who consume their catch are exposed to asymptomatic DA doses, and that

exposure is a function of the species and parts consumed, as well as storage methods and DA levels in the seawater when the fish are caught.

Introduction

Domoic acid (DA) is a hydrophilic neurotoxin produced by diatoms of the genus *Pseudo-nitzschia* and the cause of Amnesic Shellfish Poisoning (ASP) in humans (Bates et al. 1989). Humans are known to be exposed to the toxin by consuming DA-contaminated shellfish (Waldichuk 1989, Wright et al. 1989). Symptoms after ingesting DA-contaminated shellfish vary according to the ingested DA dose and include vomiting and diarrhea, with higher exposures potentially leading to neurological effects such as disorientation, seizure, short term memory loss and, in extreme cases, death (Pulido 2008). The first documented case of ASP occurred in 1987 in Prince Edward Island (PEI), Canada. At that time, 108 people who consumed DA-contaminated blue mussels (*Mytilus edulis*) were diagnosed with ASP symptoms; 3 of them died (Perl et al. 1990). The estimated DA dose ingested by victims of this event ranged between 60 and 290 mg of DA (Jeffery et al. 2004).

DA was discovered in California, USA 4 years after the PEI event. As a protective measure, the California Department of Public Health (CDPH) subsequently added DA to the phycotoxins monitored in California mussels (*Mytilus californianus*). CDPH mussels are located at sites along the coast of California, including Monterey Bay, and are the monitoring species used in the Preharvest Shellfish and Marine Biotoxin Monitoring Program. This program appears to have

been effective in protecting consumers of commercially and recreationally caught shellfish, since no ASP cases have been reported in California even though toxin levels in local waters have occasionally been high. However, DA also can be transferred from *Pseudo-nitzschia* to fish, and these can contaminate marine birds such as brown pelicans (*Pelecanus occidentalis*) and Brant's cormorants (*Phalacrocorax penicillatus*), and marine mammals including California sea lions (*Zalophus californianus*) that prey upon the contaminated fish (Work et al. 1993, Lefebvre et al. 1999, Scholin et al. 2000). Presently, there is no formal monitoring of DA in recreationally caught fish (G. Langlois, pers. com. California Department of Public Health), and yet DA has been found previously in fish at locations where anglers could harvest the toxin-contaminated fish (Fire et al. 2005). Thus, it is possible that humans may be exposed to DA not only through consumption of shellfish, but also through consumption of recreationally caught fish.

In addition, DA has been detected in fish species with differing diets that are commonly targeted by California anglers (SCCWRP & MBC 1994, RecFIN 2009 Accessed 5 Oct www.recfin.org). The planktivorous northern anchovy (*Engraulis mordax*) and Pacific sardine (*Sardinops sagax*) have been found to contain DA on the California and the Portuguese coasts, respectively (Vale & Sampayo 2001, Lefebvre et al. 2002). Larger pelagic fish, Pacific mackerel (*Scomber japonicus*) and jack mackerel (*Trachurus symmetricus*) that feed on both plankton and small planktivorous fish, have also been found to contain DA in Mexico and Southern California, respectively (Sierra-Beltran et al. 1998, Busse et al. 2006). Likewise, DA

has been documented in the viscera of several benthic-feeding flatfish species found offshore in the Central California area (Vigilant & Silver 2007).

DA contamination of fish can occur through different pathways, hence the presence of DA in fish may or may not be in phase with the local presence of toxic *Pseudo-nitzschia* in seawater. For example, planktivorous fish acquire DA by feeding directly on toxic *Pseudo-nitzschia*, but typically only when cell density exceeds approximately 1000 L⁻¹ (Lefebvre et al. 2002a). In contrast, benthic-feeding flatfish are likely to acquire DA by feeding on infaunal and/or epifaunal organisms and on DA-contaminated sediment (Vigilant & Silver 2007). Because different sources of DA have been identified for different fish species, a range of DA levels may be encountered in fish collected at the same time and their contamination may or may not be in phase with the presence of toxic *Pseudo-nitzschia* cells in local waters. Identifying an association between DA in seawater and DA in fish is important for predicting the occurrence of DA in fish. It is equally important to understand the association between DA in fish and DA in mussels, and to evaluate whether mussels used in the state's Marine Biotxin Monitoring Program can be used to predict the occurrence of such toxin in fish, since the program is already in place.

DA distribution and degradation in the fish body is also poorly understood, mostly because initial studies were focused on DA exposure of marine animals such as California sea lions, which consume whole fish. In contrast, anglers, depending on their cultural background and other factors, may prepare and consume fish in different ways, including eating different body parts, and storing their catch different

lengths of time. Thus, understanding angler consumption patterns, as well as DA distribution in the fish body and DA degradation during storage, are of paramount importance in order to evaluate potential angler exposure to such a toxin.

The goals of this study were: (1) to determine which fish species and body parts are consumed by anglers and their catch storage methods; (2) to measure DA in fish commonly caught by anglers and compare DA levels among fish species with different diets to identify those that pose the greatest threat to anglers; (3) to measure and compare DA levels in fish viscera and muscle tissue under different storage conditions to determine if DA diffusion and degradation occur during storage; and (4) to assess possible relationships between DA in fish viscera and DA in seawater (collected both near- and offshore) and DA in CDPH mussels to verify whether the presence of DA in fish can be predicted, given the occurrence of DA in seawater and in mussels harvested by CDPH.

We focused this study on the Santa Cruz Wharf (SCW) in Monterey Bay, California, because it supports a sizeable recreational fishery (W. Van Buskirk, pers. com. Pacific States Marine Fisheries Commission) and is easily accessed by anglers. Additionally, the SCW is a site where CDPH mussels are harvested, and toxic blooms of *Pseudo-nitzschia* are frequently detected.

Material and methods

Angler Survey

We conducted an intercept survey of Santa Cruz Wharf (SCW) anglers to determine whether they are at risk of exposure to DA toxins as a result of their consumption of SCW-caught fish. The survey was modeled after the Santa Monica Bay and San Francisco Bay seafood consumption studies (SCCWRP & MBC 1994, SFEI 2000) and informed by similar studies done elsewhere in the USA [e.g. the Great Lakes (West 1992), New York (Connelley et al. 1996)]. An intercept survey, a type of face-to-face interview, is recommended as the most effective survey method for SCW-like contexts because it affords more complete coverage of anglers, including those who lack a telephone or permanent address, or do not speak or read English (US EPA 1998). In addition, no fishing license is required to fish from manmade structures in California, thus, intercept surveys are the only way to reliably identify and sample the population. In addition, it facilitates the collection of more accurate and reliable data because it allows the researcher (1) to clarify questions and responses, and (2) to make on-site observations that contribute to more accurate and consistent identification of anglers' catch (US EPA 1998).

The survey protocol consisted of three components: (1) an initial census at the SCW to determine spatial and temporal patterns of angler use, (2) an initial contact with potential interviewees to determine willingness to participate and to prevent duplicate interviews; and (3) a survey questionnaire, administered as an in-person interview. The survey sought to determine the proportion of anglers that

consume their catch and their consumption patterns. Thus, the questionnaire included questions on species and parts consumed (i.e muscle tissue, viscera); and catch storage and use. A fish identification booklet was developed and used to increase the accuracy of species identification by anglers. As used here, the term “viscera” refers to all organs associated with the digestive tract, whereas the term “muscle tissue” includes the edible meat, and excludes blood, skin, head, tail, gonads and viscera organs.

The survey protocol and questionnaire were pre-tested and revised before being applied in the field for a 12-month period beginning in May 2007. The survey was conducted by a team of 2 interviewers per shift, from a total of 23 interviewers selected and trained to conduct the survey. Survey shifts lasted 2-2.5 hrs, and occurred twice per day on one weekday and one weekend day per week. Survey data were processed and analyzed using PASW Statistics 17.0 to quantify anglers who reported consuming their catch, and of those, the number of consumers by species, and the parts consumed and storage method by species.

Sample Collection

In parallel with the angler survey, fish, seawater and mussel samples were collected at the SCW weekly from January 2007 through October 2008 and in May 2009. Fish, seawater and mussel samples were not necessarily collected on the same day, but seawater and mussel samples used for comparisons were collected within 7 days of the fish capture date. Bagged mussels (*Mytilus californianus*) deployed from the SCW were collected and processed as discussed in Jester et al. (2009). Seawater

samples were collected with a surface bucket at the SCW (36.57°N, 122.01°W). Additional surface samples were collected at M1, an offshore mooring located in the center of Monterey Bay over 1000 m deep water (36.74°N, 122.02°W), because some of the fish species range more widely in the Bay outside the wharf region (Fig. 1). For determination of particulate DA in the seawater, 500 ml were filtered through Whatman GF/F filters using a low vacuum pump system. The filters were subsequently stored in a -20°C freezer for 2 months before analysis.

Fish were collected using hook-and-line gear from the SCW (total of 352 hrs of fishing effort). Twelve volunteers were trained to use angler fishing techniques. Fish caught were placed immediately in a cooler with ice. Total length, weight, and species were recorded and the fish were dissected at the wharf approximately within the next 5 hrs to prevent possible diffusion of DA from the viscera into the muscle tissue. All dissected fish and viscera were stored in a -20°C freezer for one week or for a maximum period of 2 months prior to stomach content analyses and toxin extraction, with the exception of jacksmelt muscle tissue samples, which were stored between 6 and 12 months.

In addition to the anchovies and sardines caught at the SCW, these were also acquired in March and April 2008 (anchovies), and in July and August (sardines), from commercial fishing boats based at Moss Landing Harbor (Fig.1). These fish were caught at various locations in Monterey Bay. The exact location of the catch is proprietary information of the catch vessels. The fish were kept in ice in the vessel for approximately 5 hrs until they were retrieved by scientists. Once retrieved, fish were

treated as wharf-caught fish described above, except that they were dissected in the laboratory within the next 2 hours (i.e. total of approximately 7 hrs before dissection). These additional 2 hours are considered relatively unimportant for diffusion of DA in the fish since the specimens were kept in ice continually, minimizing diffusion rates.

Fish feeding habits analyses

Fish feeding habits were determined by examining the stomach contents of each fish species. Observations were made on both a dissecting microscope (Olympus SZH Stereozoom) and a Zeiss Axiovert compound light microscope. Prey items were identified to the highest level of classification possible. After stomach content observations were completed, toxin extraction continued.

DA detection in mussels

Mussel DA data were extracted from the monthly CDPH Marine Biotxin reports(<http://www.cdph.ca.gov/healthinfo/environhealth/water/Pages/Shellfish.aspx>), accessed 10 Aug 2009. CDPH measured DA using High Liquid Performance Chromatography (HPLC), and used a reporting limit of detection (LOD) of 2.5 $\mu\text{g DA g}^{-1}$ of mussel tissue (G. Langlois, pers. com. California Department of Public Health).

DA detection in fish

DA was measured in fish viscera using two methods: HPLC and Rapid Enzyme-Linked Immunosorbent Assay (ELISA). Both methods are comparable, but ELISA is a more desirable method due to its lower LOD and faster, more cost-effective procedure (Litaker et al. 2008). Commercial ELISA kits became available in

2008 from Mercury Science, Inc. (Durham, NC27713). DA was measured with ELISA in the following fish samples: (1) all muscle tissue samples, except for anchovy collected in 2007, when DA in muscle tissue was measured using HPLC, (2) viscera of 6 sardine specimens examined in the DA degradation and diffusion experiment, and (3) fish collected in May 2009. The HPLC method was used to measure DA in all other fish viscera samples.

For both methods fish viscera samples were prepared as 4g of pooled composite from multiple fish specimens, unless the individual fish viscera weighed > 4g, in which case the viscera from a single specimen was analyzed. Samples were homogenized with a hand-held tissue homogenizer (Tissue Miser, model PNF2110, Fisher Scientific, Pittsburg, PA 15275), and after 16 ml of 50:50 MeOH:Nanopore water was added, samples were sonicated with a Sonifier Cell Disruptor (Model W185D, Branson Sonic Power Co., Danbury, CT, 06813) and centrifuged for 20 min at 3800 rpm (mid ref = 1698 Gs). The supernatant were then filtered through a 3µm polycarbonate filter.

For HPLC analyses, the filtrate was cleaned for interfering compounds using solid phase extraction (SPE) columns (J.T. Baker, Phillipsburg, NJ 08865) (Hatfield et al. 1994, Quilliam et al. 1995). DA recovery of SPE columns (lot number E03553) were determined by spike and recovery experiments using 90% pure DA reagent (Sigma Chemical Company, St. Louis, MO 63103) and 10% methanol (Fisher Scientific, Pittsburg, PA 15120). Results indicated that an average of 97% of DA was recovered ($N = 3$, $SD = 3.3$). An average of 84% ($SD = 7.4\%$, $N = 4$) of DA recovery

from SPE columns was calculated for fish viscera (i.e. anchovy and flatfish) based on previous publications that followed the same extraction procedures described here and were done during the past 8 years in our laboratory by several different researchers (Lefebvre et al. 1999, 2001, 2002a, Vigilant & Silver 2007). DA values reported here are uncorrected for possible loss during extraction and clean up. DA analyses were conducted using an isocratic gradient profile on a Hewlett-Packard 1050 High Performance Liquid Chromatographer according to Vigilant & Silver (2007). The DA LOD was $0.2 \mu\text{g ml}^{-1}$ (ppm).

For ELISA analyses, the filtrate was diluted at 1:100 and 1:1000 in the buffer solution provided in the ELISA kit. The diluted samples were used in the ELISA plates following the protocol accompanying the kit. An Emax Precision Microplate Reader (Model E10968, Molecular Devices Co., Sunnyvale, CA, 94089) was used to measure absorbance at 450nm. The ELISA detection limit was $0.1 \mu\text{g L}^{-1}$ (ppb).

DA detection in seawater

Particulate DA in phytoplankton seawater samples was measured when toxic *Pseudo-nitzschia* cell density exceeded $1000 \text{ cells L}^{-1}$, except during summer 2008 ($>100 \text{ cells L}^{-1}$; cell density data not reported here). Particulate DA was measured with HPLC in all samples collected between January 2007 and October 2008 and ELISA was used in samples collected in May 2009. Ten percent methanol (MeOH) was used to extract DA from GF/F Whatman filters. For HPLC analyses, particulate DA was analyzed by the FMOC procedure (Pocklington et al. 1990) and equipment was used as described by Vigilant & Silver (2007). The DA LOD for phytoplankton samples

was 0.06 ng ml^{-1} . For ELISA analyses, seawater sample filtrates were prepared as for fish sample filtrate described above.

DA degradation and diffusion in fish body

Vessel-caught Pacific sardine specimens were used for DA degradation and diffusion experiments in fish. To verify whether DA can diffuse from the viscera into the muscle tissue and whether DA degraded during the 11 month storage period, these specimens were stored in 2 ways: (1) dissected approximately 7 hrs after capture and stored at -20°C freezer ≤ 2 months prior to DA analyses ($N = 22$), or (2) frozen whole and dissected and analyzed for DA 11 months after capture ($N = 5$). DA levels in viscera and muscle tissue of specimens dissected 7 hrs upon capture and stored for ≤ 2 months were compared to DA levels in viscera and muscle tissue of the sardine stored whole for 11 months. We measured DA in the muscle tissue of the same specimen for which we also obtained DA estimates in the viscera.

Statistical Analyses

Systat 10.0 was used for all statistical comparisons. Chi-square with Fisher exact tests were used to test whether the presence of DA in the fish viscera was associated with the presence of DA in seawater samples (nearshore and offshore) and mussels collected within 7 days of the fish capture. ANOVA was used to determine whether DA levels varied significantly among the 3 fish feeding groups (i.e pelagic planktivores, pelagic omnivores and benthic-feeders). Tukey pairwise comparisons were subsequently used to determine which pairs of fish feeding groups had mean DA levels that significantly differed from each other. Linear regression was used to

evaluate whether DA in muscle tissue of Pacific sardines was correlated with DA levels in their viscera. Finally, ANOVA tested whether DA in muscle tissue and in viscera from sardines significantly varied between sardines stored at -20°C for 2 months and intact sardines stored for 11 months at -20°C. DA levels in muscle and viscera were log 10 +1 transformed for linear regression and for ANOVA to comply with linearity and normality assumptions of the respective tests.

Results

Angler consumption patterns

A total of 746 anglers were surveyed at the SCW over a one-year period; 565 reported fishing previously at the SCW. Of those, 58% reported consuming their catch and 56% (318 anglers) reported consuming one or more of the species tested for DA content (Fig. 2). All consumers reported eating the muscle tissue. A small percentage (between 1.4 and 7.9%) reported not removing the viscera before preparing their catch for consumption, which is important since DA may diffuse throughout the fish body depending on fish preparation method. These fish included the 2 most consumed species (i.e. jacksmelt and Pacific mackerel) and also the species with the highest DA levels detected during the present study (i.e. anchovy and sardine, discussed later) (Fig. 3). In response to a separate question, some of these individuals also reported eating the viscera (Fig. 3).

Of those anglers who consume their catch, 55% reported using their catch the same day that the fish was caught and 45% reported freezing their catch for later

consumption. Anglers reported freezing their catch whole or partitioned (i.e. remove viscera before freezing it). Among the species most consumed (i.e. jacksmelt and Pacific mackerel) and the species that contained highest DA levels (i.e. anchovies and sardines), only 1.03% of jacksmelt consumers and 0.65% of Pacific mackerel consumers reported freezing their catch whole (for an unknown period of time) and partitioning it at the time of consumption. All consumers of sardine and anchovy reported removing the viscera before freezing their catch for later consumption.

DA in fish with different feeding habits

For the toxin study, 11 species were caught: barred surfperch (*Amphistichus argenteus*), jacksmelt (*Atherinopsis californiensis*), northern anchovy, Pacific mackerel, rainbow surfperch (*Hypsurus caryi*), Pacific sardine, shiner surfperch (*Cymatogaster aggregata*), speckled sanddab (*Citharichthys stigmaeus*), spotfin surfperch (*Hyperprosopon anale*), staghorn sculpin (*Leptocottus armatus*), and white croaker (*Genyonemus lineatus*). These finfish species were assigned to the following categories according to their feeding habits, based on examination of stomach contents: (1) pelagic planktivores, (2) pelagic omnivores and (3) benthic-feeders (Table 1). Pelagic planktivores included northern anchovy and sardine and fed exclusively on plankton. Jacksmelt and Pacific mackerel were classified as pelagic omnivores and fed on a broad spectrum of prey items, which included organisms from the plankton, as well as small nekton and benthic organisms. Barred surfperch, rainbow surfperch, shiner surfperch, speckled sanddab, spotfin surfperch, staghorn sculpin and white croaker were identified as benthic-feeders, since they preyed

mainly on benthic organisms. However, some of these benthic species have also been observed to feed on planktonic organisms (Table 1) (Cailliet et al. 2000). All species caught were shown to have detectable DA at least once, except for barred surfperch, shiner surfperch, spotfin surfperch and white croaker, which did not contain measurable DA (Table 2).

DA in fish viscera and DA in seawater

DA in vessel-caught pelagic planktivores (northern anchovy and Pacific sardine) was significantly associated with DA measured in seawater samples collected offshore at M1 or nearshore at the SCW within 7 days of fish capture ($\chi^2 (1, N = 71) = 71, p < 0.01$) (Fig. 4A, B and Fig. 5A, B and C). We were unable to test if the presence of DA in wharf-caught pelagic planktivores (anchovy) was significantly associated with the presence or absence of DA at M1 or at the SCW due to the small number of times that planktivores were caught at the wharf.

DA in pelagic omnivores (jacksmelt and Pacific mackerel) was significantly associated with particulate DA measured in seawater samples collected at the SCW ($\chi^2 (1, N = 39) = 20.14, p < 0.01$), but not associated with DA measured offshore at M1 ($\chi^2 (1, N = 14) = 6.46, p = 0.142$) within 7 days of fish capture (Fig. 4A,B and Fig. 5A,D). Additionally, DA in viscera of benthic-feeders (rainbow surfperch, speckled sanddab, staghorn sculpin) was significantly associated with DA levels measured in seawater at the SCW ($\chi^2 (1, N = 31) = 5.16, p = 0.04$), and at M1 ($\chi^2 (1, N = 22) = 7.60, p = 0.02$) within 7 days of fish capture (4A, B & Fig. 5A, D).

Although some of these associations were statistically significant, DA was also detected in pelagic omnivores and benthic-feeders when no DA was detected in surface waters (i.e. false negatives). The opposite association (i.e. false positives: where DA was detected in surface waters, but not detected such fish feeding groups) was also observed (Fig. 4A, B).

In addition, ANOVA results showed that mean DA levels in the viscera of fish caught when DA was detected in the seawater significantly differed among fish feeding groups ($F(2, 71) = 122.44, p < 0.01$) (Fig. 6). Pairwise comparison (Tukey) analysis further verified that DA levels in pelagic planktivores significantly differed from DA levels in pelagic omnivores ($p < 0.01$) and benthic-feeders ($p < 0.01$), but the latter two did not significantly differ from each other ($p = 0.71$). Indeed, pelagic planktivores were the only group that had mean DA in viscera $> 20 \mu\text{g g}^{-1}$.

DA in mussels and DA in fish

DA was never detected in mussels collected within 7 days of capture of vessel- or wharf-caught DA-containing planktivorous fish (Fig. 4C). In fact, DA levels above the safety limit occurred in viscera of planktivores prior to the detection or non-detection (i.e. false negatives) of DA in mussels in April 2007 and in August 2008, respectively (Fig. 4C and Fig. 5C, D, E). Chi-square results indicated that the presence of DA in mussels collected within 7 days of fish capture was not significantly associated with the presence of DA in the viscera of benthic feeding fish ($\chi^2(1, N = 33) = 4.73, p = 0.187$). Moreover, DA was detected in benthic-feeders when DA was not detected in mussels (i.e. false negatives) in 16% of the correlated

samples (Fig. 4C). However, the detection of DA in mussels was significantly associated with the detection of DA in the viscera of pelagic omnivores ($\chi^2 (1, N = 48) = 19.2, p < 0.05$), even though DA was detected in the viscera of pelagic omnivores when no DA was detected in mussels (i.e. false negatives) (Fig. 4C and Fig. 5D and E).

DA in fish body parts

DA was detected in 31% of the muscle tissue samples from wharf-caught anchovy and 72% of the muscle tissue samples from vessel-caught sardines that were dissected within the next 7 hrs following capture, but DA was never detected in jacksmelt muscle tissue ($N = 7$). Mean DA levels in anchovy and sardine muscle tissue were $1.7 \mu\text{g g}^{-1}$ ($N = 9, SD = 4.0$) and $0.5 \mu\text{g g}^{-1}$ ($N = 22, SD = 0.6$), respectively. These values were one and two orders of magnitude lower than the mean DA levels in anchovy and sardine viscera (Table 2). In addition, linear regression results indicated DA levels measured in the viscera and muscle tissue of the same individual sardine specimens were not significantly related ($F (1, 16) = 2.90, p = 0.107$).

DA degradation and diffusion within fish

ANOVA results showed that the log of DA concentration in sardine viscera was significantly lower after storage of whole sardines for 11 months ($F (1, 26) = 129.14, p < 0.01$) than DA levels in viscera of sardines that were dissected 5 hrs upon capture (Table 3). We did not observe a significant change between the log of DA

concentration in muscle tissue ($F(1, 26) = 1.52, p = 0.22$) of sardines that were kept frozen whole for 11 months after the capture date and the log of DA concentration in muscle tissue of sardines that were dissected 7 hrs after capture (Table 3), even though mean DA levels in muscle tissue were slightly higher after storage for 11 months. In addition, total (i.e. viscera + muscle tissue) DA concentration in sardines was significantly lower after storage for 11 months at -20°C ($F(1, 26) = 19.83, p < 0.01$) than total DA levels in sardines dissected 7 hrs after capture (Table 3).

Discussion

The goal of the present study was to determine whether or not anglers are exposed to DA through the consumption of their catch, since fish are recognized DA vectors to their aquatic predators. An additional goal was to determine whether the presence of DA in local seawater or in mussels was predictive of DA in recreationally caught fish at the Santa Cruz Wharf (SCW). We conducted a study at the SCW, a heavily fished pier in California where toxic blooms of DA-producing phytoplankton have been observed frequently in the past decade (Jones 1992, Jester et al. 2009). We have shown that potential dietary exposure of SCW anglers to DA is dependent on the fish species and body parts consumed, on catch storage methods and the presence of DA in the seawater.

The variability of DA levels among fish species appears to be related to their different feeding habits. Accordingly, DA levels in viscera of pelagic planktivores (i.e. northern anchovy and Pacific sardine), which can directly feed on the DA-

producing *Pseudo-nitzschia*, were higher than and significantly different from DA levels in viscera of pelagic omnivores and benthic-feeders collected at times when DA was detected in seawater. Additionally, anchovy and sardine were the only species whose mean viscera DA levels exceeded the safety limit of 20 $\mu\text{g DA g}^{-1}$ set by the U.S. Food and Drug Administration.

DA distribution in fish viscera and muscle tissue was uneven. DA was found in viscera of anchovy, sardine and jacksmelt, but DA levels in muscle tissue were up to 2 orders of magnitude lower than in the viscera of anchovy and sardine, and were non-detectable in jacksmelt muscle tissue. Lefebvre et al. (2001) also showed that DA uptake in anchovy muscle tissue was low and generally 3 orders of magnitude less than DA levels in the viscera. Low absorption of DA from the gastrointestinal tract has also been observed in mice and monkeys (Iverson et al. 1989, Truelove et al. 1996). We further showed that DA in muscle tissue of sardines was not significantly correlated with DA levels in their viscera. The low DA levels in muscle tissue may be the residual DA after the 'first pass' renal and biliary excretion (Lefebvre et al. 2007).

Catch storage methods may alter the amount of toxin to which anglers are exposed since total DA levels in fish and in fish viscera declined significantly with time during storage (Table 3). However, muscle tissue DA levels did not significantly change after storage even though mean DA levels in muscle tissue of sardines were higher after storage. These results suggest that some of the DA may have diffused from the viscera into the muscle, but that the degradation rate may be

faster than the diffusion rate. Lefebvre et al. (2001) have also suggested that DA can diffuse in anchovy during storage. In addition, DA degradation due to storage has also been documented (1) in particulate DA samples of phytoplankton stored in GF/F filters for >2 months at +4°C and -20°C (Lane & Kudela 2007) and (2) in aqueous solutions stored at -12°C (Quilliam 2003). Although the sardine samples examined here were dissected 7 hrs after being captured and minimal diffusion and degradation may have occurred in that short amount of time, we present the first data related to measurements of DA diffusion and degradation in fish over a longer time scale (i.e. months).

The presence of DA in local waters (nearshore and offshore) was significantly associated with the presence of DA in the viscera of all three fish groups. The only non-significant association was observed between DA in pelagic omnivores and DA measured in offshore waters. This result was probably due to the small sample size. The significant associations indicate that fish, with different dietary habits and caught within 7 d of the DA detection are likely to contain detectable DA.

Moreover, sediment and benthic prey invertebrates may represent an additional source of DA contamination for pelagic omnivores and benthic feeders when DA is not detected in the water. False negatives (Fig. 4A,B) were likely observed for these fish groups since they can feed on benthic invertebrates or sediment. Sediments are effective adsorbents of DA and can act as DA reservoirs after a toxic *Pseudo-nitzschia* bloom has subsided (Burns & Ferry 2007). Sediment was found in the viscera of all pelagic omnivores and benthic-feeders analyzed in this

study, except for Pacific mackerel (Table 1). Additionally, DA has been detected in inkeeper worms (*Urechis* sp.), rock crabs, acorn barnacles (*Balanus glandula*, *Balanus nubulis*) and sea anemones (*Aiptasia* sp.) collected at SCW when no toxic *Pseudo-nitzschia* or DA were detected in the seawater column, implying that some invertebrates in Monterey Bay are able to retain DA after the DA toxic event, or that they access DA-contaminated sediment or organisms after a water column event has ended (Cheung 2007, Garcia et al. 2007, Vigilant 2007, Kvitek et al. 2008). In addition, pelagic omnivores and benthic-feeders were sometimes found not to be contaminated with DA when DA was detected in the water (i.e. false positives on Fig. 4A and B), which may further indicate that these species feed on prey that may or may not be contaminated with DA at the time of feeding.

California mussels may not reliably predict the presence of DA in fish. DA presence in mussels collected within 7 days of fish capture was not associated with DA presence in the viscera of planktivores and benthic-feeders. DA presence in mussels, however, was significantly associated with DA presence in the viscera of pelagic omnivores, though sometimes DA was present in the viscera of pelagic omnivores when no DA was detected in mussels. Three factors may explain the lack of association between DA in the viscera of fish and in mussels, and why DA was sometimes detected in omnivorous fish, but not in mussels. First, mussels have faster depuration rates (50–87% day⁻¹) and may reflect the immediate presence of DA in water, whereas fish can retain DA for longer periods (with depuration rates of 30% day⁻¹) (Novaczek et al. 1992, Lefebvre et al. 2007, Krogstad et al. 2009). Thus, the

different depuration rates may account for the dissociation between DA in mussels and fish viscera, given the same initial exposure. Second, mussels and fish feed differently: mussels are sessile benthic organisms that filter the toxic cells out of the seawater at one location, whereas fish species analyzed in this study are highly mobile and may acquire DA through routes other than by filtration (e.g. by ingesting sediment or consuming DA-contaminated organisms). Finally, DA is more readily detected in fish viscera (as it represents source material that leads to contamination) and if comparisons were done using DA levels in whole fish body instead of DA levels in fish viscera, we would have not detected DA in fish that had low viscera DA levels (i.e. pelagic omnivorous and benthic feeding fish) at times when DA was not detected in mussels.

DA exposure levels calculated for anglers were equivalent to asymptomatic doses (i.e., DA levels are measurable in fish, but below the dose that results in recognizable acute effects attributed to ASP; Table 4). The highest DA dose that anglers would likely ingest would result from consuming freshly caught whole anchovies or sardines, assuming they consumed 50g of fish (the standard amount of fish ingested by California anglers) (OEHHA 2001). Such a dose corresponds to 2.4% and 1.1%, for consumption of anchovies and sardines, respectively, of the DA dose (1 mg kg^{-1}) that can induce the lowest adverse effects of ASP (Toyofuku 2006) (Table 4). Anglers who consume only the muscle tissue of anchovy or sardine (i.e. those who remove the viscera before eating the fish) and whole jacksmelt would be exposed to

even lower DA doses, with even lower levels ingested by consumers of Pacific mackerel (Table 4).

It is unknown if the ingestion of asymptomatic DA doses can harm humans over the long term. Low DA levels (0.002- 0.11 $\mu\text{g g}^{-1}$) detected in the urine of California sea lions have been associated with a novel neurological syndrome characterized by epilepsy (Goldstein et al. 2008). More recently, Lefebvre et al. (2009) showed that DA doses below those that cause observable signs of behavioral injury in zebrafish can downregulate several genes involved in important biochemical processes, suggesting potential neurological risk associated with asymptomatic DA exposures. In contrast, experiments with rats have demonstrated that low DA doses can precondition the brain and induce tolerance against higher DA doses in young, but not in aged rats (Kerr et al. 2002, Hesp et al. 2004). Thus, whether or not exposure to asymptomatic DA doses can harm (or benefit) humans over time remains to be determined. Nevertheless, in humans, the most susceptible individuals to low DA doses may include pregnant and nursing women, since experiments with rats showed that (1) low DA doses may be transferred through milk and placenta, and accumulated in the amniotic fluid of pregnant rats and the brain of prenatals (Maucher & Ramsdell 2005, 2007) and also because (2) DA can reach the brain without restriction causing neurological and behavior effects later in the life for prenatal rats (Dakshinamurti et al. 1993, Levin et al. 2005, Maucher & Ramsdell 2007). In humans, elderly (> 65 yrs) are most susceptible, since DA is cleared from the plasma through the kidneys (Suzuki & Hierlihy 1993), and these individuals are thought to

have reduced ability to eliminate DA through the renal system (Xi et al. 1997, Pulido 2008). Additionally, higher DA levels of 275, 588 and 1,815 $\mu\text{g g}^{-1}$ have been detected in viscera of jacksmelt, sardine and anchovy caught at other times in Monterey Bay (Lefebvre et al. 2002a, b). Thus, SCW anglers could be exposed to even higher DA doses than those reported here.

Conclusions

Our study showed that SCW anglers can be exposed to asymptomatic DA doses through consumption of their catch. DA levels of exposure are dependent on the fish species and parts being consumed, as well as on storage methods and the presence of particulate DA in the water at the time the fish is caught. In addition, we determined that California mussels do not adequately predict DA in fish. To minimize DA exposure, anglers should eviscerate (clean) their fish before consumption, since the highest DA levels were found in the viscera of freshly captured fish. Furthermore, DA is heat-stable and may not be completely eliminated from fish tissue when cooked (McCarron & Hess 2006). For pelagic planktivores where DA levels were found in both viscera and muscle tissue, the removal of the viscera may not be sufficient to eliminate exposure to DA. Thus, consumption of muscle tissue of planktivorous fish caught during toxic blooms may result in low-level DA exposure; the consequences of such asymptomatic doses are worrisome, given recent evidence from studies with other mammals.

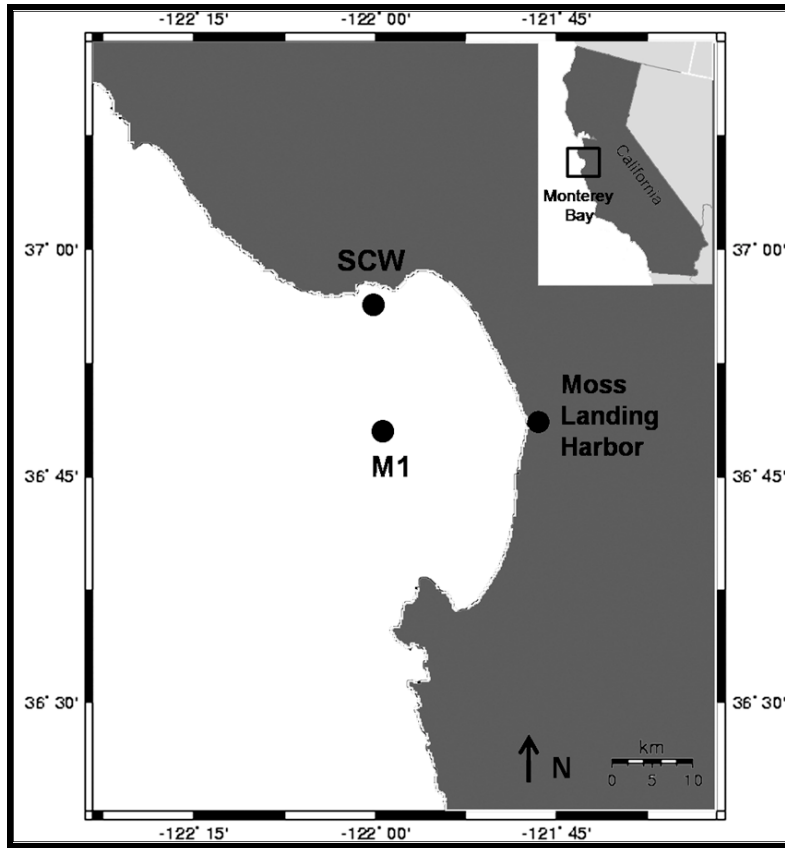


Fig. 1. Monterey Bay, California, showing sampling sites: Santa Cruz Wharf (SCW), offshore mooring (M1) and Moss Landing Harbor, where vessel-caught fish were obtained.

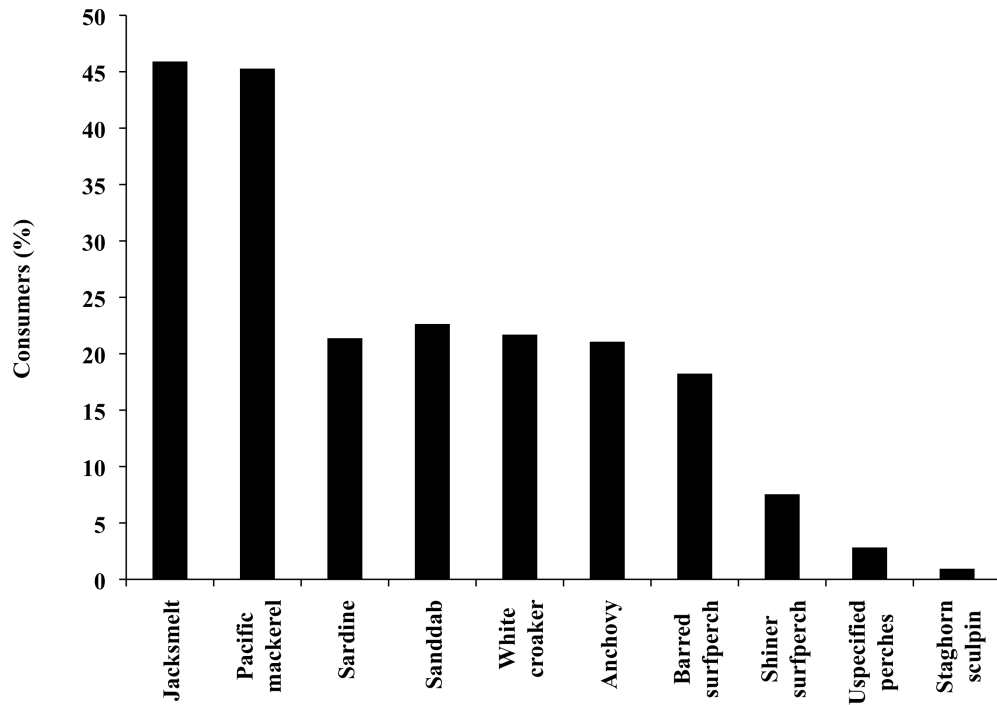


Fig. 2. Most commonly consumed fish species caught by Santa Cruz Wharf (SCW) anglers (N = 318).

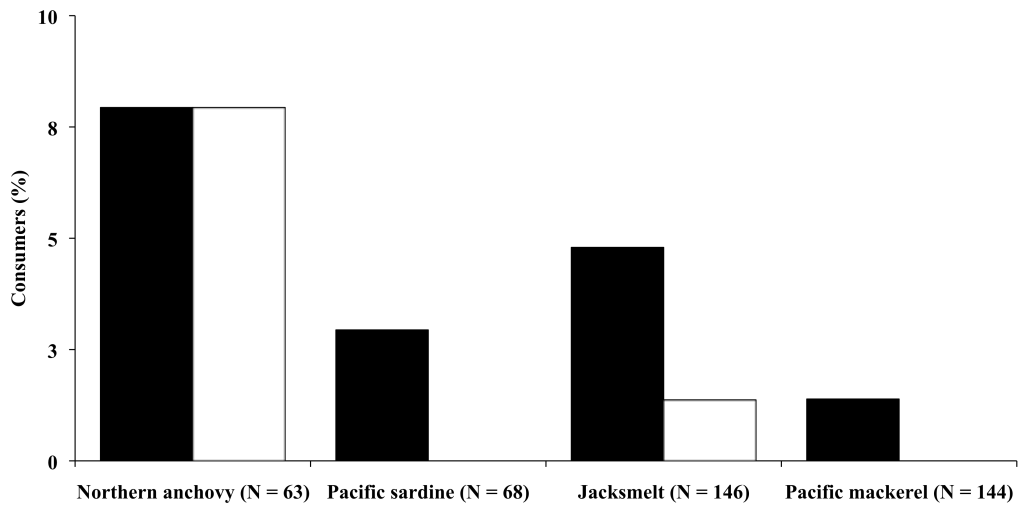


Fig. 3. Percentage of consumers of fishes caught at the Santa Cruz Wharf who do not remove viscera before preparing fish for consumption (black bars) and who eat viscera (white bars).

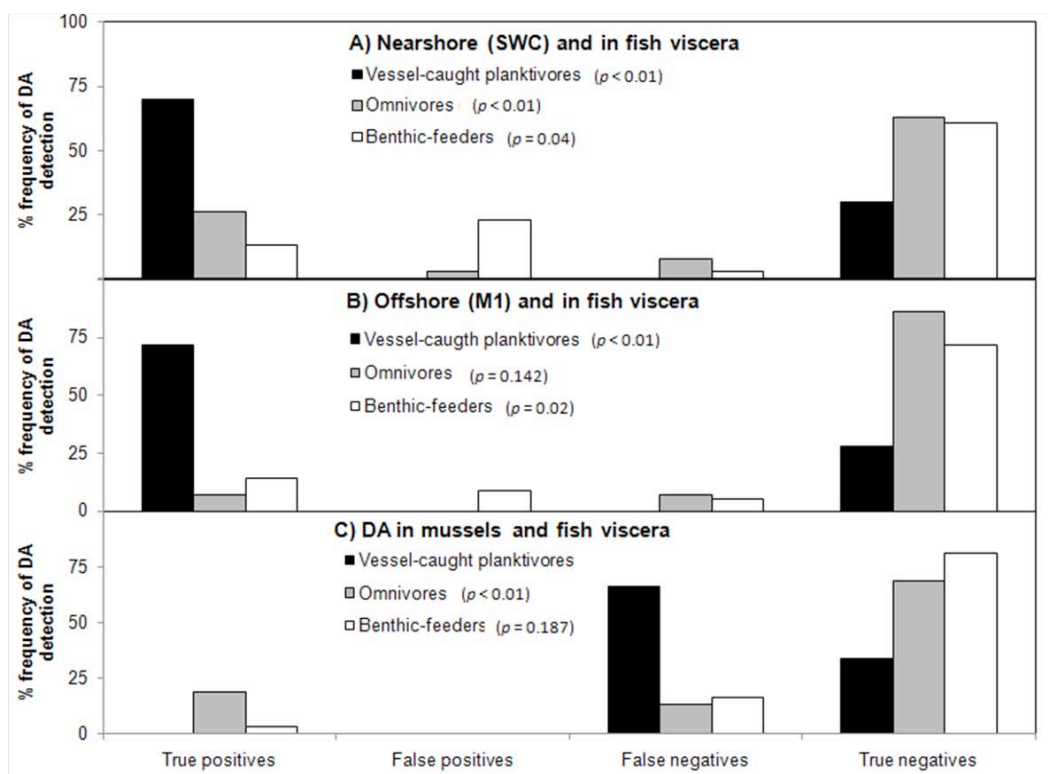


Fig. 4. Percent frequency of different associations between domoic acid (DA) detection in (A) nearshore waters (Santa Cruz Wharf [SCW]), (B) offshore waters (M1) or (C) SCW mussels and fish viscera. True positives: DA detected in water/mussel and fish; false positives: DA detected in water/mussel, but not in fish; false negatives: DA to detected in water/mussels, but detected in fish; true negatives: DA not detected in water/mussels and fish. Fisher's exact test p-values indicated whether DA detection in fish was significantly associated with DA in mussels in nearshore and offshore waters.

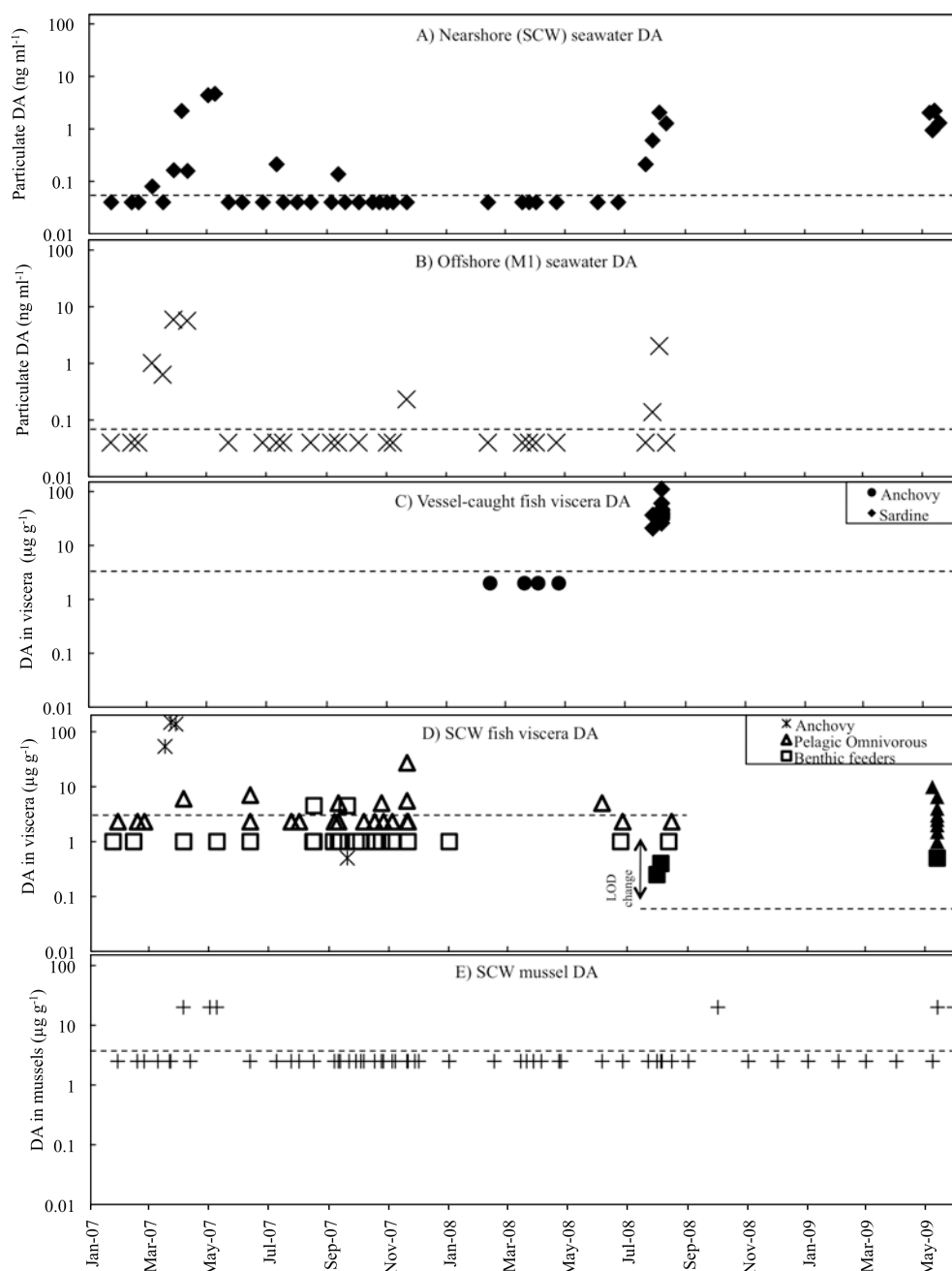


Fig. 5. Domoic acid (DA) levels in (A) seawater at the Santa Cruz Wharf (SCW), (B) seawater at M1, (C) viscera of vessel caught fish, (D) viscera of wharf-caught fish and (E) SCW mussels. Dashed line indicate HPLC limit of detection (LOD). Filled symbols in (D) show samples where ELISA was used and the step change in LOD. A DA constant, a value below the detection limit, was added to allow display of values in log format.

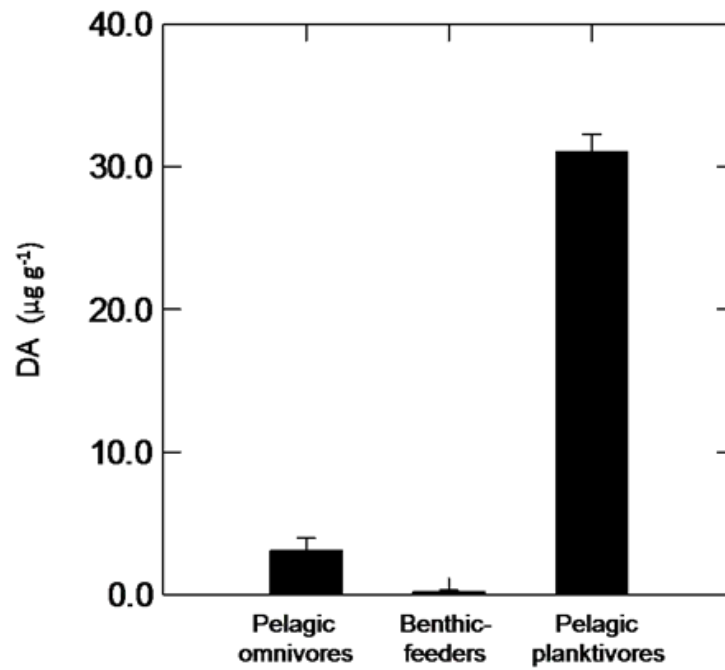


Fig. 6. Mean (\pm SE) domoic acid (DA) levels in the viscera of pelagic omnivores (N = 11), benthic feeders (N = 12), and pelagic planktivores (N = 51) caught when DA was detected in the water.

Table 1. Feeding habit groups for fish species caught, number of fish analyzed for stomach content, and typical food items observed.

Fish species grouped by feeding habit	Number of specimens analyzed	Food items observed in viscera of species in this study
Pelagic planktivores		
Northern anchovy (<i>Engraulis mordax</i>)	24	Centric and pennate diatoms (including fragments of <i>Pseudonitzschia</i> spp.), dinoflagellates, silicoflagellates, tintinnids
Pacific sardine (<i>Sardinops sagax</i>)	8	Centric and pennate diatoms (including fragments of <i>Pseudonitzschia</i> spp.)
Pelagic omnivores		
Jacksmelt (<i>Atherinopsis californiensis</i>)	21	Crustaceans (barnacles, amphipods/isopods, mysids), centric diatoms, fish bones, macroalgae, parasitic worms ^a , sediment
Pacific mackerel (<i>Scomber japonicus</i>)	5	Crustaceans, macroalgae, parasitic worms ^a
Benthic-feeders		
Barred surfperch (<i>Amphistichus argenteus</i>)	3	Clam/mussel shells, crustaceans, parasitic worms ^a , sediment
Rainbow surfperch (<i>Hypsurus caryi</i>)	1	Clam/mussel shells, crustaceans, sediment
Shiner surfperch ^b (<i>Cymatogaster aggregata</i>)	9	Clam/mussel shells, crustaceans (amphipods/isopods, mysids), copepods, foraminifera, macroalgae, parasitic worms ^a , sediment
Speckled Sanddab ^b (<i>Citharichthys stigmaeus</i>)	16	Clam/mussel shell, crustaceans (mysids), macroalgae, polychaetes, sediment
Spotfin surfperch ^b (<i>Hyperprosopon anale</i>)	1	Crustaceans
Staghorn sculpin ^b (<i>Leptocottus armatus</i>)	55	Crustaceans (isopods/amphipods, mysids), macroalgae, parasitic worms ^a , sediment
White croaker ^b (<i>Genyonemus lineatus</i>)	15	Clam/mussel shells, crustaceans (isopods/ amphipods, mysids), centric diatoms, fish bones, macroalgae, parasitic worms ^a , polychaetes, sediment

^a Parasitic worms were nematodes or flatworms, and could be part of the fish diet or parasites on the fish specimens.

^b Species reported to prey on benthic as well as planktonic organisms (Cailliet et al. 2000)

Table 2. Domoic acid (DA) levels ($\mu\text{g g}^{-1}$) in viscera samples of 11 fish species. n: number of samples with detectable levels of DA; N: total number of samples analyzed. -: not detected.

Fish species grouped						
By feeding habit	Range	Mean	Median	SD	n	N
Pelagic planktivores						
Northern anchovy	9 - 148	71	54	67	5	18
Pacific sardine	15 - 110	37	36	15	51	51
Pelagic omnivores						
Jacksmelt	2.5 - 27	7.3	4.5	7.8	9	39
Pacific mackerel ^a	1.5 - 2.3	1.9	1.9	0.3	7	9
Benthic-feeders						
Barred surfperch	-	-	-	-	0	4
Rainbow surfperch ^a	-	1.5	-	-	1	1
Shiner surfperch	-	-	-	-	0	2
Speckled sanddab	2.9 - 4.5	3.7	3.7	1.1	2	9
Spotfin surfperch	-	-	-	-	0	1
Staghorn sculpin ^a	0.25- 0.4	0.3	0.3	0.1	3	21
White croaker	-	-	-	-	0	5

^a DA analyzed by ELISA

Table 3. *Sardinops sagax*. Mean (\pm SD) domoic acid (DA) levels ($\mu\text{g g}^{-1}$) in whole and parts of fresh-caught (7h after catch, N = 22) versus frozen sardines (11mo after catch, N = 5) sardines

Sample	Fresh	Frozen	<i>F</i>_{1,26}	p
Viscera	38 (\pm 9.0)	12 (\pm 4.6)	129.14	< 0.01
Muscle tissue	0.5 (\pm 0.6)	1.2 (\pm 1.0)	1.52	0.22
Whole sardine	4.6 (\pm 1.3)	2.4(\pm 0.6)	19.83	< 0.01

Table 4. Domoic acid (DA) dose intake by Santa Cruz Wharf anglers and percentage of lowest adverse effects level dose (LOAEL) of 1mg kg^{-1} body weight that causes Amnesic Shellfish Poisoning (ASP) (Toyofuku 2006). Dose calculations of DA base on a 50g d^{-1} fish meal (standard amount of fish consumed by California anglers; OEHHA 2001).

Part consumed Species	Max DA concentration ($\mu\text{g g}^{-1}$)	DA dose (mg per 50g^{-1} of fish meal$^{-1}$)	Percent of LOAEL
Whole fish			
Northern anchovy	28.3	1.43	2.4
Pacific sardine	12.8	0.64	1.1
Jacksmelt	2.3	0.11	0.2
Pacific mackerel	0.2	0.01	0.02
Muscle tissue			
Northern anchovy	10.8	0.54	0.9
Pacific sardine	1.8	0.09	0.2

CHAPTER FOUR

A note on the detection of the neurotoxin domoic acid in beach-stranded *Dosidicus gigas* in the Southern California Bight

Abstract

The first occurrence of the neurotoxin domoic acid (DA) in Humboldt squid (*Dosidicus gigas*) during a toxic *Pseudo-nitzschia* bloom in the Southern California Bight is reported. Bloom levels of cells within the *Pseudo-nitzschia delicatissima* group were detected on 6 July 2009 at 4 nearshore collection sites in the Southern California Bight (Scripps Pier, Newport Pier, Goleta Pier and Sterns Wharf). Particulate DA was detected in all of these locations, except for Newport Pier. Stranded Humboldt squid were found south of the Scripps pier 5 days after the toxic bloom was detected. DA was measured using ELISA and low DA concentrations were detected in the stomach or mantle tissue of the stranded specimens. Stomach content analysis indicated that possible DA vectors to Humboldt squid included both pelagic (Pacific hake, *Merluccius productus*, and Pacific sardine, *Sardinops sagax*) and nearshore (pile surfperch, *Damalichthys vacca*, and shiner surfperch, *Cymatogaster aggregata*) fish species. Although low DA levels were detected in stranded squid specimens, neurological symptoms of DA toxicity were not observed and low DA concentrations alone may not have been the cause of the strandings. Further studies should focus on DA toxic effects in *D. gigas* to verify whether or not

this pelagic predator can be affected by a toxin frequently detected in pelagic ecosystems influenced by the California Current System.

Introduction

The Humboldt squid *Dosidicus gigas* is a large neritic-oceanic squid and an important link between lower trophic levels and apex predators in the pelagic food web. *D. gigas* is an opportunistic predator of small mesopelagic, pelagic and demersal fish, crustaceans and squid (Markaida and Sosa-Nishizaki 2003; Field et al. 2007) and common prey for billfish, sharks, pinnipeds, and toothed whales (Olson and Watters 2003; Ruiz-Cooley et al. 2004; Vetter et al. 2008). *D. gigas* is endemic to the eastern Pacific Ocean between 30° N and 20-25° S and 140° W (Nigmatullim et al. 2001) and can cover great horizontal distances within this range at speeds up to 30 km d⁻¹ (Gilly et al. 2006). Poleward excursions have been reported in both hemispheres, with significant range expansions taking place over the past decade (Zeidberg and Robison 2007; Field et al. 2007; Alarcón-Muñoz et al. 2008).

Large-scale (ranging from dozens to thousands of individuals) beach stranding events of *D. gigas* have taken place both historically and recently along the Eastern Pacific rim, particularly in the fringes of the squid's range and during periods of episodic high abundance (Mearns 1988; Alarcón-Muñoz et al. 2008). Beach strandings frequently result in flurries of short-term media attention and speculation into the causes of mortality, often with minimal scientific consultation. The frequency and range of reporting on these events has spiked over the past five to ten years along

the west coast of the USA and Canada (Fig.1). Among the most significant strandings in recent years include events in July, 2002 in La Jolla, CA; October, 2003 in Carmel, CA, October, 2004 in Westport, WA; January, 2005 in Los Angeles and Newport Beach, CA; March, 2005 in Oceanside, CA; October, 2008 in Westport, WA; July, 2009 in La Jolla, CA (samples reported in this manuscript); September, 2009 in Westport, WA, Seaside, OR and Vancouver Island, British Columbia (J. Field, unpublished data). The reasons for *D. gigas* mortality and strandings remain unknown.

The primary focus of this paper is to explore the hypothesis that *D. gigas* can be exposed to domoic acid (DA) during toxic *Pseudo-nitzschia* algal blooms. Investigation of this hypothesis may help understand whether domoic acid poisoning (DAP) can be considered a contributing factor to *D. gigas* mortality and strandings. DA is a neurotoxin produced by several species of the diatom *Pseudo-nitzschia* (Moestrup and Lundholm 2007) and has caused mass mortality of marine mammals and birds (Work et al. 1993; Scholin et al. 2000). However, determining that DAP is the cause of death in marine animal strandings is difficult. Studies that have come to that conclusion used a combination of observations such as (1) DA detection in hundreds of specimens in question and/or in their prey items; (2) DA detection in the water along with high concentration ($<10^4$ cells L⁻¹) of toxin producing cells; and (3) observations of typical DA neurological symptoms (i.e., seizures, ataxia, head weaving, and stereotypic scratching) and (4) histopathology to show lesion in

hippocampus brain region characteristic of DA poisoning (Work et al. 1993; Scholin et al. 2000; Gulland et al. 2002).

Accordingly, pelagic predators have been detected with DA, but DA toxicity effects and whether or not DA could cause the death of these animals have not been verified. For example, North Atlantic right whales (*Eubalaena glacialis*), pygmy sperm whales (*Kogia breviceps*) and dwarf sperm whales (*Kogia sima*) found stranded along the U.S. Atlantic coast were tested positive with low DA levels (Fire et al. 2009; Leandro et al. 2010), but DA toxicity symptoms were not determined in these studies and thus DA could not be related to the cause of stranding. However, Leandro et al. (2010) hypothesized that the observed long-term exposure of North Atlantic right whales to DA may perhaps enhance mortality due to other well-documented factors in their populations since it has been found that chronic DA exposure can impair navigational abilities of other marine mammals such as sea lions (Goldstein et al. 2007). Furthermore, high DA levels have been found in feces and prey of blue whales (*Balaenoptera musculus*) and humpback whales (*Megaptera novaeangliae*) during a toxic *Pseudo-nitzschia* bloom in Monterey Bay (California, USA) (Lefebvre et al. 2002). Although DA toxicity was not observed, the high DA doses that these whales were exposed to could lead to DA neurotoxicity effects (Lefebvre et al. 2002).

Additionally, DA has been detected in cephalopods such as market squid (*Loligo opalescens*), cuttlefish (*Sepia officinalis*) and common octopus (*Octopus vulgaris*) after feeding on DA-contaminated prey items (Costa et al. 2004; 2005;

Bargu et al. 2008). *D. gigas* could be exposed to DA through a variety of vectors. Humboldt squid are active predators of small pelagic fish such as northern anchovies (*Engraulis mordax*) and Pacific mackerel (*Scomber japonicus*) (Markaida and Sosa-Nishizaki 2003; Markaida 2006; Field et al. 2007), which have been previously identified as DA vectors to marine mammals and birds (Sierra-Beltran et al. 1998; Lefebvre et al. 1999). Krill are also a potential vector of DA, as they are a prey item of *D. gigas* (Field et al. 2007) and have been found to acquire DA (Bargu et al. 2003). Furthermore, Pacific hake consume both krill and northern anchovies (Buckley and Livingston 1997; Mackas et al. 1997), and are a key prey item of *D. gigas* in California waters (Field et al. 2007).

DA effects in cephalopods have not been confirmed. Only a few studies focused on the DA effects in invertebrates, mostly shellfish, and the results are conflicting (Maeda et al. 1987; Jones et al. 1995a; b; Dizer et al. 2001; Blanco et al. 2006; Liu et al. 2007a, b, 2008). However, *D. gigas* might be susceptible to DA neurotoxicity effects. DA is structurally similar to glutamic acid, a neurotransmitter in central nervous systems (CNS) of mammals (Nakajima et al. 1985). Such a similarity allows DA to bind to the same receptors of glutamic acid and trigger a cascade of molecular reactions inducing neuronal degradation, and consequently, DA neurotoxicity effects (Pulido 2008). Evidence indicates that DA binds with high affinity to 2 glutamate receptor subtypes: kainic acid and AMPA receptors (Hampson et al. 1992), a third receptor subtype, NMDA, is a co-participants in inducing DA neurotoxicity effects (Pulido 2008). These receptors are concentrated in the

hippocampus of mammals (Foster et al. 1981; Debonel et al. 1989; Scallet et al. 1993), a brain region responsible for memory and spatial navigation and thus, hippocampus lesions are common in mammals and humans after exposure to specific DA doses (Teitelbaum et al. 1990; Gulland et al. 2002). Cephalopods have the largest brains of any invertebrate with a complexity analogous to those of vertebrates (Messenger 1996), and it has been suggested that the arrangement of neurons in the vertical lobe of octopus is involved in memory and it has structural similarities to the vertebrate hippocampus (Boycott and Young, 1950; Young, 1965). Moreover, glutamic acid also serves as a neurotransmitter in invertebrates (Messenger, 1996) and all 3 subtypes of glutamate receptors (i.e., kainic acid, NMDA and AMPA receptors) have been detected in central and peripheral nervous systems of cephalopods (Evans et al. 1992; Messenger, 1996, Garcia 2002; Lima et al. 2003; Di Cosmos et al. 2004). The fact that cephalopods have highly developed CNS and similar glutamate receptor as mammal potentially indicate that Humboldt squid may be susceptible to DA neurotoxicity effects.

The goal of this study was to ascertain whether beach stranded *D. gigas* found in two different locations in San Diego (California, USA) were exposed to DA during the summer of 2009. Our approach was to (I) measure DA in stomach content and lining as well as in mantle tissue of 5 stranded *D. gigas*, (II) analyze surface water particulate DA in locations within 300km of the stranding site, and (III) examine the stomach contents of the stranded individuals to identify possible DA vectors.

Material and Methods

Water sample collection

Surface (~1m) seawater samples were collected weekly from five pier sampling stations as part of the Southern California Coastal Ocean Observing (SCCOOS) Harmful Algal Bloom Monitoring Program (Fig. 2). Samples were collected with surface bucket and provided subsamples for DA analysis and *Pseudo-nitzschia* identification and quantification. Cross-contamination of samples was avoided by acid washing the sampling gear and bucket with dH₂O 3 times after sample collections and 3 times with seawater before sample collections.

DA in seawater

Particulate DA concentrations were measured by filtering 200 mL of seawater onto GF/F Whatman filters. Filters were stored frozen at -80°C until shipped in dry ice and analyzed within 1 week to 5 months of sample collection, using Rapid Enzyme-Linked Immunosorbent Assay (ELISA) obtained from Mercury Science, Inc. (Durham, NC27713) at the University of Southern California following Schnetzer et al. (2007). The detection limit for the ELISA assay on water samples was 0.02 ng mL⁻¹ (ppb).

Toxic Pseudo-nitzschia identification and quantification

Abundances of two size class categories of the genus *Pseudo-nitzschia*, *seriata* group (frustule width > 3µm) and *delicatissima* group (frustule width < 3µm), were determined from settling 10-50 mL of seawater preserved with 4%

formaldehyde (Uthermöhl 1958). Cells were categorized under an inverted light microscope. DA-producing *Pseudo-nitzschia* species are found in both of these groups (Hasle and Syvertsen 1997; Moestrup and Lundholm 2007)

Humboldt squid sample collection

Stranded *D. gigas* were collected on 11 July 2009 at La Jolla Shores beach and on 12 July 2009 at La Jolla Cove. The stranding event was relatively small; only five animals were found in sufficiently good condition to be sampled. Based on media reports and conversations with beachgoers, we concluded that the animals stranded in the early morning of 11 July, and that they were alive at the time of stranding. At the time of dissection, they were dead, most likely through a combination of asphyxiation and partial predation by seagulls. We estimate that the animals dissected on 11 July had been dead for 6-8 hr, while those dissected on 12 July had been dead for 24-30 hr. The mantles were in sufficiently good condition that dorsal mantle length (DML) could be measured. Stomachs and samples of mantle tissue (approximately 2x2x2 cm) were removed and frozen at -20° C, then moved to -80° C within 48 hours. All samples were kept frozen for 3-4 months until stomach content observations and DA analysis could be performed.

Stomach content and DA analysis of Humboldt squid

The stomach contents of the five squid sampled for DA were evaluated as described in Field et al. (2007). Following these observations, DA was analyzed in stomach contents and lining (hereafter referred to as stomach) and mantle tissue using ELISA obtained from Mercury Science, Inc. (Durham, NC27713). Stomach and

mantle tissue samples were weighed and each sample was homogenized with a hand-held tissue homogenizer (Tissue Mixer, model PNF2110, Fisher Scientific). Aliquots of 4g were removed from the homogenized samples and 16ml of 50:50 MeOH:Nanopure was added to the 4g aliquots. Samples were then sonicated with a Sonifier cell disruptor (Model W185D, Branson Sonic Power) and centrifuged for 20min at 3800rpm (1698x g). The supernatant were filtered through a 3 μ m polycarbonate filter. The filtrate was diluted at 1:100 and 1:1000 in the buffer solution provided in the ELISA kit and aliquoted in 3 replicates for each sample (Table 1). Samples diluted at 1:1000 were below the detection limit of 0.1 ng mL⁻¹ (ppb) in the ELISA kit, but samples in the 1:100 dilution were within the detection limit. The diluted samples were used in the ELISA plates following the protocol accompanying the kit. An EMax Precision Microplate Reader (Model E10968, Molecular Devices) was used to measure absorbance at 450nm. Final concentrations of DA in squid samples are expressed as μ g DA g⁻¹ wet tissue mass.

Results & Discussion

DA was detected in the stomach of one *D. gigas* specimen and in the mantle tissue of one specimen found stranded in La Jolla beach (Table 1). Mantles and stomachs were not labeled individually, so it is unknown whether the specimen with DA in its mantle tissue also had DA in its stomach. Low particulate DA concentration, just above the detection limit, was recorded in surface waters at Scripps Pier 5 days before the stranding occurred (Fig. 3A). Scripps Pier is located 3

to 5 km north of the stranding locations, well within documented swimming speeds for *D. gigas* of 30 km day⁻¹ (Gilly et al. 2006). Particulate DA was also detected on Goleta Pier and Stearns Wharf, but those are located >30km north of the stranding location (Fig. 2 and 3A). DA levels at these three sites peaked on 6 July 2009 and tapered off to below detection levels within one to three weeks. Peak DA concentrations for July occurred at the same time as an increase in the abundance of cells within the *Pseudo-nitzschia delicatissima* group was observed (Fig. 3C). At these 3 locations, *P. delicatissima* group densities ranged from 5.6 x 10⁴ to 5.4 x 10⁵ cells L⁻¹, which are typical bloom levels (i.e. >10³ cells L⁻¹). Moreover, the abundance of cells within the *Pseudo-nitzschia seriata* group never exceeded 9.1 x 10² cells L⁻¹ at the Scripps Pier. This suggests that *Pseudo-nitzschia* species from the *delicatissima* group were probably responsible for the DA production at that time.

Squid stomach analyses indicated the presence of Pacific sardine, shiner surfperch (*Cymatogaster aggregata*), and pile surfperch (*Damalichthys vacca*). DA has been detected in Pacific sardine and other surfperch species (i.e. rainbow surfperch) within 7 days of the detection of DA in surface waters (Mazzillo et al. 2010), suggesting that these fish found in the stomachs of *D. gigas* could have been the DA vectors. Other prey items and potential DA sources included Pacific hake, topsmelt silversides (*Atherinops affinis*) and an unidentified species of *Gonatus* squid, future work should include the examination of these species during periods of algal blooms. Macroalgae and sand were also observed in all stomachs, suggesting other behaviors that are not typical of the class cephalopoda.

Here we showed that *D. gigas* can be exposed to DA by preying on pelagic and nearshore species when toxic *Pseudo-nitzschia* blooms are detected. However, linking the Humboldt squid strandings themselves to domoic acid poisoning (DAP) is difficult. First, DA was not detected in all stranded individuals, indicating that there could be other reasons for their death. Most of the observed prey items are frequently, if not exclusively, found in shallow water, and the nearshore species are unusual targets for *D. gigas* further demonstrating the opportunistic feeding strategy of this species. The presence of sand and algae in the stomachs also supports the hypothesis that these animals were actively foraging in shallow waters. If this is an atypical habitat for them to exploit, they may have become disoriented and accidentally swam onshore.

Second, we cannot determine from this study whether DA was absorbed from the stomach into the bloodstream and interacted with the squid nervous system via blood causing DAP, although we have evidence that DA was transferred to the mantle tissue. We measured low DA values in the squid stomach (Table 1) and DA absorption from the gastrointestinal tract to the bloodstream is known to be minimal in vertebrates (Iverson et al. 1989; Truelove et al. 1997; Lefebvre et al. 2001). The range of DA values detected in the stomach and mantle tissue of stranded squid is within the lower limit range of DA detected in body fluids of DAP stranded California sea lions. Urine and serum of California sea lions were found to contain $0.03 - 3.72 \mu\text{g DA ml}^{-1}$ and $0.17 - 0.20 \mu\text{g DA ml}^{-1}$, respectively (Scholin et al. 2000). However, Scholin et al. (2000) also measured DA levels >6,000 times higher

in fecal material, and observed common DAP neurological symptoms in addition to the detection of DA, making the DAP diagnosis in these marine mammals more certain.

Although DAP was implicated in mass mortality of marine birds and sea lions (Work et al. 1993; Scholin et al. 2000), the causes of other marine mammal strandings remain largely unknown (Bogomolni et al. 2010), and the same appears to be true of Humboldt squid strandings. Even though we report for the first time *D. gigas* exposure to DA, our results cannot be implicated as the cause of death of the stranded squid since we do not know whether the low DA concentrations measured were absorbed from the squid gastrointestinal tract into the blood and interacted with squid CNS at enough concentrations to cause neurotoxicity effects. Nevertheless, it is quite possible that *D. gigas* could be exposed to higher DA levels than those observed here, since higher DA levels have been previously detected in the waters off the coast of California and in the prey of *D. gigas* (Trainer et al. 2000; Busse et al. 2006; Anderson et al. 2009; Mazzillo et al. 2010). Additionally, cephalopods have the similar glutamate receptors sites as mammals, and as DA binds to these sites it triggers toxicity which indicates that *D. gigas* can potentially be susceptible to DA neurotoxicity effects. However, juvenile leopard sharks (*Triakis semifasciata*) appear not to be affected by DA even though it possesses kainic acid-type glutamate receptors (Schaffer et al 2006). Thus, *D. gigas* susceptibility to DA toxicity is possible, but remains an unanswered question. Laboratory studies designed to verify DA toxicity in squids could be performed using the California market squid

(*Doryteuthis opalescens*) or Atlantic longfin squid (*Doryteuthis pealei*) as models since *D. gigas* cannot be kept in captivity for more than 10 days. Laboratory experiments should include observations of squid behavioral response to DA doses, measurements of DA uptake as well as quantification of DA in brain tissue to evaluate whether or not DA toxicity occurs in squids and thus could be responsible for *D. gigas* mass strandings. Sampling larger *D. gigas* stranding events would also be desirable, as our sample size for this study was limited by the small number of stranded individuals.

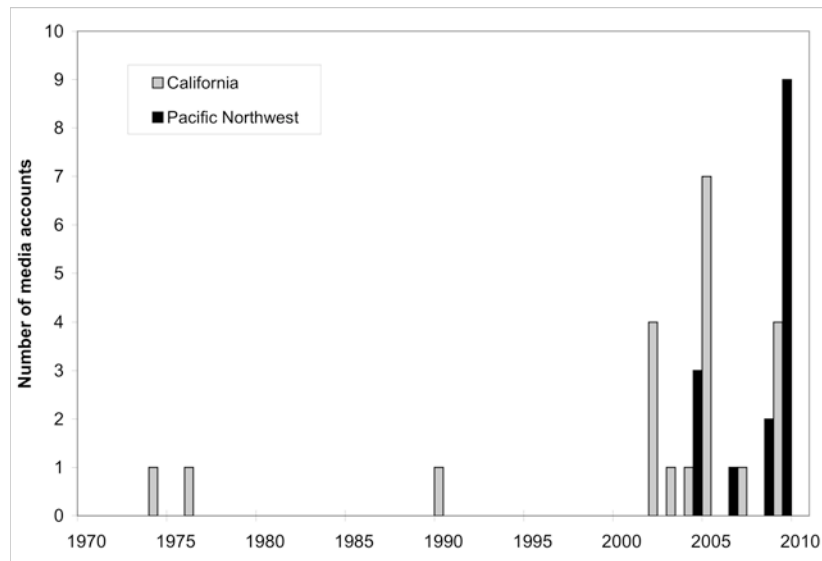


Fig. 1: Frequency of media reports of stranding events in California and the Pacific Northwest over the past forty years.

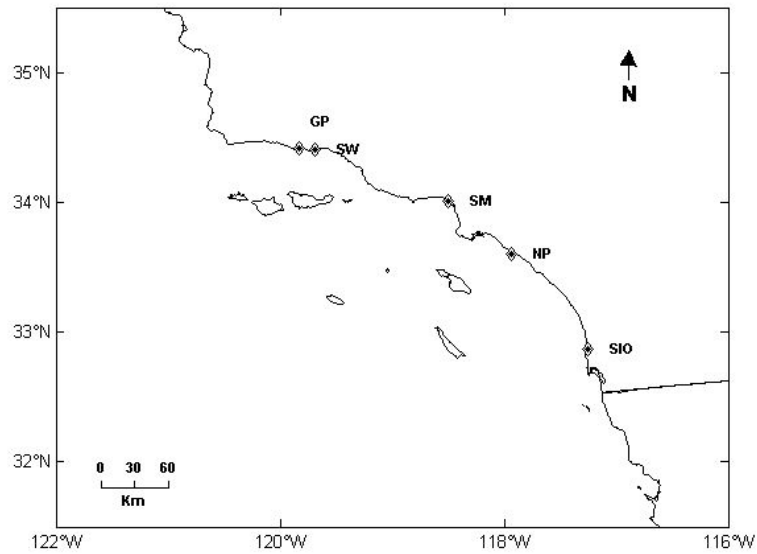


Fig 2: Particulate domoic acid sample locations: Scripps Institution of Oceanography Pier (SIO), Newport Pier (NP), Santa Monica Pier (SM), Stearns Wharf (SW), Goleta Pier (GP). Stranded squid were found between 3km and 6km south of the SIO pier.

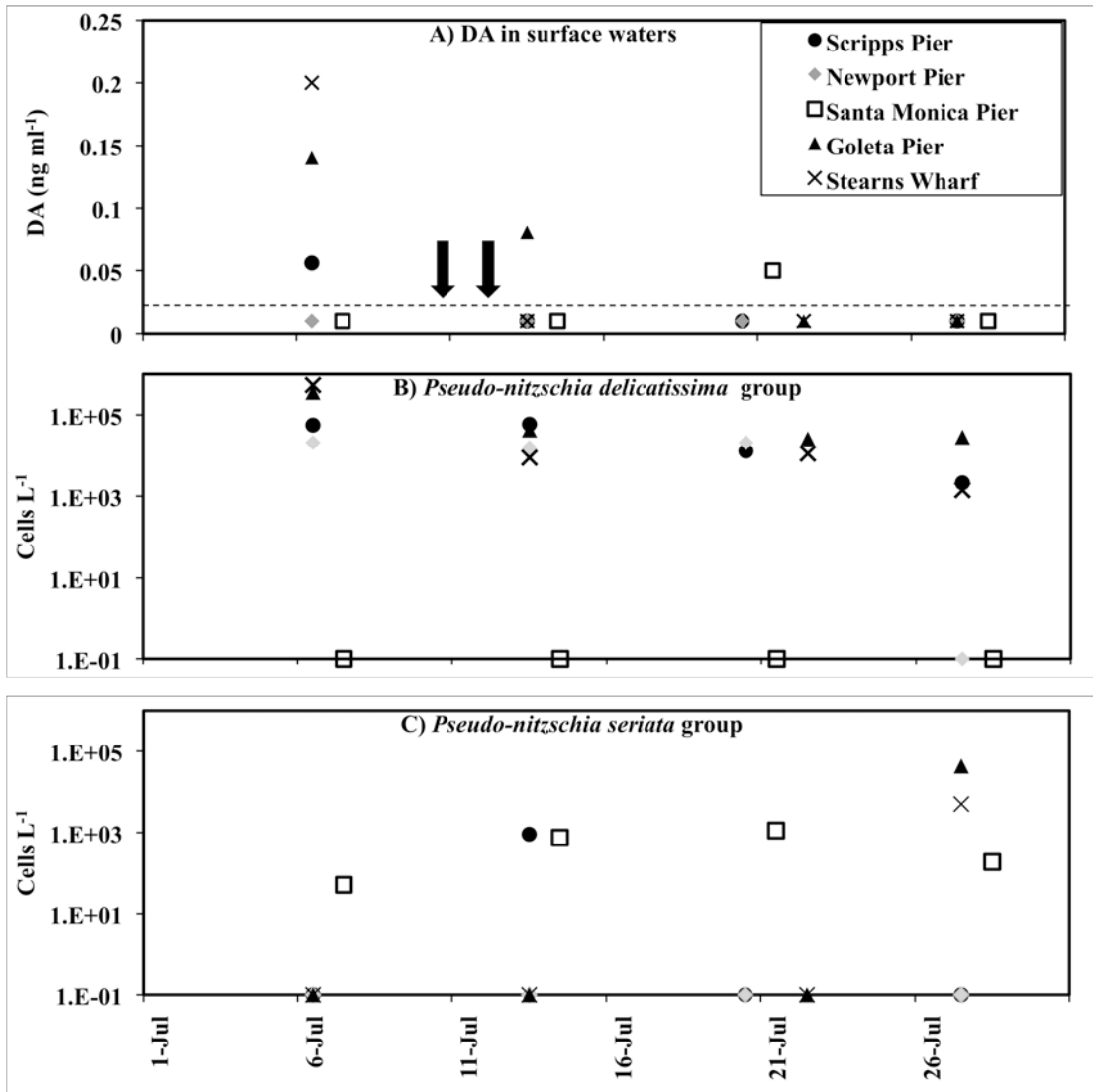


Fig.3. Particulate domoic acid (DA) levels (a), and cell density of *Pseudo-nitzschia delicatissima* group (b) and *Pseudo-nitzschia seriata* group (c) for 1-30 July 2009 from surface waters (1m) at five sites along southern the California coastline: Scripps Pier (●), Newport Pier (◆), Santa Monica Pier (□), Goleta Pier (▲), and Stearns Wharf Pier (×). Arrows in panel (a) indicate collection dates for squid analyzed here: horizontal dashed line indicates analytical detection limit.

Table 1. Domoic acid content ($\mu\text{g g}^{-1}$ or ppm) of *Doscidicus gigas* specimens collected in La Jolla, CA, on 11-12 July 2009.

Tissues analyzed for domoic acid (DA)	Mean	SD	Median	Range	Number of replicates	Date Collected	Individual Size (cm)	Sex
Stomachs	0.27	0.05	0.3	0.2-0.3	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	12-Jul-09	63	Female
	nd	nd	nd	nd	3	12-Jul-09	63	Female
Mantle tissue	0.43	0.05	0.4	0.4 – 0.5	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na

DISSERTATION CONCLUSIONS AND FUTURE WORK

This thesis showed, for the first time, the presence of an endoparasitic dinoflagellate *Amoebophrya* in 7 bloom-forming dinoflagellates species that occur throughout the California Current System (CCS). Parasitism by *Amoebophrya* may control harmful dinoflagellate blooms in Monterey Bay, California and likely blooms that occur throughout the coastal domains of the CCS. Evidence presented here also demonstrated the ecological role of parasitism in contributing to dinoflagellate species diversity levels and net phytoplankton community composition as well as temporally influencing trophic web structure during epidemic parasitic outbreaks in upwelling environments. To consider the use of this parasite as a bloom control agent, future research should focus on whether different *Amoebophrya* strains occur in the CCS and on host specificity levels. Studying the evolutionary relationship between host and parasite may also provide a better understanding of dinoflagellate bloom dynamics.

Additionally, this thesis verified that humans could be exposed to asymptomatic doses of domoic acid when consuming recreationally caught fish. The consequences of exposure to low DA levels are unknown, but worrisome given the recent findings on the effects of low DA doses in marine mammals (Goldstein et al. 2008) and the increase in the relative abundance of the domoic acid producer, *Pseudo-nitzschia* in the CCS coastal domains (Baron et al. 2010). Chapter 3 also verified that California mussels (*Mytilus californianus*) used to monitor DA toxicity

and alert consumers of shellfish may not adequately predict DA toxicity in fish.

Therefore, future work should (I) determine the effects of chronic low DA exposure of humans and (II) find an alternative monitoring strategy that could be used to effectively indicate fish toxicity levels and alert anglers from consuming DA-contaminated fish.

Finally, this research showed that the Humboldt squid (*Dosidicus gigas*) is exposed to domoic acid. However, domoic acid poisoning could not be conclusively implicated in the reported strandings of this pelagic predator, which recently invaded CCS domains. Future research should focus on the effects of DA in Humboldt squid to assess its sensitivity to a toxin that is often detected in the CCS.

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