HARMFUL EFFECTS AND RISKS SESSIONS

A Review of Feeding Preference and Deterrence in Three Faunal Species Associated with Cyanobacterial Blooms of *Lyngbya majuscula* in Southeast Queensland, Australia

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Abstract

Potentially harmful blooms of the benthic cyanobacterium *Lyngbya majuscula* have been occurring with increased frequency and longevity over the last several years in coastal regions of subtropical southeast Queensland, Australia. Biotic interactions associated with bloom proliferation and demise are poorly understood. Highly variable levels of secondary metabolites within *L. majuscula* blooms (both temporally and spatially) may lead to feeding deterrence in macro-and mesograzers. The rabbitfish *Siganus fuscescens* is a highly motile generalist herbivore, which has been known to feed upon L. *majuscula.* Speculation that over harvesting of this commercially important fish may have been a factor associated with bloom proliferation led to suggestions that captive-bred siganids could be a potential biocontrol agent for these blooms. Opisthobranch molluscs often have a 'boom-or-bust' relationship with *L. majuscula* blooms. The sea hares *Stylocheilus striatus* and *Bursatella leachii* are known consumers of *L. majuscula* and have also been suggested as potential candidates for biocontrol of these nuisance blooms.

Lyngbya majuscula Blooms in Queensland

Lyngbya majuscula blooms have been increasing in severity in southeast Queensland, Australia, over the last several years (Dennison *et al.,* 1999), particularly in Moreton Bay. Three major bloom sites in this region are Deception Bay, Eastern Banks and Adams Beach (see Fig. 1). Here, *L. majuscula* blooms appear to produce site-specific secondary metabolites: debromo-aplysiatoxin (DAT), lyngbyatoxin-a (LTA) and DAT + LTA, respectively (N. Osborne, pers. comm.). These compounds are potent tumour promoters (Fujiki *et al.,* 1984; 1990) and are cytotoxic, producing severe contact dermatitis (Grauer and Arnold, 1961; Hashimoto *et al.,* 1976; Cardellina *et al.,* 1979); respiratory problems (Dennison *et al.,* 1999); trigger eye infections (Kato and Scheuer, 1975); and intestinal haemorrhaging (Ito *et al.,* 2002). Human food poisoning incidents involving *L. majuscula* have also been reported (Sims and Zandee van Rilland, 1981; Haddock, 1993; Yasumoto, 1993; Hanne *et al.,* 1995; Nagai *et al.,* 1996; Yasumoto, 1998). Secondary metabolite levels have high variance on a small spatial scale, which may be related to nutrient levels (J. O'Neil, unpubl.). Bioavailable iron and phosphorus have been implicated as major contributing factors in bloom proliferation (Dennison *et al.,* 1999; Pillans, 1999). Moreton Bay has a high conservation value and supports a wide range of species, including the protected dugong and green turtle. Localised seagrass loss as a result of blooms could have negative consequences for many species that utilise these habitats. However, other fauna may be able to capitalise on these nuisance blooms as both a source of food and refuge.

Biotic Interactions Associated with *Lyngbya majuscula* Blooms

While there has been considerable attention given to bottom-up influences on *L. majuscula* blooms (Paerl and Millie, 1996; O'Neil, 1999; Thacker and Paul, 2001), there has been less research examining biotic interactions that might affect bloom proliferation and demise, *i.e.,* top-down effects

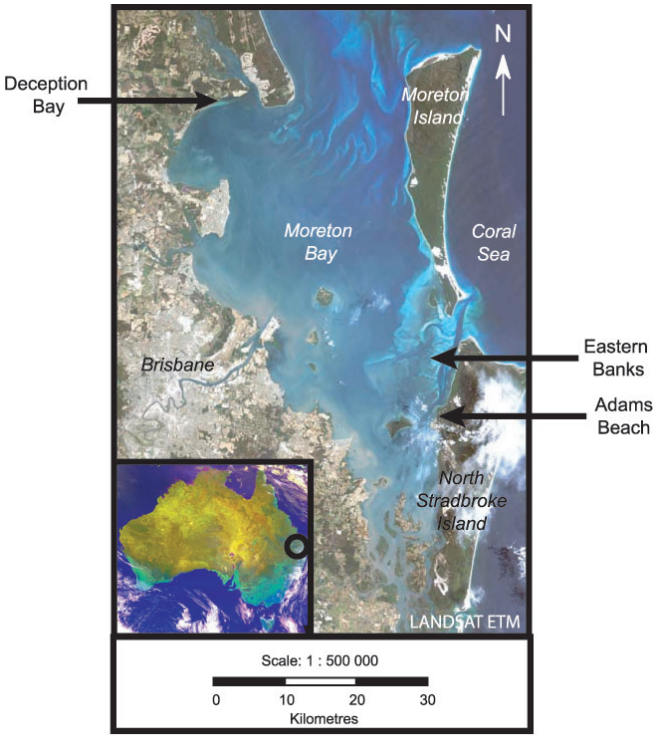

Figure 1 Major *Lyngbya majuscula* blooms sites in southeast Queensland, Australia.

(Cruz-Rivera and Paul, 2002). Of these potential interactions perhaps grazing is of primary interest, as grazers may have the ability to mediate *L. majuscula* blooms through the direct removal of the cyanobacterium at any stage of its development. However, most grazers are deterred by the secondary metabolites produced by *L. majuscula* resulting in their having only a transitory relationship with *L. majuscula* to seek either refuge or food (Cruz-Rivera and Paul, 2002). Three groups of grazers associated with cyanobacteria are of interest: micrograzing organisms, such as plankton (Boon *et al.,* 1994; Sellner, 1997; Turner and Tester, 1997; O'Neil, 1999); cryptofaunal mesograzers such

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as sea hares (Hay and Fenical, 1988; Pennings and Paul, 1993a; Pennings *et al.,* 1996), amphipods and crabs (Cruz-Rivera and Paul, 2002; 2003); and macrograzers, such as fish (Beveridge *et al.,* 1983; Klumpp and Polunin, 1989; Thacker *et al.,* 2001).

Rabbitfish (*Siganus fuscescens*)

Of the potential macrograzers in this subtropical system, the only highly motile generalist herbivore known to feed on *L. majuscula* is the rabbitfish, *Siganus fuscescens* (Hashimoto *et al.,* 1976). Siganids occupy a wide range of habitats in the coastal waters of tropical and subtropical Indian and western Pacific oceans (Hiatt and Strasburg, 1960; Lundberg and Lipkin, 1979; Gundermann *et al.,* 1983; Wassef and Hady, 1997). This selective browser species can often generate intense herbivory, dramatically depleting its preferred food sources (von Westerhagen, 1973; Bryan, 1975; Paul *et al.,* 1990). This often results in lower quality resources (such as *L. majuscula*) becoming the only available food (Thacker *et al.,* 1997). A mass mortality of juvenile siganids during pulse recruitment in Guam was attributed to a large bloom of *L. majuscula* over reef flats (Thacker *et al.,* 1997). Juvenile siganids (*S. spinus* and *S. argenteus*) are deterred by the presence of secondary metabolites in *L. majuscula* (Nagle *et al.,* 1996) and it is thought the fish probably starved to death (Thacker *et al.,* 1997). Adult siganids have, however, been known to feed upon *L. majuscula* blooms (von Westerhagen, 1973; Bryan, 1975; Hashimoto *et al.,* 1976; Lundberg and Lipkin, 1979) without exhibiting any apparent detrimental effects (Hashimoto *et al.,* 1976). However, consumption levels vary highly between species (Pitt, 1997).The relationship between secondary metabolites, diet quality and herbivore digestion is complex (Paul, 1992; Choat and Clements, 1998). Herbivores may learn to accept foods that are initially unpalatable or reject foods that are initially palatable (Hay *et al.,* 1988a; Hay and Fenical, 1988; Paul *et al.,* 1988; Irelan and Horn, 1991; Paul, 1992; Thacker *et al.,* 1997) depending on post-ingestive consequences of those foods (Paul *et al.,* 1988; Thacker *et al.,* 1997). The capacity for tolerance in the digestive system of *S. fuscescens* during the breakdown of secondary metabolites is unknown. Natives of Okinawa (Japan) refrained from consumption of *S. fuscescens* during seasonal *L. majuscula* blooms, denoting them as "poisonous" during these periods (Hashimoto *et al.,* 1976).

Siganids have often been observed feeding in areas affected by *L. majuscula* blooms in Moreton Bay (G. Savige, pers. comm.; pers. obs). Discussions suggesting captive-bred siganids be released into Moreton Bay by a local aquaculture facility to mitigate nuisance blooms led to a series of trials to assess feeding preference in both wild and captive-bred siganids (fed previously on a pellet diet). Low levels of *L. majuscula* consumption and feeding deterrence in both wild and captive-bred siganids suggest they exert little pressure as a top-down control agent and would be unsuitable as a biocontrol.

Sea Hares (*Stylocheilus striatus* and *Bursatella leachii*)

Lyngbya majuscula may be avoided by many larger macrograzers (*i.e.,* vertebrates), yet Cruz-Rivera and Paul (2001) found they had high epifaunal diversity (mainly invertebrates). Chemically defended plants potentially offer a safe haven to less mobile grazers resistant to secondary metabolites (Hay *et al.,* 1988b; Hay *et al.,* 1990; Sellner, 1997; O'Neil, 1999; Cruz-Rivera and Paul, 2002). The plants may provide not only a source of food, but also refuge to cryptofaunal species, thus minimizing predation encounters (Hay *et al.,* 1990; Cruz-Rivera and Paul, 2002).

The opisthobranch mollusc, *Stylocheilus striatus* (formerly *longicauda*; Rudman, 1999; Yonow *et al.,* 2002), appears to have a natural "boom-or-bust" relationship with *L. majuscula* blooms. *Stylocheilus* are voracious consumers of *L. majuscula* (pers. obs.) and can actively sequester some secondary metabolites (Paul and Pennings, 1991; Pennings and Paul, 1993a; 1993b; Nagle *et al.,* 1998). Use of these compounds for defence purposes by this specialist grazer have yielded ambiguous results (Pennings *et al.,* 1996; 1999; 2001) as they are stored primarily in the digestive gland and not in skin or secretions, thus reducing their capability as a predator defence mechanism (Pennings and Paul, 1993a). Compartmentalisation of toxins, however, may protect the sea hare from autotoxicity (Pennings *et al.,* 1996).

Stylocheilus striatus have a broad planktonic dispersal phase (Pennings and Paul, 1993b) and larvae appear to preferentially settle on *L. majuscula* (Switzer-Dunlap and Hadfield, 1977). The unpredictability of secondary metabolites within small patches of *L. majuscula* means newly settled *S. striatus* must be able to tolerate a wide range of chemical variation (Pennings and Paul, 1993a; Cruz-Rivera and Paul, 2002). These compounds can promote feeding stimulation or deterrence depending on concentration and potency (Nagle *et al.,* 1998; Cruz-Rivera and Paul, 2001). *Stylocheilus striatus* may utilise other host species, covering a range of algae and sponges (Cruz-Rivera and Paul, 2001), until a preferred host (*i.e., L. majuscula*) becomes available or if the local supply of *L. majuscula* is exhausted (Pennings and Paul, 1993a). However, these hosts support much lower growth rates than a monospecific diet of *L. majuscula* (Paul and Pennings, 1991). High rates of *L. majuscula* consumption, high growth rates and host specificity demonstrated by *S. striatus* may suggest its potential suitability as a biocontrol agent.

Bursatella leachii is a sea hare that has also been found recently in association with *L. majuscula* blooms in Moreton Bay (pers. obs.). It appears to feed on *L. majuscula;* however, there have been no comprehensive studies of its dietary selectivity, feeding rates or its response to *L. majuscula* secondary metabolites. At present the authors are involved in a series of studies on the food preference, growth rate, consumption rate and toxicity of these sea hares in Moreton Bay.

Potential Biocontrol Agents for *Lyngbya majuscula* Blooms

A potential biocontrol agent should 1) preferentially consume (but not necessarily assimilate) *L. majuscula* or in some other way reduce its biomass and 2) be present in sufficient quantity to impact blooms either by having (a) a large and mobile population (if they have annual reproductive cycles, slow growth and complex recruitment pathways) or (b) a relatively sedent population that can respond rapidly to bloom development (particularly through sub-annual reproductive cycles, rapid growth rates and young age at sexual maturity).

Siganids do not preferentially consume *L. majuscula,* and the quantities that they do consume are inconsistent. They are highly mobile and are probably present in relatively large numbers locally; however, for Moreton Bay there are no available estimates of their population size. This information would be required before an assessment of their potential natural impact on blooms could be made. Furthermore, the possibility that they may themselves become toxic following exposure to *L. majuscula* together with their sale in export markets suggests that further assessment of the fate of *L. majuscula* compounds in fish is required and remains a pressing health issue. Any involvement of siganids in the control of *L. majuscula* by augmenting wild populations with captive-bred animals could present a substantial risk. Moreover it remains to be assessed if captive-bred siganids raised on a non-algal (*i.e.,* pellet) diet will rapidly switch to a diet including *L. majuscula.*

In relation to the use of sea hares as a candidate for biocontrol, consideration must be given to the "boom-or-bust" relationship these organisms have with *L. majuscula.* A rapid increase in population is often observed during peak bloom phases, followed by a mass "die-off" during bloom demise (S. Albert, pers. comm. 2002). This phenomenon is often known as "purple footprints" due to ink secretions exuded at time of death. The impact of such a population crash upon scavenger species is not well studied. In Western Australia in 2002, several dog fatalities occurred after they were reported to have been feeding upon sea hares that had washed ashore en-masse (R. McKenzie, pers. comm.) It is therefore essential that a more complete understanding of the role of *L. majuscula* toxins in the food web and their potential impacts upon consumers be obtained.

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Comparative Toxicity of the Cyanobacterial Toxin Cylindrospermopsin Between Mice and Cattle: Human Implications

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Abstract

The cyanobacterial toxin cylindrospermopsin is produced by *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* in many parts of the world. A human poisoning incident occurring at Palm Island, Queensland, Australia in 1979 was subsequently ascribed to cylindrospermopsin. The structure of cylindrospermopsin, a tricyclic guanidinium moiety bridged to hydroxymethyluracil, was deduced in 1992. A number of studies have investigated the acute toxicity of cylindrospermopsin in mice. It is primarily a hepatotoxin with a 24-hour acute intraperitoneal (IP) LD_{50} of 2 mg/kg, a 5-day acute i.p. LD_{50} of 0.2 mg/kg and a 5-day acute oral LD_{50} of approximately 6 mg/kg. A human health risk assessment using data from longer-term oral dosing studies suggests a guideline value for cylindrospermopsin in drinking water of approximately 10 µg/L. We have recently studied cattle poisonings by cylindrospermopsin and detected the toxin in a number of tissues after necropsy. Concentrations of 1 mg/L or above in drinking water (dose is approximately 50 µg/kg/day) were shown to result in cattle death after short-term exposure (less than 10 days). Oral dosing of mice at levels up to 5 mg/L with cylindrospermopsin in drinking water for 90 days did not produce any significant toxicity. Human health risk assessment based on cattle however, which are much more sensitive to cylindrospermopsin than rodents, would produce a guideline for human drinking water of approximately 0.05 µg/L. A consideration of reported human poisoning incidents that implicate cylindrospermopsin suggests that humans may also be more sensitive than rodents to this toxin.

Introduction

The cyanobacterial toxin cylindrospermopsin was discovered as a result of an acute human poisoning incident in Palm Island, North Queensland, Australia, in 1979. The epidemic lasted 21 days and reached its peak by the eighth day (Byth, 1980). The illness was hepatoenteric in nature and commenced after Solomon Dam, the water supply for the island, was treated with copper sulfate to control a dense cyanobacterial bloom. An epidemiological study showed that only persons utilising the reticulated water supply became ill (Bourke *et al.,* 1983). From all available evidence, Bourke *et al.* (1983) and Hawkins *et al.* (1985) retrospectively postulated that the sickness was related to cyanobacterial toxicity. The cyanobacterium, *Cylindrospermopsis raciborskii* was believed to be the causative agent and was shown by Hawkins *et al.* (1985) to produce severe toxicity to the liver and other organs of intraperitoneally dosed experimental animals. The toxin cylindrospermopsin was later isolated and structurally identified in material from cultures of this organism (Ohtani *et al.,* 1992). Chemically this toxin is a tricyclic guanidium moiety bridged to hydroxymethyl uracil. The toxin is very zwitterionic and therefore very hydrophilic, which has implications for its distribution in drinking water reservoirs. Cylindrospermopsin has also been suggested as the cause for an illness termed "Barcoo fever" that was widespread in Northern outback Australia (Hayman, 1992). The symptoms described for Barcoo fever were very similar to those reported in the Palm Island community during the poisoning incident in 1979. More recently, cylindrospermopsin has been found in addition to microcystins in water filters in the dialysis center in Caurary, Brazil where 76 patients died of acute liver disease in 1996 (Carmichael *et al.,* 2001).

To date, acute and sub-chronic data for cylindrospermopsin toxicity in mice have been produced (Hawkins *et al.,* 1985; Ohtani *et al.,* 1992; Terao *et al.,* 1994; Falconer *et al.,* 1999a; Seawright *et al.,* 1999; Shaw *et al.,* 2001). Recently cattle mortalities have been attributed to cylindrospermopsin that was present in farm water storages featuring heavy blooms of *C. raciborskii* (Saker *et al.,* 1999; McKenzie *et al.,* 2003). To date, however, no evaluation of the comparative toxicity of cylindrospermopsin in mice and cattle has been published. In this study we have examined the death of cattle from two separate poisoning incidents involving cylindrospermopsin, have confirmed the presence of this toxin in drinking water and cattle tissues, and have histologically shown the pathology of intoxication to be typical of cylindrospermopsin poisoning in experimental animals.

Methods

Two cattle-poisoning incidents were investigated as reported in McKenzie *et al.* (2003). Case A involved the deaths of 10 cattle in Central Queensland in August 2001 and case B involved 45 cattle in Northwest Queensland in October 2001. Cylindrospermopsin in water samples from farm water supplies was analyzed using HPLC-MS/MS according to the method of Eaglesham *et al.* (1999). Toxin concentration in cattle rumen contents was determined by centrifuging to remove particulate matter and then diluting before HPLC-MS/MS analysis as for water. Liver, kidney and muscle tissue was macerated with 75%

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methanol/water and sonicated. The supernatant was removed, partitioned with hexane to remove lipid material and the aqueous layer was analyzed for cylindrospermopsin content using HPLC-MS/MS as above. Histopathology was performed on cattle organs by fixing tissues with 10% formalin and processing by standard techniques with hematoxylin and eosin staining.

Results and Discussion

Affected cattle were ill (clinical signs were lethargy and recumbency) for 3–4 days before dying. Necropsy revealed typical cylindrospermopsin toxicity with pale mottled livers and distended gall bladders. Histology revealed hepatocyte degeneration and necrosis, nephrosis and multifocal cardiac muscle degeneration. Cylindrospermopsin concentrations in water and tissues are given in Table 1.

The results demonstrate that water with a cylindrospermopsin concentration as low as approximately 1000 µg/L may be fatal to cattle. The rumen contents in case A contained cylindrospermopsin at roughly half the concentration found in the drinking water. If this relationship holds, the toxin concentration in drinking water in case B would be about 10,000 µg/L. The liver and kidney also had detectable concentrations of cylindrospermopsin. These organs are the main targets for this toxin and distribution studies using ^{14}C-labelled cylindrospermopsin (Norris *et al.,* 2001) demonstrated that they are the preferential sites for toxin accumulation. Unlike the microcystins which are transported into the liver through the bile acid transport mechanism, no active uptake mechanism appears to operate with cylindrospermopsin and uptake via passive diffusion has been suggested (Chong *et al.,* 2002). The lack of cylindrospermopsin in edible muscle of fatally poisoned cattle is of note in human health risk assessment of exposure to this toxin in meat. No international guidelines have been developed for cylindrospermopsin in drinking water to date. Using mouse data, however, it is possible to suggest human drinking water guidelines using World Health Organization protocols as described by Falconer *et al.* (1999b). The acute oral LD_{50} for mice is approximately 6000 µg/kg (Seawright *et al.,* 1999). Our data from a range of mouse-dosing studies have been reported (Shaw *et al.,* 2001), and guideline values of 1.75 µg/L were calculated from 14-day oral gavage studies. Values of 7.0 µg/L and 10.5 µg/L were calculated from the results of 28-day repetitive oral drinking water and 90-day oral drinking water studies, respectively. Due to its longer duration, the 90-day drinking water study is considered the most accurate. The no observable adverse effect level (NOAEL) in this study was 150 µg/kg/day. The results of an 11-week study involving daily oral gavage dosing of mice with cylindrospermopsin have been reported (Falconer and Humpage, 2002). That study suggests a guideline value for cylindrospermopsin in drinking water of 1 µg/L.

In the case of the cattle poisonings, the lowest concentration in drinking water that was fatal in the short-term (less than 7 days) was 1050 µg/L. Derivation of approximate guidelines for cylindrospermopsin in drinking water for humans derived from the cattle data is presented below:

- For cattle, lethal dose per day = [cylindrospermopsin] × volume water consumed / body weight
- Lethal dose = 1050 µg/L × 10L / 250 kg = 42 µg/kg/day.

Assuming a similar ratio between lethal dose and NOAEL for mice and cattle, then:

- $NOAEL_{cattle} = NOAEL_{mice} \times \text{lethal dose}_{cattle} / \text{lethal dose}_{mice}$
- $NOAEL_{cattle}$ = 150 µg/kg/day × 42 µg/kg/day / 6000 µg/kg/day = 1.05 µg/kg/day.
- Tolerable daily intake (TDI) = NOAEL / 1000 (uncertainty factors) for interspecies and intraspecies variations and less than lifetime exposure. Therefore TDI = 1.05 µg/kg/day / 1000 = 0.00105 µg/kg/day.
- Guideline value (GV) = TDI × body weight × proportion of toxin from water / daily water intake
- GV = 0.00105 µg/kg/day × 70 kg × 1 / 2L = 0.037 µg/L

The guideline value for cylindrospermopsin in human drinking water calculated from cattle data is orders of magnitude lower than that calculated from mouse data. The question remains as to whether human sensitivity to this toxin is more similar to cattle or mice. Considering the Palm Island human poisoning incident, if this was due to cylindrospermopsin toxicosis, it was an acute poisoning incident with severe symptoms requiring hospitalization. In our experience it is highly unlikely that a large drinking water reservoir would contain cylindrospermopsin at concentrations exceeding 1000 µg/L. At this concentration, the daily dose for humans would be approximately 30 µg/kg/day, which is less than the NOAEL for mice but potentially lethal to cattle. It appears that the mouse model may seri-

Table 1 Cylindrospermopsin concentrations in drinking water and cattle tissues and organs from poisoning cases.

	Cylindrospermopsin concentration*	
Sample type	Case A	Case B
Water	1050	Not analyzed
Rumen contents	570	5700
Liver	7.4–51	Not analyzed
Kidney	9.4–29	Not analyzed
Skeletal muscle	Not detected at an LOD of 0.2	Not analyzed

*Concentration in micrograms/litre for water and rumen contents and micrograms/kilogram for tissues and organs.

ously underestimate the true toxicity of cylindrospermopsin to humans and it is suggested that studies involving another animal model in place of rodents be undertaken before guideline values for cylindrospermopsin in human drinking water are established.

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Design and Use of a Harmful Algal Bloom Database for the West Coast of North America

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Abstract

The National Oceanographic Data Center (NODC) developed the Harmful Algal Bloom-Data Management System (HAB-DMS) for those interested in understanding the spatial and temporal distribution of toxic phytoplankton and shellfish toxicity events. The HAB-DMS provides scientists, managers, and the public easy access to past records of harmful phytoplankton species abundance and distribution, shellfish toxicity, and environmental data. Currently, records relating to HABs on the west coast of North America are stored in separate databases in Alaska, British Columbia, Washington, Oregon, and California. By integrating these distinct datasets into the HAB-DMS, spatial and temporal trends may be determined irrespective of state or international boundaries. Patterns can then be examined using Geographic Information System software. For this article, only a subset of the HAB data from the west coast of North America (*i.e.,* the Pacific Northwest) are discussed.

Background and Data Treatment

At present, accurate documentation of the geographical extent of HAB events on the west coast of North America is problematic for several reasons. First, HAB data are recorded in separate state and international databases, and in hardcopy format. Second, different groups, including governmental agencies and academic institutions, maintain the data. This system leads to the incomplete representation of HAB events. For example, when data from a particular state are analyzed without including data from adjacent regions, it appears that a HAB is localized (Fig. 1a,b). The goal of this project was to compile all of the available information pertaining to HABs on the west coast of North America, and store them in the HAB-DMS (Fig. 2). The data holdings comprise information on marine toxin levels and phytoplankton species abundance, as well as oceanographic measurements, *e.g.,* water temperature, salinity, and nutrient concentrations. Links to data collected by oceanographic buoys and weather stations will also be included. The resulting integrated database will be available through a website interface, providing users with the opportunity to access the data for analysis of the spatial and temporal distribution of HAB events.

Data on marine toxins in shellfish were requested from the managers of shellfish programs in Alaska, British Columbia, Washington, Oregon, and California. In order for the data to be put into the HAB-DMS, latitude and longitude coordinates were required for each sampling site. Records that did not contain essential information (*e.g.,* location, toxin levels, shellfish type, sampling dates) were excluded. When these coordinates were not available for a specific site, every effort was made to determine the location of the site. The qualifying records were compiled using Microsoft Excel (Microsoft Corp., Bellevue, WA).

Potential Database Usage

To date, data assimilation resulting from this project has provided significant spatial coverage of paralytic shellfish toxins (PSP) and domoic acid (DA) in shellfish. In addition to toxin levels in shellfish, nutrient concentrations, phytoplankton information, and physical properties for a limited number of sites have also been obtained. In the future, more records pertaining to phytoplankton assemblages and oceanographic, meteorological and environmental pa-

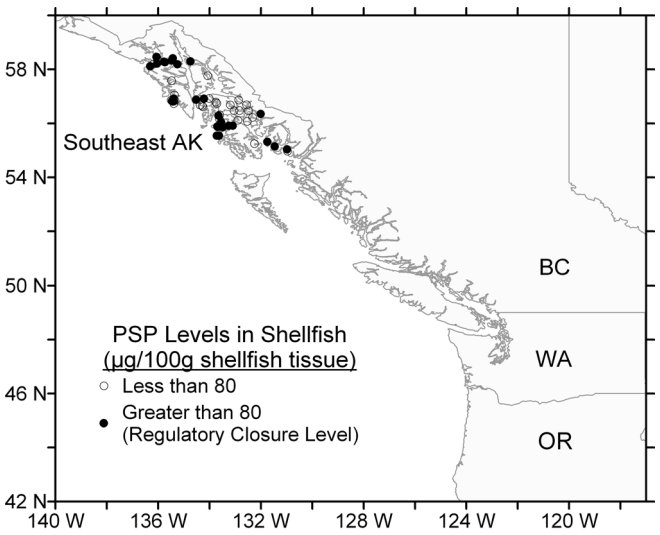

Figure 1a PSP data for Alaska during 1998.

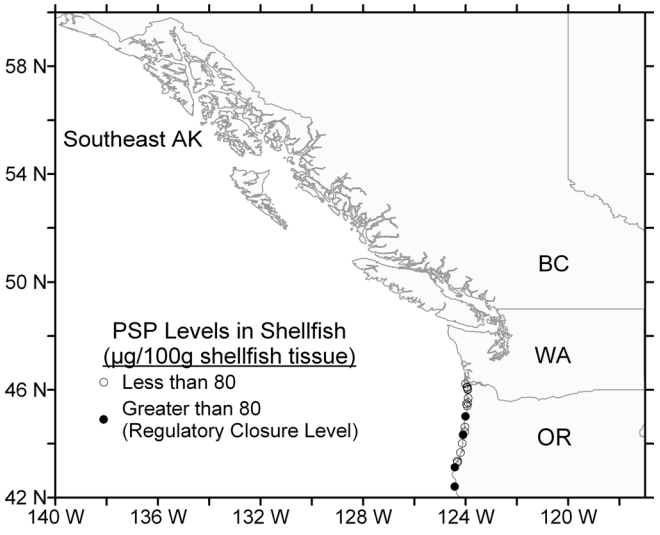

Figure 1b PSP data for Oregon during 1998.

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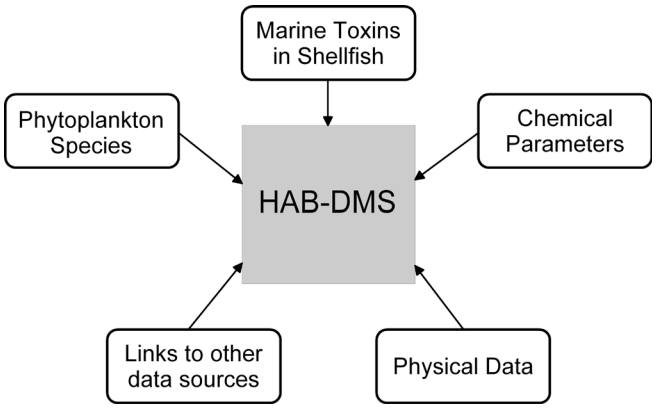

Figure 2 Schematic of data assimilation.

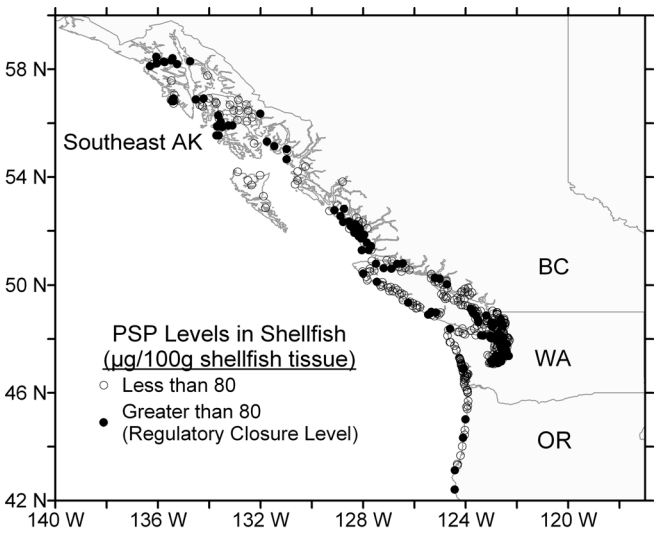

Figure 3 PSP in shellfish during 1998.

rameters will be included. When completed, the data may be used to show, for example, where levels of PSP toxins in the Pacific Northwest exceeded the regulatory limit of 80 µg per 100 grams of shellfish meat (Fig. 3). Physical and chemical parameters like water temperature and nutrient concentrations (Fig. 4) may be shown in relation to toxin levels and phytoplankton species. Atmospheric and physical properties such as wind speed or current direction during various HAB events can be accessed easily and used to identify patterns or trends that may help researchers elucidate mechanisms of bloom formation in specific regions. With all of the information accessible at one location, the user can develop and search multiple datasets. Without such a tool, one would be required to contact individuals responsible for the data in many regions in order to obtain the information necessary for the analyses. By employing a coast-wide integrated database, time-consuming inquiries are eliminated.

Conclusions

West Coast HAB data are being assimilated and input into one database. This unified database allows analysis of HABs over a greater geographical area. As a result, various types of data (*e.g.,* biological, chemical, and physical) will be consolidated, allowing for queries of multiple parameters. In the future, the database will be coupled with a web interface that will improve access to data and encourage collaboration among researchers and managers in various regions. The HAB-DMS provides a tool that will bring scientists one step closer to being able to model and predict HABs.

Acknowledgements

We thank the Alaska Departments of Environmental Conservation and Fish and Game, Fisheries and Oceans Canada, Washington State Department of Health, and the Oregon Department of Agriculture for supplying data. We also thank NOAA ESDIM Project 01-414F "Access to PACHAB data" for providing funds for these analyses and Kathi Lefebvre for helpful discussions.

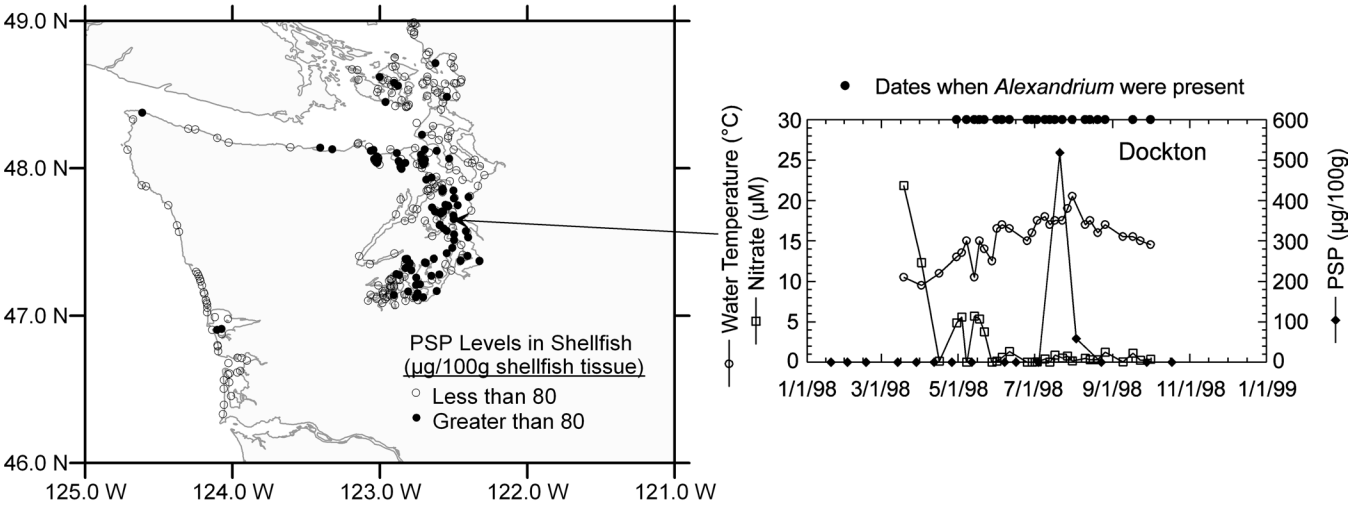

Figure 4 **A** PSP levels during 1998 in Washington State; **B** a PSP time series related to water temperature and nitrate concentration at one site in Puget Sound, WA.

Cyanobacteria Exposure, Drinking Water and Colorectal Cancer

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Abstract

The cyanobacteria (blue-green algae) represent a diverse group of organisms that produce potent natural toxins. Although there has been little epidemiologic research on toxin effects in humans, an increased association between primary liver cancer in humans and the use of surface drinking water sources was identified in a prior study. In mice, microcystins can potentially "stimulate" pre-neoplastic colorectal tumor growth. Surface drinking water supplies are particularly vulnerable to the growth of these organisms; in general, current US drinking water treatment practices do not monitor or treat for the cyanobacterial toxins. This pilot study was an ecological study using a Geographic Information System (GIS) evaluation of the risks of colorectal cancers with residence proximity to a surface drinking water treatment plant at the time of the cancer diagnosis. The study linked all colorectal cancers diagnosed in Florida from 1981–1999 with environmental databases on drinking water sources. No significantly increased risk for colorectal cancer with residence at diagnosis within the distribution area of a surface water treatment plant was found, compared to persons living in areas contiguous to the surface or ground water treatment plants, or compared to the Florida cumulative incidence rate for the study period. These findings must be interpreted in light of significant issues of latency, high population mobility, and the lack of individual exposure information. Nevertheless, the issue of chronic and acute human health effects associated with the consumption of surface waters possibly contaminated by cyanobacterial toxins merits further investigation.

Introduction

The cyanobacteria represent a diverse group of organisms that produce potent natural toxins (Fleming, 2001; Chorus, 1999). There have been case reports of severe morbidity and mortality in domestic animals through drinking contaminated water. Although there has been little epidemiologic research on toxin effects in humans, studies by Yu *et al.* (1995) and Fleming *et al.* (2002) found an increased association between primary liver cancer in humans and the use of surface drinking water sources. Humpage *et al.* (2000) showed in mice that microcystins could potentially "stimulate" pre-neoplastic colorectal tumor growth. Surface drinking water supplies are particularly vulnerable to the growth of these organisms; in general, current US drinking water treatment practices do not monitor or treat for the cyanobacterial toxins. In Florida, recent monitoring studies (SJRWMD 2000) of recreational and surface drinking water supplies with algal blooms found 87/167 (52%) samples (from 75 individual water bodies) with significant levels of toxin producing cyanobacteria; all of these samples had positive identification of cyanobacterial toxins with 80% lethality in mice. This ecological pilot study explored the possible risk of developing colorectal cancer associated with possible exposure to drinking water from surface water sources in Florida.

Methods

This pilot study was an ecological study using a GIS evaluation of the possible association of 1) residence at the time of diagnosis of colorectal cancer and 2) proximity to a surface water treatment plant for all colorectal cancers in Florida since 1981. The study used the Dept of Health (DOH) database of all colorectal cancer cases reported to the Florida Cancer Data System (FCDS) from 1981–1999, in conjunction with Florida Dept of Environmental Protection (DEP) and St. Johns River Water Management District (SJRWMD) databases on drinking water sources and treatment plants.

Disease Data The Florida Cancer Data System (FCDS) database of colorectal cancer cases (anonymous dataset with geocoded information) from 1981–1999 was obtained. The data were incorporated into the GIS database in tabular and ArcView shapefile formats.

Exposure Data From Florida DEP and other websites, geocoded information (with longitude and latitude) was acquired for the deep water wells, as well as the administrative addresses for their respective treatment plants. Deep water treatment plants in geographic coordinates were not available due to data confidentiality bioterrorism issues. Geocoded addresses of the actual surface water treatment plants, as well as accurate hardcopy maps of the water distribution service areas from these surface water treatment plants, were digitized using the UTM coordinate system (NAD 83) within ArcView GIS.

GIS Methods The geocoded data (residence at time of diagnosis) of all cases of colorectal cancer from the FCDS database included the following variables: age, date of birth, gender, and race-ethnic information. In addition, racial, ethnic, and socioeconomic data from 1990 were acquired from the US Census bureau. This information was mapped above the base layers described in the previous section. The geographic center of the actual surface water distribution service area was used as the hypothetical lo-

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cation of the surface water treatment plant. The surface water treatment plants were far removed from their actual service areas. The average size plus two standard deviations of the surface water service areas was determined and used to identify the standard area of influence for the initial analyses. A circular buffer with an area equal to the standard area of influence was created and applied to each hypothetical surface water treatment facility. Due to the absence of data, the hypothetical center for each ground water treatment plant was determined based on the average location of its corresponding wells. This calculation was based on the assumption that the ground water wells can be located within the service area to minimalize the risk of anthropogenic influence. The same circular buffer used for the surface water treatment facilities was applied to each ground water facility. Additional GIS analyses (Fig. 1) used the following: 1) the actual surface water treatment area with an actual contiguous buffer control, and 2) a circular surface water treatment area with a circular contiguous buffer control.

The geographic intersection between the 1990 Census block group data and the standard area of influence for each ground and surface water facility was determined. The demographic profile and population parameters for each area were calculated from this intersection and used as approximate denominator data. In an attempt to control for confounding bias, four (4) sets of twelve (12) ground water study areas (controls) were randomly selected from groups matching the following characteristics: Random, Similar Median Income/Rent to Surface Water Areas, Similar Race-Ethnic Distribution to Surface Water Areas, Similar Median Income/Rent and Race-Ethnic Distribution to Surface Water Areas.

The colorectal cancer cases from 1981 through 1999 were spatially joined to their corresponding service area; initial cumulative incidence rates (and confidence intervals) were calculated for each of the Surface Water Areas and for the randomly selected Ground Water Areas. In addition, the initial pooled colorectal cancer incidence rates for all study groups (surface and ground water areas) were also calculated. For the individual cumulative incidence rates, the Mann Whitney Rank Sum Test was used to compare the Surface Water Treatment Areas to the different comparison groups of the Ground Water Areas. Standardized Rate Ratios (SRRs) with confidence intervals were calculated for the pooled Surface Water Treatment Areas compared to the comparison groups of the pooled Ground Water Areas. Since these were hypothesis-generating analyses, no adjustment for multiple comparisons was made.

Results

There were 213,487 incident cases of colorectal cancer from 1981 through 1999 in Florida. There were 18 Surface Water Treatment Areas and 1249 total ground water treatment areas. There were a total of 26,635 cases of colorectal cancer in the 18 Surface Water Treatment Areas combined. The pooled Surface Water Treatment Area cumulative age-adjusted cancer rate for colorectal cancer was 60.0/100,000 (SE 0.005) for 1981–1999. The number of colorectal cancer cases in the individual Surface Water Treatment Area ranged from 0 cases to 5,717 cases; the individual cumulative age-adjusted cancer rates for colorectal cancer ranged from 0 to 142.4/100,000 (SE 0.0188). As a comparison, the cumulative age-adjusted cancer rate of colorectal cancer for all of Florida for the same time period was greater, at 53.0/100,000.

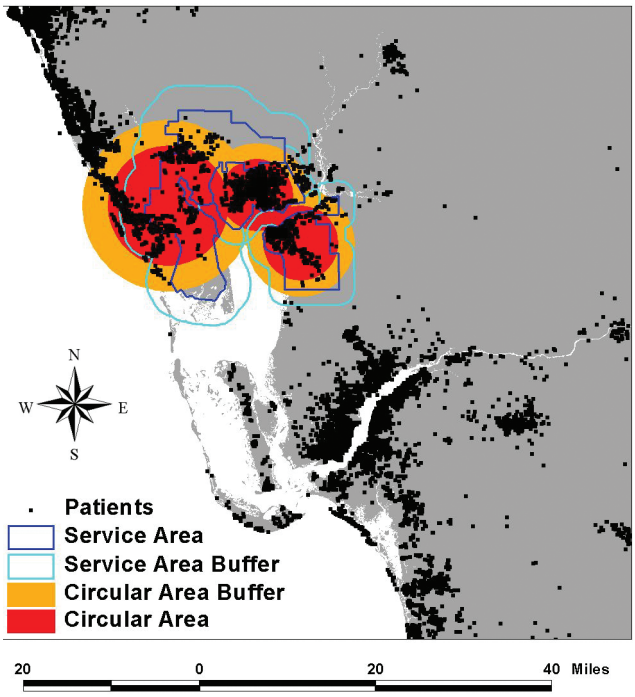

Figure 1 Example of both actual and circular surface water treatment areas and their contiguous actual and circular buffer control approximations, with colorectal cancer patients.

The Mann Whitney Rank Sum Test was applied to the individual incidence rates for the 18 surface water areas in comparison with the incidence rates of the 4 different groups of randomly selected matched ground water control areas as well as the contiguous buffer analyses. There were no significant differences between the incidence rates by the Rank Test for the actual contiguous buffer analysis. Compared to their respective comparison groups, the SRR of colorectal cancer for pooled Surface Water Treatment Areas for the actual contiguous buffer analysis was significantly decreased; the SRRs were significantly decreased for the pooled Ground Water Treatment Control Areas, the contiguous circular buffer control and for general Florida population with 95% confidence intervals.

Discussion

In this pilot ecological study using a GIS analysis, the risk of colorectal cancer was not associated with residence at the time of diagnosis in a Surface Water Treatment Area of Distribution when comparing actual service areas to GIS-created actual contiguous buffer control areas in Florida,

as well as other comparison analyses. Nevertheless, given the previously established positive association for hepatocellular cancer and the vulnerability of surface drinking water supplies to cyanobacteria and their toxins, the issue of chronic and acute human health effects associated with the consumption of surface waters possibly contaminated by cyanobacterial toxins merits further investigation. Finally, in addition to exposure monitoring and health surveillance for the cyanobacteria and their toxins, prevention of both the exposure and potential health effects needs to be a primary focus of Florida's scientific and public health community involved in the study and prevention of the harmful algal blooms.

Study Limitations This study was an ecological study of the possible association between place of residence at the time of diagnosis of colorectal cancer and location of surface and deep well drinking water plants. As an ecologic study, this study was only hypothesis-generating; it could not prove an etiological association. This study assumed that place of residence at the time of cancer diagnosis was the same place of residence at the time of cancer initiation; this was a major assumption given the significant mobility of the Florida population and the potential 15–25 year latency of colorectal cancer. It is also possible that people living close to a drinking water treatment plant did not necessarily utilize the treatment plant as their major source of drinking water.

Acknowledgements

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Acute Effects of Recreational Exposure to Freshwater Cyanobacteria– a Prospective Epidemiology Study

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Abstract

Case studies and anecdotal reports document a range of acute illnesses associated with exposure to cyanobacteria in recreational waters. Studies on the epidemiology of recreational exposure to cyanobacteria are limited and somewhat conflicting, and much uncertainty remains regarding measures of exposure, susceptibility of individuals with a history of allergic illness, and the relative contribution of cyanobacterial exotoxins to these acute illnesses. Preliminary statistical analysis of a prospective cohort study of 1,331 subjects recruited in eastern Australia and central Florida has not revealed any significant difference in specific illnesses between unexposed groups and those exposed to various levels of cyanobacteria.

Introduction

Cyanobacteria are common inhabitants of freshwater lakes and reservoirs throughout the world. Human case reports and anecdotal references dating from 1949 describe a range of acute illnesses associated with recreational exposure to cyanobacteria: hay fever-like symptoms, itchy skin rashes and gastrointestinal symptoms are most frequently reported. Some papers give convincing descriptions of allergic responses to cyanobacteria (Heise, 1949; Cohen and Reif, 1953); others describe more serious acute illnesses (Dillenberg and Dehnel, 1960; Carmichael *et al.,* 1985; Turner *et al.,* 1990; Codd and Beattie, 1991). The main public health concern with exposure to freshwater cyanobacteria relates to the understanding that some blooms can produce toxins that specifically target the liver or the central nervous system. The route of exposure for these toxins is oral, from accidental or deliberate ingestion of recreational water, and possibly by inhalation. However, observations by cyanobacteria researchers and others who use recreational waters reveal that many individuals can be exposed to high levels of cyanobacteria with no apparent acute effects.

Reports of illness following recreational exposure to cyanobacteria in the medical and scientific literature are sparse. Significant under-reporting, especially of minor, self-limiting illnesses, and a knowledge gap about cyanobacteria amongst many primary health care providers may explain this. Epidemiological studies are few in number (Philipp, 1992; Philipp and Bates, 1992; Philipp *et al.,* 1992; El Saadi *et al.,* 1995; Pilotto *et al.,* 1997). UK studies and a smaller Australian study did not find any significant hazard from exposure to cyanobacterial blooms in recreational waters, but Pilotto *et al.* (1997) reported an increase in illness amongst those exposed to fairly low levels of cyanobacteria (>5,000 cells per mL) compared to unexposed individuals.

The World Health Organization and the Agriculture and Resource Management Council of Australia and New Zealand have both published guideline levels for recreational exposure to cyanobacteria (Johnstone, 1995; WHO, 2003), yet there is concern that the current management practice in some countries of warning all users or closing access to waterbodies is overly proscriptive. Such practices cause concern among regular users of recreational waters that are affected by cyanobacteria, and can impact communities surrounding such important social and economic water resources. There is general agreement that further epidemiological studies are required to advance the understanding of acute cyanobacteria-related illnesses, so that advice and guidelines for recreational exposure can be revised and refined. Therefore we sought to conduct a prospective study of recreational water users in Australia and in the United States to compare the frequency of acute health problems among cohorts of people exposed to recreational waters with differing levels of cyanobacteria. In a prospective cohort study, participants are enrolled based on their potential to be exposed to the agent of interest. Exposure is measured prior to disease outcome, which is determined at a follow-up procedure. Subjects were initially recruited in eastern Australia; the study was expanded to include Florida because of the broad similarities in geographic latitude, climate, nuisance cyanobacteria and recreational activities across the two regions.

Materials and Methods

Members of the public were approached at various recreational water sites in southern and southeast Queensland, the Myall Lakes area in New South Wales (Australia), and central Florida (USA); those who were engaging or planning to engage in recreational activities that involved water contact were invited to participate in the study. Typical recreational activities were swimming, skiing, jet skiing

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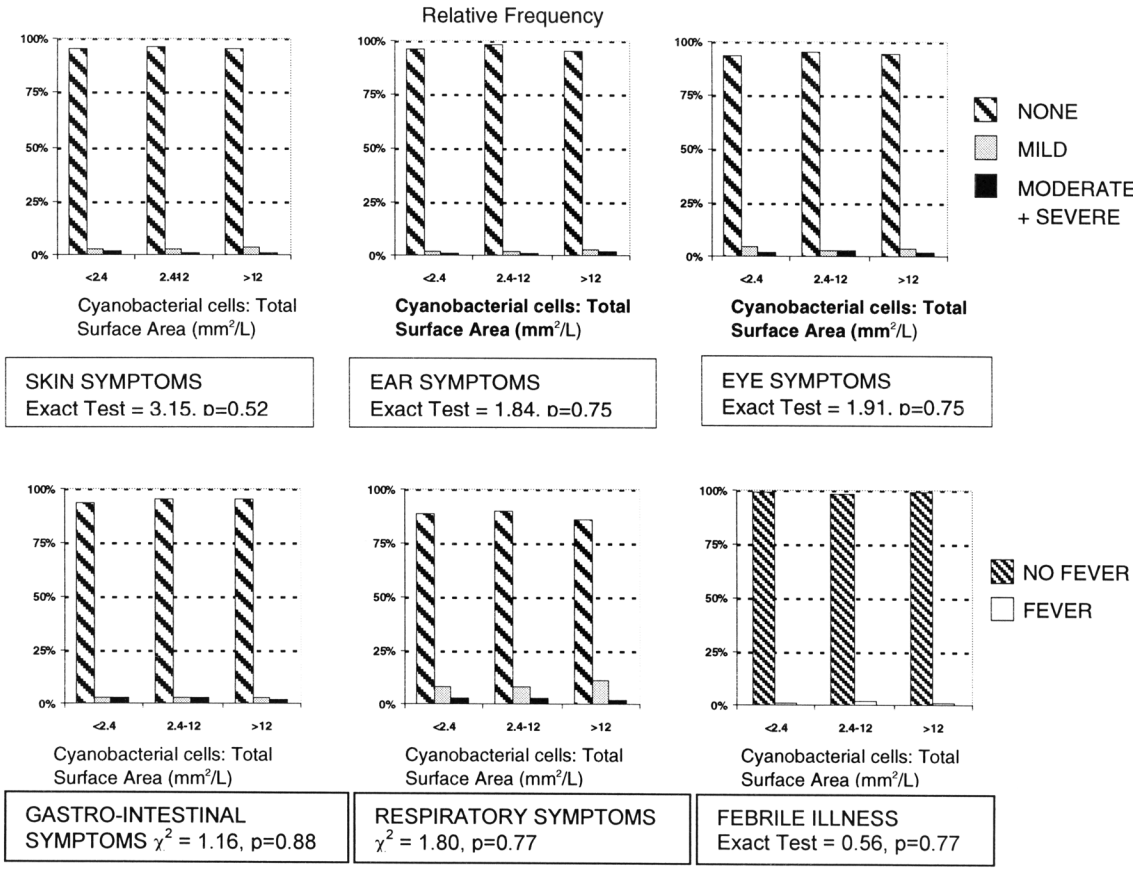

Figures 1–6 Frequency of reporting within six different symptom groups by level of cyanobacteria exposure.

and tubing. Individuals who indicated they were engaging in non-or minimal water contact activities such as boating or fishing were excluded. After they had finished their recreational activity, potential recruits were asked to complete a self-administered questionnaire on the day of recruitment, and to participate in a telephone follow-up interview after three days. The questionnaire elicited basic demographic information, details of relevant chronic illnesses (*e.g.,* asthma, dermatitis), recent acute illnesses, type and duration of recreational activity, and details of any water recreation activities during the week prior to recruitment. The follow-up interview elicited information on the occurrence of a range of specific symptoms during the three-day period following the day of recruitment. Respondents were asked to rate any symptom occurrences as mild, moderate or severe. Water samples were collected on recruitment days for 1) total phytoplankton identification and enumeration by phase-contrast microscopy using a calibrated cell-chamber; 2) cyanobacterial toxin analysis by HPLC and HPLC-MS/MS, which was run on samples in which potentially toxic species were identified during total phytoplankton analysis; and 3) faecal coliform counts. Cyanobacterial cell surface areas were used as exposure estimates for this work; surface areas were derived from cell counts and measured or documented cell dimensions (cell and/or trichome diameters and lengths). Low exposure sites were defined by total surface area less than 2.4 mm^2/L; surface areas greater than 12 mm^2/L represented high exposures. An intermediate exposure level was given for sites with surface areas between 2.4–12 mm^2/L. Data were entered into a relational database (MS Access2000) for data handling and manipulation; statistical analysis was performed using SPSS v11.5. Reported symptoms were classified into six categories (skin, eye, ear, gastrointestinal, respiratory, and febrile illness) and as absent, mild, moderate or severe. Because small numbers of subjects reported moderate or severe symptoms, these two groups were combined for analysis. The proportions of people reporting symptoms at each cyanobacteria exposure level were compared using chi-squared tests. When the expected numbers were small, the Fisher-Freeman-Halton test was used (Garson, 2003; Norušis, 2002).

Results and Discussion

1,331 subjects completed the questionnaire and follow-up interview. This number represents 37% of those who met the initial inclusion criteria. The proportions of subjects reporting symptoms that occurred within three days of water exposure are shown below in relation to their level of exposure. Overall symptom reporting was low, although we found a wide range of cyanobacteria concentrations in study waters, with cyanobacterial surface areas greater

than 300 mm^2/L at one site. Cyanobacterial toxins, when present, were generally at low levels. The frequency of reported symptoms did not vary significantly across the different cyanobacteria groups suggesting that increasing cyanobacteria exposure was not associated with acute health effects. Measuring exposure to cyanobacteria is probably the most significant source of uncertainty in this type of study, given the heterogeneity of cells in time and space within a given waterbody, the inability to determine movement of individuals within a waterbody (some will ski over a wide area of a lake, others will bathe on a small section of the lake shore), and the sometimes diverse phytoplankton profile within and across study lakes. A subsequent publication will discuss these issues in more detail, and will present further statistical analysis using different exposure measures—cell counts, cell biovolumes and chlorophyll *a*. We also plan to conduct multivariate analyses to determine the impact of cyanobacterial toxins, salinity, and faecal coliforms, and to examine whether subjects who have a history of allergic illness were differentially reporting symptoms. Cyanobacteriologists, recreational water managers and public health workers are justifiably concerned about the potential for serious illness or death from recreational or occupational exposures to cyanobacterial exotoxins, given that most of these compounds are potent systemic or neurological poisons. A recent press report of a US coroner's finding is a reminder that freshwater cyanobacteria can be extremely hazardous organisms. A teenage boy apparently died as a result of ingesting cyanobacteria containing anatoxin-a from a golf course pond. This is the first report in the world of a human fatality related to recreational exposure to cyanobacteria (Behm 2003). However, one of the difficulties with this epidemiology work is that many cyanobacteria-related illnesses appear to be mild and self-limiting. Public health priorities in this field should in the future be directed toward the epidemiology of cyanobacterial exotoxins, which have a greater potential for harm in waters where sufficient exposures may occur, compared with waters containing non-toxic strains and species, or waters where toxic strains produce low levels of toxins.

Acknowledgements

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Harmful Cyanobacteria-Invertebrate Relations: Histopathological Picture in Fall Webworm

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Abstract

New technologies in biological pest control are very significant in plant protection today. As toxic agents, cyanobacteria provide a promising direction for such pest control studies. The histopathology of cyanobacterial effects on invertebrate organisms was examined in model experiments using natural communities of the cyanobacterium *Microcystis aeruginosa* and the fall webworm *Hyphantria cunea.* Various methods of cyanobacterial application (*e.g.,* treatment of food, treatment of insects, and treatment of both food and insects) were tested to observe specific inhibitory actions and confirmed in each case by histology. Feeding larvae with cyanobacteria-treated food material correlated with irreversible alterations in the midgut, Malphigian tubes, fat body, and muscle fibres. The treatment of insects caused morphological destruction of the integument (external covering), respiratory system, and subcuticular fat body. A combination of both treatment methods increased cyanobacterial toxic action that led to the degradation of all physiological systems in the insects. Despite the prevalent intestinal activity of the cyanobacteria, external treatment also caused inhibitory effects on all of the main vital functions of the arthropods, and in particular of lepidopterous insects. The high mortality caused by the complex treatment has important practical applications in plant protection and in pest control.

Introduction

New technologies in biological pest control are very significant in plant protection today, especially in testing "non-traditional" organisms. Previous studies on the toxic activity of cyanobacteria (blue-green algae) have not focused on their potential histopathological effects on invertebrates. Nevertheless, many examples of the interactions between cyanobacteria and arthropod populations are known in the natural environment, and form a complex system of ecological and biochemical interspecific relations. There are wide and diverse connections between cyanobacteria and arthropods (*e.g.,* crustaceans, mites, and insects) that include trophic, symbiotic, antagonistic, and parasitic ties in terrestrial and aquatic ecosystems. Various effects of cyanobacteria on arthropods have been documented, from bio-stimulatory to lethal, *e.g.,* toxic action on aquatic crustaceans; their influence on the feeding, reproduction and distribution of soil-living mites, ants, other soil biota, and chironomids; and their toxic, deterrent and repellent action on herbivorous insects (Gol'din, 1982, 1999, 2000; Gol'din and Sirenko, 1998; Hiripi *et al.,* 1998; Mcelhiney *et al.,* 1998; Gol'din and Gol'dina, 2001) and blood-sucking mosquito larvae (Ilyaletdinova, 1975, 1976; Wu *et al.,* 1997). The main objective of this research is to investigate invertebrate-cyanobacterial interactions at the individual organism level. We previously established the feasibility of using cyanobacteria as a new biological pesticide for plant protection. We now try to explain the cause of the insect mortality and discuss this problem using data obtained from experimental tests.

Materials and Methods

Experiments involved natural communities of *Microcystis aeruginosa* Kütz. emend Elenk. from Ukrainian waters (mainly the Dnieper basin) and larvae of the fall webworm *Hyphantria cunea* in the second instar. The host plant (ash-leaf maple) leaves and insects were treated with 0.5% water suspension of dry cyanobacterial powder administered by syringe. Insects were kept in glass containers of 1.0 L volume with 10 specimens in each. Each experimental test included three replicates and a water treatment as the control. During the 15-day experiment, observations on the feeding and survival of the treated specimens were made. Feeding activity was estimated from the consumed fraction of the leaf surface. Various methods of cyanobacterial application (*e.g.,* treatment of food, treatment of insects, and treatment of both food and insects) were tested to observe specific inhibitory actions on the insect larvae and confirmed in each case by histology. For each treatment, 30 fall webworm in the second instar larval phase were tested. Pathological alterations in the insects were studied during a seven-day period. For histological examination, live larvae were sampled on days 3, 5, and 7. The head capsule of the larvae was also removed and fixed in Bouin's solution for 24 hours. After fixing, insect tissues were rinsed in 70% ethanol and treated using standard histological techniques: dehydration, alcohol and xylene exposure, impregnation by paraffin/xylene mixture (1:1), paraffin exposition, sectioning (thin sections 5–6 μm) and staining by haematoxylin and

Table 1 Deterrent action of *Microcystis aeruginosa* on feeding fall webworm larvae.

Version	Fall webworm larvae feeding during 5 days of testing, average leaf surface consumed per individual
Standard culture	0.67%
Supernatant	1.04%
Sediment	1.14%
Control (medium)	9.87%

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Table 2 Activity of some cyanobacterial natural populations on fall webworm larvae in relation to the treatment method (*M. aeruginosa* is prevalent, 98.0%).

	Mortality, %				
Version	3 day	5 day	7 day	10 day	15 day
Treatment of food	3.3	20.0±6.8	20.0±6.8	66.7±3.4	100.0
Treatment of insects	46.7±6.8	56.7±10.2	56.7±10.2	63.3±3.4	80.0±6.8
Treatment of food and insects	40.0±17.0	46.7±3.4	50.0	76.7±10.2	100.0
Control (water)	0	0	0	0	0

eosin. Some preparations were stained by Van Gieson to study muscle tissue (Romeis, 1953; Kiseli, 1962).

Results and Discussion

From the experimental data, we conclude that insect/arthropod mortality from exposure to cyanobacteria depends on two different factors: (1) the deterrent activity that includes inhibition of feeding (Table 1), fat synthesis, growth, and metamorphosis and (2) the action of cyanobacterial toxins that penetrate the insect.

Various methods of cyanobacterial application demonstrated specific activity in relation to the insect/ invertebrate organism, causing special inhibitory effects in each case and increased in the complex treatment assay (Table 2).

Histological examination of larvae feeding on cyanobacteria-treated food material demonstrated irreversible alteration in the midgut (Figs. 1, 2), Malphigian tubes, fat body (Fig. 2) and muscle fibres.

On the third day of the experiment, pathological changes included intensive lesions of the fat body (defined as an increase in the number of abnormal nuclei, and some nuclear and cell wall degradation), some desquamation of the midgut epithelium, caryolysis in the epithelium of the Malphigian tubes, and homogenization of the muscle tissue. On the fifth day, pathological alterations increased, with progressive desquamation of the midgut epithelium, total degradation or fragmentation of the fat body (as islet remnants) in some specimens, and dystrophy or necrosis of the Malphigian tubes and muscle fibers. A compromised excretory function led to increasing intoxication. By the seventh day, midgut degradation had progressed to complete desquamation of the epithelium and exposure of the muscle layer, the fat body was disintegrated except for some islets in the subcuticular zone, the majority of the Malphigian tubule cells were deprived of nuclei, and dystrophy of the muscle fibers had developed. Insect mortality increased each day, with the majority of the larvae dead by day ten.

The cyanobacterial treatment of insects caused morphological destruction of the integument, respiratory system, and the subcuticular fat body. On the third day, exfoliation of the cuticle and necrobiosis of some hypodermal sections was observed. By the fifth day this process had progressed and extended to the superficial parts of the trachea. Fat body size was reduced and pathological alterations appeared in the subcuticular sections. The morphology of the midgut, Malphigian tubes, and cross-striated muscle fibres had changed little. During the following days, larval mortality was slightly more prolonged than in the previous experiment.

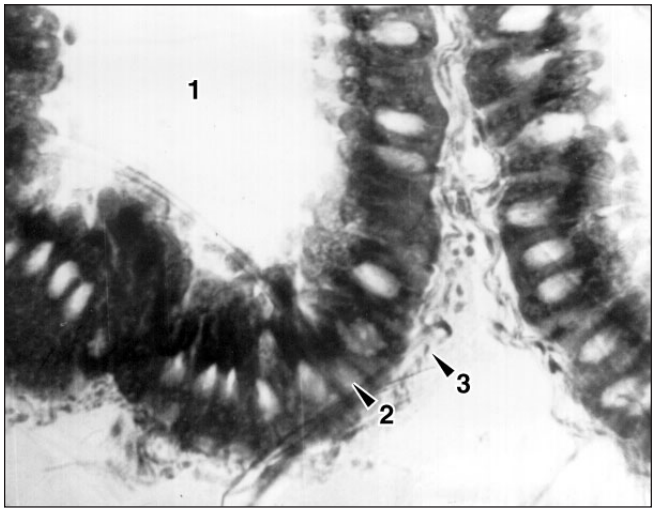

Figure 1 Normal structure of webworm midgut, 16 × 6.3, Van Gieson stain. 1, cavity of gut; 2, epithelium; 3, muscle layer.

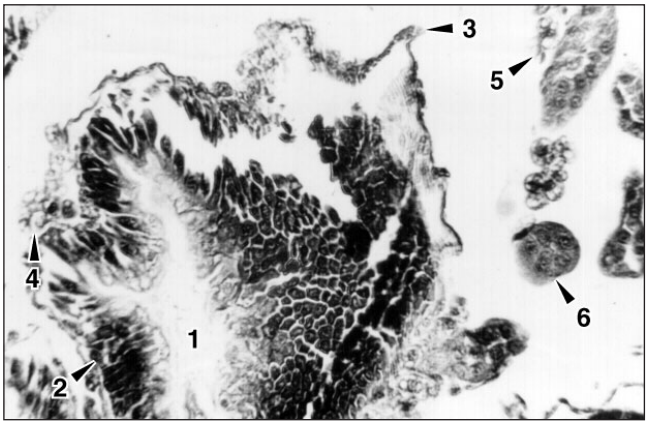

Figure 2 Webworm midgut after cyanobacterial treatment: proliferation and desquamation of epithelium and uncovering of muscle layer, 16 × 6.3, Van Gieson stain. 1, gut cavity; 2, epithelium; 3, muscle layer; 4, deep lesion in the midgut wall; 5, remains of fat body; 6, Malphigian tubes.

A combination of both treatment methods increased cyanobacterial action and led to the degradation of all insect physiological systems. Very deep disintegration was observed in the cuticle, hypodermis and superficial trachea. Disorganization, desquamation and uncovering of the muscle layer were typical for the midgut. Necrobiotic alterations caused by the external and internal effects of the cyanobacteria led to the total degradation and reduction of the fat body. Dystrophic and necrobiotic processes took place in Malphigian tube epithelium and in cross-striated muscle fibres. Despite the prevalent intestinal pathology (see Figs. 1, 2) caused by the cyanobacteria *M. aeruginosa,* the external application of the treatment also contributed to an inhibitory effect in the main vital functions of arthropods, in particular lepidopterous insects (their histological structure is very close to the fall webworm). The high mortality caused by the complex treatment has important practical applications in plant protection and in pest control.

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Effect of the Potentially Toxic Cyanobacteria *Microcystis aeruginosa* and *Nodularia spumigena* on the Survival and Reproductive Success of the Dominant Baltic Copepod Species

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Abstract

The effect of toxic cyanobacteria *Microcystis aeruginosa* and *Nodularia spumigena* on the reproductive success and survival of the calanoid copepods *Eurytemora affinis* and *Acartia bifilosa* was determined. Egg production of *E. affinis* fed a diet of the toxic cyanobacteria decreased with increasing toxicity of strains. When given toxic *N. spumigena* as food, egg production of *E. affinis* was significantly lower (average 0.4 eggs female^{-1} d^{-1}) than the control (average 5.4 eggs female^{-1} d^{-1}). Experiments with *A. bifilosa* showed a different response in egg production. In the presence of all tested strains, there was considerable variability among the experimental individuals), and results were not significantly different from the control (ANOVA; $P > 0.05$). Survival of both zooplankton species was affected by the tested cyanobacterial strains. The highest toxicity was shown by *M. aeruginosa* (strain PCC-7820). *Acartia bifilosa* was more sensitive and showed a higher mortality than *E. affinis.*

Introduction

Food availability and quality are important factors that influence zooplankton production (Rodriguez *et al.,* 1995; Schmidt *et al.,* 1998) and survival (Koski *et al.,* 1999; Ojaveer *et al.,* 2003) in aquatic ecosystems. *Eurytemora affinis* and *A. bifilosa* are dominant copepod species and they are major consumers of the primary production in the Baltic Sea. Blooms of toxic cyanobacteria have been documented from several areas around the Baltic Sea (Sivonen *et al.,* 1989; Balode and Purina, 1996; Kononen *et al.,* 1996; Kankaanpää *et al.,* 2001). This raises concern that zooplankton species may respond differently when feeding on toxic cyanobacteria.

Our objective was to investigate whether toxic cyanobacteria (*Nodularia spumigena* and *Microcystis aeruginosa*) can affect the survival and egg production of the most abundant copepod species (egg-carrying *E. affinis* and free-spawning *A. bifilosa*) in the Baltic Sea.

Materials and Methods

Sampling of zooplankton was carried out in June 2001 and September 2002. Egg production and mortality experiments were conducted using two copepod species, *E. affinis* and *A. bifilosa,* collected from the eastern part of the Baltic Sea, and three strains of toxic cyanobacteria: *N. spumigena* NSGR-2 and *M. aeruginosa* strains MAGR-2 (both isolated from the eastern part of the Baltic Sea) and PCC-7820 (isolated from Scottish waters). Before the experiments, the algal cultures were filtered through nucleopore filters to remove nutrients contained in the ASM-1 medium (Gorham *et al.,* 1964). Cultures were then concentrated ten times and resuspended in the prefiltered (GF/F) seawater from the eastern part of the Baltic Sea (salinity 5 psu). The mean density of cells of both *Microcyctis* strains was 1×10^6 cells mL^{-1}. A similar biomass of the *Nodularia* strain was obtained by equal fluorescence (3×10^3 filaments mL^{-1}). Intracellular toxin concentrations of the strains were estimated by high-performance liquid chromatography with diode array detection, HPLC-DAD (Lawton *et al.,* 1994; Anon., 1998). The concentration of nodularin in the *Nodularia* culture was 0.1 µg mg^{-1} dry weight; the concentrations of microcystins in the *Microcystis* strains (MAGR-2 and PCC-7820) were 0.2 and 5.0 µg mg^{-1} dry weight, respectively.

Copepods were collected from surface waters using a 100-µm plankton net. The samples were diluted in surface water, stored in a plastic container (5-L), and shortly after, transported to the laboratory. Experiments were performed after a 24–48 h adaptation period to a temperature of 15±1°C. Survival experiments with *E. affinis* were carried out in 96-well plates containing the desired food solutions. Eight to ten replicates were conducted for every diet. Filtered seawater (50 µm) was used as a control. The time of exposure of copepods to the toxic strains was five days. The animals were considered dead if they showed no signs of movement.

Females with egg sacs were used for egg production experiments. Egg production per female after 24 h was calculated as $P = N_e/(N_f D)$, where P is the egg production (eggs female^{-1}d^{-1}), N_e is the number of eggs at the end of experiment, N_f is the number of females, and D is the development time of eggs (D at 15°C = 2.2 d; Andersen and Nielsen, 1997).

The experiments with *A. bifilosa* were conducted after a 24-h acclimation period to the experimental temperature. Live copepods were isolated and placed in 20-mL flasks (1 female in each). For the mortality experiment, *A. bifilosa* females were incubated for five days at the experimental food concentrations (four replicate flasks per treatment). For the measurement of egg production under different experimental conditions, single healthy *A. bifilosa* females were placed in 20-mL flasks and exposed for 24 h. The contents of the flasks were rinsed into a petri dish and counted under a binocular microscope. Survival and egg production data were subjected to an ANOVA procedure.

Results and Discussion

By giving toxic *N. spumigena* as food, egg production of *E.*

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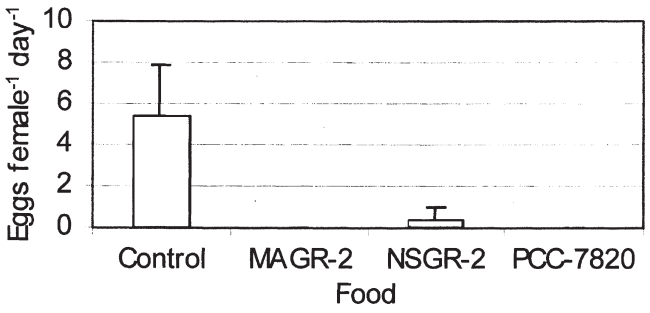

Figure 1 Mean egg production of *E. affinis*.

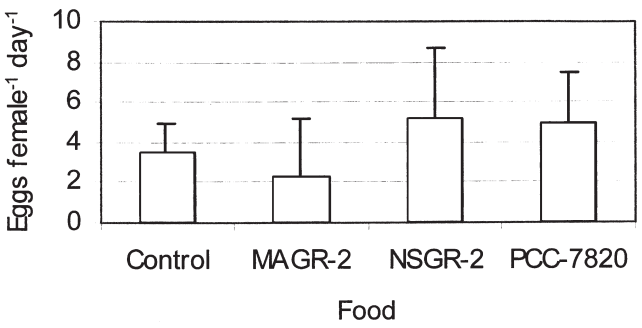

Figure 2 Mean egg production of *A. bifilosa.*

affinis was significantly lower (average 0.4 eggs female^{-1} d^{-1}) than the control (average 5.4 eggs female^{-1} d^{-1}) (ANOVA; $P < 0.01$). No egg production was observed when copepods were fed either strain of *M. aeruginosa* (Fig. 1). Treatment with toxic cyanobacteria also inhibited the formation of new egg sacs in *E. affinis.* Fifty percent of the control *E. affinis* females produced new egg sacs, compared to only 20% of those females fed *N. spumigena.* No new egg sacs were produced by females treated with the *M. aeruginosa* diets.

The effect of algal diets on egg production of *A. bifilosa* is shown in Fig. 2. In the presence of all tested strains, there was considerable variability in egg production among the experimental individuals (Fig. 2). The results were not significantly different from those of the control (ANOVA; $P > 0.05$).

Mortality of *E. affinis* in experiments with the *Microcystis* strain PCC-7820 was significantly higher than in the other experimental treatments and the control (ANOVA; $P < 0.001$; Fig. 3). Exposure to strain PCC-7820 led to a 50% mortality after 24 hours, with 100% mortality of *E. affinis* after five days. The influence of *Nodularia* and *Microcystis* strain MAGR-2 was less expressed and appeared only at the end of the experiments, causing 40% and 10% mortality of *E. affinis,* respectively.

Mortality of *A. bifilosa* fed with either *Microcystis* strains was higher than with *Nodularia.* Significant differences (ANOVA; $P < 0.05$) appeared at the end of the experiment, with 0% survival in the presence of both *Microcystis* strains and 50% using the *Nodularia* strain (Fig. 4).

Toxic *N. spumigena* and *M. aeruginosa* are low-quality food for zooplankton (Koski *et al.,* 1999; Hietala *et al.,* 1995), and they can affect the reproductive success of copepods (Lee *et al.,* 1999). The results suggest that toxic cyanobacteria *M. aeruginosa* and *N. spumigena* have a negative effect on egg production or survival of the dominant Baltic copepods *E. affinis* and *A. bifilosa.* However, a different response in egg production was observed between copepod species and toxic algal strains. *Eurytemora affinis* was able to produce a low number of eggs on an *N. spumigena* diet, while no egg production was observed with strains of *M. aeruginosa.* Therefore, the number of eggs produced by *E. affinis* decreased with increasing toxicity of strains. In *A. bifilosa,* no significant effect of the toxic algae on egg production was observed. Although *A. bifilosa* was able to produce eggs in all treatments, considerable variability amongst the experimental individuals was observed. The maximum egg production was observed in the presence of *N. spumigena* (8.5 eggs female^{-1}d^{-1}). Although previous studies have demonstrated that in the presence of toxic cyanobacteria, copepod reproduction is limited (Koski *et al.,* 1999; Reinikainen *et al.,* 2002), a recent study (Koski *et al.,* 2002) and the results presented here demonstrate that *A. bifilosa* are able to produce eggs. A high variability in the rate of egg production by the copepod *Centropages hamatus* has also been observed in the presence of toxic algae (Turner *et al.,* 1998).

The effect of *Microcystis* and *Nodularia* on the mortality of *A. bifilosa* and *E. affinis* increased over time. The highest toxicity was observed using the most toxic strain, indicating that the toxic effect depends on the toxin con-

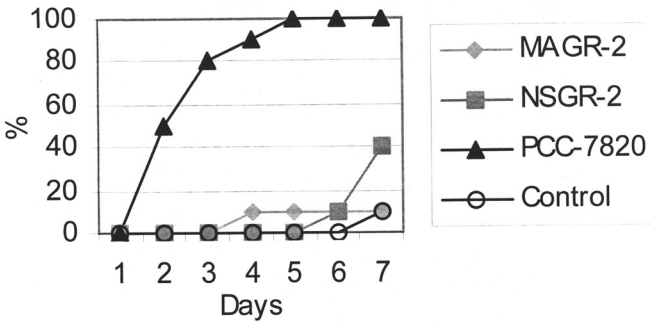

Figure 3 Mortality of *Eurytemora affinis.*

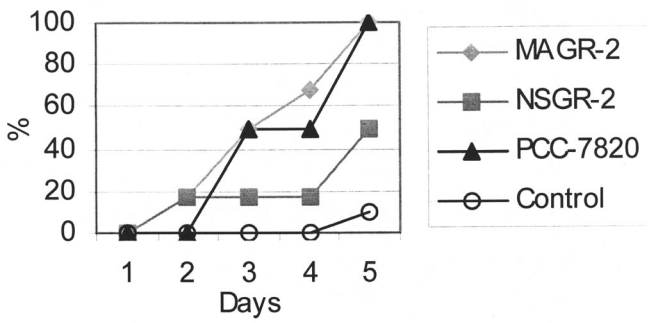

Figure 4 Mortality of *Acartia bifilosa.*

tent of the strains. Previous studies have also demonstrated that the copepods *Eurytemora* and *Acartia* are sensitive to toxic algae (Koski *et al.,* 1999; Reinikainen *et al.,* 2002).

Our study shows that the influence of toxic cyanobacteria is mainly expressed as a decrease in the reproductive capabilities of *E. affinis* and as an increase in *A. bifilosa* mortality. Our results suggest that cyanobacteria blooms can affect the ecological success of zooplankton species in the Baltic Sea.

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Paralytic Shellfish Poisoning Outbreaks in Costa Rica

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Abstract

Paralytic shellfish poisoning (PSP) outbreaks have increased in frequency and intensity over the last few years along the Pacific coast of Costa Rica. In 1999 and 2000, a harmful algal bloom dominated by *Pyrodinium bahamense* var. *compressum* ([Böhm] Steidinger, Tester, and Taylor) and *Gymnodinium catenatum* Graham 1943 affected the health of over 70 people. An episode of this magnitude had not been detected in over 20 years. The mollusk *Spondylus calcifer* Carpenter exhibited maximum levels of toxicity as determined by mouse bioassay. The bloom spread throughout the 1200 km-length Pacific coast of the country. Costa Rica's current phycotoxins monitoring program only takes into account PSP toxins in shellfish; however, the phytoplankton community is very diverse and includes a long list of potentially toxic phytoplankton species, such as *P. bahamense* var. *compressum, Gymnodinium catenatum, Alexandrium monilatum* Howell, *A. catenella* Whedon and Kofoid, *Protoceratium reticulatum* Claparède and Lachmann, *Prorocentrum lima* Ehrenberg, *Dinophysis caudata* Saville-Kent 1881, *D. mitra, Phalacroma rotundatum* Claparède and Lachmann and *Pseudo-nitzschia* spp. It is important to incorporate the detection of other phycotoxins, such as domoic acid and lipophilic toxins, into the current monitoring program.

Introduction

Costa Rica (10°N, 85°W) is a developing Central American country with a territorial extent of only 51.331 km^2 and coasts along the Caribbean Sea and the Pacific Ocean. The upper Gulf of Nicoya (Pacific Coast) is highly productive with 68% of the coastline covered by mangrove forests (Vargas, 1996; Gocke *et al.,* 2001). This gulf is one of the most important ecosystems for artisanal fishing and sustains approximately 600 families.

Among the species of mollusks commercially exploited in this region are the bivalves *Spondylus calcifer, Pinctada mazatlantica, Anadara tuberculosa,* and *Mytella guyanensis.* One hundred percent of their harvesting is carried out in natural areas of Costa Rica because there are no aquaculture projects to produce these species.

Towards the end of 1999 a toxic microalgal bloom was detected, thus obligating the government to ban the harvesting of bivalves along the central Pacific coast of Costa Rica. Unfortunately, this bloom was followed by other episodes that extended the ban by more than two years and practically included the entire Pacific coastline of the country. This situation had very important economic and social impacts. The extension of the closed season drastically affected the income of those lower income families who are dependent on the fisheries. With respect to public health, close to 70 cases of PSP were recorded, with at least six fatalities and some other victims who required hospitalization.

Because of consecutive blooms, the harvest ban was prolonged. However, the data suggest that the harmful algal bloom (HAB) events were an interrupted sequence of incidents, some of which overlapped in time and some of which showed a diversity of dominant toxic species.

The first HAB report in Costa Rica was in 1981 (Hargraves and Víquez, 1981) and was followed by Víquez and Hargraves (1995). There were no further reports until this present communication on the principal toxic phytoplankton species observed during the years 1999–2002 in the Gulf of Nicoya, Costa Rica.

Materials and Methods

Samples of phytoplankton present in the HABs that occurred from 1999–2002 were collected from the Gulf of Nicoya, central Pacific coast (10º00′N, 85º00′W), during both the rainy season (July–November) and the beginning of the dry season (December–June). Samples were obtained using a phytoplankton net (20 µm) and by a vertical Niskin bottle (1-liter) to collect surface water and water at five meters depth. Cells were enumerated with a Sedgwick-Rafter counting chamber. The samples were fixed with a modified Karnovsky solution (Karnovsky, 1965) consisting of 2.5% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer (0.1 M). After the samples were preserved, light and scanning electron microscopy were used for taxonomic identification.

Results and Discussion

Towards the end of 1999, *P. bahamense* var. *bahamense* (Fig. 1A) and *P. bahamense* var. *compressum* (Fig. 1B) blooms were observed, with the dinoflagellate *Gymnodinium catenatum* appearing later (Fig. 1C). Subsequently, as the concentration of *G. catenatum* declined, the concentration of the *Pyrodinium* varieties increased along with the concentration of the dinoflagellates *Ceratium dens, C. furca* and *C. fusus* (Fig. 1D). For several months, all of these species coexisted in the same bloom. A bloom dominated by the cyanobacterium *Trichodesmium erythraeum* was later observed, followed by a *Cochlodinium* cf. *polykrikoides* bloom (Figs. 1E,F).

In other areas of the gulf, an increase was observed in the concentration of the other species listed in Table 1.

During the period studied, concentrations of 10–60 cells per mL were determined in some species. The most significant species were *Dinophysis caudata, P. bahamense* var. *compressum,* and *P. bahamense* var. *bahamense, Gymnodinium catenatum, Pseudo-nitzschia* spp., *Noctiluca scintillans, Prorocentrum micans, Ceratium dens* and *Cochlodinium* cf. *polykrikoides.*

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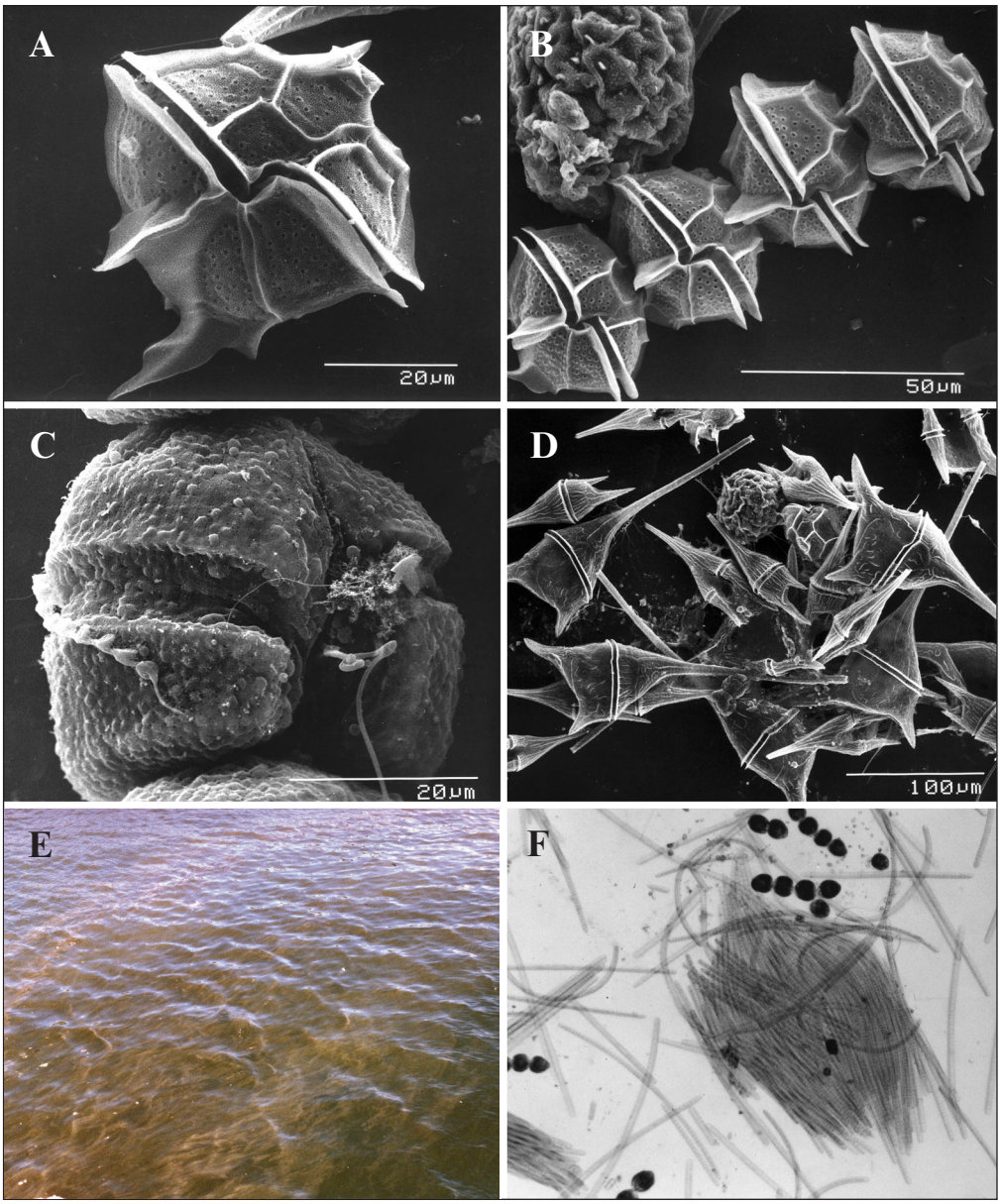

Figure 1 **A** *P. bahamense* var. *bahamense* and **B** *P. bahamense* var. *compressum* HAB producers in Costa Rica; **C** *Gymnodinium catenatum,* **D** dinoflagellates *Ceratium* spp. together at the same bloom; **E** Cyanobacteria bloom from *Trichodesmium erytraeum* and **F** *T. erytraeum* and *Cochlodinium* cf. *polykrikoides* bloom (light microscopy 40×). (Source: INCOPESCA, Costa Rica)

Over the last decade an increase in the number of HAB species along the Pacific coast of Costa Rica has been observed. A great diversity of dinoflagellates exists in this country, especially in the Gulf of Nicoya (Vargas-Montero, 2001). Many of these species have the capacity to produce potent toxins that affect human beings as well as other organisms. Furthermore, in Costa Rica there is no established monitoring plan to relate oceanographic conditions with the occurrence of toxic microalgae.

Due to the lack of resources, Costa Rica has been limited to the analysis of the PSP toxins, however HABs producing other types of toxins have been observed. For example, *Dinophysis caudata* is associated with the production of dinophysistoxins and causing Diarrhetic Shellfish Poisoning (DSP), *Pseudo-nitzschia* spp. is associated with the production of domoic acid and causing Amnesic Shellfish Poisoning (ASP), and other species produce lipophilic toxins. It is important to emphasize the need for implementing the detection of other phycotoxins into the current monitoring program.

The concentration of paralytic shellfish poisoning (PSP) found in different species of bivalves demonstrated an increase in July and October (2001) and a decrease toward the end of December (2001) and the beginning of February (2002) (Fig. 2).

Acknowledgements

The authors received special support from the University

Table 1 Estimate of the frequency of harmful species water samples from the Gulf of Nicoya from 1999 to 2002.

Microalgal Species	Occurrence	Season	Blooms	Type of Toxicity*
Dinoflagellates				
Alexandrium catenella	rare	July	Not observed	PSP (saxitoxin)
Alexandrium monilatum	very frequent	Oct	Sometimes	non toxic
Dinophysis caudata	very frequent (2002)	July	Not observed	DSP
Gymnodinium catenatum	frequent (1999–2001)	July–Sept	Frequent	PSP (saxitoxin)
Noctiluca scintillans	frequent (always)	present all year	Frequent	non toxic
Pyrodinium bahamense var. *bahamense*	frequent (1999–2002)	Nov-Jan	Frequent	non toxic
P. bahamense var. *compressum*	frequent (1999–2002)	July, Dec	Frequent	PSP (saxitoxin)
Prorocentrum lima	frequent	Jan	Not observed	non toxic
Prorocentrum micans	very frequent (2001–2002)	Feb	Not observed	non toxic
Protoceratium reticulatum	rare	Oct	Not observed	yessotoxin
Ceratium dens	very frequent (1999)	Oct, Dec	Frequent	fish kills
Ceratium furca	frequent	Oct, Dec	Sometimes	non toxic
Ceratium fusus	frequent	Oct, Dec	Sometimes	non toxic
Cochlodinium cf. *polykrikoides*	frequent (2002)	Sept–Oct	Sometimes	fish kills
Diatoms				
Chaetoceros curvisetus	rare	Apr–May, Sept	Not observed	non toxic
Chaetoceros lorenzianus	frequent (1999)	Apr–May, Sept	Not observed	fish kills
Pseudo-nitzschia spp.	frequent (1999)	Feb–Mar, Oct	Sometimes	ASP
Cyanobacteria				
Trichodesmium erythraeum	rare (2002)	May–June, Jan	Sometimes	fish kills

*Only the PSP analysis for saxitoxin was done; potential toxicity in the other species was suggested by the comparison with recent publications.

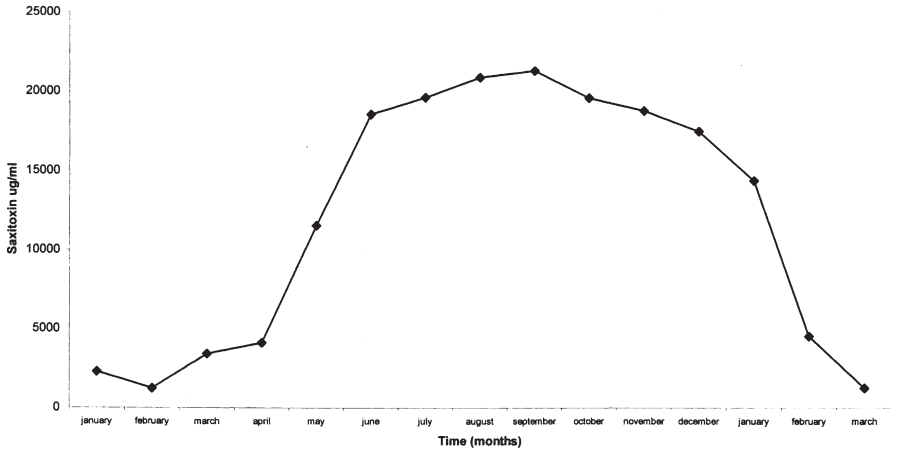

Figure 2 Distribution of saxitoxin in the mollusk *Spondylus calcifer* during 2001. (Source: Instituto Costarricense de Pesca y Acuacultura (INCOPESCA), Costa Rica, 2002)

of Costa Rica, Fundación de la Universidad de Costa Rica para la Investigación (FUNDEVI), and were the recipients of a fellowship from the Costa Rica National Council for Scientific and Technological Research Support Program to Researchers (CONICIT) and Minister for Science and Technology (MICIT). Maribelle Vargas was a recipient of a grant from the Xth International Conference on Harmful Algae, the Students' Federation of University of Costa Rica (FEUR) and Postgraduate System of Studies at the same university (SEP). We are grateful to Beatriz Reguera and Mónica Lion from the Instituto Español de Oceanografía, and Belinda Dick for their suggestions and critical review of the paper.

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Variability of Brevetoxin Accumulation Levels Within Individual Shellfish During *Karenia brevis* Blooms

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Abstract

Blooms of the toxic dinoflagellate *Karenia brevis* are common along the west coast of Florida. These blooms affect fish, marine mammals and birds, causing large epizootic events. Blooms also affect humans by direct exposure to toxic aerosols and by ingestion of contaminated shellfish, which causes neurotoxic shellfish poisoning. An officially recognized mouse bioassay is used to monitor toxicity of shellfish beds due to blooms. Very little is known about individual variation in shellfish from the same area in terms of toxin concentration. The objective of this study was to determine the extent of differences in toxicity between individuals. Shellfish were taken from natural beds during times of high *Karenia brevis* cell counts and brevetoxin was measured using the competitive ELISA. Also non-toxic oysters (*Crassostrea virginica*) and hard clams (*Mercenaria* sp.) were relocated to areas of high cell counts and exposed for several days. Each set of analyses included four individual shellfish and a homogenate of all four combined. The results compared homogenate to individuals to determine how far each individual ranged from the homogenate. Variation among individuals was less than 30% at all times. These results suggest that, within each of the two species studied, individual shellfish accumulate similar amounts of toxin when exposed to the same densities of *Karenia brevis.*

Introduction

Brevetoxins are a family of potent neurotoxins produced by the red tide dinoflagellate *Karenia brevis.* During Florida red tides, shellfish accumulate toxins to levels dangerous to humans. To prevent neurotoxic shellfish poisoning (NSP), commercial and recreational shellfish harvesting is banned when *K. brevis* densities exceed 5,000 cells/L. Shellfish beds remain closed as long as *K. brevis* densities remain higher than 5,000 cells/L and until shellfish are demonstrated to be safe using the regulatory mouse bioassay.

For ethical, safety, accuracy and precision reasons, replacing the regulatory mouse bioassay for NSP monitoring of shellfish has long been a goal of the scientific and regulatory community. Several alternative assays including the competitive ELISA (Naar *et al.,* 2002; Naar *et al.* b, this Proceedings) have been developed and are currently under evaluation as potential alternatives to the mouse bioassay (Dickey *et al.,* this Proceedings).

Figure 1 Locations of shellfish exposures and collections.

Cost-efficiency, simplicity, and rapidity of an alternative assay are important parameters to expedite shellfish bed reopenings and to minimize economic loss to the seafood industry. The challenge is to make the analysis as fast, easy, and reliable as possible. The competitive ELISA appears to meet these criteria and allows the quantification of brevetoxins and brevetoxin metabolites (Naar *et al.* a, this Proceedings) to concentrations as low as 50 ng/g of shellfish meat. However, to further reduce the sample analysis time, sample preparation could be more efficient. Currently, a large number of shellfish must be collected, shucked and blended together. This is a very lengthy process that is followed by extraction and analysis that takes longer with larger sample sizes. With the development of more sensitive methods, faster results can be obtained by analyzing smaller sample sizes.

Until now, methods were not sensitive enough to assess toxin concentrations in individual organisms. As a result, very little is known regarding feeding habits of shellfish during *K. brevis* red tides and how individuals react to brevetoxins (*e.g.,* individual variation in filtering activity). Variability of toxin concentration in individual shellfish from the same area is a key parameter for determining the necessary sample size that will provide an accurate evaluation of the toxin concentration of the area. This study seeks to determine the variability of brevetoxin concentration among individual shellfish exposed to *K. brevis* under identical conditions.

Materials and Methods

During these experiments, shellfish were either harvested

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from natural beds during red tides, or purchased live from aquaculture facilities in absence of red tides, placed in mesh bags, and relocated to areas experiencing *K. brevis* blooms (Fig. 1). Shellfish were exposed to *Karenia brevis* blooms in southwest Florida at various times between October 2001 and October 2002. Oysters (*Crassostrea virginica*) were collected from natural beds in Tampa Bay during red tides in October 2001 and in late September 2002 or were relocated from Alabama and placed in mesh cages in bloom locations (Sarasota Bay and Charlotte Harbor) during March and July 2002. Hard clams (*Mercenaria* sp.) from natural beds in Charlotte Harbor were collected in the absence of a red tide (July 2002) and relocated to an area of high cell concentrations in Sarasota Bay or were collected during a red tide in September 2002. Samples (or collections) consisted of four shellfish from a given bed or bag collected simultaneously and located within 30 cm of each other. Samples were collected over a one- or two-week period. There were a total of 12 oyster collections and four clam collections from natural beds, and 12 oyster collections and six clam collections from bags. Upon collection, shellfish were immediately shucked, individually homogenized, and blended with phosphate buffered saline (PBS, 1 g/40 mL). Additionally, aliquots (1 mL) of each of the four individuals were combined. Individual and combined homogenates were analyzed by ELISA and a comparison was then made between the brevetoxin concentration of individuals and the respective homogenates.

Results and Discussion

Total brevetoxin concentration in oysters from natural beds ranged from 0.7 µg/g of shellfish meat (below the regulatory limit for NSP) to 80 µg/g (100 times above the regulatory limit, Fig. 2). Brevetoxin concentrations in relocated oysters ranged from 0 µg/g (before relocation, data not shown) to 10.7 µg/g. Accumulation of brevetoxin in tissue was observed as soon as one day after exposure.

Overall, toxin concentration in clams collected from natural beds ranged from 0.6 to 3.1 µg/g, while the concentration in relocated clams ranged from 0.2 to 6.04 µg/g (Fig. 2). For the relocation experiments, although clams were purchased in the absence of red tide, some residual brevetoxin was present in the tissue before relocation (0.2 µg/g).

In both oysters and clams, the variability in toxin concentration never exceeded 30% among individuals collected simultaneously from the same location. These results suggest that, within each of the two species studied, individual shellfish accumulate similar amounts of toxin when exposed to the same densities of *K. brevis.* Before performing the study, we anticipated larger variation in toxin accumulation among individuals soon after relocation, as shellfish had been manipulated. We expected variation in their filtration activities and therefore variation in their toxin concentrations. In fact, no difference was observed in shellfish toxin variability throughout the exposure period. There was also no difference in variations between samples that had been relocated and those collected from natural beds.

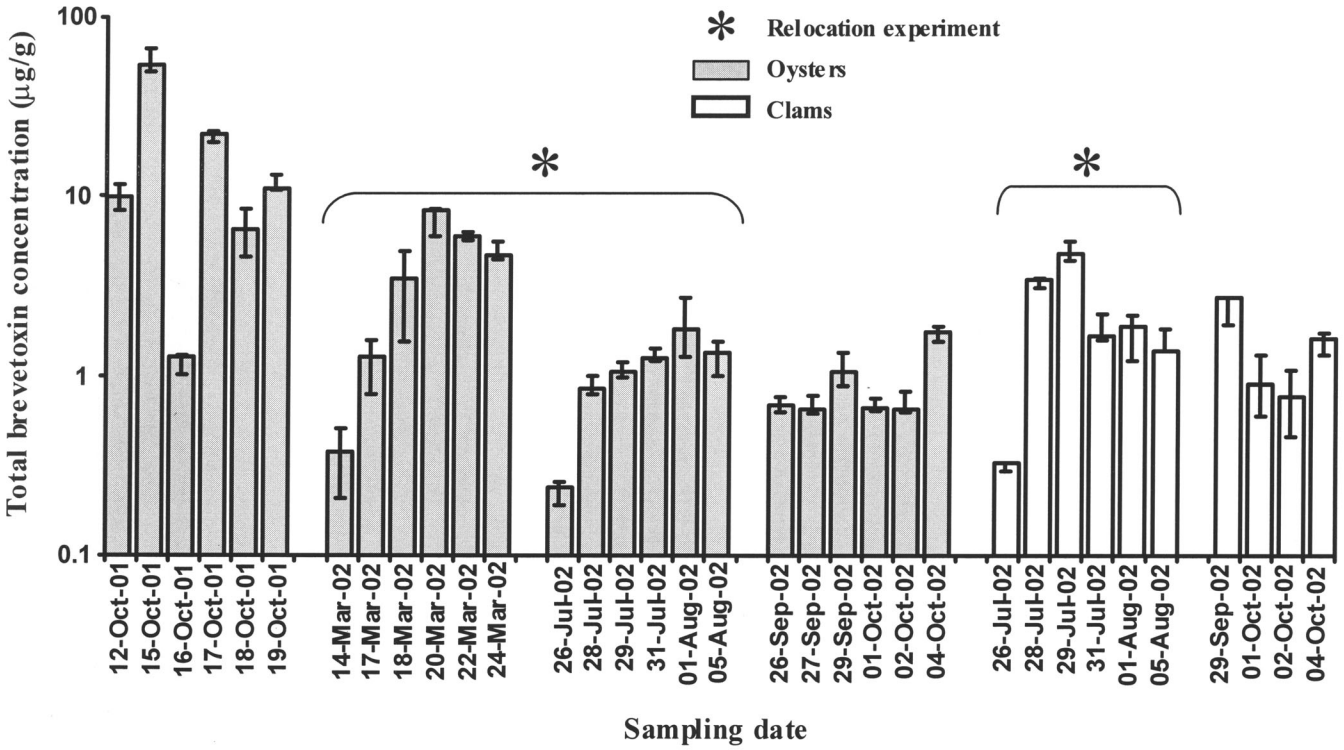

Figure 2 Brevetoxin concentration in shellfish exposed to *Karenia brevis* during natural blooms. Bars indicate the total brevetoxin concentration in each sample of four combined shellfish. Error bars represent the range of brevetoxin concentration measured in the individual shellfish.

The experiments described in this study were designed to assess the variability in toxin accumulation among individual shellfish exposed to the same conditions. Therefore, for both relocation and natural bed experiments, the four shellfish collected for each sample were less than 30 cm apart. Since *K. brevis* blooms are known to form patches and cell densities can vary, this study does not provide information on the variability of brevetoxin concentration in shellfish in large beds where oyster and clams can possibly be exposed to different densities of *K. brevis* cells. Additional studies are required to assess that critical aspect of the sampling in order to ensure a true evaluation of the brevetoxin concentration in populations of shellfish in large beds.

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Brevetoxin Depuration in Shellfish via Production of Non-toxic Metabolites: Consequences for Seafood Safety and the Environmental Fate of Biotoxins

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Abstract

During blooms of the dinoflagellate *Karenia brevis,* filter-feeders such as oysters and clams bioaccumulate brevetoxins, often to levels that are toxic to humans. In controlled aquarium experiments, we exposed live oysters to bloom levels of toxic *K. brevis,* followed by 10 weeks of exposure to non-toxic microalgae. Oysters were harvested weekly and analyzed for brevetoxins and brevetoxin metabolites to quantify toxin bioaccumulation and depuration. All of the PbTx-2 concentrated by oysters was immediately converted to a mixture of polar metabolites that were then slowly eliminated from the oysters. However, 90% of measured PbTx-3 was eliminated within two weeks of toxic exposure but without apparent biotransformation. Extracts of oysters containing high levels of PbTx-3 were toxic to mice by intraperitoneal (IP) injection. Extracts of oysters harvested after PbTx-3 had been eliminated were non-toxic despite high concentrations of PbTx-2 metabolites. Oysters collected in Florida during and after a bloom of *K. brevis* contained polar metabolites of PbTx-2 as well as PbTx-3, but no PbTx-2. Again, PbTx-3 concentration was a good predictor of mouse toxicity. One hundred percent conversion of PbTx-2 to polar metabolites was also accomplished *in vitro* by spiking oyster or clam homogenate with PbTx-2, followed by a brief incubation at room temperature. These PbTx-2 metabolites did not kill mice, either orally or by intraperitoneal injection, even at concentrations 30 times greater than toxic PbTx-3 levels.

Introduction

Neurotoxic shellfish poisoning (NSP) is a form of food poisoning caused by ingestion of shellfish contaminated with brevetoxins. A series of brevetoxin metabolites have been identified from toxic shellfish and the urine of humans suffering from NSP (Poli *et al.,* 2000). In this study, we used this competitive ELISA (Naar *et al.,* 2002), as well as HPLC and mouse bioassays, to track the bioaccumulation, derivatization, and elimination of brevetoxins and metabolites in the Eastern oyster, *Crassostrea virginica.* Experiments were conducted on oysters from commercial shellfish beds following a natural bloom of *Karenia brevis* in Florida as well as on oysters exposed to controlled levels of *K. brevis* in an aquarium. We explore the depuration of brevetoxins by shellfish and evaluate the analytical methods currently in use. The potential consequences for human health and the fisheries industry are discussed as well as the discovery of this significant environmental sink for brevetoxins into metabolites.

Materials and Methods

Oysters from Florida Oysters were collected from two shellfish beds in northwest Florida (east and central Choctawhatchee Bay) on December 18, 2000, and again on January 2, 2001. Four different samples of oysters were homogenized and subjected to one of two treatments:

1 Extraction with diethyl ether then acetone for separation of parent brevetoxins (ether extract) from polar brevetoxin metabolites (acetone post ether extract). Each 1-g aliquot was heated with 10 µL HCl/10 mg NaCl for 5 minutes. After the sample cooled to room temperature, 1–2 mL diethyl ether was added and the sample agitated for 3–5 minutes. Brief centrifugation was used to resolve the aqueous and ether layers. Three more extractions with diethyl ether followed, with all extracts being combined. These extracts are referred to as ether extracts. Residual mollusk meat was then extracted with acetone (2 × 5 mL).

2 Extraction with acetone to extract simultaneously parent brevetoxins and metabolites. Each 1-g aliquot was extracted twice with 5 mL acetone only (as described above).

Aquarium Oysters Oysters were obtained from a shellfish farm in Wilmington, NC, maintained in an aquarium, and fed *Isochrysis* for several months. Six additions of cultured *Karenia brevis* were made over three days to maintain a concentration of *K. brevis* in the aquarium of 5.0×10^5 cells/L. Oysters were harvested at the times indicated below.

In Vitro *Production of Brevetoxin Metabolites Using Oyster and Clam Homogenate* One gram of non-toxic oyster or clam meat from a local (Wilmington, NC) fish market was homogenized and spiked with up to 100 µg of pure PbTx-2 or PbTx-3. Each sample was agitated at room temperature for three hours and extracted with acetone (2 × 5 mL) as described for Florida samples above. Conversion of brevetoxins to brevetoxin metabolites was measured by HPLC followed by ELISA analysis (below).

Samples generated by the above methods were subjected to ELISA analysis, HPLC, or mouse bioassay:

ELISA Methodology ELISA analyses were performed according to Naar *et al.* (2002).

HPLC Methodology HPLC was used to separate compounds from ether extracts, acetone post-ether extracts, and acetone-only extracts. In each case extracts were dissolved in a minimum amount of methanol and injected onto a reversed-phase HPLC column (Phenomenex Inertsil ODS-2). Following injection, materials eluting from the column were collected into separate vials at 1 or 2 min intervals

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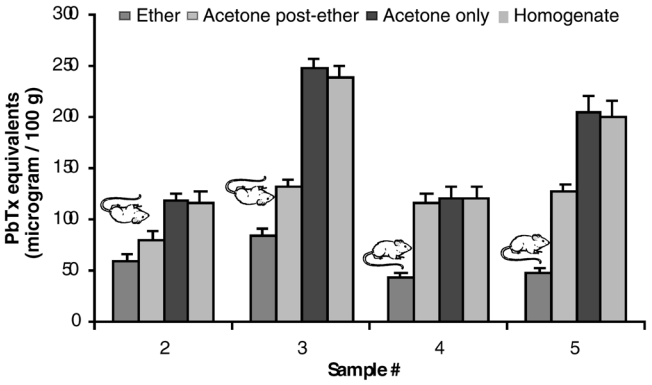

Figure 1 Brevetoxin analyses of four Florida oyster samples by ELISA. Extraction with ether only, ether followed by acetone, or acetone only. Samples 2 and 4 were collected from a commercial bed in central Choctawhatchee Bay two weeks apart. Samples 3 and 5 were collected from east Choctawatchee Bay at the same interval. ELISA analyses were performed on ether extracts (parent brevetoxins), acetone post-ether extracts (polar metabolites of brevetoxins), acetone extracts, and shellfish homogenates (parent brevetoxins and metabolites). Mouse symbols indicate 1) shellfish toxicity according to mouse bioassay (upside down) or 2) absence of toxicity according to mouse bioassay (upside up).

and subjected to analysis by ELISA.

Mouse Toxicity Assays Mouse bioassays were performed according to the regulatory protocol for shellfish monitoring (APHA, 1970).

Results and Discussion

Oysters harvested from two sites along the Florida gulf coast in December 2000 and January 2001 were deemed too toxic for human consumption using the mouse bioassay method, which involves extraction of toxins from shellfish with diethyl ether (Samples 2 and 3; Fig. 1)

There was close agreement between the mouse bioassay results, which were run using ether extracts only, and the ether-soluble brevetoxin concentrations as measured by ELISA. ELISA results on acetone post-ether extracts indicated that not all brevetoxin-like materials were extracted with ether, consistent with the findings of Dickey *et al.* (1999). Extraction with acetone or direct ELISA analysis of the homogenate without extraction appears necessary to accurately account for all of the brevetoxins and brevetoxin-like material in shellfish that has been exposed to *K. brevis.*

Ether-insoluble materials eluted earlier than the parent brevetoxins on reversed-phase columns (acetone post ether extract, Fig. 2) and, therefore, are likely to be closely related to polar metabolites of brevetoxins described by Murata *et al.* (1998) and Poli *et al.* (2000). Quantities of brevetoxin metabolites obtained in this study were insufficient for purification of individual compounds and spectroscopic analysis. In the current study, HPLC separation of shellfish constituents followed by ELISA analysis of fractions indicated that the oysters from Florida contained no PbTx-2, and that the mouse toxicity of the ether-soluble components could be attributed entirely to high levels of

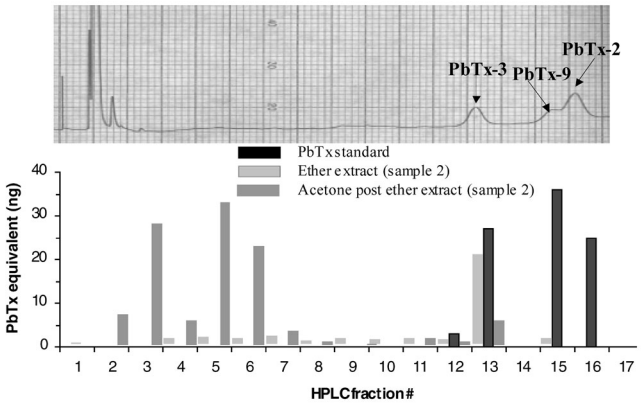

Figure 2 ELISA analysis of HPLC fractions.

PbTx-3 (Fig. 2). This supported the notion that PbTx-2 is rapidly converted to brevetoxin metabolites by shellfish (R. Dickey, pers. comm.).

Using an HPLC method similar to that described by Poli *et al.* (2000) followed by ELISA analysis of HPLC fractions, it was possible to separate parent brevetoxins (such as PbTx-2, -3, and -9) and the compounds that resisted ether extraction (see Fig. 2).

In order to directly test the hypothesis that shellfish biotransformed PbTx-2, toxins were incubated with shellfish meat and then extracted and analyzed by HPLC, followed by ELISA (Fig. 3). PbTx-3 was not biotransformed by the oyster meat, but after three hours of incubation, all PbTx-2 was biotransformed into polar metabolites. Toxicity analysis of the polar metabolites indicated that they had no acute toxicity to mice after IP injection (90 µg/mouse = 20 times LD50 of PbTx-2) or oral administration (200 mg /mouse, 20 times LD_{50} for PbTx-3).

Since the discovery of brevetoxin "metabolites" in biological fluids of people suffering from NSP (Poli *et al.,*

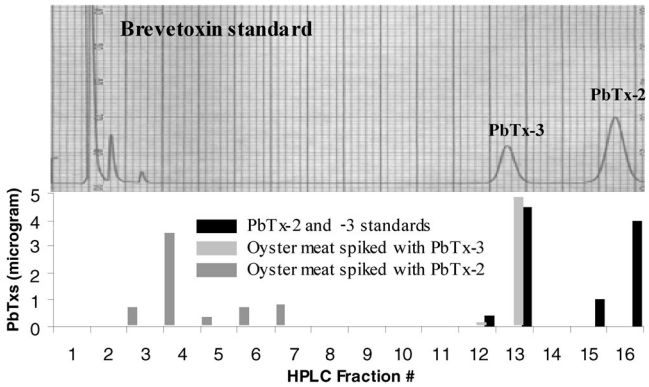

Figure 3 *In vitro* derivatization of brevetoxins by shellfish meat. HPLC fractionation of PbTx standard (5 micrograms of PbTx-2 and PbTx-3, top) and ELISA analysis of HPLC fractions. Standards (solid bars, represent analysis of fraction from HPLC chromatogram presented), oyster meat spiked with PbTx-3 (gray bars), and oyster meat spiked with PbTx-2 (striped bars) versus PbTx equivalents.

2000), detection and characterization of these compounds has been a high priority for the protection of human health. In this study, the presence of polar metabolites of brevetoxins has been demonstrated in both field oysters exposed to a bloom of *K. brevis* as well as in oysters exposed in an aquarium to bloom levels of *K. brevis* (data not shown). From the peak of a bloom to several months afterwards, these metabolites represent 60–90% of the total amount of brevetoxin-reactive compounds present in exposed shellfish. Being more polar, they are resistant to ether extraction and so are not taken into account by the regulatory agencies involved in shellfish monitoring. *In vitro* derivatization of brevetoxins by shellfish meat clearly identified PbTx-2 as a precursor of polar metabolites. This biotransformation process is rapid and efficient, with 100% transformation observed within 3 hours of incubation. Interestingly, these polar metabolites appear to be non-toxic to mice injected or fed with high doses of these compounds (200 μg/mouse). Even though these metabolites do not induce acute toxicity in mice, potential long-term effects linked to chronic exposure are possible and need to be investigated.

Acknowledgements

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Florida Red Tides, Manatee Brevetoxicosis, and Lung Models

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Abstract

In 1996, 149 Florida manatees, *Trichechus manatus latirostris,* died along the southwest coast of Florida. Necropsy pathology results of these animals indicated that brevetoxin from the Florida red tide, *Karenia brevis,* caused their death. A red tide bloom had been previously documented in the area where these animals stranded. The necropsy data suggested the mortality occurred from chronic inhalation and/or ingestion. Inhalation theories include high doses of brevetoxin deposited/stored in the manatee lung or significant manatee sensitivity to the brevetoxin. Laboratory models of the manatee lungs can be constructed from casts of necropsied animals for further studies; however, it is necessary to define the breathing pattern in the manatee, specifically the volumes and flow rates per breath to estimate toxin deposition in the lung. To obtain this information, two captive-born Florida manatees, previously trained for husbandry and research behaviors, were trained to breathe into a plastic mask placed over their nares. The mask was connected to a spirometer that measured volumes and flows *in situ.* Results reveal high volumes, short inspiratory and expiratory times and high flow rates, all consistent with observed breathing patterns.

Introduction

Mortality trends of the Florida manatee, *Trichechus manatus latirostris,* suggest that prolonged exposures to the Florida red tide, *Karenia brevis,* may be one of their leading naturally occurring causes of death as illustrated in 1996, when 149 West Indian manatees died (Bossart *et al.,* 1998; Landsberg and Steidinger, 1998). Pathology from these animals suggested chronic inhalation and/or ingestion of brevetoxins. Lesions were found in the upper respiratory tract, and immuno-histochemical demonstration suggests that a route of exposure is through inhalation (Bossart *et al.,* 1998). Development of a method to study the breathing mechanics of live manatees could provide vital information of how the inhalation of red tide affects these animals. This information would allow for the creation of accurate laboratory models to better understand the deposition and dose of the red tide toxin in the manatee lung. In addition, this information would be useful to veterinary staff that administer anesthesia to injured manatees that require surgical intervention (personal communication, Dave Murphy, Lowry Park Zoo). This new technique for measuring lung mechanics may also be useful when applied to other marine mammals.

The anatomy of the manatee lung has been well studied on dead animals (Bergey, 1986). The airways have extensive cartilage reinforcement down to the terminal bronchioles that are consistent with many marine mammals. These reinforced airways are believed to be beneficial adaptations that assist in diving, however, manatees should not have a need for them, as they are not deep divers. Observations of manatee breathing patterns suggest that they have high tidal volumes and low breathing rates. Manatees may use high flow rates to move large volumes of air in a short period of time. It has been suggested that the manatee's residual volume (RV), or the amount of air remaining in the lung after a maximal exhalation, is very low when compared to total lung capacity (TLC) (Reynolds and Odell, 1991). To determine if this is so, vital capacity (VC), the maximal exhalation following a maximal inspiration (or TLC minus [RV]) must be measured.

Materials and Methods

To date, measurement of these parameters has been through simulation of spontaneous breathing on excised lungs from deceased animals. Unfortunately, lung compliance varies and is affected by the length of time since death (Bergey *et al.,* 1987). This study presents a method to obtain lung volumes and flows from live West Indian manatees. Measurement of lung mechanics, specifically vital capacity (VC), peak expiratory flow rates (PEF), expiratory time (ET) and inspiratory

Table 1 Descriptive maximal values for Hugh and Buffett for each of the training segments.

	VC liters (L)	IVC (L)	PEF (L/sec)	PIF (L/sec)	Expiratory Time (sec)
BUFFETT					
Segment 1	12.26	10.26	12.8	8.3	2.5
Segment 2	17.81	13.67	12.2	9.7	4.0
Segment 3	19.17	15.34	12.3	10.8	2.9
HUGH					
Segment 1	10.66	11.15	13.7	8.8	3.0
Segment 2	10.73	10.73	14.9	7.4	3.2
Segment 3	11.48	11.92	14.4	8.0	2.3

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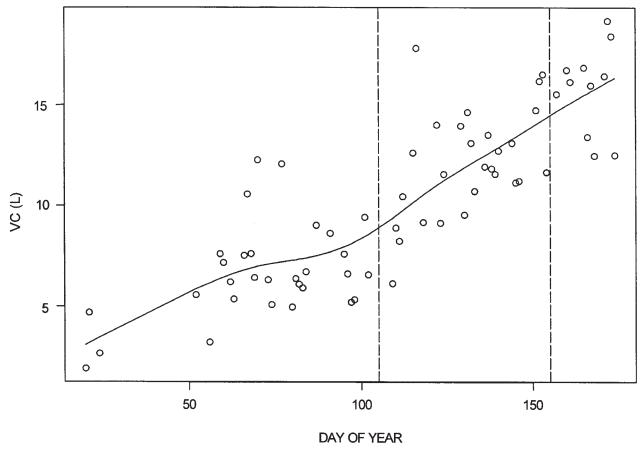

Figure 1 Buffett, Maximum VC by Day.

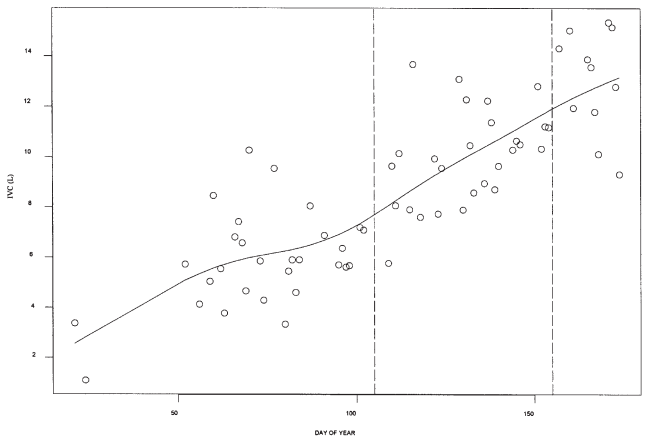

Figure 2 Buffett, Maximum IVC by Day.

vital capacity (IVC) were obtained on two male captive-born Florida manatees, *Trichechus manatus latirostris,* "Hugh" and "Buffett." Hugh was approximately 554 kg and 14 years of age and Buffett was approximately 816 kg and 12 years of age when training for these behaviors was initiated. Both manatees had been trained husbandry and research behaviors previously (Colbert *et al.,* 2001).

In-situ spirometry was obtained through training each animal to breath into a standard resuscitator mask that is typically used with adult humans. The mask, made of plastic, had an air cushion seal for comfort and optimal seal. Once each animal was reliably breathing in the mask, a spirometer (Spirometrics Flowmate LTE) was connected to it. The manatees were reinforced only if they waited for the mask to be correctly positioned over their nostrils and if they exhaled and inhaled into the mask. Manatee trainers determined the amount of time that was spent collecting these data for each session, however, a minimum goal of four breaths per manatee per session was set.

In the first segment of the study (January–April), the manatees were reinforced for all breaths captured by the spirometer. In the second segment of the study, the procedure was altered slightly so that each animal was only reinforced for breaths over 5 liters (L) (April and May). Once a minimum 5-L baseline was established, the third segment of the study was initiated. The goal of the third segment (June) was to determine the maximal flow rates and volumes for each animal. Here, the manatees were reinforced in a stair-step method such that they were only reinforced if they met increasingly greater thresholds. For example, if the first breath were a 5.2 L, the animal would have to achieve higher than a 5.2 on the next breath to be reinforced. If the next breath were a 6.5 L, the third breath would have to be higher than 6.5 L. Training of this manner will continue until the manatees reach a plateau that they cannot surpass. This stair step method will allow the research team to measure maximal effort by the animals and, therefore, measure a true vital capacity.

Spirometry ended on an arbitrary date to evaluate the data collected before continuing the research efforts.

Statistics Superimposed on each plot is a fitted regression line. A nonparametric regression method was used in order not to impose a preconceived functional relationship. Here, five degrees of freedom allowed sufficient flexibility without undue roughness in the curve. Five degrees of freedom correspond, approximately, to a possibility of five changes of direction in the fitted curve (Green and Silverman, 1993).

Results and Discussion

Table 1 shows maximum values for each subject in each of the training segments. Values for Hugh (whose weight is 60% that of Buffett) are, in general, smaller. VC is measured on exhalation and is consistently larger than IVC. This is probably due to the trainers' removing the mask prior to the end of inhalation.

Plots of the maxima for each day are shown in Figures 1–4. Delineation of the three training segments is denoted by the vertical dashed line. For Buffett, the fitted regression line for each of the variables increased steadily through segments one and two, but then, for PEF, PIF and Expiratory Time, leveled off to some extent in segment three.

For Hugh, the curves of daily maxima rose, but then actually decreased during segment three for VC, IVC and PEF. Here, very low values of the maxima for each of these variables on the last day had a strong influence on the fitted curve. If the last day is omitted, then the curves instead flattened out, but did not decrease (not shown).

The flattening of some of the curves in the plots of daily maxima in segment three may indicate that the subjects (especially Hugh) were close to achieving consistent maximum effort in this segment.

For Buffett, the largest value of VC is 19.17 L (in segment three), and for Hugh, the largest value of VC is 11.48 L (also in segment three). It is interesting to note that the ratio of these largest values of VC to body weight (in kilograms) for each of these subjects is 0.021. A similar relationship between the maxima for the other variables and body weight was not seen, however.

Maximal expiratory flow rates more closely indicated maximal effort as a more flattened curve is seen in Figure 2.

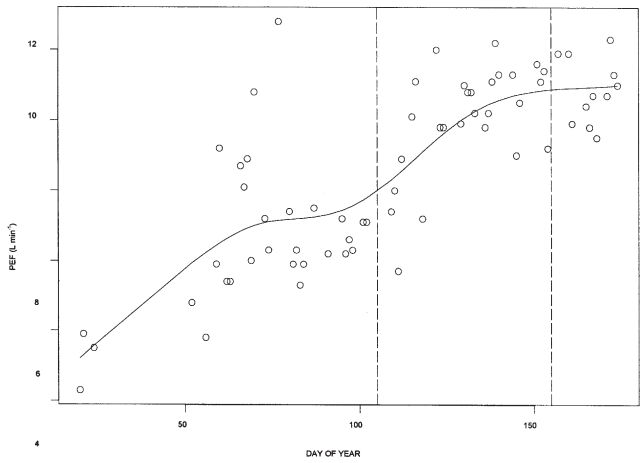

Figure 3 Buffett, Maximum PEF by Day.

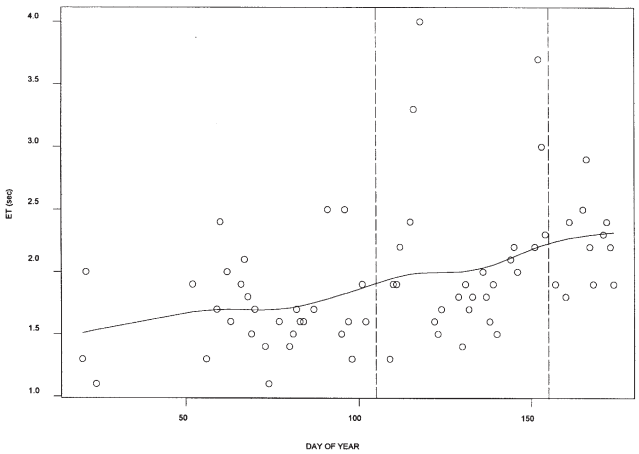

Figure 4 Buffett, Maximum ET by Day.

Discussion

Results from this study show that it is possible to measure the lung mechanics of a manatee. Since a vital capacity requires a maximal exhalation after a maximal inhalation, the results are dependent on the animal's effort, just as spirometry in the human is effort-dependent. Based on the results to date, a true vital capacity has not been reached. If vital capacity had been achieved, we would have expected the line shown in Figs. 1 and 2 to become flat. Further studies may provide maximal inspiratory and expiratory effort and allow for improved understanding of red tide aerosol toxin deposition in manatees.

Acknowledgements

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The Use of Electronic Media to Educate and Communicate with the Public During a Harmful Algal Bloom

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Abstract

Mote Marine Laboratory's website (www.mote.org) educates and communicates the research activities of the laboratory with the press, general public and scientists. From August to December 2001, the Florida west coast experienced a red tide bloom of the dinoflagellate, *Karenia brevis,* resulting in massive fish kills and reports of human respiratory irritation. During that time, the website functioned not only to inform the public of the status of the red tide, but also to solicit reports from the public concerning environmental conditions in their area, specifically fish kills and respiratory irritation. The website received 207 e-mails asking for more information and/or reporting local conditions. We reviewed the e-mails and identified two important issues: 1) the public would like more information and educational materials describing harmful algal blooms, and 2) the information describing health effects and characteristics of the bloom provided by the local community was valuable to Mote scientists in tracking the location and intensity of the red tide. The Web site e-mail method of public communication and surveillance could be valuable in addressing other harmful algal blooms (HABs) as well as other important environmental events.

Introduction

From August to December 2001, the Florida west coast experienced a red tide bloom of the dinoflagellate, *Karenia brevis,* that resulted in massive fish kills and reports of human respiratory irritation. Although red tides occur frequently along this coastline, members of the public, particularly those visiting from other areas, have many misconceptions about red tides and are often frustrated by a perceived lack of control over these events. Kusek *et al.* (1999) conducted a review of all red tide press releases in the St. Petersburg Times newspaper from 1953 to 1997. The review cited numerous articles that either misrepresented information and/or sensationalized information. Lack of information in local news media also occurs when a red tide bloom lasts over a long period of time and is no longer considered "news" (T. Behling, Mote Marine Laboratory Communications Department, personal communication, 2002). Tourists just arriving in the area do not know how to access red tide information unless there is steady information available in the media or they surf the Internet. Examples of websites that address Florida red tide are the following:

- Florida Marine Research Institute (http://www.Floridamarine.org/)
- Mote Marine Laboratory (http://www.mote.org/)
- National Marine Fisheries Service (http://www.sh.nmfs.gov/EAquaBpg.htm)
- International Society for the Study of Harmful Algae (http://www.cbr.nrc.ca/issha/)
- National Oceanic and Atmospheric Administration (http://state-of-coast.noaa.gov/bulletins/html/hab_14/hab.html)
- NIEHS Marine and Freshwater Biomedical Sciences Center, University of Miami (http://www.rsmas.miami.edu/groups/niehs/)
- Northwest Fisheries Science Center (http://www.nwfsc.noaa.gov/hab/)
- Woods Hole Oceanographic Institution (http://www.agu.org/revgeophys/anders01/anders01.html

Florida also has toll-free hotlines that provide red tide updates, fish kill information and respond to health questions. Rapid communication and accurate information availability appears to be a continuing problem in communities where red tides are endemic. Jensen (1994) cited similar problems regarding a New England red tide in 1972.

Mote Marine Laboratory's website educates and communicates the research activities of the laboratory with the press, general public and scientists, utilizing red tide status reports, fact sheets, research data, and links to other red tide sites. Specifically, the website states: "Please contact us if you have any information about dead fish or respiratory irritation." In addition, the laboratory receives numerous telephone calls from the public during active red tides. Although the websites referenced above provide excellent red tide information, only the Mote website solicits the public to report local conditions. Each e-mail receives a personal response from a staff biologist or scientist, demonstrating the laboratory's commitment to public outreach.

Methods

During the bloom, Mote's website functioned not only to inform the public of the status of the red tide, but also to solicit reports from the public concerning environmental conditions in their area, specifically fish kills and respiratory irritation. The website received 207 e-mails asking for more information and/or reporting local conditions and describing human affects. The e-mails were sorted according to their primary message or question so we could separate community feedback from beach status reports during the bloom.

Results

The total number of e-mails received was 207. However,

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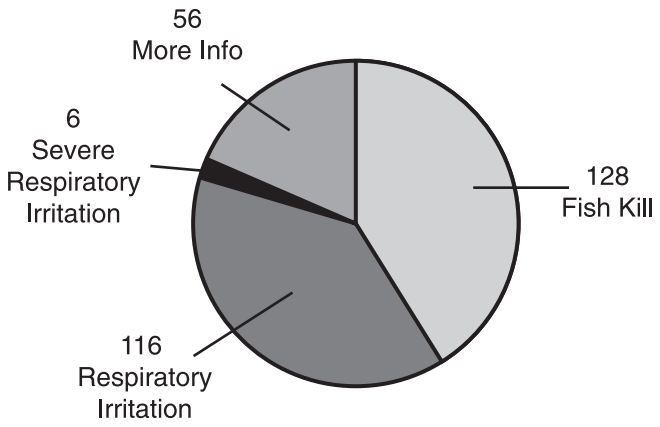

Figure 1 Analysis of e-mails.

many e-mails reported both respiratory irritation and fish kills (47), respiratory irritation and requested more information (35), or some other combination of the four major categories (Fig.1).

We noted that the e-mail identified "Severe irritation" if it cited the need for medical care soon after the exposure. We noted that the e-mail requested "More information" if it included personal requests for an e-mail response, increased press coverage, or more information on the website.

Below are some excerpts from the e-mails received:

- "I do have a major concern. We have never heard of red tide. We only learned of it after my husband asked about all the dead fish on the shoreline."
- "I'm sure all the local people are aware of this situation but what about the people who just happen upon a beach?"
- "My husband & I live in Venice & are really concerned about the red tide that just won't go away…we wanted to ask you if it's possible that this could be caused by man (a terrorist or terrorists)?"
- "I live in Venice and find it curious that nothing has been mentioned about this area in the news and updates on red tide. I saw thousands of dead fish on the shore, and a life guard wearing a dust mask."
- "We had never heard about these symptoms of this phenomenon so it seemed to be an effect of an air borne condition."
- "My son has asthma and we live in Plant City, Fl, since yesterday he has had trouble breathing. My question is "how far from the coastal area could the effects of the RED TIDE begin to effect humans."
- "We went to Casey Point and the water was thick with algae, with lots of dead fish on the water. The water almost looked like oil as it slapped against the rock and plopped back down. There were no pelicans, no fisherman, no people swimming. I was so sad I cried. I thought man had done this, like an oil spill."
- "On Venice beach, there are tons of dead fishes and the water is rusty colored."
- "I was at Siesta beach on Monday and I felt the need to throw up on many different occasions. I stayed only because of the scenery and the fact that if all these pretty girls can be out here, why can't I."

Discussion

From August to December 2001, the Mote Marine website received a large volume of e-mails from the general public. It is clear from these e-mails, that the public would like more information and educational materials describing harmful algal blooms. Since many people were staying in hotels often without Internet access, they relied on local residents and the press to relay needed information about the red tide. Many felt they did not receive adequate information and performed their own research after returning to their homes.

In addition, the information describing health effects and characteristics of the bloom provided by the local community was valuable to Mote scientists and their collaborators in tracking the location and intensity of the red tide. The NIEHS-funded red tide research project requires scientists to quickly identify beaches where airborne toxin symptoms are located and implement field studies at those sites. The information obtained from the e-mails was extremely helpful to the research project to identify the location and intensity of respiratory irritation, a needed component for implementation of the field study. This method of public communication could be valuable in addressing other important environmental events.

Acknowledgements

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Effects of Novel Antagonists of Polyether Brevetoxin (PbTx)-Induced Bronchoconstriction in Allergic Sheep

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Abstract

Florida red tide brevetoxins are potent sodium channel neurotoxins produced by the dinoflagellate *Karenia brevis* (*K. brevis*). When aerosolized, lysed cultures of *K. brevis* (crude PbTx) cause bronchoconstriction, especially in people with underlying airway diseases, such as asthma. PbTx-2 and PbTx-3 are two of the structurally related toxins present in highest concentration during the growth phase of *K. brevis*. In this study, we used sheep with airway hypersensitivity to *Ascaris suum* antigen, as a surrogate for asthmatic patients, to study the airway constrictor responses to crude PbTx, PbTx-2, PbTx-3 and hemi-brevetoxin, a truncated form of the toxin. We also determined if these constrictor responses could be blocked by clinically available drugs and two PbTx antagonists: B-Naphthoyl-PbTx-3, which antagonizes the effects of PbTx-3 *in vitro* and AJB 6.0P, a newly described antagonist, which is produced by the organism, itself. Changes in mean pulmonary airflow resistance (RL) were measured before and after inhalation challenge with increasing concentrations (20 breaths 0.1–10 pg/mL) of PbTx-2, PbTx-3 and hemi-brevetoxin or after challenge with crude PbTx (20 breaths 0.1–1.0 pg/mL). Challenge with PbTx-2 and PbTx-3 produced 226 ± 21% and 204 ± 26% (mean ± se, n = 7) increases in RL over baseline, respectively ($P < 0.05$) at 10 pg/mL, whereas 1.0 pg/mL of crude PbTx induced a 201 ± 9% (n = 4) increase. Hemi-brevetoxin produced only a 69 ± 10% (n = 4) increase. Treating the animals with the histamine H_1 antagonist diphenhydramine, blocked the responses to crude PbTx, PbTx-2 and PbTx-3 but not hemi-brevetoxin. Both B Naphthoyl-PbTx-3 and AJB 6.0P (20 breaths of increasing concentrations 15 min before toxin challenge) significantly reduced the bronchial responses to crude PbTx, PbTx-2 and PbTx-3 in a dose-dependent fashion, but had a minimal effect on the hemi-brevetoxin response. Neither of the antagonists alone affected RL. We conclude that aerosols of *K. brevis* that contain PbTx-2 and PbTx-3 are potent airway constrictors and so could adversely affect human health. The identification of clinically available drugs and new antagonists that can block the effects of these toxins provide avenues to therapies for affected individuals.

Introduction

Florida red tide is a harmful algal bloom caused by the dinoflagellate *Karenia brevis* (*K. brevis*). This organism produces a number of structurally related polyether brevetoxins (PbTx), which are potent sodium channel neurotoxins (Poli *et al.,* 1986; Baden 1989; Purkerson-Parker *et al.,* 2000). There is evidence that inhaled toxins are associated with non-productive cough and shortness of breath in humans, and toxin exposures have been reported to induce asthma attacks in patients with the disease (Watanabe *et al.,* 1988; Asai *et al.,* 1992). The symptoms induced and the exacerbations of asthma resulting from exposure to inhaled toxin suggest they pose a serious health risk, especially to individuals with compromised airways. However, there have been relatively few standardized inhalation studies using these toxins to adequately characterize their potential toxicity. To better understand the mechanisms of acute toxin-induced effects in the airways, we used sheep with airway hypersensitivity to *Ascaris suum* antigen (allergic sheep), as a surrogate for patients with compromised airways (Abraham, 2000) and compared the airway responses to aerosolized toxin from lysed cultures of *K. brevis* (crude PbTx) with those obtained with PbTx-2 and PbTx-3, two of the toxins present in the highest concentration during the organism's growth phase. We also examined the airway responses to hemi-brevetoxin, a truncated form of the molecule, which has the B, C, D, and E rings removed (Gawley *et al.,* 1995) to determine if the size of the molecule might affect the airway response. To determine if the toxin-induced airway responses could be blocked, we treated the animals with clinically available drugs before toxin challenge. In addition, for the first time *in vivo,* we tested the inhibitory activity of B-Naphthoyl-PbTx-3, a synthetic PbTx-3 antagonist (Purkerson-Parker *et al.,* 2000) and AJB 6.0P (recently renamed Brevenal), a newly described antagonist, which is produced by the organism, itself.

Materials and Methods

The Mount Sinai Medical Center Animal Research Committee approved the described studies. We measured changes in pulmonary airflow resistance (RL) to inhaled toxins using the esophageal balloon technique in intubated allergic sheep described by Abraham *et al.,* (1994). Sheep were challenged with aerosols of crude PbTx, PbTx-2, PbTx-3 and hemi-brevetoxin using the aerosol delivery system described by Abraham *et al.,* (1994). The generated aerosols were delivered directly to the animals' lungs via the endotracheal tube. Baseline RL was measured and then the sheep were challenged with 20 breaths of increasing concentrations of toxin: 0.1, 0.3, 1, 3 and 10 pg/mL of PbTx-2,

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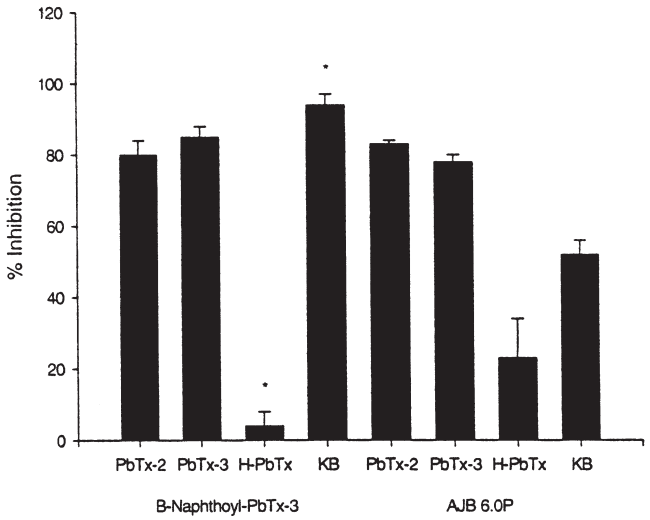

Figure 1 Effects of B-Naphthoyl-PbTx-3 and AJB 6.0P on toxin-induced bronchoconstriction. Values are mean ± se for 2-7 sheep. Data are presented as percent (%) inhibition of the increase in RL produced by aerosol challenge with 10 pg/mL of PbTx-2, PbTx-3, and H-PbTx and 1 pg/mL of KB (100% inhibition = complete blockade of the toxin-induced bronchoconstriction). The sheep were treated with 100 pg/mL of the antagonists before challenge with the pure toxins and 10 pg/mL of the antagonists before challenge with the crude toxin. * $P < 0.05$ vs. AJB 6.0P. *K. brevis* (KB), hemi-brevetoxin (H-PbTx).

PbTx-3 and hemi-brevetoxin or 20 breaths of 0.1, 0.3 and 1.0 pg/mL of crude PbTx. RL was measured after each delivered concentration. Responses to the various toxins alone were compared to those obtained after treatment with the histamine H_1 antagonist, diphenhydramine (2 mg/kg, iv, given 30 min before challenge), and the two PbTx antagonists: B-Naphthoyl-PbTx-3 (20 breaths of 1, 10, 30, 100 pg/mL and AJB 6.0P (20 breaths of 3, 10, and 100 pg/mL). The PbTx antagonists were given 15 min before the start of the toxin challenges. Repeat challenges were separated by a minimum of 48h. Data were analyzed with a one-way analysis of variance and Tukey's post hoc test. $P < 0.05$ was considered significant. Values in the text are reported as mean ± se.

Results

Inhalation challenge with crude PbTx (*K. brevis*), PbTx-2, PbTx-3 and hemi-brevetoxin all caused concentration-dependent increases in RL. At 1.0 pg/mL crude PbTx induced a 201 ± 9% (n = 4) increase in RL ($P < 0.05$), whereas challenge with 10 pg/mL PbTx-2 and PbTx- 3 produced 226 ± 21% and 204 ± 26% (n = 7) increases in R_L over baseline, respectively ($P < 0.05$). In contrast hemi-brevetoxin at 10 pg/mL only increased RL 69 ± 10% (n = 4), which suggests that the length of the molecule has some effect on constrictor potency in the airways. Pretreatment of the animals with diphenhydramine inhibited the constrictor responses to crude PbTx, PbTx-2, and PbTx-3 by 67–84%. However, the antihistamine only inhibited the response to hemi-brevetoxin by 23 ± 10% ($P < 0.05$ vs. all others). B-Naphthoyl-PbTx-3 was equally effective in inhibiting the PbTx-2- and PbTx-3-induced bronchoconstriction (Fig. 1). At 100 pg/mL, B-Naphthoyl-PbTx-3 inhibited the responses to 10 pg/mL PbTx-2 and PbTx-3 by 80 ± 4 and 85 ± 3%, respectively ($P < 0.05$). B-Naphthoyl-PbTx-3 had no effect on the hemi-brevetoxin constrictor response. AJB 6.0P also inhibited the constrictor responses to PbTx-2 and PbTx-3, but was slightly less effective against PbTx-3 than against PbTx-2. This is different from the results obtained with B-Naphthoyl-PbTx-3. Interestingly, another difference between the two antagonists was that AJB 6.0P showed a minor protective effect (23 ± 11% inhibition) against the hemi-brevetoxin-induced constrictor response (Fig. 1).

The two antagonists also showed differential effects against the crude PbTx-induced constrictor response. As illustrated in Fig. 1, treatment with 10 pg/mL B-Naphthoyl-PbTx-3 provided 94±2% inhibition of the crude PbTx response, compared to only 52± 4% inhibition with AJB 6.0P (P<0.05). Such a result would be consistent with the crude extract containing a greater proportion of PbTx-3 and the reduced activity of AJB 6.0P against this toxin.

Conclusion

Our study shows that aerosols of *K. brevis* that contain PbTx-2 and PbTx-3 are potent airway constrictors in a sheep model of asthma. The toxin concentrations (1–10 pg/mL) used in the present studies are consistent with values (40 pg/mL) that have been reported to cause respiratory irritation in man (Pierce, 1986). The bronchoconstriction can be blocked with the histamine H_1 antagonist diphenhydramine, indicating that part of the response is mediated by histamine, presumably released from airway mast cells. We also show for the first time *in vivo* that the constrictor effects of both crude and purified PbTx can be blocked with novel synthetic and natural toxin derivatives. Hemi-brevetoxin, a truncated form of the molecule, also causes bronchoconstriction, but the response is less severe, and the response is not significantly blocked by either the anti-histamine or the PbTx derivatives. This suggests that the molecular size of the PbTx is important for eliciting the maximum airway response and based on the antagonist studies could indicate that this modified molecule induces constrictor responses through alternative pathways.

These studies re-confirm the use of bronchoprovocation studies in the sheep model of asthma as a means of verifying adverse respiratory events that occur with human exposures and to test potential therapies to protect against these adverse events.

Acknowledgements

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Characterization of Red Tide Aerosol on the Texas Coast

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Abstract

The Gulf of Mexico red tide, caused by the dinoflagellate *Karenia brevis* (= *Gymnodinium breve*), occurs almost annually and has adverse economic and health effects. Exposure of people to sea spray containing aerosolized brevetoxins (PbTxs, polyether brevetoxins produced by *K. brevis*) causes irritation of the eyes, nose, and throat. Anecdotal reports suggest that exposed individuals can experience respiratory irritation and exacerbation of existing respiratory illnesses. There has been no systematic study of human exposure to red tide aerosols. In the fall of 2000, during a red tide episode on the Gulf Coast near Corpus Christi, Texas, we sampled at the Marine Science Institute (MSI) at Port Aransas on 25 October. Between 26–27 October we sampled at the Texas State Aquarium (TSA) near Corpus Christi. Two Hi-Vol samplers equipped with a filter and a five-stage impactor gave low concentrations of PbTxs, requiring us to develop methods to improve the minimum detection limit. An LC/MS/MS technique was used combining an HPLC and the API 365 MS/MS. PbTx-2 and PbTx-3 were detected at the TSA sampling location; however, PbTx was not detected in the samples from the MSI. The concentration of PbTx-2 was 1.5–4.9 ng m^{-3} but was much lower for PbTx-3. The ratio of PbTx-2 to PbTx-3 was 8.7 ± 5.2. During the highest exposure period (26–27 October), PbTx-6 was also detected. No one reported respiratory symptoms at the MSI, whereas at the TSA, several field study workers reported symptoms including nose and throat irritation, and itchy skin. A high-volume impactor was used to aerodynamically classify the particles into different size fractions. PbTx-2 was detected in all samples taken at the TSA; however, PbTx-3 was detected only between 26–27 October when the PbTx concentration was high. The mass median aerodynamic diameter (MMAD) was 7–9 mm with a relatively narrow size range (geometric standard deviation [GSD] about 1.6). In this study, much lower airborne concentrations of PbTx, 1.6–6.7 ng m^{-3} were reported, along with a few incidents of upper respiratory symptoms. Although the number of seven workers was too small for statistical analysis, the reported symptoms were consistent with no to low exposure at the MSI and detectable exposures at the TSA. This suggests that at lower environmental concentrations of about 2–7 ng m^{-3}, exposure to PbTx could result in upper respiratory symptoms. This is consistent with the particle size measurement.

Introduction

Red tides in the Gulf of Mexico are commonly formed by the fish-killing dinoflagellate, *Karenia brevis* (= *Gymnodinium breve*). Red tides off the Florida Gulf coast are almost annual events (Kirkpatrick *et al.,* in press). Environmental aerosol samples collected during red tides in Florida and North Carolina in 1987 (Pierce *et al.,* 1989; 1990) showed high concentrations of three brevetoxins (PbTx-2, -3, and -5), but measurements of particle size for the red tide aerosol were not reported. During two red tide events in 1999, PbTx levels in air and seawater were measured while conducting personal interviews and pulmonary function tests on people before and after visiting Florida beaches (Backer *et al.,* 2003). During moderate and high exposure periods, 36 ng/m^3 and 80 ng/m^3, respectively, of PbTx were detected in the air. Lower respiratory tract symptoms (*e.g.,* tightness of chest, wheezing, and shortness of breath) were reported by 8% of the people having no/low exposure, 11% with moderate exposure, and 28% with high exposure. Upper respiratory symptoms (eye and throat irritation, nasal congestion, and cough) were also increased in the moderate and high exposure groups.

In the fall of 2000, there was a red tide event near Corpus Christi, Texas. Field study teams monitored the air and water levels of PbTxs and particle size distribution. In addition, personal samples for PbTx air concentrations were obtained from a few researchers to assess exposure and response, and respiratory symptoms were noted.

Methods

Two locations on the Gulf coast near Corpus Christi, Texas were studied. Air samplers were set up at a waterfront location at the Marine Science Institute (MSI), University of Texas at Austin, Port Aransas, and samples were taken from 0900–1600 on 25 October. After 1600 the sampling equipment was moved to the Texas State Aquarium (TSA) where respiratory symptoms were reported. Discolored water was seen adjacent to the TSA, and was verified as a bloom of *K. brevis.* Air samples were collected from 1800 on 26 October and continued through 1600 on 27 October.

Two high-volume air samplers (Model G2000H, Andersen Instruments, Smyrna, GA) were placed about one meter from the water to collect large quantities of material for analysis. One was used to collect suspended particles on one filter substrate for total aerosol concentration, whereas the second sampler housed a five-stage, high-volume cascade impactor (Model SA235, Andersen Instruments). Glass fiber filters (20 cm × 25 cm) were used for the collection substrate (Whatman EPM2000; Maidstone, UK). The sampling flow rates were 1220 and 1390 L min^{-1} for the filter and impactor sampler, respectively. Personal exposure levels were measured by three volunteers who each wore a per-

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Table 1 Total air concentration (ng m^{-3}) of brevetoxins obtained by the high-volume impactor and filter samplers at Marine Science Institute (MSI) or Texas State Aquarium (TSA).

			Filter Sampler			Impactor Sampler		
Location	Date	Sampling Time	PbTx-2	PbTx-3	PbTx-6	PbTx-2	PbTx-3	PbTx-6
MSI	10/25/00	7.60 hr	0.00	0.00	0.00	0.00	0.00	0.00
TSA	10/25–26/00	15.17 hr	1.50	0.13	0.00	1.49	0.00	0.00
TSA	10/26/00	8.58 hr	2.00	0.14	0.00	4.02	0.00	0.00
TSA	10/26–27/00	15.83 hr	4.89	0.67	1.09	5.86	0.92	0.00
TSA	10/27/00	6.28 hr	1.69	0.84	0.00	2.77	0.00	0.00

sonal sampler (IOM Inhalable Dust Sampler, SKC, Inc., Eighty Four, PA) connected to a battery-operated pump (Hi Flow Sampler, Gillian Instrument, Wayne, NJ). The sampling flow rate was 2 L min^{-1}, controlled by a rotameter in the sampling pump.

Filters from high-volume filter and impactor samplers were extracted with methanol. The extract was diluted and analyzed for PbTxs by an LC/MS technique using an HPLC (SIL-DAD vp, Shimadzu Co., Kyoto, Japan), coupled with the API 365 MS/MS (Applied Biosystems Inc., Foster City, CA) run in the positive ion mode. Using PbTx standards provided by UNCW, we established standard curves for PbTx-2, -3, -6, and -9. The personal sampling filter collected small amounts of PbTx, which were analyzed by a competitive ELISA (Naar *et al.,* 2002) based on the specific activity of the goat anti-PbTx antibody.

Results

Air concentrations of PbTxs determined by the high-volume filter samples are listed in Table 1. PbTx-2 and -3 were detected in the TSA sampling location; however, PbTx was not detected in the samples from the MSI. The concentration of PbTx-2 was between 1.5–4.9 ng m^{-3}, but the concentration of PbTx-3 was much lower. The ratio of PbTx-2 and -3 was 8.7 ± 5.2. In the highest exposure period (26–27 October), PbTx-6 was also detected. No one reported respiratory symptoms at the MSI whereas at TSA, several field study workers reported symptoms including irritation in the nose and throat, and itchy skin.

A high-volume impactor was used to aerodynamically classify the particles into different size fractions. The PbTx concentration collected in each impactor stage was summed to give the total air concentrations as listed in Table 1. The air concentrations from both samplers were similar. The two samplers were located within about 20 meters of each other. PbTx-2 was detected in all samples taken at the TSA; however, PbTx-3 was detected only from 26–27 October when the PbTx concentration was high. This indicated that after fractionating the particles by size, there is not enough PbTx-3 in any size category to be detectable. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of these size distributions are listed in Table 2. The results show large particles with a mean size 7–9 mm and a relatively narrow size range (GSD about 1.6).

Personal exposure levels of PbTx were measured with personal samplers. Three personal samplers were used on 25 October and 26 October during the daytime sampling period. The total PbTx concentration was 4–18.3 ng m^{-3}. PbTx concentrations varied among the three persons by a factor of 2.0–3.9 respectively, for the two sampling days. All three volunteers were working near the waterfront, but they were not at the same location and were involved in different activities. The variability of personal samples may reflect the different activity patterns of the workers. The personal samples (ELISA analysis) represent the total PbTx concentration and were generally higher than those reported by environmental samples (Table 1).

Discussion

Red tide events in the Gulf of Mexico have been historically reported along the western coast of Florida and can occur nearly annually. Red tides along the Texas coast are much less frequent. A previous study of recreational exposure to airborne PbTxs in Florida (Backer *et al.,* 2003) showed that airborne concentrations between 36–80 ng m^{-3} correlated to increased reports of both upper respiratory symptoms (eye irritation, nasal congestion, throat irritation, and cough) and lower respiratory symptoms (chest tightness, wheezing, and a shortness of breath). In this study, much lower airborne PbTx concentrations between 1.6–6.7 ng m^{-3} were reported, along with a few reports of upper respiratory symptoms (throat irritation, nasal irritation, and itchy skin) and no reports of lower respiratory symptoms. Although the number of workers observed was too small for statistical analysis, the reported symptoms were consistent with no/low exposure at the MSI and detectable

Table 2 Aerodynamic particle size distributions of high-volume impactor samples.

Date	Sampling Time	MMAD mm	GSD
10/25–10/26	15.17 hr	9.0	1.62
10/26/00	8.58 hr	8.2	1.57
10/27/00	6.28 hr	7.0	1.60

exposures at the TSA. This suggests that at lower environmental concentrations of about 2–7 ng m^{-3}, exposure to PbTx could result in upper respiratory symptoms. This lower level of airborne PbTx concentrations could be detected using a more sensitive LC/MS technique. The detection limit could be further lowered <1 ng m^{-3} when we improve the extraction technique. Our measurements of particle size distribution with the impactor samplers are the first time that particle size of PbTx was reported. The MMAD was between 7–9 mm, a relatively large size for inhaled ambient particles. Fine particles below 2.5 mm were not detected. Inhaled particles of this size would be deposited in the upper respiratory tract (nasal, oral, and pharyngeal area) (ICRP, 1994; Yeh *et al.,* 1996), and subsequent respiratory irritation could result from the presence of the particles themselves or from toxins associated with the particles. Inhaled particles would also be deposited on the face and exposed skin, causing the skin to itch.

Acknowledgements

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Florida Red Tide: Inhalation Toxicity of *Karenia brevis* Extract in Rats

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Abstract

Brevetoxins are neurotoxins produced by the marine dinoflagellate *Karenia brevis*. Histopathologic examination of marine mammals dying following repeated exposure of brevetoxins during red tide events suggests that the respiratory tract, nervous, hematopoietic, and immune systems are potential targets for toxicity in repeatedly exposed individuals. The purpose of this experiment was to evaluate the effects of repeated inhalation of *K. brevis* extract on these potential target systems in rats. Male Sprague-Dawley rats were exposed four hours/day, five days/week for up to four weeks to target concentrations of 200 and 1000 µg/L *K. brevis* extract (approximately 50 and 200 µg/L brevetoxin-like compounds; positive neurotoxicity in a fish bioassay). Control rats were sham exposed to air. Immunohistochemical staining of pulmonary macrophages indicated deposition of brevetoxin-like compound within the lung. However, exposure resulted in no clinical signs of toxicity or behavioral changes. There were no adverse effects on hematology or serum chemistry. No histopathological changes were observed in the nose, lung, liver, kidneys, lymph nodes, spleen, or brain of exposed rats. Immune suppression was suggested by reduced responses of spleen cells in the IgM-specific antibody-forming plaque cell response assay and reduced responses of lymphocytes to mitogen stimulation *in vitro*. Differences between responses observed in rats in this study and those observed in manatees may be a function of dose or species differences in sensitivity.

Introduction

Brevetoxins (PbTxs) are potent neurotoxins produced by the marine dinoflagellate *Karenia brevis. Karenia brevis* blooms are responsible for the red tides occurring almost annually in the Gulf of Mexico and Atlantic coast of Florida (Baden, 1989). Inhalation of aerosolized PbTxs in sea spray results in almost immediate irritation of the eyes and respiratory tract that generally abates when people leave the beach area (Baden, 1989; Kirkpatrick *et al.,* in press).

Brevetoxins also produce respiratory tract responses at extremely low concentrations. Recently, Backer and colleagues (2003) correlated the extent of respiratory tract symptoms experienced by individuals recreationally exposed to aerosolized PbTxs during a *K. brevis* red tide and the brevetoxin concentration in the air. Significant increases in eye and throat irritation and cough and chest tightness were reported by individuals exposed to <10 to 36 ng total PbTx/m^3, while significant increases in nasal congestion and wheezing were reported by individuals exposed to 20–93 µg PbTx/m^3. Little is known about the long-term health effects associated with inhalation of aerosolized PbTxs during red tide events. Examination of manatees dying as a result of a *K. brevis* event suggest that the respiratory tract, nervous, immune, and hematopoietic systems are potential targets for toxicity upon repeated inhalation and/or ingestion, but dose-response relationships have not been established (Bossart *et al.,* 1998). The purpose of this study was to initiate examination of the health effects associated with inhalation of aerosolized *K. brevis* extract for up to four weeks.

Materials and Methods

The two batches of extract used for this study were prepared at the Center for Marine Sciences, University of North Carolina at Wilmington, NC, by extracting *K. brevis* cultures with chloroform (1 L chloroform per 10 L culture). The chloroform layer was removed, dried, and analyzed for total brevetoxin by ELISA (Naar *et al.,* 2002). The extracts were provided in aliquots of 10 mg of brevetoxin-positive material. High-pressure liquid chromatographic analysis (with UV detection) indicated *K. brevis* contained three main components (relative percent): PbTx2 (82), PbTx3 (12.6), and the potent PbTx antagonist, AJB6.0p (6.1). The first preparation was not chemically characterized, but the relative concentration of major components is expected to be similar to that of the second extract preparation used. Characteristics of the antagonist have recently been described (Bourdelais *et al.,* 2003).

Male Sprague-Dawley rats, five to six weeks old, were purchased from Charles River Laboratories (Wilmington, MA). The study was conducted under an IACUC-approved protocol, and animals were treated in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, 1996). The rats were randomized by weight into three groups: 1) control (sham exposed to filtered air), 2) the low dose group (50 µg brevetoxin equivalents/m^3), and high dose group (200 µg brevetoxin equivalents/m^3). For the core sub group, six rats/level were sacrificed after one and four weeks of exposure and four weeks after termination of exposure. For the immunology group, five rats/level were sacrificed after one and four weeks of exposure and four weeks after termination of exposure. The neurotoxicity subgroup consisted of three rats/level sacrificed after one and four weeks of exposure.

The rats were exposed for four hours/day, five days/week, for up to four weeks. Aerosols were generated by nebulization from a solution containing 0.67 mg brevetoxin

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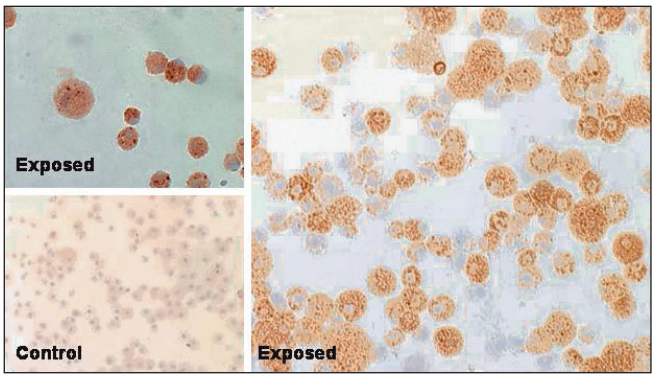

Figure 1 Immunohistochemical staining of alveolar macrophages from exposed animals (top left and right panel). Cells from control animals (bottom left panel) did not stain positive for brevetoxin.

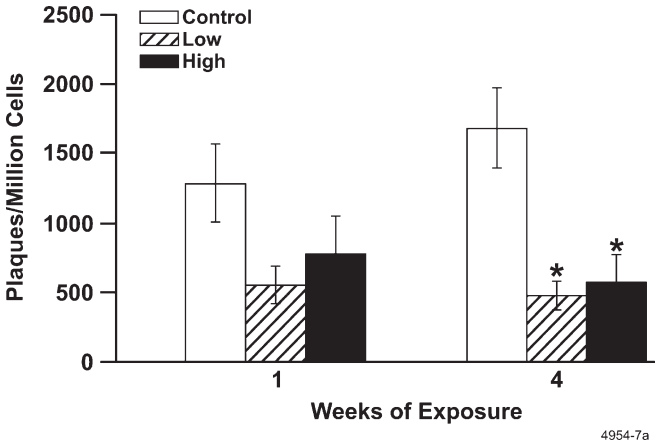

Figure 2 Effect of *K. brevis* extract exposure on numbers of plaque-forming spleen lymphocytes. Error bars represent SEM. * indicates values are significantly different from control (analysis of variance, adjusted for multiple comparisons, $P < 0.05$).

equivalents/mL of vehicle (33% ethanol in water containing 0.05% Alkamuls® EL620A). The rats were exposed in 96-port nose-only chambers. Total aerosol mass concentration was determined gravimetrically. Brevetoxin concentration was estimated by knowing the fraction of the total solute represented by brevetoxin and was confirmed by ELISA on selected filter samples (Naar *et al.,* 2002). The aerosol size distribution, volume median aerodynamic diameter (geometric standard deviations) for the low- and high-exposure concentrations were 0.66 µm (2.2) and 1.4 µm (2.5), respectively.

Body weights and detailed observations were recorded the day before exposures began and weekly thereafter. Core rats were sacrificed by intraperitoneal injection of Euthasol®. Blood was collected by cardiac puncture for evaluation of hematology (erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, platelet count, reticulocyte count, and total and differential leukocyte count), serum chemistry (blood urea nitrogen, creatinine, aspartate transaminase, alanine transaminase, alkaline phosphatase, gamma glutamyltransferase, inorganic phosphorus, cholesterol, triclycerides, total bilirubin, glucose, total protein, albumin and globulin, and electrolytes). The respiratory tract (except right lung), brain, liver, kidney, peripheral nerve, spinal chord, spleen, thymus, and thyroid were weighed and fixed in 10% neutral buffered formalin. Tissues were trimmed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin for evaluation. Before separation from the whole lung, the right lungs were lavaged via the trachea with saline. After centrifugation, the liquid portion of each lavage was analyzed for lactate dehydrogenase (an indicator of cell damage and death) and total protein (an indicator of pulmonary inflammation). Recovered leukocytes were resuspended in saline and manually counted by light microscopy using a hemocytometer. Cytospin preparations were prepared, stained with Kwik™Diff, and a differential cell count was performed. Cytospin preparations made on ProbOn™Plus sides were immunohistochemically stained for brevetoxin (Bossart *et al.,* 1998).

Rats designated for evaluation of effects on the immune system were immunized by tail vein injection of a 15% suspension of sheep red blood cells (SRBC) in phosphate buffered saline. At five days post immunization, the rats were euthanized as described above and spleens recovered. Cells were isolated from weighed portions of spleen, washed, and then resuspended in complete RPMI culture medium to a final concentration of 1×10^6 cells/mL. The IgM antibody forming cell (AFC) response to the T-cell dependent antigen, SRBC, was assessed with a modified plaque-forming assay (Cunningham and Szensberg, 1968). The number of AFC was determined by light microscopy and reported as AFC/10^6 lymphoid cells. The proliferative response of the spleen cells to the mitogen, Concanavalin A (ConA), was assessed. Spleen cells (5×10^5/100 µL complete RPMI) were incubated with 0.1, 0.3, and 1.0 µg ConA/50 µL at 37°C in the presence of 5% CO_2 for 54 hours. At that time cells were pulsed with 0.5 µCi ^{3}H-tymidine and incubated for an additional 18 hours. Cells were collected on filter paper using a cell harvester, and ^{3}H activity was determined by liquid scintillation spectrometry.

Rats in the neurotoxicity group were sacrificed and fixed *in situ* by whole body perfusion with 4% paraformaldehyde. Samples were shipped in 4% paraformaldehyde to NeuroSciences Associates (Knoxville, TN) for analysis of neuronal toxicity, as indicated by silver stain uptake.

Results and Discussion

Alveolar macrophages obtained from lungs of *K. brevis*-exposed rats, but not from control rats, stained positively for brevetoxin, indicating compound deposition in the brevetoxin-exposed rats (Fig. 1). Exposure for up to four weeks resulted in no clinical signs of toxicity or adverse effects on body weight. There were no gross lesions observed at necropsy at any sacrifice time. Weights of brain, lung, liver,

kidney, spleen, and thymus were unaffected. There were no adverse effects on hematology (including reticulocyte counts) or serum chemistry parameters (including blood urea nitrogen, aspartate transaminase, gamma glutamyltransferase, and total bilirubin). Lavage fluid endpoints were negative for cytotoxicity (lactate dehydrogenase) and inflammation (total protein, total and differential white blood cell count). There were no histopathological changes in any tissue examined. There was no evidence of neuronal toxicity by silver stain analysis in the brain. *Karenia brevis* extract inhalation reduced the number of plaque-forming lymphocytes in the spleen (Fig. 2). The effect was statistically significant after four weeks of exposure, but normal responses were observed after the four-week hold period. However, exposure did not affect lymphocyte profiles in the bronchial lymph nodes or spleen at any time.

The purpose of this study was to evaluate the effects of repeated *K. brevis* extract inhalation in rats. Endpoints focused on assessment of targets for toxicity suggested by findings in manatees exposed for extended periods by inhalation and/or ingestion to high concentrations of *K. brevis* (Bossart *et al.,* 1998). Repeated inhalation exposure to the mixture of brevetoxins at concentrations orders of magnitude greater than measured along Gulf Coast beaches (Pierce *et al.,* 2003; Cheng *et al.,* 2002) did not cause toxic effects in the respiratory, nervous, or hematopoietic systems. An important finding of this study was the significant reduction in spleen AFC response among rats exposed to even the lowest *K. brevis* extract concentration for one week in the absence of overt toxicity in the spleen. Disparity between other effects in marine mammals and rats in this study may be due to differences in dose or interspecies differences in sensitivity to the toxins. The contribution of the antagonist AJB6.0p (6.1) to the overall response is uncertain but will be the focus of future investigations.

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Philosophical Insights from an Analysis of Media Coverage of the *Pfiesteria* Controversy

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Abstract

An analysis of media coverage of the alleged biological risks posed by the dinoflagellate known as *Pfiesteria piscicida* offers several important philosophical lessons in the areas of ethics, argumentation, epistemology, and philosophy of science. The focus of this study was to examine the media's coverage surrounding the controversy over *Pfiesteria.* References to a variety of media reports warrant several conclusions: (1) With few exceptions, the media has not covered the *Pfiesteria* controversy with objectivity (Rescher, 1997; Nagel, 1986; Rabinsky, 1998). Sensationalized accounts of the alleged biohazards posed by *Pfiesteria* have skewed the public's understanding of the scientific issues involved in identifying *Pfiesteria* and the actual risks (if any) to humans; this is unethical and epistemologically flawed. (2) There is a distinction between a rigorous scientific account of *Pfiesteria* and its properties, and a popularized or sensationalized story about *Pfiesteria.* This distinction is important in light of recent work in the sociology of science which suggests that it is impossible, in principle, to draw a distinction between scientific reports and popularized accounts (Hilgartner, 1990). The present study showed, however, that there is a clear distinction between science and popularization. The study highlighted philosophical principles that are relevant for the practice of science, the philosophy of science, ethics, and public policy.

Introduction

The dinoflagellate *Pfiesteria piscicida* has gained much recent media and scientific attention, owing to new developments surrounding research into the microorganism's alleged toxicity and health hazards to humans, fish, and the environment. Various new aspects of the controversy surrounding the so-called "Cell from Hell" have been documented in the news media. In this study, our objective was to show that philosophical analysis, not just scientific expertise, is necessary for a proper understanding of the nature of the scientific controversy surrounding *Pfiesteria,* and for the ultimate resolution of such controversy. A related objective was to show that for adequate media coverage of scientific controversy, journalists and news reports (*i.e.,* the media) need to apply the tools and methods of philosophical analysis to create a proper context for the public's understanding of science (Cohen, 1992). Thus, in this paper, we did not intend to resolve any of the scientific aspects of the controversy; *e.g.,* we did not attempt to resolve here the questions of whether *Pfiesteria* is toxic, or whether the dinoflagellate has 24 life-cycles, etc. (Burkholder, Glasgow, 1999; Fleming, Easom, Rowan, 1999). Rather, we sought to illuminate conceptually the underlying philosophical assumptions and the implications of the scientific debate. Moreover, this paper deals with media analysis of scientific controversy and public understanding of science, and as a work in applied philosophy, it was informed by philosophical conceptual frameworks and theories, and developed this specific case study on the controversy over *Pfiesteria* to reach conclusions about the nature of scientific controversy in general.

Materials and Methods

For the task of reconstructing an objective account of the facts (*i.e.,* "history"—chronology, events, etc.) surrounding the *Pfiesteria* controversy, we consulted a variety of print news media with a reputation for accuracy, objectivity, and thoroughness–including the New York Times, The Associated Press, United Press International, Reuters, and C.N.N., as well as local newspapers such as the Pamlico Times (North Carolina). We collected a variety of news reports in order to assemble the public "story" (including events, chronology, etc.) about the scientific research as constructed by the media for the public's understanding. The time period of the news reports collected span the years between 1997 and 2002. The facts concerning the scientific controversy were obtained by examining a variety of news reports, which provided the data for assembling and reconstructing an objective account of the controversy. The news reports we examined offer alternative explanations and perspectives on the controversy; thus, these news reports provide the layperson with a narrative background for assessing competing (or rival) scientific views on the controversy, from an objective and impartial perspective. After reconstructing a public account of the facts, we applied the methods of conceptual analysis and argumentation theory (Walton, 1997, 1989; Rescher, 1997) to sort out various epistemological and ethical issues arising from the controversy (see Notes).

Results

Because this study was essentially philosophical in nature, the study raised a number of theoretical and conceptual questions concerning the nature of scientific inquiry, the preconditions for (and significance of) scientific consensus, the nature of scientific expertise, and the possibility for resolution of scientific controversy. Among the questions that emerged from the philosophical analysis of the scientific controversy were the following: What is an "expert," and how does one decide (in a dispute between experts) which expert opinion to accept or believe in light of conflicting claims to expertise and knowledge? Who has the logical burden of proof to demonstrate the existence of a hypothesized scientific phenomenon—the researcher(s)

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who asserts the phenomenon's existence, or the researcher(s) who is skeptical of, or denies, the existence of such phenomena? For the task of resolving rationally a scientific dispute, is there an ethical obligation (in addition to a logical and epistemological burden) to share one's data, methods, samples, results, etc., with other scientists (who may be doubtful or critical of one's conclusions)? If so, under what circumstances would there be such an ethical duty—and according to what ethical standards or norms? On the other hand, if there is no duty to share samples, materials and information with other scientists and the public, why not? These and other questions were raised by, and explored conceptually, in the present study.

One of the key ethical issues arising in the scientific controversy on *Pfiesteria* is the question of whether there is an ethical responsibility to share information and scientific materials among scientists who may disagree with each other's views and conclusions. Applying the philosophical frameworks of both utilitarianism and libertarianism (two moral and socio-political frameworks which often yield divergent results concerning decisions about which action is morally right in a given ethical dilemma or context), we arrived at the result that both of these fundamental ethical frameworks yield identical conclusions regarding the issue of whether there is a moral duty to share data, materials and information. From a utilitarian point of view (utilitarian ethical theory is based on the principle that the right action in a given context or dilemma is the action which promotes the greatest well-being for the greatest number of people affected by, or concerned with, the action), there is a moral duty for scientists to share samples, information, and materials, precisely because of the alleged nature of *Pfiesteria* itself: if *Pfiesteria* does indeed pose the serious biohazards to humans and the environment that some scientists maintain, then the public's well-being will be maximized if scientific progress is enhanced through an optimization of the free-flow of information and data among all scientists with the tools and relevant credentials to conduct an inquiry into *Pfiesteria*. Moreover, from a libertarian point of view (libertarian ethical theory is based on the principle that individuals possess inalienable natural rights—including the right to life, liberty, and property), there is a moral duty for scientists to share samples, data and information on *Pfiesteria* because research into the dinoflagellate has been funded publicly through tax dollars; thus, the rightful owners of information and samples of *Pfiesteria* is the general tax-paying public who has funded the research into *Pfiesteria*—not private individuals, corporations, or academic institutions. If all scientists who participate in the *Pfiesteria* research share their samples, data, and information, the public's opportunity to learn more about *Pfiesteria*'s actual properties will be enhanced, and scientific inquiry stands a better chance of functioning according to the scientific process, which involves putting out theories and then trying to disprove and prove them in a public forum, where dissenting points of view have the opportunity to be heard. Controversy is inherent in the scientific process, but a precondition for the reasonable resolution of scientific controversy is that all parties to the dispute have access to the same materials, data, etc. (Griffith, 1999) Finally, our results show that this condition presupposes that all parties to a scientific dispute have a common interest and willingness to revise or examine their beliefs and conclusions in light of all the available and relevant evidence.

Discussion

In this pilot study, we confirmed that, as in similar cases concerning scientific controversy, the public relies extensively on the testimony of experts to form opinions and ultimately to decide what beliefs to accept. It is in the process of relying on, and critically examining, the opinions of experts that important epistemological and logical issues arise. Difficulties in deciding what beliefs to accept, however, may arise when different experts in the same (or related) fields of inquiry judge that different beliefs should be accepted or rejected. When various (or many) experts disagree on whether a theory or belief is true, and when there is significant lack of consensus among the experts, then the public (and the experts themselves) may find it unusually difficult to determine which belief or theory to accept (Czubaroff, 1997; Coady, 1992). The lack of scientific consensus among experts, however, need not preclude the possibility of critical and analytical efforts to make epistemic progress in judging which beliefs to accept fallibly and which to reject; argumentation theorists and epistemologists (Rescher, 1997; Walton, 1997) have elucidated that a variety of epistemic criteria may be used to assess credibility, relevance, and objectivity of expert testimony.

In addition, existing case studies involving research into the nature of scientific controversy may be used to better understand the assumptions and implications of the controversy surrounding *Pfiesteria* in particular, and of the nature of scientific controversy in general. For instance, D. Walton (1997) described the scientific controversy surrounding various claims that were asserted in relation to the (alleged) health and therapeutic effects of vitamin C. In this particular case study, biochemistry Nobel laureate Linus Pauling defended the claim that consuming large doses of vitamin C is good for human health (especially for preventing the common cold), and that taking large doses of other vitamins could enhance longevity and health. In reply to Pauling's claims, various physicians and nutritionists were skeptical of (and disputed) Pauling's conclusions. One physician, Dr. Richard Rivlin, professor of medicine at Cornell University and chief of the nutrition service at Memorial Sloan-Kettering Cancer Center in New York, asserted that vitamins in excess can cause significant damage. In this scientific dispute, in which two experts disagree, the public must decide which beliefs to accept based on the analysis of expert opinion. In the present pilot study, we concur with Walton's analysis that experts with different (but significantly relevant) scientific backgrounds or areas of expertise may contribute to, and

enhance, the quality of the intellectual inquiry; one cannot simply dismiss as false the opinions of experts who are considered (by the public or other experts) to be outside the realm of "established" expertise: knowledge is fallible, and hypotheses may be either corroborated or falsified, and theories revised accordingly. A precondition for the possibility of the rational resolution to scientific debate (or any other intellectual dispute) is that the participants to the dialogue maintain a genuine effort to avoid the fallacious "Appeal to Authority"—arguments which are designed to exclude opposing or conflicting points of view by maintaining that the opinions and beliefs of those who disagree with one's own point of view are "biased" or "erroneous" simply because they do not conform to the "mainstream" or "official" view. (In the case of the dispute between Pauling and Rivlin, for example, Walton maintains—correctly, in our view—that Pauling's views on the properties of vitamin C cannot be dismissed simply because Pauling is not a physician, unlike Rivlin; although Pauling is not a nutritionist or physician, he has expertise in biochemistry—a relevant and related field of inquiry in the debate on the properties of vitamins.) Similarly, we argue that in the scientific controversy surrounding the properties of *Pfiesteria*, the public (and experts) should examine the concept of "expertise" and "appeal to expert opinion" in order to decide rationally whether the debate can be resolved within the existing framework of dialogue, or whether the assumptions underlying the present debate need to be re-examined and the conditions for dialogue revised.

Finally, an analysis of the media coverage surrounding the scientific controversy over *Pfiesteria*'s alleged properties and potential risks to humans and the environment led us to discover that the media did not address, or consider, various significant philosophical questions that arise in attempting to resolve rationally the scientific debate. Accordingly, we found that the media has not provided an adequate context for the public to think critically about and understand the nature of the *Pfiesteria* controversy (among other such scientific controversies). The media's failure to enhance the public's understanding of the controversy is due, we suggest, to its lack of philosophical analysis of the underlying scientific and epistemological assumptions concerning such concepts as "scientific expertise," "burden of proof," "reasonable dialogue," "scientific controversy," and "objectivity."

We believe that subsequent philosophical analysis of future developments on the *Pfiesteria* controversy may better illuminate various concepts first explored in this pilot study.

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Notes

One of the main developments in the philosophical tradition known as "analytic philosophy" has historically been known as conceptual analysis and the use of logical argumentation. Often, many professionals (*e.g.,* scientists, legal scholars, and others) regularly use concepts that are vague and imprecise, as well as ambiguous or inadequately defined. One of the tasks of analytic philosophy is to make our understanding of significant concepts (*e.g.,* concepts such as "knowledge," "belief," "truth," "objectivity," "bias," "expertise," etc.) as precise and rigorous as possible. In doing so, analytic philosophers seek to discover what are the necessary and sufficient conditions for a given concept to apply in a particular case. Additionally, philosophers also endeavor to defend their conceptual analyses by giving reasons (arguments) in support of their views. Presently, we used the techniques of informal logic (distinct from formal symbolic logic); whereas both formal and informal logic are concerned with discovering valid patterns of inference (*e.g.,* "If P, then Q; and P; thus Q"—a *modus ponens* structure of argument, which is deductively valid), it is only informal logic which is also concerned with fallacy theory: the study of the ways in which arguments presented in natural languages (*e.g.,* English, German, French) lead to errors in reasoning or mistakes in argumentation. For the present philosophical analysis of the *Pfiesteria* controversy, the concept of objectivity was central in our work (see the existing philosophical literature on criteria for, and analyses of, the concept of objectivity in Rescher (1997), Nagel (1986), and Rabinsky (1998)).

An Epidemiologic Approach to the Study of Aerosolized Florida Red Tides

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Abstract

Very little has been published in the scientific literature on the human health effects of Florida red tide, either as human clinical case reports or formal epidemiologic studies. In addition to the health effects associated with the ingestion of contaminated shellfish, there have been multiple anecdotal reports of respiratory irritation and possible immunologic effects associated with the inhalation of aerosolized Florida red tide. To investigate the human health effects from environmental exposure to red tide toxins, we have formed an interdisciplinary team of scientists. We have created a network of public and environmental health workers who periodically report local conditions as a red tide develops. In addition, we have access to environmental monitoring data as well as data from a surveillance program supported through the Florida Poison Information Network. When a red tide moves onshore where people might be exposed, the team rapidly assembles at the site to collect environmental samples and epidemiologic data. To assess the more long-term effects from environmental exposure to red tide toxins, we are conducting epidemiologic studies involving occupational and sensitive populations who live in areas that are regularly impacted by red tides. Other scientists are evaluating the acute and chronic respiratory effects of red tides and brevetoxins in both rat and sheep models as well as refinement of toxin measurement methodology. These models are being used to refine and validate the biomarkers of brevetoxins exposure as well as explore the pathophysiology of health effects from brevetoxins respiratory exposure. Bolstered by the additional research in rat and sheep models, this interdisciplinary scientific team is exploring the acute and chronic exposures and health effects of aerosolized Florida red tides in animal models and various human populations. In the future, this research can be applied to the understanding of exposure and effects of other aerosolized natural toxins such as cyanobacterial toxins.

Introduction

Florida red tide is an almost annual event caused by the dinoflagellate, *Karenia brevis.* This organism produces brevetoxins which cause significant fish kills as well as neurotoxic shellfish poisoning (NSP) if contaminated shellfish are consumed. There have been anecdotal reports of respiratory irritation and possibly immunologic effects associated with the inhalation of aerosolized Florida red tide. Recent die-offs of the endangered Florida manatee were associated with the inhalation of the Florida red tide toxins, and research in sheep and other laboratory animals has confirmed the ability of aerosolized red tide toxins to cause reversible bronchospasm (Kirkpatrick *et al.,* in press; red tide Group, 2002; Cheng *et al.,* in press; Backer *et al.,* 2003).

The traditional public health approach has been quite successful in preventing human cases of NSP in Florida red tide through active environmental monitoring for toxin and organisms with subsequent closure of shellfish beds, in addition to passive public health surveillance. However, faced with an intermittent annual aerosolized exposure with possible acute and chronic respiratory effects, particularly in sensitive subpopulations, in a state highly dependent on tourism and other coastal industries, a new public health and research approach must be instituted. This paper briefly describes an ongoing interdisciplinary research study of the exposure and health effects of aerosolized Florida red tide toxins on humans and animal models.

Methods

To investigate the human health effects from environmental exposure to red tide toxins, we formed an interdisciplinary team of scientists. We created a network of public and environmental health workers who periodically report local conditions as a red tide develops. We have access to environmental monitoring data (*e.g.,* cell concentrations in water samples, satellite imagery) as well as data from a surveillance program supported through the Florida Poison Information Network. When a red tide moves onshore where people might be exposed, the team assembles at the study site to collect environmental samples (air and seawater) and epidemiologic data (including pre- and post-exposure questionnaires, pulmonary function tests, and personal breathing zone monitoring). To assess the chronic effects from environmental exposure to red tide toxins, we are conducting epidemiologic studies involving occupational (including lifeguards and scientists) and sensitive populations (including elderly people with underlying respiratory dis-

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ease and children with asthma) who live in areas that are regularly impacted by red tides. Members of the interdisciplinary team are also evaluating the acute and chronic respiratory effects of red tides and brevetoxins in both rat and sheep models as well as refinement of toxin measurement methodology.

Results

The aerosolized Florida red tide toxin research group is performing research integrated between human and animal models as well as between environmental and personal sampling. The measurement in humans and animals of both exposure (*i.e.,* brevetoxins, sea water and other substances) and effects (*i.e.,* respiratory, immunologic, neurologic, and other) is the core objective of this research program.

Exposure Measurement We have examined samples of sea spray aerosol collected during red tide events and have found that the particles are predominantly large (>2.5 microns). An ELISA test for brevetoxins has been developed and used to measure brevetoxin concentrations in seawater and air (personal and environmental) samples as well as in samples of biological fluids from rats and sheep exposed to brevetoxins under laboratory conditions. Attempts to use the ELISA to measure brevetoxins in human fluids to date have not been successful except in throat swabs. An immunohistochemical stain for brevetoxins, originating from earlier research in highly exposed manatees (Bossart *et al.,* 1998), has also been successfully applied to demonstrate the presence of brevetoxins in samples from exposed laboratory rats and sheep, but not humans. In part, this may reflect the significantly lower concentrations of brevetoxins in humans exposed under natural conditions when compared with laboratory animals.

Effect Measurement Studies reveal that rats are relatively resistant to brevetoxins (*i.e.,* they demonstrate responses after being exposed to microgram levels of brevetoxins, compared with the picogram exposures in other species). Health effects being evaluated include the respiratory, immunologic, and neurologic systems. Studies in asthmatic sheep have demonstrated significant bronchoconstriction following inhalation challenge with picogram levels of brevetoxins or contaminated sea spray; furthermore, this response can be blocked by pre-treatment with a mast cell inhibitor and by antihistamines (Abraham 2001, Cheng in press). A pilot study of human recreational beachgoers (Backer 2003) found a relationship between the amount of brevetoxins in the air and seawater, with the symptoms reported by study participants. The results indicated that when the cell counts and the brevetoxin levels were higher in water and air samples, and were accompanied by strong onshore winds, people reported an increase in lower respiratory symptoms (*e.g.,* wheezing and chest tightness) after visiting the beach, compared to the symptoms reported before going to the beach. On the moderate exposure day with lower brevetoxin levels in the air and water, fewer people reported lower respiratory symptoms, and were significantly more likely to report upper respiratory symptoms (e.g.. nasal and throat irritation) after being on the beach. We also conducted spirometry tests of pulmonary function using methods and standards approved by the National Institute for Occupational Safety and Health; however, these tests did not identify any decreases in pulmonary function following exposure to aerosolized brevetoxins. We are now conducting two additional epidemiologic studies using more precise spirometry equipment. A cohort of lifeguards has been assembled as well as additional cohorts of people with asthma (>11 years) and people with Chronic Obstructive Pulmonary Disease (>44 years). The occupational cohort is being evaluated throughout several workdays, while the sensitive cohorts are evaluated during several years by conducting pulmonary function testing and requesting information about symptoms during a red tide and when there is not a red tide in the area.

In addition to the subjective symptom reporting and the spirometry data, we are developing biological markers of the effects of brevetoxins. These markers include swabs of the nose and throat to examine the inflammatory response to exposure. We are also examining other potential markers of biological effect, *e.g.,* neuropsychological testing (including auditory evoked responses) (Lu *et al.,* 2002) and other cellular markers (including epithelial cell factors and immunologic markers). If we are able to demonstrate effects in the animal models at doses comparable to human exposure, then these effect biomarkers will be evaluated in the epidemiologic cohort studies.

Discussion

The strength of our research program to evaluate human exposure to and effects from aerosolized red tide toxins is its integrated interdisciplinary approach. Animal models are being used to refine and validate potential markers of exposure and biological activity as well as to explore the pathophysiology of health effects from respiratory exposure to brevetoxins. We anticipate applying what we learn about human exposure to aerosolized brevetoxins in additional studies of the health effects associated with exposure to aerosols containing other potent natural toxins, such as those produced by cyanobacteria (blue-green algae). In addition, a number of outreach and educational materials that have been created in parallel with this research will be used as models for future materials developed to educate the public about naturally occurring toxins in their environments (www.rsmas.miami.edu/groups/niehs/redtide/; Kirkpatrick *et al.,* in press).

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Monitoring Approaches for Improved Prediction of Domoic Acid Poisoning Events in Washington State

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Abstract

The Olympic Region Harmful Algal Bloom (ORHAB, for a list of collaborators see www.nwfsc.noaa.gov/ORHAB) partnership has established a monitoring program for harmful algal blooms, resulting in more openings for commercial, subsistence, and recreational harvest of razor clams. This collaboration has resulted in reduced costs and faster analysis of shellfish samples, thereby lowering the health risks to consumers. Patterns are emerging regarding the seasonality, duration, and magnitude of *Pseudo-nitzschia* blooms that impact coastal shellfish. A simple combination of analytical techniques, including weekly microscopic determination of total *Pseudo-nitzschia* cells and levels of particulate domoic acid in seawater, give an effective early warning of shellfish toxification events. In the future, fine-scale sampling using automated devices on moorings and real-time analysis on beaches will allow detailed determination of fluctuations in biological, physical, and chemical parameters that influence HAB intensity.

Introduction

A unique problem on the outer coast of the Olympic peninsula in Washington State, USA, is that the Pacific razor clam, *Siliqua patula,* can retain high concentrations of the algal toxin domoic acid (DA) for over one year (Wekell *et al.,* 1994; Adams *et al.,* 2000). During this time, recreational, commercial, and tribal subsistence harvest of clams, valued at over $20 million annually (Anderson, 1995), is paralyzed.

Pseudo-nitzschia (Pn), the genus of diatom that can produce domoic acid, is very difficult to monitor at the species level in natural populations where several species of that same genus co-occur. Precise identification often requires tedious electron microscopy. It is not viable to identify every sample collected as part of a monitoring program using such a tedious approach. However, the ORHAB program uses a simple combination of analytical techniques, including twice-weekly microscopic determination of total Pn cells and levels of particulate DA in seawater to give an effective early warning of shellfish toxification events. This approach may assist managers in other coastal areas by providing an early warning of domoic acid problems.

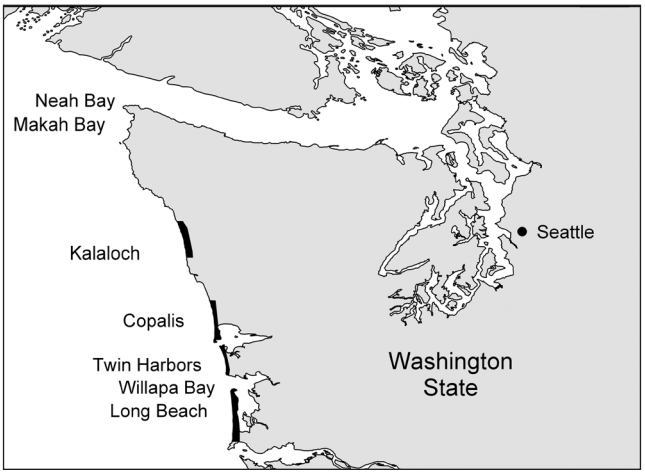

Figure 1 ORHAB sampling locations. Areas of razor clam harvest are shown as dark bars.

Materials and Methods

ORHAB technicians sample at seven locations on the Washington coast that include major areas of razor clam harvest (Fig. 1). Twice weekly, technicians identify phytoplankton by light microscopy (Adams *et al.,* 2000) and quantify numbers of Pn. Estimates of species size groups, including (1) *pungens, multiseries,* (2) *heimii, fraudulenta, australis,* and (3) *delicatissima, pseudodelicatissima* are also provided. Selected samples of Pn are also identified to the species level using scanning electron microscopy (Miller and Scholin, 1998). In addition, 1 L of seawater is filtered twice a week, and razor clams are sampled about twice a month and tested for DA content (Adams *et al.,* 2000; Hatfield *et al.,* 1994). For clarity, only results from Kalaloch beach and Long Beach are shown in this paper.

Results and Discussion

Record levels of DA in razor clams in 1998 resulted in a coastwide closure of shellfish harvest that persisted for more than one year. This bloom event resulted from Pn cell numbers reaching 17×10^6 cells/L and levels of particulate toxin in seawater up to 2500 ng/L (Adams *et al.,* 2000; Fig. 2A, event 1).

In the spring 2001 (event 2), only the southern beaches were closed, due primarily to clam exposure to numbers of *P. australis* reaching 0.5×10^6 cells/L for 1–2 weeks (Fig. 2B). Northern beaches, where the dominant species was *P. pseudodelicatissima,* were not closed to shellfishing. During event 3, in the late summer 2001 (Fig. 2B), a short-lived bloom of *P. australis* in numbers up to 0.6×10^6 cells/L was observed. No beach closures occurred; however, DA levels in razor clams were dangerously close to the regulatory limit of 20 ppm. On the same day that DA levels of 18 ppm were measured in clams, Pn cell numbers were decreasing, giving managers confidence that levels of DA in razor clams

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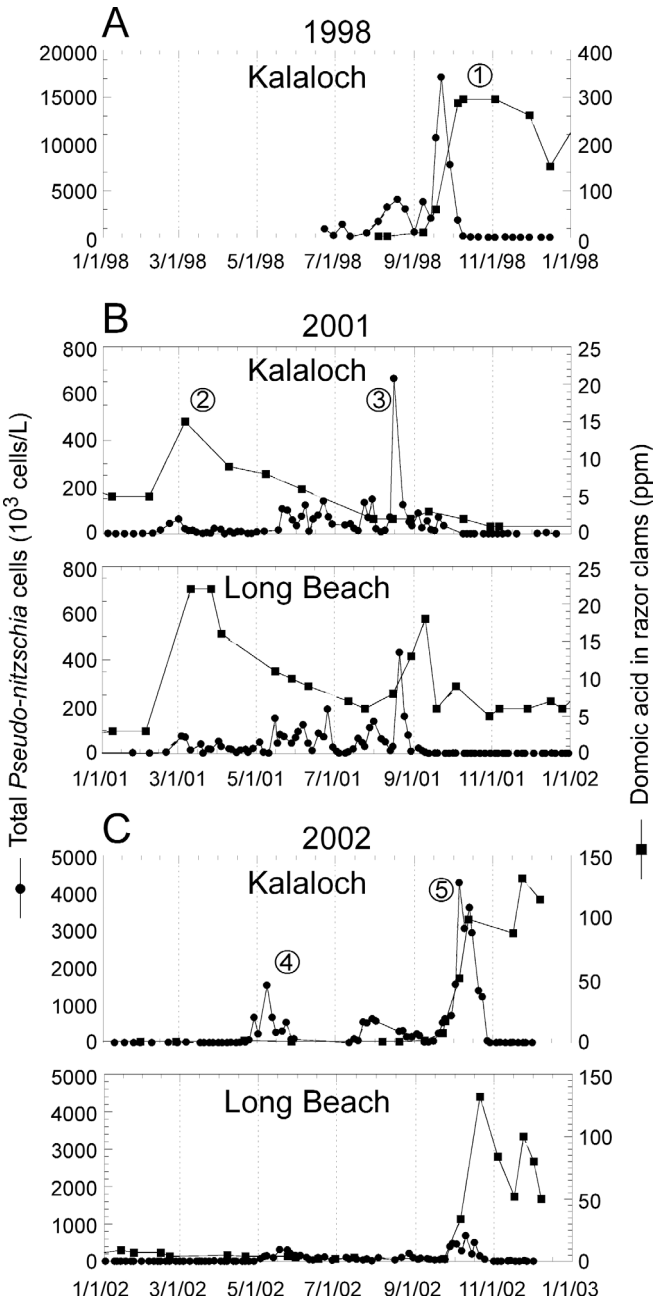

Figure 2 Pn cell numbers and DA in razor clams at Kalaloch (including 1998) and Long Beach in 2001 and 2002. Numbers correspond to selected bloom events.

Table 2 Domoic acid levels and Pn cell numbers preceding and during the late summer 2002 bloom event at Kalaloch beach.

		Domoic Acid	
Date	*Pseudo-nitzschia* (cells/L × 10^5)	Seawater (ng/L)	Razor clam (ppm)
Aug 30	1.6	5	
Sept 3	2.3	10	
Sept 5	1.9	20	
Sept 9	0.1	20	2
Sept 12	0.2	40	
Sept 16	0.4	220	
Sept 18			2
Sept 20	2.5	630	
Sept 23	5.3	2280	8
Sept 25	6.4	3260	17
Sept 30	7.2		26
Oct 3	15.6	3050	
Oct 6	42.7		52
Oct 10	30.5		
Oct 13			99

would not continue to increase. In May 2002 (event 4), *P. pseudodelicatissima* cell numbers increased to almost 1.0×10^6 cells/L for a short period of time; however, levels of DA in razor clams never exceeded 4 ppm (Fig. 2C). Finally, in September and October 2002, a coastwide closure occurred due to levels of *P. australis* up to 4×10^6 cells/L for greater than a 2-week period (Fig. 2C, event 5). Events 1–5 are summarized in Table 1.

Data from the late summer bloom event in 2002 are detailed in Table 2. On September 16, particulate DA in seawater rose to over 200 ng/L at Kalaloch beach. On September 20, Pn numbers rose to 2.5×10^5 cells/L and particulate DA measured 630 ng/L, indicating that a rise in DA levels in razor clams was likely. Indeed, on September 30, DA in razor clams increased to 26 ppm, resulting in a harvest closure. Although the level of DA in razor clams from Copalis beach (Fig. 1) was only 17 ppm, the continued increase in Pn cell numbers at all monitoring sites gave managers the confidence to invoke a coastwide closure in

Table 1 Selected Bloom Events

Event[1]	Closure	Max. Pn[2] (cells/L)	Dominant Pn species	Other Pn species[3]
1. Summer 1998	coastwide	17×10^6	psdeli[4]	heimii, pun, deli
2. Spring 2001	southern beaches[5]	0.7×10^5	aus	pun, psdeli
3. Summer 2001	none[6]	0.6×10^6	aus[7]	psdeli, heimii
4. Spring 2002	none	1.5×10^6	psdeli	aus, deli
5. Summer 2002	coastwide	4.3×10^6	aus	heimii

[1]Events correspond to numbers in Fig. 2. [2]Pn = *Pseudo-nitzschia.* Indicates maximum number of all Pn species. [3]These Pn species were present in low numbers. [4]Species abbreviations are as follows: psdeli, *P. pseudodelicatissima;* aus, *P. australis;* pun, *P. pungens;* heimii, *P. heimii;* deli, *P. delicatissima.* [5]At the northern beaches, where no closure occurred, the dominant Pn species was *psdeli.* [6]No closure occurred, but domoic acid levels in razor clams reached 18 ppm at the southern beaches. [7]*P. australis* was dominant at the southern beaches. The dominant species at the northern beaches was *P. pseudodelicatissima.*

early October of 2002. Unfortunately, because the State of Oregon does not monitor phytoplankton, the sudden rise of DA in razor clams there resulted in a costly recall of shellfish harvested in early October.

Over the past 2 years of monitoring, the ORHAB partnership has enhanced our knowledge of the seasonality, duration, and magnitude of Pn blooms associated with shellfish harvest closures on the Washington coast. This information is being used to establish an effective monitoring program that gives managers a timely warning of HAB events. Our results show that a simple combination of analytical techniques, including twice weekly microscopic determination of total Pn cells and levels of particulate DA in seawater, gives an effective early warning of shellfish toxification events. In addition, the real-time analysis of cells and toxins using rapid detection kits will increase the warning period for managers. These rapid analyses will be made possible by placement of sensors on oceanographic moorings and by detection on beaches using test kits.

Conclusion

P. australis and *P. pseudodelicatissima* are, to date, the major "problem" species of Pn on the coast of Washington State.

The northern and southern management beaches are exposed to different numbers of toxigenic Pn for different lengths of time, occasionally resulting in selective closures. The environmental factors responsible for these differences will be determined in future studies, in particular, during regional ECOHAB cruises that will begin in the summer 2003.

Measurements of increasing levels of Pn and particulate DA in seawater that supplement razor clam DA data give extra confidence to managers when establishing beach closures.

Suggested "warning" levels of Pn on the Washington coast are *P. australis* at 5×10^4 cells/L or *P. pseudodelicatissima* in excess of 1×10^6 cells/L for over 1 week. Warnings should also be released to managers when the level of particulate DA in seawater exceeds 200 ng/L.

Acknowledgements

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Ceratium furca: One Possible Cause of Mass Mortality of Cultured Blue-Fin Tuna at Baja California, Mexico

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Abstract

On 4 September 2002, a water-quality monitoring program at a blue-fin tuna (*Thunnus orientalis*) farm registered normal oxygen, nutrients and transparency levels. A "chocolate tide" had been detected 10–12 km south of the farm, but because of hurricane Hernan there was a sudden change in wind direction and the bloom patch, whose density was above 10^7 cells/L, was transported to the tuna pens. The resultant mortality of more than 500 tons of tuna in less than 48 hours caused economic damages estimated at $12–15 million. To estimate the fate and density of the bloom patches following the event, the area was observed from an aircraft. Samples were taken from inside and outside the bloom patches to evaluate the main phytoplankton species present. *Ceratium furca* was the dominant species inside the bloom patches, so we deduced that it was apparently responsible for the rise in un-ionized ammonia (as high as 1 mg/L) inside the tuna pens.

Introduction

In the Southern California Bight, water discoloration events from phytoplankton blooms occur mainly because of upwelling or in areas of urban pollutant emissions (Eppley, 1986), such as Point Loma and the ports of Santa Monica, USA, and Ensenada, Baja California (B.C.), Mexico. In the case of upwelling, the blooms are mostly dominated by diatoms. This natural enrichment gives rise to new productivity because of the input of subsurface nutrients such as nitrates, nitrites, phosphates, and silicates into the warmer surface waters. Furthermore, eutrophication is due mainly to anthropogenic influences (Orellana-Cepeda and Morales-Zamorano, 1994). Whether treated or not, this water favors the growth of dinoflagellates, which under natural conditions would dominate in this area only during late summer (Eppley, 1986). Discoloration events can appear red, green, or golden-yellow, due mainly to phytoplankton blooms of *Lingulodinium polyedrum, Prorocentrum* spp., *Akashiwo sanguinea, Pseudo-nitszchia* spp. and *Eucampia zodiacus* (Orellana *et al.,* 1993). *Ceratium furca* is a dinoflagellate that remains year-round in the plankton of this region; it can be found in Todos Santos Bay from nearly undetectable concentrations to 10^4 cells/L. *Ceratium furca* has been reported among species that generate fall blooms (Eppley, 1986). In Manzanillo, Colima, Mexico, it was reported in densities of $0.3–5 \times 10^6$ cells/L (Gómez-Aguirre, 1992). This species, though sometimes linked to anoxia problems in different parts of the world (Landsberg, 2002), was not considered among those harmful species that cause other types of problems (Taylor *et al.,* 1995). In South Africa, a massive fish and invertebrate mortality in 1994 was reportedly due to dinoflagellate phytoplankton with densities of 10^7 cells/L, including both non-toxic *Ceratium furca* (dominating) and *Prorocentrum micans,* and toxic *Alexandrium catenella* and *Dinophysis acuminata* at lower concentrations. Bacterial decomposition of the high phytoplankton biomass in Helena Bay generated anaerobic conditions (oxygen below 0.3 mg/L) and gaseous hydrogen sulphide. This event became known as "black tide." Along the 30-kilometer coast of Helena Bay, approximately 60 tons of lobster, 1,500 tons of demersal and benthic fish, and invertebrates such as sea urchins and mussels died (Matthews and Pitcher, 1996).

The first cultured blue-fin tuna mass mortality was reported in Boston Bay, Australia. An estimated mortality of 1,700 tons of caged southern blue-fin tuna, *Thunnus maccoyi,* occurred simultaneously with a *Chattonella marina* bloom, a brevetoxin-like producer, at concentrations of up 6.6×10^5 cells/L. The mortality caused by this species was attributed to the direct toxic effect of oxygen radicals on the gills in combination with the cardiotoxic effect of brevetoxins (Munday and Hallegraeff, 1998).

Materials and Methods

Puerto Escondido is located 15 miles south of Todos Santos Bay, Ensenada, Baja California, Mexico, at 31°42′30″N latitude and 116°42′00″W longitude (Fig.1). Free ammonia determinations were made with portable equipment and a laboratory HACH water quality test kit. Phytoplankton quantifications were performed with an inverted

Figure 1 Map indicating the approximate position of the blue-fin tuna farm.

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Table 1 Phytoplankton concentrations and water quality measured at the farm during the passage of the "chocolate-colored water" dominated by *Ceratium furca.* Cell densities are expressed in cells/L, and O_2 and un-ionized NH_3 are expressed in mg/L.

Date 2002	Z	Time	O_2	NH_3	Temp. °C	*Ceratium furca*	*Dinophysis* sp.	*Pseudo-nitzschia* sp.	*Prorocentrum* spp.*	Other Species	Total cells
09/04	Sur	13:30	7.5	1	16.25	10^7	$<10^3$	$<10^3$	$<10^3$	$<10^3$	1.2×10^7
09/06	Sur	13:00	9.7	<0.01	13.50	2.5×10^5	2.6×10^3	3.3×10^3	2.3×10^3	2.7×10^4	2.6×10^5
09/12	Sur	13:00	10	<0.01	15.40	1.4×10^6	2×10^3	10^3	2×10^4	1.2×10^4	1.4×10^6
	Sur	13:00	9.7	<0.01	15.40	1.4×10^6	2×10^3	10^3	2×10^4	1.2×10^4	1.75×10^6
09/18	0–3m	14:00	9.3	0.04	15.40	1.06×10^6	9.05×10^5	1.3×10^3	$<10^3$	7.7×10^4	9.0×10^5
south patch	3–9m	14:00	9.3	0.01	16.30	6.53×10^5	$<10^3$	$<10^3$	9.1×10^4	9.9×10^4	9.8×10^5

**Prorocentrum micans* + *P. gracile*

microscope (outside the patch) and during the bloom, in a Sedgewick-Rafter chamber (6 aliquots) under a Zeiss microscope (within the patch) (Throndsen, 1995). Collection of physical-chemical data (temperature and dissolved oxygen) was taken with a YSI Model 55 during and after the event. Transparency readings were taken with a Secchi disc, of 30 cm.

Results and Discussion

On 4 September 2002, the average temperature at 0800 was 14.77°C. At 1300, a patch of "chocolate-colored water" that entered the blue-fin tuna farm was measured at 16.25°C. Secchi disc readings decreased from 6 meters to 1-meter depth. Oxygen increased from 6.95 to 7.5 mg/L. The colored water patch was dominated by *Ceratium furca* (10^7 cells/L) while un-ionized ammonia was measured at 1 mg/L (Table 1). The high ammonia levels could have been responsible for the excess mucus produced on the blue-fin tuna gills. During the following 48 hours, a high and rapid mortality of over 500 tons of tuna in the pens caused economic damages estimated at $12–15 million. Detailed mortality data are not available. It took the farmer five days to clean all of the cages.

The arrival of the dense bloom patch occurred during the morning 4 September 2002, due to a change in wind intensity and direction. In the days prior to the event, the patch of "chocolate-colored water" had been detected 10–12 km south of the farm. With a sudden change in wind direction due to the effects of hurricane Hernan, the patch was transported northward, together with a high load of molecular ammonia, and entered the area of the tuna pens. Many explanations as to the causes of the mortality were considered. One speculation was a decrease in the oxygen concentration to a level anoxic for the fish, but this, however, was not observed. In fact, oxygen levels increased because of photosynthetic processes in the patch. High temperatures could also stress the fish, but in Magdalena Bay, B.C.S., the same species in pens tolerate 26°C without any apparent changes in behavior. Another speculation was to blame the rapid temperature increase (1.48°C), but on August 2, 2000, at Puerto Escondido, B.C., the same species in the farm tolerated a difference of 2.7°C with no mortality. Tuna stress in the cages was evident, and the extremely high density (10^7 cells/L) of dinoflagellates dominated by *Ceratium furca* could produce concentrations of un-ionized ammonia as high as 1 mg/L (Table 1). These levels could have led to a high and rapid mortality in the tuna pens. The sudden appearance of the bloom (only 6 hours) caused the fish to produce excess mucus on their gills. This response is consistent with the literature on cases of tuna damage due to phytoplankton (Jenkinson, 1989).

Phytoplankton blooms can cause fish mortality in three ways (Bruslé, 1995): a) oxygen depletion, resulting from the bacterial decomposition of an excess biomass of dying phytoplankton requiring a large oxygen demand and producing hypoxic conditions, which suffocates the fish; b) physical damage to the gills from diatoms and silicoflagellates that perforate the gills, causing irritation to the secondary gill lamellae and the secretion of mucus; and c) direct ichthyotoxic effects from numerous toxic phytoplankton species that directly poison and kill fish. These effects can be diagnosed by histopathological observations of fish tissues such as the gills, liver and digestive tract.

The maximum concentration of free molecular ammonia tolerated by fish established by the European Inland Fisheries Advisory Commission and by the U.S. Environmental Protection Agency is between 0.02 and 0.25 mg/L. It was reported that values above these concentrations correspond to the LC50 that kills 50% of salmonids in 48–96 hours. For an increase of one pH unit, the concentration of un-ionized ammonia will increase ten times (Alleman, 1998). For teleost fish, the toxic limit of un-ionized ammonia and total ammonia are 0.05 and 2.5 mg/L, respectively (Haywood, 1983). In prolonged experiments on salmonids, the tolerance level for un-ionized ammonia was 0.002 g/L. In marine ecosystems, the limits of ammonia concentration that are recommended to preserve good water quality are ≤0.03 mg/L in a constant manner and 0.05 mg/L in an intermittent manner (Klontz, 1991). Background ammonium concentrations in the Southern California Bight are about 0.5 μM, whereas values greater than 2–3 μM can be found

at the outfall areas (Eppley, 1986). Heaney and Eppley (1981) found that in culture, *Ceratium furca* is able to recycle nitrogen from dead cells; ammonia concentrations in the water around these cells increased to 4.4 μM. Extrapolating these laboratory results with the observations of a rapid increase in un-ionized ammonia, the high density of *Ceratium furca,* and the patch age (estimated to be about one month old), it is possible that the high ammonia concentrations measured in the patch could have originated from recycled nitrogen caused by the death of phytoplankton cells.

We conclude that the tuna in the cages could have perished because of the effects caused by the passing of a warm phytoplankton patch dominated by *Ceratium furca* at concentrations higher than 10^7 cells/L. The effects caused by this patch were rapid and the fish mortality occurred within 48 hours. The main conclusion is that even the most seemingly benign phytoplankton species, in great density, could cause serious damage to tuna in cages.

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